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Short Communication

Tracking the diversity and Mediterranean lineage of *Brucella melitensis* isolates from different animal species in Turkey using MLVA-16 genotyping

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Abstract

Brucellosis is a zoonotic disease with a high prevalence in humans and farm animals in Turkey. However, data on the genetic diversity of *Brucella* spp. circulating in Turkey and parts of the Mediterranean region are limited. In the present study, the genetic diversity of 50 *B. melitensis* isolates from seven regions of Turkey was investigated using multi-locus variable number tandem repeats analysis (MLVA-16). The profiles were compared with 163 *B. melitensis* isolates recovered from the Mediterranean basin. *B. melitensis* strains from Turkey contain 46 different genotypes and consist of two main clusters. *B. melitensis* isolates from Turkey were closely related to isolates from Greece and some Portuguese strains. The same genotypes isolated from different sites show the spread between sites. Therefore, uncontrolled animal movements and the trade of imported animals can be important factors for the spread of brucellosis. The endemic occurrence of *B. melitensis* in the Mediterranean basin is a result of socio-historical links between Mediterranean countries. Turkish strains belong to the Eastern Mediterranean line. Eradicating brucellosis in countries of the Mediterranean basin with high prevalence is a demanding need to reduce trade barriers and, more importantly, prevent human suffering.

Keywords: *Brucella melitensis*, Mediterranean basin, Turkey, Genotyping; MLVA-16

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Introduction

Brucella infection is one of the most common zoonotic diseases, causing economic losses in farm animals and public health impact on humans. Reproductive problems e.g., retained placentas and infertility, are cardinal symptoms in cattle, sheep, pigs, and goats. Although brucellosis is eradicated in Northern European countries, it is quite common in the Mediterranean basin (Wareth et al., 2020). The disease progresses with low mortality and high morbidity. Brucellae are considered potential bioterrorist agents since the mortality rate is low, symptoms tend to become chronic, and very few microorganisms in an aerosol can cause infection (Pappas et al., 2006).

Small ruminants are the primary hosts for *B. melitensis*, but it also infects a wide variety of secondary hosts such as cattle, camelids, and wild ruminants. *B. melitensis* is the most virulent species of the genus *Brucella* and has three biovars (bv). Biovars 1 and 3 are most commonly isolated from small ruminants in the Mediterranean, Middle East, and Latin America (Lucero et al., 2008; Blasco and Molina-Flores, 2011). The most common *B. melitensis* biovar isolated from humans and animals in the Mediterranean region is biovar 3 (Wareth et al., 2019).

There are very few officials or published data accessible/available on the epidemiology of brucellosis in Turkey. Although known to be endemic in Turkey, diagnosis, and reporting of *Brucella* infections are done insufficiently (Yumuk and O'Callaghan, 2012). Brucellosis is especially prevalent in the provinces of Eastern, Southeastern, and Central Anatolia (Buzgan et al., 2010). Although there is a decrease in the number of notified human cases, animal and human brucellosis is far from control (Franco et al., 2007). The report shared by the Ministry of Health of Turkey with the OIE showed that the number of human brucellosis cases in 2019 raised to 10,244, following a decrease to 4,173 cases in 2015. According to the same data, the last death was reported in 2018 (Republic of Turkey

Ministry of Health, 2019).

In a study conducted in 2011 to determine the prevalence of brucellosis in cattle and sheep in Turkey, the herd prevalence of the disease was reported to be 7.8% in cattle and 22.5% in sheep (Yumuk and O'Callaghan, 2012). Then, in a serosurvey study on sheep and goat brucellosis in 2017, the prevalence of individuals was calculated to be 2.10%, and herd prevalence was 12.34% (Republic of Turkey Ministry of Agriculture and Forestry, 2018). In Turkey, *B. melitensis* bv 3 is the most common biovar in sheep, goats, and humans (Karagul et al., 2017). The current study aimed to compare the MLVA-16 genotype profiles of Turkish *B. melitensis* isolates with a large number of *B. melitensis* isolates recovered from humans and animals in the Mediterranean basin to trace the Mediterranean lineage of Turkish *B. melitensis* isolates.

Materials and Methods

B. melitensis strains and biotyping

Fifty *B. melitensis* isolates were selected from the strain collection available in Pendik Veterinary Control Institute from 34 different cities in seven provinces in Turkey between 2009 and 2017 to represent all *B. melitensis* biovars and all regions of Turkey. Identity and validation tests of the 50 selected isolates were carried out by classical biotyping and PCR (Akar and Erganis, 2022). Briefly, different biovars were selected: *B. melitensis* bv 3 (n=23), *B. melitensis* bv 1 (n=17), atypical *B. melitensis* strains (n=8), and *B. melitensis* bv 2 isolates (n=2). Isolates were recovered from 10 cattle, 13 goats, and 27 sheep. DNA extraction was performed using a commercial High Pure FFPET DNA Isolation Kit (Roche, Germany), and all isolates were confirmed as *B. melitensis* using AMOS-PCR (Akar and Erganis, 2022). In addition, 163 previously studied *B. melitensis* isolates recovered from humans, cattle, buffaloes, goats, and sheep from the Mediterranean basin countries, including Egypt (n=49), Portugal (n=26), Italy (n=24), Greece (n=63), and Tunisia (n=1) were included in the comparison (Wareth et al., 2020).

Genetic diversity of *B. melitensis* strains from Mediterranean lineage using MLVA-16

Genotyping analysis of the 50 Turkish *B. melitensis* isolates was done as previously described (Le Flèche et al., 2006; Al Dahouk et al., 2007; Garofolo et al., 2013). The method includes 3 Panels. Panel-1 (*Bruce06*, *Bruce08*, *Bruce11*, *Bruce12*, *Bruce42*, *Bruce43*, *Bruce45*, and *Bruce55*) is used to backtrack geographic origin, while Panel-2A (*Bruce18*, *Bruce19*, and *Bruce21*) and panel-2B (*Bruce04*, *Bruce07*, *Bruce09*, *Bruce16*, and *Bruce30*) consist of eight polymorphic makers useful for highly discriminatory epidemic research. Repeated numbers for each locus were found in the MLVA database (http://mlva.u-psud.fr). The *B. melitensis* 16 M reference strain was used as control GeneMapper software 6. Cluster analysis on MLVA-16 data was performed using BioNumerics software version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium), and arithmetic averages were generated by the unweighted pair group method (UPGMA). Genetic profiles of Turkish *B. melitensis* isolates were determined, and the profiles were compared with 163 *B. melitensis* isolates from different hosts from different countries in the Mediterranean region. All strains and detailed information are shown in Table 1.

Results and Discussion

Brucellae used in this study were isolated from 34 different cities to represent all regions of Turkey. *B. melitensis* isolates have been detected in humans and other hosts than sheep and goats, which are their natural hosts, showing cross-species transmission of *B. melitensis* and its ability to establish new reservoirs. We assumed for Turkish isolates that a one-to-one MLVA match of 93.3% correlates with high pathogenicity and the ability to be transmitted between hosts (Akar and Erganis, 2022). For these reasons, molecular monitoring is extremely important in terms of control and eradication programs. With the discovery of molecular techniques, a high level of DNA similarity between classical *Brucella* species has prevented tracking the introduction. DNA-DNA hybridization studies revealed over 90% homology between the six classical species (Verger et al., 1985; Whatmore, 2009). Hence, classifying them as "pathovars" at a single species only. Species identification is essential in eradication programs, and subtyping is very valuable as epidemiological data (Bricker, 2002; Al Dahouk et al., 2007; Bounaadja et al., 2009).

Brucellosis is a notoriously endemic bacterial zoonosis in the Middle East and North African (MENA) countries (Wareth et al., 2022), resulting in substantial economic losses and significant health impacts. In Turkey, the disease is highly endemic, affecting humans and livestock (Yentur Doni et al., 2017; Babaoglu et al., 2018; Özdem et al., 2022). The prevalence of the disease in Turkey is high and reached 7.8% in cattle and 22.5% in sheep in 2012 (Republic of Turkey Ministry of Agriculture and Forestry, 2012). The prevalence in sheep was found to be 12.34% in 2018 (Republic of Turkey Ministry of Agriculture and Forestry, 2018).

This study investigated two hundred thirteen isolates, and 154 different genotypes were determined. Interestingly, 46 genotypes for Turkey, 38 for Greece, 24 for Portugal, 24 for Italy, 21 for Egypt, and 1 for Tunisia were found. The dendrogram of *B. melitensis* strains of the Mediterranean basin is shown in Figure 1. The dendrogram showed two clusters, and the similarity rate is determined to be under 29%. Cluster-I (CI) is divided into two subclusters (A and B). Turkish isolates are classified in the CIA. All isolates from Greece, 11 isolates from Portugal, and 1 isolate from Italy are included in the same group. CIB contains all Egyptian isolates, 22 Italian isolates, and one Tunisian isolate. CII contains 15 Portuguese and one Italian isolate. In the CIA, 47 Turkish isolates formed 4 groups (thirty-six, five, three, and three).

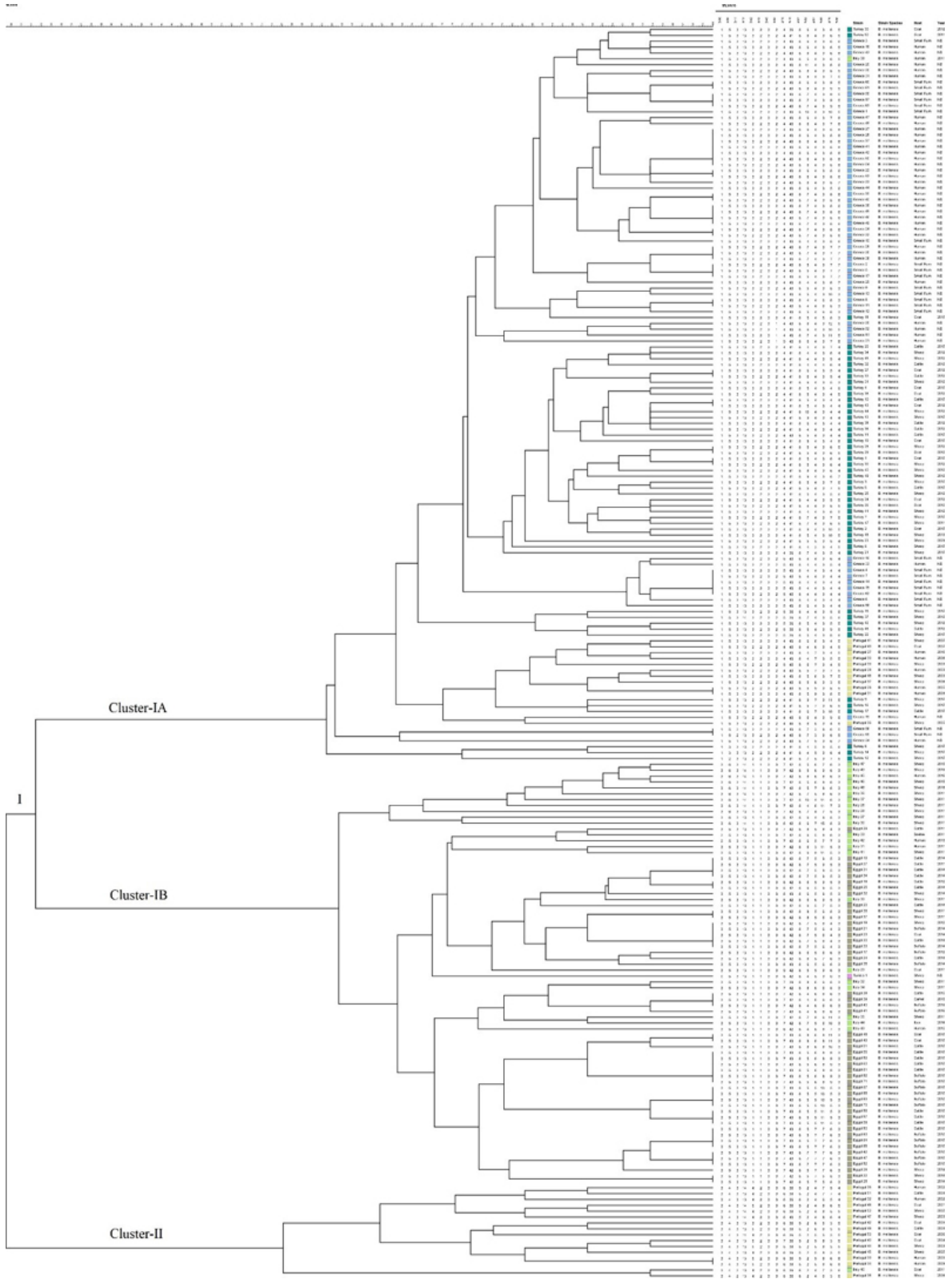

Figure 1: Dendrogram based on MLVA-16 genotyping (UPGMA method) showing the relationship between 213 *B. melitensis* isolates recovered from different animal species in the Mediterranean basin.

Table 1: Distribution of used *B. melitensis* isolates according to their countries and detailed information.

Country	*Brucella* spp	Host	Years of isolates	No. Of isolates
Turkey	*B. melitensis*	Sheep, goats, cattle	2009-2017	50
Greece	*B. melitensis*	Human, small ruminants	ND*	63
Italy	*B. melitensis*	Sheep, goats, bovine, humans, ibex	2011-2016	24
Portugal	*B. melitensis*	Sheep, goats, cattle, humans	2001-2010	26
Egypt	*B. melitensis*	Sheep, goats, cattle, buffaloes	ND*	49
Tunisia	*B. melitensis*	Sheep	2017	1
Total			2001-2017	213

*ND: not determined.

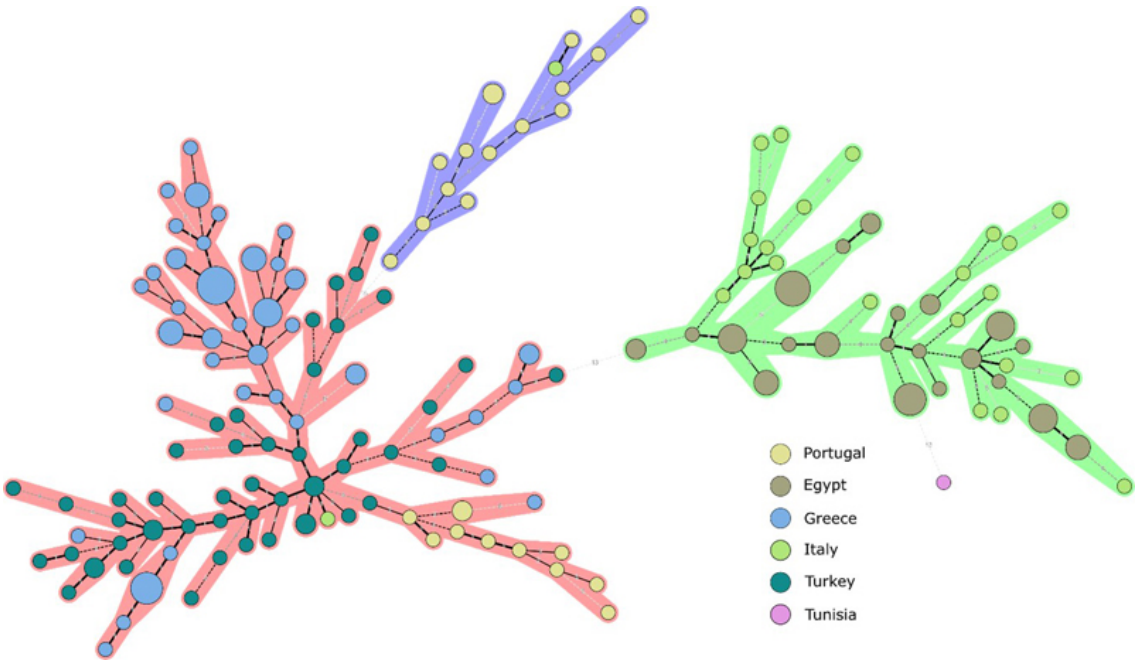

Figure 2: MLVA-16 minimum spanning tree describing the relationships of 213 *B. melitensis* isolates from countries of the Mediterranean basin. Circles represent MLVA-16 genotypes, colored according to the country of origin, and the size of the circle indicates the number of strains within that genotype.

The isolates clustered again together in separate sub-clusters. One isolate from a goat in 2017 clustered together with isolates from small ruminants from Greece. The remaining two isolates that were obtained from goats in 2011 and 2012 clustered together with isolates from humans and small ruminants from Greece and were closely related to a human Italian isolate from 2011. A strain isolated from a goat in 2012 (ID: Turkey 27) has the same genotype as a strain recovered from cattle in the same year (ID: Turkey 33). A strain isolated from a goat in 2012 (ID: Turkey 10) has the same genotype as a strain recovered from cattle in 2017 (ID: Turkey 43), representing transmission between host species. In the same context, a strain isolated from a goat in 2017 (Turkey 1) has the same genotype as a strain recovered from sheep in 2012 (ID: Turkey 39) and has a different *Bruc30* locus when compared to two isolates obtained from sheep and a goat in 2012 (Figure 1). It is worth mentioning that different *B. melitensis* isolates obtained from different host species and isolated in different years have identical genotypes, indicating the crossover of strains between hosts due to cohabitation.

The dominant profile for Turkey and Greece was genotype 43, and genotype 42 was also found in both countries. These isolates clustered in the eastern Mediterranean group of *B. melitensis*. The dominant genotypes for Italy were 51 and 49, and most of the genotypes were part of the western Mediterranean group. However, it was noted that one isolate was genotype 43 of the Eastern Mediterranean group, and one was genotype 55 of the American group. The MLVA genotype of the Tunisian isolate could not be determined. Ten Portuguese isolates were grouped in

the CIA, and the dominant genotype was 42 (Eastern Mediterranean).

Interestingly, genotype 55 was predominant in CII strains (American group). In this study of Mediterranean countries, genotypes were observed in all three described MLVA-16 groups. CI (A and B) includes the eastern Mediterranean and western Mediterranean groups, while CII includes the so-called "Americas" group. These results are similar to other MLVA studies from Europe (Al Dahouk et al., 2007; Aftab et al., 2011; Ferreira et al., 2012; Mambres et al., 2017; Vergnaud et al., 2018). Also, in the Far East and Asia, the dominant genotype belongs to the eastern Mediterranean group. The MLVA-16 minimum spanning tree of the 213 *B. melitensis* isolates from five countries of the Mediterranean basin showed again the close relationship between isolates from Turkey, Greece, Portugal, and Italy (Figure 2).

To the best of our knowledge, this study is the first comparison of *B. melitensis* isolates from Turkey with a significant number of *B. melitensis* from countries of the Mediterranean basin using MLVA-16 analysis. Tracing back the origin of zoonotic disease across borders is necessary for controlling and studying the epidemiology of transboundary disease. In spite, most Turkish isolates were grouped in separate subclusters, with a few isolates closer to Italian and Greek strains. Typing of brucellae based on whole-genome sequencing (WGS) can provide a higher resolution, and it can be supposed that brucellosis was spread by animal movement in the Mediterranean basin (Janowicz et al., 2020; Holzer et al., 2021; Ledwaba et al., 2021). Thus, it is difficult to pinpoint the source of the disease. Additional data are also needed from wildlife infections to enclose this region. These data will add another piece of knowledge to the puzzles.

Conclusion

Brucellosis is a disease that is neglected in human medicine and causes sever economic losses for the livestock industry. *B. melitensis* is the dominant species in livestock and humans in the Mediterranean region and can easily cross host species. Unrestricted movements of infected animals in Turkey may spread the infection into uninfected herds. The disease can be controlled and eradicated in the future only by strict control of animal movements and animal markets to prevent the circulation of infected animals on markets and pastures. Implementing tailored vaccine programs is a must at the beginning of such programs. As expected, the isolates from Turkey are in the eastern Mediterranean group and are closely related to those from Greece and Portugal. The close relationship to Portuguese isolates is puzzling, but maybe due to intensive trade in the past. MLVA-16 typing of *B. melitensis* isolates offers intercontinental traceability due to resolution power. The corresponding human and animal MLVA types demonstrated that brucellosis control is a One Health task; protecting animal health is fostering human health. In addition to the MLVA-16 method, the WGS method can be applied to reveal the resolution of the host-isolate relationship better.

Article Information

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Conflict of Interest. The authors have no conflict of interest to declare.

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