Evaluation of a Multilocus Variable-Number Tandem-Repeat Analysis Scheme for Typing Human Brucella Isolates in a Region of Brucellosis Endemicity†\

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Brucellosis remains an important anthropozoonosis worldwide. Brucella species are genetically homogeneous, and thus, the typing of Brucella species for epidemiological purposes by conventional molecular typing methods has remained elusive. Although many methods could segregate isolates into the phylogenetically recognized taxa, limited within-species genetic diversity has been identified. Recently, multilocus variable-number tandem-repeat analysis (MLVA) was found to have a high degree of resolution when it was applied to collections of Brucella isolates from geographically widespread locations, and an assay comprising 16 such loci (MLVA-16) was proposed. This scheme includes eight minisatellite loci (panel 1) and eight microsatellites (panel 2, which is subdivided into panels 2A and 2B). The utility of MLVA-16 for the subtyping of human Brucella isolates from geographically restricted regions needs to be further evaluated, and genotyping databases with worldwide coverage must be progressively established. In the present study, MLVA-16 was applied to the typing of 42 human Brucella isolates obtained from 41 patients recovered from 2002 to 2006 at a tertiary-care center in Lebanon. All isolates were identified as Brucella melitensis by MLVA-16 and were found to be closely related to B. melitensis isolates from neighboring countries in the Middle East when their genotypes were queried against those in the web-based Brucella2007 MLVA database (http://mlva.u-psud.fr/). Panel 2B, which comprised the most variable loci, displayed a very high discriminatory power, while panels 1 and 2A showed limited diversity. The most frequent genotype comprised seven isolates obtained over 7 weeks in 2002, demonstrating an outbreak from a common source. Two isolates obtained from one patient 5 months apart comprised another genotype, indicating relapsing disease. These findings confirm that MLVA-16 has a good discriminatory power for species determination, typing of B. melitensis isolates, and inferring their geographical origin. Abbreviated panel 2B could be used as a short-term epidemiological tool in a small region of endemicity.

Brucellae are facultative intracellular pathogens that infect a wide variety of animal species and humans. Brucellosis is the most common anthropozoonosis, with more than 500,000 cases reported annually worldwide (28). The genus Brucella currently encompasses nine recognized species (seven terrestrial and two marine mammal species) that display animal host specificity, among which three present veterinary and public health concerns (11, 27, 31). Brucella melitensis predominantly infects sheep and goats, B. abortus infects cattle, and B. suis infects swine and a range of wild animals; but cross-infection of other mammalian species, including humans, may occur (10). Animal brucellosis causes abortion and infertility in livestock (cattle, goats, and sheep), resulting in serious economic losses. Human brucellosis is a subacute or chronic febrile illness that can involve multiple organs and that can result in a wide variety of manifestations and significant morbidity if the diagnosis is overlooked and treatment is not promptly initiated (5). Animal brucellosis has been successfully eradicated in most developed countries, resulting in the virtual disappearance of the human disease in North America, Northern Europe, and Northwest Asia, where most cases are now due to either travel to areas of endemicity, accidental laboratory exposure, or occasionally, exposure to wild animals (10). However, human brucellosis remains endemic and a major public health problem in many developing countries and some developed countries in Latin America, Southern Europe, Africa, Southeast Asia, and the Middle East (28). The most common cause of human brucellosis is B. melitensis, with B. abortus and B. suis accounting for smaller proportions of cases (19, 28). Brucella spp. are considered potential military, agricultural, and civilian category B biological threat agents (30) due to their relative ease of dis-

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semination and costly eradication if they were spread in a possible bioterrorism event.

Species identification and subtyping of Brucella culture isolates is important for epidemiologic surveillance; investigation of outbreaks in regions of both endemicity and nonendemicity; and distinguishing cases of human reinfection from relapse, thereby influencing clinical therapeutic decisions (3).

Terrestrial Brucella spp. are homogeneous and harbor >80% interspecies homology by DNA-DNA hybridization studies (31, 38), identical 16S rRNA sequences (15), and >98% sequence similarity by comparative genomics (18, 29). Molecular methods commonly used for the subtyping of isolates of other bacterial species, such as multilocus enzyme electrophoresis (12), pulsed-field gel electrophoresis (4), random amplified polymorphic DNA analysis (34), enterobacterial repetitive intergenic consensus sequence PCR (26, 35), repetitive intergenic palindromic sequence PCR (25), amplified fragment length polymorphism analysis (42), and monolocus (such as omp2a and omp2b) sequence analysis (9) or multilocus sequence typing (40), are able to segregate Brucella isolates into the recognized species and certain species biovars at best. However, these methods are not sufficiently discriminatory for the routine subtyping of isolates for epidemiological trace-back purposes.

Tandemly repeated sequences used in forensic investigations have also been found in bacteria (for a review, see reference 39). Minisatellites have repeat unit sizes of 9 bp or greater, and microsatellites have repeat unit sizes of up to 8 bp (39). Combinations of minisatellite and microsatellite repeats in multilocus variable-number tandem-repeat analysis (MLVA) have proven highly discriminatory in the epidemiological subtyping of isolates belonging to monomorphic bacterial species, such as Bacillus anthracis, Yersinia pestis (8, 23), and more recently, Brucella spp. In Brucella, MLVA schemes with 21 loci (MLVA-21) and MLVA-16 (1, 22, 41) that use a combination of repeat markers distributed across the Brucella genome were able to distinguish isolates of Brucella spp. of widespread temporal and geographical origins or of very close origins (13). Furthermore, with some rare exceptions, which might be due to a lack of resolution and incorrect clustering produced by the biotyping methods, the isolates formed MLVA groups that corresponded to the known Brucella species. A study of 128 human isolates of B. melitensis by MLVA-16 identified the Americas, West Mediterranean, and East Mediterranean groups and detected remarkable degrees of diversity within each group (1). The relative stability of MLVA loci has been estimated directly by repetitive subculturing of a few strains in the laboratory (41) and by the typing of B. melitensis isolates of the Rev.1 vaccine strain obtained from different sources (13), so that the most recent MLVA data analyses have started to assign different weights to different markers, taking into account the mutation rate and the associated levels of homoplasy (1). The MLVA schemes that have been devised are very promising, but the strength of a typing method depends as much on the technique itself as on the associated genotyping database. These typing assays still need to be applied to larger numbers of isolates from as many countries as possible to validate their reliability in investigating strain relatedness both in countries of endemicity, especially where efforts to eradicate human disease may be attempted, and in brucellosis-free countries where a cluster of infections may be suspected to be due to a bioterrorism event. The primary purpose of high-resolution typing in a format compatible with the production of large-scale international databases is the long-term surveillance of infectious diseases and the early detection of abnormal events, including temporal or geographical shifts in the population structure, e.g., the emergence and spread of new clones or lineages. The current Brucella MLVA database, hosted at http://mlva.u-psud.fr, contains data derived from more than 500 animal and human Brucella isolates. It is expected that in the future, similar and compatible databases with data for isolates from wider geographical origins will be developed and made jointly and freely accessible to querying via the use of web-based analysis tools (16).

In this study, we applied the MLVA-16 scheme (1, 22) to a series of clinical isolates of Brucella accrued over a 5-year period at a single tertiary-care center in Lebanon, a small country where human brucellosis is endemic. The genotypes of these isolates were compared to the genotypes previously determined by MLVA-16 typing and are included in the Brucella MLVA database.

(Materials and Methods)

**Bacterial strains.** Forty-two clinical Brucella isolates (isolates AUB-BRUP-S1 to AUB-BRUP-S42 [isolates S1 to S42, respectively]) obtained from clinical specimens from 41 different patients submitted to the clinical microbiology laboratory at the American University of Beirut Medical Center from February 2002 to December 2006 were investigated. These represented all Brucella isolates collected during that time period. Thirty-nine of the isolates were isolated from blood, and three were from tissue specimens. Two of the 39 blood isolates were obtained from cultures of blood from one patient 5 months apart. All isolates were identified as Brucella species on the basis of colonial morphology, positive oxidase and catalase tests, positive agglutination with brucella-specific antiseraum, and positive amplification by real-time PCR, as described previously (21). In addition, all patients from whom these isolates were obtained had positive Brucella serology titers by Wright’s standard tube agglutination and the anti-human globulin Coombs test (5).

**Brucella MLVA-16 genotyping scheme.** MLVA was performed with all 42 isolates according to the scheme initially proposed by Le Flèche et al. (22), which includes 15 tandem-repeat loci (MLVA-15), and modified by Al-Dahouk et al. to include 1 additional locus, bruce19 (MLVA-16) (1). PCR amplification of eight minisatellite loci in panel 1 (the bruce06, bruce08, bruce11, bruce12, bruce42, bruce43, bruce45, and bruce55 loci), three microsatellite loci in panel 2A (the bruce18, bruce19, and bruce21 loci), and five microsatellite loci in panel 2B (the bruce04, bruce07, bruce09, bruce16, and bruce30 loci) was carried out by a protocol similar to the one described previously (1).

**DNA preparation.** DNA was extracted from one loopful of bacterial cells grown for 48 h on chocolate agar, and single colonies were isolated by using the tissue protocol of the QIAamp DNA minikit (Qiagen, Hilden, Germany). DNA concentrations were measured by UV spectrophotometry (Shimadzu, Japan).

**PCR amplification.** PCR amplification was performed in a total volume of 15 μl containing 5 ng of DNA, 1× PCR buffer, 1 U of Taq DNA polymerase, 200 μM of each deoxynucleoside triphosphate, 0.3 mM each flanking primer, and 1 M of betaine (catalog no. B2629; Sigma-Aldrich). The PCR cycling parameters were as follows: initial denaturation at 96°C for 5 min, followed by 30 cycles of denaturation at 96°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 5 min. For detection of the PCR products, 5 μl of each of the PCR amplification products was subjected to gel electrophoresis with a 2% 3:1 HRB agarose gel (catalog no. E776: Amresco) for panel 1 and a 3% HRB agarose gel for panel 2 in 0.5× Tris-borate-EDTA buffer. Electrophoresis was carried out until the bromophenol blue had run at least 20 cm. The size markers used were a 100-bp ladder (EZ Load 100-bp...
PCR molecular ruler: Bio-Rad) for panel 1 and a 20-bp ladder (EZ Load 20-bp molecular ruler; Bio-Rad) for panel 2. Gels that had been stained with ethidium bromide (0.5 μg/ml) were digitally acquired with a gel documentation system (DigiDoc-It; UVP, Upland, CA).

**Analysis of MLVA data.** Minisatellite repeat numbers (panel 1 [loci bruce06, bruce08, bruce11, bruce12, bruce42, bruce43, bruce45, and bruce55]) and microsatellite repeat numbers (panel 2A [loci bruce18, bruce19, and bruce21]) and panel 2B [loci bruce04, bruce07, bruce16, and bruce30]) were calculated on the basis of the band sizes after the gel images were normalized by use of the BioNumerics (version 5.1) software package (Applied Maths, Belgium) and the previously published allele numbering convention (22). The reproducibilities of the MLVA profiles were assessed by comparing the typing results for experiments performed independently at two laboratory testing sites in Lebanon and France. The similarity matrix for each panel was calculated with BioNumerics software by using the repeat number at each locus as a character type with the categorical similarity coefficient. This categorical approach considers all alleles at each locus and within each panel to be equally distant (37). For MLVA-16 clustering analysis, an aggregated similarity matrix was produced by using a composite data set and giving weights of 20, 5, and 1 to panels 1, 2A, and 2B, respectively, according to the principles reported previously (1). Weights were assigned to the individual loci in each panel because all loci do not evolve at the same speed but have different mutation rates, as determined empirically. Clustering was done by use of the unweighted paired group method for arithmetic averages algorithm. Genetic diversity was determined by calculating the Hunter-Gaston (or Simpson's) diversity index (HGDI) of each MLVA marker and for the combination of markers for each of panel 1, panel 2A, panel 2B, panel 2 (panel 2A plus panel 2B), and panels 1 and 2 (20). The 95% confidence intervals of the HGDI for indices of ≥0.85 were calculated by the method of Grundmann et al. (17). The MLVA-16 genotypes, defined by the combination of alleles, of the 42 isolates were compared to the corresponding data obtained for the reference strains and field isolates investigated previously (1, 13, 14, 22, 31, 32, 33). The present and previous data have been incorporated into a single database designated Brucella2007, which can be queried at http://mlva.u-psud.fr by the use of web-based analysis tools.

**RESULTS AND DISCUSSION**

The purpose of this study was to evaluate the polymorphisms of the MLVA-16 loci in a series of *Brucella* isolates and the relatedness of the strains from a geographically restricted region where *Brucella* is endemic. Furthermore, we sought to validate the established Brucella2007 genotyping database associated with MLVA-16 as a reliable investigation tool in this context. In the present study, the MLVA-16 scheme was applied to type 42 human isolates of *Brucella* obtained over a 5-year period at the largest tertiary-care center in Lebanon. Lebanon is a small country in the eastern Mediterranean region with a geographically surface area of 10,452 km², an estimated population of 4,000,000, and a reported incidence of brucellosis of 50 per 1 million inhabitants (28).

The typeability of the various MLVA markers in this series approached 1 (37). Only the bruce04 locus in strain S29 and the bruce09 locus in strain S19 could not be amplified, despite multiple attempts. All other amplification reactions were successful. The reproducibility of MLVA-16 was excellent, as experiences performed in two different laboratories in Lebanon and France by use of the same protocol and with the results interpreted separately in a blinded fashion yielded 100% concordant results. This is not surprising for a sequence-based approach and was recently confirmed by a ring trial for the panel 1 loci among 15 *Brucella* reference laboratories (http://mlva.u-psud.fr/BRUCELLA/). In that trial, the few discrepancies in typing results observed were due to errors in allele calling (24a). The 42 isolates in the series used in this study formed a homogeneous group for which the differences did not exceed 15% (Fig. 1). Limited diversity was observed with the panel 1 and panel 2A loci (Table 1), as would be expected for strains originating from a very restricted geographical area and by the use of markers that are the most phylogenetically informative. MLVA-16 yielded a total of 29 genotypes with seven clusters and 22 singleton genotypes. Previous work identified a total of 68, 52, and more than 300 genotypes for panel 1, panel 2A, and panel 2B, respectively (1, 13, 14, 22, 31, 32, 33) (see Fig. S1 in the supplemental material). Twenty-six panel 1 genotypes, numbered 41 to 66 (1, 22), corresponded to *B. melitensis*. No new panel 1 genotypes were identified in the present study. Thirty-seven isolates shared panel 1 genotype 43. Panel 1 genotypes 42, 44, and 57 were observed in a single isolate each; and genotype 60 was observed in isolates S18 and S24, which were derived from the same patient. Therefore, only five different panel 1 genotypes were observed among the 26 panel 1 *B. melitensis* genotypes observed so far. Accordingly, the bruce06, bruce11, bruce12, and bruce55 loci from panel 1 and the bruce21 locus from panel 2 showed no diversity among the 42 isolates (HGDI = 0). All five panel 1 genotypes represented in the present study correspond to the East Mediterranean group proposed previously (1) (see Fig. S1 in the supplemental material). The panel 2B markers displayed the most diversity in the present collection. This was as expected, given the high mutation rate of some panel 2B markers (13), with bruce04 and bruce16 exhibiting the highest HGDI, followed by bruce30 (HGDI > 0.7). *Brucella*07 and *Brucella*09 in panel 2B had HGDI of <0.5. Panel 2B and panels 2A and 2B yielded 27 and 28 genotypes, respectively, and had similar HGDI. The combined panel 1 and 2 markers yielded 29 genotypes and an HGDI that was only slightly higher than that for panel 2B alone. The HGDI of panel 2B, panels 2A and 2B, and panels 1 and 2 were all >0.95, which is considered optimal for a typing system (37). These data might indicate that in the setting of a local outbreak investigation, panel 1 and panel 2A could be omitted and the highly polymorphic panel 2B would be sufficient. However, typing with panel 1 and 2A is necessary before typing with panel 2B is used in order to check that the isolate is indeed of the expected local type, as it is important to keep in mind that the HGDI value is not a sufficient indicator for the selection of tandem repeat loci and validation of an MLVA assay. Three of the five panel 2B loci (bruce04, bruce09, bruce30) are part of the hypervariable octameric oligonucleotide fingerprint variable-number tandem repeats (HOOF-Prints) MLVA panel of loci that have been described by Bricker et al. (6, 7) and that have proven to be highly suitable in terrestrial *Brucella* spp. (6, 7, 36, 41). The most frequent genotype (largest cluster) comprised seven isolates (isolates S3 to S9) obtained over 7 weeks in the late spring and early summer of 2002, demonstrating an outbreak from a common source. Another genotype was shared by isolates S18 and S24, obtained 5 months apart from one patient who had no at-risk occupational history and who experienced a recurrence of his febrile illness. The two isolates from this patient differed from the rest of the isolates at the bruce45 minisatellite locus from panel 1 (panel 1 genotype 60, two versus three repeats), indicating relapsing disease in this patient. Two isolates obtained 2 months apart (isolates S37 and S41) and four pairs obtained over 1 year apart (isolates S25 and S30, S12 and S39, S2 and S23, and S28 and S42) had identical genotypes. These five seemingly unrelated pairs may represent either epidemiologi-
cally unrelated isolates with homoplasy at MLVA-16 loci (most likely panel 2B) or persistent circulating strains causing sporadic infections. All other isolates had distinct genotypes reflecting sporadic cases. In a previous study (1), two pairs of epidemiologically related human *B. melitensis* isolates in Germany displayed identical MLVA-16 profiles. One pair of isolates was obtained from one patient: one isolate was obtained before treatment for brucellosis and one isolate was obtained 3 months later when he experienced a relapse related to inadequate therapy. Another pair of isolates came from a married couple who visited Turkey, where they contracted their infection from the consumption of unpasteurized cheese. Three isolates from a small outbreak of *B. suis* infections in pigs in Spain also shared the same MLVA-16 genotype (14). The clustering of epidemiologically related isolates identified in the current and previous studies support the use of MLVA-16 as a valuable tool for investigations of outbreaks of both human and animal brucellosis.

In comparison to previous data and by the use of all 16 loci, 23 of the 29 genotypes obtained were unique (see Fig. S1 in the supplemental material). Eight isolates had genotypes identical to those of strains from Syria (*n*/H11005 2), Turkey (*n*/H11005 2), Israel (*n*/H11005 1), Lebanon (*n*/H11005 1), and Germany (*n*/H11005 2). The last two isolates probably originated from Turkey (2). Thirty-one (31) isolates were single-locus or double-locus variants of closely related *B. melitensis* isolates from neighboring countries, including Syria, Israel, and Turkey (see Fig. S1 in the supplemental material). The unique fingerprinting profiles for most of the *B. melitensis* isolates in this series and their close relatedness by MLVA compared to the relatedness of other human *B. melitensis* isolates (1) probably reflect microevolution due to few mutational events among indigenous strains derived from a common ancestor rather than the introduction of foreign strains from other areas, since most of these variations were in panel 2B loci.

Although phenotypic biovar typing was not performed in this study, previous studies have shown that the results of the MLVA-16 or the MLVA-21 typing schemes that have been...
Devised (1, 41) show no correlation with those of biovar typing for B. melitensis. Our isolates were closely related to B. melitensis isolates from various countries that belonged to all three biovars (biovars 1, 2, and 3). Neither MLVA nor multilocus sequence typing distinguished the B. melitensis biovars, which are mere serotypes (1, 40, 41). This indicates that variable-number tandem-repeat loci can be used as epidemiological tools for the resolution of strains on a regional scale but to a lesser extent. In contrast, both Brucella abortus and B. suis biovars, biovars 1, 3, and 4 (see Fig. S1 in the supplemental material). We found higher levels of diversity for bruce03 (panel 2B) and bruce19 (panel 2A) and a lower level of diversity for bruce08 (panel 1). The Sicilian isolates formed a homogeneous group that could be distinguished from the East Mediterranean group at three of the panel 1 minisatellite loci. These data illustrate the usefulness of panel 1 not only for the species identification of Brucella isolates but also for inference of their geographical origins. In contrast, panel 2 markers afford a higher discriminatory power for investigation of strain relatedness in regions of endemicity. An abbreviated panel consisting of five microsatellite loci, panel 2B, can be used as a practical short-term epidemiological tool in this setting, but the species cannot be deduced from the data produced by this panel only. Conversely, in regions of nonendemicity or whenever the introduction of an exogenous strain might be suspected, all loci should be used to pinpoint the region where the isolates may have originated. For that reason, there is a need to build expanded searchable and shared international databases of MLVA fingerprints to enable such comparisons. The currently existing Brucella2007 MLVA database (http://mlva.u-psud.fr) provides one constituent within the framework of such a concerted effort (16).

In summary, MLVA-16 displayed a good discriminatory power for the typing of B. melitensis isolates from a small region of endemicity, and either panel 2 or panels 1 and 2 may be used as epidemiological tools for the resolution of strains and for distinguishing relapses from reinfections in patients with brucellosis.

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