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DIEGO FIGUEIREDO DA COSTA

Infecção experimental por *Leptospira* sp. em ovinos deslanados da raça Santa Inês e mestiços no semiárido nordestino

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Tese submetida ao Programa de Pós-Graduação em Ciência e Saúde Animal, da Universidade Federal de Campina Grande, como requisito parcial para obtenção do grau de Doutor em Ciência e Saúde Animal.

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RESUMO

A maioria das informações sobre a leptospirose foi obtida a partir de infecções experimentais com roedores, que, apesar de relevantes, não fornecem todas as respostas sobre a patogênese da doença nos animais e seres humanos. Sabe-se que para avançar no controle e na prevenção da leptospirose é necessário conhecer melhor a interação existente entre o agente e cada hospedeiro animal. Dessa forma, buscou-se contribuir para a compreensão da interação hospedeiro-parasita, transmissão da doença e o papel portador dos ovinos da raça Santa Inês e mestiços experimentalmente infectados por *Leptospira* sp., assim como registrar alterações clínicas e hemato-bioquímicas que possam auxiliar no diagnóstico prévio da doença nessa espécie. Para isso, esta é composta de três capítulos. No Capítulo I foi realizada uma infecção experimental com *Leptospira interrogans* sorogrupo Pomona sorovar Kennewicki em ovelhas, buscando analisar um modelo natural de infecção e o comportamento da interação agente-hospedeiro de acordo com a raça de ovelhas desafiadas. No total, 12 ovelhas foram utilizadas, sendo seis mestiças e seis da raça Santa Inês. As ovelhas foram comparadas com base na sorologia, diagnóstico molecular e microbiológico. No Capítulo II, objetivou-se reproduzir a doença aguda em ovelhas da raça Santa Inês e mestiças por meio da inoculação por via intraperitoneal de *L. interrogans* sorogrupo Pomona sorovar Kennewicki, verificando possíveis diferenças na resposta à infecção nas ovelhas desafiadas. No total, 10 ovelhas foram utilizadas, sendo cinco mestiças e cinco da raça Santa Inês. As ovelhas foram comparadas com base na sorologia, diagnóstico molecular e microbiológico. No Capítulo III, procurou-se determinar a influência da infecção por *L. interrogans* sorogrupo Pomona sorovar Kennewicki nos parâmetros clínicos e hemato-bioquímicos de acordo com a via de inoculação e a raça de ovelhas experimentalmente infectadas. Utilizou-se 24 ovelhas para realização deste experimento, estas foram divididas igualmente em dois grupos: mestiças (grupo A) e Santa Inês (grupo B). Em ambos os grupos os animais foram desafiados por via intraperitoneal ou por via conjuntival. As ovelhas foram comparadas com base nos sinais clínicos, hemograma, bioquímica sérica e urinálise. Tanto a via conjuntival como a via intraperitoneal foram eficientes em reproduzir a infecção nas ovelhas infectadas, mas a via intraperitoneal apresentou alterações mais contundentes nos parâmetros hematológicos e bioquímicos. Os ovinos da raça Santa Inês apresentaram maior concentração e duração dos títulos. Nenhuma ovelha apresentou sinais clínicos compatíveis com a infecção por *Leptospira* sp, portanto a avaliação clínica se mostrou insuficiente para determinar com confiança animais infectados. Os achados enfatizam a importância do trato genital como um local extra urinário de infecção e indicam a possibilidade de transmissão venérea na espécie. É possível que tanto as ovelhas da raça Santa Inês como as mestiças possam exercer uma relação de resistência e serem capazes de participar na transmissão de cepas do sorogrupo Pomona, sendo que as mestiças parecem ser mais resistentes à infecção.

Palavras-chave: Leptospirose; Resistência; Transmissão venérea; Infecção experimental.

ABSTRACT

Most of the information on leptospirosis was obtained from experimental rodent infections, which, while relevant, do not provide all the answers about the pathogenesis of the disease in animals and humans. It is known that to advance in the control and prevention of leptospirosis it is necessary to know better on the interaction between the agent and each animal host. Thus, we sought to contribute to the understanding of host-parasite interaction, disease transmission and the carrier role of Santa Inês and crossbred sheep experimentally infected with *Leptospira* sp., as well as to register clinical and hemato-biochemical changes that may help in the previous diagnosis of the disease in this species. For this purpose, this Thesis is composed of three chapters. In Chapter I an experimental infection with *Leptospira interrogans* serogroup Pomona serovar Kennewicki was carried out in sheep, seeking to analyze a natural model of infection and the behavior of the agent-host interaction according to the breed of challenged sheep. In total, 12 sheep were used, six crossbred and six Santa Inês breed. The ewes were compared on the basis of serology, molecular and microbiological diagnosis. In Chapter II, the objective of the study was to reproduce the acute disease in Santa Inês sheep and crossbred ewes by intraperitoneal inoculation of *L. interrogans* serogroup Pomona serovar Kennewicki, verifying possible differences in response to infection in challenged ewes. In total, 10 ewes were used, five crossbred and five Santa Ines. The ewes were compared on the basis of serology, molecular and microbiological diagnosis. In Chapter III, the influence of infection by *L. interrogans* serogroup Pomona serovar Kennewicki on clinical and haemato-biochemical parameters was determined according to the route of inoculation and the breed of experimentally infected sheep. Twenty-four sheep were used to carry out this experiment, which were divided into two groups: crossbred (group A) and Santa Inês (group B). In both groups the animals were challenged intraperitoneally and conjunctivally. The ewes were compared on the basis of clinical signs, blood count, serum biochemistry and urinalysis. Both the conjunctival and intraperitoneal routes were efficient in reproducing the infection in the infected sheep, but the intraperitoneal route presented more strong alterations in hematological and biochemical parameters. Santa Inês sheep presented higher concentration and duration of the antibody titers. No sheep presented clinical signs compatible with *Leptospira* sp. infection, so clinical evaluation was insufficient to reliably determine infected animals. The findings emphasize the importance of the genital tract as an extra urinary infection site and indicate the possibility of venereal transmission in the species. It is possible that both Santa Inês and crossbred sheep can exercise a resistance relationship and be able to participate in the transmission of strains of the Pomona serogroup, with the crossbred sheep seeming to be more resistant to infection.

Keywords: Leptospirosis; Resistance; Venereal transmission; Experimental infection.

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Lista de Abreviaturas e Siglas

AST	Aspartato aminotransferase
bpm	Batimentos por minuto
DNA	Àcido Desoxirribonucleico
EDTA	Àcido Etilenodiamino Tetra-Acético
EMJH	Ellinghausen-McCullough-Johnson-Harris
FC	Frequência Cardíaca
FR	Frequência Respiratória
GGT	Gama Glutamil Transpeptidase
HE	Hematoxilina-Eosina
MAT	Microscopic Agglutination Test
OIE	Organização Mundial de Saúde Animal
PBS	Tampão fosfato-salino
PCR	Reação em Cadeia da Polimerase
MAT	Microscopic Agglutination Test
TR	Temperatura Retal
UFCG	Universidade Federal de Campina Grande

Lista de Símbolos

%	Porcentagem
½	Meio
>	Maior
<	Menor
≥	Maior ou igual
≤	Menor ou igual
m/L	Mililitro
g/dL	Gramas por decilitro
mg/dL	Miligramas por decilitro
U/L	Unidade por litro
µL	Microlitro
ºC	Graus Celsius
Mg	Miligramas
m	Metros
Kg	Quilograma
cm^2	Centímetros quadrados
µm	Micrômetros
mm	Milímetros
p/v	peso/volume
®	Marca registrada
mm^3	Milímetros cúbicos
Ery/mL	Eritrócitos por mililitro
Leu/mL	Leucócitos por mililitro

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INTRODUÇÃO GERAL

Mudanças históricas na demanda por produtos de origem animal têm ocorrido no cenário mundial, sendo indiscutivelmente uma das principais preocupações do século XXI. Segundo a Organização das Nações Unidas para Agricultura e Alimentação, a produção de alimentos terá que crescer ao menos 70% até 2050, no qual os países em desenvolvimento serão os mais exigidos (FAO, 2017). Diante disso, a agropecuária ganha um papel primordial não somente para alavancar a economia, mas como uma ferramenta social para solucionar as adversidades contemporâneas.

No contexto da pecuária, a ovinocultura tem se mostrando cada vez mais relevante, estabelecendo-se como uma alternativa economicamente viável para produtores rurais e desempenhando um importante papel social em alguns lugares do globo, como é o caso do Brasil. Em contrapartida, problemas sanitários limitam o crescimento dessa atividade e comprometem o processo produtivo. Como exemplo se destaca a leptospirose, uma doença infectocontagiosa causada por espiroquetas do gênero *Leptospira* que repercute negativamente nos índices produtivos e reprodutivos dos rebanhos, além de ser uma importante zoonose (ELLIS, 2015). Essa patologia ganha ainda mais destaque no caso do Brasil, visto que as condições de clima tropical favorecem a sobrevivência e transmissão do seu agente no ambiente (ROBERTSON et al., 2012).

No Nordeste brasileiro, houve um aprimoramento genético ao longo dos anos em que se chegou ao desenvolvimento de raças ovinas mais resistentes as adversidades climáticas da região semiárida (CEZAR et al., 2004; SOUSA et al., 2015). Contudo, essa resistência parece ser mais pronunciada quando há o cruzamento entre raças, resultando nos ovinos mestiços. Estima-se que o Brasil possua um rebanho de quase 18 milhões de ovinos, sendo que aproximadamente 65% desse efetivo estão na região Nordeste (IBGE, 2017). O estado da Paraíba, mesmo com as rigorosas secas dos últimos anos, cresceu sua produção e possui um rebanho de quase 600 mil ovinos (IBGE, 2017), compostos principalmente por animais mestiços (40%), seguido por animais da raça Santa Inês (30%), ½ sangue Santa Inês + ½ sangue dorper (25%) e ovinos da raça Dorper (5%) (GUILHERME et al., 2017). A raça Santa Inês é uma raça de ovinos deslanados que teve sua origem a partir de cruzamentos de ovinos Crioulos trazidos pelos portugueses na colonização do Brasil com ovinos deslanados oriundos do continente africano, além de ovinos da raça Bergamácia da Itália, Morada Nova, Somallis e Suffolk (SOUZA et al., 2003). Por sua vez, os ovinos mestiços compreendem aqueles

animais oriundos do cruzamento de diversas raças e que não possuem um padrão definido, no qual no Nordeste prevalecem mais as raças deslanadas.

Animais mestiços são menos sensíveis às parasitoses gastrointestinais (AMARANTE et al., 2009), portanto é provável que exista algum grau de resistência para outras doenças infectocontagiosas, como é o caso da leptospirose. Apesar da possível diferença de suscetibilidade entre as raças de pequenos ruminantes ter sido sugerida (SANTOS et al., 2012; SILVA et al., 2012; COSTA et al., 2016), ainda são apenas hipóteses. Sabe-se que algumas espécies animais podem estabelecer uma relação de adaptabilidade com estirpes de *Leptospira* sp. (PICARDEAU, 2013), sendo naturalmente mais resistentes à infecção, como é o caso de alguns roedores (ADLER e MOCTEZUDA, 2010). Embora os ovinos não apresentem com frequência a forma aguda da doença (ELLIS, 2015), pouco se sabe sobre a interação entre o agente e esse hospedeiro para afirmar que todas as raças são resistentes à leptospirose.

A relação entre o agente e o hospedeiro é bastante complexa, pois à medida que o tempo passa novas informações são obtidas e complementam ou desconstroem determinadas teorias. Por muitos anos, pequenos ruminantes foram considerados apenas como hospedeiros acidentais de leptospiras transportadas por outras espécies domésticas e silvestres (LEON-VIZCAINO et al. 1987). No entanto, vários estudos demonstraram que a infecção por leptospirose em caprinos e ovinos era comum, em que essas espécies também poderiam atuar como portadores e eliminar o agente no ambiente por longos períodos (ELLIS 1994; GERRITSEN et al. 1994; SILVA et al. 2007). No que se refere aos sorovares, não parece ser diferente. Apesar de o suíno ser o reservatório natural reconhecido de estirpes do sorogrupo Pomona (ELLIS, 2015), esse vem sendo cada vez mais detectado em rebanhos animais sem contato com suínos, sugerindo uma via independente de infecção. Por exemplo, o sorogrupo Pomona sorovar Kennewicki foi o isolado mais frequente nos bovinos em estudo recente (ZARANTONELLI et al., 2018), bem como o responsável de causar leptospirose subclínica em ovelhas (HAMOND et al., 2019). Além disso, ovinos soropositivos para o Pomona foram apontados como fator de risco para que cervos que compartilhavam o mesmo pasto reagissem positivamente para esse sorogrupo (SUBHARAT et al., 2012).

A maioria das informações sobre a leptospirose foi obtida a partir de infecções experimentais com roedores, que, apesar de relevantes, não fornecem todas as respostas sobre a patogênese da doença nos animais e seres humanos. Sabe-se que para avançar no controle e na prevenção da leptospirose é necessário conhecer melhor a interação existente entre o agente e cada hospedeiro animal. Fatores relacionados ao hospedeiro, ao agente e ao ambiente

favorecem a ocorrência dessa patologia nos rebanhos (CORTESE et al., 2014), portanto estudos que buscam desenvolver modelos de infecção experimental se tornam necessários para elucidar particularidades importantes sobre essa doença e melhor conhecer os seus mecanismos de transmissão nas diferentes espécie.

A presente Tese possui três capítulos, constituída pelo mesmo número de artigos científicos originais. O Capitulo I foi publicado no periódico Microbial Pathogenesis- Qualis B1, e descreve uma infecção experimental com *Leptospira interrogans* sorogrupo Pomona sorovar Kennewicki em ovelhas, buscando analisar um modelo natural de infecção e o comportamento da interação agente-hospedeiro de acordo com a raça. No Capítulo II, submetido ao periódico Epidemiology and Infection – Qualis A2, objetivou-se reproduzir a doença aguda em ovelhas da raça Santa Inês e mestiças por meio da inoculação por via intraperitoneal de *L. interrogans* sorogrupo Pomona sorovar Kennewicki, verificando possíveis diferenças na resposta à infecção nas ovelhas infectadas. No Capítulo III, que será submetido ao The Veterinary Journal - Qualis B1, procurou-se determinar a influência da infecção por *L. interrogans* sorogrupo Pomona sorovar Kennewicki sobre os parâmetros clínicos e hemato-bioquímicos de acordo com a via de inoculação e a raça das ovelhas experimentalmente infectadas.

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CAPÍTULO I

Susceptibility among breeds of sheep experimentally infected with *Leptospira interrogans* Pomona serogroup

(Microbial Pathogenesis, Qualis B1, Impact Factor: 2.323)

Susceptibility among breeds of sheep experimentally infected with *Leptospira interrogans* Pomona serogroup

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Abstract

Leptospirosis is a disease that negatively affects the productive and reproductive indices of ruminants. Sheep are considered highly resistant to infection, although susceptibility may vary among breeds. Thus, the aim of the present study was to analyze the susceptibility between sheep breeds to the experimental infection by leptospires of the Pomona serogroup. Pomona serogroup, Kennewicki serovar strain (1×10^7 bacteria) was inoculated via the conjunctival route in 12 sheep divided into two groups, one comprising Santa Inês ewes and the other comprising crossbred sheep. In each group, five ewes were challenged with the bacterial strain and one was used as control. All sheep were monitored for 60 days, during which blood samples were collected for serological diagnosis and urine and vaginal fluid samples for molecular and microbiological analyses. Finally, as ewes were submitted to euthanasia and necropsy, some tissues of interest were collected for microbiological, molecular, and histopathological diagnoses. The groups were compared regarding the number of positive reactions according to diagnostic tests. All sheep in each group presented antibodies to *Leptospira* in all serological analyses, except animals of the control group. However the Santa Inês sheep presented higher concentration and duration of the titers, and their positive reactions were detected earlier than those in crossbred sheep. The antibody titers in group A (median 200, geometric mean 317.48) were significantly different from the group B (median 800, geometric mean 918.96) at D60 post-infection (P = 0,032). The Santa Inês sheep presented a higher number of positive reactions than did the crossbred sheep in the

molecular diagnostic tests. According to the molecular diagnosis, the Santa Inês sheep presented more reactions (urine and vaginal fluid) compared to crossbred ewes, but there was no predominance in the detection of leptospiral DNA when comparing urine and vaginal fluid results, nor even between the number of positive kidneys and uterus. The Santa Inês sheep presented a higher number of positive bacteriological cultures. No sheep in either group presented alterations in anatomopathological and histopathological findings. Pure-bred sheep is more susceptible than crossbred sheep to infection by *Leptospira* sp. Our findings emphasize the importance of the genital tract as a site of extraurinary infection and indicate the possibility of venereal transmission in the species.

Keywords: sheep; leptospirosis; susceptibility; resistance; conjunctival route;

1. Introduction

The negative effects of leptospirosis on the productive and reproductive indices of ruminants are well understood, and this disease can lead to abortion, estrus recurrence, birth of weak calves, and decreased milk production (1). However, despite the importance of this subject, most studies on sheep only calculate the frequency of seropositive animals, and few seek to understand the status of the carrier, the dynamics of the infection, and the nuances of leptospirosis diagnosis in this species.

Leptospirosis transmission in animals has been traditionally associated with their exposure to urine contaminated with the bacteria (2). However, the constant detection of leptospiral DNA in semen and vaginal fluid samples suggest venereal transmission in small ruminants (3, 4), as has been suggested for cattle (5). The possibility of venereal transmission is quite worrying from the epidemiological point of view, because it is less influenced by environmental factors and may lead to endemism of the disease in herds (3).

Small ruminants are reportedly less susceptible to leptospirosis than other domestic animals (1). However, susceptibility may vary among breeds, as suggested by previous studies (6, 7). Knowledge of epidemiological variables is essential to enhance the control and prevention are to be advanced. Therefore, the aim of the present study was to analyze the susceptibility between sheep breeds to the experimental infection by leptospires of the Pomona serogroup.

2. Material and methods

2.1 Selection of animals

This study was approved by the Research Ethics Committee-REC of the Federal University of Campina Grande-UFCG, under Protocol No. 020/2016. The experiment was conducted at the UFCG Research Center for the Development of the Semi-Arid Tropic (Nupeárido) in Paraíba, Brazil, which is a semi-arid region where the Caatinga vegetation prevails. Twelve sheep aged 12 to 18 months with a mean live weight of 26 kg and no *Leptospira* sp. Antibodies, positive vaginal fluid and urine culture or positive polymerase chain reactions (PCR) for urine and vaginal fluid, which was verified in three tests performed before the experimental challenge (60 and 30 days after and on the day of inoculation, respectively), were used for this study. The sheep were divided into two groups according to the breed: group A, including six crossbred sheep, and group B, including six Santa Inês sheep. The animals were housed in individual covered stalls established 1.0 m from the ground (1.0 × 1.5m), with water provided *ad libitum* through individual drinking fountains. They had no access to other animals. All animals were familiarized with the facilities for 20 days before the experimental challenge.

2.2 Experimental infection

In both groups, five sheep were challenged with 1×10^7 bacteria *L. interrogans* Pomona serogroup Kennewicki serovar maintained in EMJH medium (DIFCO, BD, Franklin Lakes, NJ, USA). One animal from each group served as a control and was challenged with EMJH liquid culture medium without the bacteria. After counting, 2 ml of bacterial culture was required to achieve a bacterial count of 1×10^7. A 1 ml volume of the bacterial culture was inoculated via the conjunctival route (both eyes) using a dropper. Blood, urine, and vaginal fluid were collected from all sheep at the following intervals during the 60 days after inoculation: D4 post-infection (p.i.) (sample 1), D8 p.i. (sample 2), D15 p.i. (sample 3), D30 p.i. (sample 4), D45 p.i. (sample 5), and D60 p.i. (sample 6). Through the behavioral analysis and verification of the presence of clinical signs the ewes were evaluated by Veterinarian. The sheep were euthanized immediately after the last collection. They were first sedated with 0.2 mg/kg of intravenous xylazine hydrochloride (Bayer, Rompun®, São Paulo, SP, Brazil). Then, they were intravenously injected with 10 mg/kg of sodium thiopental (Cristália, Thiopentax®, Sao Paulo, Brazil). After the animals lost consciousness, 100 mg/kg of potassium chloride (Synth chloride Potassium PA-ACS®, Diadema, SP, Brazil) was intravenously administered, as recommended by the National Council for the Control of Animal Experimentation (8).

Finally, necropsy was performed and tissues obtained for the verification of histopathological and anatomopathological alterations.

2.3 Collection of samples

After local antisepsis with 2% iodinated alcohol solution, blood samples were collected by jugular puncture using a disposable needle and vacuum tube (without anticoagulant) with an 8 ml capacity (Vacuum Tube,Vacuette®, Porto, Portugal). The serum was drained from the blood, transferred to microtubes, and frozen at −20°C until further processing. Urine samples were extracted using a sterile urethral catheter no. 8 and collected in sterile disposable syringes. Vaginal fluid was collected in duplicate from the vaginal fornix using sterile disposable swabs (Labor Import, Ref: 25507, Osasco, SP, Brazil) with a vaginal speculum. For molecular analysis, urine aliquots (2 ml) were distributed into microtubes with 100 µl of 1X sodium phosphate buffer (PBS). The other swab with vaginal fluid was transferred to a sterile Falcon tube with 2 ml of 1X PBS, homogenized, and aliquoted into microtubes. Both urine and vaginal swab samples were immediately refrigerated and transported within a maximum of 2 h to the Laboratory of Communicable Diseases (LCD) at UFCG, where they were stored at −20 °C until DNA extraction. During necropsy of the sheep, kidney, ovary, uterine tube, uterus, bladder, lung, liver, and spleen samples were aseptically collected in triplicate ($2cm^2$). One of the samples was used for the microbiological diagnosis. The second sample was conditioned and fixed in 10% buffered formalin, followed by routine histological processing for microscopic evaluation. The third tissue sample was stored at −20°C until DNA extraction. DNA was extracted from the tissue samples obtained from the kidney and uterus.

2.4 Bacteriological culture

A portion of the urine samples and one of the vaginal fluid swabs were inoculated at a 10% concentration in an EMJH culture medium supplemented with bacterial cocktail (9). The inoculated tubes were kept at room temperature during transport to the laboratory and were incubated at 28°C in a bacteriological oven. The inoculated tubes were kept at room temperature during transport to the laboratory and were incubated at 28°C in a bacteriological oven. After 24 h in STAFF medium, the tubes were serially diluted (10^{-1}, 10^{-2}, 10^{-3}) in Fletcher's semi-solid medium (Difco, BD, Franklin Lakes, NJ, USA) with 5-fluorouracil (1 mg/ml) and incubated at 28°C. The tubes were examined weekly for 12 weeks for the presence of microorganisms similar to *Leptospira* sp. Only cultures that were further

confirmed by PCR were considered positive. During necropsy of the sheep, the tissues were immediately macerated with sterile disposable syringes and inoculated into their own culture media as described above.

2.5 Serological diagnosis

A serological diagnosis of leptospirosis was made using the microscopic agglutination test (MAT), as recommended by the World Organization for Animal Health (10). The serum samples were screened for antibodies against a battery of 24 serogroups. Sera with 50% or more agglutination at the indicated dilution were titrated in several two-fold geometric dilutions. The serum titer was the reciprocal of the highest dilution that presented a positive result.

2.6 Molecular diagnosis

DNA from *Leptospira* sp. was extracted using the Wizard® Genomic SV DNA Purification System Kit (Promega®, Madison, USA). PCR was performed as previously described (11). The primers *LipL* 32-45F (5'-AAG CAT TAC CGC TTG TGG TG-3 ') and *LipL* 32-286R (5'-GAA CTC CCA TTT CAG CGA TT-3'), which were designed by Stoddard et al. (2009) (12), were used to amplify the *LipL* 32 gene, which is specific for pathogenic leptospires. The Pomona serogroup strain and ultrapure water were used as positive and negative controls, respectively.

2.7 Histopathological diagnosis

The fragments of the collected tissues were conditioned and fixed in 10% buffered formalin, cleaved, and routinely processed for histopathological analysis, with dehydration in increasing alcohol concentrations, xylol diaphanization, inclusion in paraffin, and microtomy (section cuts of 4-5 μm). Subsequently, the slides were stained with hematoxylin and eosin (HE) and analyzed under light microscopy.

2.8 Statistical analysis

The Mann-Whitney U test was used to compare the median of antibody titers between groups in each post-infection day. The comparison of serology, PCR and culture positivity rates between groups was performed by the Chi-square test. The significance level used was 5%.

3. Results

All (100%) sheep excluding the control sheep in each group showed antibodies to *Leptospira* sp. Sheep with reactive sera presented a more frequent titer for the inoculated strain. However, at least three sheep from each group presented cross-reactivity against the serogroups Autumnalis and Australis. No sheep presented apparent clinical signs during the experiment. According to Table 1, group A presented 17 (46%) positive reactions during all the collections, while group B presented 20 (54%) reactions. Further to Table 1, 68% of the reactions indicated antibody titer ≥800 in both groups, and group B showed the highest seroconversion of antibodies.

Antibody titers were earlier detected in Santa Inês than in crossbred sheep, and persisted over the experiment (Figure 1), however, there was no significant difference in seropositivity rates between groups A and B ($P > 0.05$). On the other hand, antibody titers of the group A (median 200, geometric mean 317.48) were significantly different from the group B (median 800, geometric mean 918.96) at D60 post-infection ($P = 0,032$).

According to Table 2, the highest number of PCR positive reactions in urine and vaginal fluid was found in animals from the group B, however, there was no statistical difference ($P > 0.05$) between groups in each moment or when considering the total number of reactions. According to Table 3, the agent was detected by bacteriological culture in five urine samples throughout the experiment; however, no leptospires were recovered from vaginal fluid. The agent was detected by PCR in seven kidney and eight uterine samples in both groups, in which bacterial isolation was possible in two samples of each group, as shown in Table 4, however, there was no significant difference ($P > 0.05$) between groups regarding PCR and culture positivity rates. Regarding anatomopathological and histopathology analyzes, no gross or microscopic alterations were found.

4. Discussion

In the present study, we found that Santa Inês sheep may be more susceptible to *Leptospira* sp. than crossbred sheep, since the antibody titers were statistically different at the D60 post-infection. However, when comparing the proportions of positive samples, there was no significant difference. These findings were consistent with those of previous studies (6, 7). According to the findings of MAT, there were more positive reactions and the highest antibody titers (3200 in one of the sheep) in the group of pure-bred sheep. According to Table 2 it is possible to observe that 68% of the reactions presented antibody titers ≥800 in the two groups, different of the serological surveys conducted in sheep in Brazil, in which there is predominance of titers < 800 (7). However, apart from the limitation of exposure to a strain

that is considered unsuitable for small ruminants (1), it is worth mentioning that the amount of bacterial exposure was much higher than that in reality; this could justify the high antibody levels.

Both challenged groups in the present study presented cross-reactions to the serogroups Australis and Autumnalis, and these reactions are considered common in MAT (13). In this kind of situation, there arises a possibility of erroneous determination of the infecting serogroup in cases of insufficient strains in serological tests, and this emphasizes the importance of inserting at least one representative serovar from each serogroup in serological surveys. The beginning of antibody production and its persistence at the time of the experiment also varied between groups. The group of crossbred sheep presented detectable antibodies later, and, proportionally, their levels were identified for a period shorter than that in the group of pure-bred sheep. This indicates greater difficulty in the identification of seropositive animals among crossbred sheep.

The clinical manifestation of *Leptospira* sp. infection is believed to be determined by the strain involved in the infection (2), in which strains not adapted to a particular animal species cause the acute manifestation of the disease with its clinical manifestation (1). However, in this experiment, sheep did not present any obvious clinical signs, even being challenged by a strain considered not adapted to the species (14). The absence of clinical signs in the sheep in the present study can be associated with resistance of the species to the disease (1). It should be noted that, even without clinical signs, they were carriers of the bacterium, which is alarming because these sheep could be a source of infection to other animals and humans.

Leptospiral DNA was identified in urine or vaginal fluid samples from five Santa Inês sheep (group B) and four crossbred sheep (group A). Despite that there were no significant differences between groups regarding the positivity rates, we observed 11 reactions in group B and four reactions in group A. Therefore, it is possible that the number of challenged animals (n = 5) may have been determinant in the absence of statistical differences. In addition, it is noteworthy that the number of positive reactions in urine samples was comparable with the number of positive reactions in vaginal fluid samples, because the urinary tract is widely recognized as the main vehicle for *Leptospira* sp. excretion (2). This finding once again reinforces the importance of the genital tract as a relevant additional infection site and indicates the possibility of venereal transmission in small ruminants, as suggested by other authors (3, 4). There is also a rapidity and ease in which the inoculated strain reached the reproductive tract, group A even before the appearance of urine, as well as

a lack of clinical signs in this situation, which may lead to negligence in the detection of positive animals. The possibility of contamination of the vaginal fluid with bacteria from the urine, despite all precautions taken during collection, cannot be ruled out. However, the observation of *Leptospira* sp. DNA in the uterus of eight challenged sheep and the positive culture findings for two uterine specimens weakens this possibility and indicates the actual presence of leptospires in the genital tract.

We found that serology, bacteriological culture, and PCR showed different abilities to detect positive sheep, consistent with findings in previous studies on sheep (15, 16). Serology identified the maximum number of positive sheep in both groups, which may be related to the fact that the inoculated strain is considered to occur accidentally in the species (1), thus stimulating a higher production of antibodies and detectable titers. Despite the use of PCR for the identification of carrier animals (16), its sensitivity in the present study may have been impaired by the intermittent elimination of the bacteria in the urine, as observed in another study (17). Culture presented the lowest sensitivity among the techniques used, with only one sheep showing positivity in group A and three in group B. In absolute terms, this number was below the numbers found with PCR and serology and can be explained by the limitations inherent to the technique, such as the fastidious growth of the bacteria in artificial environments, the need for viable microorganisms for isolation, and, mainly, contamination of biological samples (18). In this particular study, it was not possible to isolate bacteria through the vaginal fluid in any sheep, probably because of sample contamination. Unlike urine, which is collected directly from the bladder, the vaginal fluid is in constant contact with the external environment and is naturally more exposed to contaminants. Therefore, we demonstrated the low efficacies of these tests when used alone, and the findings suggest the requirement for serial and paired tests as well as direct and indirect diagnostic methods.

According to the findings of necropsy, leptospirosis did not cause any anatomopathological or histopathological alterations in the sheep. However, the acute disease caused by the Pomona serovar has already been described in lambs, which present pale and icteric mucosae, hemoglobinuria, hemoglobinemia, and hepatocellular and centrilobular necrosis (14). Based on this, it is possible that acute disease with evidence of lesions is less frequent in adult animals, as well as the virulence of the strain and the breed of the animals may also influence the manifestation of clinical signs.

5. Conclusions

Pure-bred sheep is more susceptible than crossbred sheep to infection by *Leptospira* sp. Our findings emphasize the importance of the genital tract as a site of extraurinary infection and indicate the possibility of venereal transmission in the species.

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Conflict of interests

The authors have no conflict of interest to declare

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Table 1. Conversion of antibodies in microscopic agglutination test (MAT) in crossbred (Group A) and Santa Inês (Group B) sheep challenged by leptospires from the Pomona serogroup according to the post-infection day.

Group	Post-infection day	200	400	800	1600	3200	Total
Group A	D4	-	-	-	-	-	-
	D8	-	-	-	-	-	-
	D15	-	2	3	-	-	5
	D30	-	3	2	-	-	5
	D45	1	-	3	-	-	4
	D60	1	2	-	-	-	3
Group B	D4	-	-	-	-	-	-
	D8	1	1	1	-	-	3
	D15	-	-	2	2	-	4
	D30	-	-	2	1	1	4
	D45	-	-	2	1	1	4
	D60	1	-	2	1	1	5
Total (%)		4 (11)	8 (21)	17 (46)	5 (14)	3 (8)	37 (100)

Table 2. Representation of urine and vaginal fluid PCR results in crossbred (Group A) and Santa Inês (Group B) ewes challenged by leptospires from the Pomona serogroup.

Group	Sample	D4 p.i.	D8 p.i.	D15 p.i.	D30 p.i.	D45 p.i.	D60 p.i.	Total
Group A	U	-	-	-	+	-	+	2
	VF	-	+	-	-	-	+	2
Group B	U	-	+	+	+	++	+	6
	VF	-	+	+	+	+	+	5
Total (%)		-	3 (20)	2 (13)	3 (20)	3 (20)	4 (27)	15(100)

Note: p.i. (post-infection); U (urine); VF (Vaginal fluid).

Table 3. Representation of bacterial culture results of urine and vaginal fluid in crossbred (Group A) and Santa Inês (Group B) sheep challenged by leptospires from the Pomona serogroup.

Group	Sample	D4 p.i.	D8 p.i.	D15 p.i.	D30 p.i.	D45 p.i.	D60 p.i.	Total
Group A	U	-	-	-	-	-	+	1
	VF	-	-	-	-	-	-	-
Group B	U	-	-	+	+	+	+	4
	VF	-	-	-	-	-	-	-
Total (%)		-	-	1 (20)	1 (20)	1 (20)	2 (40)	5(100)

Note: p.i. (post-infection); U (urine); VF (Vaginal fluid).

Table 4. Demonstration of bacterial culture and kidney and uterus PCR results in crossbred (Group A) and Santa Inês (Group B) sheep challenged by leptospires from the Pomona serogroup.

Sample	Test	Group A	Group B	Total
Kidney	PCR	4	3	7
	Cultura	1	1	2
Uterus	PCR	4	4	8
	Cultura	1	1	2
Total (%)		10 (53)	9 (47)	19 (100)

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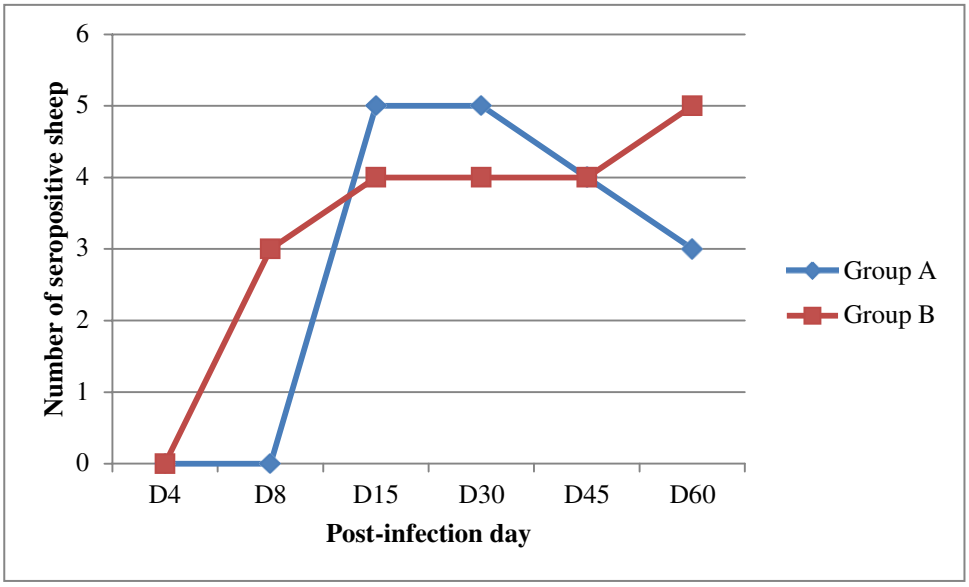

Figure 1. Positivity in MAT during the experiment according to the group of sheep inoculated with leptospires of the Pomona serogroup.

CAPÍTULO II

Dynamics of infection in sheep experimentally challenged with *L. interrogans* Pomona serogroup

(Epidemiology and Infection, Qualis A2, Impact Factor: 2.044)

Dynamics of infection in sheep experimentally challenged with *L. interrogans* Pomona serogroup

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Abstract
Most of the information about leptospirosis was obtained from experimental infections with rodents, which in spite of being relevant, do not provide all the answers about the disease in animals and human beings, given the variability of interactions which exist between the agent and the different hosts. Therefore, the objective of the present study was to verify the pathogenesis of the infection in native breeds of sheep of Brazil challenged with a strain of the serogroup Pomona. A total of 10 sheep were divided into two groups with five animals according to the breed. In each group four sheep were challenged by intraperitoneal route with a strain of the serogroup Pomona and one was used as control. Sheep were monitored for 60 days, with collection of blood for serologic diagnosis, as well as vaginal fluid and urine for microbiological and molecular analyses. After this period the animals were submitted to euthanasia and necropsy, with collection of tissues for microbiological, molecular and histopathologic diagnosis. All the challenged sheep presented anti-*Leptospira* antibodies. Crossbred sheep presented a lower concentration of titers and the antibodies were detected for a shorter period of time when compared to the Santa Inês sheep, with statistical difference in the concentration of the titers on the days 15 ($p<0.05$), 45 ($p<0.05$) and 60 ($p<0.05$) post-

infection. There was no significant difference between the groups when comparing the positivity rates of the microscopic agglutination test (MAT) ($p>0.05$). Eight positive reactions in the urine and vaginal fluid PCR were detected in both groups, being four (50%) in urine and four (50%) in the vaginal fluid, however without statistical difference ($p>0.05$). In both groups there was a greater proportion of PCR positive samples in kidneys (71.4%) in relation to uterus (28.6%), however without significant difference ($p>0.05$). There was no significant difference between the groups when comparing the positivity rates of the PCR ($p>0.05$). The isolation of leptospires from the urine and kidney of a crossbred sheep was possible. Therefore, it is possible that the native Brazilian sheep, especially the crossbred, may have a relation of adaptability with strains of the serogroup Pomona. However the intensity and duration of this relation need to be elucidated and longer-lasting investigations of natural and experimental infections are necessary in order to determine the epidemiological nature of this relation.

Key words: Adaptability; Leptospirosis; Santa Inês, Crossbred sheep*;* Pomona.

Introduction

Leptospirosis is an infectious and contagious disease caused by bacteria of the genus *Leptospira* which has a negative impact on the productive and reproductive rates of the herds, besides being an important zoonosis [1]. A recent systematic review found about one million cases of human leptospirosis per year worldwide, resulting in a mortality rate of approximately 6% [2]. The transmission of leptospirosis is mainly influenced by environmental conditions, especially rainfall indices [3]. However, in situations in which the environmental conditions are adverse the animal reservoirs have an expressive importance in the epidemiology of the disease [4]. In this context, it is assumed that in the Brazilian semiarid region the interaction between sheep and the etiologic agent happens differently from the other places, due to the uniqueness of the weather conditions and vegetation [5].

Some *Leptospira* serovars are commonly adapted to specific hosts [6], however an in-depth analysis of this adaptation and the relation of adaptability of strains with certain animal hosts may be being neglected. For a long time it was believed that sheep participated only as accidental hosts of leptospirosis and that the infection depended directly of the action of other species, mainly cattle [7]. Notwithstanding, experimental studies and field observations indicate an independence of the participation of other species in the infection [8], moreover sheep are already cited as an alternative source of maintenance of the serovar Hardjo [9, 10].

There are few reports on clinical manifestations in sheep [8], therefore, it is believed that this species may also has a possible resistance to other strains, not only to the Hardjo. The serogroup Pomona is one of the most recovered in the world and various strains have the swine as a maintenance host [11]. Although this serogroup causes an acute clinical infection and is economically significant in various animal species [12], recent reports indicate an increase in the incidence and endemism of this serogroup in sheep in some areas [13]. Furthermore, these animals have been incriminated as being risk factors for other species in infections involving this serogroup [14].

Most of the information about leptopirosis was obtained from experimental infections with rodents, which in spite of being relevant do not supply all the answers about the pathogenesis of the disease in animals and human beings, due to the variability of interactions which exist between the agent and the different hosts [15]. Therefore, knowing that the Brazilian breeds of sheep have showed to be expressive asymptomatic carriers of the agent [16], as well as the environmental cycle of the leptospires is influenced by regional factors and by the dynamic nature of the strains/animal species involved [17], the objective of the present study was to verify the pathogenesis of the infection in native sheep of Brazil challenged with the strain *L. interrogans* serogroup Pomona.

Material and methods

The present research obtained approval from the Committee of Research Ethics (CEP) at the Federal University of Campina Grande (UFCG), Protocol n. 020/2016. The trials in hamsters were performed after the approval of Animal Ethics Committee of the Universidade Federal Fluminense (protocol number 611/2015).

Selection of the animals

The experiment was conducted at the Research Center for the Development of the Semiarid Tropic (Nupeárido) of the UFCG, State of Paraíba, Brazil. For the experimental infection , 10 sheep were used aged between 12 and 18 months, average body weight of 26 Kg, not vaccinated for leptopirosis and which presented negative at serology (MAT titer ≤ 50), bacterial culture and PCR (urine and vaginal fluid) in three previous analysis with intervals of 30 days. The sheep were divided into groups according to the breed, being five crossbred (Group A) and five of the Santa Inês breed (Group B). All animals were accommodated in individual covered stalls (1.0x1.5m) without contact with other animals, distant from the soil at a height of 1m and with access to water and food *ad libitum*. The animals underwent an adaptation in the installations 20 days before the challenge.

Virulence test and experimental infection

In each of the experimental groups (A and B) four animals were challenged with 1 x 10^7 bacteria of the *L. interrogans* serogroup Pomona serovar Kennewicki (strain Fromm) isolated from pigs in the USA, supplied by Salsbury Laboratories. The virulence tests followed suggested protocols [18, 19] with slight modifications. The 3Rs policy for experimental science was applied in all steps [20] in accordance with the Brazilian Guidelines of the Federal Council of Veterinary Medicine and Brazilian Guideline for the Care and Use of Animals for Scientific and Didactic Purposes. The strain underwent four passages in

Golden Syrian hamsters as described by Silva et al. [18] and Suepaul et al. [19] with adaptations [20]. The strain was inoculated after four passages in hamster.

The strain was maintained in EMJH medium (DIFCO, BD, Franklin Lakes, NJ, EUA), whilst one animal in each group received only liquid EMJH culture media without the bacterial culture. After counting was reached a volume of 2 ml that were inoculated through intraperitoneal route (IP). Within the 60 days following the inoculation, collections of blood, urine and vaginal fluid were performed from all the sheep on days 04, 08, 15, 30, 45 and 60 post-infection (p.i.), thus totaling six collections. Throughout the trials, clinical signs (pyrexia, prostration, jaundice, hematuria, dyspnea, polypnea, tachycardia, bradycardia, dehydration and color of mucosae) were monitored. The total time of experiment and the quantity of collections was regulated by the ethics committee in animal experimentation. After the last collection the sheep were submitted to euthanasia, being previously sedated with Xylazine Hydrochloride (Bayer, Rompun®, São Paulo, SP, Brazil) at a dose of 0.2 mg/kg by endovenous route, followed by the administration of Sodium Thiopental (Cristália, Thiopentax®, São Paulo, SP, Brazil) at a dose of 10mg/kg intravenously and, after the confirmation of unconsciousness, Potassium Chloride was administered (Synth, Potassium Chloride P.A.-A.C.S®, Diadema, SP, Brazil) at a dose of 100mg/kg intravenously. Finally, the necropsy was carried out for the verification of the possible anatomopathological alterations and collection of material for the microbiological, histological and molecular diagnosis.

Collection of samples

The blood was collected by puncture of the jugular vein, using a disposable needle and a 8 ml vacuum tube (without anticoagulant) (Vacuum Tube, Vacuette®, Porto, Portugal), subsequently to the local antisepsis with 2% iodinated alcohol solution. The serum was drained from the blood, transferred to microtubes, and frozen at −20 °C until further processing. With a vaginal speculum, urine samples were collected by means of a no. 8 sterile

urethral probe, and were extracted using disposable sterile syringes. For the molecular analysis, aliquots of urine (2 ml) were distributed in microtubes containing 100 µl of PBS 1X. Samples of vaginal fluid were obtained in duplicate, collected directly from the vaginal fornix with the use of disposable sterile swabs (Labor Import, Ref: 25507, Osasco, SP, Brazil) and the aid of a vaginal speculum. The second sample of vaginal fluid collected was immersed into a sterile tube with 2 ml de PBS 1X, homogenized and aliquoted in microtubes. The aliquots were immediately refrigerated and transported within two hours to the laboratory, and were stored at -20°C until the DNA extraction was carried out.

During the necropsy, fragments ($2cm^2$) of kidney, ovary, uterine tube, uterus, bladder, lung, liver and spleen were aseptically collected in triplicate. One sample was processed and sown in proper culture media for the microbiological diagnosis, the second was stored and fixed in 10% formaldehyde buffer, being posteriorly submitted to staining with hematoxylin-eosin (HE) and microscopic evaluation, while the third sample was stored at -20°C until the DNA extraction was carried out. In the tissue samples, the DNA extraction was performed only of the kidney and uterus.

Bacteriological culture

At the time of the collections, some drops of urine and a vaginal fluid swab were sown in EMJH culture medium in the concentration of 10%, supplemented with a antimicrobial cocktail (STAFF) [21]. The seeded tubes were kept at room temperature until transport to the laboratory and then incubated at 28ºC in a bacteriological incubator. After 24 hours in STAFF medium, the tubes were submitted to serial dilution (10^{-1}, 10^{-2}, 10^{-3}) in Fletcher semi-solid medium (Difco, BD, Franklin Lakes, NJ, EUA), with the addition of 5-Fluorouracil ($1mg/ml^{-1}$) and incubated at 28°C, and were examined weekly regarding the presence of *Leptospira* sp. during the period of 12 weeks. The tissue samples obtained during the necropsy were macerated with the aid of sterile disposable needles and sown in proper culture

medium, as previously described. A culture was deemed positive when the sample was suspected at microscopy and confirmed by PCR.

Serological diagnosis

A serological diagnosis of leptospirosis was made using the microscopic agglutination test (MAT), as recommended by the World Organization for Animal Health [22]. Serum samples were researched regarding the presence of antibodies using the dilution of 1:100 against one bacterium of 24 serovars. In the analysis, the strains used were: *Leptospira biflexa:* serovars Andamana and Patoc; *Leptospira interrogans*: Kennewicki, Australis, Copenhageni, Bataviae, Bratislava, Canicola, Grippotyphosa, Hardjoprajitno, Pomona, Pyrogenes, Icterohaemorrhagiae, Hebdomadis, Wolffi; *Leptospira borgpeterseni:* Autumnalis, Castellonis, Hardjobovis Javanica, Tarassovi, *Leptospira santarosai:* Guaricura, Shermani; *Leptospira kirschneri:* Cynopteri; *Leptospira noguchii:* Panama. The sera which presented 50% or more of agglutination in the indicated dilution were titled in a series of geometric dilutions in ratio 2. The title of the serum was reciprocal of the highest dilution which presented a positive result.

Molecular diagnosis

Leptospiral DNA from the urine and vaginal fluid samples was extracted by the Wizard SV Genomic DNA Purification System (Promega, Madison, USA). For kidney and uterus samples, DNA was extracted by the Qiagen DNeasy Blood & Tissue kit. A PCR targeting the *lipL32* gene (referred as specific for pathogenic leptospires) was performed as described [23]. Primers *LipL*32-45F (5'-AAG CAT TAC CGC TTG TGG TG-3 ') and *Lip L*32-286R (5'-GAA CTC CCA TTT CAG CGA TT-3') were used to amplify the gene *LipL*32, which is specific for pathogenic leptospiras [24]. The strain *L. interrogans* serogrupo Pomona sorovar Kennewicki was used as a positive control and ultrapure water as negative control.

Sensitivity is referred to be adequate, with a detection limit around 10^2 leptospires per milliliter of sample [24]. Additionally, in order to minimize the effect of PCR inhibitors, the samples were neutralized to pH 7.6 with phosphate-buffered saline (PBS), immediately after collection [25].

Histopathological diagnosis

A part of the fragments of the tissues collected were stored and fixed in 10% buffered formalin, cleaved and routinely processed in the histopathological, going through the stages of dehydration in increasing concentrations of alcohol, diaphanization in xylol, inclusion in paraffin and microtome (cuts in sections of 4-5μm). Subsequently, the slides were stained with hematoxylin-eosin (HE) and analyzed under optical microscopy.

Statistical analysis

The Mann–Whitney U test was used to compare the median of the antibody titers between the groups in each day post-infection. The comparison of the positivity rates of in the serology, PCR and cultivation among the groups was performed by Fischer's exact test. The level of significance used was 5%.

Results

According to Table 1, all the challenged sheep presented anti-*Leptospira* antibodies at MAT, however there was no significant difference when comparing the positivity rates between the groups ($p>0.05$). Detectable antibodies were observed for a shorter period of time in the group of crossbred animals (Group A). The sheep of the crossbred group presented on D15 (median 400, geometric mean 400), D45 (median 300, geometric mean 282) and D60 (median 50, geometric mean 141) antibody titer significantly different ($p<0.05$) from Santa Inês sheep group, which presented median 800 and geometric mean 951 on D15, median 800 and geometric mean 672 on D45, and median 600 and geometric mean 565 on D60. In PCR,

there was no significant difference with relation to the positivity rate between the groups ($p>0.05$). It were detected in both groups eight positive reactions in the PCR of urine and vaginal fluid, being four of them (50%) in urine and four (50%) in the vaginal fluid, however without statistical difference when positive reactions in urine and vaginal fluid were compared ($p>0.05$). In both groups it was found a greater proportion of positive samples of kidney (71.4%) in relation to those of the uterus (28.6%), however there was no significant difference ($p>0.05$). Leptospiral DNA was found for a longer period of time in the vaginal fluid (D60) than in the urine (D45). The isolation of leptospires was possible from the urine (D30) and the kidney (D60) in one crossbred sheep (A4). Negative control animals were negative for serology, isolation and PCR. Apparent clinical signs were not identified in any of the animals during the experiment, neither anatopathological lesions suggestive of *Leptospira* sp. infection.

Discussion

The results show that there was an immunological response and invasion of the tissues by the agent in the challenged animals; however a discrepancy was identified on the level of infection between the groups. Even though statistical difference was not observed in the positivity rate of the MAT between the groups ($p>0.05$), a distinct standard in the duration and in the concentration of the antibodies was recognized. All the sheep of the Santa Inês breed were reactive in the MAT until the last collection carried out (D60), while only two of the four crossbred sheep presented positivity up to the D45 p.i., a similar result to other experimental study with sheep which used the same strain by a natural route of infection [26]. Also in sheep, experimental challenges carried out with strains of the serogroup Sejroe revealed variations of detectable titers at 22 days [27] up to 14 weeks p.i. [28], whilst that in situations of natural exposure registers of 14 and 20 months were noted for the serogroups Sejroe and Pomona, respectively [29]. The lack of correspondence between the studies with

relation to persistence of titers may be due to the virulence of the inoculated strain, infecting dose and cut-off point used; in situations of natural infection (non-controlled) the risk of re-exposure to the agent exists and thus a greater time of detection of antibodies. However, the short period of seroreactivity in crossbred animals is surprising, thereby indicating that the infection in the species may be influenced according to the breed concerned.

Concerning the antibody concentration, the animals of the Santa Inês breed registered the highest titers, with statistical difference on D15, D45 and D60. Other reports have already described high seroconversion of antibodies in pure-bred sheep caused by strains of the serogroup Pomona, either experimentally [26] or naturally infected [7]. However, the lower concentration of titers in crossbred sheep indicates a possible resistance of these animals against non-Sejroe strains. Apparently serovars of the same serogroup (Pomona, Monjakov and Kennewicki) have distinct interaction patterns which depend on the animal host and the environment [10, 11]. Therefore, it may be attributed to the fact that in some regions low frequency of antibodies for the serogroup Pomona are still reported in sheep [8], whereas in other regions this serogroup is considered to be endemic in the same species [13]. In Brazil there is no precise evidence of sheep bearing and transmitting strains of the serogroup Pomona, but due to the rusticity of the species raised in the semiarid region it cannot be ruled out that these animals may be capable, since this capacity was demonstrated in this experiment and also by an experiment which used a natural route of infection [26].

Although the serogroup Pomona has been suggested to not be adapted to sheep [7], changes in the interaction between the agent and the host have been found in other species, as for a long time cattle was incriminated basically in infections caused by Sejroe serogroups [8], however it is currently known that other strains may be maintained and eliminated by cattle [6]. Despite the antigenic similarity that exists between the serogroups, there seems to be significant differences in the pathogenicity islands [12], making a determined strain more

or less pathogenic. Furthermore, studies have demonstrated that there is a significant divergence between genotypes of the same serovar isolated in distinct species [30, 31, 32], and these differences are influenced mainly by the animal host and by the action of the environment [32]. In this circumstance, it is believed that especially in the Brazilian semiarid there is no lack of factors that influence the interaction agent-host-environment, different from what occurs in other regions. This region offers unique eco-systemic conditions and adverse to the etiological agent [5, 33], causing the weakening of the environmental action in the transmission of the disease and, by contrast, favors a higher interaction between the agent and the animal hosts [4]. Moreover, it is known that in the semiarid the practice of vaccination against Leptospirosis in sheep is rather unusual [34], favoring the maintenance of a great quantity of susceptible individuals in the species and consequently making these hosts more susceptible to the agent.

Some authors have already registered problems in sheep implied to strains of the serogroup Pomona, such as miscarriages, stillborn and birth of weak lambs [34], as well as the manifestation of acute disease in lambs presenting pale colored mucous membranes, jaundice, hemoglobinuria, hemoglobinemia and centrilobular necrosis [7]. The sheep of this experiment did not present any clinical manifestation, not even anatomopathological lesions, similar to the reported in recent studies of experimental infections in Brazilian breeds of sheep [26, 28], however the short period of the experiment may have been a limiting factor for the presentation of lesions implied to the chronic phase of the disease. It is known that, despite being infectious, the serovars have a greater pathogenicity for those hosts which are not adapted [35], therefore the absence of suggestive clinical signs may indicate a possible adaptability. It is worth highlighting that even without clinical signs the animals have shown to be healthy carriers of the agent, since the elimination of viable leptospires in the urine of a crossbred sheep was detected (A4). It must be admitted that experimental situations may not

accurately portray reality; however, there are reports of isolation of strains of *L. interrogans* serogroup Pomona serovar Kennewicki from naturally infected and asymptomatic sheep (36). From the epidemiological point of view this may be a serious problem, as sheep may serve as a silent source of infection for other animals and human beings, and this may contribute to the negligence of the disease in the herds. Therefore, seropositive sheep for the Pomona strain have already been pointed out as a risk factor for deer which shared the same pasture to also react positively to this serogroup [14].

The number of positive samples of vaginal fluid in the PCR was similar to the quantity of positive samples of urine in both groups; however it is probable that the intermittent elimination of the agent through the urine influenced negatively the evaluation of the elimination by this route [26, 27, 37]. With this in mind, this hypothesis is further reinforced by the fact that the majority of the sheep having been renal bearers of the agent. However, the detection of the agent in the vaginal fluid and in the uterus reinforces the importance of the extra-renal site of infection and the possibility of transmission in sheep, suggested previously [16, 26, 27, 38, 39]. With relation to the capability of detecting carrier animals, the results demonstrate that only one negative result in the PCR of urine and/or vaginal fluid is inadequate to trustingly consider an animal as negative, seen as the moment of colonization of the genital tract and the period of elimination by the urine are variable. Related to the agent-host interaction, it is believed that the maintenance hosts remain infected and so a balance is established, thus creating a constant reinfection cycle controlled by a peripheral immune response that can last from months to years [40]. The short period of the experiment did not allow the recording of these dynamics, however it is possible that if exposed the native Brazilian sheep may have a relation of adaptability with strains of the serogroup Pomona, even if this interaction is executed with less intensity and importance than what happens with porcine species.

Conclusion

Sheep raised in semiarid conditions, especially the crossbred ones, may have a relation of adaptability with strains of the serogroup Pomona. However, the intensity and duration of this relation need to be elucidated. Therefore, longer-lasting investigations of natural and experimental infections are necessary to determine the epidemiological nature of this relation.

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Table Lists

Table 1. Distribution of the serological (MAT), molecular (PCR) and microbiological results in different breeds of sheep experimentally infected with *Leptospira interrogans* serogroup Pomona.

Test	Group/ animal	D4	D8	D15	D30	D45	D60
MAT	A1	-	400	400	200	200	100
	A2	-	800	400	400	200	-
	A3	-	800	400	400	400	-
	A4	-	800	400	400	400	200
	B1	-	1600	1600	800	800	400
	B2	-	800	800	400	400	400
	B3	-	800	800	800	800	800
	B4	-	200	800	800	800	800
PCR/VF	A1	-	-	-	-	-	+
	A2	-	-	-	-	-	-
	A3	-	-	-	-	-	-
	A4	-	-	-	-	-	-
	B1	-	-	-	-	+	+
	B2	-	+	-	-	-	-
	B3	-	-	-	-	-	-
	B4	-	-	-	-	-	-
PCR/Urine	A1	-	-	-	+	-	-
	A2	-	-	-	-	-	-
	A3	-	-	-	-	-	-
	A4	-	-	-	+*	-	-
	B1	-	-	-	+	-	-
	B2	-	-	-	-	-	-
	B3	-	-	-	-	-	-
	B4	-	-	-	+	-	-
PCR/ Uterus	A1	NT	NT	NT	NT	NT	-
	A2	NT	NT	NT	NT	NT	+
	A3	NT	NT	NT	NT	NT	-
	A4	NT	NT	NT	NT	NT	-
	B1	NT	NT	NT	NT	NT	+
	B2	NT	NT	NT	NT	NT	-
	B3	NT	NT	NT	NT	NT	-
	B4	NT	NT	NT	NT	NT	-
PCR/ Kidney	A1	NT	NT	NT	NT	NT	+
	A2	NT	NT	NT	NT	NT	+
	A3	NT	NT	NT	NT	NT	-
	A4	NT	NT	NT	NT	NT	+*
	B1	NT	NT	NT	NT	NT	+
	B2	NT	NT	NT	NT	NT	-
	B3	NT	NT	NT	NT	NT	+
	B4	NT	NT	NT	NT	NT	-

Positive (+); Positive culture (*); Vaginal fluid (VF); Non tested (NT).

CAPÍTULO III

Influence of breed on the clinical and hemato-biochemical parameters in sheep experimentally infected with *Leptospira* sp.

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Influence of breed on the clinical and hemato-biochemical parameters in sheep experimentally infected with *Leptospira* sp.

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Abstract

Early diagnosis of leptospirosis may aid in a favorable prognosis in infected animals, but there are few reports of clinical and hematochemical changes in the ovine species, nor whether the breed exerts any influence on the response to infection. Thus, the objective of the present study was to evaluate the clinical, biochemical and hematological alterations in Santa Inês and crossbred ewes challenged with *Leptospira interrogans* serogroup Pomona serovar Kennewicki. Twenty-four sheep were used in this experiment, 12 crossbred (group A) and 12 Santa Inês (group B). In each group, sheep were conjunctivally and intraperitoneally challenged. During 60 days post-infection the ewes were evaluated for the presentation of clinical signs and the blood was collected for hemogram and serum biochemistry. Concentration of urea and creatinine; serum aspartate aminotransferase activity (AST); gamma-glutamyltransferase (GGT); total protein and albumin; total bilirubin, direct and indirect were analyzed. The urine of these sheep was collected for urinalysis. Only two Santa Inês sheep showed blood in the urine, and one of them had a dark urine color. Clinical signs implicated in *Leptospira* sp. Infection were not identified. Some sheep had anemia, especially crossbred. However, anemia may be attributed to the more effective cellular response that has been identified in crossbred animals. Only one animal presented leukocytosis with neutrophilia, while 11 presented atypical leukopenia, especially those of the Santa Inês breed. There was a decrease in total protein and albumin, as well as the increase in gammaglutamyltranspeptidase (GGT), especially in Santa Inês sheep. The level of aspartate aminotransferase (AST) remained within the normal range for the species. A significant increase ($p<0.05$) in conjugated bilirubin levels in challenged animals was detected. Only one sheep intraperitoneally challenged presented a high level of urea in the blood, but the creatinine level remained within the normal range. The intraperitoneal route was responsible for more significant changes ($p<0.05$) in the hemogram and biochemistry when compared to the conjunctival route. The results indicate that crossbred sheep have a more efficient cellular response than Santa Inês sheep, which may confer a greater resistance to infection. Clinical signs are not good parameters to follow the development of leptospirosis in crossbred and Santa Inês breed. Hematological and biochemical analyzes were useful in the detection of anemia and possible liver changes caused by leptospirosis. The intraperitoneal route was able to cause more conclusive alterations of the analyzed parameters, however, it is possible that

the alterations caused by the conjunctival route reproduce in a more faithful way what happens in a natural situation of infection.
Keywords: Leptospirosis, Intraperitoneal route, Conjunctival route, Santa Inês, breeds.

Introduction

Leptospirosis is a disease which is caused by bacteria of the genus *Leptospira* and is amongst the main zoonotic causes of worldwide morbidity and mortality, however, even with a number of deaths which exceeds other causes of hemorrhagic fever in human beings, continues to be overlooked (Costa et al., 2015). This pathology has its incidence influenced mainly by environmental factors (Martins e Lilenbaum, 2014), but the maintenance of the etiological agent in nature requires the participation of host animals, especially when the environmental conditions are unfavorable (Ellis, 2015).

Amongst the hosts involved in the epidemiology of leptospirosis, the role of the sheep seems not to have yet been elucidated. Initially this species was considered only as an accidental host (Leon-Vizcaino et al., 1987). Then, its capability to maintain and transmit some strains of the serogrup Sejroe was acknowledged (Andreani et al., 1983; Farina et al., 1996; Arent et al., 2013), and currently it is suggested that they may act as preferential hosts for the serogroup Autumnalis (Silva et al., 2007, Alves et al., 2012). The asymptomatic behavior of sheep facing the strains of the serogroup Pomona (Costa et al., 2018; Hamond et al., 2019), which had so far been referred to as accidental in the species, raises doubts and imposes challenges regarding the understanding of their role as a host.

The diagnosis of leptospirosis may be carried out by means of the evaluation of the clinical signs and by laboratorial methods, however, due to the asymptomatic characteristic of the disease in sheep, the clinical aspects are limited (Martins e Lilenbaum, 2014). It is known that the presentation of the acute disease in sheep is influenced by the virulence of the infecting strain and by the age of the affected animals (Ellis, 2015), however that a difference of susceptibility exists between the breeds (Costa et al., 2018). These particularities result in the fact that the diagnosis in the species consists mainly in the detection of antibodies, with moderate registers of isolation and direct detection of the agent (Dutra, 2017). Despite this, some studies report the acute disease, in which the animals presented loss of appetite, irritability, diarrhea, anemia, hemorrhage, miscarriage, reduction in the production of milk, hematuria, jaundice and occasionally death (Ellis, 1994; Vermunt et al., 1994).

Although the serologic diagnosis is recommended by the World Organization for Animal Health as a standard proof for the diagnosis of leptospirosis (Oie, 2014), it is believed

that this technique presents several limitations and requires the association with direct methods for the secure detection of carrier animals (Otaka et al., 2012; Costa et al., 2018). As it is a disease which may cause several reproductive disorders and, in more serious cases, the death of the animals (Ellis, 2015), the early diagnosis is crucial to avoid the serious form of the illness. The hematological and biochemical test have been helping in the fast diagnosis and prognosis of leptospirosis in human beings (Silva et al., 2014), however, there is little information about the reference values and the alterations caused by this disease in sheep. Therefore, the objective of the present study was to evaluate the clinical biochemical and hematological alterations of Santa Inês and crossbred sheep challenged by *Leptospira interrogans* serogrupo Pomona.

Material and methods

Ethical approval

Before the challenge in the sheep, tests were carried out in hamsters to evaluate the virulence of the strain. The tests in hamsters were approved by the Ethics Committee in Research in Animals of the Fluminense Federal University (under the protocol number 611/2015), whereas the tests in sheep were carried out after the approval of the Committee on Ethics in Research of the Federal University of Campina Grande-UFCG (Protocol n. 020/2016).

Animals and experimental groups

24 sheep were used in this experimental test, being 12 of them of the Santa Inês breed and 12 crossbred sheep. The animals were not vaccinated against leptospirosis and were between 12 and 18 months old, de-wormed, seronegative in the MAT (titre <50) and negative in the PCR of urine and vaginal fluid in three previous samplings with intervals of 30 days between the tests. The sheep were healthy and did not present hematological and biochemical alterations. The experiment was conducted at the Research Center for the Development of the Semi-arid Tropic (Nupeárido) of the UFCG, State of Paraíba, Brazil. All the measures were taken in order to avoid the environmental or crossed contamination between the groups. The sheep were installed in covered individual stalls (1.0x1.5m), without contact with other animals, distant from the soil at a height of 1m and with access to water and food *ad libitum*. The animals were fed concentrate (corn and soybean bran) and bulk (Tifton grass hay) in the

ratio of 60:40, going through an adaptation in the installments during 20 days prior to the challenge.

Virulence test and experimental infection

To carry this study, *L. interrogans* serogroup Pomona sorovar Kennewicki was used, isolated from swine in the United States of America (USA) and originating from the Salsbury Laboratories (Iowa, USA). Before the inoculation, virulence tests were carried out according to the suggested protocols (Silva et al., 2008; Suepaul et al., 2010). The 3-Rs policy for experimental science was applied in all the stages (Barbosa et al., 2016) according to the Brazilian Guidelines of the Federal Council on Veterinary Medicine and the Brazilian Guidelines on the Care and Use of Animals for Scientific and Learning Purposes. The strain was submitted to four passages in Golden-Syrian hamsters, according to the described by Silva et al. (2008) and Suepaul et al. (2010) with adaptations (Barbosa et al., 2016).

The sheep were divided into two groups according to the breed, being 12 animals in group A (crossbred sheep) and 12 animals in group B (Santa Inês sheep). However, within each group (A and B), there was a subdivision related to the inoculation route: subgroups ACR (crossbred/conjunctival route) and AIPR (crossbred / intraperitoneal route); subgroups BCR (Santa Inês/conjunctival route) and BIPR (Santa Inês/ intraperitoneal route). Each subgroup (ACR; AIPR; BCR; BIPR) had a control animal. With relation to the challenged sheep, the crossbred group (A) was individually identified from A1 to A10, being A1, A2, A3, A4, A5 (crossbred/conjunctival route) and A6, A7, A8, A9, A10 (crossbred/ intraperitoneal route). The same way, the Santa Inês sheep (B) were identified from B1 to B10, being B1, B2, B3, B4, B5 (Santa Inês/conjunctival route) and B6, B7, B8, B9, B10 (Santa Inês/intraperitoneal route). The sheep of the control group were challenged with EMJH sterile medium without bacterial culture, whilst the others were challenged by intraperitoneal or conjunctival route with 1×10^7 of the bacterial culture. After counting was reached a volume of 2 ml that were inoculated. The inoculation by conjunctival route (CR) was carried out with the aid of a pipette and by intraperitoneal route (IPR) using a needle (0.70x30 mm 22G 1 1/4) and sterile syringe.

Clinical Evaluation

The clinical examination was performed by a Veterinary Doctor in all the sheep before the challenge (D0) and on days D1, D2, D3, D4, D5, D6, D7, D8, D15, D30, D45 and D60 post-infection (p.i.). Throughout the tests, the presence of the following clinical signs were

assessed: prostration, jaundice, hematuria, dehydration and coloring of the mucous membrane. The analyzed physiological variables were rectal temperature (RT), respiratory rate (RR) and heart frequency (HF). The RT was measured by means of a clinical digital thermometer (Termomed, Incoterm®,Porto Alegre, Brazil), with a variation of temperature between 32 and 42°C and average error of 0.2°C. In order to obtain the RR , a flexible stethoscope was used (Rappaport, Premium®, Rio de Janeiro, Brazil) in the right thoracic region counting the number of movements per minute (mov/min^{-1}). The HF was determined with the aid of a stethoscope (Rappaport, Premium®, Rio de Janeiro, Brazil), counting the number of heart beats per minute (bpm).

Hemato-biochemistry and urinalysis

Collections of blood were carried out for the hematological and biochemical evaluation on day zero (D0) before the challenge, as well as on D4, D8, D15, D30, D45 and D60 post-infection (p.i.), totaling seven collections. The blood was collected by puncture of the jugular vein using disposable needles and vacuum tubes (without anticoagulant) of 9 mL (Vacuum tube, Vacuette®, Porto, Portugal), after the local antisepsis with 2% iodized alcohol solution. A tube (without anticoagulant) was used for the biochemical analysis, whilst another (with EDTA 10% p/v) was used for the hematological evaluation. The hemogram was performed according to the techniques described by Stockham and Scott (2011).

As the Santa Inês and crossbred sheep presented some particularities in the normal hemato-biochemical standards, the reference values were compared to those previously determined (Henriques et al., 2016; Rabassa et al., 2009; Salviano et al., 2013; Lima et al., 2015). Urea and creatinine concentration ; serum activity of the aspartate aminotransferase (AST) ; gamaglutamiltransferase (GGT); total protein and albumin; total, direct and indirect bilirubin were analyzed spectrophotometrically using commercial kinetic and colorimetric tests available for the automated biochemical analyzer (Labtest, Labtest Diagnostica S.A.®, Minas Gerais, Brazil) in a biochemical analyzer (Cobas c111 Analyzer, Roche Diagnostics®, Risch-Rotkreuz, Switzerland). Prior to the collections of blood, the sheep were submitted to an eight-hour fast, being the collections always performed in the early morning.

With the aid of a vaginal speculum, urine samples were collected by means of a sterile urethral probe number (Sterile Disposable Urethral Probe, Mark Med®, São Paulo, Brazil), collected using disposable sterile needles and analyzed immediately. Physical and chemical examinations were carried out in the urine samples, being examined as being physical

characteristics the color, the odor and the presence of turbidity. The chemical examination of the urine was carried out using reagent strips (UriAction 10, Labtest Diagnóstica S.A.®, Minas Gerais, Brazil) semiquantitative determination of leukocytes, nitrite, urobilinogen, protein, pH, blood, density, ketones, bilirrubin and glucose.

Statistical analysis

The dependent variables were crossed with each other, thus, for the comparison of the averages between the breeds, the routes and the time, variance analysis of two factors were carried out (ANOVA-two way). In all the analysis a 5% level of significance of for a type III error ($p < 0.05$). The results are presented with the values of the averages in illustrations. The statistical treatment of the data was performed using the Statistical Package for the Social Science (SPSS) version 24.0 for IBM®.

Results

Clinical evaluation and hematological variations:

None of the sheep presented clinical signs suggestive of infection by *Leptospira* sp., as well as alterations in the measured physiological parameters. With respect to the hemogram, the sheep of the control group presented results which were normal for the species. Anemia was detected in four of the (A3, A7, A8, A10) crossbred sheep and in only one (B4) sheep of the Santa Inês breed. In the crossbred sheep, the intraperitoneal route (IPR), seemed to be more efficient in causing anemia, as when the inoculation routes were compared, this one caused a significant reduction ($p \leq 0.05$) in the values of erythrocytes ($8\text{-}12 \times 10^6/mm^3$), hemoglobin (8.5-15 g/dL) and hematocrit, however the hematocrit remained within the normal values (20-38%). There was a striking reduction in these rates in the crossbred sheep, over the time of the experiment (Fig.1, Fig. 2, Fig. 3), especially when the D0 was compared to the D8 ($p \leq 0.05$) and the D15 ($p \leq 0.05$) in the erythrocytes, as well as the D0 with the D8 ($p \leq 0.05$) and the D15 ($p \leq 0.05$) in the hematocrit.

The rod cells (rare), eosinophils (0-1.000), monocytes (0-750) and basophils (0-400) remained normal during the experiment. From the 20 sheep challenged with the bacterial strain, 12 presented abnormal values in the leukocyte count (4.000-12.000). One crossbred sheep (A6) exhibited leukocytosis with neutrophilia on D8 p.i., while 11 demonstrated atypical leukopenia, being two of them crossbred (A3, A7) and nine Santa Inês (B1, B2, B3, B5, B6, B7, B8, B9, B10). There was a significant difference ($p \leq 0.05$) in the leukocytes and neutrophils count (700-6.000) between the breeds. However, it is important to highlight that

on D0 there was a significant difference ($p \leq 0.05$) between the breeds in the neutrophils count in the sheep challenged by intraperitoneal route. This greater interference on the levels of white cells seemed to be more present in the sheep on D8 p.i., when a significant difference was detected ($p \leq 0.05$) regarding the leukocyte and neutrophils count. Regarding the breeds, the inoculum had a greater influence on the leukocyte and neutrophils count from D4, remaining on D30, D45 and D60 p.i. ($p \leq 0.05$), where the crossbred sheep presented a greater cellular response (Fig. 4, Fig. 5). Likewise, there was a significant difference between the breeds ($p \leq 0.05$) on the lymphocyte count (1.000-9000) on D4 and D30 (Fig. 6).

Urinary and biochemical analysis:

The sheep of the control group did not present abnormal alterations in the biochemical and urinary analysis. Among the challenged ones, only one Santa Inês sheep (B4) presented urobilinogen (35 mg/dL) on D4, this animal also indicated a presence of blood in the urine on D4 (200 ery/µL) and on D8 (80 ery/µL). One sheep (A6) presented of blood (80 ery/µL) on D8. With regard to the detection of leukocytes in the urine, on D8 was verified (70 leu/µL) in five crossbred sheep inoculated via intraperitoneal route (A6, A7, A8, A9, A10), as well as a sheep inoculated via conjunctival route from the Santa Inês group (B3).

In relation to the total protein (6-11 g/dL), the levels decreased in the course of time until D30, including below normal values for the species (Fig. 7). Albumin (3-8 g/dL) presented a similar standard of reduction (Fig. 8). Although a not significant difference occurred in the general comparison between the breeds for total protein and albumins ($p>0.05$), on D30 was detected in the animals inoculated by intraperitoneal route ($p \leq 0.05$) for total protein. In the sheep Santa Inês challenged by conjunctival route, there also was a significant difference between the breeds in the comparison of the level of total protein on D0 with D30, D45 and D60 ($p \leq 0.05$), as well as on D15 for albumin. In both situations, Santa Inês sheep presented the lowest levels of these in relation to the crossbred sheep.

The level of the enzyme aspartate aminotransferase (AST) remained within the normal variation for the species (50-280 U/L), however there was a significant difference when the inoculation route was compared on D45 and D60 ($p \leq 0.05$), in which the animals challenged via intraperitoneal route stimulated a greater increase of this enzyme (Fig. 9). Furthermore, there was a significant increase of AST among the Santa Inês sheep when comparing D0 with D30 ($p \leq 0.05$). In relation to the levels of GGT (20-52 U/L), there was an increase of this enzyme during the period of the experiment (Fig. 10), with significant difference when D0

was compared to D45 and D60 in crossbred sheep ($p \leq 0.05$), as when D0 was compared to D8 in the Santa Inês animals ($p \leq 0.05$). The route also had a significant influence in the increase of the GGT ($p \leq 0.05$), as the sheep challenged by intraperitoneal route stimulated a greater increase. The comparison between the breeds was impaired, as the crossbred sheep had significantly higher GGT values than the Santa Inês ones on D0 ($p \leq 0.05$), even if within the normality for the species. The levels of total bilirrubin (0.5-1.5 mg/dL) and indirect bilirrubin (0.5-0.8 mg/dL) had a slight increase on D4 in relation to D0, however only the sheep of the crossbred group showed a decrease in the levels of indirect bilirrubin (Fig. 11, Fig. 12). The blood levels of direct bilirrubin (0-0.5 mg/dL) increased during the experiment (Fig. 13), with significant difference when the D0 was compared to D4 and D45 ($p \leq 0.05$). The inoculation route and the breed had a direct relation with the increase of direct bilirrubin, as the Santa Inês sheep challenged via intraperitoneal route presented a significant increase of its levels when D0 was compared to D4 ($p \leq 0.05$).

The levels of urea were elevated during the experiment (Fig. 14), however within the reference values for the species (17-60 mg/dL). The comparison between the breeds was impaired, as on D0 there was a significant difference ($p > 0.05$), as the crossbred sheep presented higher values. Both inoculation routes influenced the increase of the levels of urea, in which the crossbred sheep inoculated via intraperitoneal route presented significant difference between the D0 and D60 ($p \leq 0.05$), while the Santa Inês sheep challenged via conjunctival route between the D0 and D15. Despite the Santa Inês sheep inoculated via intraperitoneal route having had a greater elevation in the levels of urea, there was no significant difference ($p > 0.05$), possibly because one (B10) exhibited very discrepant numbers. This same sheep presented high levels of urea (>88mg/dL), followed by subnormal levels of creatinine (<0.4mg/dL) as from D15 until D60. The levels of creatinine (0.5-1.9 mg/dL) decreased up to D30 (Fig. 15).

Discussion

The sheep presented values of rectal temperature (38.5-40ºC), Heart rate (50-80 bpm) and respiratory frequency (20-34 mov/min^{-1}) within the normal variation for the species (Ribeiro et al., 2008). Although the RF and HR are not specific parameters in the diagnosis of leptospirosis, they may indicate a possible metabolic stress secondary to an infection. In contrast, fever is an important indication of an infectious process, especially resulting from high leptospiremia (Adler, 2015). Levels $>10^4$ leptospires/mL in the blood stream are associated to serious outcomes of the disease (Seguro et al. 2005), despite a study having

suggested that the leptospires with a lower virulence are capable of reaching levels in blood $>10^4$ leptospires/mL without causing serious complications (Agampodi et al. 2012). In this study, it was not possible to detect the presence and quantify the level of leptospires in the sheep's bloodstream, but as the virulence of the strain was previously attested, it is assumed that the absence of fever may be associated to a lower multiplication of the agent in the blood, especially in the crossbred sheep. As the natural resistance to gastrointestinal nematodes has been reported in crossbred sheep (Amarante et al., 2009), it is believed that this resistance also happens in leptospirosis. The sheep do not present signs of hemorrhage, jaundice or dehydration . It is known that leptospires can cause hemolysis and lesions in the endothelial cell coating of small vessels, resulting in hemorrhages, formation of thrombi and blockage in the blood supply in several organs (Adler, 2015), however, in sheep, the disease does not usually present itself in an acute form with the development of clinical signs (Martins e Lilenbaum, 2014). Despite jaundice has been described in lambs of a European breed infected by a strain of the serogroup Pomona (Vermunt et al., 1994), ruminants do not become icteric with frequency, even when there is a serious hepatocellular impairment (Pugh, 2004). Even though some sheep presented anemia and possible hepatic impairment, it may not have been sufficient to cause jaundice in these animals, therefore the clinical evaluation seems not to be so reliable to identify Santa Inês and crossbred sheep infected by *Leptospira* sp.

The infection was capable of causing significant cellular alterations in the sheep infected, especially in the white cell count. Despite humoral immunity being important, the innate immunity is primordial in the natural resistance to diseases in the species, in which the presence of certain pro-inflammatory cytokines may provide a more effective immune response of the host in the beginning of the infection (Xia et al., 2017). The macrophages are singled out as the main infiltrating cells and the phagocytes anti-*Leptospira* during the leptospirosis (Chen et al., 2017), while the neutrophils are involved in the activation, regulations and effecting of the various innate cellular and adaptive functions in the animals (Mantovani et al., 2011). In this experiment, the fact that the crossbred sheep had a significantly higher amount ($p \leq 0.05$) of white cells in comparison to the ones of the Santa Inês breed, even before the challenge, called attention. It is possible that this particularity has contributed to tackle the multiplication of the leptospires and determined a lower seroconversion of antibodies in the crossbred animals (Costa et al., 2018), as the increase of neutrophils is associated to the reduction of precocious loads of leptospires in the blood (Scharring et al., 2015). It is unlikely that there is a natural difference in the white cell count

between the Santa Ines and crossbred animals, so how crossbred sheep have a more efficient cellular response than the Santa Inês breed, so they are more resistant. However so a molecular study to identify possible differences in the differentiation groupings (cluster of differentiation) and quantification of the pro-inflammatory cytokines is necessary, in order to reach clearer conclusions about this interaction in breeds of sheep.

The infection by pathogenic leptospires is characterized by a leukocytosis with neutrophilia and anemia due to the hemolysis in the animals (Tonin et al., 2012), however in this experiment the majority of the sheep presented leukopenia, mainly in the Santa Inês sheep . Generally the leukopenia occurs in the initial stage of the disease (Greene et al., 2006), but in this study the animals had a gradual decrease of the total leukocytes. In a study with rodents, leukopenia was identified and attributed to the decrease of the production, formation of antibodies and complements against hematopoietic precursors, or even peripheric destruction by the increased depuration (Tonin et al., 2012). Thus, it is possible that such mechanisms also caused similar changes in this experiment.

The decrease of the total protein, as well as the increase of the Gamma-glutamyl transpeptidase (GGT), mainly in the Santa Inês sheep, suggests a possible hepatic damage in the affected sheep (Stockham e Scott, 2011). Despite having increased, the aspartate aminotransferase, (AST) remained within the normal limits for the species (20-52 U/L), however it is important to highlight that the increase of the transaminases in the majority of cases is discreet in the leptospirosis (Gonçalves et al., 1971). Even though the sheep in this experiment did not present hepatic lesions in the histopathological evaluation, which carried out at the end of the experiment (Costa et al., 2018), this absence can be explained as the histological lesions in the liver are generally found in patients with a clinical history of more than 30 days (Gonçalves et al., 1971). What would be unlikely to happen in the sheep as they are more resistant, especially the crossbred ones. The conjugated fraction of bilirrubin (direct bilirrubin) was significantly ($p \leq 0.05$) increased in the challenged animals. In the leptospirosis, even without jaundice, it is possible that there may be some difficulty in the excretion of billirubin by the hepatocytes as a consequence of the intrahepatic cholestasis (Tochetto et al., 2012), for this reason there was predominance of this conjugated metabolite. The intraperitoneal inoculation route seems to have influenced more in the alteration of the parameters than the conjunctival route, even though the later has been more efficient in the reproduction of the infection (Costa et al., 2018). This difference between the routes may be related to the fact that the intraperitoneal route is not a natural route for infection, thus offering less resistance to the invasion and multiplication of the etiological agent. It is

important to note that intraperitoneal inoculation is more invasive than conjunctival inoculation, which may have led to greater cellular and biochemical changes.

The renal impairment in the leptospirosis may be evidenced by the increase of the serum levels of urea and creatinine (Hagiwara et al., 2004). In this experiment, only one sheep (B10) challenged via intraperitoneal route presented a high level of urea (>88mg/dL), however the level of creatinine remained low and alteration in the urinalysis was not observed. It is known that the creatinine suffers a lower influence from extra-renal factors (Stockham e Scott, 2011), therefore it is a reliable indicator of the glomerular filtration rate. Thus, it is not possible to imply that this increase was caused by renal alteration. However, it must be registered that the values of urea and creatinine may be underestimated due to a lower metabolization of proteins (hypoproteinemia), especially the urea values. Only one Santa Inês sheep (B4) presented hemoglobinuria on D4 p.i., as well as the presence of blood in the urine on D4 (200 ery/µL) and on D8 (80 ery/µL), possibly as a result of the hemolytic anemia which it presented. This results class attention, as it was an animals challenged via conjunctival route, responsible for the minor alterations in the animals of this study. It is possible that the differentiated response to infection in this sheep (B4) happened due to an individual vulnerability, as this was the only one to present titre of 3:200, and also presented leptospiral DNA in the urine and in the kidney (Costa et al., 2018). The greatest detection of leukocytes in urine in crossbred animals inoculated via intraperitoneal route indicates the presence of the agent in the urinary tract, as well as a more effective cellular response in the crossbred sheep.

Conclusion

The results obtained with the experimental infection indicate that crossbred sheep have a more efficient cellular response than Santa Inês sheep, granting them a greater resistance to infection. The clinical signs are not good parameters to monitor the development of leptospirosis in crossbred and Santa Inês sheep. The hematological and biochemical analysis proved to be useful in the detection of anemia and possible hepatic alterations caused by leptospirosis, however only direct bilirubin appears to be a reliable parameter to diagnose leptospirosis in its early stage in sheep species. The intraperitoneal route was capable of causing more poignant alterations of the analyzed parameters; however it is possible that the alterations caused by the conjunctival route reproduce in a more faithful manner what happens in a natural infection situation.

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Lista de Figuras

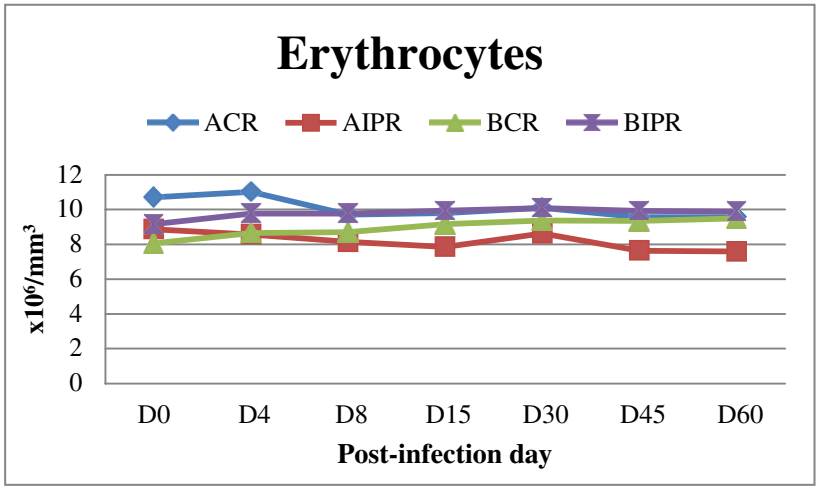
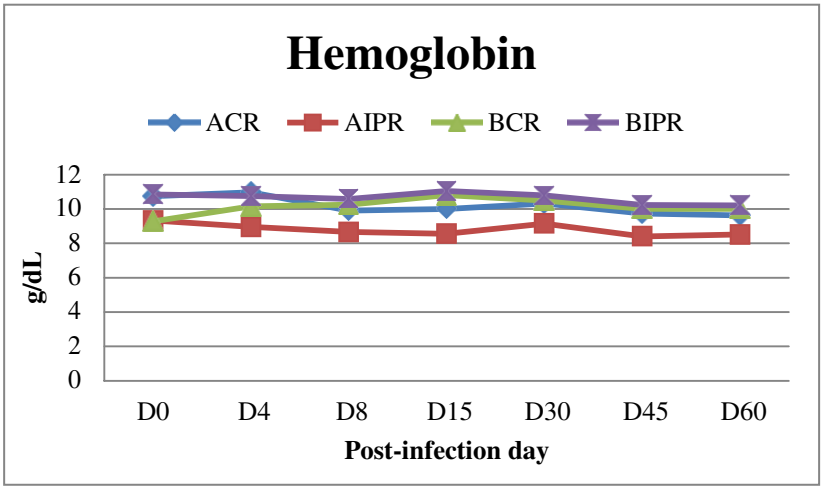


Fig. 1. Mean counts of erythrocytes according to the post-infection day in conjunctival route (ACR) and intraperitoneal route (AIPR) infected crossbred sheep, as well as Santa Inês sheep infected by the conjunctival route (BCR) and intraperitoneally route (BIPR) with *Lepstospira interrogans* serogroup Pomona.



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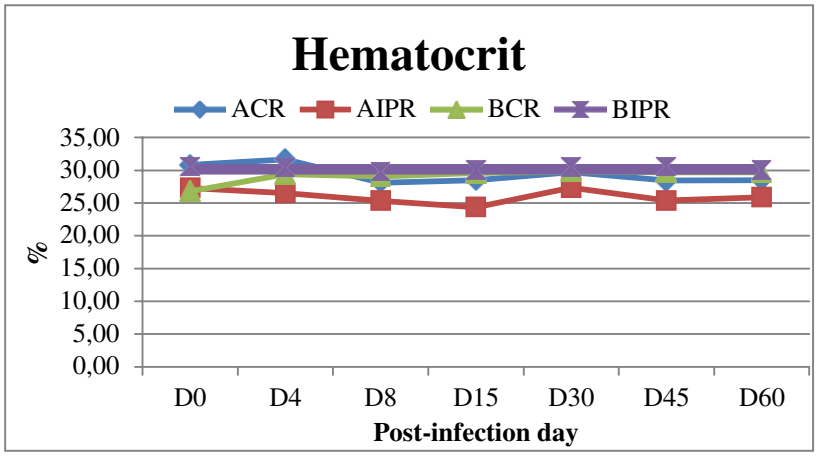

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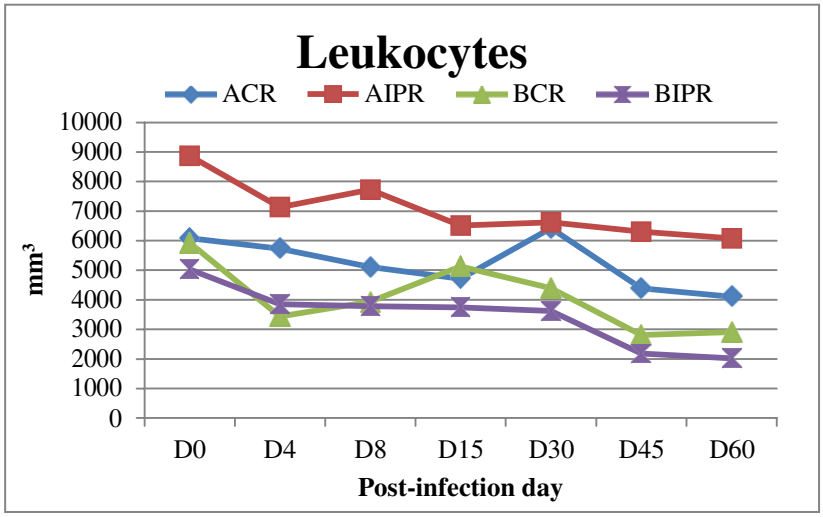

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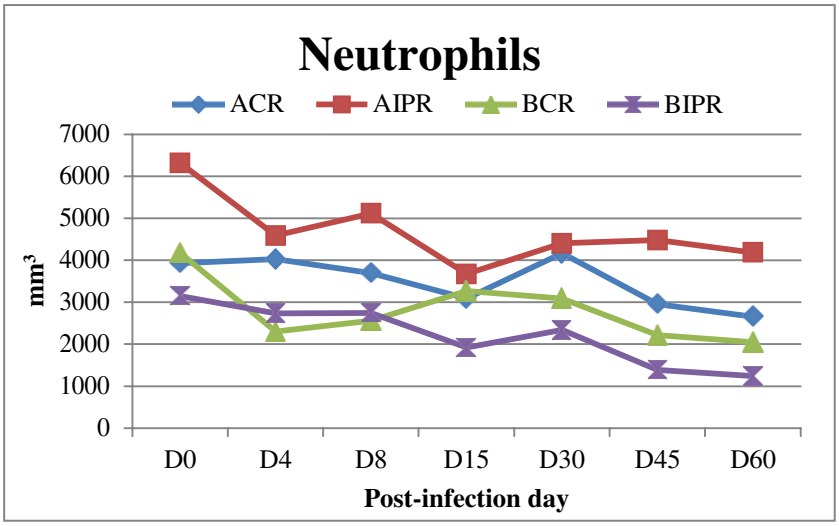
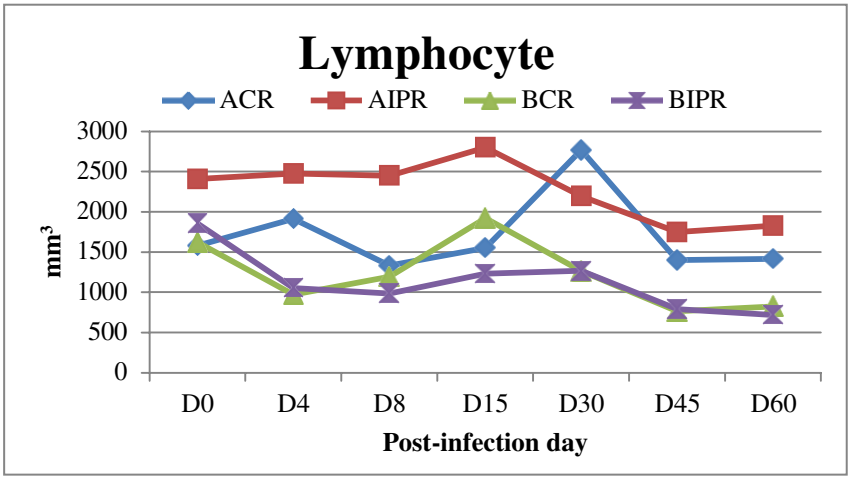


Fig. 5. Mean neutrophils count according to the post-infection day in conjunctival route (ACR) and intraperitoneal route (AIPR) infected crossbred sheep, as well as Santa Inês sheep infected by the conjunctival route (BCR) and intraperitoneally route (BIPR) with *Lepstospira interrogans* serogroup Pomona.



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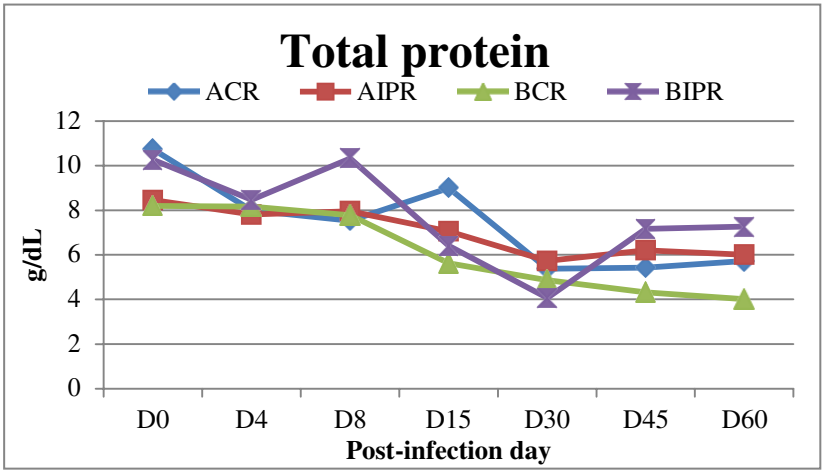

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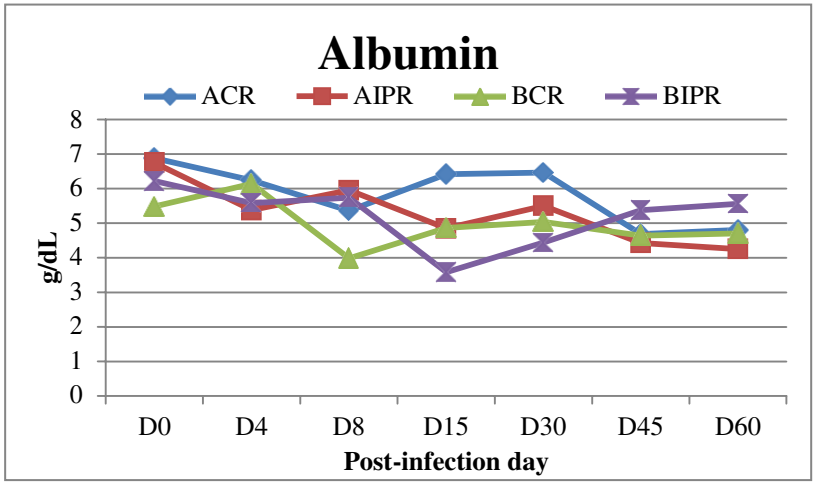

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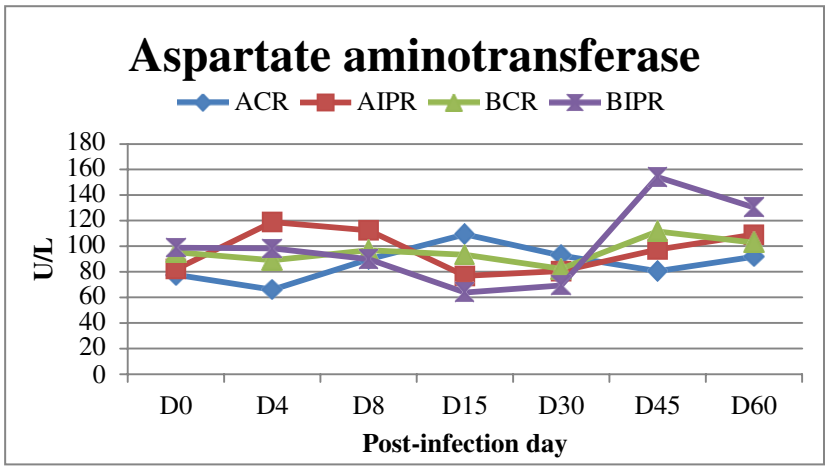

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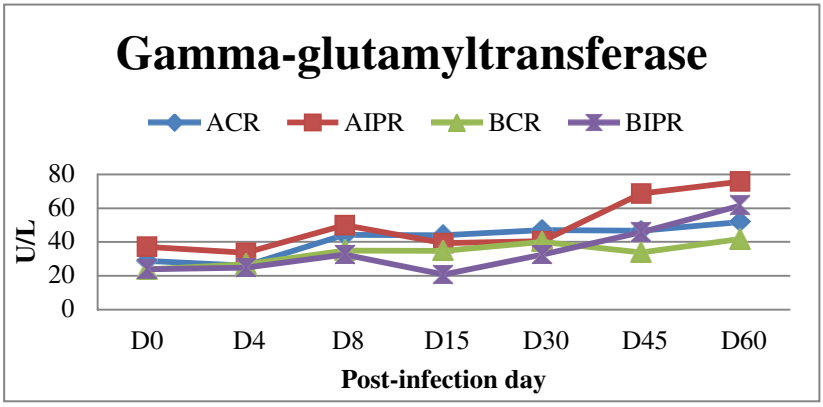

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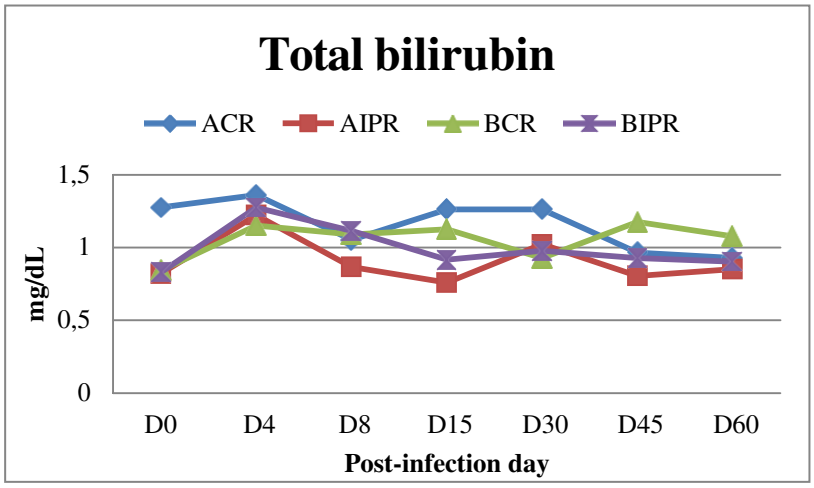
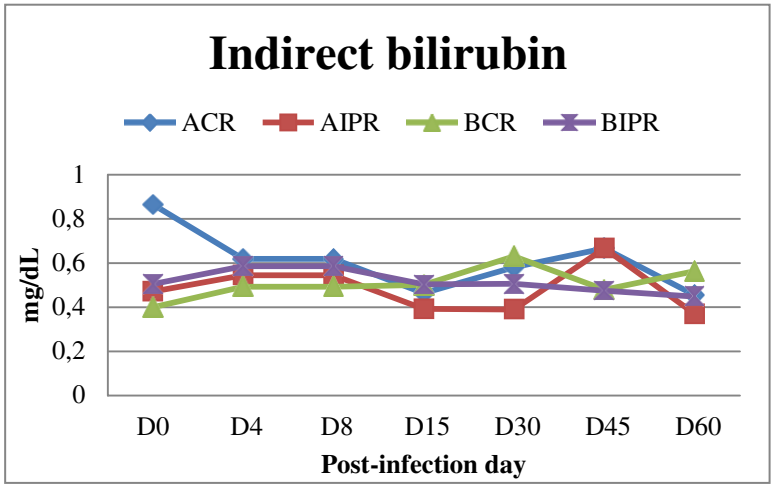


Fig. 11. Levels of total bilirubin according to the post-infection day in conjunctival route (ACR) and intraperitoneal route (AIPR) infected crossbred sheep, as well as Santa Inês sheep infected by the conjunctival route (BCR) and intraperitoneally route (BIPR) with *Lepstospira interrogans* serogroup Pomona.



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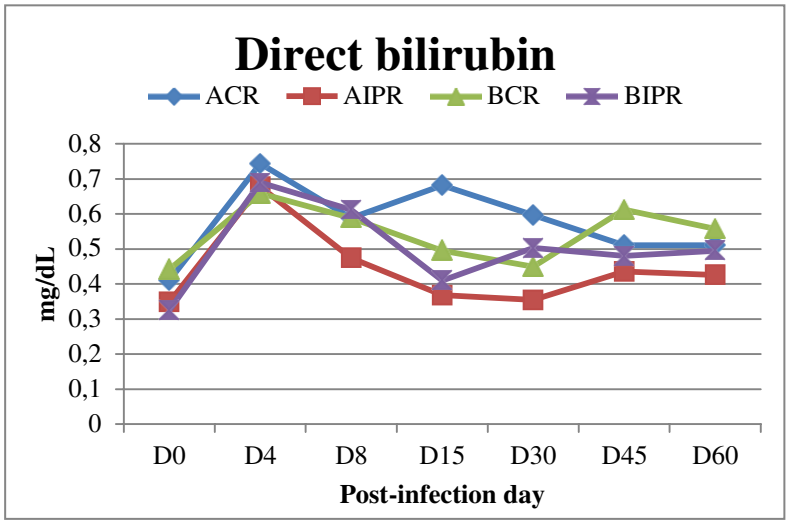

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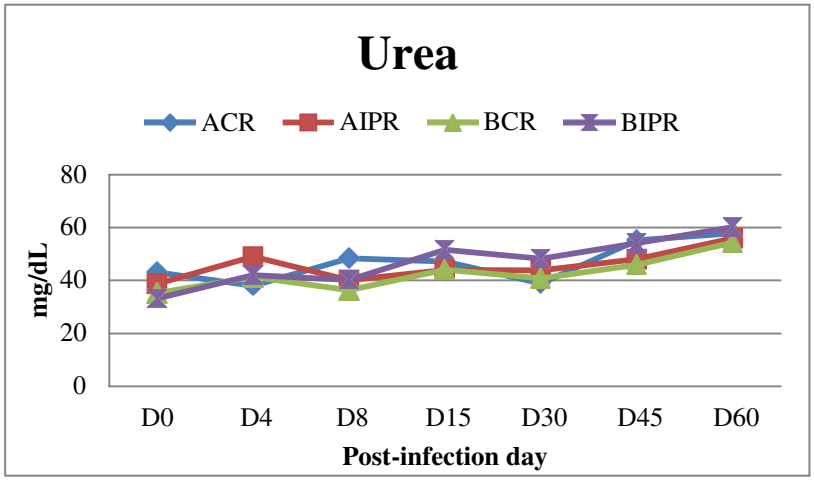

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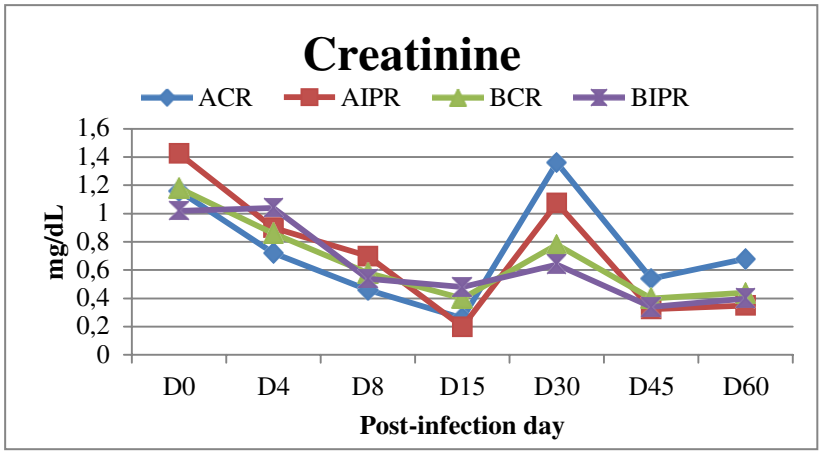

Fig. 15. Levels of creatinine according to the post-infection day in conjunctival route (ACR) and intraperitoneal route (AIPR) infected crossbred sheep, as well as Santa Inês sheep infected by the conjunctival route (BCR) and intraperitoneally route (BIPR) with *Lepstospira interrogans* serogroup Pomona.

CONCLUSÃO GERAL

Com base no que foi apresentado, os três capítulos que compõe esta Tese levaram às seguintes conclusões:

- Ambos os modelos experimentais analisados foram satisfatórios quanto à capacidade de causar a infecção nas ovelhas infectadas;
- Os achados enfatizam a importância do trato genital como um local extra urinário de infecção na espécie ovina e indicam a possibilidade de transmissão venérea nessa espécie.
- A leptospirose foi uma doença assintomática nos ovinos, portanto o diagnóstico por meio dos sinais clínicos não é confiável nessa espécie.
- As ovelhas foram portadoras e eliminaram o agente, o que sugere a capacidade dessa espécie em participar da transmissão de cepas do sorogrupo Pomona, sobretudo as de raça mestiça;
- As ovelhas mestiças apresentaram uma resposta celular mais efetiva contra *Leptospira interrogans* sorogrupo Pomona sorovar Kennewicki do que as ovelhas Santa Inês, esse pode ser o motivo de serem mais resistentes à doença;
- As análises hematológica e bioquímica se mostraram úteis na detecção de anemia e possíveis alterações hepáticas causadas pela leptospirose, inclusive a bilirrubina direta mostrou ser um importante parâmetro no diagnóstico precoce da leptospirose na espécie.

Anexo I – Aprovação pelo Comitê de Ética em Pesquisa

Universidade Federal de Campina Grande
Centro de Saúde e Tecnologia Rural
Comissão de Ética em Pesquisa
Av. Sta Cecília, s/n, Bairro Jatobá, Rodovia Patos,
CEP: 58700-970, Cx postal 64, Tel. (83) 3511-3045

Ao: Sr. DIEGO FIGUEIREDO DA COSTA (Coordenador)

Protocolo CEP nº020-2016

CERTIDÃO

ASSUNTO: Solicitação de aprovação do projeto de pesquisa intitulado "INFECÇÃO EXPERIMENTAL POR LEPTOSPIRA SPP. EM OVINOS DESLANADOS DA RAÇA SANTA INÊS E MESTIÇOS NO SEMIÁRIDO NORDESTINO".

Certificamos a V.Sa. que seu projeto teve parecer consubstanciado orientado pelo regulamento interno deste comitê e foi Aprovado, por Há de Referendum, em 05 de outubro de 2016, estando à luz das normas e regulamentos vigentes no país atendidas as especificações para a pesquisa científica.

Patos, 01 de dezembro de 2016.

Maria de Fátima de Araujo Lucena
Coordenadora do CEP