Case Report

Isolation of *Leptospira interrogans* Hardjoprajitno from vaginal fluid of a clinically healthy ewe suggests potential for venereal transmission

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A total of 15 adult ewes from one flock known to be seroreactive for leptospirosis was studied. Urine and vaginal fluid were collected from each animal to test for the presence of leptospires using bacterial culture and conventional PCR methods. One pure culture of *Leptospira* sp. was obtained from the vaginal fluid sample of a non-pregnant ewe. The isolate was characterized by DNA sequencing of the *rrs* and *secY* genes, variable-number of tandem-repeats (VNTR) analysis and serogrouping, and the isolate was typed as *Leptospira interrogans* serogroup Sejroe serovar Hardjo type Hardjoprajitno. This report indicates the presence of viable *Leptospira* in the vaginal fluid of a ewe, suggesting the potential for venereal transmission of leptospires in sheep.

Case report

A flock of sheep located in Rio de Janeiro, Brazil, was initially studied for leptospirosis by microscopic agglutination test (MAT). The flock was composed of 21 Santa Inês sheep (18 females and 3 males), which were not vaccinated against leptospirosis, semi-extensive breeding and natural mating. No reproductive failures or abortions had been reported in the last breeding season. All animals were kept together throughout the year. This study was submitted to the ethics committee on animal use of the Fluminense Federal University (9 August 2012, protocol number 225). The first screening by MAT revealed 15 seroreactive females (83.4 %) and 1 male (33.3 %) with titres ranging from 100 to 800. Antibodies against serogoroup Sejroe were predominant (81.2 % of seroreactives); however, low titres (100) against serogroup Icterohaemorrhagiae were observed in three animals (18.8 %).

Due to the high seroreactivity in this flock, it was selected for further studies. Approximately 1 month after the first sampling, urine, vaginal fluid and blood samples were collected from the 15 seroreactive females in order to confirm the leptospiral infection. Bacterial culture and PCR were performed for urine and vaginal fluid samples, while serum samples were resubmitted for serology testing (MAT); 3 of 15 (20.0 %) ewes were seronegative.

Abbreviations: *k*, kappa test; MAT, microscopic agglutination test; VNTR, variable number of tandem repeats.

Vaginal fluids were collected, after the perineum was first cleaned with water alone, using a tampon (Tampax regular) that was introduced into the vagina of the sheep. After 10 min, the tampon was removed and transferred to a sterile vial containing 20 ml PBS, as described previously (Lilenbaum *et al.*, 2008). Following the collection of vaginal fluids, 0.5–1.0 mg furosemide kg^{-1} (Teuto Laboratory) was administered intravenously and a second voiding of urine collected into sterile vials.

At the Veterinary Bacteriology Laboratory, Fluminense Federal University, the tampons were aseptically squeezed and centrifuged at 800 ***g*** for 10 min in sterile vials, and an aliquot (500 µl) of each supernatant was transferred to Fletcher and Ellinghausen–McCullough–Johnson–Harris (EMJH) media tubes (Difco). In addition, 500 µl urine samples were immediately transferred to Fletcher and EMJH media tubes. All samples were processed on the day of sampling. All cultures were incubated at 28–30 °C and examined by darkfield microscopy weekly for a period of 20 weeks. After 3 weeks, one pure culture of *Leptospira* sp. was obtained from a vaginal fluid sample of a non-pregnant ewe and was seroreactive against serogroup Sejroe (titre 400).

For analysis using PCR, DNA was extracted from the urine and supernatant vaginal fluid using the Wizard SV kit genomic DNA purification system (Promega) at the Recombinant Technology Laboratory (LATER), Brazil. Primers targeting the *lipL32* gene (thought to be present only in pathogenic leptospires) were used and PCR

performed as described previously (Hamond *et al.*, 2014). Five urine samples and four vaginal fluid samples were positive; these were not always from the same animal (Table 1). The urine and vaginal fluid PCR results were compared by kappa test (k).

A total of 46.7 % of the ewes (7/15) were positive by PCR. Overall, 26.7% of vaginal fluid samples (4/15) and 33 % urine samples (5/15) were positive by PCR. Three ewes were positive only by the urine samples, two only by the vaginal fluid samples and two others by both samples (including the ewe from which the bacterium was isolated), as shown in Table 1. PCR results of vaginal fluid and urine demonstrated a medium correlation (k=0.52).

Serogrouping of the isolate with standard rabbit antisera against all the described serogroups (provided by the Royal Tropical Institute, Amsterdam, The Netherlands) revealed high titres (12 800) against *Leptospira interrogans* serogroup Sejroe serovar Hardjo type Hardjoprajitno. Molecular characterization of the isolate was performed at the Spirochaetal Biology Unit, Institut Pasteur, Paris, France, by DNA sequencing of the *rrs* (16S rRNA) and *secY* genes and by variable-number-of-tandem-repeats (VNTR) analysis. DNA sequence analysis of the genes *rrs* and *secY* identified the isolate as a strain of *L. interrogans*. The isolate had a VNTR profile suggestive of the serovar Hardjo type Hardjoprajitno (VNTR4 500 bp, VNTR7 710 bp and VNTR10 1050 bp) (Majed *et al.*, 2005).

The predominance of seroreactions to *L. interrogans* serogroup Sejroe serovar Hardjo type Hardjoprajitno was not surprising since leptospirosis caused by this serovar has been serologically identified as the most important reproductive disease in small ruminants in this same region (Martins *et al.*, 2012). The MAT results herein did not correlate with PCR results, which is in agreement with the observations of others reported in different species backgrounds (Otaka *et al.*, 2012; Hamond *et al.*, 2012).

The Hardjo strain is known to be adapted to ruminants, including sheep and goats (Hartskeerl *et al.*, 2011; Martins *et al.*, 2012). Furthermore, strains of this serovar (mainly type Hardjobovis; *Leptospira borgpetersenii*) have been recovered from sheep worldwide (Kingscote, 1985; Gerritsen *et al.*, 1994; Cerri *et al.*, 1996; Dorjee *et al.*, 2009). Notably, the few reports concerning Hardjo recovery in Brazil report only the recovery of *L. interrogans* type Hardjoprajitno (Cosate *et al.*, 2012). Detection of leptospiral DNA has been previously demonstrated (Lilenbaum *et al.*, 2008); however, this is, to the best of our knowledge, the first study to report a viable Hardjo strain recovered from sheep in Brazil.

The possibility that leptospiral DNA or the micro-organism isolated from vaginal fluid samples was due to contamination by urine cannot be excluded, despite precautions taken during sampling. Nonetheless, if the presence of leptospires in the vaginal tract is permanent (colonization of mucosa) or transitory (by urine contamination), it is remarkable that a viable *Leptospira* was present in the vaginal fluid at the moment of sampling, as this finding has clear implications regarding the potential for venereal transmission.

Since the venereal transmission of leptospirosis from male to female is well known (Magajevski *et al.*, 2005), it is possible that the seroreactive ram could be the source of infection in this herd. Nonetheless, the possible role of the female in the reverse infection remains unclear. Leptospires have been recovered (Ellis & Thiermann, 1986; Ellis *et al.*, 1986) and detected by direct immunofluorescence (Dhaliwal *et al.*, 1996) in the female reproductive tract. Although all those cited authors have correctly suggested the possibility of venereal transmission of the infection arising of those findings, the role of the female in the venereal transmission of leptospirosis has been overlooked. It is known that leptospires may penetrate the intact mucosa, and the possibility that it penetrates the mucosa of the penis during the mating cannot be neglected. Additionally, in ovines, although leptospiral DNA has already been demonstrated in the vaginal fluid of ewes (Lilenbaum *et al.*, 2008), this is, to the best of our knowledge, the first report of the recovery of viable leptospires (*L. interrogans* serogroup Sejroe serovar Hardjo type Hardjoprajitno) from vaginal fluid, and reinforces the hypothesis that venereal transmission from female to male can occur in that species. Based on these results, it is suggested that venereal transmission of leptospirosis from female to male may occur in ovines.

Table 1. Diagnostic findings of 15 ewes initially seropositive for leptospirosis in Rio de Janeiro, Brazil

No.	MAT*	Culture (vf)	PCR (vf)	PCR (u)
1	100	−	−	+
2	200	−	−	−
3	−	−	−	−
4	200	−	−	−
5†	400	+	+	+
6	400	−	−	−
7	200	−	+	+
8	200	−	−	−
9	200	−	−	−
10	400	−	−	+
11	−	−	−	+
12	−	−	+	−
13	400	−	−	−
14	400	−	+	−
15	−	−	−	−

+, Positive; −, negative; u, urine; vf, vaginal fluid.
*Second sampling.
†Ewe from which the isolate was obtained.

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References

Cerri, D., Nuvoloni, R., Ebani, V., Pedrini, A., Mani, P., Andreani, E. & Farina, R. (1996). *Leptospira interrogans* serovar Hardjo in the kidneys and genital tracts of naturally infected sheep. *New Microbiol* **19**, 175–178.

Cosate, M. R., Barouni, A. S., Moreira, E. C., Veloso, I. F., Gomes, M. T. & Salas, C. E. (2012). Molecular characterization by LSSP-PCR and DNA sequencing of a pathogenic isolate of *Leptospira interrogans* from Brazil. *Zoonoses Public Health* **59**, 379–388.

Dhaliwal, G. S., Murray, R. D., Dobson, H., Montgomery, J. & Ellis, W. A. (1996). Presence of antigen and antibodies in serum and genital discharges of cows from dairy herds naturally infected with *Leptospira interrogans* serovar Hardjo. *Res Vet Sci* **60**, 163–167.

Dorjee, S., Heuer, C., Jackson, R., West, D. M., Collins-Emerson, J. M., Midwinter, A. C. & Ridler, A. L. (2009). Are white-spot lesions in kidneys in sheep associated with leptospirosis? *N Z Vet J* **57**, 28–33.

Ellis, W. A. & Thiermann, A. B. (1986). Isolation of leptospires from the genital tracts of Iowa cows. *Am J Vet Res* **47**, 1694–1696.

Ellis, W. A., Songer, J. G., Montgomery, J. & Cassells, J. A. (1986). Prevalence of *Leptospira interrogans* serovar Hardjo in the genital and urinary tracts of non-pregnant cattle. *Vet Rec* **118**, 11–13.

Gerritsen, M. J., Koopmans, M. J., Peterse, D. & Olyhoek, T. (1994). Sheep as maintenance host for *Leptospira interrogans* serovar Hardjo subtype Hardjobovis. *Am J Vet Res* **55**, 1232–1237.

Hamond, C., Martins, G., Lilenbaum, W. & Medeiros, M. A. (2012). PCR detection of leptospiral carriers among seronegative horses. *Vet Rec* **171**, 105–106.

Hamond, C., Martins, G., Loureiro, A. P., Pestana, C., Lawson-Ferreira, R., Medeiros, M. A. & Lilenbaum, W. (2014). Urinary PCR as an increasingly useful tool for an accurate diagnosis of leptospirosis in livestock. *Vet Res Commun* **38**, 81–85.

Hartskeerl, R. A., Collares-Pereira, M. & Ellis, W. A. (2011). Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. *Clin Microbiol Infect* **17**, 494–501.

Kingscote, B. (1985). Leptospirosis in sheep in Western Canada. *Can Vet J* **26**, 164–168.

Lilenbaum, W., Varges, R., Brandão, F. Z., Cortez, A., de Souza, S. O., Brandão, P. E., Richtzenhain, L. J. & Vasconcellos, S. A. (2008). Detection of *Leptospira* spp. in semen and vaginal fluids of goats and sheep by polymerase chain reaction. *Theriogenology* **69**, 837–842.

Magajevski, F. S., Girio, R. J. S., Mathias, L. A., Myashiro, S., Genovez, M. É. & Scarcelli, E. P. (2005). Detection of *Leptospira spp.* In the semen and urine off bulls serologically reactive to *Leptospira interrogans* serovar Hardjo. *Braz J Microbiol* **36**, 43–45.

Majed, Z., Bellenger, E., Postic, D., Pourcel, C., Baranton, G. & Picardeau, M. (2005). Identification of variable-number tandem-repeat loci in *Leptospira interrogans sensu stricto. J Clin Microbiol* **43**, 539–545.

Martins, G., Penna, B., Hamond, C., Leite, R. C., Silva, A., Ferreira, A., Brandão, F., Oliveira, F. & Lilenbaum, W. (2012). Leptospirosis as the most frequent infectious disease impairing productivity in small ruminants in Rio de Janeiro, Brazil. *Trop Anim Health Prod* **44**, 773–777.

Otaka, D. Y., Martins, G., Hamond, C., Penna, B., Medeiros, M. A. & Lilenbaum, W. (2012). Serology and PCR for bovine leptospirosis: herd and individual approaches. *Vet Rec* **170**, 338.