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

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Impacts

• Strains of *L. kirschneri* have apparently never been recovered from horses.

• Grippotyphosa has been shown to be genetically stable in various hosts and geographical zones.

• Horses may become unapparent carriers of this organism and shed leptospires in urine.

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SUMMARY

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

Keywords: *Leptospira kirschneri*; Grippotyphosa; Horse

Introduction

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

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

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

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

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

Methods

Study Design

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

leptospirosis. Blood samples for serology were collected (jugular venipuncture) into evacuated tubes (Vacutainer®, BD Diagnostics, Franklin Lakes, NJ, USA). Additionally, urine samples were collected by probing (Human nasogastric probe nº 18 – Embramed, São Paulo, SP, Brazil) and put into 50 mL sterile vials (BD, Franklin Lakes, USA) and immediately inoculated into 5 mL culture media tubes (EMJH). A 2 mL aliquot was chilled and transported to the laboratory for PCR. During the study, one mare (age 8 y), originating from Europe and living in Brazil for the last 2 y, aborted (seventh month of pregnancy). The fetus was necropsied on the following day; it had jaundice and widespread petechial haemorrhages. Samples of kidney and liver were collected for culture and PCR.

Serology

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

Bacteriological Culture

A few drops of urine from each of the 12 mares and the fetal kidney and liver were immediately inoculated into tubes containing 5 mL of EMJH liquid media (Difco Laboratories, Franklin Lakes, NJ, USA) and 5 mL semisolid Fletcher media (Difco Laboratories). At the laboratory, tubes were incubated at 28 ºC and examined under 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 *lipL*32 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 MgCl2, and 0.3
125 mM dNTP in a final volume of 2 5µL. 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

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

Results

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

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

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

Furthermore, MLVA-VNTR 4 (497 bp), VNTR 7 (372 bp) and VNTR 10 (830 bp) analysis also confirmed that our isolate was sv Grippotyphosa (Fig. 2), with a 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

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

Sequencing of *secY* in DNA extracted from the clinical isolates samples allowed a simple and rapid first-line screening and identification of the presumptive serovar. It has already been conducted on isolates from human origin (Bourhy et al., 2013). Although analysis based on a phylogenetic tree of *secY* fragments is very useful for identifying the presumptive serovar, it has not commonly been performed on isolates from animal origin and therefore, should be encouraged in future studies.

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

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

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

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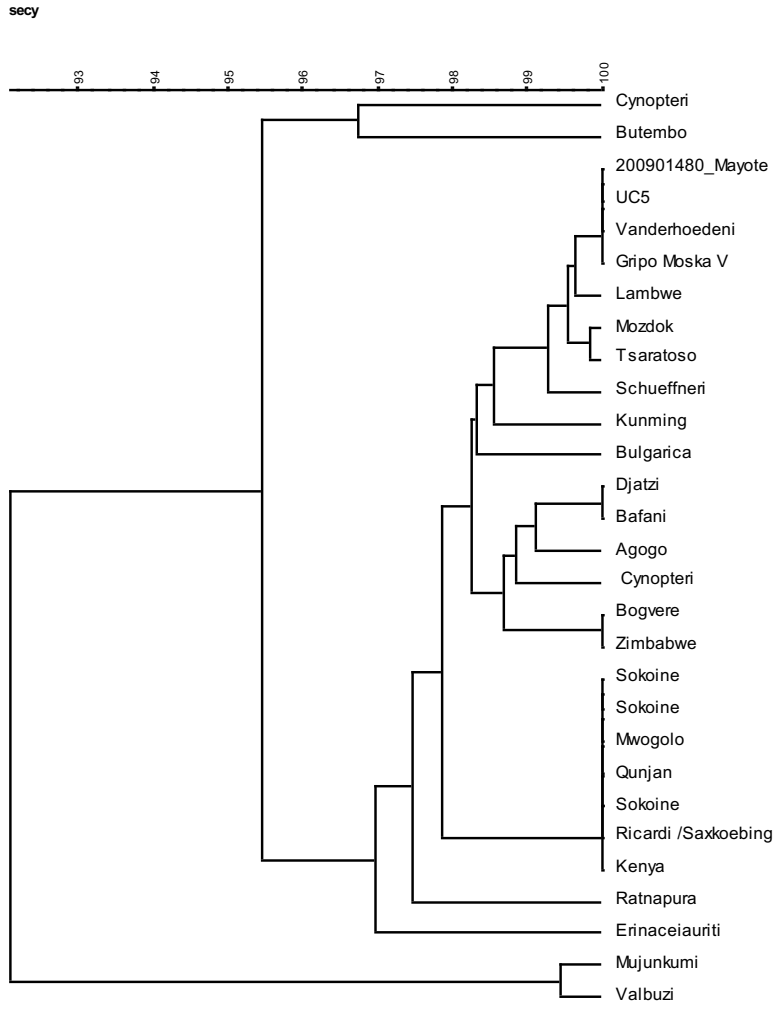
313 Fig 1. Phylogenetic tree of leptospiral *secY* partial gene sequences of reference strains

314 of *L. kirschneri* species, including the isolate obtained from a mare post-abortion (UC5).



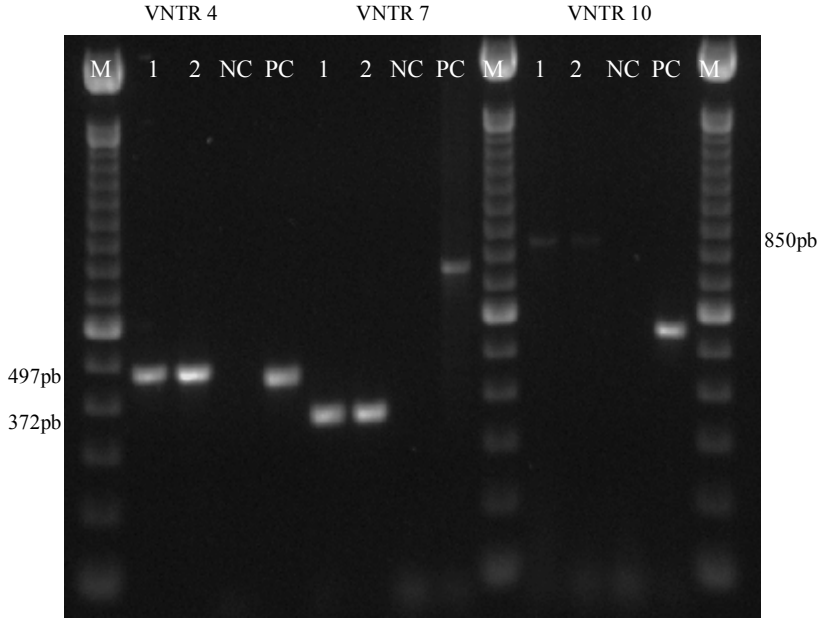
315

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.*

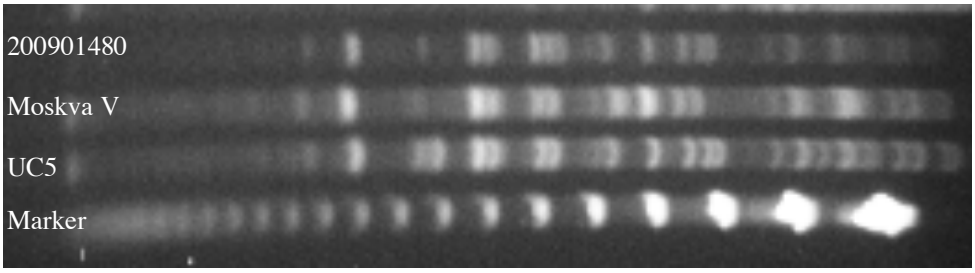
319 *kirschneri* sv Grippotyphosa Moskva V and isolate UC5 indicates *Leptospira* serovars



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.
325
326 

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328