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Amplification Fragment Length Polymorphism in *Brucella* Strains by Use of Polymerase Chain Reaction with Arbitrary Primers

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DNA heterogeneity among members of the genus *Brucella* was demonstrated with the arbitrarily primed polymerase chain reaction (AP-PCR). Simple, reproducible genomic fingerprints from DNA of 25 different *Brucella* strains were generated with five arbitrarily chosen primers, alone and in pairs, with the PCR. Reaction conditions were optimized for each primer. Several DNA segments were amplified in each sample with all of the primers. PCR products that are not shared among all strains act as polymorphic markers. Polymorphism was apparent for each primer. The *Brucella* strains can be distinguished according to the banding patterns of their amplified DNA on agarose gels, and the differences can be diagnostic of specific strains. To determine genetic relatedness among the *Brucella* strains, similarity coefficients were calculated. Statistical analysis of the similarity coefficients revealed the degrees of relatedness among strains of the genus *Brucella*.

Six species are recognized in the genus *Brucella*: *B. melitensis*, *B. abortus*, *B. ovis*, *B. suis*, *B. neotomae*, and *B. canis* (5, 11). These species have shown 96% ± 5% relatedness to the type strain of the genus, *B. melitensis* ATCC 23456, in DNA hybridizations (9, 10, 17) and might have been considered pathovars of a single species were it not for their medical importance. The species differ in pathogenicity and host preference but are not typically restricted to a single host species. Four of the six recognized species are known to infect humans, though *B. abortus* and *B. melitensis* are best known as etiologic agents of human brucellosis. Identification by standard methods is difficult and time-consuming and may be frustrated by the occurrence of intermediates. Very minor interstrain differences were seen by polyacrylamide gel electrophoresis of proteins (12) or in some analyses with restriction endonucleases (8, 14, 15). Allardet-Servent et al. (1) found DNA polymorphism in *Brucella* strains only by using low-cleavage-frequency restriction enzymes such as *Xba*I or *Not*I. These restriction fragments were separated by pulse-field electrophoresis, a laborious and time-consuming technique requiring special equipment. Epidemiological investigations would be better served by simpler and faster procedures.

Here we describe a simple and rapid procedure to detect polymorphisms in *Brucella* strains. The method is based on the recent finding that reproducible fingerprints of any complex genome can be generated by arbitrarily primed polymerase chain reaction (AP-PCR) (4, 18). AP-PCR is distinct from the standard PCR (13) because it involves amplification of genomic DNA with single primers of arbitrary sequence. No prior DNA sequence information is required. The resulting polymorphisms provide a simple means of constructing genetic maps and performing DNA fingerprinting. Polymorphic markers are also called random amplified polymorphic DNA markers (7, 19, 20). Nucleotide polymorphisms are easier to identify with random amplified polymorphic DNA markers than with restriction fragment length polymorphism (RFLP) markers. This feature makes random amplified polymorphic markers advantageous for closely related species, like those of the genus *Brucella*. In the present study five different primers were used alone and in pairs to detect polymorphisms and to reveal genomic differences among 25 different *Brucella* strains, including strains of each of the recognized species (Table 1).

MATERIALS AND METHODS

Sources of genomic DNA. Table 1 lists the sources of *Brucella* strains used in this study. The *Brucella* strains were supplied by Bill Deyoe from the National Animal Disease Center collection. The brucellae were cultured for 3 to 4 days on tryptose agar supplemented with heat-inactivated calf serum (5%) and agar (0.5%) under an atmosphere of 5% CO_2 (2). Cultures were suspended in sterile saline (0.85% NaCl) and killed by the addition of 2 volumes of methanol.

DNA was isolated from methanol-killed cells essentially as described by Anderson et al. (3). Briefly, the cells were washed, incubated on ice in the presence of lysozyme, and heated to 50°C after the addition of proteinase K. Cell lysis was brought about by the addition of *N*-lauryl sarcosyl. The lysates were treated with RNase A and extracted repeatedly with phenol. The DNA was dialyzed against TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0). The concentration of DNA was determined by measuring the A_{260}, and the purity was determined by finding the A_{260}/A_{280} ratio.

Primers. Two 20-mer primers, P1 (5′-GGACTGCATAAA ATTGGCAC-3′) and P2 (5′-CAGCAGCAGCAAGACCT TCA-3′), and three 10-mer primers, P3 (5′-CGGCCACTGT-3′), P4 (5′-CGGCCCCTGT-3′), and P5 (5′-CGGCCCCGGT-3′), were obtained from Synthetic Genetics, San Diego, Calif.

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TABLE 1. Strains used in this study

Brucella species	Biovar	Strain	ATCC no.	Source
1. *B. abortus*	1	544	23448	United Kingdom
2. *B. abortus*	1	S19		APHIS
3. *B. abortus*	1	2308S		ADS/USDA
4. *B. abortus*	1	S19R		B. Deyoe
5. *B. abortus*	1	2308R		B. Deyoe
6. *B. abortus*	1	45/25		A. D. McEwen
7. *B. abortus*	1	8-1070		California
8. *B. abortus*	2	2016		USDA
9. *B. abortus*	3	Tulya	23450	Weybridge, United Kingdom
10. *B. abortus*	4	292	23451	Weybridge
11. *B. abortus*	4	8-0840		Florida
12. *B. abortus*	5	B3196	23452	Weybridge
13. *B. abortus*	6	870	23453	Weybridge
14. *B. abortus*	7	6317	23454	Weybridge
15. *B. abortus*	9	C68	23455	Weybridge
16. *B. melitensis*	1	16M	23456	ADS/USDA
17. *B. melitensis*	2	63/9	23457	Weybridge
18. *B. melitensis*	3	Ether	23458	Weybridge
19. *B. suis*	1	1330	23444	University of Minnesota
20. *B. suis*	2	Danish sow		A. Thomsen
21. *B. suis*	3	NADC 710		B. Deyoe
22. *B. suis*	4	IAB 2597		R. A. Dietrich
23. *B. canis*		RM-6/66	23365	L. E. Carmichael
24. *B. ovis*		ANH 3572		ANDH/USDA
25. *B. noeomae*		SE-1169		M. E. Meyer

Amplification conditions. Amplification conditions for primers P1 and P2 and for P3, P4, and P5 were different. Amplification reactions for P1 and P2 were prepared in volumes of 17.5 μl containing 10 mM Tris-HCl (pH 8.3)–50 mM KCl–0.001% (wt/vol) gelatin (1× PCR buffer; Perkin-Elmer Cetus, Norwalk, Conn.), 35 μM (each) deoxynucleoside triphosphate (dNTP), 10 mM $MgCl_2$, 100 μg of bovine serum albumin per ml, 0.6 μM primer, 10 ng of genomic DNA, and 5 U of AmpliTaq polymerase Stoffel fragment (Perkin-Elmer Cetus). The template DNA was denatured at 105°C for 5 min before being added to the reaction mixture to inactivate protease and ensure DNA denaturation. To reduce evaporation, the mixture was overlaid with a drop of mineral oil. Thin-walled GeneAmp reaction tubes (Perkin-Elmer Cetus) were used to facilitate heat transfer. The temperature cycling for the amplification was performed in a Coy model 60 TempCycler as follows: cycle 1, 95.5°C for 4 min (denaturation), 37°C for 30 s (annealing), and 67°C for 2 min (extension). The next 10 cycles were as follows: 95.5°C for 1 min (denaturation), 37°C for 30 s (annealing), and 67°C for 2 min (extension). Finally, 30 cycles at 95.5°C for 1 min (denaturation), 50°C for 1 min (annealing), and 72°C for 2 min for the first 20 cycles and 3 min for the rest (extension) were performed. A similar program was executed with a Perkin-Elmer Cetus GeneAmp PCR System 9600, but the corresponding times were reduced by one-half because of the faster heating and cooling of this machine.

Amplification reactions for primers P3, P4, and P5 were prepared in volumes of 17.5 μl containing 1× PCR buffer, 35 μM (each) dNTP, 3 mM $MgCl_2$, 200 μg of bovine serum albumin per ml, 2.5 μM primer (or 1.25 μM for each primer in the multiplex reaction), 10 ng of genomic DNA, and 1 U of AmpliTaq DNA Polymerase (Perkin-Elmer Cetus). Amplification was performed in a Perkin-Elmer Cetus GeneAmp PCR System 9600 programmed for 1 cycle of 2 min at 94°C, 30 s at 36°C, and 1 min at 72°C and 39 cycles of 30 s at 94°C, 30 s at 36°C, and 1 min at 72°C, using the shortest time possible between temperature changes.

Agarose gel electrophoresis. After the amplification was complete, 5 to 10 μl of the reaction mixture was analyzed on an agarose gel consisting of 1 or 2% (wt/vol) agarose (Bethesda Research Laboratories, Gaithersburg, Md.) and 0.5 μg of ethidium bromide (Sigma) per ml in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). The samples were electrophoresed at 85 V for 1.5 to 1.8 h on a Bethesda Research Laboratories Horizon 58 electrophoresis unit. A 123-bp DNA ladder (Bethesda Research Laboratories) was used as a size standard. A 300-nm UV transilluminator was used to detect the bands, which were then photographed with a 35-mm camera equipped with a yellow filter.

Amplification fragment length polymorphism analysis. For data analysis, similarity coefficients (F) were calculated as described by Gabriel et al. (6) for RFLPs. For each of the five primers, similarity coefficients between pairs of strains were calculated with the following formula: $F = 2\,n_{xy}/(n_x + n_y) \times 100$, where n_x and n_y are the number of fragments in strains X and Y, respectively, and n_{xy} is the number of fragments in strain X that match fragments in strain Y. Band matches, which determined the similarity coefficients, were observed by direct comparison on the same gel. The values of F between strains are the means of two to five observations with each primer; only the strongly amplified DNA fragments were used for comparison with each primer. The values from pairwise experiments were not calculated. The F values in Table 2 are the mean F values from five primers. Bonferroni T tests ($p = 0.05$) were performed to determine whether the values of F for particular comparisons were significantly different (16).

RESULTS AND DISCUSSION

We observed that by using single arbitrarily chosen primers, very reproducible genomic fingerprints of different *Brucella* strains can be generated by optimized PCR. All amplifications were repeated two to five times. The number, reproducibility, and intensity of bands in a fingerprint were found to be a function of several parameters, including the concentration of salts, primer annealing temperature, template concentration, and primer length and sequence. Different optimum reaction conditions were found for the 20-mer oligonucleotide primers (P1 and P2) compared with the 10-mer primers (P3, P4, and P5). For the 20-mer primers we found that cycling at lower temperatures for 10 cycles, followed by 30 cycles of higher-stringency conditions, was sufficient to generate a pattern of bands which was characteristic of the strains. A high Mg^{2+} concentration was selected to enhance the stability of primer-template interactions. With the 10-mer primers, DNA fingerprints were produced under relaxed conditions, the Mg^{2+} concentration was 3 mM, and the annealing temperature ranged between 30 and 40°C. Annealing temperatures above 40°C in the thermal cycling profile prevented amplification by the 10-mer oligonucleotides. The results were similar even when different DNA preparations from the same strain were used, but variation was observed in the relative amounts of amplification of certain DNA fragments. This could be the result of DNA segments competing for primers, since it was found that when some minor bands were amplified to a greater amount, other DNA bands were reduced in intensity. How-

TABLE 2. Similarity coefficients (F) of combined data with P1 to P5 primers

Strain[a]	% Similarity with strain no:																							
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	83	92	72	78	86	82	81	84	84	84	84	84	76	77	85	88	80	71	83	84	72	73	72	70
2		86	83	80	78	81	81	81	84	82	82	82	82	81	77	82	82	84	87	80	65	81	73	76
3			82	77	84	83	83	83	84	82	82	87	82	78	77	80	80	82	85	86	76	86	79	85
4				77	86	89	78	83	81	79	85	88	80	81	61	74	78	68	73	68	60	71	65	65
5					89	90	88	89	90	86	86	86	91	86	83	85	93	77	89	79	58	89	74	76
6						84	85	90	88	89	92	92	87	79	76	90	90	77	93	81	67	81	75	75
7							85	84	87	89	89	86	81	79	68	79	80	68	80	70	61	74	68	68
8								90	91	86	86	86	84	80	79	73	76	77	81	67	57	75	73	65
9									92	91	91	91	89	88	72	84	83	84	87	72	65	83	74	71
10										98	92	89	91	86	73	85	85	76	88	71	65	85	75	72
11											94	90	92	84	69	83	87	87	89	73	66	86	74	76
12												93	88	84	66	83	77	75	85	71	63	82	74	73
13													88	84	63	80	80	77	82	67	60	63	71	69
14														92	75	81	85	72	87	74	67	90	74	74
15															67	73	80	63	85	69	63	81	66	65
16																73	72	77	72	83	79	79	88	92
17																	86	76	86	79	72	88	72	75
18																		74	91	77	63	81	69	73
19																			78	73	59	79	75	71
20																				66	93	75	76	84
21																					88	80	82	79
22																						71	67	70
23																							76	82
24																								86

[a] Strain numbers correspond to the strains listed in Table 1.

ever, strongly amplified DNA segments were consistently observed. Preliminary experiments indicated that polymerases from sources other than *Thermus aquaticus* (e.g., Pfu DNA polymerase) can give different amplification products due to different sensitivities to mismatches between the primer and template and to their proofreading capacities. To reproduce AP-PCR polymorphism, the reaction conditions under which the polymorphism was originally found should be duplicated exactly. With some combinations of primer and genomic DNA templates, a nondiscrete size range of amplification products appeared as a smear on agarose gels. These smears were converted to discretely sized bands by reducing the concentration of either the AmpliTaq polymerase or the genomic DNA and adjusting the Mg^{2+} concentration. The optimum reaction conditions are summarized in Materials and Methods.

The effect of varying the template concentration (from 1 pg to 100 ng) on AP-PCR patterns was also examined. We found that 10 pg of genomic DNA was the lower limit of reliable AP-PCR (data not shown). To confirm that the observed bands were amplified genomic DNA and not primer artifacts, genomic DNA was omitted from control reactions for each primer, and no amplification products were produced under the optimized conditions (see Fig. 2B and 3B, lanes 14).

Figure 1 shows the result of an experiment in which single primers P1 (Fig. 1A) and P2 (Fig. 1B) were used to amplify segments of genomic DNA from different *Brucella* strains, in this case the classical species according to the current classification scheme (11). Primers P1 and P2 were designed for other purposes and were chosen arbitrarily for these experiments. Several DNA segments were amplified in each sample, and most of these segments were common among the different species. However, some segments were amplified only in certain species. PCR products act as polymorphic markers only when they are amplified from DNA of some of the genus. Primer P1 produced 7 polymorphic bands, and 10 polymorphic bands appeared with primer P2. The arrows show some of the polymorphic bands in Fig. 1. AP-PCR polymorphisms were named on the basis of the primer name and the length of the polymorphism detected. Some polymorphisms were clear and easy to score, while others appeared ambiguous. Ambiguous polymorphism may result from poor discrimination by a primer between alternative sites of slightly different nucleotide sequences (20). Polymorphism P1-258 distinguishes *B. abortus* S19 and *B. abortus* 2308S from each other. P1-258 and P1-1172 distinguish *B. abortus* 2308S and *B. melitensis* 16M. P1-446 (Fig. 1A, lane 5) is a unique band allowing *B. canis* RM-6/66 to be differentiated from the seven other strains. *B. ovis* ANH 3572, *B. suis* 1330, and *B. neotomae* SE-1169 can be distinguished from one another on the basis of the P1-319, P1-537, P1-1010, and P1-1172 polymorphic bands. Similarly, on the basis of the 10 different P2 polymorphic bands, the corresponding *Brucella* strains can also be differentiated from one another (Fig. 1B), demonstrating the utility of the technique in strain identification.

Primers P3, P4, and P5 were designed in the absence of any sequence information on the *Brucella* DNAs. Each primer differed by a single nucleotide substitution, and the G+C content varied from 70 to 90% for P3 to P5, respectively. We found that a single nucleotide change in the primer sequence caused a complete change in the pattern of amplified DNA segments. The AP-PCR patterns of the 25 *Brucella* strains were relatively similar to one another by primer P3, and they differed the most from one another with P5. P3, P4, and P5 gave 6, 6, and 12 polymorphic bands per gel, respectively. For example, Fig. 2 shows the electrophoretic analysis of genomic fingerprints of 25 *Brucella* strains amplified with primer P5; some polymorphic bands are indicated by arrows.

Of the five primers used, P5 resulted in the most polymor-

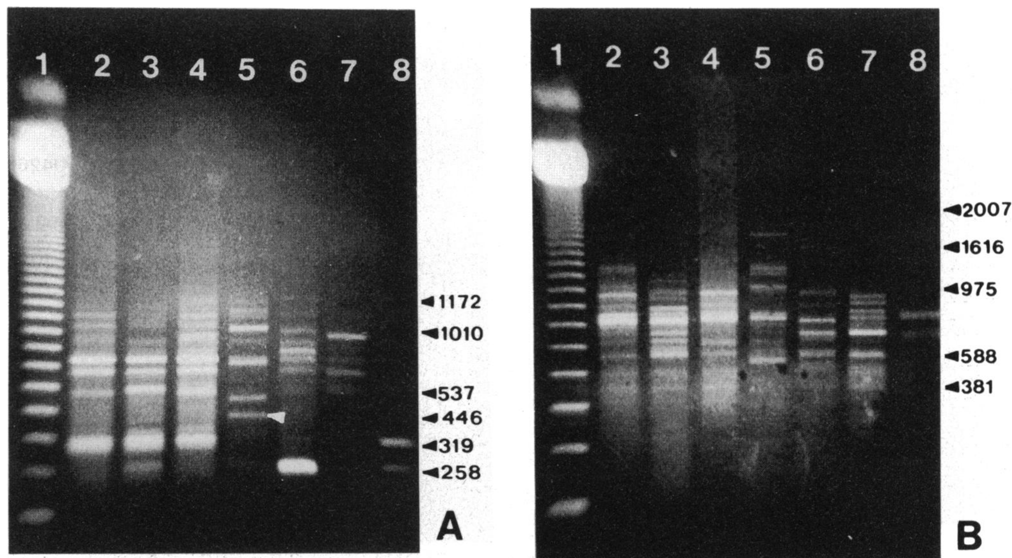

FIG. 1. Electrophoretic analysis (1% agarose gel) of genomic fingerprints of DNA from different *Brucella* strains. Genomic DNA (10 ng) was amplified with primer P1 (A) and primer P2 (B). Lanes: 1, 123-bp DNA ladder size standard; 2, *B. abortus* S19; 3, *B. abortus* 2308; 4, *B. melitensis* 16M; 5, *B. canis* RM-6/66; 6, *B. ovis* ANH 3572; 7, *B. suis* 1330; 8, *B. neotomae* SE-1169. The arrowheads show some of the polymorphic bands, and the numbers indicate the length (in bases) of the polymorphism detected.

phic fragments and was, therefore, most useful in strain identification. As each primer gave a different pattern of AP-PCR products, the data allowed the differentiation of closely related strains. These fingerprints could be tailored by changing primer length and sequence to produce patterns of varying complexity. Previously reported RFLP analyses demonstrated very little diversity within the *Brucella* strains (1, 8, 14, 15). However, we obtained a large number of different AP-PCR fingerprints. AP-PCR detected changes in a DNA sequence at arbitrary sites which were randomly distributed along the target *Brucella* genome, and the primers could flank both conserved and variable regions. Therefore, the chances to detect polymorphism in *Brucella* strains were improved compared to those with RFLP analysis. Divergence of even a fraction of a percent between two genomes can result in a different fingerprint pattern because slightly different sets of sites in the genome may have best matches with the primer. This explains the different fingerprints observed among the 25 *Brucella* strains, which were found in spite of the high degree of DNA relatedness (96% ± 5%) among all *Brucella* strains (9, 10, 17).

Multiple primers can also generate DNA fingerprints and

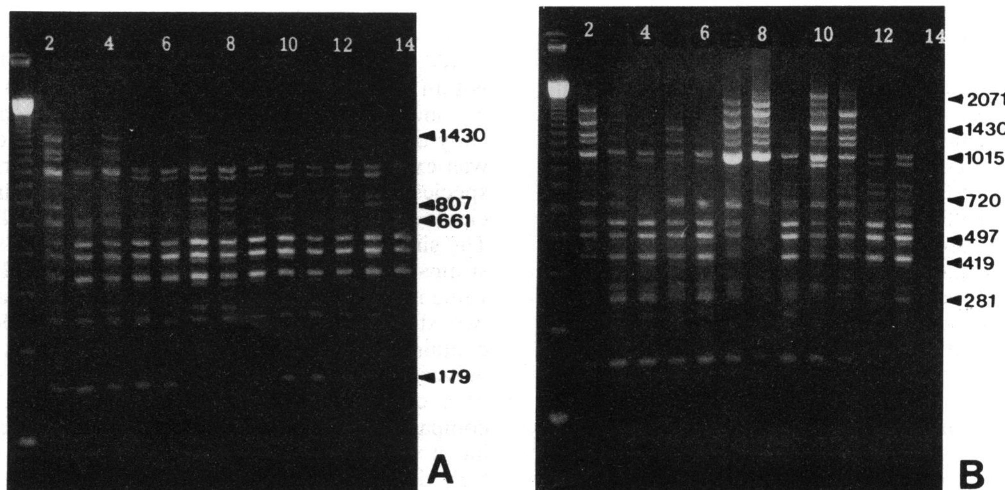

FIG. 2. Electrophoretic analysis (2% agarose gel) of AP-PCR patterns of 25 different *Brucella* strains amplified by primer P5. (A) Lanes: 1, 123-bp DNA ladder size standard; 2, *B. abortus* 544; 3, *B. abortus* S19; 4, *B. abortus* 2308S; 5, *B. abortus* 8-1070; 6, *B. abortus* 2308R; 7, *B. abortus* S19R; 8, *B. abortus* 45/25; 9, *B. abortus* 2016; 10, *B. abortus* Tulya; 11, *B. abortus* 292; 12, *B. abortus* 8-0840; 13, *B. abortus* B3196; 14, *B. abortus* 870. (B) Lanes: 1, 123-bp DNA size standard; 2, *B. melitensis* 16M; 3, *B. melitensis* 63/9; 4, *B. melitensis* Ether; 5, *B. suis* 1330; 6, *B. suis* Danish sow 160; 7, *B. suis* NADC 710; 8, *B. suis* IAB 2597; 9, *B. canis* RM-6/66; 10, *B. ovis* ANH 3572; 11, *B. neotomae* SE-1169; 12, *B. abortus* B3175; 13, *B. abortus* C68; 14, primer only, no template DNA. The arrowheads show some of the polymorphic bands, and the numbers indicate the length (in bases) of the polymorphism detected.

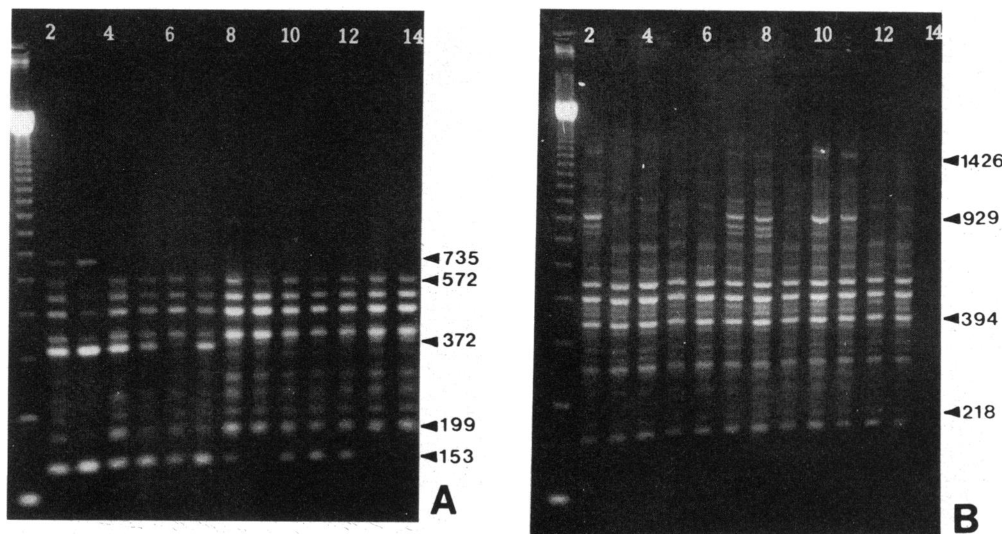

FIG. 3. Electrophoretic analysis (2% agarose gel) of genomic fingerprints of DNAs from different *Brucella* strains amplified by pairwise combination of primers. (A) Genomic DNA (10 ng) was amplified by primers P3 and P5 together. Lanes: 1, 123-bp DNA ladder size standard; 2, *B. abortus* 544; 3, *B. abortus* S19; 4, *B. abortus* 2308S; 5, *B. abortus* 8-1070; 6, *B. abortus* 2308R; 7, *B. abortus* S19R; 8, *B. abortus* 45/25; 9, *B. abortus* 2016; 10, *B. abortus* Tulya; 11, *B. abortus* 292; 12, *B. abortus* 8-0840; 13, *B. abortus* B3196; 14, *B. abortus* 870. (B) Genomic DNA (10 ng) was amplified by primers P4 and P5 together. Lanes: 1, 123-bp DNA ladder size standard; 2, *B. melitensis* 16M; 3, *B. melitensis* 63/9; 4, *B. melitensis* Ether; 5, *B. suis* 1330; 6, *B. suis* Danish sow 160; 7, *B. suis* NADC 710; 8, *B. suis* IAB 2597; 9, *B. canis* RM-6/66; 10, *B. ovis* ANH 3572; 11, *B. neotomae* SE-1169; 12, *B. abortus* B3175; 13, *B. abortus* C69; 14, primers only, no template DNA. The arrowheads show some of the polymorphic bands, and the numbers indicate the length (in bases) of the polymorphism detected.

in greater numbers than single primers (4, 20). We compared the fingerprints produced by AP-PCR using single primers (P3, P4, and P5) with the pattern generated by pairs of primers for 25 different *Brucella* strains and determined the number of polymorphisms that could be detected between the strains. P3, P4, and P5 were used in all possible pairs of primers with all of the strains. As an example, Fig. 3 shows the genomic fingerprints of *Brucella* DNAs obtained with pairs of primers P3 and P5 (Fig. 3A) and P4 and P5 (Fig. 3B). The amplification products obtained with two primers produced a fingerprint which was not merely the result of adding the amplification products obtained separately with individual primers. As expected, certain bands disappeared while few new bands were amplified. P3 and P4 together resulted in five polymorphisms that were also produced with these primers alone. Two polymorphisms detected with P3 and P5 were not found with P3 and P5 when used alone, and three among the P4 and P5 polymorphisms were the same as a polymorphism mapped with these primers alone.

The number of bands in AP-PCR fingerprints generated with primer pairs was not substantially different from the number produced by individual primers. The average lengths of the most prominent AP-PCR products were 700 bp for the three primers used individually and 450 bp when the primers were used in combination. This result was expected because the average distance between adequate matches in opposite DNA strands is less when two oligonucleotides are used as primers than when only one primer is present. Half of the AP-PCR fingerprint produced with a pair of primers would also expected to be found in one or the other pattern generated with each primer alone. The overlap was 50 to 70%. Figure 3 shows that the fingerprints of *Brucella* strains with two primers in pairs were more similar to each other than were those with single primers. In agreement with our results, Williams et al. (20) found that the efficiency of finding polymorphic bands was reduced when primers were used in pairwise combination.

Random amplified polymorphic DNA markers, like RFLPs, can be used to show genetic similarity (7, 19, 20) and thus could be analyzed in a manner similar to that for RFLPs. For RFLPs, similarity coefficients (F) are frequently employed to analyze the banding patterns (6). For closely related strains the value of F should approach 100%, and for unrelated strains the F value should approach 0%. This assumption was validated by calculating the F values for each of the five primers used with the 25 *Brucella* strains tested, including all six classical species (11). Fifteen biovars within the species of *B. abortus*, three biovars of *B. melitensis*, and four biovars of B. suis were evaluated (Table 1). Only one strain each of *B. canis*, *B. ovis*, and *B. neotomae* was examined; there are no reported biovars within these species (11). Table 2 summarizes the mean similarity coefficients of the combined data obtained with all five primers. The similarity coefficients ranged from 57 to 98%; thus, the strains were moderately to highly related. Because the F value is only an estimate of the true mean relatedness (μ) of two strains or group of strains, 95% confidence intervals containing μ were calculated (16). If groups of *B. abortus* biovar 1 (A) and *B. abortus* biovars 2 to 9 (B) were members of a common population, the mean value of F in A-A comparisons would not be significantly different from the mean value in A-B comparisons. If groups A and B were truly distinct, these mean values of F would be significantly different. Although the number of strains was limited, the relationships between the group of strains of *B. abortus* biovar 1 (A), *B. abortus* biovars 2 to 9 (B), *B. melitensis* (C), and *B. suis* (D) were examined. The data were not sufficient for statistical analysis in the case of *B. canis*, *B. ovis*, and *B. neotomae*. Bonferroni T tests ($P = 0.05$) were performed to determine whether the values of the similarity coefficients

TABLE 3. Selected mean comparisons

Type comparison[a]	Mean similarity, % (SEM)[b]	T-test value[c]
A-B	84.24 (1.35)	0.933
AB-C	78.82 (1.06)	3.353*
AB-D	74.81 (1.29)	6.353*
C-C	81.08 (4.2)	2.147
D-D	76.76 (4.2)	0.308
A-C	80.38 (1.6)	1.299
A-D	75.85 (1.75)	3.973*
B-C	77.48 (1.37)	4.582*
B-D	73.90 (1.64)	7.667*

[a] Each set of letters represents a type, designating strain comparisons in terms of the groups to which the strains belong: A, *B. abortus* biovar 1; B, *B. abortus* biovars 2 to 9; C, *B. melitensis*; D, *B. suis*.
[b] Based on data presented in Table 2.
[c] Limits of 95% confidence intervals calculated with Bonferroni T tests (16). Values followed by an asterisk are significantly different ($P = 0.05$).

(F) for particular comparisons were different. The F value in type AB-C and AB-D comparisons were significantly different at the 5% level from the F values in A-B, C-C, and D-D comparisons (Table 3). This indicates that the strains of *B. abortus* are more closely related to each other than to the strains of *B. melitensis* and *B. suis* and that the different biovars within *B. melitensis* and *B. suis* are more closely related to each other than to the different biovars of *B. abortus*. Although these subgroups were significantly different on the basis of our T tests, this result should be interpreted cautiously because of the limited number of strains in each. If a larger number of strains of other biovars were examined, the relationships among the strains might be quantitatively reflected. These fingerprints could be used to generate dendograms of taxonomic relationships by cluster analysis of the similarity coefficients. Taxonomic classification based on phenotypic properties requires the identification of those properties that remain stable and reflect a single genotype. For the genus *Brucella*, species and biotype identifications have depended on numerical analysis of unstable phenotypic features (11). The establishment of stable phylogenetic markers via genetic methods would help to better describe the genus *Brucella*. This could be performed through DNA fingerprints, which do not rely on phenotypic expression, are sensitive to minor genomic differences, and offer a precise means for characterizing species and differentiating closely related strains.

AP-PCR fingerprints revealed a great heterogeneity in the electrophoretic pattern of different *Brucella* strains. Bacteria can be distinguished according to the banding pattern, and the amplification fragment length polymorphisms can be diagnostic of specific strains. DNA fingerprints generated with arbitrary primers have potential applications in establishing genetic relationships. Conclusions from our results should be considered preliminary in view of the limited number of strains and primers tested. Currently, we are attempting to develop conditions that will increase the ability of AP-PCR to detect differences in DNA sequences, thus improving strain differentiation. Unique bands could be removed from the gel and used to hybridize back to genomic digests or clones. Since polymorphic AP-PCR bands can be used as probes, a link between genetic and physical maps can be envisioned.

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