

DETECTION OF LEPTOSPIRES IN CLINICALLY HEALTHY PIGLETS BORN FROM SOWS EXPERIMENTALLY INFECTED WITH LEPTOSPIRA INTERROGANS SEROVAR CANICOLA

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ABSTRACT

Leptospirosis is an important zoonosis that causes reproductive disorders in swine. The isolation of leptospires from aborted fetuses, stillbirths and weak piglets was obtained in several occasions, however, the bacteria was never isolated from apparently healthy piglets born from apparently healthy infected dams. Six sows of the Landrace breed with a known date of service and pregnancy confirmed by ultrasonography were infected intravenously with 5 ml of *Leptospira interrogans* serovar Canicola inoculum between 76 and 90 days of gestation, and one week after farrowing, at least one piglet per sow was euthanized and samples of liver, kidneys, lungs, heart, spleen and gastric content were taken and examined by PCR. Reproductive disorders or any clinical sign of infection were not observed in the inoculated sows. The piglets born from these animals presented no clinical signs or macroscopic lesions that could be attributed to leptospirosis. All inoculated sows presented anti-leptospires antibodies by microscopic serum-agglutination test (MAT) in the postinoculation serum samples and leptospires were not found in the urine as well as were not detected by PCR applied in this material, however, PCR accomplished in kidneys and liver from a euthanized sow presented positive results. Of the total of 12 euthanized piglets, 10 (83.3%) presented positive results by PCR in at least one of the kidney, liver, heart, spleen, lung and gastric content samples. The present study reports the vertical transmission of the infection and the detection of leptospires in clinically healthy piglets born from experimentally infected sows, which is important of the epidemiological point of view as the maintenance of clinically healthy infected animals may allow the persistence of the bacteria in the herd, exposing other animals to the risk of infection.

Key words: Experimental swine leptospirosis, *Leptospira* spp., serovar Canicola, vertical transmission

INTRODUCTION

The production and productivity indexes of swine herds can be influenced by several factors as genetic, environmental, nutritional, toxic, management and infectious. Among infectious diseases, leptospirosis occupies an important position (23). This infection, considered as reemerging in some countries, is a worldwide spread zoonose (26). Leptospires are important etiological

agents of reproductive disorders in swine and although they can cause lesions in several organs, preferentially localize in the kidneys, where they multiply and are eliminated through the urine (7,22).

The epidemiology of swine leptospirosis is potentially very complicated, since swine can be infected by any of the pathogenic serovars. Fortunately, only a small number of serovars will be endemic in any particular region or country.

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Furthermore, leptospirosis is a disease that shows a natural nidality, and each serovar tends to be maintained in specific-maintenance hosts. Therefore, in any region, pigs will be infected by serovars maintained by pigs or by serovars maintained by other animal species present in the area. The relative importance of these incidental infections is determined by the opportunity that prevailing social, management, and environmental factors provide for contact and transmission of leptospires from other species to pigs (4).

The clinical signs associated with leptospiral infection often include poor reproductive performance. Pomona and Tarassovi serovars were reported by Pritchard *et al.* (25) as causing abortions, stillbirths and the birth of weak piglets. Hathaway and Little (17) and Mousing *et al.* (21) found a strong association between serovar Bratislava and poor reproductive performance of swine. Hathaway (16) stated that serovars Hardjo and Canicola have been related in causing reproductive disorders in swine. The Icterohaemorrhagiae serogroup causes acute illness in piglets (usually with spontaneous recovery), and an association with reproductive problems in adult swine has been suspected (9,16).

Throughout the world, the *Leptospira* spp. serovars more frequent isolated from swine are Pomona, Tarassovi, Bratislava, Grippothyphosa and, with smaller predominance, Icterohaemorrhagiae and Canicola (7). The first isolations of leptospires in Brazilian swine were accomplished by Guida (14) in São Paulo state. Guida *et al.* (15), Santa Rosa (29), Santa Rosa *et al.* (30), Santa Rosa *et al.* (31), Cordeiro *et al.* (3) and Oliveira *et al.* (24) described isolations of the serovars Canicola, Pomona, Icterohaemorrhagiae and Hyos.

The pathogenesis of the reproductive disease is poorly understood, but some authors believe that transplacental infection, occurring during a very limited period of maternal leptospiremia, is the sole cause (4). The isolation of leptospires from aborted fetuses, stillbirths and weak piglets was obtained in several occasions (5,8,27), however, the bacteria was never detected in apparently healthy piglets born from apparently healthy infected dams. Although organisms belonging to the Canicola serogroup have been recovered from swine in several countries (4), little is known on the epidemiology of serovar Canicola infection in pigs. The present work describes the experimental infection of sows with *Leptospira interrogans* serovar Canicola, the vertical transmission of the infection and the detection of leptospires in apparently healthy pigs.

MATERIALS AND METHODS

Animals

Six sows of the Landrace breed with a known date of service and pregnancy confirmed by ultrasonography were used. The animals were housed in separate pens with water and commercial

ration ad libitum. During a preinoculation period of at least 3 days no fever or other sign of disease was observed in the animals. Antibodies against 24 *Leptospira* spp. serovars were not demonstrable in a 1:100 dilution of their sera at the time of inoculation.

Leptospira spp. strain

Leptospira interrogans serovar Canicola isolated from slaughtered pigs (10) and typed by monoclonal antibodies kit (Royal Tropical Institute, Amsterdam, Netherlands) was used in this study. The strain was originated from a 10% suspension (w/v) of hepatic tissue from hamsters experimentally infected and euthanized in the agonizing phase of leptospirosis. In this occasion, the strain had the characteristic of killing hamsters with jaundice and hemorrhages between four and eight days of inoculation. The leptospire counting in the suspension of hepatic tissue was performed in dark field microscopy (6), and the inoculum dilution which presented 10-20 bacteria per 200x microscopic field was used.

Experimental infection and sample collection

The sows were inoculated intravenously in the marginal vein of the ear with 5 ml of inoculum between 76 and 90 days of gestation. These animals were observed twice daily for clinical signs of disease and rectal temperature was measured three times daily for a period of seven days. Other arguments as water and food consumption and vaginal discharge were also evaluated during the experiment.

Blood samples were collected three times through the cranial vena cava, being the first sample obtained before the experimental infection. The other samples were obtained at 30 days intervals. Sera were harvested following centrifugation of clotted blood and were stored at -20°C until analysis.

Close to farrowing, urine samples (one per sow) were taken by diuretic treatment (furosemide) or natural micturition for microbiological culture and polymerase chain reaction (PCR). After farrowing, one sow was euthanized and their kidneys and liver were examined by PCR. One week after farrowing, at least one piglet per sow, totalizing 12 piglets, was euthanized and samples of liver, kidneys, lungs, heart, spleen and gastric content were taken and examined by PCR.

Serological test

For detection of anti-leptospires antibodies, the microscopic serum-agglutination test (MAT) was carried out following Galton *et al.* (11) and Cole *et al.* (2). Live cultures of 22 pathogenic and 2 saprophytic *Leptospira* spp. serovars were used: Australis, Bratislava, Autumnalis, Butembo, Castellonis, Bataviae, Canicola, Whitcombi, Cynopteri, Sentot, Grippothyphosa, Hebdomadis, Copenhageni, Icterohaemorrhagiae, Panama, Pomona, Pyrogenes, Wolffi, Hardjo, Shermani, Tarassovi, Javanica, Andamana e Patoc. The cultures were kept from 5 to

10 days at 28°C in EMJH medium enriched with sterile inactivated rabbit serum (1). All sera were initially tested at 1:100 dilution and those that presented at least 50% of agglutination at this dilution were considered positive. They were then serially diluted until the maximum positive dilution was determined. The titer of antibodies was the reciprocal of the higher positive dilution that presented 50% of agglutination.

Microbiological culture

The leptospiral isolation from urine of the experimental infected sows was performed by adding 0.1 ml of urine in 0.9 ml of sterile Sorensen buffered saline resulting in the dilution 10⁻¹. From this dilution, 2 serial 10-fold dilutions (10⁻² and 10⁻³) were prepared (13). Each dilution (0.5 ml) was transferred into tubes containing 5ml of Fletcher medium supplemented with 10% rabbit serum and incubated in aerobic atmosphere at 28°C. The cultures and subcultures were observed weekly in dark-field microscopy for up to 12 weeks (7).

Polimerase chain reaction (PCR)

DNA extraction was done by enzymatic lysis with proteinase K followed by phenol-chloroform-isoamyl alcohol from 300 µL of each sample; the DNA extraction steps were done basically as describe by Sambrook *et al.* (28). The primer used was that proposed by Mérien *et al.* (19), corresponding to nucleotides 38 to 57 (5' GGCGGCGC GTCTTAAACATG 3') and 348 to 369 (5' TCCCCCATTGAGCAAGATT 3') of the 16 S rRNA gene primary structure of the *Leptospira interrogans* serovar Canicola. DNA sample amplification was done in 500 µL microtubes, with a 50 µL final volume. The reaction mixture consisted of 18.7 µL ultrapure water, 5.0 µL 10x reaction buffer (500 mM KCl; 15 mM MgCl₂; 100 mM tris-HCl, pH 9.0), 8.0 µL dNTPs mixture (200 mM of each nucleotide [dCTP, dATP, dGTP, dTTP]), 4.0 µL each oligonucleotide (10 pmol/µL), 0.3 µL *Taq*

DNA-polymerase (5 units per µL) and 10 µL extracted DNA sample, and the mixture was placed in a thermocycler.

The amplification cycle used was the one recommended by Mérien *et al.* (19), added with an initial step at 94°C for 5 minutes. Visualization of the amplified product (330 base pairs) was done by electrophoresis in a 2.0% (w/v) agarose gel stained with ethidium bromide, using TBE 0.5 X (0.04 M tris-acetate and 0.001M EDTA, pH 8.0) as running buffer.

RESULTS AND DISCUSSION

The inoculated sows did not present significant increases in rectal temperature during the seven days postinoculation. Regarding water and food consumption and vaginal discharge, no alteration was observed during the period of the experiment. Reproductive disorders in the inoculated sows were also not observed, as well as clinical signs or lesions in the piglets born from these animals.

The results obtained with the material collected from sows are presented in Table 1. All inoculated sows presented anti-leptospire antibodies in the postinoculation serum samples. Leptospire were not found in the urine and were not detected by PCR applied to this material, however, PCR accomplished in kidneys and liver of the euthanized sow presented positive results.

The results obtained with the material collected from piglets are presented in Table 2. Of the total of 12 euthanized piglets, 10 (83.3%) presented positive results by PCR in at least one of the kidney, liver, heart, spleen, lung and gastric content samples.

In adult swine, the acute phase of leptospirosis is usually characterized by transient anorexia, pyrexia and occasionally diarrhea and hemoglobinuria (4). In the present study, all sows inoculated with *Leptospira interrogans* serovar Canicola developed a subclinical infection, which is in agreement with

Table 1. Results obtained in the sows experimentally infected with *Leptospira interrogans* serovar Canicola regarding to detection method and type of sample.

Sow identification	Detection method/type of sample						
	MAT (titers)/serum			PCR/urine	Microbiological culture/urine	PCR/kidney	PCR/liver
	BI ^a	PI-1 ^b	PI-2 ^c				
AL060	NR	400	400	-	-	+	+
AL158	NR	1600	800	-	-	NP	NP
AL026	NR	100	100	-	-	NP	NP
AL168	NR	100	200	-	-	NP	NP
TF346	NR	400	200	-	-	NP	NP
AL062	NR	800	200	-	-	NP	NP

(+) positive; (-) negative; (NP): not performed; (NR): not reactant at 1:100 dilution; ^aCollection before inoculation; ^bFirst collection postinoculation; ^cSecond collection postinoculation.

Table 2. Results obtained by polymerase chain reaction (PCR) in different materials of piglets born from sows experimentally infected with *Leptospira interrogans* serovar Canicola.

Piglet no./sow identification	Type of material					Gastric content
	Kidney	Liver	Heart	Spleen	Lung	
1/AL060	+	+	+	+	+	+
2/AL060	+	+	+	+	+	-
3/AL060	-	-	-	-	-	+
4/AL060	-	-	-	-	-	+
5/AL060	+	+	+	+	+	+
6/AL060	+	+	+	+	+	+
7/AL158	+	+	+	+	+	+
8/AL026	+	+	+	+	+	+
9/AL168	-	-	-	-	-	-
10/TF346	-	-	-	-	-	-
11/AL062	-	-	+	-	+	+
12/AL062	+	-	+	-	-	-

(+) positive; (-) negative.

Genovez *et al.* (12) and Inzana and Dawe (18). Anti-leptospire antibodies detected by MAT were found in all sows with titers ranging from 100 to 1600, and leptospire DNA was detected by PCR in kidneys and liver from the euthanized sow, however, leptospiruria did not occur in any sow. Distinct results were obtained by Michna (20), which referred a leptospiruria period of 90 days in infected swine and the ability of the serovar Canicola to survive for up to 6 days in swine urine. These observations associated to the absence of clinical signs of infection indicate a low pathogenicity of the serovar Canicola for sows. Similar results for other *Leptospira* spp. serovars as Autumnalis, Saxkoebing, Pomona and Icterohaemorrhagiae were observed by Fennestad and Borg-Petersen (8), Genovez *et al.* (12) and Inzana and Dawe (18).

Of the 12 piglets born from the infected sows, 10 (83.3%) were infected by transplacental route with PCR detection of the leptospire DNA in at least one of the following materials: kidney, liver, heart, spleen, lung and gastric content, however, none of the piglets showed any clinical signs of infection. Ellis *et al.* (5) isolated the serovar Bratislava from aborted fetuses urogenital tract of naturally infected sows. In experimental infections, Fennestad and Borg-Petersen (8) isolated leptospire from stillbirths and weak piglets. The present study reports the detection of leptospire in clinically healthy piglets born from experimentally infected sows, which is important of the epidemiological point of view, as the maintenance of clinically healthy infected animals may guarantee the persistence of the bacteria in the herd, exposing other animals to the risk of infection.

Despite the reports of leptospire isolations from swine in several countries, little is known on the epidemiology of the serovar Canicola infection in these animals. The results of this study refer the importance of serovar Canicola in swine. The fact that clinical signs of infection and the increase of rectal temperature were not observed, and that the piglets infected by transplacental route presented no clinical disorders suggest that swine and *Leptospira interrogans* serovar Canicola represent a well-adapted hosp-parasite relationship.

RESUMO

Detecção de leptospiras em leitões clinicamente saudáveis nascidos de matrizes infectadas experimentalmente com *Leptospira interrogans* serovar Canicola

A Leptospirose é uma importante zoonose que causa transtornos reprodutivos em suínos. O isolamento de leptospiras de fetos abortados, natimortos e leitões fracos foi obtido em várias ocasiões, porém, a bactéria nunca foi isolada de leitões aparentemente saudáveis nascidos de matrizes com infecção subclínica. Seis matrizes da raça Landrace com data de serviço conhecida e gravidez confirmada por ultrasonografia foram infectadas entre 76 e 90 dias de gestação por via intravenosa com 5 ml de inóculo de *Leptospira interrogans* serovar Canicola, e uma semana após o parto, pelo menos um leitão por matriz foi eutanaziado e foram colhidas amostras de fígado, rins, pulmões, coração, baço e conteúdo gástrico para exame por PCR. Transtornos reprodutivos ou qualquer sinal clínico de infecção não foram observados nas matrizes inoculadas. Os leitões nascidos destes animais não apresentaram sinais clínicos ou lesões macroscópicas que poderiam ser atribuídos à leptospirose. Todas as matrizes inoculadas apresentaram anticorpos anti-leptospiras no teste de soroadglutinação microscópica (MAT) nas amostras de soros colhidas após a inoculação, e não foram encontradas leptospiras na urina como também não foram detectadas pela PCR aplicada neste material, porém, a PCR realizada em rins e fígado de uma matriz eutanaziada apresentou resultado positivo. Dos 12 leitões eutanaziados, 10 (83,3%) apresentaram resultados positivos na PCR em pelo menos uma das amostras de rim, fígado, coração, baço, pulmão e conteúdo gástrico. O presente estudo relata a transmissão vertical da infecção e a detecção de leptospiras em leitões clinicamente saudáveis nascidos de matrizes infectadas experimentalmente, o que é importante do ponto de vista epidemiológico, pois a manutenção de animais com infecção subclínica pode permitir a persistência da bactéria no rebanho, expondo outros animais ao risco de infecção.

Palavras-chave: Leptospirose suína experimental, *Leptospira* spp., serovar Canicola, transmissão vertical

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