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Identification of *Leptospira* spp. carriers among seroreactive goats and sheep by polymerase chain reaction

W. Lilenbaum [a,*], R. Varges [a], P. Ristow [b], A. Cortez [c], S.O. Souza [c], L.J. Richtzenhain [c], S.A. Vasconcellos [c]

[a] Veterinary Bacteriology Laboratory, Department of Microbiology and Parasitology, Universidade Federal Fluminense, 24210-130 Niteroi, RJ, Brazil
[b] Laboratory of Pathology and Molecular Biology, Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, Salvador, 40296-710 Salvador, Brazil
[c] Department of Preventive Veterinary Medicine and Animal Health, Faculty of Veterinary Medicine and Zootechny, University of São Paulo, 05508-900, SP, Brazil

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ABSTRACT

Few studies were conducted on the diagnosis and control of small ruminants' leptospirosis. Thirteen goat herds and seven sheep flocks located in the state of Rio de Janeiro, Brazil, were screened for leptospirosis. From the three herds and three flocks with greatest seroreactivity by MAT (Microscopic Agglutination Test), 19 and 40 seropositive goats and sheep, respectively, were selected, and urine samples were collected for bacteriology and PCR. For both species of animals, the most prevalent reactions were due to serogroups Sejroe and Shermani. Although leptospires were observed by darkfield microscopy in eight samples, pure isolates were obtained by bacteriological culture from only two samples. However, twelve urine samples (six goats and six sheep) were positive by PCR. Based on these findings, we consider that the combined use of MAT as a screening test followed by urine PCR for the direct detection of *Leptospira* spp. DNA was adequate for the identification of carrier animals among goats and sheep. These are valuable tools for the control of leptospirosis in small ruminants.

1. Introduction

In spite of its occurrence and economic importance, leading to reproductive problems and overall impaired productivity, few studies have been conducted on the diagnosis and control of small ruminants' leptospirosis. The efficacy of control programs for leptospirosis in sheep and goats, as well as in cattle, relies mainly on the correct identification of *Leptospira* spp. carriers (Faine et al., 2000; Magajevski et al., 2005; Ruiz et al., 2005). Sheep and goats are able to develop chronic renal infection (Gerritsen et al., 1994) and maintain persistent leptospiruria, perpetuating infection in the herd (Cousins et al., 1989).

Goats and sheep are considered less susceptible to leptospirosis than other domestic farm animal species, e.g. cattle (Leon-Vizcaino et al., 1987; Ciceroni et al., 2000). They may develop the chronic form of the disease; which is characterized by impaired fertility, neonatal deaths, abortions and decreased milk production, causing substantial economic losses (Ellis, 1994). Besides, small ruminants are able to develop chronic renal infection and maintain persistent leptospiruria, disseminating bacteria to other animal species as well as to humans. A recent study in Brazil showed that 11.1% of goats were seroreactive to *Leptospira* with a predominance of serovar Hardjo (Lilenbaum et al., 2007a). Leptospires of the Grippotyphosa serogroup were isolated from dairy goats (Lilenbaum et al., 2007b), whereas *Leptospira noguchii* was recently isolated from sheep (Silva et al., 2007).

The standard serological diagnosis of leptospirosis is the microscopic agglutination test (MAT), which is recommended for the diagnosis on a herd-screening basis. It relies on the seroreaction with live bacteria representative of local serovares. Nevertheless, the correlation of serology with the presence of bacteria in the kidneys or in extra-renal locations is not evident and the direct detection of the organism is necessary to reliably identify carriers, as part of an efficacious control program (Faine et al., 2000; Magajevski et al., 2005).

Efforts to identify carriers are directed towards the detection of the agent or its DNA in urine and other tissues. Available techniques include dark field direct microscopic examination (DFM), bacterial isolation, and the detection of leptospiral DNA by PCR (polymerase chain reaction) (Lucchesi et al., 2004). Since leptospiral cultivation is laborious, time-consuming, and contamination of cultures may occur, other approaches are welcome to identify carriers of leptospires. PCR has been used to detect *Leptospira* spp. in clinical specimens as bovine urine (Magajevski et al., 2005; Bomfim and koury, 2006) and vaginal fluids/semen of goats and sheep (Lilenbaum et al., 2008), with encouraging results. The purpose of the present study was to study the prevalence of *Leptospira* infection in sheep and goats with reduced fertility, as well as to evaluate the use of urine PCR as a potential

* Corresponding author. Tel.: +55 21 2629 2435; fax: +55 21 2629 2432.
E-mail address: mipwalt@vm.uff.br (W. Lilenbaum).

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tool for the detection of *Leptospira* spp. carriers among seroreactive 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. Goat herds comprised 180–257 adult animals each, kept under intensive management system. Sheep flocks comprised 290–423 adult animals each, which were allowed to graze. Inclusion criteria included 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. Animals younger than one year or older than six years were not included in the study. Apart the age criterion, the collection of samples was blinded and we did not take in consideration individual health histories. Although it was not a specific inclusion criterion, it was noteworthy that all herds and flocks included in the study had reduced fertility. The most frequent reproductive problems identified were prolonged returns to estrus and low-conception rates (all herds/flocks), premature parturition (five herds and three flocks) and sporadic abortion (six herds and two flocks). As an initial screening step, approximately 20% of the animals in each herd/flock were randomly selected for serological screening; and, overall, 248 caprine and 292 ovine serum samples were tested by MAT.

2.2. Study design

In the second part of the study, the three herds and three flocks with the greatest proportion of seroreactive animals (>30% in each herd/flock), representing 19 goats (16 females and three bucks) and 40 sheep (26 ewes and 14 rams) with specific anti-*Leptospira* titres $\geqslant$400, were selected for further studies. From those 59 animals, urine samples, for bacteriology and PCR, and a second blood sample were collected. Goat culture and serology results were published previously (Lilenbaum et al., 2007b).

2.3. Sampling

Urine samples were collected after intravenous injection of 150 mg furosemide (Intervet Labs., Brazil) per animal, and the second voiding of urine was collected into sterile vials. Urine samples were immediately added to 10% liquid Ellinghausen-McCullough-Johnson-Harris medium (EMJH) (Difco, USA)/saline and transported to the laboratory at room temperature (Zuerner, 2005). Urine was also chilled and transported to the laboratory for PCR and DFM. Blood samples were collected into evacuated tubes by jugular venipuncture.

2.4. Laboratory procedures

2.4.1. Serology

Serum samples were processed on the same day that they were collected. Blood samples were centrifuged (1000 g for 10 min) and serum tested for *Leptospira* antibodies by MAT using international standards (Faine et al., 2000). Samples were considered reactive to the strain for which the highest titres were observed. The antigens were a panel of 24 live *Leptospira* reference strains (representing all described serogroups) grown in liquid EMJH (Difco, USA), free of contamination or auto-agglutination. Since the study was conducted in an endemic area where cross-reactions may be frequent, titres were considered positive when $\geqslant$400.

2.4.2. Bacteriology

Urine samples of the 59 animals were examined by DFM (Carl Zeiss, Germany), in order to visualize spirochetes. Bacteriological culturing was performed using the serial dilution technique to 10^{-2} and 10^{-3} dilutions into Fletcher's semisolid medium containing 300 mg/L 5-fluorouracil (Pharmacia, USA) and 20 mg/L nalidixic acid (Oxoid Ltd., United Kingdom), and incubated for 24 h at 28 °C to 30 °C. After 24 h of incubation, tubes were seeded into Fletcher's semisolid medium (Difco, USA) without antibiotics, incubated at 28 to 30 °C and examined by DFM once a week for eight weeks for the growth of leptospires (Zuerner, 2005; Lilenbaum et al., 2007b).

2.4.3. PCR

Bacterial DNA from urine samples was extracted by a phenol and guanidine thiocyanate method (Chomczynski, 1993). The PCR assay for the detection of *Leptospira* spp. was genus-specific and directed to the16S rRNA gene of *Leptospira* spp. We used a previously described pair of primers A (5′-GGCGGCGCGTCTTAAACATG-3′) and B (5′-TTCCCCCCATTGAGCAAGATT-3′) (Mérien et al., 1992) and the amplification was conducted as described (Richtzenhain et al., 2002). Briefly, the 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 of each primer, 1.5 U platinum Taq DNA polymerase (Invitrogen, USA), and 5 μL of template DNA. Ultra pure water and DNA from *Leptospira interrogans* serovar Pomona were used as negative and positive controls, respectively. The PCR was performed in a DNA thermal cycler (PTC 200 DNA engine; MJ Research, USA), with an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. Amplicons of 331 bp were visualized by electrophoresis in 2% agarose gel stained with ethidium bromide (0.5 μg/mL) under ultra-violet light.

3. Results

3.1. Serology

Of the 248 caprine and 292 ovine serum samples tested by MAT in the screening step, 52 caprine (20.9%) and 40 ovine (13.7%) were reactive. In both species, the two most common serogroups were Sejroe (represented by serovar Hardjo) and Shermani (Table 1). In the follow-up step of the study, 49 (83%) animals were still seroreactive (titres $\geqslant$400), including 14 of 16 female goats (87.5%), the three bucks, 20 of 26 ewes (76.9%), and 12 of 14 rams (85.7%). Again, serogroups Sejroe (represented by serovar Hardjo – 54.9%) and Shermani (23.5%) were the most prevalent (Table 1).

Table 1
Serogroup distribution in seroreactive goats and sheep, during the initial screening by MAT and in the follow-up examination of previously seropositive (titre >400) sera.

Serogroup	Screening		Follow-up	
	Goats (n = 248)	Sheep (n = 292)	Goats (n = 19)	Sheep (n = 40)
Sejore	19	17	9	19
Shermani	16	11	4	8
Grippotyphosa	5	4	2	4
Icterohaemorrhagiae	5	4	1	1
Autumnalis	3	3	1	–
Ballum	2	1	–	–
Australis	2	–	–	–
Total	52	40	17	32

Obs.: No sample was reactive against the other 17 tested *Leptospira* strains.