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ISOLATION AND CHARACTERIZATION OF *LEPTOSPIRA INTERROGANS* FROM PIGS SLAUGHTERED IN SÃO PAULO STATE, BRAZIL

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ABSTRACT

With the aim of isolating *Leptospira* spp., blood serum, kidney, liver and genital tract of 137 female swine (40 sows and 97 gilts) and also urine samples from 22 sows were collected in a slaughterhouse in the State of São Paulo, from April 2003 to August 2004. Four isolates were obtained from animals that presented microagglutination test (MAT) titers ≥ 100 for the serovar Pomona and one was obtained from an animal negative by MAT in which *Leptospira* was isolated from the liver and reproductive tract. The presence of leptospiral DNA was investigated by PCR, and positive results were found in kidneys of 11 females, liver of two, genital tract of two and urine of one of them. Nephrosis, interstitial multifocal nephritis, moderate to severe changing, hyalines cylinders and hemorrhagic focuses, hepatic and uterine horns congestion were histological lesions observed in higher frequency in animals positive for leptospira. The silver impregnation (Warthin Starry) confirmed the presence of spirochetes in renal tubules of four females with positive leptospira cultures from kidneys. The serogroup of the five isolates was identified as Pomona by cross agglutination with reference polyclonal antibodies. Molecular characterization of the isolates was carried out by variable-number tandem-repeats analysis. All the isolates revealed a pattern distinct from the *L. interrogans* Pomona type strain, but identical to a previously identified pattern from strains isolated in Argentina belonging to serovar Pomona.

Key-words: Pomona. Swine. VNTR. Culture. PCR. Genital Tract.

INTRODUCTION

Pigs are one of the most important sources of leptospirosis infection for man and other domesticated animal species. Frequently, swine do not show signs of infection but shed large amounts of leptospires in their urine for periods of up to one year following infection (13,24-26,28,36). Pomona, Tarassovi, Canicola and Bratislava serovars of *Leptospira interrogans* and *L. borgpetersenii* have been isolated from pigs in different countries. Until now the serovars isolated from genital tract of swine were Bratislava and München (7-11). Leptospires have been frequently recovered from aborted fetuses, stillborn and weakly born piglets in several countries (51), however in Brazil, the number of studies with isolation of leptospires from swine are scarce (18,42,49,51).

In recent years, direct diagnosis of leptospirosis has been facilitated by the use of molecular techniques such as PCR from urine samples, semen and organs of suspected animals

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(17,26,46,61), however, in spite of presenting high sensitivity and specificity when compared to the bacterial isolation, the indirect diagnosis by demonstration of antibodies against *Leptospira* by MAT is still the most frequently used method. Histopathology by the hematoxilin-eosin and Warthin-Starry staining has been used to demonstrate leptospires and structural lesions in affected organs, although there is no association between them, as the lesions are usually non specific (5,42,48,53, 56). After isolation in appropriate culture media, the bacterium must be characterized by serological methods for serogroup and serovar determination (6,31). More recently, a molecular typing method was developed and used to characterize *L. interrogans* strains at serovar level (33). This method is based on the analysis by PCR of variable-number tandem repeats (VNTR), using seven loci (VNTR 4, VNTR 7, VNTR 9, VNTR 10, VNTR 11, VNTR 19 and VNTR 23). Pavan *et al.* (43) used six of these VNTR loci to examine 16 strains of *L. interrogans* serovar Pomona isolated from animals and humans in Argentina, and their strains were classified as a genotype genetically distinct from the reference strain.

The aim of this investigation was to isolate *Leptospira* spp. and to correlate MAT results with the demonstration of leptospires and lesions in kidneys, liver and genital tract of apparently healthy female swine. The leptospiral isolates obtained in this study were characterized by cross agglutination with polyclonal antibodies and by VNTR analysis.

MATERIALS AND METHODS

Animals and Materials Collected

One hundred and thirty seven female swine (40 sows and 97 gilts) were allocated into 11 groups identified by letters A, B, C, D, E, F, G, H, I, J e K. The samples were collected from April 2003 to August 2004, in a slaughterhouse located in São Paulo State, Brazil.

Microscopic Agglutination Test (MAT)

MAT (4,16) was performed firstly in the 1:100 screening dilution with 24 live reference serovars: Australis, Bratislava, Autumnalis, Butembo, Castellonis, Batavie, Canicola, Whitcombi, Cynopteri, Grippotyphosa, Hebdomadis, Copenhageni, Icterohaemorragiae, Javanica, Panama, Pomona, Pyrogenes, Hardjoprajitino, Wolffi, Shermani, Tarassovi, Andamana, Patoc e Sentot. The second step was the titration of the positive samples by two fold dilutions. After 2-4 h of incubation (28 to 30ºC), the titers were determined as the reciprocal of the highest dilution presenting 50% of agglutination.

Isolation of Leptospires

Samples of 10 g of kidneys, liver, uterus, oviducts and ovaries were collected and homogenized in 50 mL of Sorensen saline (48). One hundred microliters of 10^{-1}, 10^{-2} and 10^{-3} dilutions were inoculated into culture tubes in duplicates containing modified EMJH medium (DIFCO/USA) (1) enriched with 15% rabbit serum, 5-fluorouracil and nalidixic acid, according to Miraglia *et al.* (37). For urine samples, after collection (22 sows), the urine was diluted in Sorensen solution and 100 µL of 10^{-1}, 10^{-2} and 10^{-3} dilutions (48), were inoculated into culture tubes in duplicate (37). The cultures were checked once a week over 4-6 months.

PCR Analysis

A 10% tissue suspensions (w/v) and urine samples (v/v) were prepared by homogenization in Sorensen solution (48). For 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 homogenized for 10 sec and centrifuged at 13000 × *g* for 5 min. The pellet was suspended in 400 µL of TE buffer, vortexed and boiled for 15 min. The suspension was purified by mixing an equal volume of saturated phenol and vortexing for three min. After centrifugation at 13000 × *g* for 5 min, the upper phase was carefully transferred to another microtube and extracted with a half volume of phenol: chloroform: isoamyl alcohol (25:24:1), followed by ethanol precipitation. The precipitate was collected by centrifugation, dried and then resuspended in 30 µL of TE buffer and stored at -20ºC until used for DNA amplification. The primer set used was that proposed by Mérien *et al.* (19), corresponding to nucleotides 38 to 57 (5' GGCGGCGCGTCTTAAACATG 3') and 369 to 348 (5' TTAGAACGAAGTTACCCCCCTT 3') of the 16S rRNA gene. DNA amplification was carried out in a total of 50 µL containing 1 x PCR buffer, 200 mM of each dNTP, 1.5 mM $MgCl_2$, 25 pmol of each primer, 2.5 U of Taq DNA Polymerase and 10 µL of extracted DNA. Amplifications were performed in a thermocycler with an initial denaturation step at 94ºC (3 min), followed by 35 cycles of denaturation at 94ºC (1 min), annealing at 60ºC (1 min) and extension at 72ºC (1 min). *L. interrogans* pure cultures were used as positive control. Negative control tissue suspensions were collected from a non-inoculated hamster. The specific amplicon of 330 bp fragment was visualized after electrophoresis in 2% agarose gel in the presence of ethidium bromide (0.5 µg/mL).

Histopathology (Staining Methods)

The tissues were examined histologically by Warthin-Starry and Hematoxilin-eosin staining methods (38,60).

Serological Identification

Serogroup identification of the isolates was carried out by cross agglutination technique described by Faine (13). The preparation of rabbit antiserum was performed using two animals for each isolate. Rabbits weighing 3-4 kg were injected intravenously at weekly intervals with live bacteria (density of $2x10^8$/mL) in doses of one, two, four, six and six milliliters, respectively. One week after the last injection the MAT titer

from rabbit serum was found to be at least 12800. The rabbits were bled by cardiac puncture two weeks after the last injection. The cross agglutination was performed between isolates and reference polyclonal antibodies (representative recognized serogroups) from Budesinstitut für Gesundheitlichen Verbraucherschultz und Veterinäermedizin (bgvv) – Berlin/ Germany: Australis, Bratislava, Autumnalis, Castellonis, Batavie, Canicola, Cynopteri, Grippotyphosa, Hebdomadis, Copenhageni, Javanica, Panama, Pomona, Pyrogenes, Hardjoprajitino, Wolffi, Tarassovi and Patoc. Twenty-four live reference serovars were also used: Australis, Bratislava, Autumnalis, Butembo, Castellonis, Batavie, Canicola, Whitcombi, Cynopteri, Grippotyphosa, Hebdomadis, Copenhageni, Icterohaemorragiae, Javanica, Panama, Pomona, Pyrogenes, Hardjoprajitino, Wolffi, Shermani, Tarassovi, Andamana, Patoc and Sentot.

Molecular Typing

The molecular characterization of the isolates was performed by VNTR analysis with seven discriminatory markers (VNTR4, VNTR7, VNTR9, VNTR10, VNTR11, VNTR19 and VNTR23), using the primers described by Majed *et al*. (33). Genomic DNA was extracted using the GFX Genomic Blood DNA Purification Kit following the protocol for Gram-negative bacteria recommended by the manufacturer (GE healthcare). The extracted DNA was submitted to 0.8% agarose gel electrophoresis in order to quantify and evaluate its integrity, and stored at – 20ºC. Amplification was achieved with *Taq* DNA polymerase (Invitrogen), using one cycle of denaturation (94ºC for 5 min) followed by 35 cycles of amplification consisting of denaturation (94ºC for 30 sec), annealing (55ºC for 30 sec), and extension (72ºC for 1 min 30 sec) and a final extension of 10 min at 72ºC. The amplified products were analyzed by 1.5% agarose gel electrophoresis. The size of the amplified products was estimated by comparison with a 50 bp DNA ladder (Invitrogen).

RESULTS AND DISCUSSION

Attempts to isolate *Leptospira* spp. from 137 apparently healthy pigs slaughtered in São Paulo, resulted in five positive cultures (3.5%) from two collection dates (groups G and K - gilts) (Table 1). PCR analysis revealed positive reaction from 11 kidneys, two livers, two genital tracts and from urine of one animal (Table 2). PCR failed to detect the presence of leptospiral DNA at more than one organ from the same animal. There was a positive correlation ($P<0.05$) between culture positive and PCR positive from renal tissue, determined by Mc Nemar's test (55). As expected, PCR analysis was more sensitive (11/137) than culture (4/137), similar to what was found by other authors (11, 37,52,53). The analysis of agreement adjusted (kappa indicator) (58), when it was compared the direct methods of leptospire´s research (PCR e cultivo), showed low agreement between PCR and culture from kidney (k=0.27), liver (k=0.00), reproductive tract (k = - 48.65) and urine (k=0.00).

MAT, the standard method of serologic diagnosis of leptospirosis, revealed seropositivity with titers ≥ 100 of 39.4% of the animals, 45.0% of sows and 37.1% of gilts (Table 1). It

Table 1. Number of samples obtained from female pigs according SAM≥100, to the groups and number of leptospira isolations.

Groups	MAT Results according female swine[1]		Positive Isolates
	Sows	*Gilts*	Gilts
A	06/08	00/00	00/08
B	06/10	00/00	00/10
C	00/01	00/00	00/01
D	02/04	00/00	00/04
E	00/00	02/15	00/15
F	01/08	04/06	00/14
G	00/00	14/15	02/15
H	03/09	01/06	00/15
I	00/00	00/15	00/15
J	00/00	5/25	00/25
K	00/00	10/15	03/15
Subtotal	**18/40 (45,0%)**	**36/97 (37,1%)**	
Total	**54/137 (39,4%)**		**05/137 (3,6%)**

(1) positive female/examinated.

Table 2. Number of pigs with positive PCR for leptospires according to groups and kind of material examined.

Groups	**Number of pigs with PCR positive**			
	Kidney	Liver	Genital tract	Urine
A	0	0	0	0/3
B	0	0	1	0/8
C	0	0	0	0/1
D	0	0	1	1/1
E	0	2	0	—
F	2	0	0	0/4
G	9	0	0	—
H	0	0	0	0/5
I	0	0	0	—
J	0	0	0	—
K	0	0	0	—
TOTAL*	**11/137 (8.0%)**	**2/137 (1.46%)**	**2/137 (1.46%)**	**1/22 (4.5%)**

— not done/ not collected - absent urine; * number of pigs with positive PCR for leptospira/number of pigs examined.

was expected to find a higher number of reactors by MAT as the immune response persists long after the bacteria have been cleared from the organism. Four out of five culture positive animals for *Leptospira* showed high titers for the Pomona serovar (Table 3). The animal that was culture positive, but had no antibodies against leptospira and no lesions was probably recently infected. The Pomona serovar is frequently associated to leptospirosis in swine all over the world (13,36). Previous serological surveys carried out in Brazil also identified Pomona as the most prevalent serovar in swine. Giorgi *et al.* (18) and Santa Rosa *et al.* (50) reported serology against Pomona in São Paulo, Oliveira (41) in Santa Catarina and Rio Grande do Sul, Ramos *et al.* (45), in Rio de Janeiro, Fávero (14) in Rio Grande do Sul, Rio de Janeiro and Pernambuco.

Microscopic lesions in kidneys, evidenced by the hematoxilin-eosin, were found in higher frequency in animals positive for leptospires (culture and/or PCR) than in animals without evidence of leptospires. The lesions were classified from moderate (05/G and 09/K) to severe with hemorrhagic focuses (11/K) and glomerulonephritis. The presence of the spirochetes in the kidneys of animals with positive cultures for leptospires (05G, 06G, 09K and 11K) was confirmed by Warthin-Starry staining. The animal with leptospires isolated from the liver (10K) had also structural lesions of hepatic congestion. Similar findings were reported by Freitas *et al.* (15). The animal 10K presented congestion of uterine horns and focuses of mononuclear cells in its genital tract, as described by Delbem *et al.* (5) and Schönberg *et al.* (55).

Titers ≥ 100 in the cross agglutination between antisera and antigens are shown in Table 4. The cross agglutination between the isolates and reference polyclonal antibodies (bgvv) resulted in titers of 6400 with Pomona. Cross agglutination of rabbit antisera resulted titers ≥ 100 with 11 out of 24 live antigens. The titers with Pomona antigen (POM) were: 3200 (KR11), 6400 (GR5, KR9, KF10) and 12800 (GR6).

The results of the molecular typing by the VNTR technique of the five isolates (GR5, GR6, KR9, KF10, KR11), as well as three reference strains (*L. interrogans* serovars Djasiman, Pomona and Kennewicki) are shown in Fig. 1 and Table 5. Interestingly, a single genotype, different from the reference strains tested, was obtained with the isolates. The three reference strains were chosen because they share the same banding size obtained with the isolates for some of the VNTRs analyzed. This direct comparison was necessary to ascertain that the difference was not a miscalculation of the size of the band, what would have lead to an erroneous result. In addition, serovar

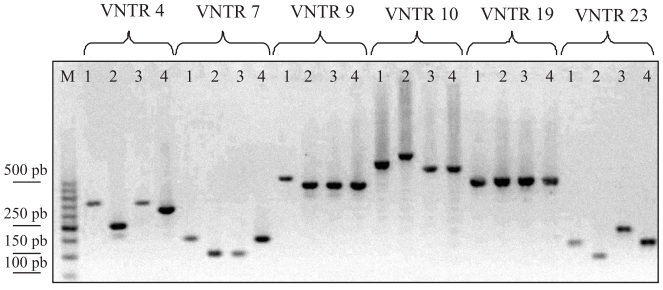

Figure 1. Agarose gel electrophoresis of PCR amplified fragments from the six VNTR loci used to characterize the *L. interrogans* isolates. Lane 1, serovar Djasiman stain Djasiman (reference); lane 2, serovar Pomona, strain Pomona (reference); lane 3, serovar Kennewicki strain LT 1026 (reference); lane 4, one of the five isolates obtained from swine. Note distinct profile obtained with the isolates. Analysis of the VNTR 11 was also carried out, but it was not included in this gel.

Table 3. Resume of Serology, PCR and histopathology results of animals that had positive culture for *Leptospira* spp.

Pig (Gilts)	Culture positive for *Leptospira*	Serology (MAT) / Serovar	Positive PCR for *Leptospira*	Histopathology - HE
05G	kidney*	6400 Pomona	kidney	Interstitial nephritis
06G	kidney*	3200 Pomona	kidney	NephrosisInterstitial nephritisHemorrhagic focusLesion glomerulousCongestion liver
09K	kidney*	1600 Pomona	none	Severe interstitial nephritisCongestion liver
10K	Liver	3200 Pomona	none	NephrosisInterstitial nephritisHemorrhagic focus Glomerular lesionsCongestion liverInflammatory cells (genital tract)
	genital tract		nome	
11K	kidney*	negative	none	none

***** Warthin-Starry staining positive; HE: Hematoxilin-Eosin.

Table 4 Titers ≥ 100 in the cross agglutination test between antisera and antigens.

Antisera	Titers ≥ 100															
	Antigens															
	BRA	AUT	BUT	WHI	CYN	GRI	COP	PAN	POM	PYR	SHE	GR5	GR6	KR9	KF10	KR11
GR5		100							6400			6400				
GR6		400		800			100		12800				6400			
KR9	400	1600	200	800		400		100	6400	100	400			25600		
KF10	100	1600	200		200				6400						25600	
KR11	100	800	100	100		100		100	3200	100	200					25600
Pomona (bgvv)									12800			6400	6400	6400	6400	6400

Bratislava (BRA); Autumnalis (AUT); Butembo (BUT); Whitcombi (WHI); Grippothyphosa (GRI); Copenhageni (COP); Panama (PAN); Pomona (POM); Pyrogenes (PYR); Shermani (SHE); Cross agglutination with other antisera and antigens used: not reagent.

Table 5. VNTR analysis of five *L. interrogans* isolates and three reference strains. Copy number of VNTR locus.

Isolates	VNTR 4	VNTR 7	VNTR 9	VNTR 10	VNTR 11	VNTR 19	VNTR 23
GR5	4	1	6	10	2	8	2
GR6	4	1	6	10	2	8	2
KR9	4	1	6	10	2	8	2
KF10	4	1	6	10	2	8	2
KR11	4	1	6	10	2	8	2
Pomona*	2	0	6	14	2	8	1
Kennewicki**	5	0	6	10	2	8	3
Djasiman***	5	1	1	13	3	8	2

* *L. interrogans* Pomona serovar Pomona strain Pomona (reference)
** *L. interrogans* Pomona serovar Kennewicki strain LT1026 (reference)
*** *L. interrogans* Djasiman serovar Djasiman strain Djasiman (reference)

Pomona and serovar Kennewicki belong to Pomona serogroup, therefore either of them would be compatible with the serological characterization.

The genotype presented by the isolates was recently described by Pavan *et al*. (43) in *L. interrogans* serovar Pomona isolated in Argentina, and named Genotype A. Interestingly, this genotype differs from the serovar Pomona type strain in four VNTR loci. Pavan *et al.* (43) characterized 16 *L. interrogans* serovar Pomona strains isolated from animals and humans in Argentina, and identified four distinct genotypes. Genotype A, comprising 12 strains, is identical to the genotype identified for the isolates characterized in this study. The other 3 genotypes have a distinct number of tandem repeats in VNTR4, but share the same number in the other VNTRs. Apparently, *L. interrogans* serovar Pomona from Brazil and Argentina have a similar genetic profile, distinct from the reference strain. A larger number of serovar Pomona strains have to be typed to confirm if this profile is specific for this region, or if it is also found in other parts of the world.

Another important observation was that serovar Kennewicki strain LT1026 has three tandem repeats in VNTR 23, and not two as originally reported by Majed *et al*. (33). It is conceivable that this difference is due to polymorphism that this strain has undergone upon successive passages in semi-solid medium. However, there are evidences that these VNTR loci are relatively stable over time. Similarly, in serovar Djasiman strain Djasiman, VNTR9 showed only 1 repeat and not 7 as previously reported (33). It remains to be elucidated if this difference is due to a misinterpretation of the result, or if it represents a polymorphism that occurred over time in these strains.

The results presented here show that *L. interrogans* serovar Pomona is prevalent in apparently healthy swine. The implication of this observation in productive parameters remains to be determined, however, as it is a zoonosis, the presence of animals shedding leptospires constitute a potential risk of infection to humans and other domestic animals. The isolates obtained showed a distinct genetic profile, similar to Pomona strains isolated in Argentina.

RESUMO

Isolamento e caracterização de *Leptospira interrogans* de suínos abatidos no Estado de São Paulo, Brasil

Amostras de soro sanguíneo, rim, fígado e trato genital de 137 fêmeas suínas (40 matrizes e 97 marrãs) e de urina de 22 matrizes foram colhidas em abatedouro no Estado de São Paulo, no período de abril de 2003 a agosto de 2004 tendo como objetivo

o isolamento de *Leptospira* spp. Quatro estirpes foram isoladas de animais que apresentaram títulos, no teste de soroaglutinação microscópica (SAM) ≥ 100, para o sorovar Pomona e de um animal, não reagente na SAM, em que houve isolamento de leptospiras do fígado e aparelho reprodutor. A presença do DNA de leptospira foi investigada pela técnica da PCR e foram observados resultados positivos nos rins de 11 fêmeas, no fígado de duas, no aparelho reprodutor de duas e na urina de uma delas. Nefrose, nefrite intersticial multifocal variando de moderada a severa, cilindros hialinos e focos hemorrágicos, congestão hepática e de cornos uterinos foram lesões histológicas evidenciadas com freqüência mais alta em animais positivos para leptospira. A impregnação argêntica (Warthin Starry) confirmou a presença de espiroquetas nos túbulos renais das quatro fêmeas onde houve cultura positiva para leptospiras dos rins. O sorogrupo dos cinco isolados foi identificado como Pomona pela técnica de aglutinação cruzada com anticorpos policlonais de referência. A caracterização molecular dos isolados foi realizada pela análise do número variável de repetições em tandem (VNTR). Os mesmos revelaram um padrão distinto da estirpe padrão de *L. interrogans* sorovar Pomona, porém idêntico a um padrão previamente identificado em estirpes isoladas na Argentina, pertencentes ao sorovar Pomona.

Palavras-chave: Pomona. Suínos. VNTR. Isolamento. PCR. Trato Genital.

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