

Multiple-Locus Variable-Number Tandem-Repeat Analysis Genotyping of Human *Brucella* Isolates from Turkey^{▽†}

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A multiple-locus 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 to 2008). The isolates were genotyped by using an MLVA assay developed in Orsay, France (MLVA-16_{Orsay}) including eight minisatellite (panel 1) and eight microsatellite (panel 2, subdivided into 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_{Orsay} yielded 105 genotypes; 73 were represented by a unique isolate, and 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 *Brucella melitensis* isolates were most closely related to the neighboring countries' isolates included in the East Mediterranean group.

Brucellosis is the most common anthroponozoonosis, 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, the Arabian Peninsula, the Indian subcontinent, Asia, Africa, and Central and South America (19, 26).

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 lives 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 (24). 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, socioeconomic 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 in the number of annually reported human cases, from 18,563 cases in 2004 to 9,818 cases in 2008 (17).

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, 9, 25). Recently, a selection of 16 variable-number tandem repeats has been proposed for fingerprinting *Brucella* isolates (7, 14, 25). This multiple-locus variable-number tandem-repeat analysis (MLVA) genotyping system, MLVA-16_{Orsay}, comprised eight minisatellite markers (panel 1, Bruce06, Bruce08, Bruce11, Bruce12, Bruce42, Bruce43, Bruce45, and Bruce55) for species identification and eight complementary microsatellite markers (panel 2A, Bruce18, Bruce19, and Bruce21; panel

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2B, Bruce04, Bruce07, Bruce09, Bruce16, and Bruce30) for further subspecies differentiation. The MLVA-16_{Orsay} 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, 14). This method has been reported to be highly discriminatory to distinguish strains within a local outbreak, and to some extent phylogenetically relevant (1, 11, 14, 16, 18, 23) and typing data from several hundred isolates can be queried and accessed via the Internet (<http://mlva.u-psud.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_{Orsay} assay was applied to investigate epidemiological relationships among human brucellosis isolates collected from all regions of Turkey and to determine the most common genotypes among *Brucella* strains in Turkey.

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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 the species and biovar levels were enrolled in this study. Two patients experienced relapse episodes, and two isolates were obtained from each of these patients. A total of 160 of these isolates were collected over an 8-year period (from 2001 to 2008) at various tertiary care centers in Turkey. One isolate of cerebrospinal fluid in 1998 (BRU-S131) and one from the 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 the Mediterranean, 14 from the Black Sea, 11 from the 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 the genus level by conventional microbiological methods and biotyped as previously described based on requirement of CO_2 for growth, urease activity, H_2S production, sensitivity to the fuchsin and thionin dyes (20 and 40 $\mu\text{g/ml}$), lysis by Tbilisi phage, and agglutination with monospecific antiserum for A and M antigens (2).

MLVA-16_{Orsay} genotyping. (i) **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 a McFarland standard of approximately 0.5. Bacterial suspensions were heated at 100°C for 10 min and then centrifuged at $13,000 \times g$ for 10 min to obtain a clear nucleic acid-containing supernatant. Two microliters of the supernatant was used as the template in the PCR assays.

(ii) **PCR amplification.** The PCR was performed as previously described (1, 14) 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 (14) and ensures unambiguous interpretation. Primer concentrations were adjusted as follows: dPCR1, 0.2 μM Bruce06, 0.45 μM Bruce12; dPCR4, 0.35 μM Bruce45, 0.4 μM Bruce55; dPCR2, 0.4 μM Bruce08, 0.4 μM Bruce42; and dPCR3, 0.4 μM Bruce11, 0.4 μM Bruce43. PCR amplification was performed in a total volume of 25 μl containing $1 \times$ Gold buffer, 0.25 mM deoxynucleoside triphosphate (dNTP) mix, 2 mM MgCl_2 , 0.16 mg/ml bovine serum albumin, 2.5% dimethyl sulfoxide, 1 U AmpliTaq Gold (Applied Biosystems), appropriate concentrations of each flanking primer, and 2 μl of thermolysate solution. The amplification was run in a QB-96 cyler (Quanta Biotech Ltd., United Kingdom). The initial denaturation step (96°C for 5 min) was followed by 30 cycles of 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, 16 M, ATCC 23456; bv. 2, 63/9, ATCC 23457; bv. 3, ether, ATCC 23458) and *Brucella abortus* bv. 1 reference strain (544; ATCC 23448) as well as *Brucella 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 version 3.0.05 (Qiagen, Germany), and fragment sizes converted to repeat unit numbers were imported into BioNumerics (Applied Maths, Belgium) as a character data set. The cluster analysis was performed using the UPGMA (unweighted pair group method with arithmetic mean) algorithm and the categorical (or Hamming's) distance. Genetic diversity (Hunter-Gaston diversity index [HGDI]) and confidence intervals were calculated using online tools at www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl. Values of the HGDI can range from 0 (no polymorphism) to 1 (all samples are different). The MLVA-16_{Orsay} genotypes of *B. melitensis* isolates were compared to the corresponding data obtained for the reference strains and field isolates investigated previously (15). Chi-square analysis was used to correlate patient characteristics with genotype.

RESULTS

Patient characteristics. The mean age of the 159 patients was 34.2 years (range, 0 to 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 to 79 years) were slightly older than male patients (mean age, 30.6 years; range, 0 to 85 years). Most patients ($n = 147$; 92.5%) presented with acute brucellosis (<2 months of illness), and 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. A total of 162 *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 midsummer (peak in June or July), with a gradual decrease in autumn and winter. These characteristics confirm the representativity of the present strain collection.

MLVA-16_{Orsay} 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 HGDI in panel 1 was highest (0.329) at Bruce42. Loci Bruce06, Bruce11, and Bruce45 showed 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 (15) is shown in Fig. S1 in the supplemental material 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 (numbers 83, 84, and 85) observed in a single strain. The seven others (numbers 42, 43, 44, 57, 61, 62, 63) were previously observed. In particular, panel 1 genotypes 42 (27 strains) and 43 (109 strains) are the most common genotypes (see Table S1 in the supplemental material).

MLVA-11_{Orsay} (composed of the eight panel 1 and three panel 2A loci) discriminated 14 genotypes, 6 of which (numbers 100, 102, 109, 113, 119, and 121) were not previously observed. Eighty-five percent of the isolates belong to

TABLE 1. Numbers of alleles and HGDI values of 161 *B. melitensis* isolates from Turkey

Locus	No. of alleles	Tandem repeat copy no.	HGDI ^a	Confidence interval
Panel 1				
Bruce06	1	1	0.000	0.000–0.036
Bruce08	3	3, 4, 5	0.049	0.018–0.096
Bruce11	1	3	0.000	0.000–0.000
Bruce12	3	12, 13, 14	0.084	0.026–0.142
Bruce42	3	1, 2, 3	0.329	0.252–0.406
Bruce43	3	2, 3, 4	0.049	0.0019–0.096
Bruce45	1	3	0.000	0.000–0.044
Bruce55	2	1, 2	0.012	0.000–0.037
Total	10		0.437	
Panel 2A				
Bruce18	3	4, 5, 6	0.161	0.0768–0.222
Bruce19	4	18, 20, 21, 23	0.182	0.115–0.270
Bruce21	1	8	0.000	0.000–0.000
Total (panels 1 and 2A)	14		0.522	
Panel 2B				
Bruce04	7	3, 4, 5, 6, 7, 8, 9	0.778	0.7513–0.806
Bruce07	6	3, 4, 5, 6, 7, 12	0.465	0.369–0.544
Bruce09	8	3, 4, 5, 6, 7, 8, 9, 14	0.165	0.097–0.255
Bruce16	8	3, 4, 5, 6, 7, 8, 9, 11	0.778	0.743–0.814
Bruce30	6	3, 4, 5, 6, 7, 8	0.740	0.702–0.777
MLVA-16 _{Orsay}	105		0.991	

^a HGDI, Hunter-Gaston diversity index.

MLVA-11 genotypes 116 (27 isolates) or 125 (109 isolates). The genotype of *B. abortus* strain BRU-S093 was not identical to the previously described *B. abortus* genotypes (see Fig. S1 in the supplemental material). Distribution of MLVA-11_{Orsay} genotypes showed variation in different geographical regions. MLVA-11 genotype 125 is observed all over the country, whereas genotype 116 was isolated mainly in the central Anatolia region. The isolates with the genotypes 103 and 104 were primarily observed in the Black Sea region, and the genotype 104 was essentially found in patients from the Aegean region (Fig. 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 frequencies of the two most frequent genotypes were essentially identical in male and female patients (54.1% versus 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_{Orsay} genotype group (genotype 125) were isolated in all age groups. However, the isolates in the second largest genotype group (genotype 116) were not isolated from adults between the ages of 20 and 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 clustered within the Eastern Mediterranean group (Fig. 2).

Cluster analysis for Turkish *B. melitensis* genotypes. MLVA-16_{Orsay} 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

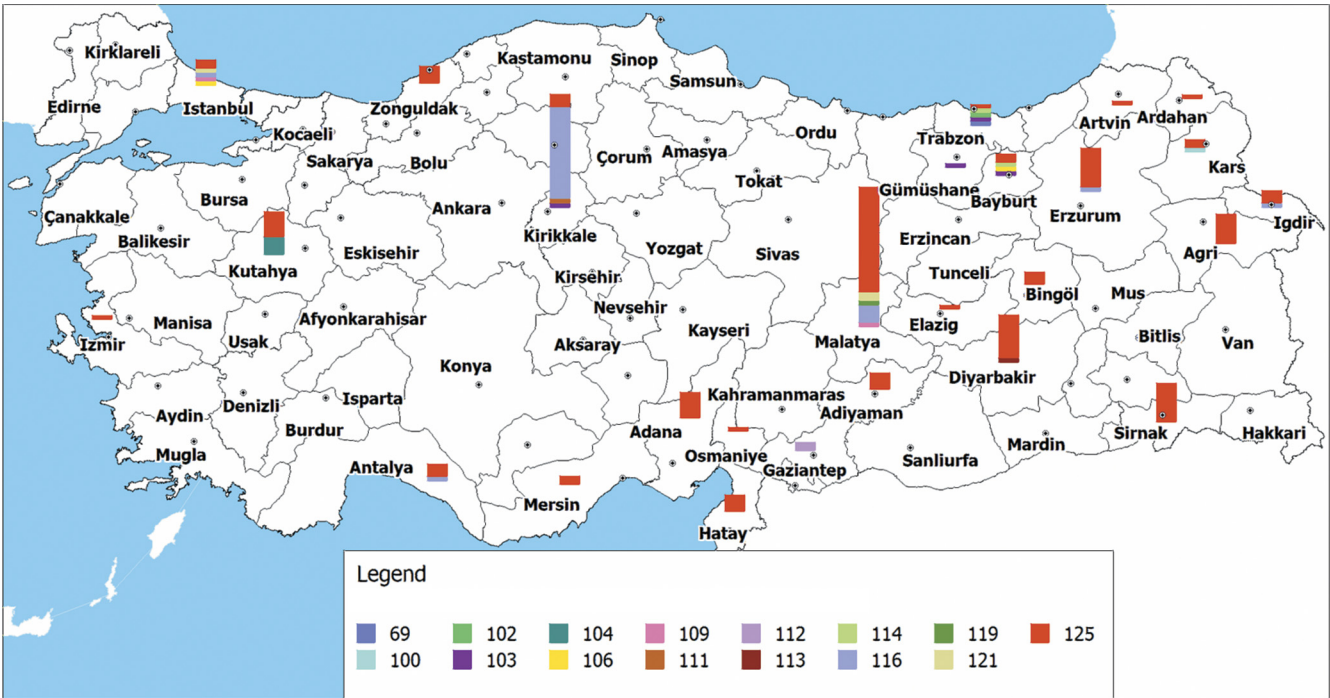


FIG. 1. Geographic distribution of panel 1 and 2A genotypes (genotype 69, *B. abortus* bv. 3). The bar size is proportional to the number of isolates. Each color corresponds to a different MLVA-11_{Orsay} genotype. (Adapted from a map available at www.basarssoft.com.tr with permission of Basarssoft Ltd.)

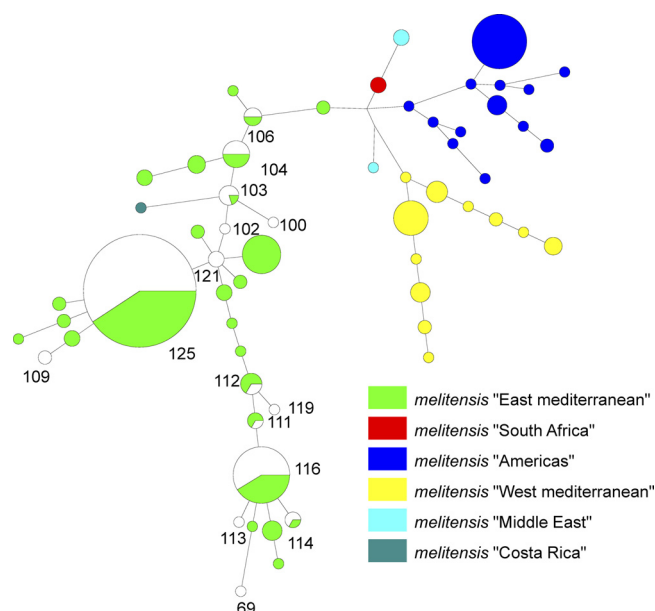


FIG. 2. Minimum spanning tree analysis of published *B. melitensis* isolates using the MLVA-11_{Orsay} data. Color codes are associated with the main *B. melitensis* MLVA clusters. The published data for *B. melitensis* strains were recovered from the compilation by Maquart et al. (15). The 161 Turkish *B. melitensis* isolates, which were representing 14 MLVA-11_{Orsay} genotypes, are associated with the green East Mediterranean *B. melitensis* strains. Turkish isolates are shown in white. The numbers represent the 14 MLVA-11_{Orsay} genotypes found in this study. The size of the shapes indicates the number of strains described in the genotype. Each of the circles showing white and green colors included the Turkish genotype (white) and the genotypes found in Eastern Mediterranean *B. melitensis* isolates (green).

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_{Orsay} genotyping for patient management and source identification. The MLVA-16_{Orsay} 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's isolates showed identical MLVA-16_{Orsay} patterns (Fig. 3, genotype 35, BRU-S157, BRU-S158, BRU-S159).

The MLVA-16_{Orsay} typing assay also showed very high concordance with available epidemiological data. For example, genotypes 22 (BRU-S080 and BRU-S081), 29 (BRU-S066, BRU-S075, BRU-S078), 43 (BRU-S094 to BRU-S097), 54 (BRU-S126 and BRU-S127), and 100 (BRU-S145 and BRU-S146) were each recovered from patients of the same family that 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 the MLVA panel to 11 isolates from an epidemiologically linked *B. melitensis* outbreak observed in June 2003 in a small village in the Kirikkale province from the central Anatolia region yielded six genotypes. Genotypes 22, 23, and 25 each comprised two isolates, and 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; genotype 30, BRU-S083) obtained from family members who did not share the same MLVA-16_{Orsay} genotype may either represent 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 ± 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* (bv. 3). Previous studies conducted in different regions of Turkey found that human brucellosis was almost exclusively caused by *B. melitensis*, accounting for 99% of the total cases, and *B. melitensis* bv. 3 was the biovar most frequently isolated in humans (4–6, 8, 12, 13, 22). The data obtained in Turkey are consistent with the results obtained in the Mediterranean region (1, 16, 21, 26). 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_{Orsay} yielded a total of 105 genotypes. Panel 2B markers in MLVA-16_{Orsay} loci displayed very high discriminatory power, while panels 1 and 2A showed limited diversity. MLVA genotypes did not show significant differences 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, and epidemiologically related isolates were of identical or very closely related genotypes.

MLVA-11_{Orsay} (combined panel 1 and 2A markers) yielded 14 genotypes, whereas the added panel 2B increased the number of genotypes 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) loci. This may reflect microevolution via a stepwise mutational event of the most variable loci from a very limited number of ancestors. In agreement with previous molecular studies (1, 11), 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_{Orsay} and MLVA-11_{Orsay} is very low for evaluation of the cross-transmis-

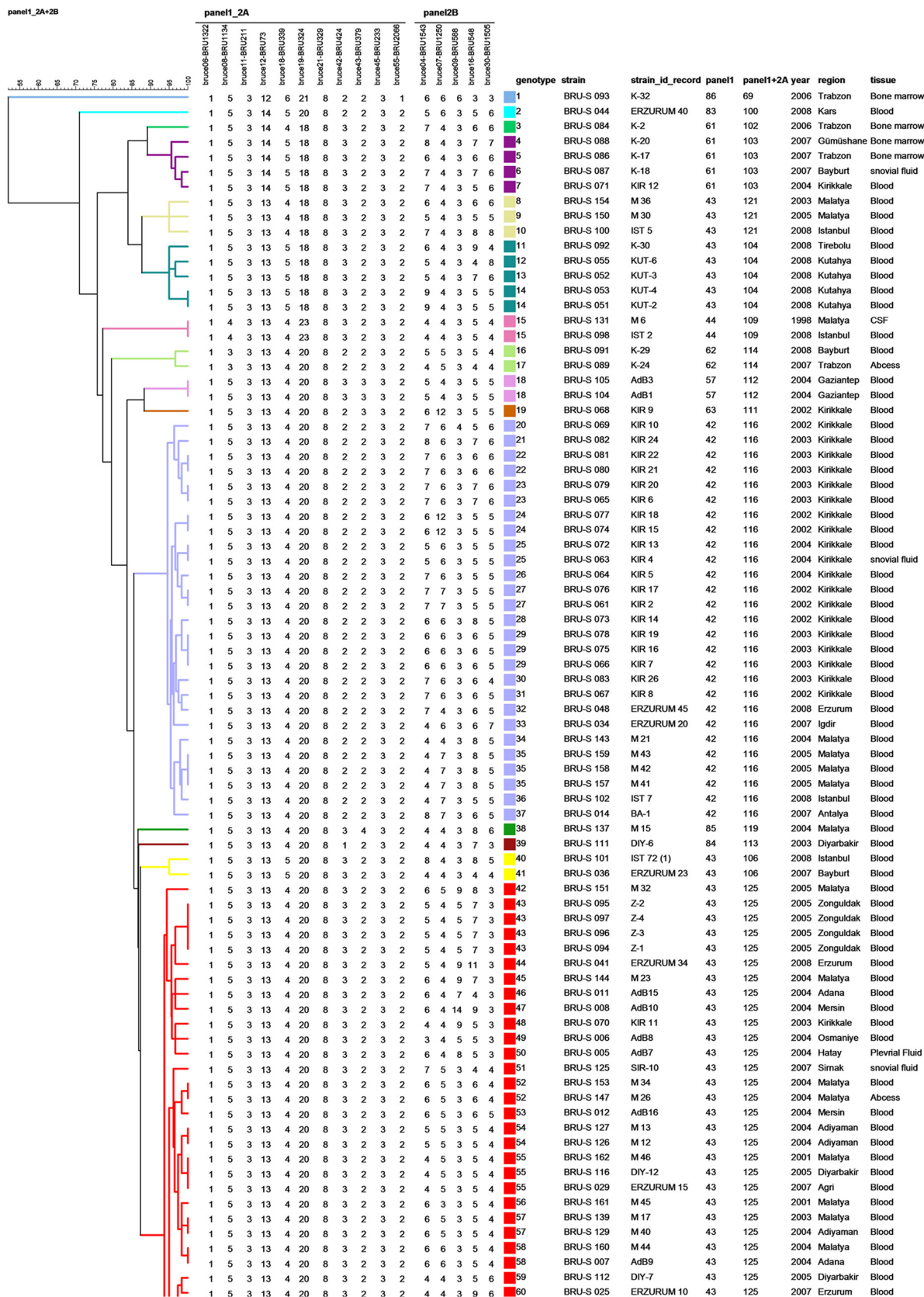


FIG. 3. Cluster analysis for 162 human isolates of *Brucella* and Rev1 vaccine strain based on the data set of MLVA-16_{Orsay}. In the columns, the following data are indicated: genotype, strain, strain ID, MLVA-8_{Orsay} (panel 1), and MLVA-11_{Orsay} (panels 1 and 2A) genotypes corresponding to each 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 MLVA-16_{Orsay} loci and the numbers of tandem-repeat units for each isolate. A total of 105 genotypes were observed. The color code reflects the MLVA-11_{Orsay} genotype and is identical to the Fig. 1 color code.

1	5	3	13	4	20	8	3	2	3	2	6	6	3	5	4	58	BRU-S 007	AdB9	43	125	2004	Adana	Blood
1	5	3	13	4	20	8	3	2	3	2	4	4	3	5	6	59	BRU-S 112	DIY-7	43	125	2005	Diyarbakir	Blood
1	5	3	13	4	20	8	3	2	3	2	4	4	3	9	6	60	BRU-S 025	ERZURUM 10	43	125	2007	Erzurum	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	8	6	61	BRU-S 049	EGE-1	43	125	2003	Izmir	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	5	6	62	BRU-S 136	M 11	43	125	2001	Malatya	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	5	6	62	BRU-S 109	DIY-4	43	125	2005	Diyarbakir	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	5	6	62	BRU-S 106	DIY-1	43	125	2006	Diyarbakir	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	5	6	62	BRU-S 024	ERZURUM 9	43	125	2005	Erzurum	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	4	6	63	BRU-S 023	ERZURUM 7	43	125	2005	Erzurum	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	9	6	64	BRU-S 021	ERZURUM 5	43	125	2007	Erzurum	Blood
1	5	3	13	4	20	8	3	2	3	2	8	4	3	7	7	65	BRU-S 110	DIY-5	43	125	2003	Diyarbakir	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	8	7	66	BRU-S 108	DIY-3	43	125	2003	Diyarbakir	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	4	7	67	BRU-S 047	ERZURUM 44	43	125	2008	Igdir	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	5	7	68	BRU-S 090	K-27	43	125	2007	Trabzon	Blood
1	5	3	13	4	20	8	3	2	3	2	8	4	3	5	7	69	BRU-S 031	ERZURUM 17	43	125	2007	Bayburt	Blood
1	5	3	13	4	20	8	3	2	3	2	8	4	3	6	6	70	BRU-S 056	KUT-7	43	125	2008	Kutahya	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	6	7	71	BRU-S 085	K-7	43	125	2006	Artvin	Bone marrow
1	5	3	13	4	20	8	3	2	3	2	5	4	3	6	7	71	BRU-S 059	KUT-10	43	125	2008	Kutahya	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	6	7	71	BRU-S 058	KUT-9	43	125	2008	Kutahya	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	6	4	72	BRU-S 099	IST 4	43	125	2008	Istanbul	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	6	4	72	BRU-S 037	ERZURUM 24	43	125	2007	Erzurum	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	6	4	72	BRU-S 035	ERZURUM 22	43	125	2007	Agri	Blood
1	5	3	13	4	20	8	3	2	3	2	6	4	3	6	4	73	BRU-S 123	SIR-8	43	125	2007	Simak	Blood
1	5	3	13	4	20	8	3	2	3	2	6	4	3	6	7	74	BRU-S 103	IST 8	43	125	2008	Istanbul	Blood
1	5	3	13	4	20	8	3	2	3	2	6	4	3	6	8	75	BRU-S 018	ERZURUM 2	43	125	2004	Erzurum	Blood
1	5	3	13	4	20	8	3	2	3	2	5	3	3	5	4	76	BRU-S 152	M 33	43	125	2005	Malatya	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	9	4	77	BRU-S 142	M 20	43	125	2003	Malatya	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	9	4	77	BRU-S 128	M 37	43	125	2004	Adiyaman	Blood (IE)
1	5	3	13	4	20	8	3	2	3	2	6	4	3	5	4	78	BRU-S 149	M 29	43	125	2005	Malatya	Blood
1	5	3	13	4	20	8	3	2	3	2	8	4	3	5	4	79	BRU-S 057	KUT-8	43	125	2008	Kutahya	Blood
1	5	3	13	4	20	8	3	2	3	2	7	4	3	5	4	80	BRU-S 062	KIR 3	43	125	2003	Kirikkale	Blood
1	5	3	13	4	20	8	3	2	3	2	7	4	3	5	4	80	BRU-S 010	AdB14	43	125	2004	Adana	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	5	4	81	BRU-S 138	M 16	43	125	2003	Malatya	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	5	4	81	BRU-S 115	DIY-11	43	125	2005	Diyarbakir	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	5	4	81	BRU-S 114	DIY-9	43	125	2003	Diyarbakir	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	5	4	81	BRU-S 004	AdB6	43	125	2003	Hatay	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	7	4	82	BRU-S 140	M 18	43	125	2003	Malatya	Blood
1	5	3	13	4	20	8	3	2	3	2	6	4	3	7	4	83	BRU-S 060	KIR 1	43	125	2002	Kirikkale	Blood
1	5	3	13	4	20	8	3	2	3	2	8	4	3	4	4	84	BRU-S 040	ERZURUM 31	43	125	2008	Kars	Blood
1	5	3	13	4	20	8	3	2	3	2	6	4	3	4	4	85	BRU-S 122	SIR-7	43	125	2007	Simak	Blood
1	5	3	13	4	20	8	3	2	3	2	6	4	3	4	4	85	BRU-S 118	SIR-2	43	125	2007	Simak	Blood
1	5	3	13	4	20	8	3	2	3	2	6	4	3	4	4	85	BRU-S 046	ERZURUM 43	43	125	2008	Igdir	Blood
1	5	3	13	4	20	8	3	2	3	2	6	4	3	4	4	85	BRU-S 030	ERZURUM 16	43	125	2007	Bingöl	Blood
1	5	3	13	4	20	8	3	2	3	2	6	4	3	4	4	85	BRU-S 028	ERZURUM 14	43	125	2005	Agri	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	4	4	86	BRU-S 156	M 39	43	125	2004	Malatya	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	4	4	86	BRU-S 121	SIR-5	43	125	2007	Simak	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	4	4	86	BRU-S 043	ERZURUM 39	43	125	2008	Bingöl	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	4	4	86	BRU-S 042	ERZURUM 38	43	125	2008	Kars	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	4	4	86	BRU-S 038	ERZURUM 25	43	125	2007	Bingöl	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	4	4	86	BRU-S 033	ERZURUM 19	43	125	2007	Agri	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	4	4	86	BRU-S 027	ERZURUM 12	43	125	2005	Agri	Blood
1	5	3	13	4	20	8	3	2	3	2	9	4	3	4	4	87	BRU-S 020	ERZURUM 4	43	125	2005	Agri	Blood
1	5	3	13	4	20	8	3	2	3	2	4	4	3	8	4	88	BRU-S 119	SIR-3	43	125	2007	Simak	Blood
1	5	3	13	4	20	8	3	2	3	2	4	4	3	7	4	89	BRU-S 117	SIR-1	43	125	2007	Simak	Blood
1	5	3	13	4	20	8	3	2	3	2	4	4	3	7	4	89	BRU-S 017	BA-4	43	125	2008	Antalya	Blood
1	5	3	13	4	20	8	3	2	3	2	4	4	3	9	4	90	BRU-S 013	AdB17	43	125	2006	Adana	Blood
1	5	3	13	4	20	8	3	2	3	2	4	4	3	4	4	91	BRU-S 148	M 28	43	125	2004	Malatya	Blood (IE)
1	5	3	13	4	20	8	3	2	3	2	4	4	3	4	4	91	BRU-S 135	M 10	43	125	2002	Malatya	Blood
1	5	3	13	4	20	8	3	2	3	2	4	4	3	4	4	91	BRU-S 133	M 8	43	125	2003	Malatya	Blood
1	5	3	13	4	20	8	3	2	3	2	4	4	3	4	4	91	BRU-S 132	M 7	43	125	2002	Malatya	Blood
1	5	3	13	4	20	8	3	2	3	2	4	4	3	4	4	91	BRU-S 130	DIY-29	43	125	2009	Elazig	Blood
1	5	3	13	4	20	8	3	2	3	2	4	4	3	4	4	91	BRU-S 113	DIY-8	43	125	2004	Diyarbakir	Blood
1	5	3	13	4	20	8	3	2	3	2	4	4	3	4	4	91	BRU-S 022	ERZURUM 6	43	125	2007	Bayburt	Blood
1	5	3	13	4	20	8	3	2	3	2	4	4	3	4	4	91	BRU-S 002	AdB4	43	125	2003	Hatay	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	8	5	92	BRU-S 124	SIR-9	43	125	2007	Simak	Blood
1	5	3	13	4	20	8	3	2	3	2	4	4	3	4	5	93	BRU-S 120	SIR-4	43	125	2007	Simak	Blood
1	5	3	13	4	20	8	3	2	3	2	4	4	3	4	5	93	BRU-S 054	KUT-5	43	125	2008	Kutahya	Blood
1	5	3	13	4	20	8	3	2	3	2	6	4	3	4	5	94	BRU-S 045	ERZURUM 41	43	125	2008	Igdir	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	4	5	95	BRU-S 134	M 9	43	125	2002	Malatya	Blood
1	5	3	13	4	20	8	3	2	3	2	5	4	3	4	5	95	BRU-S 039	ERZURUM 28	43	125	2008	Ardahan	Blood
1	5	3	13	4	20	8	3	2	3	2	7	4	3	4	5	96	BRU-S 141	M 19	43	125	2004	Malatya	Blood
1	5	3	13	4	20	8	3	2	3	2	7	4	3	7	5	97	BRU-S 016	BA-3	43	125	2005	Antalya	Abcess
1	5	3	13	4	20	8	3	2	3	2	7	4	3	7	5								

sion among the cases, the results of these panels provide useful information about distribution of the genotypes among countries. With regard to MLVA-8_{Orsay} genotypes, the most common genotypes (42 and 43) found in the current study were also observed in other parts of the world (1, 11, 18, 23). In contrast, those of the typical West Mediterranean family, including MLVA-8_{Orsay} 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 proportion of strains being in clusters 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 epidemiologically unrelated sporadic characteristics.

In agreement with the previous investigations (1, 11, 18), the MLVA-16_{Orsay} 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 identical MLVA-16_{Orsay} genotypes. In the current study, MLVA-16_{Orsay} typing enabled us 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 farmland people residing in the southeastern, eastern, and central Anatolia regions of Turkey. These traditional food habits also lead to a higher incidence of brucellosis in these regions than in 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 from sharing improperly processed milk products among households and relatives. MLVA-16_{Orsay} divided the 11 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 one or two 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 then with the genotypes observed in human isolates as described in the present study.

Conversely, some isolates recovered from separate re-

gions and with no known direct epidemiological links displayed identical MLVA-16_{Orsay} profiles (genotypes 7, 13, 15, 16, 24, 55, 85, 86, and 91). This observation might reflect homoplasmy 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, the most prevalent MLVA genotypes found in Turkey are typically from the East Mediterranean region. Molecular typing confirmed that more than half of the human brucellosis cases resulted from either very close cross-transmission in a location or persistent and ongoing transmission among the different regions. MLVA-16_{Orsay} 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 *Brucella* 2010 release) at <http://mlva.u-psud.fr>.

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