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Comparison of Multiple-Locus Variable-Number Tandem-Repeat Analysis with Other PCR-Based Methods for Typing *Brucella suis* Isolates[▽]

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Multiple-locus variable-number tandem-repeat analysis (MLVA), multiplex PCR, and PCR-restriction fragment length polymorphism analysis were compared for typing *Brucella suis* isolates. A perfect concordance was obtained among these molecular assays. However, MLVA was the only method to demonstrate brucellosis outbreaks and to confirm that wildlife is a reservoir for zoonotic brucellosis.

Brucella suis, the causative agent of swine brucellosis, is classified in five biovars that preferentially infect different animal hosts: biovars 1 and 3 affect domestic pigs and wild boars, biovar 2 also affects hares, biovar 4 infects reindeer and caribou, and biovar 5 infects only rodents. In contrast to biovars 1 and 3, biovar 2 has been isolated rarely from humans, and its zoonotic role is questioned (9). The increasing number of brucellosis outbreaks in pig farms is becoming a serious problem all over Europe (9, 13). Hares and wild boars are widely infected with *B. suis*, and spillover from wildlife to domestic pigs and cattle has been reported (3, 10, 11, 13). Accurate diagnostic and typing procedures are essential for epidemiological studies aimed at *B. suis* control or eradication.

The identification of the *B. suis* biovars is currently performed by standard bacteriological methods (2). However, these tests lack specificity and are not straightforward particularly for the identification of biovars 1, 2, and 3 (7). With the objective of improving the typing of these biovars, different PCR-based assays have been proposed. The most widely used are the PCR-restriction fragment length polymorphism (RFLP) analysis of genes *omp2a* and *omp2b*, which can differentiate between reference biovars 1, 2, and 3 (6), and of gene *omp31*, which is able to differentiate biovars 1 and 3 from biovar 2 (15). In addition, a modification of the original AMOS-PCR multiplex assay (4), including a new pair of primers derived from the *ery* locus (AMOS-*ery*-PCR [14]), can differentiate biovar 1 from others. Recently, a new "fingerprinting" approach based on a PCR method for multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) has been developed for molecular typing of *Brucella* (5, 12, 16). The high variability of VNTR loci has already been applied in a large panel of animal and human *Brucella* isolates, proving a high discriminatory power for typing purposes (1, 8, 12). However, the concordance of VNTR analysis with other molecular typing assays has not been directly investigated yet. To do this, a representative collection of *B. suis* field isolates was tested; 35 strains were isolated from pigs, 13 from wild boars, 8 from hares, and 2 from horses, and the strains were isolated from different regions of Europe during more than 20 years to ensure adequate diversity (Fig. 1). All isolates were typed according to standard bacteriological procedures (2), and 38 strains were identified as biovar 2, and 20 were identified as biovar 1 (6 of these strains showed atypical fuchsin resistance). Growth and harvesting of *Brucella* cells and bacterial DNA were performed as described elsewhere (2, 8, 12). All isolates were subjected to five different PCR-based typing techniques: PCR-RFLP analysis for *omp2a*, *omp2b*, and *omp31* genes (6, 15), multiplex AMOS-*ery*-PCR (14), and *Brucella* MLVA using 15 genetic markers (8, 12).

Figure 1 summarizes the molecular typing results and represents a dendrogram construct from MLVA genotyping assay data on the *B. suis* isolates. Strains of the reference biovars 1, 2, and 3 could be differentiated clearly according to the RFLP identified in *omp* genes and to MLVA results. By contrast, the AMOS-*ery*-PCR assay was unable to differentiate between biovars 2 and 3. Twenty field strains typed as biovar 1 were also grouped together using the different PCR-based techniques (S1 pattern). All these strains gave the same results that the reference biovar 1 strain, suggesting a very high genetic homogeneity for this biovar. Interestingly, this is the first evidence showing that *B. suis* biovar 1 is a cause of brucellosis in hares. Neither PCR-RFLP nor AMOS-*ery*-PCR assays were able to differentiate the six biovar 1 strains showing atypical fuchsin resistance (strains S-80, S-87, S-88, S-92, S-93, and S-94 in Fig. 1). In contrast, these six strains were placed in very closely related MLVA clusters (Fig. 1). A similar correlation between this phenotypic characteristic and VNTR clustering has been recently reported in *Brucella melitensis* (1). This is also the first

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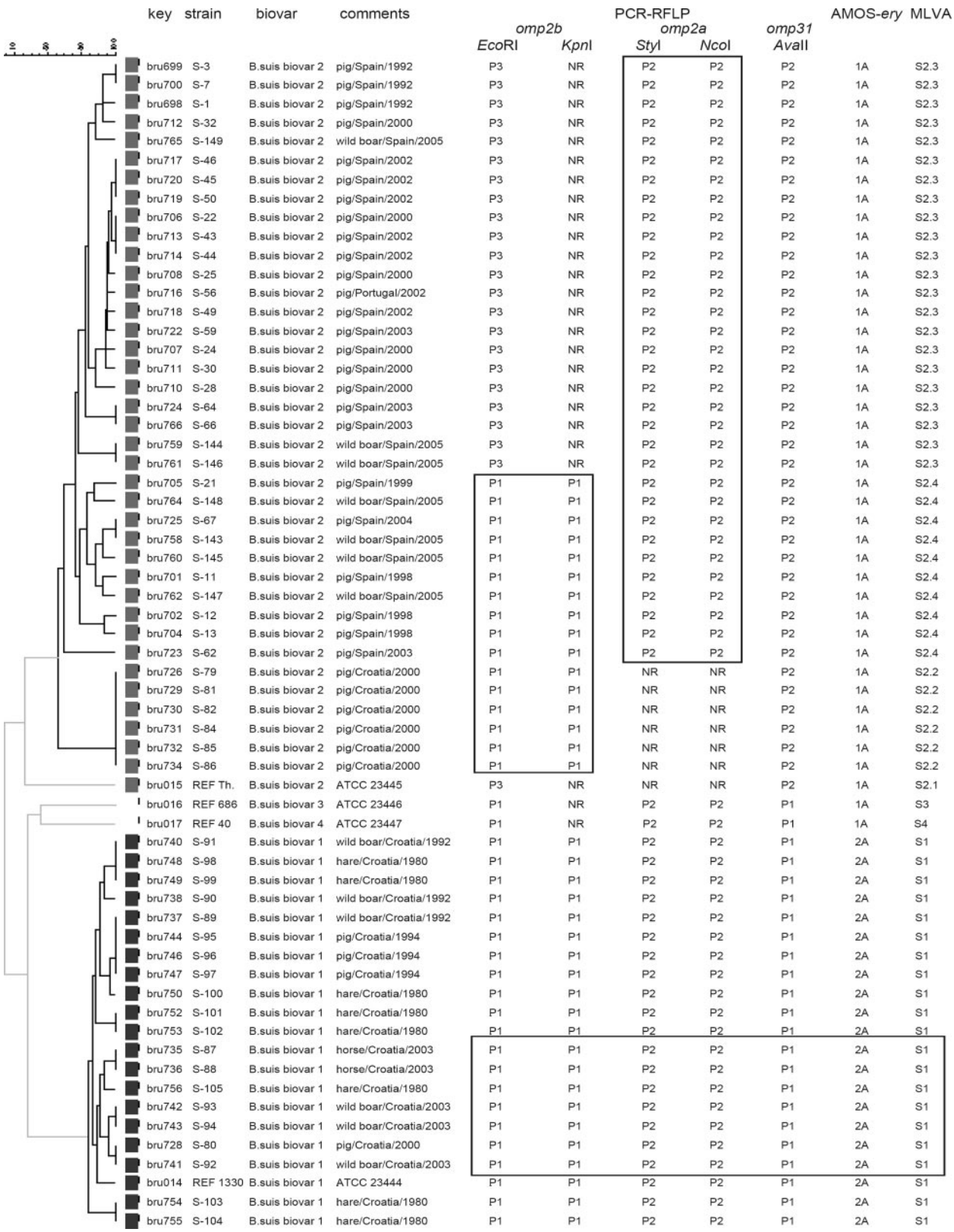

FIG. 1. Dendrogram constructed from MLVA testing and molecular typing results of representative *B. suis* isolates. The dendrogram was generated using a distance matrix calculated with the categorical coefficient and the unweighted-pair group method using average linkages as previously described (12). An identical weight was given to each marker. The 58 animal *B. suis* isolates investigated clustered (indicated by boxes) in 35 different MLVA genotypes. In the columns the following data are presented: DNA batch (key); strain identification; biovar, according to standard bacteriological procedures; animal host, geographic origin, and year of isolation (comments); *omp2b*, *omp2a*, and *omp31* PCR-RFLP patterns, as defined originally in references 5 and 14; AMOS-*ery*-PCR patterns, as defined originally in references 4 and 13; and MLVA patterns, see text for details. *B. suis* biovar 1, 2, 3 and 4 ATCC reference strains were included as controls. Based on detailed background information collected from the veterinary records, strains S-3 and S-7, strains S-45, S-46, and S-50, strains S-64 and S-66, strains S-87 and S-88, strains S-95, S-96, and S-97, and strains S-144 and S-146 corresponded to specific outbreaks.

TABLE 1. Repeat copy numbers at each locus in the MLVA assay for 58 *B. suis* representative isolates

Marker[a]	No. of repeat copies at each locus in the following pattern[b]:						
	S1	S2.1	S2.2	S32.3	S2.4	S3	S4
bruce04	2–7	9	2	2	2–7	7	4
bruce06	2	2	2	2	2	2	2
bruce07	0–8	9	5	0–7	0–10	5	5
bruce08	5	4	7	7	7	3	3
bruce09	0–8	18	3	0–21	5–17	10	9
bruce11	6	8	8	8	8	4	9
bruce12	10	15	15	9	9	11	11
bruce16	0–5	2	2	2	2	4	6
bruce18	0–4	6	4	5–6	5–6	4	5
bruce21	0–9	9	9	9	9	9	9
bruce30	3	4	8	0–8	0–8	5	3
bruce42	4	6	5	5	5	3	3
bruce43	1	1	1	1	1	1	1
bruce45	5	5	5	5	5	5	5
bruce55	3	2	8	0–5	5–6	2	2

[a] MLVA markers are defined as described in references 8 and 12.
[b] Patterns are defined as described in the legend to Fig. 1. See text for details.

evidence showing the existence of fuchsin-resistant *B. suis* biovar 1 strains in Europe. When testing the 38 strains identified as biovar 2, the *omp31* PCR-RFLP and AMOS-*ery*-PCR assays resulted in patterns identical to those obtained with the reference biovar 2 strain (S2 patterns). However, the RFLP patterns of the *omp2a* and *omp2b* PCR-amplified product (Fig. 1) demonstrated additional polymorphism within this biovar, and at least four genetic subgroups were identified (S2.1, S2.2, S2.3, and S2.4), which were also identified in separate clusters by the MLVA assay (Table 1). Subgroup S2.1 included only the reference biovar 2 strain, which was also clearly differentiated from the remaining S2 clusters by MLVA. MLVA results were in perfect agreement with those of standard typing procedures and clearly differentiated these 38 strains from the rest of the patterns (S1, S3, and S4).

Table 1 summarizes the repeat copy numbers at each locus in the MLVA assay. Four markers (bruce06, bruce21, bruce43, and bruce45) proved to be almost identical in all *B. suis* strains tested. In contrast, markers bruce04, bruce07, bruce09, bruce12, bruce18, bruce30, and bruce55 were highly discriminatory. The four *B. suis* biovars tested were clearly differentiated with markers bruce11 and bruce16.

There was a perfect correlation of classical typing, *omp31* PCR-RFLP, AMOS-*ery*-PCR, and MLVA assays. However, the MLVA was the only assay able to evidence epidemiological relationship between strains. For example, some strains that clustered together after the MLVA analysis had been isolated from different animal hosts. As an example, strain 89 (from a wild boar) and strains 95, 96 and 97 (from domestic pigs) share exactly the same genotype. These results suggest the spread of particular *B. suis* strains from one animal species to another and that spillover from wildlife to domestic pigs seems to be a frequent event (3, 10, 11, 13). In addition, the MLVA assay demonstrated the same genotype in strains isolated from the same outbreak: strains 45, 46, and 50 from Spain, for instance. In conclusion, the MLVA is the most sensitive assay suitable for simultaneously typing *B. suis* and epidemiologically tracing the infection.

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