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SHORT COMMUNICATION

Detection of pathogenic leptospira as a cause of abortion in cattle-observations on diagnosis

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ABSTRACT. Leptospirosis is a zoonotic infectious disease caused by members of the genus *Leptospira,* which affects domestic and wild animals. Cases of abortion in cattle have been associated with this infection, but these are often not adequately confirmed. To determine the best diagnostic strategy for leptospirosis-associated cases of abortion, we evaluated some of the techniques used in the veterinary laboratory and found that the key issues are sample type and timing. In a retrospective anatomical and histopathological analysis, we studied 42 aborted foetuses with lesions consistent with leptospirosis to check for the presence of pathogenic leptospira by qPCR, as well as ascertaining the serologic status of the cows. In addition, in a prospective analysis, cows that had aborted foetuses were analysed within 2 days of the event by MAT and qPCR using blood and urine samples. Analysis of the foetuses indicated that only 14.3% of the selected cases (6 of 42) gave a positive qPCR result. Regarding cows that had recently aborted foetuses, 4 out of 11 sampled showed a positive qPCR, while MAT tests showed only negative results. The evidence provided in this study indicates that the time that has elapsed since a clinical event has occurred and the type of clinical sample taken are key elements in the successful confirmation of pathogenic leptospira as the cause of abortion.

Key words: Leptospirosis, pathogenic leptospira, abortion, dairy cattle, diagnostic.

INTRODUCTION

Leptospirosis is probably one of the most widespread and prevalent zoonotic diseases worldwide (Hartskeerl *et al.*, 2011). This infectious disease is caused by a group of spirochetes of the genus *Leptospira,* called pathogenic leptospira.

Pathogenic leptospira infection in cattle, which is associated with reproductive failure, is considered a major cause of economic loss (Bolin & Alt, 2001). In dairy cattle, abortion and stillbirth are the most serious clinical events caused by pathogenic leptospira infection (Ellis, 2015), followed by "Milk Drop Syndrome" (Alonso-Andicoberry *et al.*, 2001; Bolin, 2003). The negative economic impact can also be attributed to the cost of treatment, increases in culling and low pregnancy rates (Dhaliwal *et al.*, 1996). Gädicke and Monti (2013), have estimated that the economic losses in Chile could be as high as US$143 per lactation when a case of abortion occurs. Leptospirosis is often difficult to diagnose, and frequent misdiagnosis probably makes it the most neglected infectious disease in cattle (Martins & Lilenbaum, 2017). This is particularly significant when the diagnosis of a clinical case must be confirmed. Identification of the aetiology is essential to establish proper control and mitigation measures in the herd. Furthermore, an early and accurate diagnosis is of paramount importance to establish appropriate antibiotic treatment when pathogenic leptospira infection is suspected (Adler & De la Peña Moctezuma, 2010).

This brings to light a frequent and widespread diagnostic problem, namely confirmation of the aetiology of cattle abortion due to pathogenic leptospira. In southern Chile, most veterinary practitioners take a serum sample to detect antibodies in an aborted cow and the result obtained is interpreted as confirmation of pathogenic leptospira (Elder *et al.*, 1985). Although less frequent, the Pathology Department of our Faculty has also received requests for histopathological analysis as a diagnostic method through the identification of lesions, mainly in the foetus, consistent with infection from/by pathogenic leptospira in cases of abortion.

In the present study, we aimed to ascertain the type of diagnostic tool, as well as the clinical specimen and sampling time that should be used to accurately establish the aetiology of a case of abortion in cattle once pathogenic leptospira infection is suspected.

MATERIAL AND METHODS

To accomplish the objective of the present study, we organised the methodology in two independent but complementary observational surveys (I and II):

DESIGN SURVEY I

To assess pathogenic leptospira detection in an abortion case, a retrospective study was carried out. Primarily, this included information on all cases of aborted cattle recorded in the Veterinary Anatomical Pathology laboratory at

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the Institute of Animal Pathology, Faculty of Veterinary Sciences, Universidad Austral de Chile, between 2010 and 2019. As a second inclusion criterion, the samples of aborted foetuses that underwent further analysis were those displaying gross lesions consistent with pathogenic leptospira infection, such as jaundice, foci of necrosis in the liver, as well as signs of histopathological lesions, such as accumulations of mononuclear cells in the liver and/or kidney, areas of necrosis in the liver, vacuolization in the liver and/or kidney. In addition, the result of the microscopic agglutination test (MAT) of the cow was also taken into account.

For each selected sample, 10 to 15 sections of 5 µm thick tissue were taken from the paraffin-embedded tissue samples using a precision rotation microtome (Jung®), intended for DNA extraction. The paraffin sections were stored in 1.7 mL Eppendorf tubes. After this, the deparaffinization procedure was carried out according to Miller *et al.* (1997). Deparaffinized tissue samples were subjected to a DNA extraction-purification protocol using the High Pure PCR Template Preparation Kit (Roche, Indianapolis, IN, USA), following the manufacturer's instructions. The DNA templates obtained from the above protocol were analysed in a qPCR system (Roche LightCycler 2.0) using a TaqMan probe and targeting the *LipL32* gene which is specific only to pathogenic leptospira species (Stoddard *et al.*, 2009).

DESIGN SURVEY II

To show evidence of active infection by pathogenic leptospira in live cattle that have recently aborted foetuses, a field cross-sectional survey was performed.

Between January and December 2020, cows that had aborted within the previous 48 hours were selected and sampled for this study. The sampling was carried out from five dairy cattle herds located in three different districts of the Los Ríos region, Chile. To assess the infection status of the animals studied, urine samples (5-20 mL) were taken through direct stimulation of the vulvar area. The urine was collected in sterile 50 mL Falcon tubes. Also, to detect the pathogen in the whole blood, individual blood samples (5 to 10 mL) were taken by venipuncture of the coccygeal vein of each animal, using vacutainer tubes with anticoagulant and, in parallel, without coagulant for the detection of antibodies in the blood serum, using individual needles for each animal in both cases. Both types of samples were kept at room temperature until they were transferred to the Laboratory of Infectious Diseases, Institute of Preventive Veterinary Medicine, Universidad Austral de Chile. The sampling was carried out in strict accordance with the Universidad Austral de Chile's Guide for the Use of Animals for Research. (www.uach.cl/direccion/investigacion/uso_animales.htm).

Urine samples were pretreated using an immunomagnetic separation (IMS) protocol coupled to real time PCR (qPCR), according to a published protocol (Tomckowiack *et al.*, 2020). A 25 mL aliquot of each urine sample was centrifuged at 4,000 g for 15 min and the pellet was resuspended in 1 mL of phosphate buffered saline (PBS) [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 (pH 7)] and then transferred to a 1.5 mL microcentrifuge tube and recentrifuged at 11,000 g for 5 min. Finally, the supernatant was discarded, the pellet was resuspended in 1 mL of PBS and a 100 µL aliquot was submitted to be used in the IMS protocol (Tomckowiack *et al.*, 2020), before proceeding with DNA extraction by High Pure DNA Template Preparation Kit protocol (Roche, USA).

From a whole blood sample, an aliquot of 200 µL of blood was taken (with EDTA) from Vacutainer tubes (Becton Dickinson, USA), from which DNA was extracted using the High Pure DNA Template Preparation Kit (Roche, USA). This was performed as described in survey I.

Pathogenic leptospira cell numbers (genome equivalents) detected by qPCR were estimated according to a published protocol used in Tomckowiack *et al.* (2020), using the molecular weight of the genome of *Leptospira interrogans* serovar Hardjo type prajitno strain Hardjoprajitno (GenBank accession number EU357983.1) to establish a standard curve for the estimation of leptospira numbers by qPCR, according to a published algorithm (Dzieciol *et al.*, 2010).

Sera were tested for the presence of antibodies against six reference leptospira serovars, according to the published protocol (Salgado *et al.*, 2014), using the microagglutination test (MAT).

For survey I, the association between cow MAT and foetal tissue PCR results was evaluated by the McNemar test. For survey II, the proportion of positive results obtained by the diagnostic tests used for each of the clinical samples were compared. To do this, the ratio test was performed, using the Z statistic. The R software (R Core Team, year 2016®) was used, considering a significance level of 5%.

RESULTS AND DISCUSSION

To propose an adequate solution to the problem of pathogenic leptospira infection in cattle, we must first have a thorough understanding of the biology of this infection. Infection by pathogenic leptospira begins with bacteremia and the infection can then migrate to organs such as the liver and kidney, which is followed by leptospira urine shedding and, weeks later, antibody titers (Adler & De la Peña Moctezuma, 2010; Adler, 2014).

Because pathological analysis is also used as a diagnostic method, through the identification of lesions consistent with infection by pathogenic leptospira in cases of abortion, it seemed pertinent to us to retrospectively analyse a significant number of possible cases of abortions due to pathogenic leptospires. A total of 247 aborted bovine foetuses met the inclusion criteria in the retrospective study. Macroscopic and/or microscopic findings associated with pathogenic leptospira infection, as well as MAT results of the (mother) cow, led us to select a total of 42 aborted

foetuses. Overall, information from 7 (16.7%) of them showed only macroscopic lesions (enlarged liver, small areas of necrosis, jaundice). In addition, 10 (23.8%) displayed only microscopic lesions (inflammatory mononuclear (cell) infiltration), cloudy swelling) and 13 (30.9%) showed both types of lesions consistent with pathogenic leptospira infection. Finally, in 12 (28.6%) cases the sera showed MAT positive results without lesions. Although the lesions identified in these foetuses correspond with what has been considered suggestive for pathogenic leptospira infection, in most cases these lesions can be seen in other pathologies (Sebastian *et al.*, 2005; Smyth *et al.*, 1999). In this regard, Schlafer and Foster (2016) reported other findings, such as lesions in the placenta and lungs, which were not observed in the cases we studied because the majority of foetuses displayed a state of autolysis that made it difficult to identify these lesions (Smyth *et al.*, 1999). Therefore, these anatomical and histological findings should only be interpreted as presumptive information that requires a second confirmatory tool.

Therefore, in order to confirm those presumptive cases, the PCR technique on fixed tissue was used. Only 6 (14.3%) out of the 42 cases showed PCR positive results (table 1). Of these 6 cases, 1 sample case showed both microscopic and macroscopic lesions, while 2 showed only macroscopic lesions, and finally, 3 foetal sample cases did not show any visible lesions but exhibited high MAT antibody titers. However, molecular identification by PCR in paraffin-embedded samples may be underestimated due to a decrease in PCR efficiency. The study by Einerson *et al.* (2005) showed a loss of detection of up to 50% in their samples, attributed to the effect of the fixing reagents on the efficiency of the PCR.

Since the most commonly used diagnostic technique for leptospira infection is MAT (Thiermann, 1984), it seemed reasonable to monitor the serological status of the aborted cow. Thirty-one of the 42 aborted fetuses monitored (73%) produced positive serology results for one or more leptospira serovars. Hardjo was the predominant serovar, with 24 cases (77.4%), followed by Ballum, with 9 cases (29%), whilst the Canicola and Pomona serovars were identified in 5 cases (16.1%), and Autumnalis showed up in just 1 case (3.2%). The antibody titers ranged between 1:100 to 1:3200 (data not shown). There was a high percentage of selected foetuses with lesions consistent with pathogenic leptospira infection, although unconfirmed by PCR, with a positive MAT result for the mother. Any valid interpretation drawn from this finding must take into account the fact that, in southern Chile, there is a high pathogenic leptospira seroprevalence in dairy herds (Salgado *et al.*, 2014). Besides, MAT is useful for a herd-level diagnosis and may not be reliable for individual diagnoses (Otaka *et al.*, 2012), since most cows that show seroreactivity with low titers show no direct evidence of pathogenic leptospira shedding (Hamond *et al.*, 2014). So, samples with positive MAT from the mother indicate exposure, and not necessarily active infection, as a cause of abortion, thereby explaining the lack of a significant relationship ($P<0.05$) between MAT and PCR.

A key aspect that would allow us to determine the cause of abortion is the status of active infection by pathogenic leptospira in the mother through direct detection of the pathogen at the genomic level. In this way, there would be a confirmatory relationship between infection by this pathogen and a case of abortion. In survey II, the sampling of cows that had recently aborted was successful due to the valuable collaboration of veterinary practitioners. We found only 11 cows that had aborted foetuses and were sampled within 48 hours during the study period. None of the sera in the cows that had aborted showed positive MAT results. Of the 11 cows, 4 cows showed positive results which suggest an active infection status due to pathogenic leptospira. In the case of urine samples, only 1 out of 11 (9.1%) samples showed a positive result with a low concentration of 8.77 leptospira per mL. However, a higher proportion of positive results was observed when

Table 1. Aborted foetuses with a history associated with leptospirosis identified in the records of the Laboratory of Veterinary Anatomic Pathology with positive PCR (n=6).

ID animal	Ab titers	Serovar	Macroscopic lesion	Microscopic lesion	qPCR
781-11	1:400	Hardjo	Pale red mucous membranes and musculature	Liver and kidney: degenerative conditions	(+)
101-13	1:400 1:100	Hardjo Pomona	–	–	(+)
188-13	1:1600	Canicola	–	–	(+)
272-13	1:800 1:200	Ballum Hardjo	–	–	(+)
363-13	1:400	Hardjo	–	Liver: necrotic foci	(+)
395-14	1:400 1:100	Hardjo Ballum	–	Kidney: degenerative conditions	(+)

Table 2. Results of the analysis of the 11 cows for pathogenic leptospira infection.

Nº	ID animal	qPCR (Whole Blood) pathogenic leptospira per mL	IMS-qPCR (Urine) pathogenic leptospira per mL	MAT
1	3806	–	–	–
2	6487	$4.86 \cdot 10^2$	–	–
3	8731	–	$8.77 \cdot 10^0$	–
4	8732	–	–	–
5	8327	–	–	–
6	8238	$2.11 \cdot 10^4$	–	–
7	8042	-	-	-
8	8726	$6.14 \cdot 10^3$	-	-
9	8523	-	-	-
10	6693	-	-	-
11	9980	-	-	-

blood samples were used ($P<0.05$). Three out of 11 blood (27.3%) samples were positive, ranging in concentration from $4.86 \cdot 10^2$ to $2.11 \cdot 10^4$ pathogenic leptospira per mL (table 2). The reported finding regarding the detection of pathogenic leptospira in whole blood is consistent with published experimental investigations (Zuerner *et al.*, 2012) that studied the infection in golden hamsters by injecting this pathogen intraperitoneally and could be detected in blood vessels around 48 hours post inoculation, giving a graphic indication of the biology of this infection. The biology of infection indicates that leptospiremia is observed up to the first week after exposure, which is followed by pathogen migration to the target organs (Adler & De la Peña Moctezuma, 2010). As pathogenic leptospira decrease in concentration in the blood, the antibodies start to rise, reaching a detectable level between 7-14 days (Adler, 2014).

It seems that blood samples do not have a negative effect on polymerase efficiency, unlike urine samples which is likely to be due to PCR inhibitors (Rosenstraus *et al.*, 1998). The presence of PCR inhibitors in urine samples makes the analysis more difficult and expensive where DNA extraction protocols should consider inhibitor removal. To solve this problem, immunomagnetic separation (IMS) has been described as a technique to provide inhibitor-free PCR samples and improve their analytical sensitivity (Olsvik *et al.*, 1994; Taylor *et al.*, 1997). Recently, Tomckowiack *et al.* (2020) developed an immunomagnetic separation (IMS) protocol as pretreatment for qPCR that offered a cost-effective tool for urine analysis and solved the false negative problems of low bacterial loads in the specimen, allowing 30% higher detection of positive results than a conventional system. In the present study, the low bacterial load detected by the IMS-qPCR system in the positive urine sample (8.44 bacteria/mL) suggests that the animal has an initial kidney infection post bacteremia, where it would possibly have been classified as negative in a conventional molecular detection system without the pre-step of immunoseparation.

Most of the abortions caused by leptospira occur in the last trimester of pregnancy and are associated with a chronic infection in the individual aborting animal (Adler *et al.*, 2014; BonDurant, 2007). The present study reported the absence of antibody titers in MAT and the presence of pathogenic leptospira in blood, which could indicate a status of early or acute infection with initial bacteremia, and in this infectious scenario, an abortion is not an unexpected result in a pregnant cow.

This study showed that anatomical and histopathological information must be considered as a presumptive and non-confirmatory tool for pathogenic leptospira as a cause of abortions in cattle. Also, the detection of pathogen DNA in blood may provide new evidence of abortions in the early stages of this infection, suggesting that the evaluation of antibodies alone is not an accurate diagnostic strategy for the cause of abortion in cattle. Besides, it has been suggested that a chronic event and MAT values are either static flailing or are not detectable (Ellis *et al.*, 1982). The evidence provided in this study indicates that the time elapsed since a clinical event has occurred and the type of clinical sample taken are key elements in the successful confirmation of pathogenic leptospira as the cause of abortion.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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