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Original Article

Characterization of *Leptospira interrogans* serovar Pomona isolated from swine in Brazil

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Abstract

Introduction: *Leptospira interrogans* swine infection is a cause of serious economic loss and a potential human health hazard. In Brazil, the most common serovars associated with swine infections are Pomona, Icterohaemorrhagie and Tarassovi. Cross-reactions among serovars and the failure of infected animals to seroconvert may complicate the interpretation of serological tests. Molecular methods with better discriminatory powers are useful tools for swine leptospirosis characterization and diagnosis.

Methodology: This study evaluated nine *L. interrogans* isolates from the States of Sao Paulo and Minas Gerais during different time periods. Isolates from diseased and apparently healthy swine were characterized by microscopic agglutination tests with polyclonal antibodies and were genotyped by VNTR, PFGE and MLST techniques. Broth microdilution was used to determine the minimal inhibitory concentration of the antimicrobials of veterinary interest.

Results: The strains were identified as *L. interrogans* serogroup Pomona serovar Pomona Genotype A, while MLST grouped all of the isolates in sequence type 37. The PFGE analysis resulted in two pulsotypes with more than 70% similarity, distinguishing serovar Pomona isolates from the serovar Kennewicki reference strain. All of the isolates presented low MIC values to penicillin, ampicillin, ceftiofur and tulathromycin. High MIC values for fluoroquinolones, tiamulin, gentamicin, tetracyclines, neomycin, tilmicosin and sulfas were also observed.

Conclusions: All molecular techniques were concordant in *L. interrogans* serovar Pomona identification. This serovar may have a different antibiotic susceptibility profile than previously reported for *Leptospira* isolates.

Key words: *Leptospira interrogans*; serovar Pomona; antimicrobial resistance; PFGE; MLST.

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Introduction

Leptospirosis is an important infectious disease, which is reemerging in some developing countries, and is a globally spread zoonosis [1]. *Leptospira interrogans* infection in swine is a cause of serious economic losses and a potential human health hazard [2]. Swine are commonly infected with serovars Pomona, Tarassovi, Grippotyphosa, Bratislava, Sejroe, Icterohaemorrhagiae and Canicola, and the clinical signs of swine leptospirosis vary with the infecting serovar [3].

In Brazil, the most common serovars described are Pomona, Icterohaemorrhagie and Tarassovi. Infection with serovar Pomona is characterized by high antibody titers, variable clinical signs and a renal carrier state [4]. Icterohaemorrhagiae infection is associated with an increased risk of weak newborn piglets and stillbirths in Brazil [5]; serovar Tarassovi is associated with dead piglets [2]. Seroprevalence may vary with management practices, the presence of surface water and a farm rodent-control program [6,7]. Cross-reactions among serovars, diagnostic titer uncertainty, and failure of some infected animals to seroconvert cause considerable difficulty in serological test interpretation [8]. Thus, molecular methods with

higher discriminatory capabilities are useful tools for swine leptospirosis characterization and diagnosis.

Genotyping methods, such as pulsed-field gel electrophoresis (PFGE), have been standardized for *Leptospira* characterization [9] in addition to simple PCR techniques, such as variable number tandem-repeat analysis (VNTR) [10]. The increasing number of sequenced genomes enables the use of sequence-based approaches such as multilocus sequence typing (MLST) for concrete serovar identification [11]. A microdilution technique was developed for antibiotic selection and leptospirosis treatment [12].

This study aimed to characterize *Leptospira interrogans* serovar Pomona isolated from swine in Sao Paulo and Minas Gerais states during different time periods by serotyping, variable number tandem-repeat analysis (VNTR), pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and broth microdilution techniques.

Methodology

Bacterial isolates and culture conditions

Nine *Leptospira interrogans* isolates were studied. The isolates originated from the Laboratory of Bacterial Zoonosis – University of Sao Paulo bacterial collection. They were isolated from diseased and apparently healthy swine in Sao Paulo and Minas Gerais states at three different time periods (Table 1).

For isolation, 5 g of kidney (7) or abortion samples (2) were collected and homogenized in 50 mL of Sorensen saline; 100 µL of 10^{-1}, 10^{-2} and 10^{-3} dilutions were inoculated into culture tubes in duplicate containing modified EMJH medium (Difco-BBL, Detroit /USA) enriched with 15% rabbit serum, 5-fluorouracil and nalidixic acid [13]. Once isolated, cultures were stored in EMJH semi-solid media at 30°C and maintained in EMJH liquid medium until serotyping, microdilution, and molecular analysis.

Isolates GR5, GR6, KR9, KF10 and KR11 were first described by Miraglia *et al*. [14]. The *L. interrogans* serogroup Pomona serovar Pomona reference strain 13A (1937, Australia) and *L. interrogans* serogroup Pomona serovar Kennewicki reference strain LPF (1985, Brazil) were used in this study as internal and quality controls.

Serotyping

The isolates were typed at the WHO/FAO/OIE and National Collaborating Centre for Reference and Research on Leptospirosis (Kit Biomedical Research, Amsterdam, Netherlands). For the serogroup determination, the isolates were subjected to microscopic agglutination tests (MAT) using a panel of 43 rabbit anti-*Leptospira* sera representative of pathogenic and saprophytic serogroups. The polyclonal rabbit antisera were prepared as previously described [15,16]. The MAT was performed using a microtiter plate with serial 2-fold rabbit antisera dilutions, starting with a serum dilution of 1:10. Equal volumes of viable leptospiral strains and antisera dilutions were mixed. After incubation at 30°C for 2 hours, the mixtures were investigated for agglutination using dark-field microscopy. High agglutination rates of the leptospiral strain with one particular antisera, were used to identify the presumptive serogroup of the strain [17].

Broth microdilution

Broth microdilution was performed as previously described [12] and adapted for use with the Sensititre Standard Susceptibility MIC Plate BOPO6F (Thermo Fisher Scientific – Waltham, USA). For the inoculum, cultures were grown at 30°C for 7 days and diluted to an optical density at 420 nm of 0.32 (approximately 10^8 CFU/mL), followed by serial dilution using EMJH medium to achieve a final concentration of approximately 2×10^6 CFU/mL. Fifty microliters of the inoculum was added in each Sensititre MIC Plate well, and after 3 days of incubation, 5 µL of 10X alamarBlue (Thermo Fisher Scientific – Waltham, USA) was added to each well. The MICs were assessed visually as the lowest antibiotic concentration in the wells without alamarBlue color change at the fifth day of incubation.

Variable number tandem-repeat analysis (VNTR)

VNTR analysis was performed with 7 discriminatory markers for VNTR loci 4, 7, 9, 10, 11, 19 and 23 as previously described [10] at the Biotechnology Centre, Federal University of Pelotas, Rio Grande do Sul, Brazil. The PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide, and the molecular weights were estimated with a 100-bp DNA ladder.

Pulsed-field gel electrophoresis (PFGE)

The *Leptospira* isolates were incubated at 30°C in EMJH medium for 7 days and centrifuged at 5000 rpm for 20 minutes. The supernatant was discarded and the pellet was resuspended in 10 mL of PETT IV solution (10 mmol/L Tris-HCl [pH 8.0], and 1 mol/L NaCl, 10 mmol/L EDTA).

Table 1 Source and origin characteristics and molecular profile of *L. interrogans* serogroup Pomona isolates

Isolate	Specie	Year	State	Description	Serotyping	Profile									
						VNTR 4	VNTR 7	VNTR 9	VNTR 10	VNTR 11	VNTR 19	VNTR 23	VNTR - serovar	PFGE	MLST (ST[1])
M7/87	Swine – *Sus scrofa domestica*	1987	SP	-	serogroup Pomona serovar Pomona	4	1	6	10	2	8	2	Pomona (Genotype A)	P1	37
BOT-10D	Swine – *Sus scrofa domestica*	2002	SP	-	serogroup Pomona serovar Pomona	4	1	6	10	2	8	2	Pomona (Genotype A)	P1	37
GR6	Swine – *Sus scrofa domestica*	2004	SP	Apparently healthy female	serogroup Pomona serovar Pomona	4	1	6	10	2	8	2	Pomona (Genotype A)	P1	37
GR5	Swine – *Sus scrofa domestica*	2004	SP	Apparently healthy female	serogroup Pomona serovar Pomona	4	1	6	10	2	8	2	Pomona (Genotype A)	P1	37
M12/04	Swine – *Sus scrofa domestica*	2004	MG	Abortion	serogroup Pomona serovar Pomona	4	1	6	10	2	8	2	Pomona (Genotype A)	P1	37
M13/04	Swine – *Sus scrofa domestica*	2004	MG	Abortion	serogroup Pomona serovar Pomona	4	1	6	10	2	8	2	Pomona (Genotype A)	P1	37
KR9	Swine – *Sus scrofa domestica*	2004	SP	Apparently healthy female	serogroup Pomona serovar Pomona	4	1	6	10	2	8	2	Pomona (Genotype A)	P1	37
KF10	Swine – *Sus scrofa domestica*	2004	SP	Apparently healthy female	serogroup Pomona serovar Pomona	4	1	6	10	2	8	2	Pomona (Genotype A)	P1	37
KR11	Swine – *Sus scrofa domestica*	2004	SP	Apparently healthy female	serogroup Pomona serovar Pomona	4	1	6	10	2	8	2	Pomona (Genotype A)	P1	37
LPF[2]	Swine – *Sus scrofa domestica*	1985	-	-	serogroup Pomona	5	0	6	10	2	8	3	Kennewicki	P2	37
13A[3]	-	1937	-	-	serogroup Pomona serovar Pomona	2	0	6	14	2	8	1	Pomona	P1	37

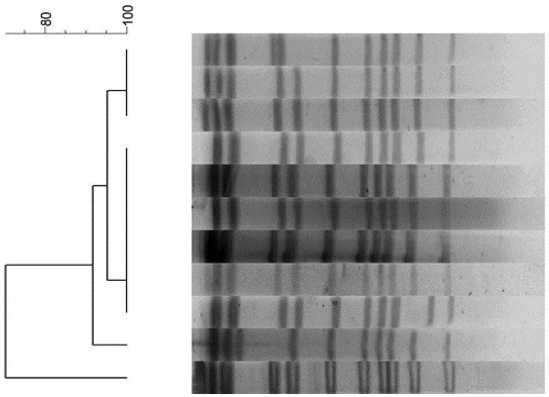
[1] ST – Sequence type; [2] *L. interrogans* serogroup Pomona serovar Kennewicki strain Fromm; [3] *L. interrogans* serogroup Pomona serovar Pomona. SP-Sao Paulo. MG-Minas Gerais.

Table 2 Minimal inhibitory concentration (MIC) values of antimicrobials against *L. interrogans* serogroup Pomona isolates

	MIC (µg/mL)[a]																
Isolate	Ceftiofur	Tiamulin	Gentamicin	Florfenicol	Chlortetracyclin	Oxytetraciclin	Penicilin	Ampicillin	Danofloxacin	Neomycin	Trimethoprim / sulfamethoxazole	Spectomycin	Tylosin tartrate	Tulathromycin	Tilmicosin	Clindamycin	Sulphadimethoxine	Enrofloxacin
M7/87	≤ 0.25	2.0	16.0	2.0	>16.0	>16.0	≤ 0.125	≤ 0.25	>2.0	>64.0	>4/72	≤ 8.0	≤ 0.5	≤ 1.0	>128.0	1.0	>512.0	>4.0
BOT-10D	≤ 0.25	2.0	16.0	4.0	>16.0	>16.0	≤ 0.125	≤ 0.25	>2.0	>64.0	>4/72	>128.0	≤ 0.5	≤ 1.0	>128.0	1.0	>512.0	>4.0
GR6	≤ 0.25	2.0	16.0	4.0	>16.0	>16.0	≤ 0.125	≤ 0.25	>2.0	>64.0	>4/72	>128.0	≤ 0.5	≤ 1.0	>128.0	1.0	>512.0	>4.0
GR5	≤ 0.25	2.0	8.0	1.0	2.0	2.0	≤ 0.125	≤ 0.25	>2.0	>64.0	>4/72	>128.0	≤ 0.5	≤ 1.0	>128.0	>32.0	>512.0	>4.0
M12/04	≤ 0.25	2.0	16.0	1.0	>16.0	>16.0	≤ 0.125	≤ 0.25	>2.0	>64.0	>4/72	>128.0	≤ 0.5	≤ 1.0	>128.0	1.0	>512.0	>4.0
M13/04	≤ 0.25	2.0	16.0	2.0	>16.0	>16.0	≤ 0.125	≤ 0.25	>2.0	>64.0	>4/72	>128.0	≤ 0.5	≤ 1.0	>128.0	1.0	>512.0	>4.0
KR9	≤ 0.25	2.0	16.0	2.0	>16.0	>16.0	≤ 0.125	≤ 0.25	>2.0	>64.0	>4/72	>128.0	≤ 0.5	≤ 1.0	>128.0	1.0	>512.0	>4.0
KF10	≤ 0.25	2.0	16.0	2.0	>16.0	>16.0	≤ 0.125	≤ 0.25	>2.0	>64.0	>4/72	>128.0	≤ 0.5	≤ 1.0	>128.0	1.0	>512.0	>4.0
KF10	≤ 0.25	2.0	16.0	2.0	>16.0	>16.0	≤ 0.125	≤ 0.25	>2.0	>64.0	>4/72	>128.0	≤ 0.5	≤ 1.0	>128.0	1.0	>512.0	>4.0
13A[1]	≤ 0.25	2.0	8.0	2.0	>16.0	>16.0	≤ 0.125	≤ 0.25	>2.0	>64.0	>4/72	≤ 8.0	≤ 0.5	≤ 1.0	>128.0	0.5	>512.0	2.0
LPF[2]	≤ 0.25	2.0	16.0	8.0	>16.0	>16.0	≤ 0.125	≤ 0.25	>2.0	>64.0	>4/72	32.0	≤ 0.5	≤ 1.0	>128.0	1.0	>512.0	>4.0

[a] The MICs of penicillin are given in units/mL. [1] *L. interrogans* serogroup Pomona serovar Pomona; [2] *L. interrogans* serogroup Pomona serovar Kennewicki strain Fromm.

Figure 1. A dendrogram showing the relationship among the *L. interrogans* serogroup Pomona isolates PFGE patterns.



Ident	Year	Origin	Specie	Serogroup	Serovar
43 (KR9)	2004	SP	interrogans	Pomona	Pomona
44 (KF10)	2004	SP	interrogans	Pomona	Pomona
13 A	1937	Australia	interrogans	Pomona	Pomona
35 (BOT-10D)	2002	SP	interrogans	Pomona	Pomona
36 (GR6)	2004	SP	interrogans	Pomona	Pomona
41 (M12/04)	2004	MG	interrogans	Pomona	Pomona
42 (M13/04)	2004	MG	interrogans	Pomona	Pomona
45 (KF11)	2004	SP	interrogans	Pomona	Pomona
05 (M7/87)	1987	SP	interrogans	Pomona	Pomona
37 (GR5)	2004	SP	interrogans	Pomona	Pomona
39 (LPF)	1986	Brazil	interrogans	Pomona	Kennewicki

The bacterial suspension was centrifuged at 1500 rpm for 10 minutes, the supernatant was discarded, and the pellet was suspended in 1 mL of lysis buffer (1 mol/L NaCl, 10 mmol/L Tris [pH 8.0], 200 mmol/L EDTA, 0.5 % sarcosyl, and 0.2 % sodium deoxycholate). Agarose SeaKem Gold 2% (Lonza Group Ltd – Basel, Switzerland) was prepared in 0.5X Tris Borate EDTA buffer. A 400 µL bacterial suspension aliquot was heated to 40°C and was added to 20 µL of 100 mg of lysozyme/mL (LGC Biotecnologia, Sao Paulo, Brazil) and 400 µL of a heated 2% agarose solution. The mixture was immediately dispensed into wells and chilled for 10 minutes at 4°C. Plugs were placed in 2.5 mL of lysis buffer, and 70 µL of proteinase K (20 mg/ml; LGC Biotecnologia) were added before incubation at 56°C for 20 hours. The plugs were rinsed once in 1 mL of Tris EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA). The plugs were washed with 5 mL of Tris EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA) twice for 30 minutes, each time at 37°C, and then stored in 1 mL of Tris EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA) at 4°C. The DNA was cleaved with 5 U of *Not*I enzyme (New England BioLabs, Ipswich, MA, USA) per microgram of DNA for 4 hours at 37°C.

PFGE was performed as previously described [9]. The gels were stained with 1x Sybr Safe (Invitrogen Corporation, Carlsbad, USA) for 40 minutes and photographed with a Gel Doc XR System (Bio-Rad Laboratories, Hercules, USA). The DNA fragments were identified using a Lambda DNA-PFGE marker (New England BioLabs, Ipswich, USA). For the PFGE analysis, the isolates were categorized into different pulsotypes when differing by 4 or more bands [18]. Fingerprint patterns were analyzed by comprehensive pairwise comparison of restriction fragment sizes using the Dice coefficient. The mean values obtained from Dice coefficients were employed in UPGMA using BioNumeric 6.6 (Applied Maths NV, Saint-Martens-Latem, Belgium) to generate dendrograms.

Multilocus sequence typing (MLST)

MLST was performed according to the protocol which analyzes *pntA*, *sucA*, *pfkB*, *tpiA*, *mreA*, *glmU* and *fadD* genes sequences polymorphisms [19]. Purified DNA was recovered according to the protocol of DNA extraction described by Boom and colleagues (1990) [20] and stored at -20ºC. The PCRs (50 µL) used 5 µL of genomic DNA, MilliQ water, 10X PCR buffer, 1.5 mM $MgCl_2$, 200 µM of dNTPs (Fermentas Inc, USA.), 200 µM of each primer and 1.25 U of Taq-DNA-polymerase (Fermentas Inc, USA). The amplified products were stained with BlueGreen (LGC Biotecnologia, Sao Paulo, Brazil) and separated by electrophoresis in 1.5% agarose gels. Molecular weights were estimated using a 100-bp DNA Ladder (Fermentas Inc, USA).

Amplified fragments were purified using the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare do Brasil Ltda, São Paulo, Brasil) and sequenced directly by the Human Genome Research Center (University of Sao Paulo, Brazil). The BIOEDIT *Sequence Alignment Editor* 7.0.9 [21] was used for sequence editing. Sequences were submitted to the *Leptospira* MLST database (http://www.mlst.net) to obtain isolates allelic profiles and respective sequence types (ST).

Nucleotide sequence accession numbers

All DNA sequences from this study were deposited in GenBank under accession numbers KJ885311 to KJ885373.

Results

The nine isolates were serotyped as *Leptospira interrogans* serogroup Pomona serovar Pomona. The variable tandem repeat number profile in the seven VNTR loci (VNTR4, VNTR7, VNTR9, VNTR10, VNTR11, VNTR19, VNTR23) obtained were 4, 1, 6, 10, 2, 8 and 2, respectively. This profile is different from the serovar Pomona reference strain (2, 0, 6, 14, 2, 8, 1) at four VNTR loci and corresponds to the *L. interrogans* serovar Pomona Genotype A [22].

PFGE analysis resulted in two pulsotypes (P1 and P2). The pulsotype P1 grouped all of the studied isolates, including the serovar Pomona reference strain 13A, with more than 90% genetic similarity; P2 only represented the reference strain LPF (Figure 1). The serovars Pomona and Kennewicki corresponding to the pulsotypes P1 and P2, respectively, had over 70% genetic similarity. All of the isolates presented the same allelic profile (3, 3, 3, 2, 3, 4, 5) and sequence type (ST 37) obtained from the MLST analysis.

The MIC values are presented in Table 2. All of the isolates had high MIC values to tiamulin, gentamicin, chlortetracyclin, oxytetraciclin, neomycin, tilmicosin, trimethoprim/sulfamethoxazole, spectinomycin, sulfadimethoxine. High fluoroquinolone MIC values were also observed. All of the isolates appear to be sensitive to penicillin, ampicillin, ceftiofur, tylosin tartrate and tulathromycin. MIC variability was observed for florfenicol. Isolate GR5 presented a slightly different

profile with low MIC values for gentamicin, florfenicol, chlortetracyclin and oxytetracycline, while it appeared to be resistant to clindamycin.

Discussion

We characterized *L. interrogans* serovar Pomona isolates from diseased and apparently healthy swine. Despite the distinct sample types, origin and three different isolation time periods, the studied isolates presented an identical genotype and ST and a similar antibiotic susceptibility profile.

The serotyping presents the same result as the molecular techniques, with the identification of serovar Pomona; however, molecular approaches gave us more information identifying genotype A strains, which differs from the *L. interrogans* serovar Pomona reference strain. Furthermore, serological methods still are laborious, especially for antisera production that involves animal use [8].

Regarding molecular typing methods in this study, VNTR analysis allowed us to identify the serovar Pomona genotype A and clustered the isolates into a single group. This genotype was first isolated from Argentinian cow, pig and human samples [22]. Genotype A is dominant among the Argentinian territory and has been isolated since 1960 with a 55% prevalence [23]. Despite not fully understanding the epidemiological importance of this genotype, we can conclude that it is present in South America and that it can be isolated from both healthy and diseased animals.

The PFGE technique enabled the isolates differentiation in only two pulsotypes. The field isolates presented over 90% similarity with reference strain 13A and grouped in pulsotype P1, while P2 represented only the serovar Kennewicki reference strain. Therefore, PFGE enabled the distinction of serovars Pomona and Kennewicki with more than 70% genetic similarity. Although serovar Kennewicki is no longer separated from serovar Pomona [24], PFGE is a useful method for serovar identification, as previously reported [9].

While PFGE distinguished closely related serovars, the MLST technique grouped all of the isolates into one ST. ST 37 was expected for the Pomona isolates because it had already been assigned this serovar, independently of its origin [19,9,11]. As MLST does not always specify *Leptospira* serovars [9], the same ST 37 also encompasses serovar Canicola when using the scheme previously proposed [19,9,11]. This is also observed with the new MLST protocol [25] in which serovar Pomona can be assigned to STs 38 and 140 together with serovars Gem and Guaratuba, respectively. Although MLST techniques enable *Leptospira* species differentiation [9,25], serovar assignment still needs improvement. Only when molecular methods ensure species and serovar identification, will they completely replace serological methods.

Although a standardized *Leptospira* antibiotic susceptibility guideline has not been established by the Clinical and Laboratory Standards Institute, *Leptospira* susceptibility studies have been developed to enhance leptospirosis treatments [26]. However, few field isolates and reference strains have been studied to generate an understanding of the variability of susceptibilities among *Leptospira* species and serovars. *L. interrogans* is the most studied species, but there are no data regarding the variability of *L. interrogans* serovars antibiotic susceptibilities.

Our results corroborate previous studies that presented high MIC values for tetracycline and chloramphenicol-florfenicol [27,12,28,29]. All of the isolates were also resistant to trimethoprim/sulfamethoxazole and sulfadimethoxine, as expected. However, the higher MIC values for tiamulin, gentamicin, neomycin and spectomycin were not expected and the veterinary usage of these antibiotics requires attention. Regardless of the susceptibility to penicillin and ampicillin that consists of a classical leptospirosis treatment, all serovar Pomona isolates appear to be resistant to fluoroquinolones, which have been indicated as an alternative empirical treatment [26].

These results indicate that *L. interrogans* serovar Pomona genotype A has a different antibiotic susceptibility profile compared to other *L. interrogans* serovar [27,28,29,30] and although they are sensitive to penicillin and ampicillin, they also have high MIC values for important veterinary medicine antibiotics, such as tetracycline, chloramphenicol-florfenicol, aminoglycosides and tiamulin. Fluoroquinolone (danofloxacin and enrofloxacin) resistance in Brazilian leptospires has been reported by Miraglia *et al.* [30] and contradicts previous publications from other research laboratories.

Conclusions

Molecular techniques were concordant in *L. interrogans* serovar Pomona identification. This serovar presents a different antibiotic susceptibility profile compared to other *Leptospira interrogans* strains previously reported. Further studies with large

numbers of strains are necessary to define the susceptibility profile of serovar Pomona.

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