

Comparison of Two Multiple-Locus Variable-Number Tandem-Repeat Analysis Methods for Molecular Strain Typing of Human *Brucella melitensis* Isolates from the Middle East[∇]

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***Brucella* species are highly monomorphic, with minimal genetic variation among species, hindering the development of reliable subtyping tools for epidemiologic and phylogenetic analyses. Our objective was to compare two distinct multiple-locus variable-number tandem-repeat analysis (MLVA) subtyping methods on a collection of 101 *Brucella melitensis* isolates from sporadic human cases of brucellosis in Egypt ($n = 83$), Qatar ($n = 17$), and Libya ($n = 1$). A gel-based MLVA technique, MLVA-15_{IGM}, was compared to an automated capillary electrophoresis-based method, MLVA-15_{NAU}, with each MLVA scheme examining a unique set of variable-number tandem repeats. Both the MLVA_{IGM} and MLVA_{NAU} methods were highly discriminatory, resolving 99 and 101 distinct genotypes, respectively, and were able to largely separate genotypes from Egypt and Qatar. The MLVA-15_{NAU} scheme presented higher strain-to-strain diversity in our test population than that observed with the MLVA-15_{IGM} assay. Both schemes were able to genetically correlate some strains originating from the same hospital or region within a country. In addition to comparing the genotyping abilities of these two schemes, we also compared the usability, limitations, and advantages of the two MLVA systems and their applications in the epidemiological genotyping of human *B. melitensis* strains.**

Brucellosis is a common zoonotic disease causing infections in economically important livestock, such as cattle, goats, sheep, and pigs (22). In humans, this highly diverse illness, also known as Malta or undulant fever, initially presents as a fever, malaise, and myalgia and may later develop into a chronic illness affecting various organs and tissues (21, 25). Brucellosis is usually transmitted to humans through consumption of contaminated and untreated milk products or by direct contact with infected animals. There are nine phenotypically recognized species in the *Brucella* genus: *Brucella melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae*; two new marine species, *B. ceti* and *B. pinnipedialis* (11); and *B. microti* (27), isolated from the common vole. Each species has distinctive host preferences, pathogenicity, and epidemiology.

Although some developed countries have eradicated *B. abortus* from cattle through vaccination campaigns, *B. melitensis*, *B. abortus*, and *B. suis* remain the principal causes of human brucellosis worldwide and are major public health problems, primarily in developing countries (7). Brucellosis is prevalent in the Mediterranean basin, the Middle East, Africa, Asia, and areas of Latin America where people are economically dependent on ruminant livestock (23, 28). Over the past decade, the global epidemiology of human brucellosis has changed, due in

part to the implementation of national and international surveillance programs, animal vaccination campaigns, and socioeconomic changes (22).

Current microbiological (4) and low-resolution molecular typing methods are useful for identifying *Brucella* isolates and determining species and biovar designations; however, they have limited value for epidemiological trace-back investigations (6, 15). High-resolution genetic subtyping tools that could provide important information about disease transmission patterns and the molecular epidemiology of *Brucella* have been difficult to develop and implement due to the genetically monomorphic nature of *Brucella* species (12). Since the genome sequencing of four *Brucella* strains, *B. abortus* 2308 (26), *B. abortus* 9-941 (14), *B. suis* 1330 (24), and *B. melitensis* 16M (9), multilocus sequence typing (30) and multiple-locus variable-number tandem-repeat analysis (MLVA) have been developed (16, 18, 29). Recent studies have shown that MLVA, targeting multiple repeat regions with higher mutation rates than other genomic markers, demonstrates higher genetic resolution when applied to *Brucella* species than assays targeting other, more monomorphic molecular markers, such as the outer membrane proteins (8, 10), the *rpmB* gene (19), and insertion sequence regions (6).

In efforts to improve epidemiological surveillance and better evaluate the utility of MLVA as a genotyping tool for *Brucella* strains, the present study compared the discriminatory power of two distinct MLVA methods (16, 18) in their ability to distinguish geographic origin and resolve relationships within a group of 101 human *B. melitensis* isolates from Egypt, Qatar,

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TABLE 1. *Brucella melitensis* isolates examined in this study

Country of origin and region	Hospital location ^a	No. of isolates
Egypt		
Upper Egypt	AST	5
	SOH	2
	QEN	4
	ASW	7
Delta region	SHB	16
	ZAG	2
	BEN	2
	FAY	4
	MAL	13
Cairo metropolitan area	ABS	12
Coastal region	ALX	15
	PRS	1
Libya		1
Qatar	Doha	17
Total		101

^a ABS, Abbassia; ALX, Alexandria; ASW, Aswan; AST, Assiut; BEN, Benha; FAY, Fayoum; MAL, Mahalla; PRS, Port Said; QEN, Qena; SHB, Shebin; SOH, Sohag; ZAG, Zagazig.

and Libya. Each MLVA typing technique examined polymorphisms at 15 different tandem repeat loci and employed two different separation techniques. This study also compares the usability of the two MLVA systems with regard to resources, technological needs, scientific expertise, and time consumption.

MATERIALS AND METHODS

The laboratory work for the gel-based MLVA (MLVA-15_{IGM} [MLVA with 15 VNTR loci from the Institut de Génétique et Microbiologie]) method was performed at the NAMRU-3 laboratory facilities in Cairo, Egypt, while data from the automated MLVA (MLVA-15_{NAU} [MLVA with 15 VNTR loci from Northern Arizona University]) assay were generated at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA.

Strain collection and template preparation. All 101 isolates used in the study were of human origin and were identified as *B. melitensis* by the AMOS-PCR assay (6). They originated from Egypt (*n* = 83), Qatar (*n* = 17), and Libya (*n* = 1). The Egyptian isolates were collected from an acute-febrile-illness laboratory-

based surveillance program implemented in a network of infectious-disease hospitals throughout Egypt from 1999 to 2003 (1, 2), and the Libyan isolate was recovered from a patient who traveled to Libya in 2006 (20). Human *B. melitensis* isolates from Qatar, collected in August 2006, were donated by the Hamad Medical Corporation, Doha, Qatar. The details of all isolates used in this study are presented in Table 1. Bacterial isolates were cultured on Trypticase soy agar with 5% sheep blood (BBL Microbiology Systems, Cockeysville, MD) at 37°C for 48 to 72 h. Total genomic DNA was extracted either using a Qiagen (Valencia, CA) DNeasy blood and tissue kit by following the manufacturer's protocol for extraction of genomic DNA from gram-negative bacteria or by whole-cell lysis as described by Gee et al. (13). The resulting lysates were stored at -20°C until needed.

VNTR identification. The methods for selecting the variable-number tandem repeats (VNTRs) used in the MLVA-15_{NAU} and MLVA-15_{IGM} techniques have been described previously (16, 18). Table 2 shows the characteristics of the VNTRs examined in both MLVA schemes. All primers used in this study were obtained from Sigma Genosys (The Woodlands, TX).

VNTR analysis by the MLVA-15_{NAU} technique. PCR amplification of 15 VNTR loci was performed as described previously (16) with the following modifications. Forward primers were designed with either a 6-carboxyfluorescein (FAM) or a HEX (*N*-hexachloro-fluorescein cyanoethyl phosphoramidite) fluorescent label. Four multiplex PCRs (AI, BI, AII, and BII) were performed to amplify 15 VNTR loci in a final volume of 10 µl containing 1× PCR buffer, 2 mM MgCl₂, 2 mM deoxynucleoside triphosphate mix, 0.04 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 1 µl DNA template, and the following primer combinations: for AI, Bruce VNTR 21 F-HEX-Bruce VNTR 21 R, Bruce VNTR 28 F-FAM-Bruce VNTR 28 R, and Bruce VNTR 16 F-HEX-Bruce VNTR 16 R; for BI, Bruce VNTR 03 F-FAM-Bruce VNTR 03 R, Bruce VNTR 20 F-HEX-Bruce VNTR 20 R, Bruce VNTR 31 F-FAM-Bruce VNTR 31 R, Bruce VNTR 29 F-HEX-Bruce VNTR 29 R, and Bruce VNTR 33 F-FAM-Bruce VNTR 33 R; for AII, Bruce VNTR 14 F-FAM-Bruce VNTR 14 R, Bruce VNTR 01 F-HEX-Bruce VNTR 01R, Bruce VNTR 07 F-FAM-Bruce VNTR 07R, and Bruce VNTR 27 F-HEX-Bruce VNTR 27 R; and for BII, Bruce VNTR 25 F-FAM-Bruce VNTR 25 R, Bruce VNTR 30 F-HEX-Bruce VNTR 30 R, and Bruce VNTR 02 F-FAM-Bruce VNTR 02 R. All reactions were performed on an ABI 9700 thermocycler using the primer concentrations and thermal cycling conditions reported previously (16).

Multiplex PCRs were pooled and diluted to a final dilution of 1:20 with molecular-grade water. PCR products were denatured and resolved by capillary electrophoresis on an ABI Prism 3130 automated fluorescent capillary DNA sequencer (Applied Biosystems, Foster City, CA). Fragments were sized by comparison to a ROX (carboxy-X-rhodamine)-labeled molecular ladder (MapMaker 1000; BioVentures Inc., Murfreesboro, TN) with GeneMapper, version 4.0 (Applied Biosystems, Foster City, CA), software. Appropriate VNTR designations of the fragments were assigned based on size calling through internal software binning capabilities.

VNTR analysis by the MLVA-15_{IGM} technique. PCR primers for the MLVA-15_{IGM} technique have been reported previously (18). PCR amplification of the 15 VNTR loci occurred in a final volume of 25 µl containing final concentrations

TABLE 2. Characteristics of the VNTRs of the two MLVA-15 assays

VNTR	MLVA-15 _{NAU} VNTR characteristics				VNTR	MLVA-15 _{IGM} VNTR characteristics			
	Genomic location in <i>B. melitensis</i> 16M ^a	Repeat size (bp)	No. of alleles	Simpson's DI		Genomic location in <i>B. melitensis</i> 16M ^a	Repeat size (bp)	No. of alleles	Simpson's DI
BruceVNTR 16	339714-339950	8	4	0.49	Bruce 18	339785-339930	8	6	0.79
BruceVNTR 3	1249996-1250345	8	19	0.93	Bruce 07	1250093-1250250	8	8	0.77
BruceVNTR 28	1543941-1544121	8	9	0.79	Bruce 04	1543967-1544118	8	6	0.64
BruceVNTR 30	588314-588862	8	12	0.89	Bruce 09	588256-588681	8	9	0.82
BruceVNTR 27	548529-548905	8	9	0.84	Bruce 16	548571-548722	8	7	0.64
BruceVNTR 1	1938531-1938766	8	16	0.91	Bruce 21	328936-329083	8	2	0.18
BruceVNTR 2	1940192-1940778	8	17	0.92	Bruce 30	1505073-1505223	8	2	0.32
BruceVNTR 7	275628-275961	8	1	0	Bruce 06	1322650-1323057	134	2	0.20
BruceVNTR 21	574937-575041	6	2	0.06	Bruce 08	1134883-1135230	18	3	0.25
BruceVNTR 29	1915916-1916471	8	2	0.02	Bruce 11	211361-211617	63	1	0
BruceVNTR 31	344488-344993	5	6	0.77	Bruce 12	73619-74010	15	2	0.26
BruceVNTR 33	18596-19246	8	19	0.93	Bruce 42	424317-424855	125	4	0.25
BruceVNTR 14	396453-396581	9	1	0	Bruce 43	379369-379550	12	2	0.37
BruceVNTR 20	542226-542683	12	7	0.27	Bruce 45	233374-233524	18	2	0.37
BruceVNTR 25	216642-217140	15	1	0	Bruce 55	2066378-2066650	40	3	0.25

^a Determined by NCBI nucleotide primer BLAST.

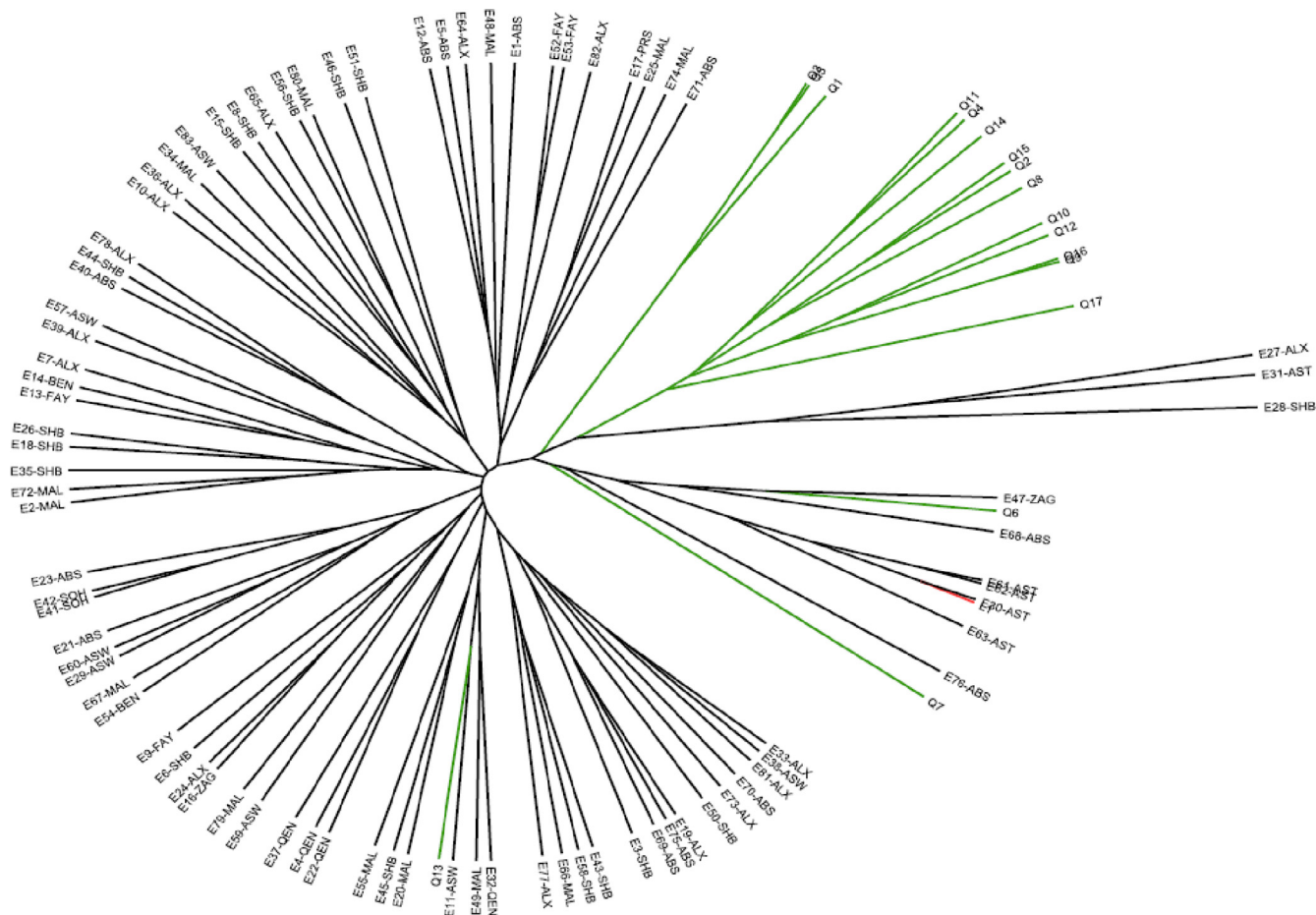


FIG. 1. UPGMA analysis of 101 human *B. melitensis* isolates examined by the MLVA-15_{NAU} scheme. Isolate designations indicate the country of origin (E, Egypt; Q, Qatar; L, Libya), followed by the location within the country (ABS, Abbassia; ALX, Alexandria; ASW, Aswan; AST, Assiut; BEN, Benha; FAY, Fayoum; MAL, Mahalla; PRS, Port Said; QEN, Qena; SHB, Shebin; SOH, Sohag; ZAG, Zagazig).

of 2.5 mM MgCl₂, 1× PCR buffer, 0.4 mM deoxynucleoside triphosphate nucleotide mixture, 1.25 U *Taq* DNA polymerase (Promega Corporation, Madison, WI), 0.5 μl DNA template, and 0.5 μM forward and reverse primers. All PCRs were performed on an MJ Research PTC-200 thermocycler with the thermal cycling parameters reported previously. PCR amplicons were analyzed by gel electrophoresis as described previously (3, 18); agarose gels were then normalized; and band sizes were estimated using BioNumerics version 4.61 (Applied Maths, Belgium). Band size estimates were then converted to repeat units by following the published allele numbering system that can be found at <http://mlva.u-psud.fr/>.

Analysis of MLVA data. Simpson's index of diversity was calculated (5) for each tandem-repeat locus based on a sample size of 101 *B. melitensis* isolates (Table 2).

Data from both MLVA schemes were converted into character data sets within the BioNumerics software (version 5.1; Applied Maths, Saint-Martens-Latem, Belgium). Cluster analysis was performed on both data sets using the categorical coefficient and UPGMA (unweighted-pair group method using arithmetic averages) analysis. The resulting UPGMA similarity matrices were used to generate unrooted trees in FigTree, version 1.1.2 (<http://tree.bio.ed.ac.uk/software/figtree>) (see Fig. 1 and 2).

RESULTS AND DISCUSSION

The two MLVA schemes evaluated in our study were selected based on the availability of published MLVA schemes at the time of the study design, their abilities to type human *B. melitensis* isolates, and the availability of the resources, technology, and technical expertise required to perform the tech-

niques. As mentioned above, the gel-based MLVA assay was carried out in Cairo, Egypt, and data from the automated MLVA system were collected at the CDC in Atlanta, GA. Thus far, there has been no study demonstrating the typing abilities of different MLVA systems for a common collection of *Brucella* isolates. The objective of our study was to compare the resolving powers of two different MLVA techniques on a common collection of 101 clinical *B. melitensis* isolates from the Middle East and to provide an assessment of the limitations, advantages, and field applications of the two MLVA assays for genotyping *B. melitensis* isolates.

The MLVA techniques compared in this study were developed for different purposes, which are reflected in the unique combination of tandem-repeat markers that makes up each scheme. Originally intended for forensic purposes, the MLVA-15_{NAU} assay contains 12 markers that have small repeat units less than or equal to 8 bp; 8 of these markers have diversity indexes (DIs) greater than 0.75 in our study. The MLVA-15_{IGM} assay, developed for epidemiological typing, contains two panels: a panel of eight conserved markers with larger repeat units (>8 bp) (panel 1), exhibiting DIs less than 0.40 in the strains we examined, and a panel of seven more-diverse loci (panel 2) with small repeat units (<8 bp).

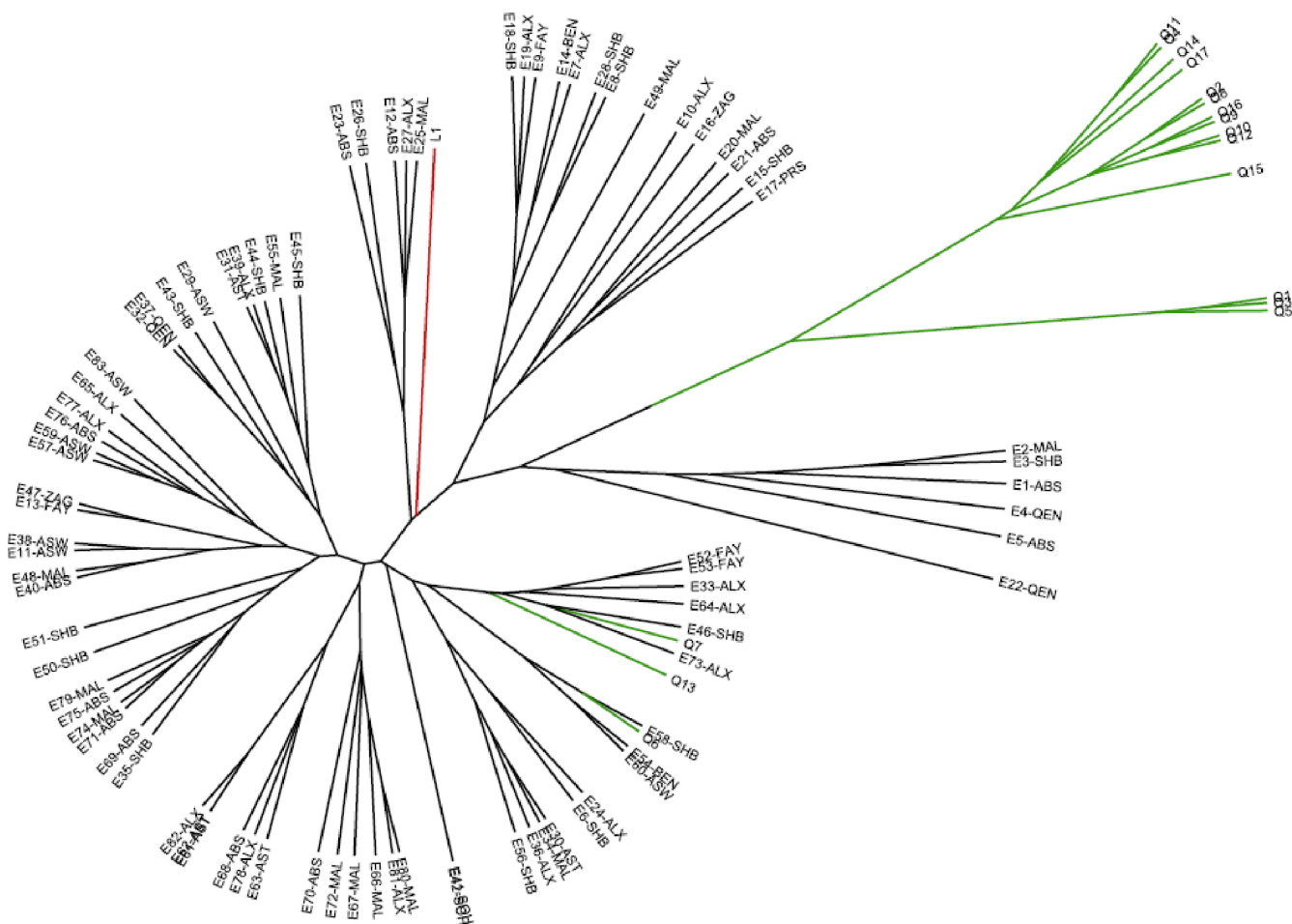


FIG. 2. UPGMA analysis of 101 human *B. melitensis* isolates examined by the MLVA-15_{IGM} scheme. For isolate designations, see the legend to Fig. 1.

Overall, the VNTR markers in the MLVA-15_{NAU} assay presented a broader number of alleles and higher DIs than the markers examined by the MLVA-15_{IGM} scheme. Six of the 15 VNTR loci in the MLVA-15_{NAU} scheme exhibited DIs of >0.80 for the 101 *B. melitensis* isolates examined, with allele numbers ranging from 9 to 20 (Table 2). The Bruce VNTR 21 and Bruce VNTR 29 markers display very low genetic DIs of 0.06 and 0.02, respectively, while the Bruce VNTR 7, Bruce VNTR 14, and Bruce VNTR 25 loci were monomorphic in the strains we examined. The invariance we observed at these loci is most likely due to the localized geographic origins of our isolates and represents a level of strain-to-strain conservation within a geographic area that can be better evaluated in a larger population of isolates from similar locations. The monomorphic nature of the Bruce VNTR 7 and Bruce VNTR 25 loci in the Middle Eastern strains could represent geographically specific alleles, since these markers have been reported to exhibit higher DIs in other *B. melitensis* strains, from the United States ($n = 85$) (16). The Bruce VNTR 14 locus is consistently monomorphic in our strains and in other *B. melitensis* strains examined by Huynh et al. (16) and could be examined as a species-specific marker. Bruce VNTR 02, 20, 28, 31, and 33 were initially difficult to amplify in certain isolates.

However, all VNTR loci were successfully amplified in all isolates except for Bruce VNTR 33 in isolate E5 and Bruce VNTR 02 in isolate E53.

Only one of the 15 VNTR markers from the MLVA-15_{IGM} assay had a DI of >0.80 in the 101 strains we examined. The Bruce 04, 07, 09, 16, and 18 VNTR markers exhibit the highest DIs, ranging from 0.64 to 0.82, among the 15 markers (Table 2). The remaining 10 tandem-repeat markers ranged in diversity from 0.18 to 0.37. The Bruce 11 VNTR marker is the only monomorphic marker in the Middle Eastern strains we examined. Two of the 101 *B. melitensis* isolates (E21 and E26) exhibited null alleles at the Bruce 09 locus; however, all other markers were successfully amplified in all isolates.

The two MLVA schemes evaluated in this study were able to correlate similar genetic relationships among the 101 *B. melitensis* strains we examined. The MLVA-15_{NAU} scheme, exhibiting greater DIs, resolved 101 unique genotypes, while the MLVA-15_{IGM} assay discriminated 99 genotypes. Dendrograms generated by UPGMA clustering analysis (Fig. 1 and 2) show similar groupings of *B. melitensis* strains by the MLVA methods, such as the clustering of Qatar isolates Q1, Q3, and Q5 with each other and the genetic similarity of Q6, Q7, and Q13 strains to the Egyptian genotypes. Also, the Libyan strain

consistently clusters with the Egyptian genotypes by both MLVA methods. Because none of the strains we examined were epidemiologically linked, since they were sporadic cases from various regions of Egypt and Qatar, we did not expect to see a great deal of homogeneity by either MLVA method. However, by the MLVA-15_{IGM} scheme, two strains, E41 and E42, which were isolated in the same hospital in Sohag, Egypt, 6 weeks apart from each other, had identical genotypes. Two other strains, E61 and E62, isolated 2 years apart from each other from another hospital, in Assiut, Egypt, also had identical genotypes. The same strains (E41 and E42; E61 and 62) did not have identical genotypes by the MLVA_{NAU} assay but diverged by a mutation at one or both of the hypervariable markers VNTR 01 and VNTR 33. By both MLVA schemes, it is possible to see some strains originating from the same hospital location or the same region (see Table 1 for regions) within Egypt that have very similar but not identical genotypes, which is expected for nonoutbreak strains.

The MLVA schemes evaluated in this study have different logistical and technical requirements. The gel-based MLVA method requires more time for typing from start to finish, requires less technical expertise, and involves less-expensive materials, although it does require BioNumerics software, which is quite costly and requires technical training. The use of multiple individual PCRs and agarose gels for typing one strain is cumbersome and requires considerable subjectivity in the stages of analysis of the raw data. The automated system is much faster and has a high throughput capacity; however, the instrumentation and proprietary reagents make this method more expensive. The use of an automated sequencer and the genetic analysis software require a skilled technician; however, the data output is more reliable, and once properly optimized, the method is very reproducible.

Both MLVA schemes examined in this study can be used to epidemiologically type human *B. melitensis* strains and can be used for high- and low-resolution typing by the inclusion and exclusion of highly variable markers. We evaluated the two MLVA schemes and compared their abilities to resolve relationships between geographically localized strains from the Middle East, where *B. melitensis* is the primary etiologic agent of human brucellosis. Other studies have looked at globally diverse and localized *Brucella* strains using the MLVA_{IGM} assay (3, 17–19). However, it would be interesting to demonstrate the capability of the MLVA_{NAU} method for differentiating global genotypes in *Brucella* species. Although we currently utilize the MLVA-15_{NAU} assay for genotyping *Brucella* isolates in our reference lab at the CDC and prefer the automated platform for its reproducibility, the MLVA-15_{IGM} scheme is used widely in the European community and collaborating laboratories. The comparable geographic and in-country resolution of the two MLVA systems, targeting different VNTR regions, validates the utility of a combination analysis system, such as MLVA, for genotyping genetically monomorphic bacteria. With the increasing use of multiple MLVA systems for typing *Brucella* species and the increasing need to share data, it would be effective to investigate the discriminatory value of all the currently utilized VNTR markers in these combination systems by using statistical methods such as principal-component analysis in order to establish one streamlined

MLVA system that serves the global need for an epidemiological genotyping tool to improve brucellosis surveillance.

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