A multilocus variable number tandem repeat analysis assay provides high discrimination for genotyping *Leptospira santarosai* strains

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INTRODUCTION

Leptospirosis, a worldwide zoonosis, is a re-emerging infectious disease caused by pathogenic *Leptospira* species. Infected animals are mostly subclinical and may act as reservoir hosts to a particular serovar of leptospires, shedding the bacteria through their urine for prolonged periods of time. There is a wide range of animal hosts, including wild and domestic animals, especially rodents, small marsupials, pigs, horses, dogs and cattle (Roqueplo et al., 2013).

In cattle, the infection has been established as a major infectious disease, due to its negative impacts on reproduction (abortion, embryonic death, stillbirths and infertility), decreased milk production and growth rates, as well as indirect costs associated with treatments and veterinary assistance (Mughini-Gras et al., 2014). Whilst *Leptospira borgpetersenii* sv. Hardjo subtype Hardjobovis (Hardjobovis) has been referred as the main causative agent of bovine leptospirosis in Australia, New Zealand and Chile (Salgado et al., 2014), *Leptospira interrogans* sv. Hardjo subtype Hardjoprajitno seems to prevail in other regions (Carmona et al., 2013). In this context, the real role of other leptospiral species remains to be elucidated.

Other pathogenic *Leptospira* species include *Leptospira santarosai*, which has been mostly isolated in the Americas, particularly in Latin America (Nalam et al., 2010). Strains have been isolated from different hosts, including humans as well as domestic and wild animals (see the collections of *Leptospira* reference strains at the Leptospirosis Reference Centers at the Institute Pasteur, France, http://www.pasteur.fr/recherche/Leptospira/LeptospiraF.html, and KIT Biomedical Research, The Netherlands, http://www.kit.nl/biomedical-research/product-category/leptospira-strains/). In ruminants, to our knowledge, only five *L. santarosai* strains have been isolated until now, including four from the Americas: two from Brazil ([*L. santarosai* strain Bov-G (serogroup Hebdomadis) from a cow (Santa Rosa et al., 1980)] and [*L. santarosai* sv. Guaricura (serogroup Sejroe) from a buffalo (Vasconcellos et al., 2001)], and two from cattle in Peru (Rivera et al., 2012).

Multilocus variable number tandem repeat (VNTR) analysis (MLVA) is a molecular method used for the DNA fingerprinting of pathogenic *Leptospira*, and is currently available for *L. interrogans*, *Leptospira kirschneri* and *L. borgpetersenii* species (Slack et al., 2003; Salaün et al., 2006). Nevertheless, it has never been applied to *L. santarosai* strains.

In this context, considering the importance of *L. santarosai* in the Americas and the scarce information about the species, we aimed to apply a multilocus variable number tandem repeat (VNTR) analysis (MLVA) for the molecular typing of *L. santarosai* isolates from various sources. Amplification of three VNTR loci selected from *L. santarosai* genome sequences resulted in a wide range of sizes for the amplified products amongst the 21 *L. santarosai* strains analysed. This suggested a variation in tandem repeat copy numbers in the VNTR loci. secY sequencing also showed a high nucleotide diversity, confirming the MLVA data. In conclusion, this novel MLVA analysis assay provides high discrimination for *L. santarosai* isolates, and this new typing tool could be used to investigate leptospirosis in regions where *L. santarosai* predominates.
species, we aimed to develop an MLVA assay for the molecular typing of *L. santarosai* isolates from various sources.

**METHODS**

**Studied strains and DNA.** A total of 21 *L. santarosai* isolates were studied, 15 of them obtained in the Americas. Seven belonged to the *Leptospira* Collection of Veterinary Bacteriology Laboratory of Universidade Federal Fluminense and had been obtained from cattle form Rio de Janeiro, Brazil, in 2013. The other 14 strains were from the collection of the French National Reference Center and WHO Collaborating Center for Leptospirosis (http://www.pasteur.fr/recherche/Leptospira/), and were obtained from humans (seven strains) and wild animals (seven strains) (see Table 2 below). Additionally, DNA of 10 reference strains (*L. interrogans* sv. Autumnalis strain Akiyami, *L. interrogans* sv. Copenhageni strain Winberg, *L. kirschneri* sv. Grippotyphosa strain Moskva V, *L. kirschneri* sv. Cynopteri strain 3522C, *L. borgpetersenii* sv. Hardjobovis strain Sponselee, *L. borgpetersenii* sv. Tarassovi strain Perpeletisín, *Leptospira* noguchii sv. Panama strain CZ214K, *L. noguchii* sv. Louisiana strain LUC 1945, *Leptospira biflexa* sv. Patoc strain Patoc 1 and *Leptospira weilli* sv. Sarmin strain Sarmin) was used to check the specificity of the primers.

**Molecular and serological characterization of Leptospira isolates.** The strains were characterized by serogrouping (Bourhy et al., 2013; Hamond et al., 2015) (21 strains), and by sequencing of *rrs* (Mérien et al., 1992) (21 strains) and *secY* (Ahmed et al., 2006) (18 isolates) genes. All molecular epidemiological data were stored and analysed with Bionumerics software (version 6.5; Applied-Maths).

**Identification of VNTR-like regions.** The nucleotide sequences of the draft genomes of *L. santarosai* sv. Shermanni strain 1342KT and *L. santarosai* sv. Shermanni strain LT 821 (Chou et al., 2012) were analysed using the Tandem Repeats Database (http://tandem.bu.edu/cgi-bin/trdb/trdb.exe).

The GenBank accession numbers of the genome sequences of strains 1342KT and LT 821 are NZ_AOHB00000000.2 and NZ_ADOR00000000.1, respectively. The genome sequence of *L. santarosai* sv. Shermanni strain 1342KT is part of the ‘*Leptospira Genomics and Human Health*’ project from the J. Craig Venter Institute Genomic Center for Infectious Diseases (http://gsc.jcvi.org/).

Ten VNTR-like loci were initially selected after comparing the length (67–76 bp), sequence identity (>88%) and copy number (between three and 10 copies) of repeats. These criteria were considered to allow a simple and easy determination of the size of the PCR products on agarose gels following electrophoresis (length of PCR products should be <1 kb). Primer pairs flanking the VNTR loci (called VNTR S1, S2, etc.) were then designed and PCR was performed with the 10 selected VNTR loci on genomic DNA of six reference strains (see conditions below). VNTR loci (i.e. VNTR S1, S2 and S9) that exhibited PCR products whose size could be easily determined in an agarose gel electrophoresis for the six reference strains were further evaluated with a larger panel of strains.

**PCR-VNTR.** For VNTR, the DNA was amplified using *Taq* polymerase (Illustra; GE Healthcare) under the following conditions: one denaturation cycle at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min; and a final elongation at 72°C for 10 min. The amplified products were analysed by 1% agarose gel electrophoresis. The sizes of the amplicons were estimated by comparison with a 100 bp ladder (Invitrogen).

**RESULTS**

Based upon the genome sequences of *L. santarosai*, 10 VNTR-like loci were analysed by PCR. Analysis of the amplified products of PCR-VNTR by agarose gel electrophoresis revealed size variations for most of the primer pairs. However, either no amplification (VNTR S6 and S7) or amplification for only a minority of strains (VNTR S3, S4, S8 and S10) was obtained for several primer pairs, which were therefore excluded from this study (data not shown). The three most discriminative VNTR loci were VNTR S1, S2 and S9, which are shown in Table 1. VNTR S2 showed a profile containing multiple bands and was used to differentiate between genetically related strains. VNTR S9 amplified more than one band for two of the studied strains. The developed *L. santarosai* VNTR scheme did not amplify DNA obtained from *L. interrogans*, *L. kirschneri*, *L. borgpetersenii*, *L. noguchii*, *L. biflexa* or *L. weilli* species.

When PCR-VNTR was performed for the 21 *L. santarosai* strains with the three selected VNTR loci, the sizes of the amplified products displayed a high polymorphism. This suggested a variation in tandem repeat copy numbers in the VNTR S1 (Fig. 1), S2 (Fig. 2) and S9 (Fig. 3) loci. Using VNTR S1 and S9, 10 different profiles (designated profiles A–J) could be identified, including one (profile C) which was predominant (six strains). Other profiles included four (A, three (B)), two (F) and one (D, E, G, H, I and J) strains. When VNTR S2 was included, a higher level of discrimination was obtained and 17 profiles were observed. The results are summarized in Table 2.

Sequencing of the *secY* gene enabled genotyping of *Leptospira* strains at the subspecies level (Fig. 4). The MLVA patterns were in close agreement with the alleles determined by *secY* sequencing (Table 2). For example, the genotype of strain 201103640 was unique amongst the strains tested by both MLVA and *secY* sequencing, and strains 2013_U152 and 2013_U278 were indistinguishable by both molecular techniques.

**DISCUSSION**

*L. santarosai* is found almost exclusively in the Americas, particularly in Latin America. This species has been isolated mostly from humans and wild animals, but has also been reported in ruminants in Brazil (Santa Rosa et al., 1980; Vasconcellos et al., 2001). Therefore, it is not surprising that seven of the strains isolated from cattle in Brazil were from *L. santarosai*. Amongst these isolates, members of serogroup Grippo-pyphosa are usually associated with environmental contamination and are maintained by wildlife species. From that serogroup, Bananal is a local serovar that has already been isolated in Brazil from mice, capybaras and goats (Lilenbaum et al., 2014), but had never been recovered from cattle. Serogroup Sejroe is the most common in ruminants worldwide, as well as in Brazil (Cosate et al., 2012). Nevertheless, although serovar Hardjo
is the most frequently reported, the epidemiological role of serovar Guaricura, a local strain that was first obtained from a buffalo, remains to be elucidated. In contrast, L. santarosai strains belonging to serogroups Sarmin, Shermani and Tarassovi had never been reported from cattle in Brazil, and may have been neglected as agents of bovine leptospirosis. These findings, considering both the species and serogroups of the isolates, suggest that the aetiology of bovine leptospirosis in the Americas may be much more variable and complex than previously thought.

Detailed characterization of leptospiral isolates is important for understanding the epidemiology of leptospirosis (Bourhy et al., 2013). Identification of leptospiral isolates is usually based on serological and molecular methods (Benacer et al., 2013). However, molecular characterization of Leptospira strains does not always match with the

Table 1. VNTR loci from the L. santarosai sv. Shermani genome used in this study

<table>
<thead>
<tr>
<th>VNTR locus</th>
<th>Primer (5'→3')</th>
<th>GenBank accession no.</th>
<th>Position (bp)</th>
<th>Unit length (bp)</th>
<th>Copy no.</th>
</tr>
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<tbody>
<tr>
<td>VNTR S1</td>
<td>1F_CGAGTGAACTGCTGGCACA</td>
<td>NZ_AOHB00000000.2</td>
<td>11431–11653</td>
<td>71</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1R_CGCTCTAGGTATGCTGGGA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VNTR S2</td>
<td>2F_AAGAACCTCAAAGCCCTTC</td>
<td>NZ_AOHB00000000.2</td>
<td>29481–29773</td>
<td>75</td>
<td>6</td>
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<tr>
<td></td>
<td>2R_TCCATACCCCGTGCTCTTTACT</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>VNTR S9</td>
<td>9F_GCGAAAAATGGTGATGAGGA</td>
<td>NZ_ADOR00000000.1</td>
<td>177151–177848</td>
<td>67</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>9R_AACTCACAACCCCTCCCCGAA</td>
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Table 2. Characterization of 21 strains of L. santarosai from different origins and hosts according to serogrouping and MLVA results

<table>
<thead>
<tr>
<th>ID</th>
<th>Serogroup</th>
<th>Serovar</th>
<th>Strain</th>
<th>Country</th>
<th>Host</th>
<th>Estimated locus copy no.</th>
<th>Profile</th>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>VNTR S1 VTRN S2 VNTR S9</td>
<td>secY</td>
</tr>
<tr>
<td>1</td>
<td>Shermani</td>
<td>Shermani</td>
<td>1342KT</td>
<td>Panama</td>
<td>Spiny rat</td>
<td>4 1 10</td>
<td>A1 4</td>
</tr>
<tr>
<td>2</td>
<td>Mini</td>
<td>Beye</td>
<td>1537 U</td>
<td>Panama</td>
<td>Spiny rat</td>
<td>2 2 10</td>
<td>B1 ND</td>
</tr>
<tr>
<td>3</td>
<td>Tarassovi</td>
<td>Atlantae</td>
<td>LT 81</td>
<td>USA</td>
<td>Opossum</td>
<td>4 3 10</td>
<td>A2 4</td>
</tr>
<tr>
<td>4</td>
<td>Mini</td>
<td>Georgia</td>
<td>LT 117</td>
<td>USA</td>
<td>Raccoon</td>
<td>3 4 11</td>
<td>E 8</td>
</tr>
<tr>
<td>5</td>
<td>Tabaquite</td>
<td>TRVL 3214</td>
<td></td>
<td>Trinidad</td>
<td>Human</td>
<td>2 5 11</td>
<td>C1 6</td>
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<td>6</td>
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<td>2013_U152</td>
<td>Brazil</td>
<td>Cattle</td>
<td>2 6 6</td>
<td>F 9</td>
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<tr>
<td>7</td>
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<td>1 7 –</td>
<td>I 10</td>
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<td>2 8 11</td>
<td>C2 11</td>
</tr>
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<td>Sejroe</td>
<td>Guaricura</td>
<td>2013_U214</td>
<td>Brazil</td>
<td>Cattle</td>
<td>2 9 4</td>
<td>G 3</td>
</tr>
<tr>
<td>10</td>
<td>Grippotyphosa</td>
<td>Bananal</td>
<td>2013_U233</td>
<td>Brazil</td>
<td>Cattle</td>
<td>2 10 11</td>
<td>C3 1</td>
</tr>
<tr>
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<td>Shermani</td>
<td>Unidentified*</td>
<td>2013_U278</td>
<td>Brazil</td>
<td>Cattle</td>
<td>2 6 6</td>
<td>F 9</td>
</tr>
<tr>
<td>12</td>
<td>Grippotyphosa</td>
<td>Bananal</td>
<td>2013_U280</td>
<td>Brazil</td>
<td>Cattle</td>
<td>2 10 10</td>
<td>B2 1</td>
</tr>
<tr>
<td>13</td>
<td>Shermani</td>
<td>Shermani</td>
<td>LT 821</td>
<td>Panama</td>
<td>Rat</td>
<td>4 3 10</td>
<td>A2 ND</td>
</tr>
<tr>
<td>14</td>
<td>Ballum</td>
<td>Peru</td>
<td>MW 10</td>
<td>Peru</td>
<td>Opossum</td>
<td>3 11 10</td>
<td>D 5</td>
</tr>
<tr>
<td>15</td>
<td>Bataviae</td>
<td>Bataviae</td>
<td>Schoolby</td>
<td>Panama</td>
<td>Human</td>
<td>4 3 10</td>
<td>A2 ND</td>
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<td>16</td>
<td>Grippotyphosa</td>
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<td>CZ 188</td>
<td>Panama</td>
<td>Spiny rat</td>
<td>2 9 10</td>
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<tr>
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<td>Tabaquite</td>
<td>201103484</td>
<td>French West</td>
<td>Indies Human</td>
<td>2 – 10</td>
<td>B4 6</td>
</tr>
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<td>Tabaquite</td>
<td>201103460</td>
<td>French West</td>
<td>Indies Human</td>
<td>13 12 10</td>
<td>H 2</td>
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<tr>
<td>19</td>
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<td>Tabaquite</td>
<td>201102932</td>
<td>French West</td>
<td>Indies Human</td>
<td>2 13 10</td>
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<td>21</td>
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<td>201101963</td>
<td>French West</td>
<td>Indies Human</td>
<td>2 14 –</td>
<td>J 7</td>
</tr>
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</table>

ND, Not done.
*Possibly refers to new genotype/serovar.
original serological classification. Identification of serovars, which is defined by the complex, laborious and expensive cross-agglutinin absorption test, may not be accurate to cluster phylogenetically and/or epidemiologically related strains (Bourhy et al., 2013).

The VNTR analysis rendered 18 genotypes amongst the isolates. Importantly, genetic analysis did not match serology. Strains from the same serogroup (e.g. Shermani) rendered different genotypes, whilst strains from the same genotype (e.g. A2) were found to belong to different serogroups.
Discrepancies between serology and genetic classification are not unexpected. Several authors have commented on this, and there is a consensus that more isolates and loci are needed to obtain an improved typing method for *Leptospira* characterization (Nalam *et al.*, 2010; Caimi *et al.*, 2012). Thus, a combined molecular (VNTR, multilocus sequence typing) and serological analysis seems to be a better approach for the typing of *Leptospira* isolates in order to obtain a greater insight into the evolutionary biology and epidemiology of this important pathogen.

In this study, secY sequencing and MLVA were used to characterize the *L. santarosai* isolates. MLVA showed a strong correlation with the secY sequence analysis, which was previously used for taxonomic and epidemiological purposes (Ahmed *et al.*, 2006; Perez & Goarant, 2010; Paiva-Cardoso *et al.*, 2013). Amongst the molecular techniques, genotyping *L. interrogans* strains by MLVA is increasingly being utilized for identification and epidemiological studies (Slack *et al.*, 2005; Li *et al.*, 2012; Caimi *et al.*, 2012). In the present study, MLVA of *L. santarosai* showed that serovars from either the same serogroup or the same geographical area were not grouped together. In contrast, strains belonging to distinct serovars (e.g. Atlantae, Shermani and Bataviae) showed identical VNTR patterns (Table 2). The discrepancies between serovar and VNTR patterns may reflect a higher stability of the genome of serovars/genotypes with specific animal reservoirs or ecological niches over time and geographical distribution.

Altogether, serological and molecular tools allowed a reliable characterization of the isolates, and represent a contribution to the understanding of the epidemiology of *L. santarosai* in various hosts. Using these tools, a deeper understanding on the transmission of the disease in cattle is possible. The role of cow-to-cow transmission in an infected herd and the impact of the environment or wild...
animals in tropical regions have been speculated, but are still uncertain under many conditions. Understanding the epidemiology is mandatory for an adequate control program, and molecular tools developed for *L. santarosai* may contribute to its understanding and control, particularly in Latin America. In this context, the MLVA scheme described here can be applied to *L. santarosai* isolates and this new typing tool can contribute to a better understanding of the epidemiology of circulating strains in the Americas.

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