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MICROBIOLOGY

Molecular demonstration of intermittent shedding of *Leptospira* in cattle and sheep and its implications on control

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ABSTRACT: *For a long time, it has been stated that urine leptospiral shedding is intermittent, which was observed primarily by culturing. However, culturing presents serious limitations, mainly low sensitivity, and failure on detection of leptospires cannot be neglected. PCR presents several advantages, mainly higher sensitivity. The present study aimed to analyze the occurrence of intermittency on leptospiral shedding by PCR in naturally and experimentally infected animals. In this study two experiments were conducted, the first with 60 cows naturally infected from an endemic herd. The second one was conducted in three sheep experimentally infected, each one with a different strain of Leptospira (strains Copenhageni L1-130, Canicola LO-4 and Pomona Fromm). Considering cattle, 43.3% presented negative in all tests, the remaining (56.7%) were positive at least once. From these, only one (1.6%) was positive in all samples, and seven (11.8%) were positive only in the last sampling, making it impossible to evaluate the intermittency. Noteworthy, 26 cows (43.3%) presented the typical intermittent pattern of leptospiral shedding in urine. In sheep, all experimentally infected animals presented the typical intermittent shedding patterns, independently of the inoculated leptospiral strain. We considered that a careful serial analysis of urine samples for a more definitive and reliable individual diagnosis would be required for a successful antimicrobial therapy and control of leptospirosis on a herd.*
Key words: *cows, intermittent, PCR, sheep, strain.*

Demonstração molecular da intermitência eliminando *Leptospira* em vacas e ovelhas e suas implicações no controle

RESUMO: *Durante muito tempo, foi afirmado que a eliminação de leptospiras na urina era intermitente, o que havia sido demonstrado principalmente por meio do cultivo microbiano. No entanto, a cultura apresenta graves limitações, principalmente com relação à baixa sensibilidade. Em contraste, a PCR apresenta várias vantagens em relação ao cultivo bacteriológico para leptospiras, sendo esta ferramenta cada vez mais utilizada para o diagnóstico de animais eliminadores da bactéria em diversos sítios. Assim, o presente estudo teve como objetivo analisar a ocorrência de intermitência na eliminação de leptospiras por meio de PCR em animais natural e experimentalmente infectados. Para este estudo foram realizados dois experimentos, sendo o primeiro com 60 vacas naturalmente infectadas de um rebanho sabidamente endêmico e o segundo em três ovelhas experimentalmente infectadas, cada uma com uma estirpe diferente de Leptospira (estirpes Copenhageni L1-130, Canicola LO-4 e Pomona Fromm). Considerando-se os bovinos, 43,3% apresentaram negatividade em todos os testes, sendo os demais 56,7% positivos ao menos uma vez. Destes, apenas um (1,6%) foi positivo em todas as amostras, e sete (11,8%) foram positivos somente na última coleta, o que impossibilitou a avaliação da intermitência. Não obstante, 26 vacas naturalmente infectadas (43,3%) apresentaram o padrão de eliminação tipicamente intermitente de leptospiras na urina. Das três ovelhas experimentalmente infectadas, todas apresentaram eliminação intermitente da bactéria na urina, independentemente da estirpe inoculada. Consideramos que seria necessária uma cuidadosa análise seriada de amostras de urina para um diagnóstico individual mais definitivo e confiável para uma terapia antimicrobiana bem-sucedida e o controle da leptospirose em um rebanho.*
Palavras-chave: *vacas, intermitência, PCR, ovelhas, estirpes.*

INTRODUCTION

Leptospirosis is a zoonotic disease of worldwide distribution, affecting both wildlife and domestic animals. In livestock it often presents as a silent and asymptomatic infection (DIRECTOR et al., 2014). Leptospirosis in livestock leads to reproductive failure, such as oestrus repetition, abortion, stillbirths and weak offspring (ELLIS, 2015). The real impact of these affections has not yet been estimated, but it is well known that these reproductive symptoms are related to economic losses (AYRAL, 2013).

Leptospires penetrate the host for lesions on the skin and mucous membranes. After penetration, the bacteria invade the circulation (leptospiremia), spreading throughout the animal (ADLER, 2014). After

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this initial phase, it is known that leptospires lodges in the renal tubules of infected animals, being shed for long periods (leptospiruria), contaminating the environment and other animals (ELLIS, 2015). In this context, it has been reported that naturally infected cattle may shed the bacteria for about 40 weeks, while experimentally infected calves shed for up to 32 weeks (LEONARD et al., 1992). Independently of the use of host-adapted (e.g. Hardjo in ruminants), or incidental (e.g. Pomona in ruminants) serovars for experimental infections, renal colonization has been reported, in general 10-25 days after the infectious challenge (SLEIGHT et al, 1964; LITLE & SALT, 1976; RINEHART et al, 2012).

For a long time, it has been stated that urine leptospiral shedding to be intermittent (FAINE et al., 2000). It was first determined by the inconstant recovery of this microorganism by culturing (INADA et al., 1916). Nevertheless, it must be considered that culturing of leptospires presents serious limitations, mostly low sensitivity (CHIDEROLI et al., 2016), so failure on detection of leptospires cannot be neglected. Thus, it may be unclear if intermittence really occurs or if the failure on recovering leptospires from infected animals is a reflex of the low sensitivity of culturing. PCR has been widely used for the diagnosis of leptospirosis, with high sensibility and specificity (PICARDEAU, 2013; TAYLOR et al., 2015). It is a rapid and reliable method that may be used in large-scale. Additionally, frozen samples may be used for PCR without compromising the reaction (HAMOND et al., 2014). Considering the advances of PCR on the detection of carriers in leptospirosis, this study aimed to analyze the occurrence of intermittency on leptospiral shedding in naturally and experimentally infected animals.

MATERIALS AND METHODS

Study Design

In this study two experiments were conducted, both with the approval of the Ethics Committee of Universidade Federal Fluminense (UFF), Brazil (number 814/2016). The first one was conducted in naturally infected cows belonging to the Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Gado de Leite - Valença, Rio de Janeiro, while the other was conducted on experimentally infected sheep in the Unidade de Pesquisa Experimental em Caprinos e Ovinos (UniPECO) on the school farm of the Universidade Federal Fluminense (UFF) - Cachoeiras de Macacu, Rio de Janeiro.

Experiment I

In the first experiment (naturally infected cows), 60 adult cows from a herd known to be endemically infected were studied. The animals remained altogether in the same pasture. Six urine samplings were made from each cow, at weekly intervals, totaling 360 samples. Urine was collected after intravenous furosemide administration, in sterile conical tubes (50ml) and then 1ml aliquots were transferred into microtubes, identified, conditioned at 4°C and sent to the laboratory.

Experiment II

The second experiment was conducted in three experimentally infected ewes. Each one received a single dose ($\pm 10^8$ leptospires) of different strains of *Leptospira* (strains Copenhageni L1-130, Canicola LO-4 and Pomona Fromm). Animals were infected by intraperitoneal route and were kept in separate pens. Animals were observed daily by veterinarians. Urine samples were collected as described in cows, but during eight times, at three days intervals, totaling 24 samples. Thus, the total number of samples (cows and ewes) was 384.

PCR

The DNA was extracted using the Promega Wizard SV Genomic DNA Purification System® (Promega, Madison, WI, USA). Primers were targeted to the *lipL32* gene (regarded as present only in pathogenic leptospires), LipL32_45F - 5′-AAG CAT TAC CGC TTG TGG TG-3′ and LipL32_286R - 5′-GAA CTC CCA TTT CAG CGA TT-3′, which generate a fragment of 242pb (STODDARD et al., 2009). Briefly, primers were used in concentrations of 0.6µM, 1.0U Taq polymerase, 2.4µM MgCl2, and 0.3m MdNTP in a final volume of 25µL. One cycle of initial denaturation at 94°C for 2min, followed by 35 cycles of denaturation at 94°C for 30s, annealing primers to 53°C for 30s and 1min extension with 72°C and final extension cycle at 72°C for 5min. Strain *L. interrogans* serovar Copenhageni, Fiocruz L1-130 (ATCC BAA-1198) was used as a positive control.

RESULTS

Considering cattle, 26/60 (43.3%) animals tested negative in all samples and were considered as non-infected. For the remaining 34 (56.7%) cows that were PCR-positive at least once, only one (1.6%) presented positive in all samples, and seven (11.8%) were positive only in the last sampling, making impossible to evaluate the intermittency. Noteworthy, 26 of the naturally infected cows (43.3%) presented the typical intermittent pattern of urine leptospiral shedding (Table 1). In relation to the three experimentally infected sheep, all of them

Table 1 - Status of naturally infected cows and experimentally infected sheep for leptospiral urine detection after serial PCR results.

Status Species	Positive on all samplings	Negative on all samplings	Intermittent shedding	Inconclusive
Cows	1 (1.7%)	26 (43.3%)	26 (43.3%)	7 (11.7%)
Sheep	0 (0%)	0 (0%)	3 (100%)	0 (0%)

presented the typical intermittent pattern of urine leptospiral shedding, independently of the inoculated leptospiral strain (Table 1).

DISCUSSION

The results confirmed that when employing molecular tools there was intermittent shedding of leptospires, independently of the leptospiral strain, host species, natural or experimental infection. This shedding intermittency may have strong implications for the control of leptospirosis on livestock, mainly on the strategic decision about employing or not antimicrobial agents. Although, the whole-herd treatment approach has been recommended (MUGHINI-GRAS et al., 2014) it may be expensive and economically non-feasible. Besides the cost, other important aspects should be reminded, as the grace period of milk/meat after the usage of antibiotic therapy, as well as the environmental impact of the antibiotics usage. Therefore, employing antibiotic therapy cannot be performed indiscriminately and must be proceeded by a thorough identification of infected animals (MARTINS & LILENBAUM, 2017). Isolation of leptospires in tissues, urine and blood is considered the gold standard for the definitive diagnosis of leptospirosis (OIE, 2014). However, this isolation is usually achieved after weeks or months (VERMA et al., 2012), what makes this method not ideal for a rapid diagnosis (ADLER & DE LA PEÑA MOCTEZUMA, 2010). In contrast, PCR seems to be an alternative for a rapid and direct diagnosis of the infection. Nevertheless, this method is a poor indicator for the infecting serovar in a herd, and its implication for epidemiological studies is limited, expensive and difficult (ELLIS, 2015).

Results of our experiments clearly demonstrated that after negative results on PCR or culture of one single urine sample, animals cannot be reliably considered as non-infected, since infected animals may not be detected due to the intermittency. Considering the recent reduction of costs on molecular diagnostic methods, we consider that a careful serial analysis of urine samples for a more definitive and reliable individual diagnosis would be required for a successful control program of leptospirosis on a herd.

The low number of experimentally infected animals used in the presented study represents a limitation. Despite that, intermittence was clearly observed in all infected ewes. Other possible bias of this study was the possible interference of environmental conditions in naturally infected animals. Transmission of leptospirosis is influenced by environmental conditions, such as rainfalls (CORREIA et al., 2017), which may interfere in the exposure of those animals to leptospires, also influencing the reinfection and intermittency.

In conclusion this is the first study to describe the intermittent shedding of leptospires in urine by PCR. We suggest that control strategies may incorporate a serial analysis of urine samples for a more reliable individual diagnosis and treatment.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

This study was approved by the Ethics Committee of Universidade Federal Fluminense (UFF), Brazil (number 814/2016).

REFERENCES

ADLER B. Pathogenesis of leptospirosis: cellular and molecular aspects. **Veterinary Microbiology**, v.172, p.353-358, 2014. Available from: <http://www.sciencedirect.com/science/article/pii/S0378113514002958>. Accessed: Nov. 4, 2016. doi: 10.1016/j.vetmic.2014.06.015.

ADLER, B.; DE LA PEÑA MOCTEZUMA, A. *Leptospira* and leptospirosis. **Veterinary Microbiology**, v.140, p.287-296, 2010. Available from: <http://www.sciencedirect.com/science/article/pii/S0378113509001163>. Accessed: Nov. 4, 2016. doi: 10.1016/j.vetmic.2009.03.012.

AYRAL, F. La leptospirose dans les cheptels bovins laitiers en France: impact économique de l'infection. **Bulletin des GTV**, v.69, p.61-67, 2013.

CHIDEROLI, R.T. et al. Isolation and molecular characterization of *Leptospira borgpetersenii* serovar Hardjo strain Hardjobovis in the urine of naturally infected cattle in Brazil. **Genetics and Molecular Research**, v.19, p.15, 2016. Available from: <http://www.geneticsmr.com/articles/6041>. Accessed: Oct. 13, 2016. doi: 10.4238/gmr.15018473.

CORREIA, L. et al. Effects of rainfall on incidental and host-maintained leptospiral infections in cattle in a tropical region. **Veterinary Journal**, v.220, p.63-64, 2017. Available from: <http://www.sciencedirect.com/science/article/pii/S1090023317300011>. Accessed: Mar. 15, 2017. doi: 10.1016/j.tvjl.2016.12.016.

DIRECTOR, A. et al. Isolation of *Leptospira interrogans* Hardjoprajitno from vaginal fluid of a clinically healthy ewe suggests potential for venereal transmission. **Journal Medicine Microbiology**, v.51, p.1234-1236, 2014. Available from: <http://jmm.microbiologyresearch.org/content/journal/jmm/10.1099/jmm.0.065466-0#tab2>. Accessed: Nov. 6, 2016. doi: 10.1099/jmm.0.065466-0.

ELLIS, W.A. Animal leptospirosis. Current topics. **Microbiology and Immunology**, v.387, p.99-137, 2015.

FAINE, S. et al. A brief overview of the disease, leptospirosis. In: FAINE, S.; ADLER, B.; BOLIN, C.; **PEROLAT, P. *Leptospira* and *leptospirosis***. Melbourne: MedSci, 2000. p.68.

HAMOND, C. et al. Urinary PCR as an increasingly useful tool for an accurate diagnosis of leptospirosis in livestock. **Veterinary Research Communications**, v.38, p.81-85, 2014. Available from: <http://link.springer.com/article/10.1007%2Fs11259-013-9582-x>. Accessed: Nov. 4, 2016. doi: 10.1007/s11259-013-9582-x.

INADA, R. et al. The etiology, mode of infection, and specific therapy of Weil's Disease (*Spirochaetosis Icterohaemorrhagica*). **Journal of Experimental Medicine**, v.23, p.377-402, 1916.

LEONARD, F.C. et al. Duration of urinary excretion of leptospires by cattle naturally or experimentally infected with *Leptospira interrogans* serovar hardjo. **Veterinary Records**, v.131, p.435-439, 1992. Available from: <http://veterinaryrecord.bmj.com/content/131/19/435>. Accessed: Jan. 2, 2016. doi:10.1136/vr.131.19.435.

LITTLE, T.A.; SALT, G.F. The experimental infection of calves with a British leptospire of the Pomona serogroup. **Research Veterinary Science**, v.21, p.363-364, 1976.

MARTINS, G.; LILENBAUM, W. Control of bovine leptospirosis: aspects for consideration in a tropical environment. **Research Veterinary Science**, 2017. In press.

MUGHINI-GRAS, L. et al. Application of an integrated outbreak management plan for the control of leptospirosis in dairy cattle herds. **Epidemiology and Infection**, v.142, p.1172-1181, 2014. Available from: <https://www.cambridge.org/core/journals/epidemiology-and-infection/article/application-of-an-integrated-outbreak-management-plan-for-the-control-of-leptospirosis-in-dairy-cattle-herds/0C3A318DB5D39C56A8F411573F0E0D25>. Accessed: Nov. 3, 2016. doi: 10.1017/S0950268813001817.

OIE. Leptospirosis. IN: **Manual of diagnostic tests and vaccines for terrestrial animals**. 7.ed. Paris: W. Organism for Animal Health, 2014. v.1, p.598.

PICARDEAU, M. Diagnosis and epidemiology of leptospirosis. **Médecine et Maladies Infectieuses**, v.43, p.1-9, 2013. Available from: <http://www.sciencedirect.com/science/article/pii/S0399077X12003198>. Accessed: Feb. 12, 2017. doi: 10.1016/j.medmal.2012.11.005.

RINEHART, C.L. et al. Efficacy of vaccination of cattle with the *Leptospira interrogans* serovar hardjo type hardjoprajitno component of a pentavalent *Leptospira* bacterin against experimental challenge with *Leptospira borgpetersenii* serovar hardjo type hardjo-bovis. **American Journal Veterinary Research**, v.73, p.735-740, 2012. Available from: <http://avmajournals.avma.org/doi/abs/10.2460/ajvr.73.5.735?url_ver=Z39.88-2003&rfr_id=ori:rid:crossref.org&rfr_dat=cr_pub%3dpubmed>. Accessed: Apr. 2, 2017. doi: 10.2460/ajvr.73.5.735.

SLEIGHT, S.D. et al. Experimental *Leptospira pomona* infection in bulls. **American Journal Veterinary Research**, v.25, p.1663-1668, 1964.

STODDARD, R.A. et al. Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the LipL32 gene. **Diagnostic Microbiology and Infectious Disease**, v.64, p.247-255, 2009. Available from:<http://www.sciencedirect.com/science/article/pii/S0732889309001059>. Accessed: Jan. 7, 2017. doi: 10.1016/j.diagmicrobio.2009.03.014.

TAYLOR, A.J. et al. A systematic review of the mortality from untreated leptospirosis. **PLoS Neglected Tropical Diseases**, v.25, p.9, 2015. Available from:<http://journals.plos.org/plosntds/article?id=10.1371/journal.pntd.0003866>. Accessed: Apr. 2, 2017. doi: 10.1371/journal.pntd.0003866.

VERMA, A.K. et al. Leptospirosis-persistence of a dilemma: an overview with particular emphasis on trends and recent advances in vaccines and vaccination strategies. **Pakistan Journal of Biological Sciences**, v.15, p.954-963, 2012. Available from: <http://docsdrive.com/pdfs/ansinet/pjbs/2012/954-963.pdf>. Accessed: Jan. 2, 2017. doi: 10.3923/pjbs.2012.954.963.