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## Molecular Characterization and Serology of *Leptospira kirschneri* (Serogroup Grippotyphosa) Isolated from Urine of a Mare Post-Abortion in Brazil

C. Hamond, G. Martins, S. Bremont, M. A. Medeiros, Pascale Bourhy, W. Lilenbaum

### ► To cite this version:

C. Hamond, G. Martins, S. Bremont, M. A. Medeiros, Pascale Bourhy, et al.. Molecular Characterization and Serology of *Leptospira kirschneri* (Serogroup Grippotyphosa) Isolated from Urine of a Mare Post-Abortion in Brazil. *Zoonoses and Public Health*, 2015, 63 (3), pp.191-195. 10.1111/zph.12224 . pasteur-02945392

**HAL Id: pasteur-02945392**

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Submitted on 22 Sep 2020

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1           Molecular characterization and serology of *Leptospira kirschneri* (serogroup  
2           Grippotyphosa) isolated from urine of a mare post-abortion in Brazil

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5   Camila Hamond<sup>a</sup>, Gabriel Martins<sup>a</sup>, Sylvie Bremont<sup>c</sup>, Marco Alberto Medeiros<sup>b</sup>, Pascale  
6                                   Bourhy<sup>c</sup>, Walter Lilenbaum<sup>a</sup>

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8   <sup>a</sup>Laboratory of Veterinary Bacteriology, Department of Microbiology and Parasitology,  
9   Universidade Federal Fluminense, Niterói-RJ, Brazil

10   <sup>b</sup>Bio-Manguinhos, Oswaldo Cruz Foundation, Brazilian Ministry of Health, Rio de  
11   Janeiro-RJ, Brazil

12   <sup>c</sup>Institut Pasteur, Unité de Biologie des Spirochètes, National Reference Center, Paris,  
13   France.

14  
15   **Impacts**

- 16  
17   • Strains of *L. kirschneri* have apparently never been recovered from horses.  
18  
19   • Grippotyphosa has been shown to be genetically stable in various hosts and  
20   geographical zones.  
21  
22   • Horses may become unapparent carriers of this organism and shed leptospire in urine.

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25   **Correspondence:**

26   W. Lilenbaum, Department of Microbiology and Parasitology, Universidade Federal  
27   Fluminense, Rua Hernani de Melo 101, Niterói-RJ, 24210-130, Brazil

28   E-mail: [mipwalt@vm.uff.br](mailto:mipwalt@vm.uff.br)  
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31 **SUMMARY**

32           A strain of *Leptospira kirschneri* (serogroup Grippotyphosa) was cultured from  
33 urine of a mare post-abortion in Brazil, and characterized by serogrouping, MLVA,  
34 PGFE, and sequencing of genes *rrs* and *secY*. Strains of *L. kirschneri* have apparently  
35 never been recovered from horses in tropical area, only in Europe and USA. Knowledge  
36 of local epidemiology is important to interpret genetic profiles of leptospires circulating  
37 in an area.

38

39 **Keywords:** *Leptospira kirschneri*; Grippotyphosa; Horse

40

41

## 42 **Introduction**

43 Leptospirosis is an important infectious disease in livestock caused by spirochetes  
44 belonging to the genus *Leptospira*, which is reported worldwide, particularly in tropical  
45 countries (Martins and Lilenbaum, 2013). Infected animals (as cattle, sheep, goats, pigs  
46 and horses) often present a chronic form, with impaired fertility, abortion, stillbirth, and  
47 decreased milk production. Although most reported cases of urban leptospirosis in  
48 humans in Brazil are caused by *Leptospira interrogans*, particularly serovar  
49 Copenhageni (Silva et al., 2009), infection in livestock seems to be majorly determined  
50 by other serogroups, as Sejroe for ruminants and Australis for horses (Martins and  
51 Lilenbaum, 2013). Leptospirosis regarded as a zoonosis, humans become infected  
52 through either direct contact with the urine or other biological materials from the  
53 infected animals or indirect contact with water, soil and vegetation polluted with urine  
54 from animals harbouring pathogenic leptospires (Foronda et al. 2009).

55 Leptospirosis is considered a neglected zoonosis. Since leptospirosis  
56 transmission to humans can occur through contact with urine of animal reservoirs or  
57 exposure to an environment contaminated with leptospires, the contemporary concept of  
58 "One Health" is particularly appropriate for these organisms, due to its epidemiology,  
59 especially in tropical countries (Chappel and Smythe, 2012). In that regard, increases  
60 and expansion of human populations, including encroachment on wildlife habitat,  
61 increase opportunities for animal-human interactions. Various biomes and ecosystems  
62 promote exposure to various *Leptospira* strains, as rural and urban environments.  
63 Furthermore, global climate change is also apparently promoting the spread of  
64 leptospirosis. In that regard, increased temperatures may enhance survival of leptospires  
65 in neofomed environments and may result in an expansion of the habitats occupied by  
66 animal reservoirs of the bacterium (Lau et al. 2010).

67           Leptospiral infection can be host-maintained, if transmitted readily among  
68 members of host species, or incidental when such transmission does not normally occur.  
69 Incidental infections are usually more severe than those in maintenance hosts (Chappel  
70 and Smythe, 2012). Detecting carrier animals is vital to understanding enzootic and  
71 epizootic leptospirosis in a particular environment (Foronda et al., 2009). Generally the  
72 incidence in various hosts as well as the infecting serovars varies considerably among  
73 geographical regions (Arent et al., 2013; Chappel and Smythe, 2012; Verma et al.,  
74 2013).

75           Horses may become unapparent carriers and shed leptospire [in](#) urine, thereby  
76 serving as reservoirs and sources of infection for other animals, including humans  
77 (Hamond et al., 2013). The reproductive syndrome of equine leptospirosis was recently  
78 reviewed; the most common serogroup recovered from equine abortions was Pomona,  
79 whereas other serogroups (Australis, Icterohaemorrhagiae, Sejroe) have also been  
80 isolated from aborted equine fetuses in several countries (Verma et al., 2013; Hamond  
81 et al., 2014). However, *Leptospira kirschneri* serogroup Grippotyphosa has apparently  
82 never been recovered from horses in [tropical area](#).

83           Therefore, the purpose of this study was to describe recovery and characterization of  
84 *Leptospira kirschneri* (serogroup Grippotyphosa) from urine of a mare post-abortion  
85 (and the aborted fetus), as well as serological findings in this mare and her herd mates.

86

## 87 **Methods**

### 88 **Study Design**

89           Twelve mares (aged 7-12 y) from the same herd (extensive breeding) in the state  
90 of Rio de Janeiro, Brazil, were studied. These mares had a history of reproductive  
91 problems (mainly abortions) and had not been vaccinated [and](#) nor treated for

92 leptospirosis. Blood samples for serology were collected (jugular venipuncture) into  
93 evacuated tubes (Vacutainer<sup>®</sup>, BD Diagnostics, Franklin Lakes, NJ, USA).  
94 Additionally, urine samples were collected by probing (Human nasogastric probe n° 18  
95 – Embramed, São Paulo, SP, Brazil) and put into 50 mL sterile vials (BD, Franklin  
96 Lakes, USA) and immediately inoculated into 5 mL culture media tubes (EMJH). A 2  
97 mL aliquot was chilled and transported to the laboratory for PCR. During the study, one  
98 mare (age 8 y), originating from Europe and living in Brazil for the last 2 y, aborted  
99 (seventh month of pregnancy). The fetus was necropsied on the following day; it had  
100 jaundice and widespread petechial haemorrhages. Samples of kidney and liver were  
101 collected for culture and PCR.

## 102 **Serology**

103 For detection of anti-*Leptospira* antibodies, a microscopic agglutination test  
104 (MAT) was used, with a complete panel (28 serovars representing 24 serogroups;  
105 Institute Pasteur - Paris, France), according to international standards (World  
106 Organization for Animal Health, 2012). The serogroup (serovar) with the highest titre  
107 was regarded as infective. Samples were considered reactive when for titers  $\geq 200$ , and  
108 whereas titres  $\geq 800$  were considered strongly reactive and indicative of an acute  
109 infection (Martins and Lilenbaum, 2013).

110

## 111 **Bacteriological Culture**

112 A few drops of urine from each of the 12 mares and the fetal kidney and liver were  
113 immediately inoculated into tubes containing 5 mL of EMJH liquid media (Difco  
114 Laboratories, Franklin Lakes, NJ, USA) and 5 mL semisolid Fletcher media (Difco  
115 Laboratories). At the laboratory, tubes were incubated at 28 °C and examined under  
116 darkfield microscopy once weekly for 20 wk (Faine et al., 2000).

## 117 **PCR protocol**

118 All DNA samples (urine from the 12 mares and liver/kidney from the aborted  
119 foal) were extracted using the Promega Wizard SV Genomic DNA Purification System®  
120 (Promega, Madison, WI, USA). Primers used were targeted to the *lipL32* gene  
121 (regarded as present only in pathogenic leptospires) as described (LipL32\_45F - 5'AAG  
122 CAT TAC TTG CGC TGG TG 3' and LipL32\_286R - 5'TTT CAG CCA GAA CTC  
123 CGA TT 3'), which generate a 242 bp fragment (Stoddard et al. 2009). Briefly, primers  
124 were used in a concentration of 0.6 µM, 1.0 U Taq polymerase, 2.4 µM MgCl<sub>2</sub>, and 0.3  
125 mM dNTP in a final volume of 25 µL. One cycle of initial denaturation at 94 °C for 2  
126 min, was followed by 35 cycles of denaturation at 94 °C for 30 s, annealing the primers  
127 to 53 °C for 30 s, 1 min extension at 72 °C, and a final extension cycle at 72 °C for 5  
128 min. Strain *Leptospira interrogans* serovar Copenhageni, Fiocruz L1-130 (ATCC BAA-  
129 1198) was used as a positive control. To minimize false-negative results, an internal  
130 DNA control was designed and synthesized (IDT – Integrated DNA Technologies,  
131 Coralville, IA, USA). The synthetic gene had a 121 bp portion of the *lipL32* gene in  
132 each extremity, whereas in the middle has a gene part of the sequence *ligB*, yielding a  
133 total DNA sequence of 754 bp (Hamond et al., 2014).

134

## 135 **Characterization of the isolate**

136 The isolate was serogrouped using a panel of 32 specific antisera provided by the  
137 Royal Tropical Institute (KIT, Amsterdam; Faine et al., 2000). Furthermore, its DNA  
138 was extracted and a partial sequence of the *rrs* (Merien et al., 1993) and *secY* genes  
139 (Ahmed et al., 2006) were amplified by PCR and sequenced. The latter procedure was  
140 done at the Genotyping of Pathogens and Public Health Platform (*Institut Pasteur*,  
141 Paris, France). All molecular epidemiological data were stored and analyzed with

142 Bionumerics software (Version 6.5; Applied-Maths, Sint-Martens-Latem,  
143 Belgium). Genotyping was also performed by multiple-locus variable-number tandem  
144 repeat analysis (MLVA) using the loci VNTR4, VNTR7, and VNTR10, as described  
145 (Salaun et al., 2003). According to the analysis of partial sequencing of the gene *secY*,  
146 PFGE was conducted using *Not* I restriction enzyme (Herrmann et al., 1992), to  
147 compare DNA of the isolate to that of other strains of the same species and serogroup.

148

## 149 **Results**

150 Four of the 12 (33%) tested sera were reactive, all of them against serogroup  
151 Grippotyphosa (sv. Grippotyphosa). The mare that had aborted had a titre of 400,  
152 whereas the three others had titres of 200.

153 In this study, PCR detected leptospiral DNA in the urine of three of 12 (25%)  
154 mares, of which two were seroreactive, although the remaining mare was seronegative.  
155 From those mares, only the urine of the mare that had aborted yielded a pure culture of  
156 leptospire.

157 Based on serogrouping, the isolate belonged to Grippotyphosa serogroup (titre  
158 12,800), whereas sequencing products of *rrs* and *secY* partial genes characterized it as  
159 *Leptospira kirschneri* genomospecies. Furthermore, based on *secY* nucleotide sequences  
160 (Fig. 1), it was similar to sv Grippotyphosa strains Moskva V (isolated from humans in  
161 Russia) and 200901480\_Mayotte (from humans in Mayotte), as well as to sv  
162 Vanderhoedeni strain Kipod 179 (from a hedgehog in Israel).

163 Furthermore, MLVA-VNTR 4 (497 bp), VNTR 7 (372 bp) and VNTR 10 (830  
164 bp) analysis also confirmed that our isolate was sv Grippotyphosa (Fig. 2), with a  
165 profile closely related to the reference strain Moskva V. Finally, PFGE of the isolate

166 with other *L. kirschneri*/Grippotyphosa strains, such as Moskva V and Grippotyphosa  
167 strain 200901480, showed a very close profile among the three strains (Fig 3).

168

## 169 **Discussion**

170 The occurrence of anti-*Leptospira* agglutinins for serogroup Grippotyphosa was  
171 unexpected, since horses from the same region are typically seroreactive for serogroups  
172 Icterohaemorrhagiae or Australis (Hamond et al. 2013), as are many humans in Brazil  
173 (Silva et al., 2009).

174 It is not clear if the mare that had aborted has acquired the infection in Brazil or  
175 if it was a chronic infection acquired in Europe, at least 2 y before the abortion  
176 occurred. [Nevertheless, that mare](#) had already delivered a healthy foal in the past year,  
177 and that other mares from the same herd also seroreacted against that serovar, we  
178 inferred it was more likely to be a locally acquired infection. Although common in  
179 Europe (Arent et al., 2013), Grippotyphosa serogroup is not the most prevalent in  
180 tropical regions, where members of Icterohaemorrhagiae serogroup seem to be  
181 predominant, as well as serogroup Australis (sv Bratislava), regarded as adapted to  
182 horses (Hamond et al., 2014). Grippotyphosa is usually associated with environmental  
183 contamination, and is maintained by various wildlife species (de Carvalho et al., 2014).  
184 There are reports regarding isolation of that serogroup from animals, including  
185 abortions in sheep in Canada (Kingscote, 1985), cattle in the USA (Hanson et al., 1964),  
186 [horse with uveitis in Europe \(Hartskeerl et al., 2004\) and abortion in US \(Erol et al.,](#)  
187 [2015\).](#)

188 Although bacteria cultures were negative, PCR detected leptospiral DNA from  
189 the kidney of the aborted foal, thereby confirming the cause of the abortion.

190 Furthermore, *L. kirschneri* was detected by PCR in the tissues of a premature foal

191 (Vemulapalli et al., 2005), but recovery of that species in pure culture, as well as  
192 molecular characterization of the isolate, has apparently never been reported in horses in  
193 tropical area.

194 Sequencing of *secY* in DNA extracted from the clinical isolates samples allowed  
195 a simple and rapid first-line screening and identification of the presumptive serovar. It  
196 has already been conducted on isolates from human origin (Bourhy et al., 2013).

197 Although analysis based on a phylogenetic tree of *secY* fragments is very useful for  
198 identifying the presumptive serovar, it has not commonly been performed on isolates  
199 from animal origin and therefore, should be encouraged in future studies.

200 The MLVA analysis is a simple and rapid PCR-based method for identification  
201 of most serovars of *L. interrogans* and *L. kirschneri* (Bourhy et al. 2013; Zilber et al.  
202 2014). Therefore, its use should also be encouraged for a fast and simple DNA-based  
203 characterization of leptospiral isolates.

204 The PFGE analysis of *Not* I-digested genomic DNA revealed a very close profile  
205 among the three strains, as Moskva V and Grippytyphosa strain 200901480, which is  
206 remarkable, given the distances (South America, Russia and Indian Ocean) and distinct  
207 hosts (man and horse). Notwithstanding, that finding reinforces the One Health concept  
208 regarding human/animal leptospirosis, which should be considered in future approaches  
209 regarding the diagnosis and control of leptospirosis worldwide.

210 In conclusion, this was apparently the first report of *L. kirschneri* to have been  
211 isolated from a horse in tropical area. In addition, it is noteworthy that characterizing the  
212 genetic profile of the leptospirosis strains circulating in an area is very important to  
213 interpret local epidemiology of this organism.

214

215

216 **Acknowledgements**

217 This study was supported by CAPES, CNPq and FAPERJ. WL and MAM are CNPq  
218 fellows. The authors thank C.P. Pestana and R. Lawson-Ferreira (Fiocruz) for their  
219 assistance. [The authors are thankful for the English editing and critical review](#)  
220 [performed by Prof. John Kastelic, form University of Calgary.](#)

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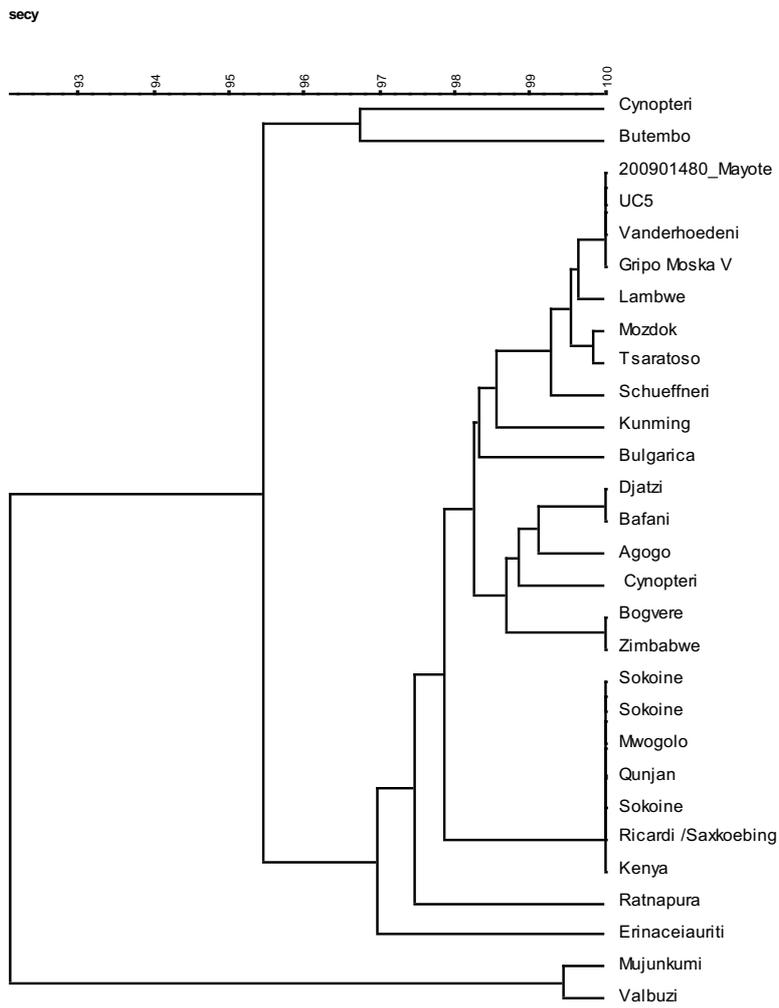
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- 312

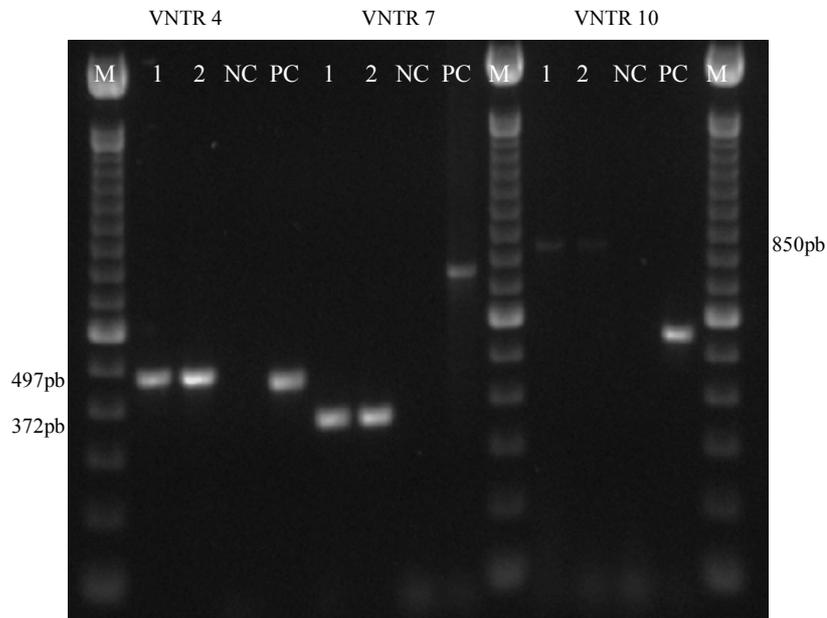
313 Fig 1. Phylogenetic tree of leptospiral *secY* partial gene sequences of reference strains  
 314 of *L. kirschneri* species, including the isolate obtained from a mare post-abortion (UC5).



315

316

317 Fig 2. PCR analysis of the polymorphism of two representative VNTR loci.  
318 Amplification was performed on the VNTR4, VNTR7 and VNTR10 loci of *L.*  
319 *kirschneri* sv Grippotyphosa Moskva V and isolate UC5 indicates *Leptospira* serovars



M- 100pb DNA ladder 1-UC5; 2-*L.kirschneri* sv Grippotyphosa Moskva V; NC- Negative Control; PC – Positive control (*L.interrogans* sv Canicola)

320

321

322 Fig 3. PFGE (using *Not* I restriction enzyme) from the recovered sample (UC5)  
323 compared to other *Leptospira kirchneri* serogroup Grippotyphosa, as Moskva V and  
324 strain 200901480.

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