Journal of
Clinical Microbiology

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J. Clin. Microbiol. **2008, 46(1):296. DOI: 10.1128/JCM.01496-07.**
Published Ahead of Print 21 November 2007.

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JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2008, p. 296–301 Vol. 46, No. 1
0095-1137/08/$08.00+0 doi:10.1128/JCM.01496-07
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Real-Time PCR Assays of Single-Nucleotide Polymorphisms Defining the Major *Brucella* Clades[▽]

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Received 25 July 2007/Returned for modification 21 October 2007/Accepted 4 November 2007

Members of the genus *Brucella* are known worldwide as pathogens of wildlife and livestock and are the most common organisms of zoonotic infection in humans. In general, brucellae exhibit a range of host specificity in animals that has led to the identification of at least seven *Brucella* species. The genomes of the various *Brucella* species are highly conserved, which makes the differentiation of species highly challenging. However, we found single-nucleotide polymorphisms (SNPs) in housekeeping and other genes that differentiated the seven main *Brucella* species or clades and thus enabled us to develop real-time PCR assays based around these SNPs. Screening of a diverse panel of 338 diverse isolates with these assays correctly identified each isolate with its previously determined *Brucella* clade. Six of the seven clade-specific assays detected DNA concentrations of less than 10 fg, indicating a high level of sensitivity. This SNP-based approach places samples into a phylogenetic framework, allowing reliable comparisons to be made among the lineages of clonal bacteria and providing a solid basis for genotyping. These PCR assays provide a rapid and highly sensitive method of differentiating the major *Brucella* groups that will be valuable for clinical and forensic applications.

Brucella spp. are pathogenic bacteria that infect a wide variety of mammalian hosts worldwide, often causing reproductive failure. The genus *Brucella* has classically been divided into six species based on host specificity, including *B. abortus* (cattle and bison), *B. melitensis* (goats and sheep), *B. suis* (pigs), *B. canis* (dogs), *B. neotomae* (desert woodrat), and *B. ovis* (sheep) (12). Two new species have been discovered recently in marine mammals (*B. cetaceae* in dolphins and whales and *B. pinnipediae* in seals) (10). Taxonomic limits of the marine clade, however, are not fully defined, and this group may represent one to three species (8, 18). *B. abortus*, *B. melitensis*, *B. suis*, and *B. canis* are well-characterized zoonotic pathogens, annually infecting >500,000 people worldwide (26). In the United States, the first three of these species are defined as select agents due to their pathogenicity and potential use as biological weapons (11).

Despite host-based segregation, *Brucella* spp. have proven challenging to differentiate using molecular techniques. *Brucella* genomes are highly conserved, with >90% homology among species based on DNA-DNA hybridization (35), identical 16S rRNA sequences among all species (15), and >90% of genes sharing >98% sequence identity (16, 27). Serological methods and biochemical testing of isolates allow differentiation of species and biovars. However, PCR-based methods have been used increasingly due to their accuracy, sensitivity, and speed of identification and the ability to work with DNA as opposed to highly infectious live cultures. A wide array of genetic polymorphisms can be assayed for the differentiation of *Brucella* spp., including the insertion element IS*711* (2, 3, 29, 31) and genes of outer membrane proteins (7, 10, 20), and other assay techniques may be used, such as whole-gene differentiation (30), infrequent restriction site PCR (6), and amplified fragment length polymorphisms (37). PCR-based assays for identifying *Brucella* were recently reviewed (1).

Improved resolution among *Brucella* isolates for the purposes of genotyping and epidemiology has been obtained by using more rapidly evolving markers. For example, variable-number tandem repeats (VNTR) incorporated into multilocus VNTR analysis (MLVA) successfully differentiate even closely related isolates and provide fairly accurate species-level resolution (1a, 17, 21, 39). Rapidly evolving VNTR markers often suffer from homoplasy, i.e., the appearance of the same genetic alteration in two or more branches of a phylogenetic tree. These phenomena can disrupt and confound the accurate phylogenetic placement of all isolates within a single MLVA tree and prevent the accurate species-level designation of some isolates.

For distinguishing bacteria with clonally derived population structures (such as *Brucella* [38]), single-nucleotide polymorphisms (SNPs) can be used to accurately describe the phylogenetic framework of a species (28, 33). SNPs can be discovered through either whole-genome comparisons or multilocus sequence typing (MLST) of housekeeping genes. In *Brucella*,

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▽ Published ahead of print on 21 November 2007.

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TABLE 1. Primers used to amplify portions of housekeeping genes and other loci in *Brucella*

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Fragment size (bp)
abc	TAGGCCGAATAATTGCCTTC	GACCGCTACAACGAGCTGAT	419
aroE	ATGGAAGGCAAGATCGTCAA	CTGGCACAGTTCGTCAACAG	498
cysW	CTTCCCTGCACTTCCATCAG	ACCAATCTCATCCAGGCAAG	546
gdh	GATGGTGTCGGTTTTGTGC	GATATGCTGGTGCATTGTGG	557
groEL	CTGGACGACAGCTTCGAGA	GGCTTCCAAGACCAACGATA	485
pip	CGGTCAGGCGCTTGTAAT	CGCATTCATGTCGAGCAAT	484
omp25	TGGTGGCTATACCGGTCTTT	AGGATGTTGTCCGTCAGCTT	384
omp25	AAGTCAAGCAGGGCTTTGAA	ACCGGATGCCTGAAATCCTT[a]	395

[a] Same primer designated as 25B and used by Cloeckaert et al. (9). A portion of the omp_{25} gene was sequenced with two overlapping primer sets. The fragment size is approximate due to a tandem repeat at the 3′ end of the sequence.

these stable and slowly evolving markers provide the initial resolution within taxonomic trees. A nested hierarchical approach involving SNP analysis to define the major branches, followed by analysis using more rapidly evolving VNTR markers, will generate unambiguous differentiation of isolates into major clades, with maximum resolution at the individual isolate level (19). SNP-based differentiation of clades can then be incorporated into real-time PCR assays, providing a quick method for determining specific groupings (23, 32, 34).

We discovered SNPs that use nucleotide sequences from housekeeping genes and published gene sequences, and we developed real-time PCR assays to identify the seven main *Brucella* species. These TaqMan assays contained probes specific to each allele and were screened against a large and diverse collection. Our assays provide a reliable and rapid method for the identification of *Brucella* species that can readily be incorporated into clinical, forensic, or evolutionary applications.

MATERIALS AND METHODS

SNP discovery. To identify species-specific SNPs for the development of real-time PCR assays, we initially selected five housekeeping gene products: the ABC (*abc*) transporter ATP-binding protein, shikimate 5-dehydrogenase and shikimate dehydrogenase (*aroE* and *gdh*) family proteins, and the chaperonin (*groEL*), proline iminopeptidase (*pip*), and sulfate ABC transporter (*cysW*) proteins. Our primary goal was not to run a full MLST analysis but to find suitable SNPs for species differentiation. We also utilized SNPs in genes coding for an outer membrane protein (omp_{25}), the RNA polymerase beta-subunit (*rpoB*), and the anthranilate synthase (*trpE*) (polymorphisms previously described in references 22, 36, and 38, respectively). We designed PCR primers to amplify a portion of each gene (Table 1).

Cellular DNAs were extracted using heat soaks or genomic preparations and were diluted to roughly 0.1 to 1 ng/μl for assay screening. We amplified fragments in 10-μl PCR mixtures consisting of 1× PCR buffer, 2.8 mM $MgCl_2$, 0.6 μM of each primer, 0.8 mM of each deoxynucleotide triphosphate, and 1 U of Platinum *Taq* (Invitrogen, Carlsbad, CA). We used the following cycling profile at 94°C for 5 min: 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min, with a final extension of 72°C for 7 min. We cycle sequenced 1 μl of product in a 10-μl reaction mixture with Applied Biosystems Big Dye 3.1 and then purified with 2.5 μl of 125 mM EDTA and followed with washes of 100% and 70% ethanol. Sequences were run on an AB 3730 model automated sequencer. We edited and aligned sequences using SEQUENCHER 4.6 software (Gene Codes Corporation, Ann Arbor, MI). Sequences were then compared in silico to those of whole-genome sequences from GenBank or unpublished sources from *B. abortus* 2308 (4), *B. abortus* 9-941 (16), *B. melitensis* 16 M (13), *B. suis* 1330 (27), *B. ovis* 63/290 (The Institute for Genomic Research accession numbers CP000708 and CP000709), and *B. canis* RM6/66 (Los Alamos National Laboratory, NM).

Real-time PCR development. Real-time PCR assays were developed with six genes, containing species-specific SNPs: *abc*, *cysW*, omp_{25}, *pip*, *rpoB*, and *trpE*. We designed primers and probes with Primer Express TaqMan minor groove binding (MGB) for Allelic Discrimination version 3.0 (Applied Biosystems, Foster City, CA) software. Primers amplified the region containing the SNP, which binds to either of the two probes for the specific allele of the SNP state (Table 2). Assays were run on an ABI Prism 7900HT sequence detection system (Applied Biosystems). Each 10-μl PCR mixture contained 1× TaqMan Universal master mixture (Applied Biosystems), 0.9 μM of each primer, and 0.2 μM of each probe. In addition, 0.2 U of Platinum *Taq* (Invitrogen) per reaction mixture was added to increase the efficiency of the amplification, except for the *rpoB* assay, which used 0.25 U of Platinum Taq. For the *trpE* assay, the PCR was slightly modified to reduce amplification of the nonmarine mammal probe. The quantity of the probes was set to 0.06 μM of probe 1 (using 6-carboxyfluorescein [FAM] dye) and 0.14 μM of probe 2 (using VIC dye) to optimize the reaction. We ran each assay under standard conditions consisting of a 2-min inactivation at 50°C and a 10-min hot start at 95°C, followed by 40 cycles of a 15-s denaturation and 1 min of annealing at 60°C.

We tested the limits and sensitivity of each assay, using serial 10-fold dilutions. Starting at a concentration of 1 ng/μl, we progressively diluted down to 1 fg/μl. Each sample was run in duplicate, and five samples were tested for each assay. We also quantified the efficiency of the reaction at 1 ng/μl with these 10 samples, as expressed by the cycle threshold values. DNA concentrations were quantified by UV spectroscopy by NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and PicoGreen assays (Molecular Probes, Eugene, OR).

***Brucella* DNA samples.** We screened a diverse collection of 338 *Brucella* DNA samples for each assay. These samples included 165 isolates of *B. melitensis*, 85 isolates of *B. abortus*, 53 isolates of *B. suis*, 11 marine mammal isolates (from eight seals and three dolphins), 10 isolates of *B. canis*, 8 isolates of *B. ovis*, and 6 isolates of *B. neotomae* (Table 3). Samples were known to contain all recognized biovars except for *B. abortus* biovar 3 and *B. suis* biovars 3 and 5. We also included four samples from *Ochrobactrum anthropi*, a closely related soil bacterium, to test the specificity of the assays. All *Brucella* samples had initially been identified using phenotypic, biochemical, and serological tests, including tests such as Gram stain morphology, lack of motility, oxidase positivity, and agglutination in *Brucella* antiserum (5). Over 75% of the samples had also undergone PCR testing for species designation by following procedures described in reference 3.

Nucleotide sequence accession numbers. We deposited all sequences in GenBank under accession numbers EU001373 to EU001657.

RESULTS

SNP identification. We compared sequences for the presence of six housekeeping genes for at least 36 isolates across all seven *Brucella* species. Nineteen SNPs were identified, including four specific to *B. abortus*, three for *B. canis*/*B. suis*, two for *B. ovis* and one for *B. melitensis*. Four of these SNPs were developed into species-specific assays for each species/clade, including two SNPs in the *abc* gene and two SNPs in the *cysW* gene. Additional genes were necessary for species-specific assays for *B. canis*, *B. neotomae*, and the marine mammal clade. No SNPs were found that defined *B. suis* exclusively.

Allelic discrimination and assay screening. All seven SNP assays correctly identified all samples (n = 338) belonging to the appropriate species. However, one sample we believe contained mixed DNA, although we did not have the cell culture to test this hypothesis. In this instance, both species-specific (*B.*

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TABLE 2. Oligonucleotide sequences for primers and TaqMan MGB probes for *Brucella* clade identification

SNP clade	Assay name	Primers (5′–3′)	Probes (5′–3′)[a]	Genome position[b]	SNP identity[c]
B. abortus	*abc*_205	F-GTTTCCGCATCCAAAT GGTT	FAM-AAGCAGAA**T**CTTG CACA	573136	**T**, *B. abortus*
		R-TTCGGGCGGTGAAAAGC	VIC-AAAGCAGAA**G**CTTG		**G**, other spp.
B. suis/*B. canis*	*abc*_246	F-AGCCTGACCTGCTGCT TCTC	FAM-ATGAGCC**C**ACCAAC	573177	**C**, *B. suis*/*B. canis*
		R-GAGCCAGGCTGTGGT TTCC	VIC-ATGAGCC**G**ACCAAC		**G**, other spp.
B. ovis	*cysW*_234	F-CCGGGAAAGCGGAATTTC	VIC-CGAAAGC**G**ATGT TGAT	696894	**G**, *B. ovis*
		R-GCTGACCGCAATCGT TGTC	FAM-CGAAAGC**C**ATGT TGAT		**C**, other spp.
B. melitensis	*cysW*_288	F-GGAAAAAGGTATCTCCAC GAAGGT	VIC-AGCCT**G**CGTCCGGG	696948	**G**, *B. melitensis*
		R-CGTGGCTGGTGACGA AATT	FAM-TGAGGAGCCT**T**CG		**T**, other spp.
B. canis	*omp25*_256	F-GGCTGGCGCCTTTGCT	FAM-AACTTCCAG**A**A GGAC	1297911	**A**, *B. canis*
		R-GGCCCAGGAATAACCT GCAT	VIC-ACTTCCAG**C**AGGACC		**C**, other spp.
B. neotomae	*rpoB*_2673	F-CATCCTGGCGACATTCT TGTC	VIC-AAGATCACGCC**G** AAGG	776263	**G**, *B. neotomae*
		R-GGCGTCATCGGGCTTTC	FAM-TCACGCC**T**AAGGG		**T**, other spp.
Marine mammals	*trpE*_290	F-ACGAGGATTCCTTCGTCC ATAC	FAM-CCAATTATTTCC**A**CC AGAC	468289	**A**, marine mammal
		R-AACGCACGGTGGAAA CCTT	VIC-TTGCCAATTATTTCC **G**CCA		**G**, other spp.

[a] TaqMan probes with a minor groove binder (MGB). Each probe was fluorescently labeled with either VIC or FAM dye.
[b] Based on the genome of *B. melitensis* 16M chromosome I (GenBank accession no. AE008917).
[c] The SNP identities are listed for the primer letters in bold.

melitensis and *B. suis*/*B. canis*) assays indicated a mixed sample, with both alleles amplifying almost equally, suggesting equal amounts of DNA from the corresponding species. We identified the correct species/clade of 11 blind samples and 6 incorrectly labeled samples whose identities were discovered only after the analyses. These samples were identified with their species by conventional biochemical testing and/or were assigned to species by MLVA (17).

Six of the seven assays exhibited rapid amplification of the allele containing the corresponding SNP, with the alternate allele either failing to amplify or weakly amplifying (Table 4). Amplification of the alternate allele above the minimum cycle threshold (C_T) value occurred only in the assays for *B. abortus* and marine mammals. The difference in the C_T values between probes (ΔC_T) in the *B. abortus* assay was 12.3 ($n = 8$), providing easy differentiation. In our initial screening of the marine mammal assay, using standard probe concentrations (0. 9 μM, each probe), the allele for nonmarine mammal samples rapidly amplified at a C_T value of 20.2 ± 0.9 ($n = 5$), and the alternate allele was not detected when nonmarine mammal samples were run. However, cross-hybridization of probes occurred when marine mammal samples were run ($\Delta C_T = 1.6$). Thus, we optimized probe concentrations to improve the differentiation of alleles. When the altered concentrations were used with the marine mammal assay, the ΔC_T was 4.7 between the primary and the alternate probes for marine mammals and 9.9 for samples from nonmarine mammals ($n = 14$).

All assays detected at least one sample with a DNA concen-

TABLE 3. *Brucella* species isolates used in screening assays[a]

Species	No. of isolates of indicated biovar or strek										Total
	1	2	3	4	5	6	7	9	Type strain	None	
B. abortus	41	2	2	4	2	1	1	1	2	29	85
B. canis									1	9	10
B. melitensis	49	15	20						1	80	165
B. neotomae									2	4	6
B. ovis									1	7	8
B. suis	28	1		4					1	19	53
Marine mammal										11	11

[a] *Brucella* species isolates ($n = 338$) used to screen assays are shown. The biovar is given when available.

TABLE 4. Cycle threshold values and detection concentrations for seven *Brucella* species assays[a]

SNP clade	Assay name	C_T value ± SD		Detection concn (fg/μl)[b]
		Allele 1	Allele 2	
B. abortus	*abc*_205	21.1 ± 1.4	19.5 ± 0.8	>10
B. suis/*B. canis*	*abc*_246	19.3 ± 1.1	20.1 ± 0.9	>10
B. ovis	*cysW*_234	17.5 ± 1.3	18.4 ± 1.3	>10
B. melitensis	*cysW*_288	20.9 ± 0.1	21.2 ± 0.5	>10
B. canis	omp_{25}_256	19.3 ± 0.7	19.2 ± 1.1	>10
B. neotomae	*rpoB*_2673	21.3 ± 0.1	26.3 ± 1.2	>100
Marine mammals	*trpE*_290	20.2 ± 0.9	21.2 ± 0.2	>10

[a] C_T values ± standard deviations and detection concentrations are shown for seven *Brucella* species assays. C_T values are from assays run at a concentration of 1 ng/μl ($n = 8$ to 10 isolates).
[b] We considered the positive detection of a sample when the majority of samples successfully amplified within 40 cycles. For the marine mammal assay, detection concentrations were assessed at standard (equal) probe concentrations.

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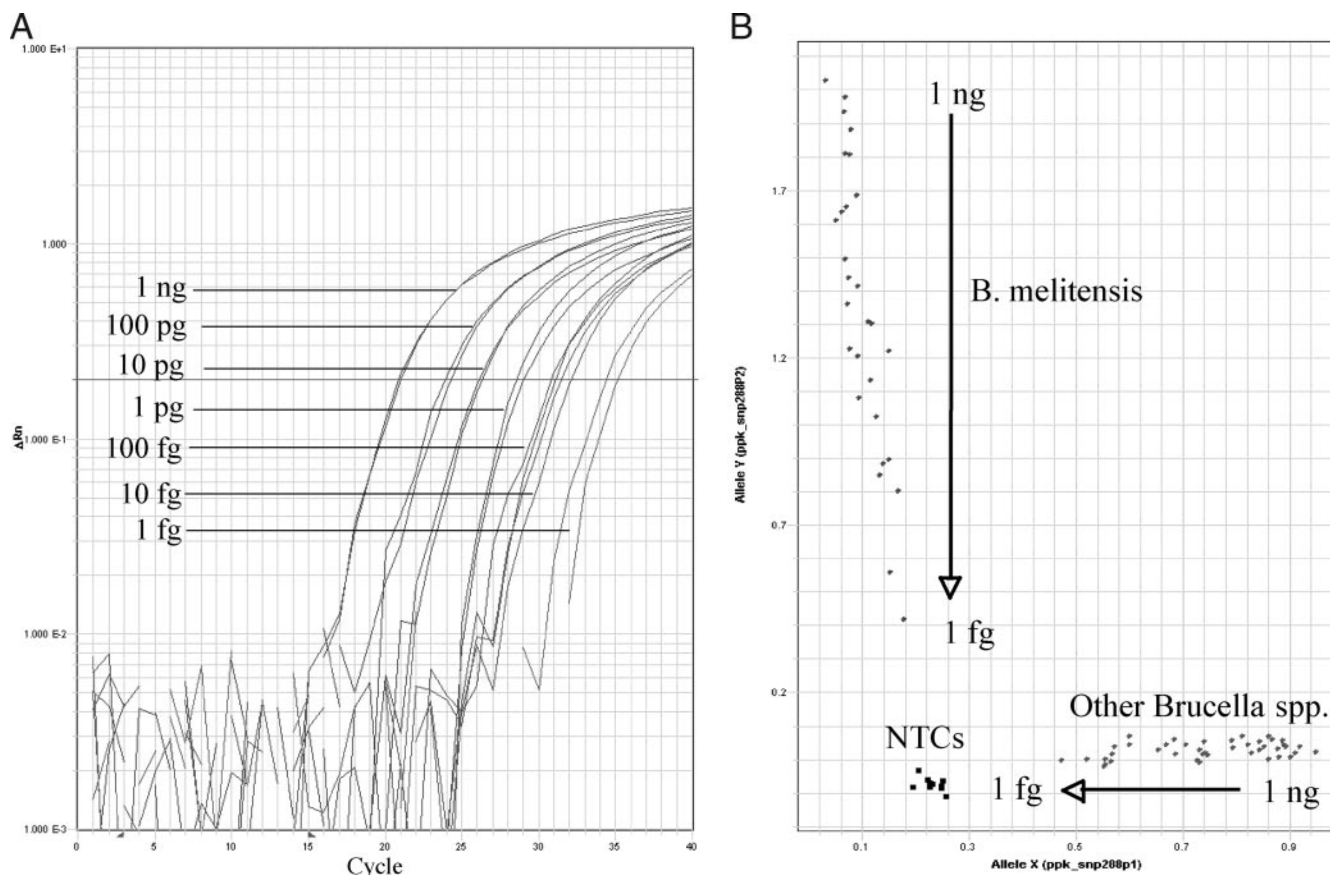

FIG. 1. Real-time PCR and allelic discrimination plots from TaqMan MGB assays for an SNP defining *Brucella melitensis*. Ten samples (4 *B. melitensis* and 6 other *Brucella* species samples) were run at concentrations decreasing from 1 ng/μl to 1 fg/μl. (A) Amplification curves for *B. melitensis* samples run in duplicate. (B) Allelic discrimination plots for all samples. Samples at the top left are *B. melitensis* and at the bottom right are other *Brucella* spp., and at the bottom left, squares near the plot origin are no-template controls (NTCs; $n = 8$). Numbers on the axes represent degrees of differentiation of points and are based on the threshold values of the reactions.

tration of less than 10 fg/μl, with reliable amplification for all assays at 100 fg/μl and greater (Fig. 1). In fact, the majority of samples at a concentration of 1 fg/μl were successfully amplified in six of the seven assays (the assay for *B. neotomae* was less sensitive). In every instance, the amplification was clearly distinguishable from the no-template controls. Samples from the closely related soil bacterium *Ochrobactrum anthropi* failed to amplify in five of the seven assays. In the *B. abortus* assay (*abc*_205), the alternate allele (i.e., all non-*B. abortus* samples) amplified strongly, and in the *B. neotomae* assay (*rpoB*_2673), the alternate allele amplified weakly.

DISCUSSION

The taxonomic description of the *Brucella* species has been accomplished using a broad range of microbiological and molecular approaches. In a clinical setting, working with *Brucella* requires the handling of highly infectious agents, expertise with culturing bacteria on a variety of media, and at least 5 to 7 days under standard microbiological practices. Current molecular genetic techniques for species-level identification are technically challenging and/or are limited to only a few species. Thus, development of quick and reliable methods for identifying *Brucella* species from limited amounts of DNA is crucial for today's clinical and epidemiological applications. Furthermore, determination of the *Brucella* species will allow implementation of appropriate public health interventions in a timely manner.

Our real-time PCR assays provide rapid identification of the seven major *Brucella* clades: *B. abortus*, *B. melitensis*, *B. canis*, *B. suis*/*B. canis*, *B. neotomae*, *B. ovis*, and *Brucella* in marine mammals. SNPs defining *B. suis* exclusively have yet to be found, but this species can be identified using the *B. suis*/*B. canis* assay to assign a sample to this clade and a *B. canis* assay to rule out that it is *B. canis*. Each assay that we have presented is binary within the *Brucella* genus; that is, either the sample is in a particular *Brucella* clade or it is one of the other *Brucella* species. One of the closest known relatives, *Ochrobactrum anthropi*, served as a good negative control and never amplified as the primary allele in any assay. Although the strains used were biochemically similar to those of other fastidious gram-negative coccobacilli, such as *Bordetella bronchiseptica*, *Oligella ureolytica*, or other *Brucella* "mimics" (5), GenBank BLAST searches of the real-time PCR sequences failed to produce significant homology to any genus besides *Brucella*. Thus, it is very unlikely that broader specificity testing with other bacteria would amplify the primary allele in any of the assays.

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The consistent sensitivity of the assays at 10 fg/μl or less represents detection of less than three genome equivalents of DNA. In fact, most of our assays detected DNA at concentrations of 1 fg/μl (less than a genome's equivalent), which is close to the theoretical limit of detection. Similar results have been achieved with *B. abortus* assays with detections of roughly two genome copies of DNA (25). At extremely low concentrations of DNA, a failure of at least 37% of reactions is expected based on Poisson statistics and the likelihood of sampling error (14). Low-level detectability is essential for forensic applications, as well as for environmental sampling and clinical detection. Detecting minute quantities in blood is important for clinical work relating to *Brucella* infections, where small amounts of bacteria may be circulating in the blood. Diagnostic PCR assays from blood must deal with PCR inhibitors such as heme and/or leukocyte DNA, but this can be alleviated through specific lysis and washing procedures (24). We believe our assays can be modified and incorporated into clinical tests to detect and subtype *Brucella* DNAs from human or animal blood samples. Because a false negative could have serious consequences regarding laboratory exposure, when running our assays to test for the presence of *Brucella*, a 16S rRNA control should be included to confirm the presence of bacterial DNA.

Sequencing of whole genomes, housekeeping genes for MLST, and specific other loci have provided the SNPs necessary for the differentiation of various *Brucella* species. To increase our confidence that the SNPs chosen could accurately place each isolate into its appropriate species or clade, assays were tested against a large and diverse collection of 338 isolates. Each additional sample that is screened increases the confidence in the assay. Providing that the assays successfully amplify each allele with minimal cross-hybridization of probes, SNP-based real-time PCR assays are a very reliable method for differentiating species. The conserved distribution of the SNPs used in this study with a relatively large collection of diverse *Brucella* subtypes suggests that these sites are nonhomoplastic. This idea is supported by previous studies that indicate sparse mutation density and rare recombination events and suggests a primary clonal population structure for brucellae (16, 27, 38). The conserved *Brucella* phylogeny is reminiscent of the extensively characterized *Bacillus anthracis* genome, the status of 1,000 SNPs in 27 diverse isolates revealed only a single homoplastic event (33). A more definitive estimate of the extent of homoplasy in *Brucella* organisms can eventually be obtained by similar comparative genomic analysis and SNP discovery in the *Brucella* genomes. The hypothesis that a limited number of SNPs (canonical SNPs) along a conserved phylogenetic branch can represent a large subset of SNPs in *B. anthracis* (19, 28, 33) appears to be similar to the scenario for *Brucella*, for which limited but powerful SNP-based assays provide a strong phylogenetic framework and, combined with MLVA, provide a highly resolved genotyping scheme across a broad spectrum of isolates. Again, SNPs resolve the major *Brucella* species, and MLVA provides finer-scale resolution and genotyping that can be used for epidemiological purposes (1a).

Our samples may or may not have contained isolates from the biovars *B. abortus* biovar 3 (Tulya) and *B. suis* biovars 3 and 5. Of these, *B. suis* biovar 5 represents the most likely challenge to our *B. suis*/*B. canis* assay because strong differentiation of this biovar from other *B. suis* species has been shown by both MLVA (21, 39) and MLST (38). These papers also suggest that *B. suis* is the most diverse clade within the *Brucella* genus. Furthermore, finding SNPs that separate *B. canis* from *B. suis* is challenging due to a high degree of sequence homology that indicates a recent split between these species.

In conclusion, we present an efficient method of determining all currently recognized *Brucella* species that should have broad clinical and forensic applications. Furthermore, samples can be distinguished near the limits of detection. Multiplexing of reactions appears possible in the future so that discrimination of *Brucella* can be achieved with one test. Nonetheless, in many areas of the world, the *Brucella* species that cause infection are likely known, and this test will rapidly confirm species identification.

ACKNOWLEDGMENTS

We thank numerous contributors of DNA to our *Brucella* collection, including Bryan Bellaire, Robert Burgess, Ted Hadfield, Wally Buchholz, Brian Bell, and William Slanta. Tom Brettin of the Los Alamos National Laboratory allowed access to the unpublished *B. canis* genome. James Schupp, Judy Lee, Dawn Birdsell, Jodi Beaudry, Ray Auerbach, Shalamar Georgia, and Tal Pearson provided invaluable technical support.

Funding was provided by an HSARPA grant to Northern Arizona University by the U.S. Department of Homeland Security Science and Technology Directorate.

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JOURNAL OF CLINICAL MICROBIOLOGY, July 2008, p. 2474 Vol. 46, No. 7
0095-1137/08/$08.00+0 doi:10.1128/JCM.00909-08

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ERRATUM

Real-Time PCR Assays of Single-Nucleotide Polymorphisms Defining the Major *Brucella* Clades

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Vol. 46, no. 1, p. 296–301, 2008. Page 298, Table 2, column 6, line 12: "**G**, *B. melitensis"* should read "**G**, other spp."

Page 298, Table 2, column 6, line 14: "**T**, other spp." should read "**T**, *B. melitensis*."

Page 298, Table 3, spanner over columns 2 to 11: "No. of isolates of indicated biovar or strek" should read "No. of isolates of indicated biovar or strain."