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Identification of an IS*711* Element Interrupting the *wboA* Gene of *Brucella abortus* Vaccine Strain RB51 and a PCR Assay To Distinguish Strain RB51 from Other *Brucella* Species and Strains

RAMESH VEMULAPALLI,[1] JOHN R. MCQUISTON,[1] GERHARDT G. SCHURIG,[1] NAMMALWAR SRIRANGANATHAN,[1] SHIRLEY M. HALLING,[2] AND STEPHEN M. BOYLE[1]*

Department of Biomedical Sciences and Pathobiology, Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0342,[1] and National Animal Disease Center, ARS, USDA, Ames, Iowa 50011[2]

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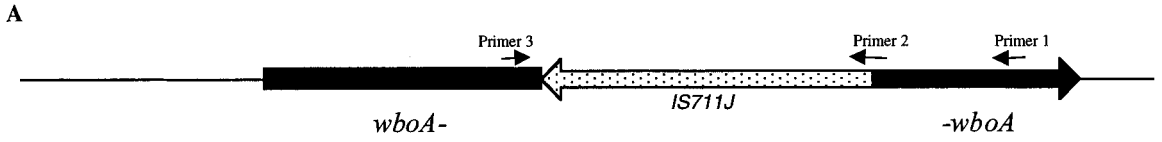
***Brucella abortus* vaccine strain RB51 is a natural stable attenuated rough mutant derived from the virulent strain 2308. The genetic mutations that are responsible for the roughness and the attenuation of strain RB51 have not been identified until now. Also, except for an assay based on pulsed-field gel electrophoresis, no other simple method to differentiate strain RB51 from its parent strain 2308 is available. In the present study, we demonstrate that the *wboA* gene encoding a glycosyltransferase, an enzyme essential for the synthesis of O antigen, is disrupted by an IS*711* element in *B. abortus* vaccine strain RB51. Exploiting this feature, we developed a PCR assay that distinguishes strain RB51 from all other *Brucella* species and strains tested.**

Brucella abortus is one of six well-recognized species of the genus *Brucella* which infects cattle as well as a variety of other mammals including humans (1, 12). Infection with *B. abortus* leads to abortions and reduced fertility in cattle. Vaccination with live, attenuated *B. abortus* strains has been effective in preventing *B. abortus* infections and abortions in cattle. Until recently, strain 19 (S19), a naturally occurring smooth and attenuated strain of *B. abortus*, had been used as the vaccine for cattle brucellosis. Similar to virulent *B. abortus* strains, the lipopolysaccharide of S19 also contains O side chain, which is responsible for an immunodominant antibody response after vaccination or infection with field strains. S19 vaccination usually causes the appearance of a transient serologic titer of antibody to *Brucella* O antigen, and in some vaccinated cattle, these titers of antibody do persist (30). Hence, at least in a few cases, conventional serological techniques cannot be used to clearly distinguish field-infected from S19-vaccinated cattle. *B. abortus* vaccine strain RB51 is a stable, rough, and attenuated mutant that was derived from strain 2308, a smooth and virulent strain of *B. abortus* (25). *B. abortus* RB51 was approved in the United States in 1996 for use as a vaccine for cattle, replacing S19. Since the lipopolysaccharide of *B. abortus* RB51 is devoid of O side chain, antibodies induced by vaccination with this strain do not interfere with the conventional serology (27). The stability and vaccine efficacy of *B. abortus* RB51 have been well studied and documented (8, 9, 16, 18, 22). However, the genetic bases for the rough phenotype and attenuation in this strain are not known. Also, except for a pulsed-field gel electrophoresis-based assay (16), no other DNA-based method to distinguish *B. abortus* RB51 from its parent strain 2308 or similar field strains is available. Previously, we characterized the *wboA* gene of *B. abortus* that encodes glycosyltransferase, an enzyme essential in the biosynthesis of O antigen (19). We also demonstrated that disruption of the *wboA* gene in smooth strains *B. abortus* 2308, *Brucella melitensis* 16M, and *Brucella suis* biovar 4 resulted in conversion to a rough phenotype (19, 29 [in reference 29, the *wboA* gene was designated *rfbU*]). We have discovered that the *wboA* gene in *B. abortus* RB51 is disrupted by an IS*711*-like element. Based on this genetic feature, we have developed a PCR assay that can distinguish RB51 from other *Brucella* species and strains, including its parent, virulent strain 2308.

Interruption of the *wboA* gene by an IS*711* element in *B. abortus* RB51. The *wboA* gene along with the flanking nucleotide sequences was amplified by PCR from the genomic DNAs of *B. abortus* RB51 and 2308. *B. abortus* genomic DNAs were extracted and purified as described previously (14). The primers (forward primer, 5′ GGATGTCGACCAGCCCTCCACA TCAATAGC 3′; reverse primer, 5′ TTGCGGATCCTTTAC TCGTCCGTCTCTTAC 3′) used for the amplification were designed based on the previously described nucleotide sequence of the *wboA* gene from strain 2308 (19) (GenBank accession no. AF107768). PCR was performed with Ready-To-Go PCR beads (Pharmacia Biotech) and a thermal cycler (Hybaid). Each PCR tube contained 0.5 μM (each) primer and 5 ng of genomic DNA in a total volume of 25 μl. Amplification was performed for 40 cycles, each cycle comprising denaturation at 95°C for 1 min, annealing at 53°C for 30 s, and extension at 72°C for 1 min. The amplified products were separated by electrophoresis on a 0.8% agarose gel, stained with ethidium bromide, and viewed under UV light. The amplified product from RB51 genomic DNA was ~3 kb in size, which was ~900 bp larger than that from 2308 (data not shown). The PCR products were cloned in a pCR2.1 vector (Invitrogen, Inc.), and the nucleotide sequences of both strands were determined at the DNA Sequencing Facility of the Iowa State University (Ames). Computer analysis of the nucleotide sequence from RB51 revealed that the *wboA* gene was interrupted by an 842-bp fragment (Fig. 1). A BLAST search (2)

* Corresponding author. Mailing address: Department of Biomedical Sciences and Pathobiology, Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0342. Phone: (540) 231-4641. Fax: (540) 231-3426. E-mail: smboyle@vt.edu.



B

Primer 1 : 5' TTAAGCGCTGATGCCATTTCCTTCAC 3'

Primer 2 : 5' TTTAGTTTGCCGTAATATAGGTCTAGAACCTGTC 3' (*wboA* / *IS711J*)

Primer 3 : 5' GCCAACCAACCCAAATGCTCACAA 3'

FIG. 1. (A) Schematic diagram showing the interruption of the *wboA* gene by an IS*711* element (IS*711J*) in *B. abortus* RB51 and the location of primers (small arrows) used in the PCR assay. (B) Nucleotide sequences of the primers used in the PCR assay.

(BLASTN program) indicated that the 842-bp fragment was almost identical to the previously described *Brucella* IS*711* element (Fig. 2). IS*711* is an insertion sequence of 842 bp initially found in *Brucella ovis* downstream to the gene encoding BCSP31 (13). This element was also discovered and sequenced by Ouahrani and colleagues (20), who designated the element IS*6501*. The element is present in five or more copies in *Brucella* spp. and appears to be quite stable in number and position in the chromosome (5, 6, 14). However, differences in the number of elements have been reported. *B. abortus* biovar 1 has at least six copies of IS*711*, but *B. abortus* 2308 and RB51 have tandem IS*711* copies at one locus (6).

Sequence features of the IS*711* element. The IS*711* element present in the *wboA* gene of *B. abortus* RB51 was designated IS*711J*. Comparison of IS*711J* with the IS*711* element of *B. ovis* indicated 98.6% identity with specific nucleotide sequence differences (Fig. 2). The IS*711J* element is consistent with the IS*711* elements with regard to insertion within the sequence 5′ CTAG 3′ and duplication of the sequence 5′ TA 3′ (13).

Minor sequence variation among the IS*711* copies exists in *B. ovis* (13). The sequence variation occurs at specific loci within the element, with the ends of the elements being much more polymorphic than the coding regions (6a, 13). All the polymorphisms were at sites identified previously by sequencing the common copies of the element in brucellae (4, 6a). Only one of these sites, bp 747, differentiates the sequence of IS*711J* from that of other *B. abortus* IS*711* copies. All the IS*711* copies in *B. abortus*, including IS*711J*, are distinct from the other *Brucella* spp. IS*711* elements because they have an A at positions 2 and 3 in one end of the element. All the rest of the elements have G or C at these positions. Transposition of the IS*711* elements in brucellae does not appear to be limited to a specific copy or originate from a single locus, as unique copies

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IS711     ----------Ggg CTTGTCTGCAtTCaAGGATTCCCTTTTGTACGAAATTCTGATTCAAGGTTGTTaAAGGAGAACAGCCgTGAGCAGACGAAGCCTTAC  90
IS711J    TATAGGTCTAGaa CCTGTCTGCAaTCgAGGATTCCCTTTTGTACGAAATTCTGATTCAAGGTTGTTgAAGGAGAACAGCCtTGAGCAGACGAAGCCTTAC 100

IS711     AGATGAGCAATGGAACCGGATCGAAGCATATCTTCCGGGGCGAGTTGGTACGCCCGGCCGCAGTGGCGTTGATAACCGATTATTTGTCGACGCCATCTTG 190
IS711J    AGATGAGCAATGGAACCGGATCGAAGCATATCTTCCGGGGCGAGTTGGTACGCCCGGCCGCAGTGGCGTTGATAACCGATTATTTGTCGACGCCATCTTG 200

IS711     TGGATGGCTGCCAATGCAGCGCACTGGCGCGATCTGCCTGCGACCTTCGGCAAATGGACAGCGGTTCATGCCCGCTTTCGGCGCTGGTCGCACGCCGGTG 290
IS711J    TGGATGGCTGCCAATGCAGCGCACTGGCGCGATCTGCCTGCGACCTTCGGCAAATGGACAGCGGTTCATGCCCGCTTTCGGCGCTGGTCGCACGCCGGTG 300

IS711     TATGGGAAAGGCTTTTCCATGCCCTGGCTGATACGCCGGACTTTGAATATGTCCTCATTGAtAGCACCATATCGAAAGTCCACGCAGATGCGGCGGGCGC 390
IS711J    TATGGGAAAGGCTTTTCCATGCCCTGGCTGATACGCCGGACTTTGAATATGTCCTCATTGAcAGCACCATATCGAAAGTCCACGCAGATGCGGCGGGCGC 400

IS711     AAAAGGGGGGCTGAAGCTGCCTGCATCGGTCGCTCGCGCGGTGGATTGACGACCAAGCTGCATGCTGTTGTCGATGCTATCGGCCTACCGCTGCGAATAA 490
IS711J    AAAAGGGGGGCTGAAGCTGCCTGCATCGGTCGCTCGCGCGGTGGATTGACGACCAAGCTGCATGCTGTTGTCGATGCTATCGGCCTACCGCTGCGAATAA 500

IS711     AGCCAACACCCGGCCATTATGGTGACTGTCCGCAAGCTTCAAGCCTTCTATCCGGCTTGAAGGGTGTGGGGCATGTCATTGCTGATGCAGCCTATGATGC 590
IS711J    AGCCAACACCCGGCCATTATGGTGACTGTCCGCAAGCTTCAAGCCTTCTATCCGGCTTGAAGGGTGTGGGGCATGTCATTGCTGATGCAGCCTATGATGC 600

IS711     CGATCACTTAAGGGCCTTCATTGCCAGCaATCTCAAGGCAACGGCTCAGATCAAGGtCAATCCAACACGTTCCAGTGtCCCAACAATCGACTGGAGGCTG 690
IS711J    CGATCACTTAAGGGCCTTCATTGCCAGCgATCTCAAGGCAACGGCTCAGATCAAGGcCAATCCAACACGTTCCAGTGcCCCAACAATCGACTGGAGGCTG 700

IS711     TACAAGGAACGCCATCAGATTGAATGCTTTTTTAACAAGTTGAAACGCTATCGTCGtATTGCGCTGCGATGCGAGAAAACATTGACCGCATTCATGGGcT 790
IS711J    TACAAGGAACGCCATCAGATTGAATGCTTTTTTAACAAGTTGAAACGCTATCGTCGcATTGCGCTGCGATGCGAGAAAACATTGACCGCATTCATGGGtT 800

IS711     TCGTCCATCTCGCATGCGCTATGATCTGGTTaCGTTGAATGCAGACACGCCC----------  842
IS711J    TCGTCCATCTCGCATGCGCTATGATCTGGTTgCGTTGAATGCAGACACGCCCTAGCGCTTGA  862
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FIG. 2. Comparison of the sequences of IS*711* (accession no. M94960) and the IS*711*-like element, IS*711J*, interrupting the *wboA* gene in *B. abortus* RB51. Ten base pairs of sequence flanking IS*711J* is also shown. Nucleotide residues varying between the two elements are indicated by lowercase, boldface, and underlining.

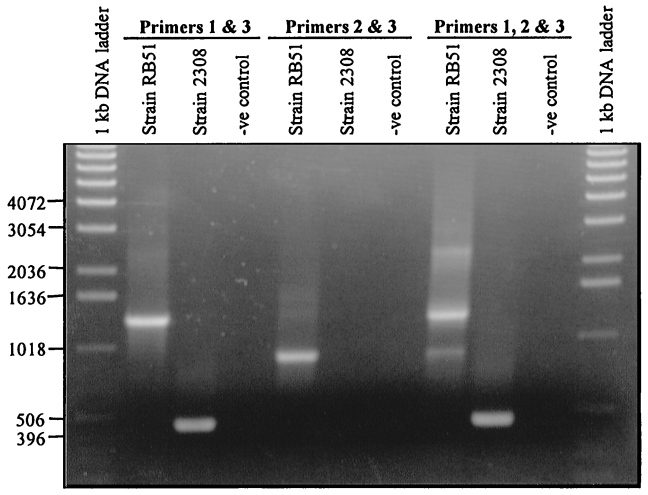

FIG. 3. Differentiation of *B. abortus* RB51 from its parent strain 2308 by a *wboA* gene-based PCR assay. PCR amplifications with the indicated primer pairs were performed with the purified genomic DNA from strains RB51 and 2308 as templates. Negative (−ve) controls contained no template DNA. The amplified products were separated on a 0.8% agarose gel, stained with ethidium bromide, and photographed under UV light. Numbers at left indicate the 1-kb DNA ladder fragment sizes in base pairs.

of the element in *B. ovis* and IS*711* of *B. abortus* vary in sequence.

***B. abortus* RB51-specific PCR assay.** Exploiting the nature of *wboA* gene disruption by IS*711*, we developed a PCR assay that can distinguish *B. abortus* RB51 from all other *Brucella* species and strains. Based on computer analysis (Primer Select program, LaserGene software; DNAStar Inc.), two primers, primers 1 and 3 (Fig. 1), were selected so that the amplified fragment from strain RB51 is ~1,300 bp and the fragments from all other *Brucella* species (assuming an intact *wboA* gene) are ~400 bp. An additional primer, primer 2 (Fig. 1), was selected manually to encompass the junction between the *wboA* gene and the 5′ end of IS*711*. Five nanograms of purified *Brucella* genomic DNA was used as template for the PCR amplification. In some cases (see Table 1), a medium-sized (2 mm in diameter) bacterial colony was taken from an agar plate and resuspended in 200 μl of sterile distilled water, incubated in a boiling water bath for 15 min, and centrifuged for 5 min at 10,000 × *g*, and 10 μl of the supernatant was used as template. PCR amplifications were performed in a 25-μl total volume with Ready-To-Go PCR beads. Amplification was performed for 40 cycles, each cycle comprising denaturation at 95°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1.5 min. These parameters were selected after several trials to optimize the conditions for appropriate stringency (as determined by the absence of any undesired nonspecific bands) and better yield of the amplified product(s). Three different PCR amplifications were performed with primer combinations of primers 1 and 3; primers 2 and 3; and primers 1, 2, and 3. In reaction mixtures containing two primers, 0.5 μM (each) primer was included. Whereas in reaction mixtures containing all three primers, 0.5 μM (each) primers 1 and 2 and 1 μM primer 3 were included. Initial PCR amplifications were performed with genomic DNA from strains RB51 and 2308. As shown in Fig. 3, different sizes of fragments were amplified when primers 1 and 3 were used (~1,300-bp fragment from RB51 and ~400-bp fragment from 2308). Primers 2 and 3 amplified a 900-bp fragment from the RB51 genomic DNA but none from that of 2308.

When all three primers were used in the reaction, fragments of expected sizes were amplified (400 bp from 2308 and 900 and 1,300 bp from RB51). In addition, a band of ~2.3 kb in size was also amplified in RB51 (Fig. 3). The 900-bp and the

TABLE 1. Bacterial strains used in the PCR assay[a]

Bacterial strain	Description	Source	PCR template
B. abortus RB51	Rough, derived from 2308 (25)	VPI, Blacksburg, Va.	Bact,[b] DNA
B. abortus 2308	Smooth, virulent	VPI, Blacksburg, Va.	Bact, DNA[b]
B. abortus 19	Smooth, attenuated	VPI, Blacksburg, Va.	Bact,[b] DNA
B. abortus 45/20	Intermediate[c]	VPI, Blacksburg, Va.	Bact
B. abortus field strains			
Biovar 2	Smooth	NADC, Ames, Iowa	DNA
Biovar 3	Smooth	NADC, Ames, Iowa	DNA
Biovar 4	Smooth	NADC, Ames, Iowa	DNA
Biovar 5	Smooth	NADC, Ames, Iowa	DNA
B. melitensis 16M	Smooth	VPI, Blacksburg, Va.	Bact, DNA[b]
B. melitensis Rev1	Smooth	VPI, Blacksburg, Va.	Bact,[b] DNA
B. melitensis B115	Rough, O antigen in cytoplasm (11)	VPI, Blacksburg, Va.	Bact,[b] DNA
B. melitensis biovar 3	Smooth	NADC, Ames, Iowa	DNA
B. suis biovar 2	Smooth	NADC, Ames, Iowa	DNA
B. suis biovar 3	Smooth	NADC, Ames, Iowa	DNA
B. suis biovar 4	Smooth	NADC, Ames, Iowa	DNA
B. canis RM6/66	Rough	VPI, Blacksburg, Va.	Bact
B. ovis	Rough	VPI, Blacksburg, Va.	Bact
Brucella neotomae	Smooth	NADC, Ames, Iowa	DNA
Ochrobactrum anthropi strains			
49237		ATCC, Manassas, Va.	Bact, DNA
49188		ATCC, Manassas, Va.	Bact, DNA
Yersinia enterocolitica O:9		VPI, Blacksburg, Va.	Bact

[a] Abbreviations: Bact, bacteria; VPI, Virginia Polytechnic Institute and State University; NADC, National Animal Disease Center; ATCC, American Type Culture Collection.

[b] Templates used for the PCR amplifications are shown in Fig. 3 and 4.

[c] Strain 45/20 is referred to as intermediate since this strain expresses variable amounts of O side chain (24) and can convert to the smooth phenotype (10).

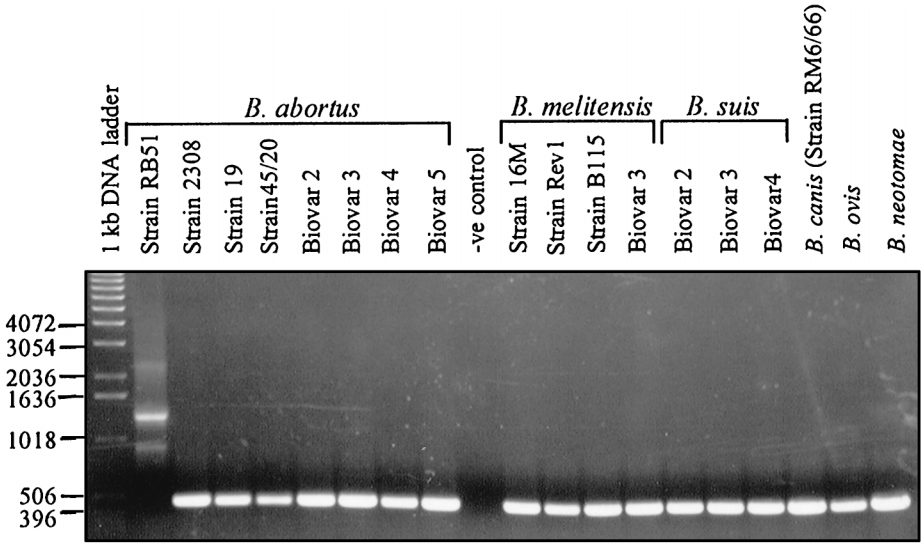

FIG. 4. PCR assay to differentiate *B. abortus* RB51 from all other *Brucella* species and strains. Primers 1, 2, and 3 were used in all PCR amplifications. Genomic DNA from the indicated *Brucella* strains was used as template. Negative (−ve) controls contained no template DNA. The amplified products were separated on a 0.8% agarose gel, stained with ethidium bromide, and photographed under UV light. Numbers at left indicate the 1-kb DNA ladder fragment sizes in base pairs.

2.3-kb bands in strain RB51 were of lower intensity, indicating that there was some inhibition in the amplification. Adjustments of several parameters, including the concentration of Mg^{2+}, primers, deoxynucleoside triphosphates, or changes in annealing temperature, did not result in either enhancement of the amplified products or absence of the 2.3-kb fragment. The low level of amplification of the 900-bp fragment is most probably due to the 5′→3′ exonuclease activity of *Taq* DNA polymerase; while extending primer 1, *Taq* DNA polymerase could have degraded the DNA strand that was being extended from primer 2 (primers 1 and 2 bind to the same template in strain RB51 [Fig. 1]) (15). Some of the single-stranded DNA fragments resulting from the degradation of the DNA strand that was initiated by primer 2 might have primed for the amplification of the 2.3-kb fragment. This appears likely, since the ~2.3-kb band appeared only when all three primers were used in the amplification reaction. No such problem is encountered with strain 2308, since primer 2 cannot bind to the template; again this supports, though indirectly, the above hypothesis for the low level of amplification of the 900-bp fragment and the appearance of the 2.3-kb band in the case of RB51. We tested the specificity of this strain-specific PCR assay with various *Brucella* strains (Table 1). As shown in Fig. 4, when the three primers were used for the PCR assay, all the other *Brucella* strains tested gave an amplified product of 400 bp in size; identical results were obtained by using only primers 1 and 3 (data not shown). No amplified products were detectable when the template was genomic DNA from bacteria that are closely related to *Brucella* species, *Ochrobactrum anthropi* 49237 and 49188. Also, no products could be amplified from the genomic DNAs of *Yersinia enterocolitica* O:9, which synthesizes O antigen that is identical to that of *Brucella* (reference 7 and data not shown). Based on these results, we recommend the PCR assay with primers 1 and 3 to distinguish strain RB51 from all other *Brucella* strains. It should be mentioned that several attempts to clone the 2.3-kb fragment present in the amplified products of strain RB51 were unsuccessful. Amplification of the 400-bp fragment from *B. ovis* and *Brucella canis* indicates that these naturally rough species contain the *wboA* gene sequences. However, further studies are needed to confirm the intactness and functionality of the *wboA* gene in these species, since the mere presence of a gene sequence does not necessarily result in expression of a functional product.

Even though the stability of RB51 is well proven in vivo and in vitro, the actual genetic mutation(s) that contributed to the rough phenotype and attenuation of this strain has not been identified until now. This study describes the first such mutation in the *wboA* gene. It is clear from previous studies that deletion of the *wboA* gene in *B. melitensis*, *B. abortus*, and *B. suis* leads to the rough phenotype and attenuation (19, 29). Ongoing studies in our laboratory indicated that complementation of RB51 with a functional *wboA* gene resulted in O antigen production but did not result in reversion to the smooth phenotype and did not affect attenuation (unpublished data). This suggests that RB51 contains an additional genetic mutation(s) that probably affects either the export of O antigen to the bacterial surface, the coupling of O antigen to core lipopolysaccharide, or both.

Recently, several PCR assays to detect or differentiate various *Brucella* strains have been reported (3, 5, 6, 17, 21, 23, 26, 28). However, none of these assays could distinguish RB51 from its parent, virulent strain 2308. A PCR assay with primers 1 and 3, as described in this paper, can be used to quickly identify RB51; hence, it should be useful in studies where detecting the presence of RB51 is needed, such as those with the risk of potential abortions, which can occur if pregnant animals are vaccinated with RB51, and in studies where the fate of RB51 has to be determined after vaccination of bison and other wild as well as domestic animals. We successfully used this PCR assay to quickly verify the presence or absence of RB51 among field *Brucella* isolates cultured from aborted bovine fetuses (unpublished data).

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