MLVA GENOTYPING OF HUMAN *BRUCELLA* ISOLATES FROM TURKEY.

Running Title: *BRUCELLA* MLVA GENOTYPES FROM TURKEY

Selçuk Kılıç1*, Ivan N. Ivanov2, Rıza Durmaζ1,3, Mehmet Refik Bayraktar4, Ergin Ayaşlıoğlu5, Hamidullah Uyanık6, Hikmet Alishan7, Ekrem Yaşar8, Gülçin Bayramoğlu9, Ahmet Arslanturk1,10, Gilles Vergnaud11,12, Todor V. Kantardjiev2.

1 Refik Saydam National Public Health Agency, Ankara, Turkey
2 National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria
3 Department of Microbiology and Clinical Microbiology, Inonu University, Malatya, Turkey
4 Department of Microbiology and Clinical Microbiology, Harran University, Sanliurfa, Turkey
5 Department of Infectious Diseases and Clinical Microbiology, Kirikkale University, Kirikkale, Turkey
6 Department of Microbiology and Clinical Microbiology, Ataturk University, Erzurum, Turkey
7 Department of Microbiology and Clinical Microbiology, Baskent University, Adana, Turkey
8 Microbiology Laboratory, Children’s Hospital, Diyarbakır, Turkey
9 Department of Microbiology and Clinical Microbiology, Karadeniz Technical University, Trabzon, Turkey
10 Microbiology Laboratory, State Hospital, Kutahya, Turkey
11 Université Paris Sud 11, CNRS, UMR 8621, Institut de Génétique et Microbiologie, Orsay, France
12 DGA/MRIS- Mission pour la Recherche et l’Innovation Scientifique, Bagneux, France

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**Correspondance:**

Selçuk Kılıç
Mailing address: Refik Saydam National Public Health Agency,
Department of Communicable Diseases research,
Bacterial Zoonoses Research and Reference Laboratory,
(National Brucellosis Reference Laboratory)
Cemal Gursel street 18, 06100 Sihhiye Ankara, Turkey
Phone: 00 90 312 458 21 69
Fax: 0090 312 458 24 04
E-mail: selcuk.kilic@rshm.gov.tr
ABSTRACT:

A multiple loci variable-number tandem-repeat analysis (MLVA) was applied to investigate the epidemiological relationship and genetic diversity among 162 human *Brucella* isolates collected from all geographic regions of Turkey in an 8-year period (2001-2008). The isolates were genotyped by using MLVA-16\textsubscript{UPSUD} including eight minisatellite (panel 1) and eight microsatellite (panel 2 subdivided in 2A and 2B) markers. Panels 1 and 2A distinguish 14 genotypes; two of these represented 85% of the strains. Panel 2B displayed a very high discriminatory power. Three loci from panel 2B had diversity index values higher than 0.74. MLVA-16\textsubscript{UPSUD} yielded 105 genotypes; 73 were represented by a unique isolate, 32 included two to eight isolates. The isolates from different patients within the same outbreak or from the same patient before first-line therapy and after relapse showed identical genotypes. A number of MLVA genotypes appeared to be partially restricted to some geographic areas and displayed no annual variation, possibly reflecting persistence of genotypes in certain areas for a time span of at least a decade. This study representing the first molecular typing results of human *Brucella* isolates from Turkey indicated that Turkish human *B. melitensis* isolates were most closely related to the neighboring countries’ isolates included in the “East Mediterranean” group.

Key words: Brucellosis, Genotyping, MLVA-16\textsubscript{UPSUD}, *Brucella melitensis*, Turkey

INTRODUCTION

Brucellosis is the most common anthropozoonosis with more than 500,000 cases annually. While the disease was eradicated in the vast majority of industrialized regions around the world, it remains a significant public health concern
mainly in the Mediterranean littoral, the Middle East, Arabian Peninsula, the Indian subcontinent, Asia, Africa, and Central and South America (8, 20).

Turkey is a relatively large country in the eastern Mediterranean region with a geographical surface of 783,562 km², and comprises seven regions. It has a population of 72 million, 70% of which live in cities and 30% in rural areas. Brucellosis is endemic and approximately 10,000 human brucellosis cases are reported annually. The reported incidence is 150 cases per 1 million inhabitants (25). Its prevalence varies widely from region to region due to several factors including food habits, milk processing methods, husbandry practices, nomadism, social customs, climatic conditions, socio-economic status, and environmental conditions. A steady increase of reported human cases was observed from 1986 (3.03/100,000 population) until 2004 (25.65/100,000). Livestock vaccination, elimination of infected animals, control of animal movements, and education induced a decline of the number of annually reported human cases from 18563 cases in 2004 to 9818 cases in 2008) (19).

Rapid and accurate typing procedures are crucial for epidemiologic surveillance, investigation of outbreaks, and follow-up of a control program. Many molecular typing methods commonly used for the subtyping of isolates of other bacterial species are not appropriate for routine typing of Brucella strains and none has proven to be fully satisfactory for epidemiological trace-back investigations of brucellosis (1, 11, 26). Recently, a selection of 16 variable-number tandem-repeats has been proposed for fingerprinting Brucella isolates (7, 16, 26). This MLVA-16 UPSUD genotyping system comprised eight minisatellite markers (panel1: bruce06, Bruce08, Bruce11, Bruce12, Bruce42, Bruce43, Bruce45 and Bruce55) for species identification and eight complementary microsatellite markers (panel 2A: bruce18,
bruce19 and bruce21 and 2B: Bruce04, bruce07, bruce09, bruce16 and bruce30) for further subspecies differentiation. The MLVA-16\textsubscript{UPSUD} assay has been shown to be an appropriate method for species identification in the Brucella genus, for discriminating isolates originating from restricted geographic sources at the subspecies level, and for trace-back analyses (1, 16). This method has been reported to be highly discriminatory to distinguish strains within a local outbreak and to some extend phylogenetically relevant (1, 12, 13, 16, 17, 24) and typing data from several hundred isolates can be queried and accessed via internet (http://mlva.u-
upsud.fr). The genetic diversity of Brucella strains isolated from human and animal infection has not yet been investigated in Turkey. In the present study, the MLVA-
16\textsubscript{UPSUD} assay was applied to investigate epidemiological relationship among human brucellosis isolates collected from all regions of Turkey and to determine the most common genotypes among Brucella strains in Turkey.

**Materials and methods**

**Brucella strains**

A total of 162 presumptive Brucella isolates from 159 patients (isolates BRU-S001 to BRU-S162) submitted to the Refik Saydam National Public Health Agency for a precise identification at species and biovar level were enrolled in this study. Two patients experienced relapse episodes, and two isolates were obtained from each of these patients. One hundred sixty of these isolates were collected over an 8-years period (from 2001 to 2008) at various tertiary-care centers in Turkey. One isolate of cerebra-spinal fluid in 1998 (BRU-S131) and one from blood culture of a preterm baby with congenital brucellosis in 2009 (BRU-S130) were also included in the study. The number of Brucella isolates analyzed from each region (64 isolates from Eastern Anatolia, 26 from South-Eastern Anatolia, 24 from Central Anatolia, 17
from Mediterranean, 14 from Black Sea, 11 from Aegean, 6 from Marmara) was roughly in proportion to brucellosis incidence. Brucella strains isolated in early years were recovered from freeze-dried stocks whereas more recently isolated strains were stored at -80° C in 10% skim-milk.

The isolates were identified at genus level by conventional microbiological methods and biotyped as previously described based on requirement of CO₂ for growth, urease activity, H₂S production, sensitivity to the fuchsin and thionin dyes (20 and 40 µg/ml), lysis by Tbilisi phage and agglutination with monospecific antiserum for A and M antigens (2).

**MLVA-16**<sub>UPSUD</sub> **genotyping**

**DNA sample preparation:** Brucella DNA samples were prepared by a simple thermolysate procedure. A loop of bacterial colony was suspended into 200 µl TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). Turbidity was adjusted to approximately 0.5 McFarland. Bacterial suspensions were heated at 100°C for 10 min and then centrifuged at 13,000xg for 10 min to obtain a clear nucleic acid containing supernatant. Two microliters of the supernatant were used as template in the polymerase chain reaction (PCR) assays.

**PCR amplification:** The PCR was performed as previously described (1, 16) with a slight modification. In brief, Panel 1 primers were combined and run into four duplex PCR (dPCR). The primer multiplexing was arranged in a manner that avoids overlapping of the resulting PCR fragments according to published allele size ranges (16) and ensures unambiguous interpretation. Primer concentrations were adjusted as follows: dPCR1-0.2 µM Bruce06/0.45 µM Bruce12; dPCR4-0.35 µM Bruce45/0.4µM Bruce55; dPCR2-Bruce08/Bruce42; and dPCR3-Bruce11/Bruce43 primers were at 0.4 µM each. PCR amplification was performed in a total volume of
25 µl containing 1x Gold buffer, 0.25 mM dNTP mix, 2 mM MgCl₂, 0.16 mg/ml bovine serum albumine, 2.5% dimethyl sulphoxide, 1U AmpliTaq Gold (Applied Biosystems, USA), appropriate concentrations of each flanking primer and 2 µl of thermolysate solution. The amplification was run in a QB-96 cycler (Quanta Biotech ltd., UK). Initial denaturation step (96°C for 5 min) was followed by 30 cycles denaturation at 96°C for 30 s, primer annealing at 60°C for 30 s, and extension at 70°C for 30 s, with a final extension step at 70°C for 5 min. Electrophoretic separation was performed by applying the M500 method of the QIAxcel capillary electrophoresis system coupled with a high resolution cartridge (Qiagen, Germany).

Three *B. melitensis* reference strains, (biovar (bv) 1: 16M, ATCC 23456; bv 2: 63/9, ATCC 23457; bv 3: Ether, ATCC 23458) and *B. abortus* biovar 1 reference strain (544; ATCC 23448) as well as *B. melitensis* Rev-1 vaccine strain (BRU-S163) were included as control strains.

**Data analysis:**

Analysis of electrophoresis patterns was carried out with the BioCalculator software v.3.0.05 (Qiagen, Germany) and fragment sizes converted to repeat unit numbers were imported into BioNumerics (Applied Maths, Belgium) as a character dataset. The cluster analysis was performed using the UPGMA (unweighted pair group method with arithmetic mean) algorithm and the categorical (or Hamming's) distance (20). Genetic diversity (Hunter-Gaston diversity index (HGDI)) and confidence intervals were calculated using on-line tools at [www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl](http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl). Values of the HGDI index can range from 0 (no polymorphism) to 1 (all samples are different). The MLVA-16UPSUD genotypes of *B. melitensis* isolates were compared to the corresponding data obtained for the reference strains and field isolates investigated previously (18). Chi-square analysis
RESULTS

**Patient characteristics:** The mean age of the 159 patients was 34.2 years (range 0 and 85 years), and the ratio of males (n=86) to females (n=73) was 1.17. Female patients (mean age, 38.6 years; range 2 and 79 years) were slightly older than male patients (mean age, 30.6 years; range 0 and 85 years). Most patients (n = 147; 92.5%) presented with acute brucellosis (<2 months of illness), five (3.1%) had subacute, four (2.5%) had chronic, and two (1.3%) had relapse brucellosis. One (0.6%) case had acute and subacute phases of a single illness episode.

**Brucella isolates:** One hundred sixty-two *Brucella* isolates were identified as *B. melitensis* bv 3 (161 isolates) and *B. abortus* bv 3 (one isolate). The seasonal distribution of isolates in the present collection was in agreement with the global epidemiology of *Brucella* in Turkey, i.e. an increased incidence from late spring (May) to mid summer (peak in June or July) with a gradual decrease in autumn and winter. These characteristics confirm the representativeness of the present strain collection.

**MLVA-16 UPSUD genotyping results:** PCR amplification products were obtained for all 162 isolates. Panels 1 and 2A showed limited diversity whereas panel 2B displayed a very high discriminatory power. The HGDIs in panel 1 was highest (0.329) at Bruce42. Loci Bruce06, Bruce11 and Bruce45 show only one allele (HGDI=0). The HGDI values ranged from 0.0 to 0.182 in panel 2A, and from 0.165 to 0.788 in panel 2B. In panel 2B, Bruce04, Bruce16, and Bruce30 had the highest variability (0.778, 0.778 and 0.740 respectively, see Table 1).

Clustering analysis with previously published typing data from more than 500 isolates (18) is shown in supplementary Figure 1 and confirms the species
identification deduced from biotyping. Panel 1 loci gave 10 different genotypes among 161 *B. melitensis* strains. Three of those were new genotypes (numbered 83, 84 and 85) observed in a single strain. The seven others (42, 43, 44, 57, 61, 62, and 63) were previously observed. In particular, panel 1 genotypes 42 (27 strains) and 43 (109 strains) are the most common genotypes (see supplementary Table 1).

MLVA-11<sub>UPSUD</sub> (composed of the 8 panel 1 and 3 panel 2A loci) discriminated 14 genotypes, 6 of which (numbered 100, 102, 109, 113, 119, and 121) were not previously observed. Eighty-five percent of the isolates belong to MLVA-11 genotype 116 (27 isolates) or 125 (109 isolates). Genotype of *B. abortus* strain (BRU-S093) was not identical to the previously described *B. abortus* genotypes (see supplementary Fig. S1). Distribution of MLVA-11<sub>UPSUD</sub> genotypes showed variation in different geographical regions. MLVA11 genotype 125 is observed all over the country, whereas genotype 116 was isolated mainly in Central Anatolia region. The isolates with the genotype 103 and 104 were primarily observed in Black Sea region and the genotype 104 was essentially found in patients from the Aegean region (Figure 1).

The distribution of the main genotypes was not associated with a specific period of time. Genotypes 116 and 125 were isolated during respectively six and eight (*i.e.* throughout the study period) years. The relative frequency of the two most frequent genotypes was identical in male and female patients (54.1% vs. 45.9%, chi-square= 0.002 and *P* = 0.96). There was also no significant difference between the spectrum of genotypes isolated from children and those isolated from adults.

The isolates in the largest MLVA-11<sub>UPSUD</sub> genotype (genotype 125) was isolated in all age groups. However, the isolates in the second largest genotype (genotype 116) were not isolated from adults between the ages of 20–30 (weak significance,
chi-square=4.14, P = 0.24). In the Minimum Spanning Tree clustering using previously published *B. melitensis* typing data, all strains analyzed in the current study clustered within the Eastern Mediterranean group (Fig. 2).

**Cluster analysis for Turkish *B. melitensis* genotypes.** MLVA-16 yielded a total of 105 genotypes; 73 of which were represented by a unique strain. The remaining 32 genotypes included the 88 clustered strains (clustering rate was 54.6%) (Fig. 3). The most frequently observed genotype comprised eight isolates obtained from five separate provinces in three geographic regions over 7 years (from 2002 to 2009). The seven strains in the second most frequent genotype were isolated from five separate provinces in two geographic regions between 2004 and 2008.

**Relevance of MLVA-16<sub>UPSUD</sub> genotyping for patient management and source identification.** The MLVA-16<sub>UPSUD</sub> genotypes of the isolates obtained from two patients (genotype 24 and genotype 27) during the acute phase and after relapse were identical. Additionally, two isolates cultured from one patient’s blood samples during the acute and subacute phases of a single illness episode, and one isolate corresponding to a laboratory-acquired infection from this patient’ isolates showed an identical MLVA-16<sub>UPSUD</sub> pattern (Fig. 3, genotype 35, BRU-S157, BRU-S158, BRU-S159).

MLVA-16<sub>UPSUD</sub> typing assay also showed very high concordance with available epidemiological data. For example, genotypes 22 (BRU-S080/081), 29 (BRU-S066, BRU-S075, BRU-S078), 43 (BRU-S094 to S097), 54 (BRU-S126 and BRU-S127), and 100 (BRU-S145 and BRU-S146), were each recovered from patients of the same family having contracted brucellosis from unpasteurized dairy products. The
genotype 71 patients (isolates BRU-S058 and BRU-S059) have a common history of occupational exposure to infected animals.

Application of MLVA panel to eleven isolates from an epidemiologically-linked *B. melitensis* outbreak observed in June 2003 in a small village in Kirikkale province from the Central Anatolia region yielded six genotypes. Genotypes 22, 23, and 25 each comprised two isolates, genotype 29 was shared by three isolates. The outbreak genotypes typically comprised family members and patients who were presumed to have contracted brucellosis from a common point source (consumption of homemade cheese). Two isolates (genotype 21, BRU-S082 and genotype 30, BRU-S083) obtained from family members who did not share the same MLVA-16\textsubscript{UPSUD} genotype may represent either persistent circulating strains causing sporadic infections or be the result of mutation events in the course of the outbreak. Genotypes 21 to 25, 29 and 30 differ only by + or − 1 repeat unit at one or two of the most variable loci, Bruce04, Bruce16 or Bruce30 (Fig. 3).

**DISCUSSION**

In the present study, a total of 162 human *Brucella* isolates collected from different parts of Turkey during an 8-year period was evaluated by bacteriological, epidemiological, and molecular typing characteristics. All isolates but one were *B. melitensis* (biovar 3). Previous studies conducted in different regions of Turkey found that human brucellosis was almost exclusively caused by *B. melitensis*, accounting for 99% of total cases and *B. melitensis* bv-3 was the biovar most frequently isolated in humans (4-6, 9, 14, 15, 23). The data obtained in Turkey is consistent with the results obtained in the Mediterranean region (1, 8, 17, 22). These results reveal that human brucellosis in Turkey seems to be related more to ovicaprine than to cattle infection, which may be partly attributed to the virulence of the
organism. In addition, brucellosis control measures such as the financial compensation of owners of slaughtered seropositive cattle may play a significant role. No such measure exists for sheep or goats.

MLVA-16_{UPSUD} yielded a total of 105 genotypes. Panel 2B markers in MLVA-16_{UPSUD} loci displayed very high discriminatory power, while panels 1 and 2A showed limited diversity. MLVA genotypes did not show significant difference among gender or different age groups. The frequency of different MLVA genotypes varied among the seven geographical regions. There was good correlation between molecular typing results and epidemiological data, epidemiologically related isolates were of identical or very closely related genotypes.

MLVA-11_{UPSUD} (combined panel 1 and 2A markers) yielded 14 genotypes whereas the added panel 2B increased the number of genotype to 105. These findings showed that the genotypic variation of Turkish isolates was mostly associated with the highly variable panel 2B loci and to a much lesser extent panel 2A (locus Bruce19) and panel 1 (loci Bruce01, 42, and 55). This may reflect micro-evolution via a step-wise mutational event of the most variable loci from a very limited number of ancestors. In agreement with previous molecular studies (1,12), in the setting of a local outbreak investigation, the highly polymorphic panel 2B might be sufficient for a rapid and low-cost result.

Although the discrimination power of MLVA-8_{UPSUD} and MLVA-11_{UPSUD} is very low for evaluation of the cross-transmission among the cases, the results of these panels provide useful information about distribution of the genotypes among countries. With regard to MLVA-8_{UPSUD} genotypes, the most common genotypes (42 and 43) found in the current study were also observed in other parts of the world (1, 12, 13, 24). In contrast, typical 'West Mediterranean family' including MLVA-8
genotypes 49 and 51 were not detected in this study. These data indicate that human Brucella isolates in Turkey form a highly homogeneous group belonging to the East Mediterranean group.

The rate of strains being in cluster suggests that a significant proportion of brucellosis in Turkey is due to multiple contaminations from a single source. The large clusters included strains from different provinces and different regions. For instance, the largest cluster included eight isolates collected over an 8-year period (from 2002 to 2009) from five separate provinces in three geographic regions. The seven strains in the second most frequent genotype were isolated from five separate provinces in two geographic regions between 2004 and 2008. These data show that ongoing transmission of human brucellosis has continued for a long period not only in a specific region but also among the regions in Turkey. Additionally, the 73 isolates showing distinct genotypes reflected that more than 45% of the brucellosis in Turkey had epidemiological unrelated sporadic characteristics.

In agreement with the previous investigations (1, 12, 13) the MLVA-16 genotyping results showed good correlation with the epidemiological data. The present findings also confirmed relapses, laboratory-acquired brucellosis, and intrafamilial brucellosis resulting from food sharing. The isolates of two patients from the acute and relapse stages showed an identical MLVA-16 genotype. In the current study, MLVA-16\textsubscript{UPSUD} typing enabled to identify the source of laboratory-acquired brucellosis in a laboratory worker who was exposed to Brucella while processing a blood culture specimen. MLVA genotype also confirmed intrafamilial brucellosis in many cases, in whom brucellosis most probably resulted from traditional food habits, including the consumption of homemade cheese and cream which are particularly common in rural areas or farm-land people residing in Southeastern, Eastern and
Central Anatolia regions of Turkey. These traditional food habits also lead to higher incidence of brucellosis in these regions compared to other parts of Turkey (3).

We detailed a small outbreak in a village, where a major part of the population was occupied with agriculture and/or livestock-farming. It was supposed that this outbreak originated from a single source with sharing improperly processed milk products among households and relatives. MLVA-16 divided the eleven cases investigated here into six genotypes (genotypes 21, 22, 23, 25, 29, 30). These six genotypes were very closely related and differ by single repeat unit differences at up to 2 of the most variable loci. One hypothesis is that independent contamination occurred from different sources contaminated by historically very closely related strains. A more precise investigation of the strains circulating in the animal reservoir in this village will be required to answer this question. For such purposes, we have planned a project to characterize the genotypes circulating in livestock. We will then be able to compare with the genotypes observed in human isolates as described in the present study.

Conversely, some isolates recovered from separate regions and with no known direct epidemiological links displayed identical MLVA-16 profiles (genotypes 7, 13, 15, 16, 24, 55, 85, 86, and 91). This observation might reflect homoplasy and convergent evolution. Alternatively, some of the associated isolates may result from either the lack of control of animal movements between regions or the circulation of improperly processed milk products or household products in the market.

In agreement with its location most prevalent MLVA genotypes found in Turkey are typical from the East Mediterranean region. Molecular typing confirmed that more than half of the human brucellosis resulted from either very close cross-transmission in a location or persistent and ongoing transmission among the
different regions. MLVA-16 proved to be highly discriminatory among related human *Brucella* isolates that could not be differentiated by conventional microbiological methods. Hence, MLVA can significantly contribute to epidemiological trace-back analysis of *Brucella* infections and may advance surveillance and control of brucellosis in Turkey. The data produced in this investigation can be queried in the Brucella MLVA database release (starting from the Brucella2010 release) at [http://mlva.u-psud.fr](http://mlva.u-psud.fr).

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Figure Legends

**Figure 1.** Geographic distribution of Panel 1+2A genotypes (genotype 69: *B. abortus* bv 3). The bar size is proportional to the number of isolates. Each color corresponds to a different MLVA11\textsubscript{UPSUD} genotype.

**Figure 2.** Minimum spanning tree analysis of published *B. melitensis* isolates using the MLVA11\textsubscript{UPSUD} data. Color codes are associated with the main *B. melitensis* MLVA clusters. The published data for *B. melitensis* strains were recovered from the compilation by Maquart et al., 2009 (18). The 161 Turkish *B. melitensis* isolates, which were representing 14 MLVA11\textsubscript{UPSUD} genotypes are associated with the green East Mediterranean *B. melitensis* strains. Turkish isolates are shown in white. The numbers represent the 14 MLVA11\textsubscript{UPSUD} genotypes found in this study. The size of the shapes indicates the number of strains described in the genotype. Each of the circle showing white and green colors included Turkish genotype as a white color and the genotypes found in Eastern Mediterranean *B. melitensis* as a green color.

**Figure 3.** Cluster analysis for 162 human isolates of *Brucella* and Rev1 vaccine strain based on the data set of MLVA16\textsubscript{UPSUD}. In the columns, the following data for isolates are indicated: genotype, strain, strain ID, MLVA8\textsubscript{UPSUD} (panel 1) and MLVA11\textsubscript{UPSUD} (1+2A) genotypes corresponding to each isolate in the database for each set of loci; isolation date (year), geographic region and the specimen source. Under panels 1-2A, and 2B are shown the individual MLVA16\textsubscript{UPSUD} loci and the number of tandem repeats units for each isolate. One hundred and five genotypes were observed. The color code reflects the MLVA11\textsubscript{UPSUD} genotype and is identical to the Figure 1 color code.
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Table 1. Number of alleles and Hunter-Gaston Diversity Index (HGDI) of 161 B. melitensis isolates from Turkey.

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<td>0.049</td>
<td>0.0019 - 0.096</td>
</tr>
<tr>
<td>Bruce45</td>
<td>1</td>
<td>3</td>
<td>0.000</td>
<td>0.000 - 0.044</td>
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<td>2</td>
<td>1,2</td>
<td>0.012</td>
<td>0.000 - 0.037</td>
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<tr>
<td>Total</td>
<td>10</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Bruce18</td>
<td>3</td>
<td>4,5,6</td>
<td>0.161</td>
<td>0.0768 - 0.222</td>
</tr>
<tr>
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<td>4</td>
<td>18,20,21,23</td>
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<td>0.115 - 0.270</td>
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<tr>
<td>Bruce21</td>
<td>1</td>
<td>8</td>
<td>0.000</td>
<td>0.000 - 0.000</td>
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<tr>
<td>Total (panel 1+2A)</td>
<td></td>
<td></td>
<td>14</td>
<td>0.522</td>
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<tr>
<td>Panel 2B</td>
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</tr>
<tr>
<td>Bruce04</td>
<td>7</td>
<td>3,4,5,6,7,8,9</td>
<td>0.778</td>
<td>0.7513 - 0.806</td>
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<tr>
<td>Bruce07</td>
<td>6</td>
<td>3,4,5,6,7,12</td>
<td>0.465</td>
<td>0.369 - 0.544</td>
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<tr>
<td>Bruce09</td>
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<td>3,4,5,6,7,8,9,14</td>
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<td>0.097 - 0.255</td>
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<tr>
<td>Bruce16</td>
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<td>3,4,5,6,7,8,9,11</td>
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<td>0.743 - 0.814</td>
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<tr>
<td>Bruce30</td>
<td>6</td>
<td>3,4,5,6,7,8</td>
<td>0.740</td>
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<td>MLVA-16</td>
<td>105</td>
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<td>0.991</td>
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</tr>
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\(^a\)HGDI: Hunter-Gaston diversity index.
\(^b\)CI: confidence interval.