

Molecular detection of leptospiral carriers in sheep under tropical field conditions

Detecção molecular de ovinos carreadores de Leptospira em ambiente tropical

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Abstract

The purpose of this study was to analyze the usefulness of PCR for the detection of leptospiral carriers in sheep under tropical field conditions. Two flocks, previously reported as seroreactive (A) and seronegative (B), were selected for this study. From those, the totality of animals of each flock, urine and vaginal fluid (VF)/semen were collected for bacteriological culture and PCR, as well as serum samples for serology. Serology confirmed the previous status of the two flocks. Culture was negative for all the samples. In PCR, animals of Flock A presented 26.7% (VF), 33.3% (semen) and 38.9% (urine) of positivity. Flock B presented 40.0% (VF), 33.3% (semen) and 5.6% (urine) of positivity by PCR. In conclusion, PCR was important to identify carriers of leptospires, including animals from a seronegative flock, what reinforces the advantages of the usage of this tool for the detection of carriers in sheep as part of control programs of leptospirosis under tropical field conditions.

Keywords: Leptospirosis. PCR. Sheep. Control.

Resumo

O objetivo do presente estudo foi analisar a aplicabilidade da PCR na detecção de ovinos carreadores de *Leptospira* em ambiente tropical. Dois rebanhos ovinos, previamente reportados como sororeativo (A) e soronegativo (B) foram selecionados para este estudo. Da totalidade de animais dos rebanhos, amostras de urina e fluido vaginal (FV)/sêmen foram colhidas para cultura bacteriológica e PCR. Além disso, amostras de soro foram colhidas e utilizadas na sorologia (teste da soroaglutinação microscópica). Essa técnica confirmou o estado prévio dos dois rebanhos. Nenhuma amostra pura de leptospirosas foi obtida no cultivo. Já na PCR, animais do Rebanho A apresentaram 26,7% (FV), 33,3% (sêmen) e 38,9% (urina) de amostras positivas. O Rebanho B apresentou 40,0% (FV), 33,3% (sêmen) e 5,6% (urina) de positividade pela PCR. Em conclusão, a PCR foi uma importante ferramenta na identificação de carreadores de leptospirosas, incluindo animais do rebanho soronegativo, o que reforça as vantagens do uso desta técnica para a detecção de ovinos portadores como parte dos programas de controle da leptospirose em ambiente tropical.

Palavras-chave: Leptospirose. PCR. Ovinos. Controle.

Introduction

Sheep are ubiquitous and important for subsistence, economic and social sustenance of the human population, mainly in developing countries (FAOSTAT, 2011). In this scenario, infectious diseases play a dramatic role determining important economic hazards in flocks worldwide (GIANGASPERO et al., 2013). From those, leptospirosis has been reported as one of the major reproductive infections, impairing productivity and leading to important hazards in small ruminants breeding (MARTINS et al., 2012; SUEPAUL et al., 2011).

There is a lack of apparent clinical symptoms in leptospiral infection in sheep, which are frequently subclinical. Moreover, laboratorial tests are essential to achieve an accurate diagnosis of the infection, and the Microscopic agglutination test (MAT) is

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the recurrent test for the diagnosis of leptospirosis (ELLIS; THIERMANN, 1986; OTAKA et al., 2012). Nevertheless, the correlation between serology and the status of carrier is not evident in an individual basis and the direct detection of the organism or its DNA may be necessary to identify reliably reservoirs (GAMAGE et al., 2011; OTAKA et al., 2012).

Carriers are considered as a key of the transmission chain of leptospires (LILENBAUM et al., 2008). In this scenario, host-adapted strains (as Hardjo in ruminants) are indirectly or directly transmitted from a reservoir to a susceptible animal (SUEPAUL et al., 2011). In this context, a reliable diagnostic test is required to identify carriers, as a part of a comprehensive control program of leptospirosis under field conditions. In this regard, PCR have been described as a sensitive and specific molecular tool for the detection of carriers of leptospires in different specimens, such as urine, semen and vaginal fluid, presenting encouraging results (GAMAGE et al., 2011; HAMOND et al., 2012; LILENBAUM et al., 2008). Nevertheless, the usage of PCR in animal leptospirosis is still limited to a small number of samples or herds/flocks and generally into the research scenario, with questionable application under field conditions. The purpose of this study was to analyze the usefulness of PCR for the detection of leptospiral carriers in sheep under tropical field conditions.

Materials and Methods

Two flocks located in Rio de Janeiro, Brazil were selected for the study, based on previous serology. The seroreactive group (Flock A, 33.3% seroreactivity, serovar Hardjo) was composed by 15 ewes and three rams (n=18), and the seronegative one (Flock B, 100% seronegative) consisted also of 15 ewes and three rams (n = 18). From the totality of animals of each flock (n = 36), urine and vaginal fluid (VF)/ semen were collected for bacteriological and molecular assays, as well as serum samples for MAT.

Blood samples were collected into evacuated tubes by jugular venipuncture, centrifuged (550 x G for 10

min) and sera examined for *Leptospira* antibodies by MAT, as described (OIE, 2012). The antigens were a panel of 24 strains of live *Leptospira* representing all the known serogroups, grown in liquid medium EMJH (Difco, Detroit, MI, USA), and free of contamination or self-agglutination.

For VF collection, the perineum was cleaned with water (without soap), and a woman's tampon (Tampax® Regular, Procter & Gamble, São Paulo, SP, Brazil) was introduced into the vagina. After 10 minutes, tampons were removed and transferred to sterile vials (50 mL) containing PBS, as described (LILENBAUM et al., 2008) and transported to the laboratory at room temperature. Semen samples were collected by electro-ejaculation (Eletropulsador TK-300 - TK Reprodução, Uberaba, Brasil) into sterile vials, after cleaning with water the prepuce and adjacent areas. Following collection of VF and semen, sheep were given 0.5-1.0 mg/kg furosemide (Teuto Brasileiro, Anápolis, Brazil) intravenously, and the second voiding of urine was collected into sterile vials (50 mL). Semen and urine samples were added to cryotubes with 100 µL PBS for PCR, chilled and transported to the laboratory.

All samples were processed in average three hours after sampling. The tampons were aseptically squeezed and centrifuged for 10 min (800 G) in sterile vials, and an aliquot (500 µL) of supernatant, as well as 500 µL of urine, was transferred to Fletcher and EMJH media tubes (Difco, Detroit, MI, USA). Tubes were incubated at 28-30°C and examined (darkfield microscopy) weekly for 20 weeks.

For PCR, DNA from VF, semen and urine was extracted with Promega Wizard SV kit genomic DNA Purification System® (Promega, Fitchburg, WI, USA). The employed primers were LipL32-45F (5'-AAG CAT TAC CGC TTGTGG TG-3') and LipL32-286R (3'-GAA GTC TAA AAT TAA GGG ACG G-5') targeting amplification of the *LipL32* gene, which is present only in pathogenic strains and has been referred as 100% specific for leptospires (HAMOND et al., 2012).

Results

Serology confirmed the status of the two flocks, with slight variation when compared to the previous test. Flock A was still seroreactive (38.9% seroreactivity, serovar Hardjo) and Flock B remained 100% seronegative. Culture was negative for all the samples.

Considering PCR results, animals of Flock A were positive in 4/15 (26.7%) VF, 1/3 (33.3%) semen and 7/18 (38.9%) of urine samples. In Flock B, PCR was positive on 6/15 (40.0%) VF, 1/3 (33.3%) semen and 1/18 (5.6%) urine samples. Results of PCR of the different samples are demonstrated on figure 1.

Discussion

Microscopic agglutination test (MAT) is the most widely used serological test for leptospirosis. Furthermore, it is the test recommended by World Organization for Animal Health for several animal species (OIE, 2012). The major advantage of MAT is its high specificity in a serogroup level. Nevertheless, it has been demonstrated that many animals may present leptospire in their kidneys (and in other sites) without presenting a detectable titre at MAT (GAMAGE et al., 2011; OTAKA et al., 2012).

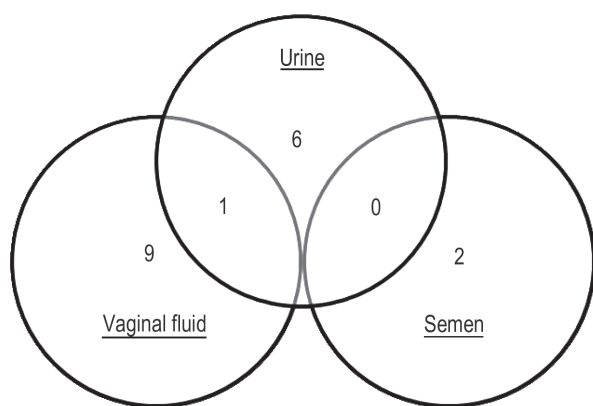


Figure 1 - Venn diagram demonstrating the distribution of positive animals by PCR in urine (n = 36), vaginal fluid (n = 30) and semen (n = 6) samples of sheep from two flocks in Rio de Janeiro - Brazil

Source: (DIRECTOR et al., 2014)

Although unexpected, results of the current study corroborate this scenario. Seven out of 18 animals from Flock B were seronegative twice at MAT but presented leptospiral DNA in all the specimens (urine/VF/semen), what demonstrates that MAT was not able to detect the presence of the bacterium among the animals of that flock. Bacteriological culture, which is considered the gold-standard method for the detection of leptospiral infections, was ineffective in both flocks, since none of the studied samples yielded a pure culture. Although disappointing, it was not an unexpected finding, since the limitations of leptospiral culturing, which is laborious and difficult to perform, have been widely exposed in many reports (HAMOND et al., 2012; OIE, 2012).

Ruminants are referred as reservoirs of the serovar Hardjo, spreading the microorganism and playing a direct role on the transmission of the infection (SUEPAUL et al., 2011). Identification of carriers by direct demonstration of the agent is important for the control of leptospiral infections (GAMAGE et al., 2011), avoiding economic hazards in livestock.

Although the usage of PCR for leptospirosis in veterinary medicine has been rapidly increasing, the majority of reports were conducted on experimental conditions, and not focused on the detection of carriers in order to improve control programs (GAMAGE et al., 2011; HAMOND et al., 2012). Despite a few disadvantages, as the possibility of detecting leptospiral DNA of non-viable bacteria or the limitations regarding the required equipment, the cost of this technique has enormously decreased in the past years, and the possibility of a rapid and reliable direct diagnostic must encourage a wide usage of that molecular tool as part of control programs under field conditions.

An interesting outcome of the present study refers to the location from where leptospiral DNA was detected, particularly VF and semen. The possibility of venereal transmission of leptospirosis has been first suggested in cattle (ELLIS; THIERMANN, 1986) and more recently a study of our group has suggested it that

it may also occur in small ruminants (LILENBAUM et al., 2008). Noteworthy that PCR results of urine and VF/semens were not coincident except for one ewe (Figure 1); it reinforces that many animals, by analogy to cows, may present leptospires not only in their kidneys but also in other sites, as reproductive tract.

In conclusion, PCR was important to identify carriers of leptospires, including animals from a seronegative flock, what reinforces the advantages of the usage of this tool for the detection of carriers in sheep as part of control programs of leptospirosis under tropical field conditions.

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Bioethics and Biosecurity Committee Approval

This study was approved by the Comissão de Ética no Uso de Animais - PROPPi - UFF, under the number 255/2012.