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Presence of leptospires on genital tract of mares with reproductive problems

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ABSTRACT

Leptospirosis is a zoonotic disease of global importance, and has a worldwide distribution. Equine leptospirosis is commonly manifested by recurrent uveitis, reproductive disorders, as abortions, embryonic absorption, stillbirth and the birth of weak foals. The aim of this study was to verify the presence of *Leptospira* sp or its DNA in genital tract of mares with reproductive problems. A total of 38 mares with reproductive problems were studied. All the mares were sampled for blood (for serology), urine (for culturing and qPCR), vaginal fluid-VF and endometrial biopsy-EB (for culturing, qPCR and indirect immunofluorescence). PCRs products were sequenced for *secY* gene. Seventeen (44.7%) serum samples were reactive, predominantly against serogroups Australis (76.4%) and Pomona (23.6%). No positive culture was obtained, but DNA was detected by qPCR on urine samples (26.3%), VF (44.7%) and EB (18.4%) collected 2 months or longer following diagnosis of early fetal death and endometritis. *Leptospira* cell aggregations were visible by indirect immunofluorescence on 57.1% (4/7) EBs and 17.6% (3/17) VFs. A total of 18 amplicons showed interpretable sequences. Out of those 18 amplicons, 15 presented 100% of identity with the species *L. interrogans* (sv Bratislava and Pomona), while three were *L. borgpertersenii*. This study suggests the presence of leptospires in the uterus of mares with reproductive problems. Moreover, serology was shown not to be indicated for the diagnosis of presumptive *Leptospira* infection in early gestation. The most common agent of the genital infection in those mares was *L. interrogans*, most probably sg Australis.

1. Introduction

Equine leptospirosis is commonly manifested by recurrent uveitis and reproductive disorders, as abortions, embryonic absorption, stillbirth or neonatal mortality and the birth of weak foals (Ellis, 2015). The mechanisms involved in leptospiral pathogenesis are not well elucidated (Adler, 2014). Although leptospires are usually reported colonizing the kidneys, it has been demonstrated that they can also be present in the genital tract of cattle and small ruminants, leading to reproductive problems (Arent et al., 2013; Ellis and Thiermann, 1986; Farina et al., 1996). In mares, evidence of leptospiral DNA in vaginal fluid was recently reported by our group (Hamond et al., 2014). Furthermore, the presence of leptospiral DNA was also found in the placenta and fetal fluids contained in the uterus of mares (Donahue and Williams, 2000; Erol et al., 2014). However, leptospiral DNA has never been reported in uterus samples, and it is uncertain if its presence on vaginal fluid is of genital or urinary origin.

Although late-term abortion is the most commonly reported aspect of equine reproductive leptospirosis, the infection may also lead to other reproductive problems such as early embryonic death (Pinna et al., 2014). Endometritis, mainly its subclinical form, is a major cause of infertility in the mare and it is influenced by the pathogen's type and the mare's subsequent immunological response (Buczkowska et al., 2014). Although never reported in equine leptospirosis, endometritis has been associated to leptospirosis in cows (Gonzalez et al., 1985).

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In this context, the aim of this study was to verify the presence of *Leptospira* sp or its DNA in genital tract of mares with reproductive problems.

2. Material and methods

2.1. Animals

Of the 206 horses (6 stallions, 105 mares and 95 foals), thirty-eight mares (4–21 y) of different races with reproductive problems were studied, all from the same farm in Rio de Janeiro, Brazil. As inclusion criteria, all the studied mares had presented reproductive problems repeat breeding, endometritis, early embryonic death ([≤45 days of pregnancy] or abortion [from 45 days to term]) during the last breeding season (October 2013–April 2014). The samples were collected from July to August (2014) in the period of anestrus. All mares were vaccinated against herpesvirus only. Additionally, during the study, no specific treatment for leptospirosis was conducted.

Mares were teased daily from the beginning of the breeding season for detection of estrus; once the estrus was detected they were daily submitted to rectal palpation for monitoring of follicular growth. The insemination took place when the mare presented ovarian follicle with 3.5 cm in diameter. For semen collection, artificial vagina method, following Botucatu model (Biotech Botucatu/ME Ltd., Botucatu, SP, Brazil) was employed. After collection, semen was evaluated for motility, vigor and sperm concentration. Semen was diluted with extender Botu-semen© (Biotech Botucatu, Botucatu, SP, Brazil), in a final concentration of 800 million sperm per insemination dose (20 mL). Insemination was performed on alternate days until the verification of ovulation. Semen wasn't tested for the presence of *Leptospira* or its DNA.

Reproductive tract examinations were done with transrectal palpation and ultrasound (US) (Aloka SSD-500, Aloka Co., Ltd., Tokyo, Japan). Mares were examined after insemination when necessary by ultrasonography during the post breeding period, and at approximately 12, 30, 45, 60, 90, 180, and 270 days of pregnancy. Embryonic death and endometritis were detected by US. The first was determined by a positive result followed by a negative result in between 12 and 45 days post insemination. The later was determined by the presence of fluid in the uterus.

Two months after the end of the breeding season (June 2014), blood (serology), urine (culturing and qPCR), and vaginal fluid/endometrial biopsy (culturing, qPCR and direct immunofluorescence—DI) were obtained from all the mares (Table 1).

2.2. Collection and processing samples

Blood—blood samples were collected into evacuated tubes (Vacutainer®, BD Diagnostics, Franklin Lakes, USA) by jugular venipuncture. All samples were refrigerated and transported to the Leptospirosis Laboratory of Universidade Federal Fluminense, Niteroi, RJ, Brazil, to be tested as a batch.

Endometrial biopsy (EB)—prior to sample collection, the mare's tail was bandaged, and the vulva and perineum were cleaned and dried with a paper towel. Endometrial biopsy was collected using alligator-jawed (rounded) biopsy forceps 55–70 cm in length with a basket 20 × 4 × 3 mm in size (Biotech®, Botucatu, SP, Brazil), and taken from the base of the uterine horn (Buczkowska et al., 2014). The biopsy forceps was washed with running water and soap and kept in Kilol-L solution, according to the recommendations of the manufacturer, in between each collection (Quinabra, Qúimica Natural Brasileira LTDA—São José dos Campos, SP-Brazil). After collection it was flambéed with alcohol 70% (Rioumina, São Paulo, SP-Brazil), and then cooled with sterile saline. Each sample was divided into two parts. The first was immediately inoculated into 5 mL of culture media (EMJH) and the other one was used for DI-imprints (Chagas-Junior et al., 2012) and then kept in a 2 mL tube containing 1 mL of RNAlater (Ambion, Huntingdon, UK) for DNA extraction (qPCR).

Vaginal fluids (VF)—immediately after biopsy collection, the vulva and perineum were cleaned once more and VF was collected from each mare using three commercially available Cytology Brushes (CB-PlasticWay®, São Paulo, Brazil). While one CB was immediately inoculated into 5 mL of culture media (culturing), the other two were transferred to 15 mL sterile vials (BD, Franklin Lakes, USA) containing 5 mL of stock media (Saline solution 0.15 M, TrisHCl pH7.6 and BSA 2%), for DI and qPCR.

At the laboratory, VF from stock media was centrifuged 16,000 g for 20 min, the supernatant was discarded and pellet was re-suspended with 200 μL of PBS. VF smears (duplicate) for imprint were prepared by placing 100 μL of VF onto a poly-L-lysine-coated glass slide (Pinne and Haake, 2011). Imprint slides were dried at room temperature and fixed in 1% formaldehyde for 3 min.

Urine—after collection of VF urine samples were collected by probing. Urine was collected in 50 mL sterile vials (BD, Franklin Lakes, USA) and immediately inoculated into 5 mL of culture media (EMJH). A 2 mL aliquot was refrigerated and transported to the laboratory for qPCR.

Table 1
Reproductive problems, serology (MAT), qPCR and Indirect immunofluorescence assay (IFA) results on 38 mares with reproductive problems studied for leptospirosis in Rio de Janeiro, Brazil.

ID	Reproductive problem	MAT	qPCR			IFA	
			EB	VF	U	EB	VF
1	Early embryonic death	+	–	+	–	ND	–
2	Endometritis	+	–	–	–	ND	ND
3	Endometritis	–	–	+	–	ND	–
4	Endometritis	+	–	–	+	ND	ND
5	Early embryonic death	–	–	+	–	ND	–
6	Repeat breeding	+	–	–	–	ND	ND
7	Early embryonic death	+	–	+	+	ND	–
8	Early embryonic death	–	–	–	–	ND	ND
9	Early embryonic death	–	–	–	–	ND	ND
10	Repeat breeding	–	+	+	–	+	ND
11	Endometritis	–	+	+	–	+	+
12	Endometritis	–	+	+	–	–	–
13	Endometritis	–	+	+	–	–	–
14	Early embryonic death	+	–	–	–	ND	ND
15	Endometritis	+	+	+	–	+	–
16	Abortion	+	+	+	+	ND	–
17	Repeat breeding	–	–	–	–	ND	ND
18	Early embryonic death	–	–	+	–	ND	–
19	Early embryonic death	+	–	–	–	ND	ND
20	Endometritis	–	+	+	+	+	–
21	Repeat breeding	–	–	–	–	ND	ND
22	Early embryonic death	–	–	–	–	ND	ND
23	Repeat breeding	–	–	–	–	ND	ND
24	Early embryonic death	+	–	–	+	ND	ND
25	Endometritis	+	–	+	+	ND	–
26	Repeat breeding	–	–	–	–	ND	ND
27	Repeat breeding	–	–	–	–	ND	ND
28	Early embryonic death	+	–	–	–	ND	ND
29	Early embryonic death	+	–	–	–	ND	ND
30	Endometritis	–	–	+	+	ND	+
31	Early embryonic death	+	–	–	+	ND	ND
32	Endometritis	+	–	–	+	ND	ND
33	Early embryonic death	–	–	+	–	ND	–
34	Endometritis	+	–	+	–	ND	–
35	Endometritis	+	–	–	–	ND	ND
36	Early embryonic death	–	–	+	–	ND	–
37	Endometritis	–	–	–	–	ND	ND
38	Repeat breeding	+	–	–	+	ND	ND

+ Positive, – Negative, ND—not determined.