Brucella, a Monospecific Genus as Shown by Deoxyribonucleic Acid Hybridization

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A total of 51 strains (including type, reference, vaccine, and field strains) representing all species and biovars of *Brucella* formed a single deoxyribonucleic acid-deoxyribonucleic acid hybridization group (S1 nuclease method). Accordingly, we propose that only one species, *Brucella melitensis*, be recognized in the genus. We recommend that other specific epithets formerly associated with the generic name *Brucella* be used in a vernacular form for biovar designation (e.g., *Brucella melitensis* biovar Abortus 1).

At present, the genus *Brucella* Meyer and Shaw 1920 contains six species, *Brucella melitensis* (Hughes 1893) Meyer and Shaw 1920, *Brucella abortus* (Schmidt 1901) Meyer and Shaw 1920, *Brucella suis* Huddleson 1929, *Brucella ovis* Buddle 1956, *Brucella neotomae* Stoenner and Lackman 1957, and *Brucella canis* Carmichael and Bruner 1968 (8, 20). These species were proposed on the basis of differential tests and different preferential hosts (8).

The identification of these bacteria at the species level is difficult and best suited to reference laboratories.

In 1968, Hoyer and McCullough (13, 14) studied six strains of *Brucella*, one of each species, by deoxyribonucleic acid (DNA)-DNA hybridization, using the DNA-agar method and a filter method. These authors (13, 14) found that their strains are highly related. Unfortunately, the work of Hoyer and McCullough (13, 14) had a limited impact on the taxonomy of the genus *Brucella*, probably because DNA hybridization technology and its interpretation had not reached the state of development which it now has, too few strains were studied, and only two of the strains were type strains (*B. melitensis* 16M and *B. canis* RM6/66).

The purpose of the present study was to apply modern DNA-DNA hybridization methods to 51 strains (including type strains) representing all present species and biovars of *Brucella*. Our results give no support to the division of the genus *Brucella* into more than one species. Consequently, we propose that only one species, *B. melitensis*, be recognized in this genus.

MATERIALS AND METHODS

Bacterial strains. The 51 strains used in this study are listed in Table 1. All strains were kept freeze-dried in the *Brucella* Culture Collection, INRA-Nouzilly, Monnaie, France. Cultures were grown on Trypcase Soy Agar (Bio-Mérieux, Marcy l'Etoile, France) supplemented with 0.1% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.) (TSAYE medium). For fastidious strains (*B. abortus* biovar 2 and African biovar 3 and *B. ovis*), sterile equine serum (Bio-Mérieux) was added to TSAYE medium to a final concentration of 5% (vol/vol) (TSAYES medium). The strains were checked for purity and species and biovar characterization by using standard procedures (1).

DNA preparation. For each strain, six Roux flasks containing 250 ml of TSAYE or TSAYES medium were inoculated

with a 5-ml suspension in saline (pH 6.8) made with a 1-day culture on TSAYE or TSAYES medium slopes. After incubation for 72 h at 37°C under air (supplemented with 10% [vol/vol] CO₂ if required), the growth from the six Roux flasks was harvested with 120 ml of 0.05 M tris(hydroxymethyl)-aminomethane–0.05 M ethylenediamine-tetraacetic acid–0.1 M NaCl buffer (pH 8). This suspension was checked for purity by streaking onto TSAYE or TSAYES medium. Then 2.6 ml of a 25% (wt/vol) aqueous solution of sodium dodecyl sulfate (BDH, Poole, England) and 0.4 ml of a 2% (wt/vol) pronase (Sigma Chemical Co., St. Louis, Mo.) solution were added. The mixture was vigorously shaken and then incubated with slight rotary shaking (120 cycles per min) overnight at 37°C to maximize cell lysis. The sodium dodecyl sulfate mixture was then placed in a water bath at 60°C for 1 h to kill the surviving *Brucella* cells.

DNA was extracted and purified from the cell lysate by using previously described procedures (4). Samples of DNA preparations were sheared by sonication (4). Sheared DNAs were dialyzed overnight against 0.042 M NaCl and stored at 4°C over a layer of chloroform.

DNA-DNA hybridization. Native DNA was labeled in vitro by nick translation (12). The S1 nuclease method (9) was used; we used a procedure (S1 nuclease-trichloroacetic acid) described elsewhere (12), except that all volumes were reduced by one-half. Labeled DNA (about 10 ng) and unlabeled DNA (75 µg) were heat denatured, adjusted to 0.42 M NaCl (final volume, 0.5 ml), and incubated at 60°C for 16 h.

The temperature (T_m) at which 50% of the reassociated DNA became hydrolyzable by the S1 nuclease was determined (9). The difference (ΔT_m) between the T_m of the homologous reaction (homoduplex) and the T_m of the heterologous reaction (heteroduplex) is an estimate of the divergence between two DNAs (2).

RESULTS

The DNA relatedness results obtained with labeled reference DNA from *B. melitensis* strain 16M^T (= ATCC 23456^T [T = type strain]) and unlabeled DNAs from 50 other *Brucella* strains are shown in Table 1. The 50 strains formed a homogeneous DNA relatedness group that was $96 \pm 5\%$ (mean $\pm$ standard deviation) related to strain 16M^T, with ΔT_m values below 1°C.

Reciprocal DNA relatedness data for the type strains of the six conventional *Brucella* species are shown in Table 2.

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TABLE 1. Reassociation of labeled DNA from *B. melitensis* 16M^T with DNAs of *Brucella* strains representing all species and biovars presently recognized within the genus *Brucella*^a

Source of unlabeled DNA					
Species	Species Biovar or type of isolate	Strain ^b	Host or source	Geographic origin	Relative binding ratio (%) at 60°C
<i>B. melitensis</i>	Biovar 1	16M ^T (= BCCN R1 ^T = ATCC 23456 ^T)	Goat	United States	100 (0.0) ^c
		BCCN 83225	Human	France	102
		H38 (= BCCN V3)	Human	Mexico	103
		Rev1 (= BCCN V4a)	Goat	Mexico	104
		Rev1 (= BCCN V4b)	Goat	Mexico	107
		Rev1 (= BCCN V4c)	Goat	Mexico	101
		Rev1 (= BCCN V4d)	Goat	Mexico	103
	Biovar 2	63/9 (= BCCN R2 = ATCC 23457)	Goat	Turkey	93
		BCCN 82.59	Human	Spain	93
		BCCN 84.3	Dog	Costa Rica	93 (0.6)
	Biovar 3	Ether (= BCCN R3 = ATCC 23458)	Goat	Italy	91
		BCCN 82.11	Sheep	France	92 (0.7)
	Rough isolate	BCCN R19	Goat	Malta	95
		544 ^T (= BCCN R4 ^T = ATCC 23448 ^T)	Cattle	England	87 (0.7)
<i>B. abortus</i>	Biovar 1	BCCN 83.29	Cattle	France	91
		544.CO ₂ independent (= BCCN R4 CO ₂ ⁻)	Laboratory variant of strain 544 ^T		87
		B19 (= BCCN V1)	Cattle	United States	103
		86/8/59 (= BCCN R5 = ATCC 23449)	Cattle	England	94
	Biovar 2	BCCN 83.86	Cattle	France	94
		BCCN 78.7	Human	France	91
		Tulya (= BCCN R6 = ATCC 23450)	Human	Uganda	90
		BCCN 83.74	Cattle	France	101
	Biovar 3	BCCN 83.20	Cattle	Niger	97
		BCCN 76.166	Cattle	Senegal	93 (+0.8)
		BCCN 83.31	Cattle	Rwanda	92
		BCCN 80.204	Cattle	Togo	94
	Biovar 4	BCCN 77.79	Human	France	92
		292 (= BCCN R7 = ATCC 23451)	Cattle	England	100
		BCCN 80.190	Cattle	France	99
		B3196 (= BCCN R8 = ATCC 23452)	Cattle	England	94
	Biovar 5	BCCN 84.16 (= CVL 201/78)	Cattle	Scotland	96
		870 (= BCCN R9 = ATCC 23453)	Cattle	Africa	98
	Biovar 6	BCCN 80.238	Human	France	98
		C68 (= BCCN R11 = ATCC 23455)	Cattle	England	94
	Biovar 9	BCCN 83.9	Cattle	Belgium	93
		45/20 (= BCCN V2)	Cattle	England	96
<i>B. suis</i>	Rough isolate	1330 ^T (= BCCN R12 ^T = ATCC 23444 ^T)	Swine	United States	105 (0.9)
		BCCN 78.157	Human	France	102
	Biovar 1	Thomsen (= BCCN R13 = ATCC 23445)	Swine	Denmark	96
		BCCN 82.4	Hare	France	101 (0.4)
	Biovar 2	686 (= BCCN R14 = ATCC 23446)	Swine	United States	97
		BCCN 83.210	Wild rodent	Australia	85
	Biovar 3	40 (= BCCN R15 = ATCC 23447)	Reindeer	USSR	94
		BCCN RT1	Caribou	Alaska	84
	Biovar 4	BCCN 82.75 (= CVL ELT80)	Human	England	93
		5K33 ^T (= BCCN R16 ^T = ATCC 23459 ^T)	Desert wood rat	United States	99 (0.5)
<i>B. neotomae</i>	Biovar 5	BCCN N1	Desert wood rat	United States	92 (0.0)
		63/290 ^T (= BCCN R17 ^T = ATCC 25840 ^T)	Sheep	Africa	92 (0.5)
	Biovar 6	BCCN O1	Sheep	France	98 (+1.5)
<i>B. ovis</i>	Biovar 7	RM6/66 ^T (= BCCN R18 ^T = ATCC 23365 ^T)	Dog	United States	99 (0.9)
		BCCN C1 (= CIP 74.36)	Dog	Madagascar	91 (0.5)

^a *B. abortus* biovar 7 was discarded from this study, as Food and Agriculture Organization/World Health Organization reference strain 63/75 (= ATCC 23454) is not a pure culture and no authentic isolate has been reported for many years. In 1978 *B. abortus* biovar 8 was deleted by the Subcommittee on Taxonomy of *Brucella* from the list of biovars as no authentic isolate of this biovar had been reported for many years and no reference strain was available (6).

^b ATCC, American Type Culture Collection, Rockville, Md.; BCCN, *Brucella* Culture Collection, Nouzilly, France; CIP, Collection of Pasteur Institute, Paris, France; CVL, *Brucella* Culture Collection, Central Veterinary Laboratory, Weybridge, England. Type strains and biovar reference strains are listed first by their Food and Agriculture Organization/World Health Organization designations and then by their BCCN and ATCC numbers. The reference strain of *B. suis* biovar 5 recently proposed by the Subcommittee on Taxonomy of *Brucella* (7) has not been chosen yet. Vaccine strains are listed first by their worldwide known designations (i.e., H38, Rev1, B19, and 45/20) and then by their BCCN numbers. Because of some differences in growth characteristics, antibiotic resistance, and protective activity, vaccine strain Rev1 originated from four different laboratories. Rev1 (= BCCN V4a) is the original strain of S. S. Elberg; this strain is a nondependent reverse clone of a streptomycin-dependent mutant of a virulent *B. melitensis* strain (strain 6056 of M. Ruiz-Castañeda, Mexico City, Mexico). Rev1 (= BCCN V4b) is the strain Rev1 used at the Centro Panamericano de Zoonosis, Buenos Aires, Argentina. Rev1 (= BCCN V4c) is the strain Rev1 used at the Veterinary Research Institute, Onderstepoort, South Africa. Rev1 (= BCCN V4d) is the strain Rev1 which has been maintained at Nouzilly, France, since 1970 and originated from Madison, Wis. Field strains are listed by their BCCN numbers and, for some, also by their CVL or CIP numbers.

^c The numbers in parentheses are ΔT_m values (in degrees Celsius).

TABLE 2. Levels of DNA relatedness among the type strains of the six conventional *Brucella* species

Unlabeled DNA from strain:	% DNA relatedness with labeled DNA from strain:					
	<i>B. melitensis</i> 16M ^T	<i>B. abortus</i> 544 ^T	<i>B. suis</i> 1330 ^T	<i>B. neotomae</i> 5K33 ^T	<i>B. ovis</i> 63/290 ^T	<i>B. canis</i> RM6/66 ^T
<i>B. melitensis</i> 16M ^T	100	93	92	89	99	97
<i>B. abortus</i> 544 ^T	87	100	100	94	89	103
<i>B. suis</i> 1330 ^T	105	100	100	98	93	110
<i>B. neotomae</i> 5K33 ^T	99	104	102	100	103	99
<i>B. ovis</i> 63/290 ^T	92	94	100	90	100	110
<i>B. canis</i> RM6/66 ^T	99	96	102	92	94	100

The level of relatedness among all species was more than 87%.

DISCUSSION

The genus *Brucella* is not significantly related to *Bordetella* (14), *Alcaligenes* (14), *Francisella* (13), *Haemophilus* (21), *Moraxella* (21), or the *Enterobacteriaceae* (13, 14), as determined by DNA-DNA hybridization. As determined by DNA-ribosomal ribonucleic acid hybridization, the genus *Brucella* belongs to ribosomal ribonucleic acid superfamily IV (10), which contains *Agrobacterium*, *Rhizobium*, *Mycoplana*, *Phylobacterium*, and Centers for Disease Control group Vd (10).

The identification of the present nomenspecies of *Brucella* can be difficult, and intermediate strains are occasionally found. Tests for substrate oxidation, although reliable for identification (8), require the use of a Warburg apparatus (or thin-layer chromatography) and are limited to reference laboratories. Other biochemical tests (CO₂ requirement, H₂S production, susceptibility to dyes) do not allow safe identification of nomenspecies, but are necessary for biovar identification (8). Nonrough strains cross-react in agglutination tests with antisera, and antigenic differences among biovars are quantitative rather than qualitative. Tests of lysis by a battery of four to six phages allow identification of *Brucella* nomenspecies (8).

In the taxonomy of any other genus, species behaving like *Brucella* nomenspecies would be treated as biovars or phagovars. Controversy has occurred concerning the classification of some *Brucella* taxa. Rough strains of *Brucella* isolated from dogs were proposed either as a biovar of *B. suis* (17) or as a separate species, *B. canis* (5). The latter proposal has been followed by the Subcommittee on Taxonomy of *Brucella* (6), and the nature of the animal host weighed heavily in this decision. *Brucella* strains isolated from reindeer presently constitute biovar 4 of *B. suis* (6), although Russian authors currently use the species name "*Brucella rangiferi*" (18) for these strains. Thus, there have been no scientific criteria to delineate species. In fact, present *Brucella* nomenspecies are only groups of biovars. This conclusion was reached 27 years ago by Renoux (19).

The levels of homology among all *Brucella* "species" are very high despite the fact that the S1 nuclease method is known to give lower homology values than all other DNA hybridization methods (12, 15). The range of ΔT_m values is similar to that observed within a subspecies (3, 11). Clearly, the 51 strains which we studied belong to a single genomic species with no subdivision deducible from DNA data. Overall, our results confirm and extend those published 17 years ago by Hoyer and McCullough (13, 14). However, the 2 to 3% deletion in the genome of a *B. ovis* strain that Hoyer and McCullough (13, 14) deduced from their reciprocal experiments was not confirmed by our study. It should be

recalled that the standard error of DNA relatedness results is currently about 3% (12).

We believe that taxonomy is a science and that nomenclature should be in accord with this science. Therefore, we propose that only one species, *B. melitensis* (Hughes 1893) Meyer and Shaw 1920, be recognized in the genus *Brucella*. *B. abortus*, *B. suis*, *B. neotomae*, *B. ovis*, and *B. canis* are thus subjective synonyms of *B. melitensis* (the type species). We suggest that specific epithets, other than the specific epithet of *B. melitensis*, formerly associated with the generic name *Brucella* be used in a vernacular form (i.e., not italicized) to refer to biovars (e.g., *B. melitensis* biovar Abortus 2). Intrasubspecific taxon designation is not regulated by the *International Code of Nomenclature of Bacteria* (16) and is the domain of the International Committee on Systematic Bacteriology Subcommittee on Taxonomy of *Brucella*.

The amended description of the single species in the genus *Brucella*, *B. melitensis*, is exactly that presently given for the genus *Brucella* (8). The type strain remains strain 16M (= ATCC 23456).

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