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SCIENTIFIC COMMUNICATION

POSITIVE PCR FOR *LEPTOSPIRA* SPP. IN A SOW FROM A GERMAN HERD PRESENTING ANIMALS WITH MAT TITRES FOR *LEPTOSPIRA INTERROGANS* SEROVAR BRATISLAVA

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ABSTRACT

Reports based on serological data indicate *Leptospira* (L.) *interrogans* serovar Bratislava infection in pigs in parts of Germany and other countries. Two sows of a pig breeding herd located in Germany and which showed titres of 200 and 800 against L. *interrogans* serovar Bratislava in MAT were followed up under experimental conditions with microbiological and PCR methods for detection of leptospires. Cultures of urine and organs were negative for leptospires in both pigs. The PCR was also negative in all samples of one pig, but was positive in samples from uterus, oviduct and ovary of the other animal. The histological investigation of the kidneys from this pig showed an interstitial glomerulonephritis. The positive results of the PCR demonstrated the tropism of leptospires for the genital tract. This fact may be responsible for more unexpected abortion and infertility in pig breeding herds.

KEY WORDS: Leptospirosis, swine, serology, PCR, genital tract.

RESUMO

PCR POSITOVA PARA *LEPTOSPIRA* SPP. EM UMA PORCA DE UMA CRIAÇÃO DA ALEMANHA APRESENTANDO ANIMAIS COM TÍTULOS PARA *LEPTOSPIRA INTERROGANS* SOROVAR BRATISLAVA, NO TESTE DE SOROAGLUTINAÇÃO MICOSCÓPICA. Inquéritos sorológicos indicaram a presença de infecção por *Leptospira interrogans* sorovar Bratislava em partes da Alemanha e em outros países do mundo. Duas porcas de uma criação de suínos da Alemanha que apresentaram títulos de 200 e 800 para *L. interrogans* sorovar Bratislava no teste da soroaglutinação micoscópica (SAM) foram monitoradas em condições experimentais com o emprego de métodos microbiológicos e moleculares (PCR) para a detecção de leptospira. Os cultivos de urina e órgãos foram negativos para leptospiras nos dois animais. A PCR também foi negativa em todas as amostras de uma das porcas, porém foi positiva em amostras de útero, oviduto e ovário do outro animal. As investigações histológicas dos rins desta porca apresentaram lesões de glomerulonefrite intersticial. Os resultados positivos da PCR demonstraram o tropismo das leptospiras para o trato genital. Este fato pode ser responsável por transtornos reprodutivos como abortamentos e infertilidades nos rebanhos.

PALAVRAS-CHAVE: Leptospirose, suínos, sorologia, trato genital.

Leptospira interrogans serovar Bratislava was originally isolated from a hedgehog in Czechoslovakia in 1953 and this animal has been considered as the maintenance host for the serovar in Europe (SEBEK & ROSICKY, 1975). Results of serological studies indicated Bratislava infection in pigs in parts of Germany and other countries. Nevertheless, isolation sometimes failed due to the fastidious cultivation of pig-adapted strains of Bratislava. Early reports about antibodies of strains belonging to the Australis serogroup in pig sera, in particular for the Bratislava serovar, dismissed such finding as being due to non-specific factors (DOBSON, 1974; WEBER, 1978). The first isolation of *L. interrogans* serovar Bratislava strain Berlin 406, from

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a pig in Germany was reported in 1987 (Schönberg et al., 1992). Serological reactions against Bratislava in connection with unsolved problems of infertility in recent years highlighted the necessity to get more information about clinical significance and epidemiology of leptospires infection in the German pig population (Ortmann & Schönberg, 1996; Schönberg et al., 1999).

A pig-breeding herd received female pigs from another breeder. In the microscopic agglutination test (MAT) 2 sows (age: 7 months) of these received animals showed titres of 200 (n 476) and 800 (n 468) against *L. interrogans* serovar Bratislava. These 2 animals were followed up under experimental conditions. Urine samples were collected every two weeks during 6 months of observation after injection of furosemide (furosemide-ratiopharm^R 20, ratiopharm GmbH). For urine collection 0.5 mg / kg furosemide was injected (Hahn, 1987). The middle stream urine of the second miction was collected. Different media were used for cultural isolation (Table 1). The basic medium consisted of Tween / Bovine Serum Albumin completed with rabbit serum, inhibitors and 0.15% agar (Hahn, 1987; Schönberg et al., 1992).

After collection of the urine samples dilutions were prepared in bovine serum albumin diluent (BSAD) (Ellinghausen, 1973). Two hundred microlitres of 10^{-1} and 10^{-2} dilutions were inoculated into culture tubes in duplicate. The culture was checked once a week over 4-6 months.

The MAT was repeated after three months and before slaughtering. The sows 476 and 468 were slaughtered respectively at 6 and 8 months after beginning of the study. Samples of kidneys and genital tract (uterus, oviduct, and ovary) were investigated by cultivation and Polymerase Chain Reaction (PCR). In addition, the organs including the liver were examined histologically.

Three samples of 10 g were taken from each kidney and homogenized with a Colworth Stomacher 400 for 1 min. in 50 mL BSAD. From the other organs one sample as far as available was homogenized in the same way. Two hundred microlitres of 10^{-2} and 10^{-3} dilutions were inoculated into culture tubes in duplicate. The culture was checked in the same way as described for urine samples.

For the PCR 20% tissue suspensions were prepared by homogenization using PBS (e.g. 20 g kidney in 100 mL PBS). Suspensions (1.8 mL) were transferred into tubes and stored at -20º C until carrying out the PCR.

The PCR, primers were used as proposed by Mérien et al. (1992), according to the protocol of Heinemann et al. (2000) and Richtzenhain et al. (2002):

DNA extraction: 400 µL of TE (Tris-HCl 10 mM, EDTA 1 mM, pH 8,0) were added to 200 µL of the tissue suspension. The suspension was shaken for 10 sec. and centrifuged at 13000xg for 5 min. The pellet was suspended in 400 µL of TE buffer, vortexed and boiled for 15 min. The obtained DNA was purified by mixing with an equal volume of saturated phenol and vortexed for 3 min. After centrifugation at 13000xg for 5 min the supernatant was carefully transferred to another microtube and mixed with a half volume of phenol: chloroform: isoamyl alcohol (25:24:1). The pellet was resuspended in 30 µL TE buffer and stored at -20º C until used for DNA amplification.

DNA amplification: Enzymatic amplification was carried out in a total of 50 µL containing 1 x PCR buffer, 200 mM of each dNTPs, 1.5 mM $MgCl_2$, 25 pmol of each primer Lep 1 (5'GGC GGC GCG TCT TAA ACA TG 3') and Lep 2 (5'TTA GAA CGA AGT TAC CCC CTT 3'), 2.5U of Taq DNA Polymerase and 10 µL of extracted DNA. Amplifications were performed in a thermocycler with an initial denaturation step at 94º C (3 min.), followed by 35 cycles of denaturation step at 94º C (3 min.), followed by 35 cycles of denaturation at 94º C (1 min.), annealing at 60º C (1 min) and extension at 72º C (1 min.).

Leptospira interrogans pure cultures obtained from reference laboratory (CDC-EUA) were used as positive control. Leptospires negative control tissue suspensions were collected from a non-inoculated hamster.

The specific amplicon of 330bp fragments was visualized after electrophoresis in 2% agarose gel in the presence of ethidium bromide.

Table 1 - Media for cultivation of leptospires.

		Inhibitory Substances / mL				
n	Medium	Rifampicin	5-Fluorouracil	Amphotericin B	Rabbit Serum	Agar
1	EMJH	-	-	-	0.4%	-
2	EMJH	10 µg	100 µg	2 µg	0.4%	-
3	EMJH	10 µg	100 µg	2 µg	0.4%	0.15%
4	Tween 40 / 80[1]	10 µg	100 µg	2 µg	0.4%	0.15%
5	Tween 40 / 80[1,2]	-	100 µg	-	0.4%	0.15%

[1]According to Ellis (1986).

[2]According to Ellis (1986) used as transport medium by Brem et al. (1987).

Table 2 - Results of different methods for the detection of leptospires in urine and organs from two pigs.

Pig	Serology MAT	Cultivation		PCR	Pathology	
		Urine samples	Organs samples		Histology	PAP Tech.
476	200 to 100	negative	negative	positive (uterus, oviduct, ovary)	positive nephritis	negative
468	800 to 200	negative	negative	negative	negative	negative

The Warthin-Starry staining method and the peroxidase-antiperoxidase (PAP) technique (BOURNE, 1983) were carried out for histological investigation. *L. interrogans* serovar Bratislava antiserum was used prepared by immunization of rabbits with the isolate from a pig, strain 406 (SCHÖNBERG *et al.*, 1992).

The results are summarized in Table 2. The MAT titres of pig 476 and 468 dropped respectively from 200 to 100 and from 800 to 200, before slaughtering.

All urine and organ samples were negative.

The PCR was also negative in all samples of pig 468, however, the PCR was positive in samples from uterus, oviduct and ovary of pig 476 (Fig. 1).

Histological lesions were not detected in the samples of pig 468. However, the histological investigation of the kidneys from pig 476 showed an interstitial glomerulonephritis. Histological lesions were not detected in the genital tract and liver. The PAP technique gave also negative results.

Swine frequently are not infected with clinical symptoms but shed leptospires with the urine in large amounts for a long period up to one year after infection (FAINE, 1982). In pigs, infected with serovar Bratislava, the elimination with the urine is irregular and the number of separated leptospires is low (ELLIS, 1991). Pigs may harbour serovar Bratislava in their genital tract for a long period after infection (ELLIS et al., 1995; ELLIS et al., 1986b, ELLIS & THIERMANN, 1986c; ELLIS, 1991).

SCHÖNBERG et al. (1992) reported the first isolation of *L. interrogans* serovar Bratislava from a pig in Germany in 1987. This finding initiated more detailed studies on the clinical significance and epidemiology of serovar Bratislava infection in the German pig population. Results confirm that the Bratislava serovar is extremely fastidious and difficult to isolate (BOLIN & CASSELIS, 1990).

The negative results in isolation of leptospires from urine and organs do not prove that the pigs are free from leptospires. Furthermore, the positive results of the PCR in samples from uterus, oviduct and ovary of pig 476 demonstrated the tropism of the infective strain to the genital tract. This is very important under the consideration of the fact that leptospires could be transmitted by the venereal pathway (ELLIS et al., 1986 d).

Various reports (ORTMANN & SCHÖNBERG, 1986; SCHÖNBERG et al., 1999) demonstrated that serovars of the Pomona and Sejroe serogroup were responsible for abortion in pigs. The serovars, strains Vehlefanz 1 and 2, of the Pomona serogroup could be identified as *L. kirschneri* serovar Mozdok.* The pig herd, in which abortions were caused by *L. kirschneri* serovar Mozdok in 1999 had abortion in 2002, too. Kidneys from 2 sows, that aborted, were prepared for isolation, with leptospires being isolated from both animals. These isolates were classified as serovars of the Pomona serogroup, perhaps also *L. kirschneri* serovar Mozdok.

1. molecular weight 100 bp
2. kidney, left side
3. uterus
4. oviduct
5. ovaries
6. negative DNA extraction control
7. positive amplification control
8. negative amplification control

Fig. 1 - Results of PCR for detection of leptospires from pig 476.

*Kidneys from 2 sows that aborted were preoared for isolation, with Dr. Hartskeerl, Royal Tropical Institute Amsterdam.

All these results show, that *L. interrogans* serovar Bratislava could not be identified as source for abortion until now, although the first isolation of serovar Bratislava from a pig in Germany was in 1987. However, the serological reactions against serovar Bratislava in pig herds and the positive PCR results in the genital tract might be an indication that abortions can be caused by this *Leptospira interrogans* serovar.

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