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Research article

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. 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.

1. 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 and Lilenbaum,

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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 and 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 et al., 1994; Vermunt et al., 1994).

Although the microscopic agglutination test (MAT) 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.

2. Material and methods

2.1. 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 (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 number 020/2016).

2.2. 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, dewormed, 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 and crossed contamination between the groups. The sheep were installed in covered individual stalls (1.0 × 1.5m), 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.

2.3. 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 (0.70 × 30 mm 22G 1 1/4) and sterile syringe.

2.4. 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).

2.5. 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 8-h 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 (Sterile Disposable Urethral Probe number 8, 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.

After last collection sheep were submitted to euthanasia with previous sedation 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 10 mg/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 100 mg/kg intravenously.

2.6. 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®.

3. Results

3.1. 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 (8–12 $\times$ 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), 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. 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. 2).

3.2. 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

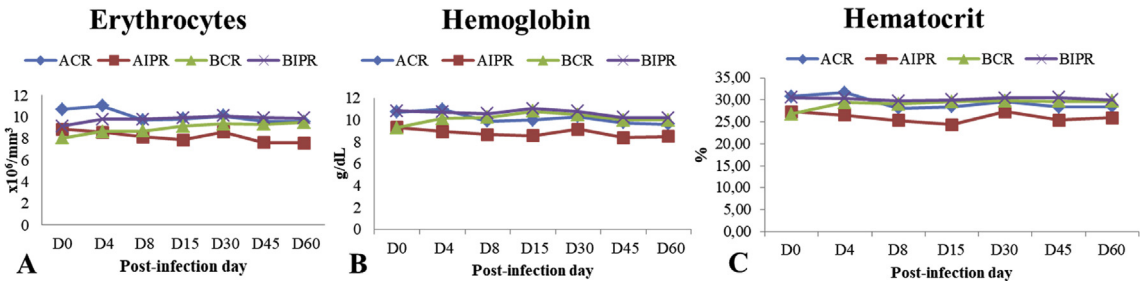

Fig. 1. Mean counts of erythrocytes (A), hemoglobin concentration (B) and percentage of hematocrit (C) according to the post-infection day in conjunctival route (ACR) and intraperitoneal route (AIPR) infected crossbred sheep, as well as Santa Inês sheep infected by the conjunctival route (BCR) and intraperitoneally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

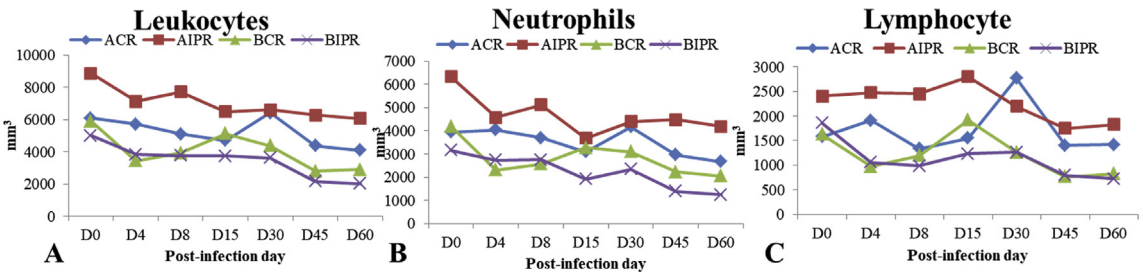

Fig. 2. Mean leukocytes (A), neutrophils (B) and lymphocyte (C) count according to the post-infection day in conjunctival route (ACR) and intraperitoneal route (AIPR) infected crossbred sheep, as well as Santa Inês sheep infected by the conjunctival route (BCR) and intraperitoneally route (BIPR) with *Leptospira interrogans* serogroup Pomona.

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. Albumin (3–8 g/dL) presented a similar standard of reduction (Fig. 3). 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. 3). 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. 3), 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. 4). The blood levels of direct bilirrubin (0–0.5 mg/dL) increased during the experiment (Fig. 4), 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. 5), 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 (>88 mg/dL), followed by subnormal levels of creatinine (<0.4 mg/dL) as from D15 until D60. The levels of creatinine (0.5–1.9 mg/dL) decreased up to D30 (Fig. 5).

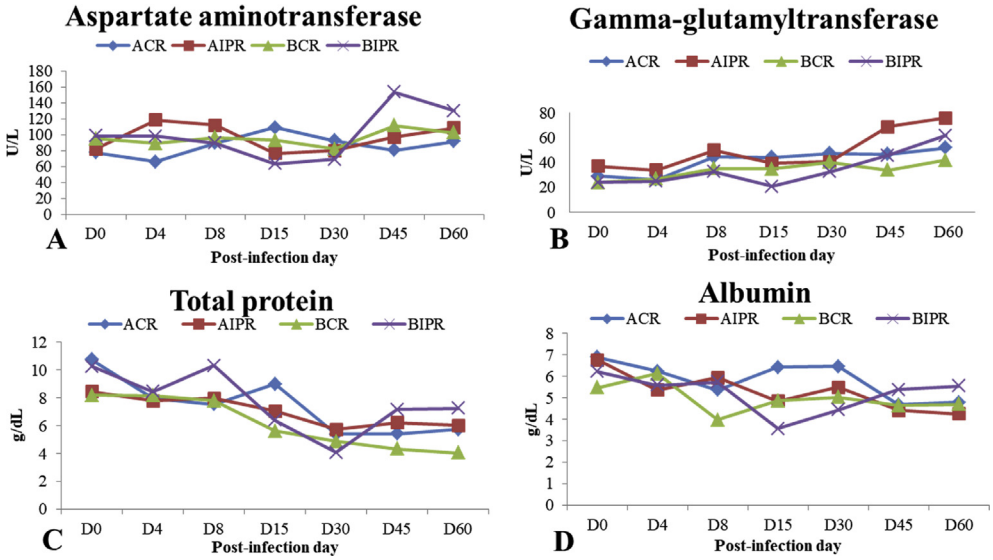

Fig. 3. Levels of aspartate aminotransferase (A), gamma-glutamyltransferase (B), total protein (C) and albumin (D) according to the post-infection day in conjunctival route (ACR) and intraperitoneal route (AIPR) infected crossbred sheep, as well as Santa Inês sheep infected by the conjunctival route (BCR) and intraperitoneally route (BIPR) with *Leptospira interrogans* serogroup Pomona.

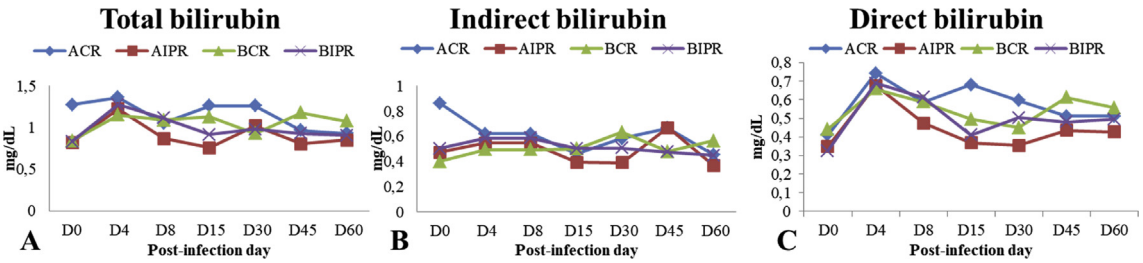

Fig. 4. Levels of total bilirubin (A), indirect bilirubin (B) and direct bilirubin (C) according to the post-infection day in conjunctival route (ACR) and intraperitoneal route (AIPR) infected crossbred sheep, as well as Santa Inês sheep infected by the conjunctival route (BCR) and intraperitoneally route (BIPR)) with *Leptospira interrogans* serogroup Pomona.

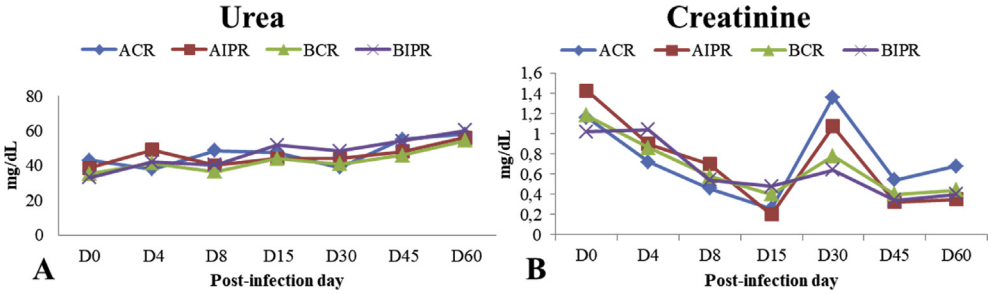

Fig. 5. Levels of urea (A) and creatinine (B) according to the post-infection day in conjunctival route (ACR) and intraperitoneal route (AIPR) infected crossbred sheep, as well as Santa Inês sheep infected by the conjunctival route (BCR) and intraperitoneally route (BIPR)) with *Leptospira interrogans* serogroup Pomona.

4. Discussion

The sheep presented values of rectal temperature (38.5–40 °C), 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 (Segura 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. As clinical leptospirosis is poorly reported in Santa Inês and crossbred sheep in Brazil (Martins and Lilenbaum, 2014), there is a strong possibility that the animals' rusticity influenced the results of the experiment. It is noteworthy that the animals reproduced the infection, but subclinically, confirmed by the serological and molecular evaluation previously published by this research group (Costa et al., 2018). However, more studies are needed to reach concrete conclusions, such as the identification of genetic markers involved in any resistance.

4.1. 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 and 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 (Scharrig 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 and 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 (>88 mg/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 and 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 animal 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 presented 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 suggest the presence of the agent in the urinary tract, as well as a more effective cellular response in the crossbred sheep.

5. 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.

Declarations

Author contribution statement

Diego Figueiredo da Costa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Pedro Jorge Àlvares de Faria, Denise Batista Nogueira, Laura Honório de Oliveira Tolentino: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Maira Pôrto Viana, José Devedê da Silva: Performed the experiments; Analyzed and interpreted the data.

Antônio Fernando de Melo Vaz, Sergio Santos de Azevedo: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Severino Silvano dos Santos Higino: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Clebert José Alves: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

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The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

Adler, B., 2015. History of leptospirosis and *Leptospira*. Curr. Top. Microbiol. Immunol. 387, 1–9.

Agampodi, S.B., Matthias, M.A., Moreno, A.C., Vinetz, J.M., 2012. Utility of quantitative polymerase chain reaction in leptospirosis diagnosis: association of level of leptospiremia and clinical manifestations in Sri Lanka. Clin. Infect. Dis. 54, 1249–1255.

Alves, C.J., Alcino, J.F., Farias, A.E.M., Higino, S.S.S., Santos, F.A., Azevedo, S.S., Costa, D.F., Santos, C.S.A.B., 2012. Epidemiological characterization and risk factors associated with leptospirosis in the Brazilian semiarid. Pesqui. Vet. Bras. 32, 523–528.

Amarante, A.F., Susin, I., Rocha, R.A., Silva, M.B., Mendes, C.Q., Pires, A.V., 2009. Resistance of Santa Ines and crossbred ewes to naturally acquired gastrointestinal nematode infections. Vet. Parasitol. 165, 273–280.

Andreani, E., Tolari, F., Farina, R., 1983. Experimental infection in sheep with *Leptospira interrogans* serotype hardjo. Br. Vet. J. 139, 165–170.

Arent, Z., Frizzell, C., Gilmore, C., Mackie, D., Ellis, W.A., 2013. Isolation of Leptospires from genital tract of sheep. Vet. Rec. 173, 582–583.

Barbosa, C.S., Martins, G., Lilenbaum, W., 2016. Blood collection by gingival puncture on hamsters reduces animal number in leptospirosis virulence tests. ALTEX 33, 322–323.

Chen, X., Li, S.J., Ojcius, D.M., Sun, A.H., Hu, W.L., Lin, X., Yan, J., 2017. Mononuclear-macrophages but not neutrophils act as major infiltrating anti-leptospiral phagocytes during leptospirosis. PLoS One 12, e0181014.

Costa, F., Hagan, J.E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M.S., Stein, C., Abela-Ridder, B., Ko, A., 2015. Global morbidity and mortality of leptospirosis: a systematic review. PLoS Neglected Trop. Dis. 9, e0003898.

Costa, D.F., Silva, M.L.C.R., Martins, G., Dantas, F.M., Melo, M.A., Azevedo, S.S., Linlenbaum, W., Alves, C.J., 2018. Susceptibility among breeds of sheep

experimentally infected with *Leptospira interrogans* Pomona serogroup. Microb. Pathog. 122, 79–83.
Dutra, F., 2017. Leptospirosis aguda en cordero. Archivo Veterinario del Este 20, 15–16.
Ellis, W.A., 2015. Animal leptospirosis. Curr. Top. Microbiol. Immunol. 387, 99–137.
Ellis, G.R., Partington, D.L., Hindmarsh, M., Barton, M.D., 1994. Seroprevalence to *Leptospira interrogans* serovar hardjo in merino stud rams in South Australia. Aust. Vet. J. 71, 203–206.
Farina, R., Cerri, D., Renzoni, G., Andreani, E., Mani, P., Ebani, V., Pedrini, A., Nuvoloni, R., 1996. *Leptospira interrogans* in the genital tract of sheep. Research on ewes and rams experimentally infected with serovar hardjo (hardjobovis). New Microbiol. 19, 235–242.
Gonçalves, A.J.R., Lins, D.O., Susuki, L.E., Duarte, F., Ferreira, M., Andrade, J., 1971. O fígado nas leptospiroses. Rev. Soc. Bras. Med. Trop. 5, 67–98.
Greene, C.E., Sykes, J.F., Brown, C.A., Hartmann, K., 2006. Leptospirosis. In: Infectious Diseases of the Dog and Cat, 3° edition. Saunders Elsevier, St. Louis, Missouri, U.S.A, pp. 402–415.
Hagiwara, M.K., Lustosa, M., Kogika, M.M., 2004. Leptospirose canina. Vet. News 11, 7–8.
Hamond, C., Silveira, C.S., Buroni, F., Suanes, A., Nieves, C., Salaberry, X., Aráoz, V., Costa, R.A., Rivero, R., Giannitti, F., Zarantonelli, L., 2019. *Leptospira interrogans* serogroup Pomona serovar Kennewicki infection in two sheep flocks with acute leptospirosis in Uruguay. Transbound. Emerg. Dis.
Henriques, L.C.S., Gregory, L., Rizzo, H., Hasegawa, M.Y., Meira Jr., E.B.S., 2016. Avaliação dos fatores etários sobre a função renal de ovelhas Santa Inês1. Pesqui. Vet. Bras. 36, 642–646.
Leon-vizcaino, L., Mendoza, M.H., Garrido, F., 1987. Incidence of abortions caused by leptospirosis in sheep and goats in Spain. Comp. Immunol. Microbiol. Infect. Dis. 10, 149–202.
Lima, M.B., Monteiro, M.V.B., Jorge, E.M., Campello, C.C., Rodrigues, L.F.S., Viana, R.B., Monteiro, F.O.B., Costa, C.T.C., 2015. Blood reference intervals and the influence of age and gender on hematologic and biochemical parameters of Santa Ines sheep bred in the eastern Amazon. Acta Amazonica 45, 317–322.
Mantovani, A., Cassatella, M.A., Costantini, C., Jaillon, S., 2011. Neutrophils in the activation and regulation of innate and adaptive immunity. Nat. Rev. Immunol. 11, 519–531.
Martins, G., Lilenbaum, W., 2014. Leptospirosis in sheep and goats under tropical conditions. Trop. Anim. Health Prod. 46, 11–17.
World Organization for Animal Health (OIE), 2014. Leptospirosis. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. World Organization for Animal Health, Paris.
Otaka, D.Y., Martins, G., Hamond, C., Penna, B., Medeiros, M.A., Lilenbaum, W., 2012. Serology and PCR for bovine leptospirosis: herd and individual approaches. Vet. Rec. 170, 338.
Pugh, D.G., 2004. Clínica de ovinos e caprinos. Rocca, São Paulo, p. 513p.
Rabassa, V.R., Tabeleão, V.C., Schneider, A., 2009. Metabolic evaluation of ewes kept on native pasture during autumn/winter. Rev. Bras. Agrociência. 15, 125–128.
Ribeiro, N.M., Furtado, D.A., Medeiros, A.N., Ribeiro, M.N., Silva, R.C.B., Souza, C.M.S., 2008. Evaluation of the thermal comfort index, physiological parameters and thermal gradient on native sheep. Agric. Eng. 28, 614–623.
Salviano, M.B., Sousa Junior, A., Moura, W.L., 2013. Hematologic sheep of Santa Inès adults not pregnant. Rev. Cient. Elet. Med. Vet. 20, 1–12.
Scharrig, E., Carestia, A., Ferrer, M.F., Cedola, M., Pretre, G., Drut, R., Picardeau, M., Schattner, M., Gomez, R.M., 2015. Neutrophil extracellular traps are involved in the innate immune response to infection with *Leptospira*. PLoS Neglected Trop. Dis. 9, e0003927.
Segura, E.R., Ganoza, C.A., Campos, K., Ricaldi, J.N., Torres, S., Silva, H., Cespedes, M.J., Matthias, M.A., Swancutt, M.A., Lopez, Linan R., Gotuzzo, E., Guerra, H., Gilman, R.H., Vinetz, J.M., 2005. Clinical spectrum of pulmonary involvement in leptospirosis in a region of endemicity, with quantification of leptospiral burden. Clin. Infect. Dis. 40, 343–351.
Silva, É.F., Brod, C.S., Cerqueira, G.M., Bourscheidt, D., Seyffert, N., Queiroz, A., Santos, C.S., Ko, A.I., Dellagostin, O.A., 2007. Isolation of *Leptospira noguchii* from sheep. Vet. Microbiol. 121, 144–149.
Silva, E.F., Santos, C.S., Athanazio, D.A., Seyffert, N., Seixas, F.K., Cerqueira, G.M., Fagundes, M.Q., Brod, C.S., Reis, M.G., Dellagostin, O.A., Ko, A.I., 2008. Characterization of virulence of *Leptospira* isolates in a hamster model. Vaccine 26, 3892–3896.
Silva, N.L., Niloofa, M., Fernando, N., Karunanayake, L., Chaturaka, R., Silva, J., Premawansa, S., Handunnetti, S.M., Rajapakse, S., 2014. Changes in full blood count parameters in leptospirosis: a prospective study. Int. Arch. Med. 7, 1–4.
Stockham, S.L., Scott, M.A., 2011. Fundamentos de Patologia Clínica Veterinária, 2a edição. Editora Guanabara Koogan, Rio de Janeiro, p. 744.
Suepaul, S.M., Carrington, C.V., Campbell, M., Borde, G., Adesiyun, A.A., 2010. Study on the efficacy of *Leptospira* vaccines developed from serovars isolated from Trinidad and comparison with commercial vaccines using a hamster model. Vaccine 28, 5421–5426.
Tochetto, C., Flores, M.M., Kommers, G.D., Barros, C.S.L., Fighera, R.A., 2012. Aspectos anatomopatológicos da leptospirose em cães: 53 casos (1965-2011). Pesqui. Vet. Bras. 32, 430–433.
Tonin, A.A., Salva, A.S., Azevedo, M.I., Franca, R.T., Paim, F.C., Schaefer, P.C., Martins, J.L.R., Badke, M.R.T., Sonia, T.A.L., 2012. Hematologic and biochemical alterations in Wistar rats experimentally infected by *Leptospira interrogans*. Comp. Clin. Pathol. 21, 833–838.
Vermunt, J.J., West, D.M., Cooke, M.M., Alley, M.R., Collins-Emerson, J., 1994. Observations on three outbreaks of *Leptospira interrogans* serovar Pomona infection in lambs. N. Z. Vet. J. 42, 133–139.
Xia, B., Sun, L., Fan, X., Xiao, H., Zhu, Y., Qin, J., Cai, C., Zhao, W., Chang, Y., Guo, X., He, P., 2017. A new model of self-resolving leptospirosis in mice infected with a strain of *Leptospira interrogans* serovar Autumnalis harboring LPS signaling only through TLR4. Emerg. Microb. Infect. 6, e36.