Molecular diagnosis of *Leptospira* spp. in culled sows

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Abstract

Leptospirosis diagnosis was performed through molecular, histopathological and serological tests in 30 culled sows in Rio Grande do Sul, Brazil. The objectives were to compare the efficiency of the three methods, to verify the sensitivity of a PCR methodology using a single primer based on the sequence of a repetitive element of *Leptospira interrogans* genome, as well as to verify the possible detection of Leptospira in several tissue including the genital tract of sows. The animals were selected based on the microscopic agglutination test in order to have sows with negative and positive results, presenting low and higher serologic titers. The higher frequency (90 % of the positive sows) and titers (100 to 800) was observed for *L. interrogans* serovar *bratislava*. Leptospira was detected by histopathology in nine sows only, all presenting higher serologic titers (at least 100). A PCR product of 438 bp was observed in all animals (25 kidneys, 24 uterus and 9 oviduct) fragments. Similar PCR product was obtained for DNA from cultures of other pathogenic leptospires, while the pattern observed for the non-pathogenic *L. patoc* was distinct. No *Leptospira spp* DNA amplification product was detected in *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella sp, Streptococcus sp* and *Staphylococcus aureus* DNAs obtained from cultures, or in blood DNA samples of two piglets. The molecular system was therefore specific and the most effective to detect low pathogen levels, being able to differentiate pathogenic from non-pathogenic leptospires.

Key-words:
Molecular diagnosis.
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Introduction

Before 1989 the genus *Leptospira* had been considered as having only two species, *Leptospira interrogans* and *Leptospira biflexa*. The former included the pathogenic species involving at least 200 serovars and 23 serogroups, while the other comprised the non-pathogenic species[1].

Through molecular hybridization ten different species were demonstrated[2] and a new one described, *L. kirschner*[3]. Recently 16 species could be defined[2,3] including those previously described and indeed pathogenic and non pathogenic serovars occurs within the same species[4,5].

The microscopic agglutination test (MAT) is the reference method to leptospirosis diagnosis. However its interpretation is complicated by the high degree of cross-reaction that occurs between different serogroup, especially in acute-phase samples[6] and the presence of several common antigens among leptospires[7].

Molecular diagnosis has been attempted and several primers have been

described based on rRNA genes for PCR detection of leptospires and leptospiral DNA has been amplified from serum, urine, aqueous humor and tissues[8]. However few PCR systems have been shown to amplify leptospiral DNA from either human or veterinary clinical samples[8,9] and primers derived from rRNA had shown low specificity and sensitivity[10]. Barocchi et al.[11] described new primers based on a Leptospira specific repetitive element and although *L. interrogans* serovar *icterohaemorrhagiae* and *copenhageni* presented the same pattern it was possible to distinguish eleven Leptospira species in humans patients.

The present paper compared the efficiency of molecular, histopathological (silver staining of leptospires into renal tissue) and serological methods, to verify the sensitivity of a PCR methodology using a single primer based on the sequence of a repetitive element of *Leptospira interrogans* genome, as well as verified the possible detection of *Leptospira sp* in several tissue including the genital tract of sows.

Material and Methods

Thirty culled sows discarded from Rio Grande do Sul state farms were selected among 288 other analyzed in a previous paper[12]. Negative and positive samples analyzed by microscopic agglutination test (MAT), with low and high title levels to only one or more serovars were included in this study. Samples of kidney, uterus and oviduct had been obtained during slaughtering and stocked at –20 °C until the molecular tests. Blood of each animal was collected for serology, and sera was stored also at –20 °C, until MAT.

Kidney samples were also fixed in 10% formalin for the histopathological tests, performed according to the classical Warthin Starry method, for visualization of Leptospira in the tubules by silver staining.

Serum samples were analyzed by MAT[13] using live antigen suspension of *Leptospira australis, L. autumnalis, L. bratislava, L canicola, L. grippotyphosa, L. hardjo, L. hebdomadis, L. icterohaemorrhagiae, L. pomona, L. pyrogenes, L. tarassovi* and *L. wolffi.*

Genomic DNA was isolated from swine blood, kidney, uterus and oviduct using the phenol:chloroform: isoamyl alcohol protocol[14]. The same methodology was used for DNA isolation from pattern cultures of positive (*Leptospira interrogans* sorovar *L. icterohaemorrhagiae, L. copenhageni, L. pomona, L. bratislava* and *L. patoc*) and negative controls (*Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella sp, Streptococcus sp* and *Staphylococcus aureus*). Genomic DNA was also obtained from blood samples of 6 alive pigs, 4 adults and 2 piglets (7 days and 4 months old) as control.

Molecular analysis were performed by polymerase chain reaction using as primer a sequence surrounding a repetitive element of the *L. interrogans,* sorovar *copenhageni* genome (5´- GCGGACTCATACCCGCT – 3´)[11]

The amplification program consisted of an initial denaturation of 94º C for 5 min., 35 cycles of 94º C for 30 seg., 50º C for 1.5 min., and 72º C for 4 min., followed by a final extension of 72º C for 7 min.[11].

The PCR products of 438 bp were analyzed on horizontal electrophoresis on 2% agarose gel, with ethidium bromide staining and UV visualization.

Results

Table 1 presents the MAT, molecular tests and histopathological results of 30 culled sows. Thirty-three per cent of the animals presented negative results in serological and histopathological tests but positive results to PCR, 37% were negative by histopathology and positive by serology and PCR and the others presented positive results in all of the tests. *Leptospira bratislava* was the most frequent positive reaction being observed in 90% of the positive reactors. Among the 6 alive pigs analyzed as negative controls four presented the 438bp product and only two piglets were in fact negative.

The same PCR product (438bp) was

Table 1 - Results of molecular, histopathological and serological tests

Sows *	PCR kidney	PCR uterus	PCR oviduct	Histopathology	Serology**
2	POS	-	-	NEG	NEG
3	-	POS	POS	NEG	NEG
1	-	POS	-	NEG	NEG
2	POS	POS	-	NEG	NEG
1	POS	POS	POS	NEG	NEG
1	POS	-	POS	NEG	NEG
7	POS	POS	-	NEG	100 (b)
1	POS	POS	-	POS	100 (b)
1	POS	POS	-	POS	100 (i)
1	POS	POS	-	NEG	100 (b, g, a)
1	POS	-	-	NEG	200(b,i);100 (gh)
1	POS	POS	-	POS	200 (b, h, i)
1	POS	-	-	POS	200 (b, g, py)
1	POS	POS	-	NEG	200 (b); 100 (h)
1	POS	POS	-	POS	400 (b); 100 (g)
1	POS	POS	POS	POS	400 (b); 100 (i,p)
1	POS	POS	POS	POS	400 (py)
1	POS	POS	POS	POS	800 (i); 200 (b) 100 (py)
1	POS	POS	-	POS	800 (b); 200(gh)
1	POS	POS	-	NEG	800 (b, h)

(*) = culled sows, grouped according to the results
(**) = *Leptospira sp* serovars and titers by MAT: (a) = *L. australis,* (b) = *L.bratislava*, (c) = *L. canicola*, (g) = *L. grippotyphosa,* (h) = *L. hardjo* , (i) = *L. icterohaemorrhagiae*, (p) = *L. pomona*, (py) = *L. pyrogenes*
POS = positive results; NEG = negative results; (-) = not tested

seen in genomic DNA obtained from culture of *Leptospira interrogans* sorovar, *L. icterohaemorrhagiae, L. copenhageni, L. pomona* and *L. bratislava*. However no *Leptospira sp* DNA product was detected in DNAs obtained from cultures of *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella sp, Streptococcus sp* and *Staphylococcus aureus*, while a different PCR product was observed for the non-pathogenic *L. patoc.*

Discussion

Leptospirosis is one of the most important reasons for reproductive problems in swine, resulting in abortions, stillborns and debilitated piglets that die soon after delivery. Contamination could result from direct contact with the urine, oral or nasal mucous membrane, conjunctive and skin of infected animals or through ingestion of contaminated water or food, by the contact with infected soil or by genital canal[15].

The most common serovars detected in swine all over the world are *pomona, icterohaemorrhagiae, canicola, tarassovi, grippotyphosa* and *bratislava*[16], *L. pomona* being the most frequent in Brazil[17,18]. However, other serovars have been described more recently in this country, such as *bratislava, djasiman, grippotyphosa, hardjo, icterohaemorrhagiae*[19,20,21].

The serological tests performed in the present sample indicated high frequency of *L. interrogans* sorovar *bratislava* (90 % of the

positive sows), with titers from 100 to 800, which is in accordance with the data previously described for Rio Grande do Sul[12,21]. As expected the histological test presented the lower sensivity since a negative result in it does not exclude the occurrence of the pathogen in other parts of the tissue.

The PCR test was more sensitive than the serological or histopathological and indicated a very high frequency of leptospirosis in culled sows in Rio Grande do Sul, since positive results were obtained for all animals except two piglets. Leptospiral DNA from the different organs showed the same product (438 pb) obtained also in DNA samples of pathogenic *Leptospira* sp. and was identified in 25 kidneys as well as in 24 uteri and 9 oviduct fragment. This is the first description of the detection of *Leptospira* sp in genital tract of pigs in Brazil, and confirms the serologic positives results of *L. bratislava* previously described [19]. Although the PCR test here employed did not identify the serovar it could discriminate very well between pathogenic and non-pathogenic leptospires. The DNA sequences of six samples were similar to that of *L. interrogans* genome confirming the PCR results.

The PCR test seems to be more effective to detect low levels of infection. However it is possible that animals with negative serology but with positive PCR results are in fact non symptomatic carriers of leptospires.

Conclusions

The comparison of molecular, histopathological (silver staining of leptospires into renal tissue) and serological methods to detect *Leptospira sp* in culled sows from Rio Grande do Sul indicated high efficiency of the PCR method which seems to be more effective to detect low levels of infection. However it is possible that animals with negative serology but with positive PCR results are in fact non-symptomatic carriers of leptospires. Although the PCR test here employed did not identify the serovar it could discriminate very well between pathogenic and non-pathogenic leptospires. Leptospiral DNA from the different organs showed the same product (438 pb) obtained also in DNA samples of pathogenic *Leptospira* sp. and was identified in 25 kidney as well as in 24 uterus and 9 oviduct fragments, being the first description of the detection of *Leptospira* sp in genital tract of pigs in Brazil.

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Diagnóstico molecular de *Leptospira spp* em matrizes suínas descartadas

Resumo

O Diagnóstico de leptospirose foi efetuado através de método molecular, histopatológico e sorológico em 30 matrizes suínas, descartadas, no Rio Grande do Sul, Brasil. Os objetivos foram comparar a eficiência dos 3 métodos, verificar a sensibilidade de um método de PCR que utiliza um primer único baseado na seqüência de um elemento repetitivo do genoma de *Leptospira interrogans,* bem como verificar a possível detecção de leptospiras em vários tecidos, incluindo o trato genital. Os animais foram selecionados com base no teste de aglutinação microscópica para incluir tanto animais negativos como positivos e com baixos e altos títulos sorológicos. As maiores freqüências (90 % dos aniamis positivos) e títulos (100 to 800) foram observados para *L. interrogans* serovar *bratislava*. Leptospiras

Palavras-chave:
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Porcas descartadas.

foram detectadas por histopatologia em apenas 9 matrizes, todas com altos títulos (pelo menos 100). Um produto de PCR de 438 bp foi observado em todos os animais (fragmentos de 25 rins, 24 úteros e 9 ovidutos). Produtos de PCR similares foram obtidos em DNA de culturas de leptospiras patogênicas, enquanto a não patogênica, *L. patoc* apresentou um padrão distinto. Nenhum produto de amplificação de DNA de *Leptospira spp* foi detectado em DNA de culturas de *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella sp, Streptococcus sp* and *Staphylococcus aureus*, ou de sangue de dois leitões. O método molecular foi, assim, específico e o mais eficiente para detectar baixos níveis de patógeno, sendo capaz de diferenciar leptospiras patogênicas e não patogênicas.

References

1 FAINE, S.; STALLMAN, N. D. Amended descriptions of the genus *Leptospira* Noguchi 1917 and the species *L. interrogans* (Stimson, 1907) Wenion 1926 and *L. biflexa* (Wolbach and Binger, 1914) Noguchi 1918. **Int. J. Syst. Bacteriol.,** v. 32, p. 461-463, 1982.

2 YASUDA, P. H. et al. Deoxyribonucleic acid relatedness between serogroups and serovars in the family *Leptospiraceae* with proposals for seven new *Leptospira* species. **Int. J. Syst. Bacteriol.,** v. 37, p. 407-415, 1987.

3 RAMADASS, P. et al. Genetic characterization of pathogenic *Leptospira* species by DNA hybridization. **Int. J. Syst. Bacteriol.,** v. 42, n. 2, p. 215-219, 1992.

4 BRENNER, D. J. et al. Further determination of DNA relatedness between serogroups and serovars in the family *Leptospiraceae*, with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. **Int. J. Syst. Bacteriol.**, v. 49, Pt. 2, p. 839-858, 1999.

5 FEREZU, S. B.; STEIGERWALT, A. G.; BRENNER, D. J. DNA relatedness of *Leptospira* strains isolated from beef cattle in Zimbabwe. **Int. J. Syst. Bacteriol.**, v. 49, Pt. 3, p. 1111-1117, 1999.

6 ADLER, B.; FAINE, S. The antibodies involved in the Human immune response to leptospiral infection. **J. Med. Microbiol.** v. 11, p. 387-400, 1978.

7 CHAPMAN, A. J.; ADLER, B.; FAINE, S. Genus-specific antigens in Leptospira revealed by immunobloting. **Zentbl Bakteriol,** v. 264, p. 279-283, 1987.

8 BROWN, P. D. et al. Evaluation of the polymerase chain reaction for early diagnosis of leptospirosis. **J. Medical Microbiology**, v. 43, n. 2, p. 110-114, 1995.

9 MERIEN, F.; BARANTON, G.; PEROLAT, P. Comparison of polymerase chain reaction with microagglutination test and culture, for diagnosis of leptospirosis. **J. Infectious Diseases,** v. 172, n. 1, p. 281-285, 1995.

10 WAGENAAR, J. et al. Comparison of polymerase chain reaction assays with bacteriologic culture, immunofluorescence and nucleic acid hybridization for detection of *Leptospira borgpetersenii* serovar *hardjo* in urine of cattle. **American Journal of Veterinary Research**, v. 61, n. 3, p. 316-320, 2000.

11 BAROCCHI, M. A. et al. Identification of new repetitive element in *Leptospira interrogans* serovar *copenhageni* and its application to PCR-based differentiation of *Leptospira* serogroups. **Journal of Clinical Microbiology,** v. 39, n. 1, p. 191-195, 2001.

12 OLIVEIRA, S. J. Comprovação de infecção por *Leptospira sp* em matrizes suínas descartadas, através do exame sorológico e da detecção dos microorganismos em rins, útero e oviduto. In: XI CONGRESSO BRASILEIRO DE VETERINÁRIOS ESPECIALISTAS EM SUÍNOS; 11. Goiânia, 2003. **Anais...** Goiânia: [s. n.], 2003. p. 143-144.

13 COLE, J. R.; SULZER, C. R.; PURSSEL, A. R. Improved microtechnique for the leptospiral microscopic agglutination test. **Applied Microbiology**, v. 25, n. 6, p. 976-980, 1973.

14 SAMBROOK, J.; RUSSELL, D. W. **Molecular cloning.** A laboratory manual. 3rd ed. USA: Cold Spring Harbor Laboratory Press, 2001.

15 ELLIS, W. A.; MONTGOMERY, J.; CASSELS, J. A. Dihydrostreptomycin treatment of bovine carriers of *Leptospira interrogans* serovar *hardjo*. **Research in Veterinary Science**, v. 39, n. 3, p. 292-295, 1985.

16 ELLIS, W. A.; BRYSON, D. G.; THIERMANN, A. B. Isolation of leptospires from the genital tract and kidneys of aborted sows. **The Veterinary Record**, v. 118, n. 11, p. 294-295, 1986.

17 OLIVEIRA, S. J. DE; FALLAVENA, L. C. B.; PIANTA, C. Leptospirose em suínos no Rio Grande do Sul. Isolamento e caracterização dos agentes; estudos em suínos abatidos em frigoríficos e em granjas com problemas de reprodução. **Arquivo Brasileiro de Medicina Veterinaria e Zootecnia**, Belo Horizonte, v. 39, n. 5, p. 641-650, 1983.

18 CORDEIRO, F.; RAMOS, A. A.; BARBOSA, M. Aglutininas anti-leptospira em soros de suínos criados em regime semi-selvagem no interior do Estado da

Bahia. **Arquivos da Escola de Veterinaria da Universidade Federal de Minas Gerais,** v. 27, n. 1, p. 59-62, 1975.

19 OLIVEIRA, S. J. DE; LIMA, P. C. R.; BARCELLOS, D. E. S. N.; BOROWSKI, S. M. Sorologia para diagnóstico de leptospirose em suínos no Rio Grande do Sul: resultados obtidos de granjas com e sem problemas de reprodução. **Pesquisa Agropecuária Gaúcha,** Porto Alegre, v. 1, n. 2, p. 263-267, 1995.

20 LANGONI, H. Inquérito soroepidemiológico para leptospirose suína. CONGRESSO BRASILEIRO DE VETERINARIOS ESPECIALISTAS EM SUÍNOS, 7., Blumenau, SC, 1995. **Anais...** Blumenau: [s. n.]**,** 1995. p. 153.

21 OLIVEIRA, S. J. DE; SIMÕES PIRES NETO, J. A. Aspectos etiológicos e de diagnóstico das leptospiroses. **Revista do Conselho Federal de Medicina Veterinária**, v. 10, n. 33, p. 36-46, 2004.