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Determination of Stability of *Brucella abortus* RB51 by Use of Genomic Fingerprint, Oxidative Metabolism, and Colonial Morphology and Differentiation of Strain RB51 from *B. abortus* Isolates from Bison and Elk

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Brucella abortus **RB51 and isolates from cattle, bison, and elk were characterized by pulsed-field gel electrophoresis and standard techniques for biotyping *Brucella* species, which included biochemical, morphological, and antigenic techniques, phage susceptibility, and antibiotic resistance. The objectives were to ascertain the stability of RB51 and to differentiate RB51 from other brucellae. Genomic restriction endonuclease patterns produced by pulsed-field gel electrophoresis demonstrated a unique fingerprint for RB51 relative to other brucellae. Comparisons of the oxidative metabolic profiles of RB51 after time in vivo (14 weeks) and in vitro (75 passages) showed no change in characteristic patterns of oxygen uptake on selected amino acid and carbohydrate substrates. Strain RB51 was biotyped as a typical rough *B. abortus* biovar 1 (not strain 19) after animal passage or a high number of passages in vitro and remained resistant to rifampin or penicillin and susceptible to tetracycline. No reactions with A or M antiserum or with a monoclonal antibody to the O antigen of *Brucella* lipopolysaccharides were detected; however, RB51 agglutinated with R antiserum. The results indicate that the genomic fingerprint and rough colonial morphology of RB51 are stable characteristics and can be used to differentiate this vaccine strain from *Brucella* isolates from cattle, bison, and elk.**

Brucella abortus RB51 has been proposed as a vaccine to replace strain 19, which is currently used in the eradication program against brucellosis in domestic cattle. Cattle vaccinated with RB51 are protected from abortion following experimental challenge (9) and have cell-mediated immune responses similar to those of cattle vaccinated with strain 19 (34). In contrast to strain 19, RB51 does not induce antibodies against the O polysaccharide of *Brucella* lipopolysaccharides (LPS) that react immunologically in tests for detecting naturally acquired brucellosis (7, 9, 31, 33). In addition, unlike strain 19-vaccinated cattle, cattle vaccinated with RB51 fail to develop cutaneous reactions against brucellin thought to be prompted by LPS (8).

Smooth strains of *Brucella* have an LPS (endotoxin) which is implicated in the pathogenesis of brucellosis (1, 21). Strain RB51 has a rough colonial morphology and lacks the O polysaccharide of LPS (31). Changes in colonial morphology (i.e., smooth to rough) in *Brucella* species are associated with changes in infectivity and antigenicity (4). Reversion to a smooth colonial morphology and virulence has been reported for the rough strain 45/20 (18, 35) but not for RB51.

Survival of the vaccine strain is crucial for cattle to develop a protective cellular immune response against *B. abortus* (7). After vaccination, RB51 persists for several weeks in superficial cervical lymph nodes of cows. In mice, the organism persists up to 4 weeks postinoculation (31). Reversion of RB51 has not been demonstrated after 15 passages in mice (31), after experimental infection in pregnant goats (29), and even after 93 passages in vitro (31). However, the ability of RB51 to remain stable after long periods of time in cattle has not been determined.

Brucellae are identified by routine techniques that are used for biotyping (4). Each species of *Brucella* has a characteristic pattern of oxygen uptake on selected amino acid and carbohydrate substrates (20). Recently, genomic fingerprinting by pulsed-field gel electrophoresis (PFGE) of *Xba*I-digested DNA has been reported to provide a reliable means of differentiating RB51 from field isolates of *B. abortus* biovars 1, 2, and 4 from cattle and several isolates of biovar 1 from bison and elk (16).

Bison (*Bison bison*) and elk (*Cervus elaphus*) are significant reservoirs of *B. abortus* (22, 27, 37, 38, 41); because of the potential social impact of brucellosis on bison and elk in the national parks and the economic relevance of possible transmission of the disease from bison or elk to nearby domestic livestock, this study was designed to evaluate the phenotypic and genotypic characteristics of the proposed vaccine strain, RB51. The objectives of this study were to assess the stability of RB51 as determined by the use of genomic fingerprint, oxidative metabolism, and colonial morphology and to differentiate this strain from isolates of *Brucella* primarily from bison and elk.

MATERIALS AND METHODS

Brucellae. *B. abortus* RB51 was obtained from G. G. Schurig (Virginia Polytechnic Institute and State University, Blacksburg, Va.). The seed was expanded to produce a new master seed stock, referred to as ARS/1. This stock has been used since 1990 for experiments conducted in the Brucellosis Cattle Test Program at the National Animal Disease Center, Ames, Iowa.

The U.S. Department of Agriculture National Veterinary Services Laboratories (Ames, Iowa) routinely obtain cultures and tissue samples from wildlife in the United States for the identification of *Brucella* species. Included in this study

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TABLE 1. Characteristics of *B. abortus* RB51, 19, and typical biovar 1

Characteristic	*B. abortus*					
	Typical biovar 1	RB51	RB51/HP	RB51/cow	RB51/bison	19
CO_2 for growth	±	−	−	−	−	−
H_2S produced	+	+	+	+	+	+
Urease	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Growth on medium containing:						
Basic fuchsin	+	+	+	+	+	+
Thionin	−	−	−	−	−	−
Thionin blue	+	+	+	+	+	−
Penicillin	+	+	+	+	+	−
Erythritol	+	+	+	+	+	−
Antigen	A	R	R	R	R	A
Lysis by phage	+	−	−	−	−	+

were samples from the states of Wyoming, Montana, South Dakota, and North Dakota. A total of 86 isolates of *B. abortus*, which includes 51 from bison and 35 from elk, were obtained and characterized. Stock cultures of each isolate were passaged no more than three times before freezing (−80°C) or lyophilization in World Health Organization preservation medium (4). *B. abortus* 19 was obtained from R. D. Angus (National Veterinary Services Laboratories). The following strains were obtained from the National Animal Disease Center culture collection: *B. abortus* 2308, 2308R, 19R, and 45/20; *B. melitensis* 16M; *B. canis* RM-666; and *B. ovis* 63/290.

Isolates of RB51 were recovered from animal tissues sampled during vaccination projects with cattle (7) and bison (25). The animals were part of experiments to determine the effects of vaccination with RB51 against infection and abortion attributable to *B. abortus*. Strain RB51 was isolated from biopsies of superficial cervical lymph nodes of animals vaccinated with RB51 (7). In vitro passage of RB51 was 75, 96, or 159 transfers on artificial medium established during a period of 1.5 to 2.5 years.

Growth of bacteria. Working cultures of brucellae were grown for 44 h at 37°C on potato infusion agar or on tryptose agar (Difco Laboratories, Detroit, Mich.) containing 5% bovine serum (TSA) before storage at 5°C. Brucellae from tissue samples were grown on TSA; on TSA with antibiotics (cycloheximide [30 μg/ml], bacitracin [7.5 U/ml], and polymyxin B sulfate [1.8 U/ml]) (4); on TSA with antibiotics and ethyl violet (1:800,000) (4); on Ewalt's medium (11); and on Farrell's medium (12).

Typing of brucellae. Brucellae were identified by routine typing techniques used for *Brucella* biotyping (4). Conventional methods used for characterizing the growth of *Brucella* isolates were performed as recommended by the Food and Agriculture Organization of the United Nations and the World Health Organization (4). Characteristics of routine typing included tests for urease, H_2S production, catalase, CO_2 dependency, sensitivity to lysis by Tbilisi (Tb) bacteriophage, agglutination reactivity with A-, M-, and R-monospecific antisera, and growth in the presence of basic fuchsin, thionin, thionin blue, penicillin, or erythritol.

Antibiotic sensitivity testing was done by using modifications of the agar diffusion method (5). Discs containing antibiotics (Difco) were placed on TSA, and zones of inhibition were determined after 48 h of growth at 37°C. The antibiotics used were penicillin (10 U), rifampin (5 μg), amikacin (30 μg), ampicillin (10 μg), carbenicillin (100 μg), doxycycline (30 μg), gentamicin (10 μg), tetracycline (30 μg), kanamycin (30 μg), streptomycin (10 μg), novobiocin (30 μg), trimethoprim-sulfamethoxazole (1.25 and 23.75 μg, respectively), and tobramycin (10 μg).

DNA fingerprinting. DNA fingerprinting was done as previously described (16). *Brucella* DNA was extracted by using modifications of the methods of Allardet-Servent et al. (2). *Brucella* DNA in the agar inserts was digested with 10 U of restriction endonuclease for 48 h at 37°C in 200-μl volumes containing the restriction endonuclease in the appropriate buffer, according to the instructions of the manufacturer (GIBCO Laboratories, Grand Island, N.Y.). The restriction endonucleases (GIBCO) *Xba*I, *Afl*II, *Xho*I, *Spe*I, *Ssp*I, and *Dra*I were used. DNA preparations in agarose were stored in 0.05 M EDTA (pH 8.0) at 4°C.

Digested DNA was electrophoresed by PFGE (32) in 1.5% agarose (Stratagene, La Jolla, Calif.) with TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M Na_2EDTA) (30) and the clamped homogeneous electric field system (CHEF-DRII; Bio-Rad Laboratories, Richmond, Calif.). The pulse times were 0.3 to 1.2, 0.3 to 10, or 1 to 20 s for 40 h at 200 V. Lambda concatemers (New England Biolabs, Beverly, Mass.), chromosomes of *Saccharomyces cerevisiae* (Bio-Rad), and lambda phage digested with *Hin*dIII (Bio-Rad) were used as size markers. DNA visualized with UV illumination after staining with ethidium bromide (0.5 mg/ml) was photographed. Fragment sizes were determined with a computer-assisted analysis program by BioImage Products (Millipore Corporation, Ann Arbor, Mich.).

Oxidative patterns. Conventional manometric techniques were used to determine oxygen uptake for brucellae (20). Cells grown in tryptose agar roux flasks that had been incubated for 48 h at 37°C in 10% CO_2 in air were suspended in Sorenson's buffer, washed, and spectrophotometrically standardized to 45% transmittance ± 2% (optical density at 600 nm). Substrates were dissolved in Sorenson's 0.06 M phosphate buffer and adjusted to pH 7.0 when necessary. The standardized cell suspensions were then added to individual flasks. Each flask contained 1.0 ml of cell suspension, 0.5 ml of buffer containing 5 mg of the desired substrate, 1.4 ml of buffer, and 0.1 ml of 20% KOH in the center well. Data were presented as microliters of oxygen used per hour per milligram of cell nitrogen [$QO_2(N)$], with endogenous respirator rates subtracted.

Colonial morphology. Monitoring for the degree of smoothness was accomplished by using obliquely reflected light (15), acriflavine (6), crystal violet (40), specific antiserum (3, 4), and Tb bacteriophage (17).

Individual clones of RB51 were examined to ascertain if inducibility into smooth colonial forms would occur. This was done by growing RB51 on TSA for 72 h at 37°C. The resulting growth was harvested in 10.0 ml of 0.85% NaCl and adjusted to contain 2×10^3 CFU/ml. This suspension (0.5 ml) was plated onto 10 plates of TSA containing 0, 0.9, 1.8, or 3.6 U of polymyxin B per ml. After 72 h of incubation, colonies were examined with obliquely reflected light, plates were flooded with crystal violet (40) for 15 to 20 s. Approximately 30 unstained colonies were selected and inoculated onto separate slants, and each was examined as a separate strain. This procedure was repeated.

Micro-dot blot. Detection of the O antigen of smooth *Brucella* LPS was done by immunoblotting. Bacteria were fixed overnight in 67% methanol, dotted (0.5 μl) onto nitrocellulose membranes (0.45-μm-pore size; Schleicher & Schuell, Inc., Keene, N.H.), and allowed to dry. Membranes were immersed in 10 mM phosphate-buffered saline (PBS; pH 7.2) containing 0.05% Tween 20 (TPBS) and 0.9% gelatin (Difco) and incubated for 30 min at room temperature. A monoclonal antibody to LPS (26) was applied, and the membranes were incubated for 1 h at 37°C. Immunocomplexes were detected with biotinylated rabbit anti-mouse immunoglobulin G (Vector Laboratories, Burlingame, Calif.) followed by Vectastain ABC reagent (Vector) with 4-chloro-1-naphthol (Bio-Rad) as the substrate. *B. abortus* 2308 and *B. melitensis* 16M were used as positive controls, and *B. canis* RM-666 and *B. ovis* 63/290 were used as negative controls.

RESULTS

Bacteriological analysis. *Brucella* isolates from bison and elk were biotyped as *B. abortus* biovar 1 (not strain 19). Strain RB51 and laboratory and animal passages of RB51 differed from the typical *B. abortus* biovar 1 (Table 1) in that they did not contain the A or M antigen, were not lysed by Tb bacteriophage, and lacked the O antigen as determined by immunoblot. Field isolates of *B. abortus* biovar 1 usually required CO_2 for growth, whereas strains RB51, 19, and 2308 did not. The production of H_2S was weak in 2308 compared with that in field isolates. Strain 19 did not grow in the presence of thionin blue, penicillin, and erythritol.

The determination of antibiotic susceptibility by using discs showed that RB51 remained resistant to rifampin after in vivo

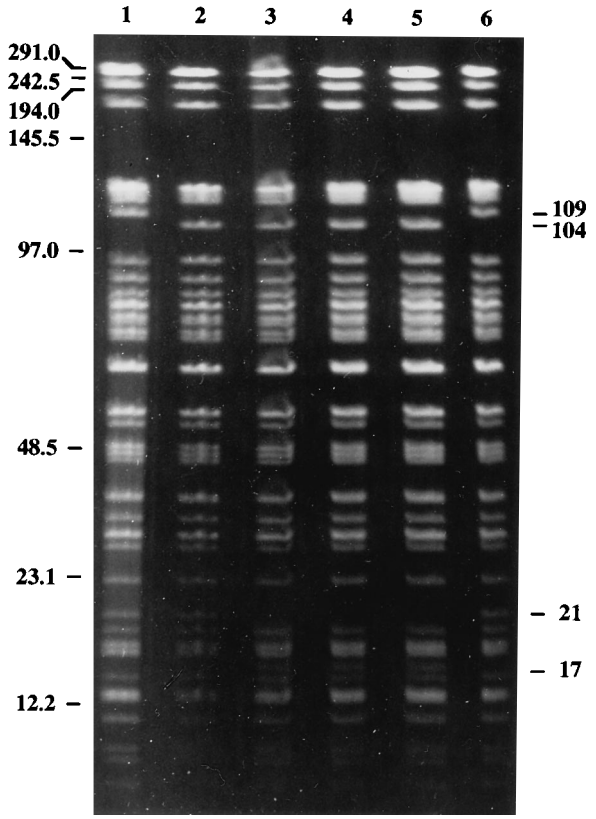

FIG. 1. PFGE (pulse time, 0.3 to 10 s) of *Xba*I digests of DNA from *B. abortus* strains. Lanes: 1, 45/20; 2, 2308; 3, RB51; 4, RB51 from cattle (8 weeks postvaccination); 5, RB51 from cattle (10 weeks postvaccination); 6, 19 from cattle (8 weeks postvaccination). The sizes (in kilobases) of discerned fragments are indicated on the right. The positions of size markers (in kilobases) are indicated on the left.

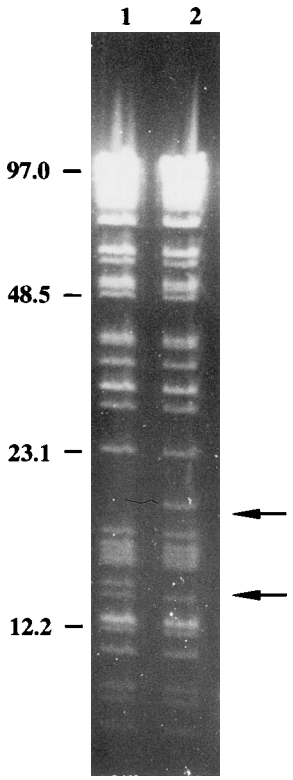

FIG. 2. PFGE (pulse time, 0.3 to 1.2 s) of *Xba*I digests of DNA from *B. abortus* RB51 (lane 1) and 2308 (lane 2). Arrows indicate differentiating bands. The positions of size markers (in kilobases) are indicated on the left.

and in vitro passages. These isolates did not show cross-resistance to structurally unrelated therapeutic agents. Strain RB51 showed susceptibility to tetracycline, amikacin, carbenicillin, doxycycline, gentamicin, kanamycin, penicillin, streptomycin, and tobramycin, was resistant to trimethoprim-sulfamethoxazole, and had intermediate susceptibility to ampicillin and novobiocin. However, isolates of RB51 before and after in vivo and in vitro passages grew on tryptose agar containing penicillin (5 U/ml), whereas strain 19 did not.

Restriction enzyme analysis. The restriction endonucleases that were used varied in their ability to enable discrimination between isolates. The DNA fingerprints of strains RB51, 19, and 2308 were specifically compared by using the restriction endonucleases *Xba*I, *Afl*II, *Xho*I, *Spe*I, *Ssp*I, and *Dra*I. Among these enzymes, only *Xba*I enabled the differentiation of RB51 from 19 and 2308 (Fig. 1). No discriminative profiles were detected when *Afl*II, *Xho*I, *Spe*I, *Ssp*I, and *Dra*I were used (data not shown); therefore, *Xba*I was used throughout the study. The reproducibility of the technique was established by repeat testing, which yielded identical fingerprint patterns.

Gels that ran at different pulse times gave similar comparative results and provided better separation for a given size range. Shorter pulses resolved smaller fragments of DNA (Fig. 2); longer pulses resolved the larger fragments. The consistency of the profile for RB51 was determined by testing the same isolate on four separate occasions.

Analysis using large restriction fragments produced by digesting genomic DNA with *Xba*I and separating these fragments by PFGE allowed the visual differentiation of RB51 from *Brucella* field isolates. Macrorestriction fingerprinting with the use of *Xba*I produced three fragments in the size range of 150 to 250 kb, and multiple fragments smaller than 125 kb were generated. Fragment patterns were conserved among the field isolates of *B. abortus* from cattle, bison, and elk (data not shown). Differentiation of RB51 and 2308 from field isolates and strain 19 was based on variable bands at 109, 104,

TABLE 2. Characteristics distinguishing *B. abortus* RB51 from strains 2308 and 19 and typical biovar 1 strains

Characteristic	*B. abortus*			
	RB51	2308	Typical biovar 1	19
*Xba*I fragment				
109 kb	−	−	+	+
104 kb	+	+	−	−
21 kb	−	+	+	+
17 kb	+	−	−	−
Colonial morphology	Rough	Smooth	Smooth	Smooth
Rifampin susceptibility	−	+	+	+
Lysis by phage	−	+	+	+
Agglutination in serum				
A	−	+	+	+
M	−	−	−	−
R	+	−	−	−
Immunoblot[a]	−	+	+	+
CO_2 requirement	−	−	±	−

[a] Immunoblot response to monoclonal antibody to LPS.

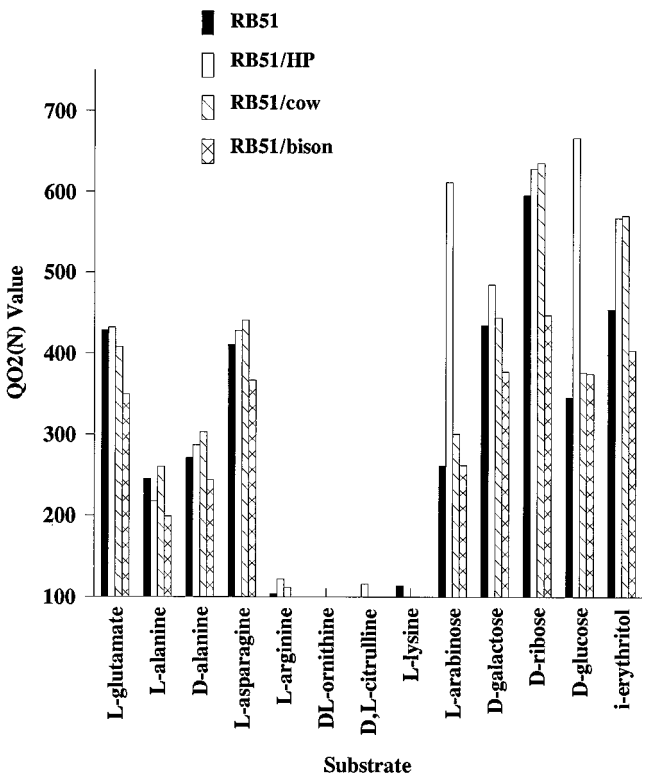

FIG. 3. Oxidative metabolic profiles of *B. abortus* RB51 before and after in vivo (in cattle or bison) or in vitro (high number of passages [HP]) passages. Rates of oxygen uptake on selected amino acid and carbohydrate substrates are reported as $QO_2(N)$ values (microliters of oxygen uptake per milligram of nitrogen per hour).

21, and 17 kb (Fig. 1; Table 2). Strain RB51 differed from 2308 in that it had a 17-kb fragment instead of a 21-kb fragment (Fig. 2). The profiles of strains 19 and RB51 remained the same for isolates from vaccinated cattle or bison.

Long-term laboratory passage of RB51, 19R, and 2308R produced no detectable changes in the restriction endonuclease patterns (data not shown). Animal passage of RB51 did not show any change in the profile (Fig. 1).

Oxidative metabolism. Graphic representation of the oxidative metabolic profiles of RB51, RB51 after a high number of passages (RB51/HP), RB51 from cattle (RB51/cow), and RB51 from bison (RB51/bison) had similar profiles (Fig. 3). These isolates had the oxidative metabolic pattern typical of *B. abortus* on 13 of 13 substrates which are currently used for differentiating species and biovars of *Brucella*. The $QO_2(N)$ rates of RB51 were compared with those of strain 19 and field isolates of *B. abortus* biovars 1, 2, and 4 (Table 3).

Strain RB51, whether of repository origin (ARS/1) or isolated from animals, utilized L-glutamic acid, L-alanine, D-alanine, and L-asparagine. The substrates of the urea cycle (L-arginine, DL-ornithine, and D,L-citrulline) and L-lysine were not substantially oxidized by any of the RB51 isolates. The carbohydrates tested (L-arabinose, D-galactose, D-ribose, D-glucose, and i-erythritol) were all utilized by the RB51, RB51/cow, RB51/bison, 2308, and *B. abortus* biovars 1, 2, and 4. Strain 19 is distinct from the rest of the isolates of *B. abortus* biovars 1, 2, and 4 in that it typically fails to utilize i-erythritol. The oxidative metabolic rate for the high-passage in vitro RB51 utilizing L-arabinose or D-glucose was approximately twice the rate for RB51 and RB51 passaged in cattle or bison (Fig. 3).

Colonial morphology. Colonies of RB51 (before and after in vivo and in vitro passages) examined with obliquely reflected light were dry, granular, and yellow to yellow-white in color. In contrast, strains 19 and 2308 had smooth colonies that were round, glistening, and blue to blue-green in color. Colonies of 19 and 2308 did not stain with crystal violet and failed to agglutinate in the presence of acriflavine. The isolates of RB51 agglutinated with acriflavine, and colonies were stained by crystal violet. These isolates did not react with A or M antiserum but did react with R antiserum. The smooth bacteriophage (Tb) failed to lyse them. The isolates did not react by immunoblotting with a monoclonal antibody to LPS, whereas 2308 and 19 did.

Growth of RB51 was completely inhibited on TSA containing 3.6 U of polymyxin B per ml (data not shown). The number of CFUs of RB51 on TSA with antibiotics (i.e., [polymyxin B] = 1.8 U/ml) was reduced by approximately 85%. In contrast, strain 19 and the parental strain 2308 were not inhibited on TSA containing 3.6 U of polymyxin B per ml. Strain RB51 passaged 159 times on TSA with antibiotics ([polymyxin B] = 1.8 U/ml) was not inhibited on TSA containing 1.8 U/ml but failed to grow on TSA containing 3.6 U/ml. Direct observation of RB51 and high-passage RB51 with obliquely reflected light did not reveal smooth colonial forms. Colonies stained with crystal violet, agglutinated in the presence of acriflavine, and were not lysed by Tb bacteriophage. The presence of the O antigen of *Brucella* smooth LPS was not detected by microdot blot. The R antigen was present, but the A- and M antigens were not present. Strains 19 and 2308 retained the character-

TABLE 3. Oxidative metabolic values [$QO_2(N)$] of *B. abortus* for selected amino acids and carbohydrates

Isolate[a]	Mean $QO_2(N)$ value for growth on indicated substrate[b]												
	D-Alanine	L-Alanine	L-Asparagine	L-Glutamate	DL-Ornithine	D,L-Citrulline	L-Arginine	L-Lysine	L-Arabinose	D-Galactose	D-Ribose	D-Glucose	i-Erythritol
RB51	271	245	411	428	83	68	104	114	261	435	596	346	454
RB51/HP	287	217	428	432	70	116	122	56	612	485	629	667	568
RB51/cow	303	260	441	408	68	92	112	74	301	444	636	377	571
RB51/bison	244	199	367	350	95	92	78	64	262	378	447	375	404
B. abortus biovar													
1	152	150	215	250	50	39	50	42	156	270	376	206	315
2	156	119	237	201	15	8	11	8	139	192	379	257	498
4	166	160	234	191	30	35	46	28	150	251	481	268	410
19	96	176	216	616	93	47	85	30	126	246	294	388	25

[a] HP, high passage.
[b] $QO_2(N)$ values are expressed in microliters of oxygen uptake per milligram of nitrogen per hour.

istics of smooth forms. Repeated attempts to isolate smooth colonial forms of RB51 from cattle and bison were not successful.

DISCUSSION

The profile of RB51 is unique among *Brucella* isolates from cattle, bison, and elk on the basis of comparison of total DNA fingerprints. Macrorestriction genomic fingerprinting by PFGE encompasses the overall organization of the bacterial genome and may be an indicator of clonal origin and genetic relatedness. The absence of noticeable differences in the banding patterns of *B. abortus* from bison and elk indicates that these isolates are clonally related. The patterns were similar to those reported for *B. abortus* biovars 1, 2, and 4 isolated from cattle (16). Three other profiles were reported for *B. abortus* from (i) *B. abortus* 11 (biovar 1), (ii) *B. abortus* biovar 3, and (iii) *B. abortus* biovars 5, 6, and 9 (16).

Correlation of PFGE patterns with biotyping of *Brucella* isolates indicates that a low level of genetic diversity exists within *B. abortus* biovar 1 isolates from bison and elk. The striking feature is the monomorphism in *B. abortus* from bison and elk and in *B. abortus* biovars 1, 2, and 4 from cattle (16), regardless of geographical origin (within the United States) or host (cattle, bison, or elk). The presence of genomic fragments that are similar in size does not prove that the fragments are the same but does suggest that strains of *B. abortus* which are limited in nature to the bovine host are closely related to the strains which can replicate repeatedly in bison or elk.

The oxidative metabolic profiles for RB51 before and after in vivo or in vitro passage were characteristic of the profile reported for *B. abortus* (39). There are three levels of utilization for *Brucella* species (39). The first level involves the use of four substrates, L-glutamic acid, L-alanine, D-alanine, and L-asparagine, which are always utilized by *B. abortus* and *B. melitensis*. For the second level the four substrates used are L-arginine, DL-ornithine, D,L-citrulline, and L-lysine, three of which are amino acids from the urea cycle and are usually utilized by *B. suis* but not by *B. abortus* or *B. melitensis*. Utilization of the carbohydrate substrates varies from species to species. Profiles produced by *B. ovis*, *B. canis*, and *B. neotomae* differ from each other and from the profiles of the other three species (*B. abortus*, *B. melitensis*, and *B. suis*).

There were no definite differences in the magnitude or relationships of the oxidative rates between the isolates of RB51 and typical field isolates of *B. abortus* biovars 1, 2, and 4. However, the oxidative metabolic rate for the high-passage in vitro RB51 utilizing L-arabinose or D-glucose was approximately twice the rate for RB51. Brucellae maintained under laboratory conditions retain characteristic species metabolic patterns but may have oxidative rates on some substrates higher than those of recent isolates (19).

Strain RB51 maintains the characteristics of the rough form after in vivo and in vitro passages as shown in this study and by others (29, 31). The A, M, or O antigen of smooth strains of *Brucella* was not demonstrated after passage, but RB51 did retain reactivity to R antiserum. Resistance to rifampin was consistent, and no cross-resistance to structurally unrelated therapeutic agents was demonstrated, as was reported for spontaneous rifampin-resistant variants recovered from rifampin-containing medium (10). The colonial morphology of RB51 remained the rough form.

In this study, polymyxin B was used as a selective agent in the attempt to obtain smooth colonial forms of RB51 as this strain is more susceptible to polymyxin B than its parental strain, 2308. *Brucella* LPS is unique in that it does not bind polymyxin B (23, 24), unlike LPS from many other gram-negative bacteria (28). Polymyxin B binds electrostatically to outer membranes, disrupting the structure of phospholipid and LPS components because of its cationic detergent-like properties (13). Farrell (12) has reported that the MIC of polymyxin B sulfate was 10 to 160 U/ml for 95 smooth *Brucella* strains typed as *B. abortus* biovar 1 to 6 or 9; *B. melitensis* biovar 1, 2, or 3; or *B. suis* biovar 1. In addition, the rough *B. canis* is resistant to polymyxin B (36); however, the selective medium recommended for isolation of rough *B. ovis* from field samples does not contain polymyxin B (4).

The LPS in the outer membrane of *Brucella* spp. may be responsible for excluding interactions of polymyxin B with the phospholipids (e.g., phosphatidylcholine [14]) of the outer leaflet of the outer membrane. When phospholipids of this leaflet are exposed, as with RB51, the outer membrane is susceptible to polymyxin. The inability of RB51 to grow on media containing higher levels of polymyxin B indicated that the smooth LPS was absent, thus allowing the antibiotic to interact with the cell membrane.

Although strain-to-strain differences are small, they permit discrimination of *B. abortus* RB51 from other brucellae. Since the reliability of bacterial markers for epidemiological investigations depends on diversity and reproducibility, this method of genomic fingerprinting by means of PFGE provides an effective and highly specific epidemiological tool for pursuing the source, transmission, and spread of the vaccine strain RB51. The results of the present study indicate that the genomic fingerprint, oxidative metabolism, and rough colonial morphology of RB51 are stable characteristics and can be used to differentiate this vaccine strain from other isolates of *Brucella* from infected cattle, bison, and elk.

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