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Biovar distribution of *Brucella* strains isolated from livestock in Turkey between 2010-2015

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ABSTRACT

According to 'the Regulation of Reporting of Notifiable Animal Diseases in Turkey', the valid diagnosis for Brucellosis in livestock, which is in the list of notifiable animal diseases, is the isolation and identification of *Brucellae* as the gold standard as mentioned in 'the Regulation of the Fight with *Brucella*'. In the context of 'The Brucellosis Control and Eradication Program' in Turkey, where mass vaccination is practised as a part of this program in livestock, serological diagnosis is not considered to be valid except for *Brucella* free herds. While most of the current molecular techniques can differentiate *Brucella* organism at the genus level, they generally cannot make differentiation at the biovar-level. The primary purpose of this study is to determine the most prevalent *Brucella* biovars and the biovar distribution of *Brucella* isolates from the abort cases of livestock between 2010 and 2015 in Turkey. In this study, 5203 *Brucella* field isolates sent to our laboratory from different parts of Turkey for *Brucella* species and biovar identification between 2010 and 2015 were biotyped through conventional biotyping procedures. According to the results showing the percentages of dominant biovars causing *brucellosis* in livestock, the most common biovar was *B. abortus* biovar-3 in cattle and *B.melitensis* biovar-3 in sheep and goats. Vaccine strains isolated from goats were not included in biovar distribution in this study.

Key words: Biotyping, Biovar, *Brucella*, Isolation, Mass Vaccination.

INTRODUCTION

Brucellosis is one of the most common zoonotic diseases caused by *Brucella spp*. (Songer and Post, 2012). World Health Organization (WHO hereafter) considers Brucellosis as a worldwide zoonotic infection, which leads to important health and economic problems. (Godfroid *et al.*, 2005; Yumuk and O'Callaghan, 2012).

This disease has a particular socio-economic effect on the countries where animal husbandry contributes to rural income (Kushwaha *et al.*, 2016). According to WHO, there are 500,000 reported Brucellosis cases annually worlwide (Kumar *et al.*, 2010;Pérez-Sancho, *et al.*, 2015). WHO laboratory biosecurity manual also describes *Brucella* organisms as belonging to risk group 3 microorganisms (WHO, 2004; OIE, 2009a; Pérez-Sancho *et al*., 2015).

Brucellae are settled in the genitals and udders of animals like cattle, sheep, goats, pigs, and dogs and lead to abortion, infertility, and chronic infectious and necrotic inflamatory infection and complications such as mastitis, orchitis, and arthritis (Alton *et al.*, 1988; Aydin, 1997). *B.abortus*, *B.melitensis* and *B.suis* can be classified into biovars based on their cultural and serological features. There are 7 biovars belonging to *B.abortus*. However, *B.melitensis* includes 3 biovars and *B.suis* has 5 biovars. Brucellosis in cattle is generally caused *by B.abortus* biovars whereas in sheep and goats, it is primarily caused by one of the 3 biovars of *B.melitensis* and *B.ovis* (OIE, 2009a, 2009b; OIE, 2012). Each *Brucella* species and even each biovar has got a specific epidemiologic feature and there has been an increase in the complexity of the interaction between *Brucella spp.*, animals and humans. Moreover, new *Brucella* strains or species can emerge and the already existing ones may adapt to the social, cultural, travel and agricultural environments, which are continuously changing (Godfroid, *et al.*, 2005).

The main aim of this study is to determine the most prevalent *Brucella* biovars and the biovar distribution of *Brucella spp.* from the abort cases of livestock between 2010 and 2015 in Turkey.

MATERIALS AND METHODS

Reference strains and test isolates: *B.melitensis* 16M (*B.melitensis* bv-1 ATCCC 23456), *B.abortus* S19, *B.melitensis* Rev1, *B.abortus* 544 (*B.abortus* bv-1 ATCC 23448), *B.abortus* Tulya (*B.abortus* bv-3 ATCC 23450), *B.melitensis* Ether (*B.melitensis* bv-3 ATCC 23458), which

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were a part of the Veterinary Control Institute culture collection, were utilized as reference strains. 5203 *Brucella* field test isolates from the abort cases of sheep, goat and cattle submitted to National Brucella Reference Laboratory for species and biovar (bv) determination were utilized.These isolates were identified as *Brucella spp*. by Veterinary Control Institutes of Turkish Ministry of Food, Agriculture and Livestock and sent to our National Brucellosis Laboratory for species and biovars identification. Out of test isolates, 3008 were isolated from cattle, 987 from sheep and 1208 from goats.

Biotyping of *Brucella* cultures: *Brucella* cultures were examined by standard procedures for the identification of species and biotype level. Tryptic soy agar (BD 236950) supplemented with heat-inactivated bovine serum (N4762, SIGMA) (5%, v/v) (TSA) was employed as the basal medium for all culture work. Inoculated plates were incubated at 37ºC in normal atmospheric conditions and with the addition of 10% CO_2 for 4-5 days.

In this study, 5203 *Brucella* field isolates sent to our laboratory from different parts of Turkey for *Brucella* species and biovar identification between 2010 and 2015 were biotyped through conventional biotyping procedures (Alton *et al*., 1988; Cloeckaert *et al*., 2002; OIE, 2012). Biovar identification of isolates was implemented according to CO_2 requirement, H_2S production (Lead acetate paper, Fluka 37104), growth in media containing thionin (T3387, SIGMA) (20µg/ml), basic fuchsin(115937, Merck) (20µg/ml), safranine (S2255, SIGMA) (100µg/ml), penicillin, streptomycin, and i-erythritol sensitivity, lysis with Tibilisi (TbØ 10^4 RTD) and R/C phages and agglutination with monospecific A and M antisera. Media including streptomisin (A1852, Applichem)(2,5 µg/ml), penicilin (A1837, Applichem)(5IU/ml) i-erythritol(E7500, SIGMA)(1 mg/ml) were used in the identification of vaccine strains. *B.melitensis* and *B.abortus* biovar properties are listed in Table 1.

RESULTS AND DISCUSSION

Of 5203 *Brucella* spp. isolates, 2872 were identified as *B.abortus* biovar-3, 1332 as *B.melitensis* Rev.1, 748 as *B.melitensis* biovar-3, 120 as *B.abortus* biovar-1, 98 as *B. melitensis* biovar-1, and 30 as *B.abortus* S19. One of the isolates was identified as *B.melitensis* biovar-2, 1 isolate was *B.abortus* biovar-2, and 1 isolate was *B.abortus* biovar-9.

When the results were evaluated according to the animal species, out of 3008 cattle isolates, 2812 (94.5%) were identified as *B.abortus* biovar-3, 117 (4%) *B.abortus* biovar-1, 37 (1.2%) *B.melitensis* biovar-3, 28 *B.abortus* S-19 vaccine strains, 7 *B.melitensis* biovar-1, 5 Rev1, 1 *B.abortus* biovar-2, and 1 isolate was *B.abortus* biovar-9. Of 987 sheep isolates, 631 (88%) were identified as *B.melitensis* biovar-3, 46 (6.4%) *B.abortus* biovar-3, 35 (5%) *B.melitensis* biovar-1, 3 *B-abortus* biovar-1, 1 *B.abortus* S19, 1 *B.melitensis* bv-2, and 270 as *B.melitensis* Rev-1 vaccine strains.

Of 1208 goat isolates, 1057 were identified as *B.melitensis Rev-1*, 80 (53.3%) as *B.melitensis* bv-3, 56 (37.3%) *B.melitensis* biovar-1, 14 (9.3%) *B.abortus* bv-3, and one of them was S-19 vaccine strain. Biovar distribution according to animal species were illustrated in Figures 1, 2, and 3.The percentages of biovars according to the geographical regions are given in Table 2.Vaccine strain identifications were not included in the charts and in this table as vaccine induced abortions are temporary situations and they do not reflect prevailing biovars in a given country.

Biotyping is considered as a perfect way of finding and clarifying epidemiological data (Thimm, 1982). The fact that biovar distribution might be different among regions and even in the same region was emphasized and it was considered to be a good source of useful epidemiological information (Sayan and Erdenlig Gurbilek, 2014).

In this study, which aims to show the biovar distribution and the most prevalent biovars, different results were obtained for cattle, sheep, and goats. The findings illustrated in Figure 1 show that the most prevalent biovar for sheep and goats is *B.melitensis* bv-3 and it is *B.abortus* bv-*3* for cattle. *B.melitensis* bv-1 is also a common biovar for goats.

These results have similarities with previous studies carried out in Turkey (Erdenlig, *et al.*, 2009; Erdenlig, *et al.*, 2011). However, the other biovars identified very small

Table 1: *B.melitensis* and *B.abortus* biovar properties.

Biovar	CO_2	H_2S	Thionin	B.fuksin	A	M	Tb	R/C
B.melitensis bv-1	-	-	+	+	-	+	-	-
B.melitensis bv-2	-	-	+	+	+	-	-	-
B.melitensis bv-3	-	-	+	+	+	+	-	-
B.abortus bv-1	+	+	-	+	+	-	+	-
B.abortus bv-2	+	+	-	-	+	-	+	-
B.abortus bv-3	+	+	+	+	+	-	+	-
B.abortus bv-4	+	+	-	+	-	+	+	-
B.abortus bv-5	-	-	+	+	-	+	+	-
B.abortus bv-6	-	-	+	+	+	-	+	-
B.abortus bv-9	-, +	+	+	+	-	+	+	-

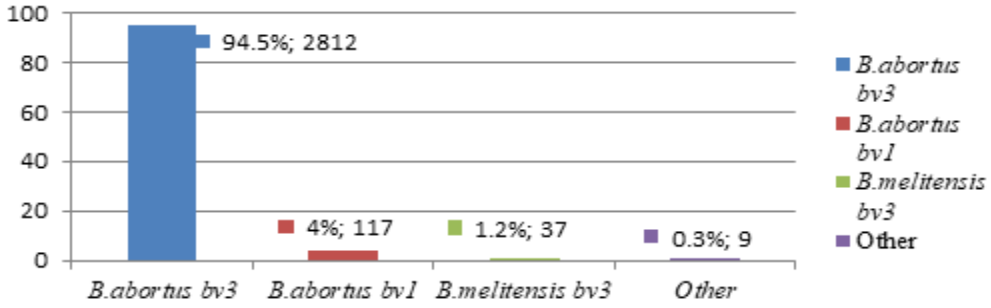

Fig 1: Biovar distribution of cattle between the years 2010 and 2015.

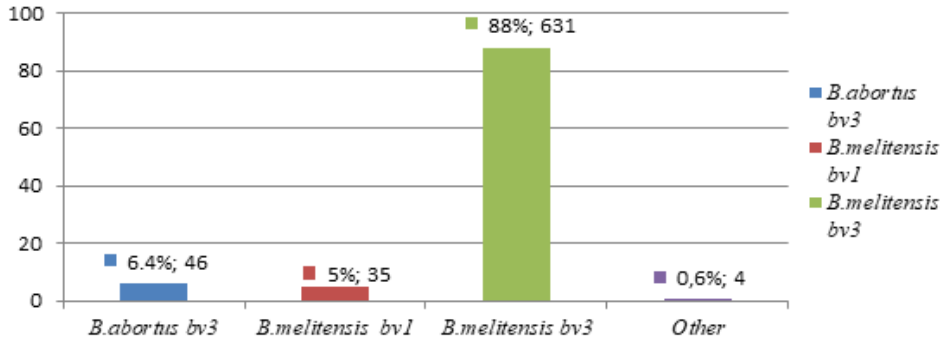

Fig 2: Biovar distribution of sheep between the years 2010 and 2015.

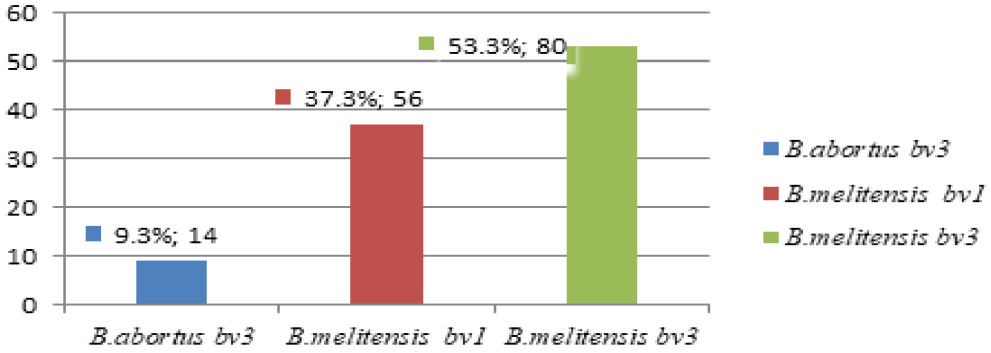

Fig 3: Biovar distribution of goats between the years 2010 and 2015.

Table 2: Biovar distribution in different regions of Turkey (%).

Biovar	Marmara	Mediterranean	Aegean	Blacksea	Southeast	Central Anatolia	Eastern Anatolia
B.abortus bv3	16.52	35.82	59.04	64.46	81.81	72.38	94.68
B.melitensis bv3	65.7	54.01	18.08	29.54	9.09	21.14	3.52
B.melitensis bv1	16.52	6.95	14.36	1.44	6.06	0.99	0.06
B.abortus bv1	0.82	3.2	8.51	4.54	3.03	5.22	1.67

in number in this study are not considered to be epidemiologically important in the emergence of the disease. Among the isolates sent from South Eastern and Eastern Turkey, *B.abortus* bv-3 is the most prevalent biovar with the percentages of 81.81% and 94.68%, respectively. In a study by Beytut *et al*., (2009), *B.abortus* bv-3 was isolated in 4 of 11 seropositive animals in Kars region.

In another study conducted in Kars region of Turkey, all the *Brucella* strains isolated from the milk samples and vaginal swabs of aborted cattle were biotyped as *B.abortus biovar-3* (Celebi and Otlu, 2011).These studies are compatible with our results that showed the high percentage of *B.abortus* bv-3 existing in Eastern Turkey including Kars.

In central Anatolia, Black Sea Region and Aegean Region, too, *B.abortus* bv-3 is the most prevalent biovar. However, *B.melitensis* bv-3 has a significant percentage in these 3 regions. Ica *et al.,* (2012), identified 17 *B.abortus* bv-3 and 12 *B.melitensis* bv-3 out of 29 isolates in their study conducted in Kayseri located in central Anatolia. They also identified all of the cattle isolates as *B.abortus* bv-3 and all the sheep isolates as *B.melitensis* bv-3. These findings of the study were in line with our results of biovars distribution regarding the livestock and region. Sahin *et al.,* (2008) investigated the bovine brucellosis in North Eastern Turkey and identified 45 *B.abortus* bv-3 and only 3 *B.abortus* bv-1 out of 48 *Brucella* isolates. These findings supported our results obtained from Black Sea Region, which is located in North Eastern Turkey.

In both Marmara and Mediterrenean Regions, *B.melitensis* bv-3 is the most prevalent biovar. *B.abortus* bv-3 and *B.melitensis* bv-1 are the second most common biovars in Marmara Region while *B.abortus* bv-3 is the second most common biovar in Mediterranean Region. In a study conducted in the Thrace Region of Turkey, of 13 aborted cattle fetus samples, 69% of them were biotyped as *B.abortus bv-3* and 31 % of them were as *B.abortus bv-1* (Erdogan*et al.,*1993). Furthermore, in the North Eastern Turkey including Marmara Region, 16 *B.melitensis* bv-3, 6 *B.melitensis* bv-1, and 1 *B.melitensis* bv-2 were biotyped from 21 small ruminant abort cases (Buyukcangaz *et al.,* 2009). These results were parallel with our findings belonging to Marmara Region.

Among the *B.abortus* biovars, biovar 1,2,3,4, and 9 were reported as the most commonly reported biovars in the world (Aparicio, 2013). Besides, the differences between *B.abortus* biovars, *B.abortus* biovar-1 was documented as the most common biovar in the world (Adesiyun *et al.,* 2011). The most frequently circulated biovar in Italy and Poland is biovar-6, in Israel it is biovar 1,2, and 6, and in Iran, Malta and Egypt, it is biovar 3 (Crawford *et al.,* 1990).

For *B.melitensis* species, however, biovar-1 and biovar-3 are the most commonly isolated biovars in the Mediterranean Region, Middle East, and Latin America (Blasco, 2010; Aparicio, 2013).

The identification of *B.abortus* S-19 and *B.melitens is* Rev-1 vaccine strains in the vaccine induced abortions is an expected result when pregnant animals are vaccinated during the mass vaccination program. Particularly, vaccine induced abortions emerged more frequently in goats in 2013 as they are more susceptible to Rev-1 vaccine than sheep (Saytekin *et al.,* 2015). Therefore, S-19 and *B.melitensis* Rev1 vaccine strains do not represent the circulated field strains and only the field strains were included in the biovar distribution charts.

Brucellosis caused by *B.melitensis* was reported to be common in domestic and wild animals, which are susceptible to the disease and bred together with sheep and goats in enzootic regions (OIE, 2009a). Different cases of the isolation of *B.melitensis* in cattle were reported from different parts of the world (Corbel, 1989). Similarly, in this study, a very small percentage of the cattle isolates were biotyped as *B.melitensis* biovars. In Northern Europe and Western Asia, where cattle, sheep and goats are herded together, cattle infection was reported to be caused by *B.melitensis* (OIE, 2012).

Similarly, a very small percentage of sheep and goat isolates were biotyped as *B.abortus* biovars. Although *B.abortus* infection is rare in sheep, *B.abortus*-induced sheep abortions were reported in different countries (Ochali, *et al.,* 2005). Even if the preferred hosts for *B.abortus* include cattle and water buffaloes, host preference can include other species as a spill-over host in enzootic areas (Nyirenda *et al*., 2016). Husbandry management, which includes different animal species being herded together, increases the risk of *Brucella* transmission among animals (Godfroid *et al.,* 2013). It was suggested that sporadic infections induced by *B.suis* and *B.abortus* were observed in sheep and goats although such cases are rare. (OIE, 2009a).

Another important finding of this study is the identification of the biovars, which have never been identified before in Turkey. They include one *B.melitensis* bv-2 from sheep isolates and one *B.abortus* bv-2 and *B.abortus* bv-9 from cattle isolates. The identification of these strains for the first time shows the significance of monitoring biovars circulated in the country.

Turkey's geographical location carries some risks due to the possibility of the spread of infectious diseases that mostly come from its neighbours in the east and northeast. It was revealed that Brucellosis was endemic in all the countries surrounding Turkey (Yumuk and O'Callaghan, 2012). In a study investigating Brucellosis in sheep and goats in Iran, most of the Brucellosis isolates were

identified as *B.melitensis* bv-1, followed by *B.melitensis* bv-2, and *B.abortus* bv-1 (Behroozikhah *et al.*, 2012).

In another study on Brucellosis incidence in the Near East, *B.melitensis* bv-3 was reported to be the most commonly isolated strain in Egypt, Jordan, Israel, and Tunusia. It was also maintained that there were reports related to the existence of *B.abortus* bv-1 in Egypt, *B.abortus* bv-*2* and bv-3 in Iran, and *B.melitensis* bv-1 in Libya, Israel, and Oman (Refai, 2002).

Behroozikhah *et al.*, (2012) stated that the identification of biovars in animal Brucellosis is an important step for its epidemiological characterization in a country and it is also the first requirement to design control-eradication programs. Focusing on research on biovar identification and search for their origin and monitoring the sharp increases of less-frequently encountered biovars are beneficial approaches for epidemiological studies and control-eradication projects.

To this end, to continue biotype identification and monitor prevailing biovars will both enable the detection of atypical and newly identified strains and support the control and eradication programs. Finally, as the findings of this study summarize the distribution of existing biovars and also form the basis for prospective epidemiological studies, it will enable other researchers to discuss the concrete results presented here.

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