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# Multiple-Locus Variable-Number Tandem-Repeat Analysis of the Swine Dysentery Pathogen, *Brachyspira hyodysenteriae*▽†

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**The spirochete *Brachyspira hyodysenteriae* is the causative agent of swine dysentery, a severe colonic infection of pigs that has a considerable economic impact in many swine-producing countries. In spite of its importance, knowledge about the global epidemiology and population structure of *B. hyodysenteriae* is limited. Progress in this area has been hampered by the lack of a low-cost, portable, and discriminatory method for strain typing. The aim of the current study was to develop and test a multiple-locus variable-number tandem-repeat analysis (MLVA) method that could be used in basic veterinary diagnostic microbiology laboratories equipped with PCR technology or in more advanced laboratories with access to capillary electrophoresis. Based on eight loci, and when performed on isolates from different farms in different countries, as well as type and reference strains, the MLVA technique developed was highly discriminatory (Hunter and Gaston discriminatory index, 0.938 [95% confidence interval, 0.9175 to 0.9584]) while retaining a high phylogenetic value. Using the technique, the species was shown to be diverse (44 MLVA types from 172 isolates and strains), although isolates were stable in herds over time. The population structure appeared to be clonal. The finding of *B. hyodysenteriae* MLVA type 3 in piggeries in three European countries, as well as other, related, strains in different countries, suggests that spreading of the pathogen via carrier pigs is likely. MLVA overcame drawbacks associated with previous typing techniques for *B. hyodysenteriae* and was a powerful method for epidemiologic and population structure studies on this important pathogenic spirochete.**

*Brachyspira hyodysenteriae* is a Gram-negative, oxygen-tolerant, anaerobic spirochete that colonizes the porcine large intestine to cause swine dysentery. This condition is characterized by a severe mucohemorrhagic diarrhea that primarily affects animals during the growing-finishing period and has been reported from all major pig-rearing countries. The enzootic nature of swine dysentery increases the economic impact of the disease, which arises from decreased rates of growth, poor feed conversion, deaths, costs of medication and treatments, preventive measures, and restrictions on movements of stock (16, 17).

Carrier pigs play a main role in the epidemiology of swine dysentery and are considered the major source of transmission between herds (16). Moreover, *B. hyodysenteriae* survives in the environment for long periods, especially in liquid feces contained in pits and lagoons, where it may remain infective for up to 60 days (16). This spirochete also can naturally colonize mice, rheas, chickens, and mallards (9, 30), and together with mechanical vectors or fomites, this increases the ways in which *B. hyodysenteriae* may be spread within and between herds.

Different typing tools have been developed to discriminate between *B. hyodysenteriae* field isolates and provide a better understanding of the molecular epidemiology of the pathogen. The methods used have included serotyping (3), DNA restriction endonuclease analysis (REA) (6), random amplification of polymorphic DNA (RAPD) (8), restriction fragment length polymorphism of DNA (21), pulsed-field gel electrophoresis (PFGE) (2), multilocus enzyme electrophoresis (MLEE) (25), and multilocus sequence typing (MLST) (24). These techniques provide different levels of discrimination between isolates, and those that are highly discriminating present associated drawbacks such as difficulties in comparing results between laboratories (for example, RAPD and PFGE). On the other hand, MLEE is extremely time-consuming while MLST has high associated costs that are not compatible with routine use in veterinary clinical diagnostic laboratories. Hence, a highly discriminatory method that is time- and cost-effective and yields portable results which allow interlaboratory comparison is still lacking for the typing of *B. hyodysenteriae* isolates.

In the last few years, multiple-locus variable-number tandem-repeat analysis (MLVA) has been developed as an important epidemiologic tool for strain typing of pathogenic microorganism (26). MLVA is based on PCR amplification of multiple loci of minisatellites called variable numbers of tandem repeats (VNTRs). This sort of minisatellite consists of unique direct head-to-tail DNA repeats which can be found in all bacterial genomes and can be used to define specific isolates of bacterial species (35). In addition, VNTRs have been used to infer the bacterial population structure and phylogeny of diverse bacteria species (22, 29, 33). MLVA has the potential to be a highly discriminatory typing technique, being fast, cost-effective, and easy to implement in laboratories with PCR

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TABLE 1. Primers and thermocycling programs used for MLVA of *B. hyodysenteriae*

| Locus | Primer direction,[a] sequence (5′→3′) | Thermocycling program[b] |
|---|---|---|
| Bhyo_6 | F, AAATATAACTCATATTCATAACAAG<br>R, AGAGAACTTCAAAAAACTTC | 30 × (94°C for 20 s, 52°C for 20 s, 72°C for 30 s), 72°C for 5 min |
| Bhyo_7 | F, AAGTAATAAATTAAAAAATCTCTAGGGTGG<br>R, GGTTTGGTAGAACAATCTGC | 30 × (94°C for 20 s, 59.5°C for 20 s, 72°C for 30 s), 72°C for 5 min |
| Bhyo_12 | F, CGTATGATTATTTTACTTGTCAG<br>R, TTTTATTACAGCAACTTTACTC | 30 × (94°C for 30 s, 59°C for 30 s, 74°C for 40 s) |
| Bhyo_17 | F, TTTTTGCCATAAATATCTCTC<br>R, GAAATGCCGTCCTTCTTAG | 30 × (94°C for 30 s, 59°C for 30 s, 74°C for 40 s) |
| Bhyo_21 | F, AAAATAATGATGAAGTATCTAATG<br>R, AAGTATCAGGTAAAGGTAAATC | 30 × (94°C for 20 s, 52°C for 20 s, 72°C for 30 s), 72°C for 5 min |
| Bhyo_22 | F, AGATTAAAAACTGACGGAG<br>R, AGCACAAGAACCTTCAAAC | 30 × (94°C for 30 s, 55°C for 30 s, 72°C for 60 s), 72°C for 5 min |
| Bhyo_10 | F, CTCTCTTTTATATTTTTTATTATAGTTG<br>R, TTGATGAAATTAGACCATTC | 30 × (94°C for 30 s, 55°C for 30 s, 72°C for 40 s), 72°C for 5 min |
| Bhyo_23 | F, CACCCTTTAGACTTATTATTTTATTTTG<br>R, TTGTTCTGCGTGCGTGTAG | 30 × (94°C for 30 s, 55°C for 30 s, 72°C for 40 s), 72°C for 5 min |

[a] F, forward; R, reverse.
[b] Thermocycling programs included a first step of 5 min at 95°C and 30 cycles under the conditions show in parentheses.

technology. Moreover, multiplexing the PCR in combination with capillary electrophoresis of fluorescently labeled primers may allow a higher sample throughput.

In this study, we aimed to develop a simple and reproducible MLVA typing method for use in veterinary clinical microbiology laboratories equipped with either basic PCR technology or more sophisticated capillary electrophoresis equipment. We then applied the method to analyze an international collection of isolates to provide new information about the molecular epidemiology and population structure of this important pathogenic spirochete.

## MATERIALS AND METHODS

**Bacterial strains and DNA preparation.** A set of 172 porcine *B. hyodysenteriae* isolates and strains was used in this study, including the three reference strains B204$^R$ (ATCC 31212), B234$^R$ (ATCC 31287), and WA1$^R$ (ATCC 49526) and the type strain B78$^T$ (ATCC 27164). Duplicates of the B204$^R$ and B78$^T$ strains were obtained from the bacterial collections held at the University of León and Murdoch University. The strains and field isolates were from Spain ($n$ = 115), Australia ($n$ = 36), Canada ($n$ = 3), the United States ($n$ = 7), the United Kingdom ($n$ = 4), and Netherlands ($n$ = 7) and had been recovered from the 1970s to 2009 (see Table S1 in the supplemental material). Twenty-three isolates were recovered from Iberian pigs, a local Spanish breed. These pigs contribute to the preservation of the "dehesa," a specific Mediterranean ecosystem located in the western regions of the country (Castilla y León, Extremadura, and Andalucía), where they are traditionally reared in extensive units. The field isolates were recovered from different herds, except for 26 Spanish isolates that were additionally isolated from 11 herds on different sampling occasions. *B. hyodysenteriae* isolates from the University of León and Murdoch University bacterial collections were identified and cultured, and DNA was extracted in each supplying laboratory by previously reported methods (19, 24). Working dilutions of extracted DNA were prepared by adjusting them to 1 to 20 ng/μl using a NanoDrop 1000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE).

**Identification of tandem repeats and primer design.** The chromosomal DNA sequence of *B. hyodysenteriae* WA1$^R$ was retrieved from GenBank (accession no. NC_012225) and investigated for potential tandem repeats using the default parameters of the Tandem Repeat Finder program (5), available as a Web service (http://tandem.bu.edu/). The selected tandem-repeat loci were ranked by consensus length, and those with lengths between 25 and 300 bp were used to design primers within the flanking regions. Loci were named Bhyo, followed by the repeat length ranking number (from 1 to 23), separated by an underscore.

**Tandem-repeat screening and MLVA setup.** In a preliminary step, DNA extracted from *B. hyodysenteriae* strain B204$^R$ was used to estimate the empirical annealing temperature of the 23 selected primer pairs in a gradient PCR. The PCR was run in a Mastercycler Gradient (Eppendorf Scientific Inc., Westbury, NY) with an initial step of 95°C for 5 min, followed by 30 cycles of a three-step cycle protocol consisting of 94°C for 30 s, 56 ± 8°C for 30 s, and 72°C for 1 min and a final extension step of 72°C for 10 min.

To screen the usefulness of the 23 selected loci as epidemiological markers, DNA samples of *B. hyodysenteriae* strains B204$^R$ and B78$^T$ and isolates 3, 19, 23, 53, 64, H9, and H72, which have been shown to have genetic differences by PFGE and RAPD in a previous investigation (19), were used. In addition, tandem-repeat data generated for *B. hyodysenteriae* strain WA1$^R$ were taken into account. Each locus was amplified individually, and the length of the product was analyzed by conventional agarose gel electrophoresis using a 100-bp DNA ladder (Invitrogen, Carlsbad, CA). Loci were selected according to their length polymorphism and their ability to generate amplicons for most of the DNA samples tested. To confirm the length of the PCR product, as well as the number of repeats, the consensus patterns, and the sizes of the flanking regions, amplicons were purified using the AxyPrep PCR Cleanup kit (Axygen Biosciences, Union City, CA) and sequenced by using fluorescently labeled dideoxynucleotide technology according to the manufacturer's recommendations (Applied Biosystems, Foster City, CA). On this basis, eight VNTR loci were selected to be used in the final typing tool.

**PCR amplifications for MLVA.** The isolates obtained with the bacterial collection selected for this study were analyzed by independently amplifying the eight selected VNTR loci in a Mastercycler apparatus (Eppendorf). The thermocycling conditions and primers used are described in Table 1. PCR mixtures were prepared using 0.2-ml sterile tubes containing 1× PCR buffer (20 mM Tris HCl [pH 8.4], 50 mM KCl), 5 mM $MgCl_2$, 1 U of Platinum *Taq* DNA polymerase (Invitrogen), 200 μM deoxynucleoside triphosphate mix (Invitrogen), 0.2 μM each forward and reverse primers, 2 μl of the DNA working dilution, and sterile distilled water up to a final volume of 50 μl. PCR products were resolved in agarose gels, and their allelic sizes were estimated using a 100-bp DNA ladder (Invitrogen). Amplicons of alleles not detected in the setup step were sequenced as described above. In addition, in order to ensure the repeatability of the technique, 28 DNA samples were randomly selected and tested again. Reproducibility between laboratories was assessed by independent determination of

the VNTR types of 14 isolates at the University of León and Murdoch University.

**Multiplexing and capillary electrophoresis.** Thirty-six Australian *B. hyodysenteriae* isolates and the type strain B78$^T$ were used to develop a capillary electrophoresis-based tool for MLVA. For this purpose, the eight primer pairs used in the individual PCRs were grouped into two sets (set 1 and set 2); labeled fluorescently with 6-carboxyfluorescein (6-FAM), VIC, PET, or NED (Applied Biosystems) at the 5′ end of the forward primers; and pooled as indicated below prior to performing a multiplex PCR using the Qiagen Multiplex PCR kit according to the manufacturer's recommendations (Qiagen, Germantown, MD). Primer set 1 was composed of Bhyo_7 (6-FAM), Bhyo_12 (VIC), Bhyo_17 (NED), and Bhyo_22 (PET) primer pairs at final concentrations of 0.25 μM, 0.25 μM, 0.15 μM, and 0.15 μM, respectively. Primer set 2 was pooled at final primer concentrations of 0.25 μM for Bhyo_6 (6-FAM), 0.25 μM for Bhyo_10 (PET), 0.15 μM for Bhyo_21 (VIC), and 0.15 μM for Bhyo_23 (NED). A 25-μl volume was used for multiplex PCR amplification with a thermal cycling protocol of 95°C for 15 min; 30 three-step cycles of 94°C for 30 s, 55/53°C (set 1/set 2) for 90 s, and 72°C for 90 s; and a final extension step of 72°C for 10 min. Multiplex PCR products were diluted 1:10 in distilled water before 1 μl of this dilution was mixed with 0.5 μl of 1200 LIZ Size Standard (Applied Biosystems) and 10.5 μl of formamide. After the mixture was heated for 3 min at 96°C and rapidly cooled on ice, GeneScan analysis was performed using an ABI 3730 DNA analyzer (Applied Biosystems). The freely available program Peak Scanner Software v1.0 (Applied Biosystems) was used to size the PCR fragments for each locus.

**Analysis of data.** The number of repeats was calculated according to the following formula: Number of repeats = [Fragment size (bp) − Flanking regions (bp)]/Repeat size (bp). The results were approximated to the nearest lower integer and sequentially scored (Bhyo_6, Bhyo_7, Bhyo_12, Bhyo_17, Bhyo_21, Bhyo_22, Bhyo_10, and Bhyo_23) to create a numerical profile that defined each strain. When PCR amplification was undetectable, the assigned number of repeats was 99. MLVA profiles were ascribed to MLVA types by assigning a whole number. Isolates were considered genetically identical when the numerical profiles for all eight loci matched.

The Hunter-Gaston diversity index was used to measure the polymorphism of individual loci and the index of discrimination of the MLVA typing method for the eight combined VNTR loci (20). Approximate 95% confidence intervals (CI) were calculated as described by Grundmann et al. (14). Redundant isolates ($n$ = 26) were removed prior to calculating the previous indexes. The Sequence Type Analysis and Recombinational Tests (START2) program (23), available for free at http://pubmlst.org/software/analysis/start2/, was used to analyze the MLVA profiles and types of the spirochetes tested. A phylogenetic tree of the MLVA types was constructed based on the unweighted-pair group method using average linkages (UPGMA) clustering strategy. A bootstrap analysis for 1,000 replicates was undertaken using FreeTree (15) at http://web.natur.cuni.cz/flegr/programs/freetree.htm. The goeBURST algorithm (12), available at http://goeburst.phyloviz.net/#Software, a global implementation of the eBURST algorithm (10), was used to identify groups of related genotypes of *B. hyodysenteriae* at single-, double-, and triple-locus variant levels.

Population structure was tested as proposed by Smith et al. (32), taking into account the modifications proposed by Haubold et al. (18) for the calculation of the critical value ($L_{MC}$) of the distribution of the variance of the pairwise differences ($V_D$), and expressed as a standardized index of association ($I^{SA}$).

## RESULTS

**Identification of VNTR markers.** Investigation of the chromosomal sequence of *B. hyodysenteriae* WA1$^R$ with the Tandem Repeat Finder program identified 404 repeats in tandem through the whole chromosome, with 135 repeats/Mbp. Subsequent selection of the most suitable tandem-repeat markers decreased the number to be included in the MLVA to 23, which were consecutively named Bhyo_1 to Bhyo_23 and used to design primers within the flanking regions. Fifteen loci that were monomorphic or failed to amplify all or most of the nine selected isolates with the specific primers were discarded. The remaining eight loci were polymorphic, with different allele sizes. Sequencing of the PCR products confirmed that the length polymorphism was due to differences in the copy number of tandem repeats and that the consensus pattern, its period size, and the flanking regions were stable (Table 2). Therefore, eight loci (Bhyo_6, Bhyo_7, Bhyo_12, Bhyo_17, Bhyo_21, Bhyo_22, Bhyo_10, and Bhyo_23) were included in the MLVA scheme for *B. hyodysenteriae*. These loci were distributed from position 1236667 to position 2949421 of the WA1$^R$ genome (Table 2). Four loci, Bhyo_6, Bhyo_10, Bhyo_21, and Bhyo_22, were placed in open reading frames encoding hypothetical proteins, while the other four were located in intergenic regions. Bhyo_7 was placed between the genes for methyl-accepting chemotaxis protein McpA and a hypothetical protein. Bhyo_12 was between the genes for a putative glycosyltransferase family 2 protein and a hypothetical protein. Bhyo_17 was between the genes for glycerol 3-phosphate dehydrogenase and ferredoxin. Bhyo_23 was between the genes for a hypothetical protein and putative RarR, predicted to be a permease.

TABLE 2. Features of the loci included in the MLVA

| Locus | Size (bp) of: | | Position[a] |
|---|---|---|---|
| | Repeat | Flanking region | |
| Bhyo_6 | 156 | 78 | 1236667–1237672 |
| Bhyo_7 | 135 | 177 | 1818959–1819765 |
| Bhyo_10 | 111 | 88 | 1754196–1755095 |
| Bhyo_12 | 105 | 59 | 2949083–2949421 |
| Bhyo_17 | 76 | 175 | 1690628–1691034 |
| Bhyo_21 | 33 | 195 | 1396843–1397034 |
| Bhyo_22 | 30 | 153 | 2597474–2597543 |
| Bhyo_23 | 26 | 102 | 1838685–1838736 |

[a] Location of the VNTR loci in the chromosome of the reference strain *B. hyodysenteriae* WA1$^R$.

**MLVA typing.** The set of eight VNTR markers was used to type the full collection of 174 *B. hyodysenteriae* strains and isolates recovered from pigs in several countries (including the duplicates of B78$^T$ and B204$^R$). The strains and isolates were efficiently amplified, and the lengths of the PCR products were converted into numbers of repeats. Sequencing of new alleles that were identified at this stage confirmed that the length differences represented variations in the number of the previously detected repeat motifs.

The marker Bhyo_10 was the most diverse VNTR, with eight different numbers of repeats (99, 2, 3, 5, 6, 7, 8, and 10), with an assigned number of repeats of 99 because of a lack of amplification. Seven numbers of repeats were detected for locus Bhyo_17, while markers Bhyo_6, Bhyo_7, and Bhyo_21 each presented six numbers of repeats. Loci Bhyo_12 and Bhyo_22 showed a discontinuous distribution of four numbers of repeats. VNTR marker Bhyo_23 showed less diversity, with only two different numbers of repeats, 1 and 2, detected (see Table S2 in the supplemental material).

An accurate estimation of the degree of polymorphism of the loci was achieved by means of the Hunter-Gaston diversity index, with the discrimination powers of the loci ranging from 0.141 to 0.764. Locus Bhyo_10 was the most discriminatory, with a value of 0.764, followed by loci Bhyo_7, Bhyo_6, Bhyo_17, and Bhyo_21, with values of 0.761, 0.718, 0.71, and 0.699, respectively. Loci Bhyo_12 and Bhyo_23 had diversity indexes of 0.472 and 0.318, respectively, while the most conserved locus was Bhyo_22, with a polymorphism index of 0.141.

The Hunter-Gaston discriminatory index of the MLVA typing method at eight loci for 146 strains and isolates from different herds was 0.938 (95% CI, 0.9175 to 0.9584).

Analysis of the combination of the eight VNTR loci for all of the *B. hyodysenteriae* isolates and strains showed 44 MLVA types (see Table S2 in the supplemental material), which differed by at least one repeat for one of the eight loci among two different types. The MLVA types of the reference strains were type 35 for WA1$^R$, type 23 for B204$^R$, and type 10 for B234$^R$, while the type strain B78$^T$ was assigned to MLVA type 28. Analysis of the different MLVA types in each country showed the existence of considerable diversity. There were 15 types (1, 2, 3, 5, 9, 11, 12, 13, 14, 18, 19, 20, 22, 24, and 37) found among the 89 Spanish isolates from different herds, 16 types (15, 16, 17, 25, 26, 31, 32, 33, 34, 35, 36, 38, 39, 42, 43, and 44) among the 36 Australian isolates, 2 types (21 and 27) for the three Canadian isolates, 3 types (3, 6 and 41) for the seven from Netherlands, 4 types (3, 8, 29, and 30) for the four strains from the United Kingdom, and 6 types (4, 7, 10, 23, 28, and 40) for the seven isolates and strains from the United States. MLVA type 3 was shared by isolates from Spain, the United Kingdom, and Netherlands. The MLVA types were stable for the herds where more than one isolate was recovered on different sampling occasions.

*B. hyodysenteriae* strain WA1$^R$ showed a mismatch for locus Bhyo_6 between the length of the PCR product, 780 bp (four numbers of repeats), and the data derived from the sequenced genome, 1,092 bp (six numbers of repeats).

Isolates and strains included in the repeatability and reproducibility tests had the same MLVA types at the different testing times. Moreover, each of the duplicates of the *B. hyodysenteriae* type and reference strains, B78$^T$ and B204$^R$, from the University of León and Murdoch University collections, generated the same MLVA patterns.

**Capillary electrophoresis of multiplexed VNTR markers.** GeneScan analysis to determine the lengths of the VNTR markers included in set 1 and set 2 worked satisfactorily (results not shown). Different loci were clearly distinguished by color in the electropherograms, and use of the internal ladder allowed accurate measurement of their sizes. Differences in allele lengths for all of the loci were recorded compared to allele sizes obtained by sequencing. Loci Bhyo_7, Bhyo_23, and Bhyo_10 were 4 bp shorter and Bhyo_21, Bhyo_17, and Bhyo_12 were 1 to 2 bp shorter by capillary electrophoresis than the expected sizes based on sequencing. Peak sizes for locus Bhyo_22 were 1 bp longer, while the Bhyo_6 locus contained an additional 6 bp. No corrections were needed to calculate the numbers of repeats for loci Bhyo_6, Bhyo_10, Bhyo_12, Bhyo_17, Bhyo_21, and Bhyo_22 because they had incomplete repeats, which made the number of repeats invariable after rounding. As Bhyo_7 and Bhyo_23 were composed of exact copy numbers of repeats, 4 bp were added in order to calculate the number of repeats. MLVA types for all of the bacterial samples typed by GeneScan analysis were congruent with the previously established types.

**MLVA types and bacterial population analysis.** An evolutionary tree based on MLVA profiles and constructed according to the UPGMA clustering strategy for the 44 MLVA types of *B. hyodysenteriae* determined in this study is shown in Fig. 1.

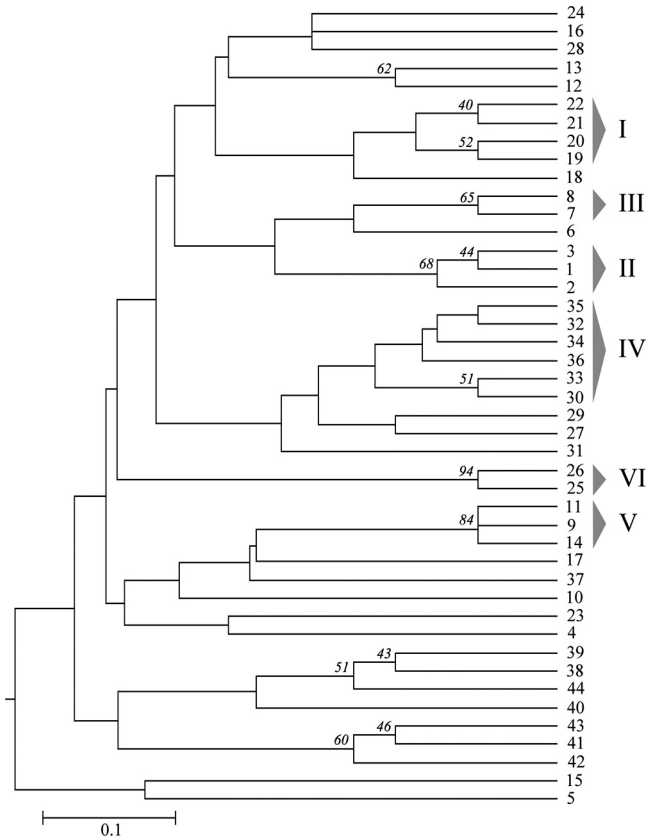


FIG. 1. Dendrogram of the 44 *B. hyodysenteriae* MLVA types found in the present study and clustered using UPGMA. Roman numerals I to VI indicate clonal complexes defined at the single-locus variant level. The scale bar represents genetic distance as the absolute number of differences in marker alleles among genotypes. Bootstrap values of ≥40% are shown.

MLVA type relationships at the single-, double-, and triple-locus variant levels depicted with the goeBURST algorithm are shown in Fig. 2. Six clonal complexes (I to VI) were established at the single-locus variant level. Three new groups appeared when investigating double-locus variants, while three single-locus variant groups (II, III, and IV) were clustered together at this level. When high-level edges were displayed to study relationships at the triple-locus variant level, a large cluster appeared which included groups I to IV, and group V was expanded by two more types. MLVA types 4, 5, 10, and 15 were not linked with any of the other types detected at any of the levels studied. Population linkage disequilibrium was detected for the 146 isolates from different herds ($I^{S_A}$ = 0.1359; $P < 0.001$) and for the different MLVA types ($I^{S_A}$ = 0.0336; $P = 0.005$).

## DISCUSSION

In this study, we established and used MLVA as a novel typing method for the pathogenic spirochete *B. hyodysenteriae*. Many of the MLVA tools for pathogenic bacteria are based on short repeats (26), whereas the current technique was based on repeats of greater than 25 bp. These long repeats are unlikely to exist alone by chance, and hence, they could have an important effect on the biology of *B. hyodysenteriae* (31). The

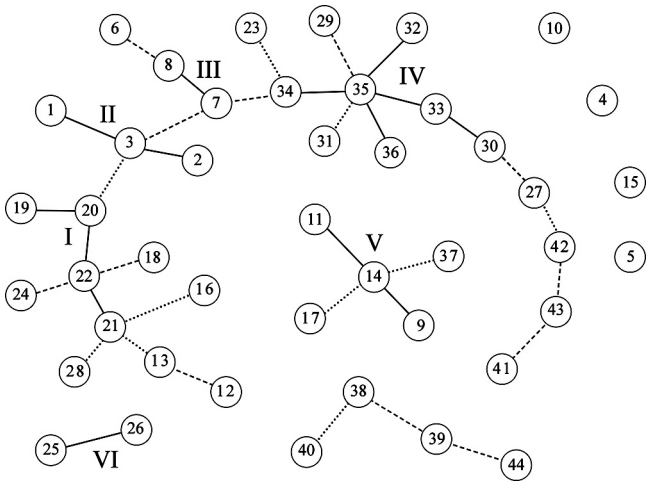


FIG. 2. MLVA types (circled) and relationships found among them according to the goeBURST algorithm. Solid lines show the single-locus variant level, dashed lines show the double-locus variant level, and dotted lines show the triple-locus variant level. Groups at the single-locus variant level are indicated by roman numerals I to VI.

tandem-repeat loci included in this study were placed in intergenic regions or in regions encoding hypothetical proteins; thus, their functions are unknown and deserve further investigation. Long repeats have the potential to be resolved and accurately sized by agarose gel electrophoresis, not requiring the use of sophisticated technology to measuring the differences in length, as is required for short repeats. The use of capillary electrophoresis with the technique, however, enables more rapid throughput. Mismatches between the length in capillary electrophoresis and the size obtained by sequencing have been reported for other bacteria and have been explained by the fact that electrophoretic mobility of DNA is sequence dependent (28). In the current study, the use of long repeats minimized their impact and they were easily corrected and did not affect the general performance of the technique.

The MLVA technique distinguished 94 of 100 isolates chosen at random, exceeding the Hunter-Gaston diversity index acceptance level of 0.9 previously proposed when developing new typing schemes (20). There are few published Hunter-Gaston diversity index results for other typing techniques using large nonlocal collections of *B. hyodysenteriae* strains. However, a recent study by MLST reported a diversity index of 0.974 for 111 strains (24), and a previous MLEE study performed on 231 isolates gave a haplotypic diversity of 0.94 (34). Although no confidence limits were defined in the previous studies to enable an accurate evaluation of the techniques (14), MLST seems to be slightly more discriminatory than MLVA. In general, MLVA clustering also was in agreement with the groups defined by MLST, MLEE, REA, and PFGE, demonstrating the consistency of this new typing technique.

The index of diversity at individual loci indirectly reflects the mutation rate and homoplasy. Accordingly, markers with higher homoplasy have a lower phylogenetic value (13). A Hunter-Gaston diversity index cutoff value of 0.9 has been used for other bacteria to detect hypervariable loci (1, 13). However, none of the loci selected for the *B. hyodysenteriae* MLVA exceeded this value, indicating that the technique is sufficiently robust to perform phylogenetic studies.

In spite of the strict biosecurity measures that are observed in modern European piggeries, in this study, one newly defined clone of *B. hyodysenteriae* (MLVA type 3) was found in Spain, Netherlands, and the United Kingdom. This clone was different from the clone of unusual indole-negative *B. hyodysenteriae* isolates that previously has been detected in pigs from Spain, Germany, and Belgium (11, 19) and which were located in MLVA types 19, 20, and 22 in clonal complex I in the current study. The European spread of clones like this could have been the result of past movements of carrier pigs of high genetic value, particularly within the larger commercial pig-producing companies.

The links at the single-locus variant level were sufficiently strong to establish epidemiologic connections between strains and define clonal complexes that were in agreement with the clusters found in the evolutionary study. However, the goeBURST higher-level definitions (double- and triple-locus variant levels) should be considered together with epidemiologic data before linking isolates. Consequently, the combination of the data produced in the phylogenetic analysis together with the established relationships at different locus variants allowed the study of associations between strains. This analysis revealed several international connections between strains, based on a common ancestry. For example, four Spanish types (18, 19, 20, and 22) had the same origin as Canadian isolate FMV89.3323 (type 21) from the late 1980s. Isolates from the United Kingdom and the United States were strongly linked (clonal complex III) and were likely related to Dutch strain B5 (type 6). As mentioned above, these findings could be explained by the past movement of carrier pigs, particularly those of high genetic value. In agreement with this, an isolate from the United Kingdom was included in clonal complex IV, composed mainly of Australian isolates. The within-country spread of clonal complexes II and V in Spain and clonal complex VI in Australia is probably the result of adaptive responses of a common ancestral strain to specific herd conditions in these countries, resulting in the current different detectable types.

It is relatively rare for two different strains of *B. hyodysenteriae* to be present in the same herd (7). However, a microevolution phenomenon resulting in minor changes between isolates has been reported for *B. hyodysenteriae* isolates within herds when more than one per herd were tested by PFGE or MLST (2, 19, 24). It is not known if these minor changes reflect the true status of the bacterial population in the herd or are a result of an inherent variability of the techniques that can depend on a single nucleotide change. On the other hand, the stability of the MLVA types within herds could indicate that tandem repeats are less susceptible to undergoing these minor changes that can negatively affect the epidemiologic follow-up of strains between herds. Some of the Spanish field isolates had stable MLVA types over 8 years (types 3 and 13), and this chronologic stability is in agreement with previous observations based on PFGE and RAPD (11, 19). The stability of *B. hyodysenteriae* under field conditions could reflect the way this pathogen has adapted to survive in the specialized ecological niche represented by the hindgut of the pig (4).

A previous study based on MLEE data concluded that *B. hyodysenteriae* is a recombinant species with an epidemic structure (34). In contrast, another study using MLST analysis

found that the population structure appeared clonal (24). Both studies were based on the index of association proposed by Smith et al. (32), which was later improved by Haubold et al. (18) and used for the current study. The analysis of the data generated by MLVA indicated that the population was in linkage disequilibrium, consistent with a clonal population. Even when analysis of MLVA types and subgroups based on phylogenetic analysis was performed, the population was clonal at all levels. However, there are two situations which are likely to bias the clonality of a population: the spatial isolation of lineages and the existence of mechanism for recombination (32). Modern pig farming uses spatial isolation to protect pigs from diseases and avoid their spread. We attempted to circumvent this by examining isolates from Iberian pigs reared in extensive units with limited biosecurity measures. In this system, geographic isolation is minimized while the production characteristics, with access to open field areas and regular movements of animals of uncertain sanitary status between farms, enhance opportunities for transmission. However, linkage disequilibrium persisted under these circumstances, further supporting the clonality of the species. It is known that *B. hyodysenteriae* is able to horizontally transfer genetic information *in vitro* by using a prophage-like mechanism (27), but under field conditions, this mechanism does not seem to be sufficient to destroy linkage disequilibrium.

In conclusion, MLVA is a low-cost and simple epidemiologic tool for typing and tracking *B. hyodysenteriae* isolates. It has a high phylogenetic value and can be used with other techniques such as MLST if more strain discrimination is needed.

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## REFERENCES

1. **Al Dahouk, S., P. Le Flèche, K. Nöckler, I. Jacques, M. Grayon, H. C. Scholz, H. Tomaso, G. Vergnaud, and H. Neubauer.** 2007. Evaluation of *Brucella* MLVA typing for human brucellosis. J. Microbiol. Methods **69:**137–145.
2. **Atyeo, R. F., S. L. Oxberry, and D. J. Hampson.** 1999. Analysis of *Serpulina hyodysenteriae* strain variation and its molecular epidemiology using pulsed-field gel electrophoresis. Epidemiol. Infect. **123:**133–138.
3. **Baum, D. H., and L. A. Joens.** 1979. Serotypes of beta-haemolytic *Treponema hyodysenteriae*. Infect. Immun. **25:**792–796.
4. **Bellgard, M. I., P. Wanchanthuek, T. La, K. Ryan, P. Moolhuijzen, Z. Albertyn, B. Shaban, Y. Motro, D. S. Dunn, D. Schibeci, A. Hunter, R. Barrero, N. D. Phillips, and D. J. Hampson.** 2009. Genome sequence of the pathogenic intestinal spirochete *Brachyspira hyodysenteriae* reveals adaptations to its lifestyle in the porcine large intestine. PLoS One **4:**e4641.
5. **Benson, G.** 1999. Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res. **27:**573–580.
6. **Combs, B., D. J. Hampson, J. R. Mhoma, and J. R. Buddle.** 1989. Typing of *Treponema hyodysenteriae* by restriction endonuclease analysis. Vet. Microbiol. **19:**351–359.
7. **Combs, B. G., D. J. Hampson, and S. J. Harders.** 1992. Typing of Australian isolates of *Treponema hyodysenteriae* by serology and by DNA restriction endonuclease analysis. Vet. Microbiol. **31:**273–285.
8. **Dugourd, D., M. Jacques, M. Bigras-Poulin, and J. Harel.** 1996. Characterization of *Serpulina hyodysenteriae* isolates of serotypes 8 and 9 by random amplification of polymorphic DNA analysis. Vet. Microbiol. **48:**305–314.
9. **Feberwee, A., D. J. Hampson, N. D. Phillips, T. La, H. M. van der Heijden, G. J. Wellenberg, R. M. Dwars, and W. J. Landman.** 2008. Identification of *Brachyspira hyodysenteriae* and other pathogenic *Brachyspira* species in chickens from laying flocks with diarrhea or reduced production or both. J. Clin. Microbiol. **46:**593–600.
10. **Feil, E. J., B. C. Li, D. M. Aanensen, W. P. Hanage, and B. G. Spratt.** 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J. Bacteriol. **186:**1518–1530.
11. **Fellström, C., M. Karlsson, B. Pettersson, U. Zimmerman, A. Gunnarsson, and A. Aspan.** 1999. Emended descriptions of indole negative and indole positive isolates of *Brachyspira* (*Serpulina*) *hyodysenteriae*. Vet. Microbiol. **70:**225–238.
12. **Francisco, A. P., M. Bugalho, M. Ramirez, and J. A. Carriço.** 2009. Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. BMC Bioinformatics **10:**152.
13. **Gorgé, O., S. Lopez, V. Hilaire, O. Lisanti, V. Ramisse, and G. Vergnaud.** 2008. Selection and validation of a multilocus variable-number tandem-repeat analysis panel for typing *Shigella* spp. J. Clin. Microbiol. **46:**1026–1036.
14. **Grundmann, H., S. Hori, and G. Tanner.** 2001. Determining confidence intervals when measuring genetic diversity and the discriminatory abilities of typing methods for microorganisms. J. Clin. Microbiol. **39:**4190–4192.
15. **Hampl, V., A. Pavlícek, and J. Flegr.** 2001. Construction and bootstrap analysis of DNA fingerprinting-based phylogenetic trees with the freeware program FreeTree: application to trichomonad parasites. Int. J. Syst. Evol. Microbiol. **51:**731–735.
16. **Hampson, D. J., R. F. Atyeo, and B. G. Combs.** 1997. Swine dysentery, p. 175–209. *In* D. J. Hampson and T. B. Stanton (ed.), Intestinal spirochaetes in domestic animals and humans. CAB International, Wallingford, United Kingdom.
17. **Hampson, D. J., C. Fellström, and J. R. Thomson.** 2006. Swine dysentery, p. 785–805. *In* B. E. Straw, J. J. Zimmerman, S. D'Allaire, and D. J. Taylor (ed.), Diseases of swine, 9th ed. Blackwell Publishing, Ames, IA.
18. **Haubold, B., M. Travisano, P. B. Rainey, and R. R. Hudson.** 1998. Detecting linkage disequilibrium in bacterial populations. Genetics **150:**1341–1348.
19. **Hidalgo, Á., A. Carvajal, M. Pringle, P. Rubio, and C. Fellström.** 2010. Characterization and epidemiological relationships of Spanish *Brachyspira hyodysenteriae* field isolates. Epidemiol. Infect. **138:**76–85.
20. **Hunter, P. R., and M. A. Gaston.** 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. **26:**2465–2466.
21. **Jensen, N. S., and T. B. Stanton.** 1993. Comparison of *Serpulina hyodysenteriae* B78, the type strain of the species, with other *S. hyodysenteriae* strains using enteropathogenicity studies and restriction fragment length polymorphism analysis. Vet. Microbiol. **36:**221–231.
22. **Johansson, A., J. Farlow, P. Larsson, M. Dukerich, E. Chambers, M. Byström, J. Fox, M. Chu, M. Forsman, A. Sjöstedt, and P. Keim.** 2004. Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. J. Bacteriol. **186:**5808–5818.
23. **Jolley, K. A., E. J. Feil, M. S. Chan, and M. C. Maiden.** 2001. Sequence type analysis and recombinational tests (START). Bioinformatics **17:**1230–1231.
24. **La, T., N. D. Phillips, B. L. Harland, P. Wanchanthuek, M. I. Bellgard, and D. J. Hampson.** 2009. Multilocus sequence typing as a tool for studying the molecular epidemiology and population structure of *Brachyspira hyodysenteriae*. Vet. Microbiol. **138:**330–338.
25. **Lee, J. I., D. J. Hampson, B. G. Combs, and A. J. Lymbery.** 1993. Genetic relationships between isolates of *Serpulina* (*Treponema*) *hyodysenteriae*, and comparison of methods for their subspecific differentiation. Vet. Microbiol. **34:**35–46.
26. **Lindstedt, B. A.** 2005. Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. Electrophoresis **26:**2567–2582.
27. **Matson, E. G., R. L. Zuerner, and T. B. Stanton.** 2007. Induction and transcription of VSH-1, a prophage-like gene transfer agent of *Brachyspira hyodysenteriae*. Anaerobe **13:**89–97.
28. **Pasqualotto, A. C., D. W. Denning, and M. J. Anderson.** 2007. A cautionary tale: lack of consistency in allele sizes between two laboratories for a published multilocus microsatellite typing system. J. Clin. Microbiol. **45:**522–528.
29. **Pourcel, C., F. André-Mazeaud, H. Neubauer, F. Ramisse, and G. Vergnaud.** 2004. Tandem repeats analysis for the high resolution phylogenetic analysis of *Yersinia pestis*. BMC Microbiol. **4:**22.
30. **Råsbäck, T., D. S. Jansson, K. E. Johansson, and C. Fellström.** 2007. A novel enteropathogenic, strongly haemolytic spirochaete isolated from pig and mallard, provisionally designated "*Brachyspira suanatina*" sp. nov. Environ. Microbiol. **9:**983–991.
31. **Rocha, E. P., A. Danchin, and A. Viari.** 1999. Functional and evolutionary roles of long repeats in prokaryotes. Res. Microbiol. **150:**725–733.

32. **Smith, J. M., N. H. Smith, M. O'Rourke, and B. G. Spratt.** 1993. How clonal are bacteria? Proc. Natl. Acad. Sci. U. S. A. **90:**4384–4388.
33. **Supply, P., R. M. Warren, A. L. Bañuls, S. Lesjean, G. D. Van Der Spuy, L. A. Lewis, M. Tibayrenc, P. D. Van Helden, and C. Locht.** 2003. Linkage disequilibrium between minisatellite loci supports clonal evolution of *Mycobacterium tuberculosis* in a high tuberculosis incidence area. Mol. Microbiol. **47:**529–538.
34. **Trott, D. J., S. L. Oxberry, and D. J. Hampson.** 1997. Evidence for *Serpulina hyodysenteriae* being recombinant, with an epidemic population structure. Microbiology **143:**3357–3365.
35. **van Belkum, A.** 2007. Tracing isolates of bacterial species by multilocus variable number of tandem repeat analysis (MLVA). FEMS Immunol. Med. Microbiol. **49:**22–27.