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Detection of *Leptospira* spp. in semen and vaginal fluids of goats and sheep by polymerase chain reaction

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Abstract

Thirteen goat herds and seven sheep flocks in the state of Rio de Janeiro, Brazil were screened for leptospirosis. From the three herds and three flocks with greatest seroreactivity, 19 goats (16 females and three bucks) and 40 sheep (26 ewes and 14 rams) that were seropositive (specific anti-*Leptospira* titres ≥400, based on a microscopic agglutination test), were selected for more detailed studies. From those animals, samples of vaginal fluids or semen were collected for bacteriological and molecular assays. For both species of animals, the most prevalent reactions were to serovars Hardjo, Shermani, and Grippotyphosa. Although leptospires were detected by darkfield microscopy in three vaginal fluid samples (from two goats and one ewe), pure isolates were not obtained by bacteriological culture of vaginal fluids or semen. However, seven vaginal fluid samples (from four goats and three ewes) and six semen samples (all from rams) were positive on polymerase chain reaction (PCR). Based on these findings, in addition to analogous findings in cattle, we inferred that there is potential for venereal transmission of leptospirosis in small ruminants.

Keywords: Leptospirosis; Venereal transmission; Reproductive disease; Sheep; Goat

1. Introduction

Goats and sheep are considered less susceptible to leptospirosis than other domestic farm animal species (e.g. cattle [1]). Leptospirosis in small ruminants may present in an acute form, with pyrexia, anorexia, depression, jaundice, and anemic or hemorrhagic syndromes [2]. Nevertheless, the chronic form with impaired fertility, neonatal deaths, abortions and decreased milk production occurs more frequently, causing substantial economic losses [3,4]. In several states of Brazil, the disease has been reported for approximately 40 years [5]. In Rio de Janeiro state, leptospirosis is a major infectious disease affecting the reproductive performance of dairy goats. In a recent study, 11.1% of goats tested were seroreactive, predominantly due to serovar Hardjo [4]. Leptospires of the Grippotyphosa serogroup have been recently isolated from dairy goats [6], whereas *Leptospira noguchii* was recently described in sheep in Brazil [7].

The presence of leptospires in the genital tract of cows has been reported since 1986, when Ellis et al. [8] described genital Hardjo infection in naturally infected cattle. Since then, the genital tract, especially the

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vagina, was recognized as an important extra-urinary site of *Leptospira* infection. It was also suggested that venereal transmission could play a role in the epidemiology of this organism. The same research group [9] described the isolation of Hardjo serovar from the genital tract of naturally infected bulls and discussed the role of venereal transmission and AI in the epidemiology of bovine leptospirosis. Leptospires were also detected by immunofluorescence and darkfield microscopy in genital secretions of cows; that that it was not isolated was attributed to the difficulties inherent in culturing this organism [10].

Serological diagnosis of leptospirosis is usually done with a microscopic agglutination test (MAT), which relies on the use of live cultures as antigens, and is usually performed using a panel of antigens representative of local serovars. Although widely employed as diagnostic method on a herd basis, the absence of detectable levels of serum antibodies in some infected animals make indirect methods less sensitive [2,11]. Furthermore, that there was no consistent association between serology and the presence of bacteria in the kidneys or extra-renal locations, direct evidence of the presence of the organism is required to reliably identify carriers, as part of an efficacious control program [2].

The principle of carrier identification is detection of the agent or agent-specific DNA in urine and other tissues. Available techniques include direct examination for leptospires, bacterial culture, detection of leptospiral antigens with antibodies, and detection of leptospiral DNA with homologous nucleic acid sequence probes, with or without amplification by polymerase chain reaction (PCR) [12]. Bacterial cultivation is laborious and may take 8 weeks, with uncertain results [20]. Molecular approaches, e.g. PCR, have been used to detect *Leptospira* spp. in bovine urine [13] and in bovine semen [11], with encouraging results. The purpose of the present study was to evaluate the use of PCR for the detection of *Leptospira* sp. in semen and vaginal fluids of goats and sheep.

2. Materials and methods

2.1. Study population

Thirteen goat herds and seven sheep flocks located in Rio de Janeiro, Brazil were screened for leptospirosis. The existence of a reliable individual-animal identification system and the absence of a vaccination program against leptospirosis (to prevent false positive results on serology) were the only inclusion criteria. Although it was not a specific inclusion criterion, it was noteworthy that all affected herds and flocks apparently had reduced fertility. The most frequent reproductive problems identified were returns to estrus and low-conception rates (all herds/flocks), premature parturition (eight herds/flocks) and sporadic abortion (eight herds/flocks). As an initial screening step, approximately 20% of the animals in each herd/flock were randomly selected; overall, 248 caprine and 292 ovine serum samples were tested by MAT.

2.2. Study design

In the second part of the study, three herds and three flocks with the greatest proportion of seroreactive animals (>30% in each herd/flock), 19 goats (16 females and three bucks) and 40 sheep (26 ewes and 14 rams) with specific anti-*Leptospira* titres ≥400 were selected for further study. From those 59 animals, vaginal fluids/semen samples (for bacteriological and molecular assays) and a serum sample were collected.

2.3. Sampling

Semen samples were collected (by electroejaculation) into sterile vials. For collection of vaginal fluids from females, the perineum was cleaned, and a female tampon (Tampax Super®, Procter and Gamble, São Paulo, SP, Brazil) was introduced into the vagina via a sterile, disposable speculum. After 10 min, tampons were removed and placed in a sterile vial containing 20 mL of PBS. For all animals, blood samples were collected into evacuated tubes, by jugular venipuncture. All samples were chilled and transported to the Leptospirosis Laboratory of Universidade Federal Fluminense, Niteroi, RJ, Brazil.

2.4. Laboratory procedures

2.4.1. Serology

All samples were processed on the same day that they were collected. In the laboratory, semen samples were diluted (final concentration, 10%) in sterile PBS and vaginal tampons were aseptically squeezed. Diluted semen and vaginal fluid samples were centrifuged ($3000 \times g$ for 10 min) and the supernatant used for both molecular and bacteriological assays. Blood samples were also centrifuged ($1000 \times g$ for 10 min) and examined for *Leptospira* antibodies by MAT, as described [2]. The antigens were a panel of 24 strains (representing all described serogroups) of live *Leptospira* grown in liquid medium EMJH (Difco, Detroit, MI, USA), and free of contamination or self-agglutination.

Table 1
Seroreactive goats and sheep and distribution of *Leptospira* serovars during an initial screening (first collection) and in a followup examination of previously seropositive (titres ≥400) animals (second collection)

Serovar	First collection		Second collection	
	Goats (n = 248)	Sheep (n = 292)	Goats (n = 19)	Sheep (n = 40)
Hardjo	19	17	9	19
Shermani	16	11	4	8
Grippotyphosa	5	4	2	4
Icterohaemorrhagiae	5	4	1	1
Autumnalis	3	3	1	–
Castellonis	2	1	–	–
Bratislava	2	–	–	–
Total	52	40	17	32

2.4.2. Bacteriology

Before culturing, all samples were examined with darkfield microscopy (Carl Zeiss, Germany), in order to visualize spirochetes. Samples were seeded for bacteriological culturing, using a serial dilution technique (to 10^{-2} and 10^{-3} dilutions), into Fletcher's semisolid medium, which also contained 300 mg/L 5-fluorouracil (Pharmacia, Kalamazoo, MI, USA) and 20 mg/L nalidixic acid (Oxoid Ltd., Basingstoke, United Kingdom) and incubated for 24 h at 28 °C. After 24 h of incubation, tubes were seeded into Fletcher's semisolid medium (Difco, Detroit, MI, USA) without antibiotics, incubated at 28–30° C and examined (darkfield microscopy) once weekly (for 6 weeks) [6].

2.4.3. PCR

Bacterial DNA was extracted by a phenol and guanidine thiocyanate method [14]. The PCR assay for the detection of *Leptospira* spp. is genus-specific and was conducted as described [15], based on a previously described protocol [16], that employed the primers Lep1 (5′-GGCGGCGCGTCTTAAACATG-3′) and Lep2 (3′-TTAGAACGAAGTTACCCCCCTT-5′). The expected size of the amplicon was 331 bp from the 16S rRNA gene of *Leptospira* spp. The amplification reaction mixture was prepared in a volume of 50 μL, containing 200 μM of each deoxynucleoside triphosphate, 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.5 μM each primer Lep1 and Lep2, 1.5 U platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 5 μL of template DNA. The reaction was performed in a DNA thermal cycler (PTC 200 DNA engine; MJ Research, Watertown, MA, USA). For each set of samples, ultrapure water was used as a negative control, whereas DNA extracted from *Leptospira interrogans* serovar Pomona was used as a positive control. After an initial denaturation at 95 °C for 3 min, the PCR profile was set as follows: 30 s of template denaturation at 94 °C, 30 s of primer annealing at 60 °C and 30 s of primer extension at 72 °C, for a total of 35 cycles, with a final extension at 72 °C for 5 min. Samples were analyzed by electrophoresis in a 2% agarose gel, stained with ethidium bromide (0.5 μg/mL), and DNA bands visualized under UV light.

3. Results

3.1. Serology

Of the 248 caprine and 292 ovine serum samples tested by MAT in the first (screening) step, 20.9 and 13.7%, respectively, were reactive. In both species, the two most common serovars were Hardjo and Shermani (Table 1). In the second step of the study, 49 (83%) of the animals sampled were still seroreactive (titres ≥400), including 14 of 16 female goats (87.5%) and all three bucks, 20 of 26 ewes (76.9%), and 12 of 14 rams (85.7%). In the second sample, Hardjo (54.9%) and Shermani (23.5%) remained the most prevalent serovars (Table 1).

3.2. Bacteriology

Although leptospires were detected by darkfield microscopy in three vaginal fluid samples (from two goats and one ewe), pure isolates were not obtained from sample of vaginal fluid or semen.

3.3. PCR

The PCR was positive in six of the semen samples (all from rams) and in seven samples of vaginal fluid (four goats and three ewes). Of the six positive rams, five were from the same flock as the three positive ewes

Table 2
Direct evidence of *Leptospira* spp. in body fluids collected from sheep and goats that were seropositive (titres ≥400) during an initial screening

Herd or flock	Animal	Darkfield	Culture	PCR
1	Goat	♀: 1/7 ♂: 0/1	♀: 1/7 ♂: 0/1	♀:1/7 ♂:0/1
2	Goat	♀: 1/4 ♂: 0/1	♀: 0/4 ♂: 0/1	♀:0/4 ♂:0/1
3	Goat	♀: 0/5 ♂: 0/1	♀: 0/5 ♂: 1/1	♀:3/5 ♂:0/1
4	Sheep	♀: 1/14 ♂:0/10	♀: 0/14 ♂: 0/10	♀: 3/14 ♂: 5/10
5	Sheep	♀: 0/5 ♂: 0/1	♀: 0/5 ♂: 0/1	♀: 0/5 ♂: 0/1
6	Ovine	♀: 0/7 ♂: 0/3	♀: 0/7 ♂: 0/3	♀: 0/7 ♂: 1/3
Total		♀: 3/42 ♂: 0/17	♀: 1/41 ♂: 1/17	♀: 7/41 ♂: 6/17

and one from another flock where no other animal was positive on PCR (Table 2). The positive goats belonged to two herds.

Many animals that were positive on PCR had reproductive problems (particularly females). Regarding the four positive goats, two aborted the previous year, both near the end of pregnancy; the other two required three consecutive breedings to achieve pregnancy. Two of the three positive ewes had also aborted (late in pregnancy) in the previous year. Furthermore, all herds/flocks from which these animals were derived had impaired fertility.

4. Discussion

In the present study, detection (by PCR) of molecular evidence of leptospires in vaginal fluids from goats and ewes confirmed the presence of the agent in the genital tract. Similarly, leptospires in the genital tract of non-pregnant cows [8], as well as the presence of specific immunoglobulins in the cervico-vaginal mucus of heifers [10], indicated that these organisms persisted in the genital tract of cattle. Although infected heifers had low-pregnancy rates and increased services per conception [10], to our knowledge, there are no similar reports for female goats or ewes. *Leptospira* sp. serovar Hardjo was also isolated from the postpartum vaginal discharge of experimentally infected cows that aborted or calved [17], providing further indications of the association between leptospires in the female genital tract and impaired reproductive efficiency of cattle. Based on the demonstration of the presence of *Leptospira* spp. in vaginal fluid of both goats and ewes, we inferred that these organisms could persist in the reproductive tract of female small ruminants.

Infection in males, especially bulls, is often subclinical. Therefore, the absence of clinical signs in the infected rams was not surprising. Bulls may carry leptospires in the genital tract, mainly in the seminal vesicles, making venereal transmission a key means of spread [9]. Although the role of semen in the transmission of bovine leptospirosis is well established [11], its role in the transmission of the disease in sheep or goats has not been investigated. The possibility that leptospiral DNA found in semen was due to urine contamination of the urethra and not from the male genital tract, cannot be excluded. Nevertheless, in our opinion, the presence of *Leptospira* spp. in the semen of six from 14 subclinical but seropositive rams (42.8%), strongly suggested a role for the male in the transmission of leptospirosis in this species.

Although transmission of leptospires has traditionally been associated with exposure to infected urine, evidence of its presence in semen and vaginal fluids suggests that, as in cattle, venereal transmission may also occur in small ruminants. In cattle [8], it is well established that venereal transmission may play an important part in the epidemiology of the infection, and that subclinically infected bulls are frequently implicated as a source of infection [9]. Transmission occurred with semen used for AI [2], as well as with oocytes used for IVF. The presence of leptospires in the reproductive tract of cows has also contaminated embryos [18].

Transmission due to direct or indirect exposure to infected urine is usually associated with specific seasonal or environmental factors that facilitate such exposure [19]. Under those conditions, strains maintained by other reservoirs, such as Bataviae, Icterohaemorrhagiae or Grippotyphosa become important and there may be abortion storms [2]. Conversely, venereal transmission is less influenced by seasonal or environmental factors and may lead to endemic leptospirosis, making it much more difficult to control; this was well described in flocks infected by serovar Hardjo [8], the most frequent serovar detected in the present study.

The seroprevalence of 20.9 and 13.7% for goats and sheep, respectively, and the predominance of Hardjo serovar, were expected for this population. These findings were consistent with recent reports of seroprevalence of leptospirosis in Brazil [5], although

they seemed higher than the 11.1% prevalence recently reported in the same region [4]. However, instead of a large epidemiological survey, the present study focused on flocks with varying degrees of impaired fertility, what may have increased the seroreactivity rate, although not altering the serovar distribution. Serological results of the second step of the study, performed in samples collected in the same animals of the bacteriological/molecular samples, confirmed the results. All flocks remained seroreactive, with the majority (83%) of the selected animals still seropositive. It was noteworthy that the present study used a minimum titres of 400, in order to identify animals from which recovery of leptospires would be more probable. Since the study was performed in suspect flocks from an endemic area, titres <400 could be interpreted as nonspecific reactions.

Based on the high seroprevalence in these animals, we concluded that impaired fertility, as well as other reproductive problems in these flocks, could be attributed to the disease. In an endemic scenario, as that observed in those flocks, subclinically infected animals are common and play an important role in the epidemiology of the disease [10]. Leptospirosis was recently identified as the major reproductive infectious disease in goats in Rio de Janeiro, Brazil [4], with similar findings reported for sheep in other regions of the country [7].

In spite of the molecular evidence of the presence of leptospires in both semen and vaginal fluids, the agent was not isolated by bacteriological culture. However, culture has specific limitations, including fastidious growth of this organism in artificial media, contamination, and poor sensitivity [2]. Leptospires have been isolated from goat urine samples within 6 weeks of incubation [6]; however, a longer incubation period, as well as the employment of different culture media might be more appropriate [8]. Furthermore, although PCR can detect 100 bacteria/mL in bovine semen, this is below the sensitivity of microbiological culture [11]. In addition to a lower detection limit, PCR is much faster than *Leptospira* culture and it may detect DNA from lysed or inactive organisms, following association with local antibody (IgG or IgA) produced in the genital tract. In that regard, local antibody was detected in the vaginal mucus of cattle infected with *L. interrogans* serovar Hardjo [20]. Nevertheless, one major disadvantage of the PCR method is that it is only genus specific. Although very useful for diagnostic purposes, it does not identify the causative serovar, an extremely important limitation to study the epidemiology of the infection. However, the combined use of PCR for direct evidence of *Leptospira* DNA in selected samples, with serology (MAT) for a comprehensive epidemiological investigation, may be useful.

In summary, we demonstrated the presence of *Leptospira* spp. in semen and vaginal fluids of goats and sheep by PCR. Based on these findings, we inferred that there is potential venereal transmission of leptospirosis in small ruminants.

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