Development of a Novel Differential Duplex PCR for Pathogenic and Intermediate Leptospire: First Report of Intermediate Leptospira in Animal Sera in Argentina

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Abstract

Background: Even though leptospirosis is one of the most important zoonoses in the world, many of its aspects remain unknown, such as the distribution of different species and their pathogenicity for animals and humans.

Results: Intermediate leptospiral DNA was detected in bovine sera samples from animals with abortion history from Argentina. We designed a novel duplex PCR, which differentiates pathogenic and intermediate leptospires in one single step. This new diagnostic method allowed the detection of intermediate leptospiral DNA in 4.38 % ($n = 5/114$) of the total analyzed bovine samples.

Conclusions: These results highlight the need of further studies of intermediate species in our country, especially regarding their association to clinical disease in animals. Remarkably, this is the first report of detection of intermediate leptospires circulating in livestock animals in Argentina and the second report in the entire world.

Background

Leptospirosis is the most prevalent zoonosis in the world, with a considerable number of severe or fatal human cases. The global burden of the disease is hardly known, due to the existence of mild or subclinical forms that vanish without diagnosis or specific medical treatment. Nevertheless, it is estimated that approximately 60.000 people die annually from this disease¹, due to renal or hepatic failure or pulmonary hemorrhage. Although rats and other small rodents are the main reservoirs of these bacteria, domestic animals such as cattle, pigs, dogs and horses may get infected and become a source of infection for humans^{2,3}.

This disease also causes substantial economic losses, due to reproductive failures in livestock animals (mainly in bovine and swine production). In Argentina, meat production is one of the main sources of national income, and it is estimated that up to 10% of the abortions in cattle may be caused by leptospiral infection⁴. To the time of writing, the only species reported to infect cattle in our country are *L. interrogans* and *L. borgpeterseni*⁴⁻⁶.

The diagnosis of this disease is usually complicated, especially in animals. Bacteriological isolation has no clinical use, since leptospiral culture is time-consuming (cultures may take up to 6 months to grow) and of low sensitivity (it is hard to recover bacteria from samples due to the presence of contaminants)⁷. Therefore, diagnosis is usually made by serological tests. The most used serological technique is the Microscopic Agglutination Test (MAT) which uses live cultures as antigens to detect specific antibodies in the sample. Although being the gold standard test for leptospirosis, it is a cumbersome method for it requires the constant maintenance of a large ceparium. Also, it is usually not possible to diagnose the disease with one single sample because of a broad cross-reactivity between different serovars in the acute phase, but rather paired sera are required for definitive diagnosis. Seroconversion of at least

fourfold increases in titers must be observed between acute and convalescent serum samples for confirmation⁸. Moreover, the interpretation of the serological reaction is subjective and may vary between observers². These facts make MAT unpractical to use in the diagnosis of a large group of animals.

The genus *Leptospira* spp. is traditionally classified into three different phylogenetic clades^{7,9}. The pathogenic clade is widely known to cause human and animal disease, while the saprophytic clade is found only in the environment, with no human or animal infection reported. The newest group of *Leptospira* spp. is represented by the intermediate clade, and the first intermediate species reported was *L. inadai* in 1986 which was, at that time, considered to belong to the pathogenic clade¹⁰. In the following years, more species were found: *L. fainei* in 1998¹¹, *L. broomii* in 2006¹², *L. licerasiae* in 2008¹³, *L. wolffii* also in 2008¹⁴, *L. venezuelensis* in 2017¹⁵ and several new candidates in 2018 and 2019^{16,17}. These species were detected in different mammals like rats and livestock animals and in many environmental samples^{11,13,15,18-25}. Also, many recent studies revealed that species belonging to the intermediate clade may be associated with clinical disease in humans^{13,14,18,26-28}. Despite these new findings, little is known about the intermediate clade and its pathogenicity to animals and humans remains unclear, since it was not possible to reproduce the disease in a hamster model^{19,20}.

In Latin America, there are very few studies about these species and its detection has been reported only in the following countries: Perú, Ecuador, Colombia, Argentina, Venezuela and Brazil^{13,15,19,21,27,29}. In Argentina, the only study available reports the detection of intermediate leptospiral DNA in human samples²⁸, but there is no further research until the present day, mainly due to the lack of an accurate diagnostic test.

Another hurdle that prevents obtaining more information about these leptospires is the complexity of the currently available diagnostic methods. Intermediate leptospires can be detected in clinical or environmental samples through isolation followed by genetic sequencing (e. g. whole genome sequencing or 16S rRNA gene sequencing)^{19,26,27}, through qPCR, which requires expensive equipment³⁰, or through several consecutive steps as nested-PCR followed by RFLP and sequencing¹⁸.

In this study we describe the detection of intermediate leptospires DNA in animal sera (bovines) for the first time in Argentina using a newly developed end-point polymerase chain reaction (end-point PCR) with two sets of primers (duplex PCR) targeting both pathogenic and intermediate species in one single step. The aim of developing this PCR was to obtain an inexpensive and simple differential diagnostic method for clinical samples, applicable even in developing countries.

Materials And Methods

Samples:

Bovine sera from animals with clinical signs compatible with leptospirosis (mainly abortion history) were selected. The selection criteria were presence of clinical signs with a negative or a low titration result (1/400 or lower) to the MAT, and thus a negative or dubious diagnosis of leptospirosis. Higher titers were not considered because they imply a positive or a highly probable diagnosis of leptospirosis caused by the pathogenic serovars used in the MAT (high titers in MAT stand for the presence of large quantities of antibodies against specific serovars). The MAT cutoff titer used in our Laboratory for bovines is 1/200³³. The sera were selected from a pool of samples that are routinely received by the Laboratory of Leptospirosis (OIE Reference Laboratory) of the National Institute of Agricultural Technology (INTA) in Buenos Aires, Argentina, for the diagnosis of Leptospirosis, during 2017–2018. Most of the samples proceeded from the central region (Pampa Region) of Argentina. From more than 2500 bovine sera received during these years, 114 bovine samples followed the selection criteria. Sera were stored at -20°C until use.

Primers design:

16S rRNA (*rrs*) and *secY* genes from *Leptospira* spp. were selected as target genes for duplex PCR amplification to differentiate in one single step the intermediate and the pathogenic group of this genus (Table 2). The *secY* gene was amplified using the set of primers G1/G2³⁴ to detect all the currently circulating pathogenic species in livestock animals in our country^{4–6}. The *rrs* gene was amplified using the primers 16SFw/16SRv, especially designed for this duplex PCR to detect all the intermediate species. The *rrs* gene was selected as target gene as it is widely used as bacterial housekeeping genetic marker to study phylogeny and taxonomy.

Table 2
– Primers used to detect intermediate and pathogenic *Leptospira* spp.

Target gene	Primer	Sequence (5'-3')	Product size	Detected species	Source
secY	G1	CTGAATCGCTGTATAAAAGT	285 bp	<i>L. interrogans</i> , <i>L. borgpetersenii</i> , <i>L. weilii</i> , <i>L. noguchii</i> , <i>L. santarosai</i> , <i>L. meyeri</i>	Gravekamp <i>et al.</i> , 1993 ³⁴
	G2	GGAAAACAAATGGTCGGAAG			
rrs	16SFw	TAAAGCACCGGCTAACTACG	640 bp	Intermediate <i>Leptospira</i> spp.	Present study
	16SRv	ACTNAATGGTAGCAACATACGATAG			

Specifically, the 16SRv primer sequence was designed in such a way as to make it specific for the intermediate leptospires species. The 16S rRNA sequences of *Leptospira* spp. were downloaded from Nucleotide database of NCBI, and a multiple sequence alignment was carried out with BioEdit program³⁵. The primers sequences were designed manually to amplify the nucleotide sequence from 440 to 1079 base pairs (bp) of 16S rRNA gene of *Leptospira fainei* serovar Hurstbridge strain BUT6 (NR_043049.1). In

silico amplification was checked with Primer3 program³⁶, Primer-BLAST tool³⁷ and SILVA database³⁸. The partial 16S rRNA sequences alignment of the most representative *Leptospira* spp. and the sites of hybridization of the primers are shown in Fig. 2.

To confirm the sequences amplified with 16SFw/16SRv primers, PCR products from *L. fainei* serovar Hurstbridge strain BUT6 and *L. licerasiae* serovar Varillal strain VAR010 were sequenced in both directions and analyzed with BioEdit program.

As one-step duplex PCR was designed, all the primers were also checked for possible primer-dimer formation, for their secondary structures and thermodynamic properties, using Multiple Primer Analyzer (ThermoFisher Scientific) and PrimerDimer (<http://www.primer-dimer.com>) online tools. Specificity of the newly designed 16S primer pair was determined *in silico* by comparison to the GenBank nr database using Primer-BLAST³⁷ searches and also by PCR using different leptospiral species and other bacteria that are commonly found in animal samples, such as: *L. interrogans*, *L. borgpetersenii*, *L. kirschneri*, *Escherichia* spp., *Pseudomonas* spp., *Borrelia* spp., *Brucella abortus* 2308, *Salmonella* spp., *Clostridium* spp. and *Mycobacterium* spp. (data not shown).

PCR amplification:

DNA was extracted from sera samples following a previously described protocol³⁹ using Chelex-100 resin (BioRad). As positive controls for the pathogenic clade, *L. interrogans* serovar Pomona strain Pomona DNA was used whereas *L. fainei* serovar Hurstbridge strain BUT6 DNA was used as positive control for the intermediate clade.

The master mix was prepared using Taq Pegasus (5 U/μL, PB-L) and PCR was performed under the following conditions: 1 cycle of 94°C for 5 minutes, 35 cycles of 94°C for 1,5 minutes, 57°C for 1 minute and 72°C for 2 minutes, and a final extension at 72°C for 10 minutes (MyCycler, BioRad). PCR products (15 μL) were revealed by electrophoresis in a 2% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) with ethidium bromide at 100 V for 60 minutes (PowerPac Basic, BioRad). Amplified DNA bands were visualized upon UV light exposure (UVI Tec transilluminator BTS-20.M). Amplicon sizes were estimated using DNA molecular marker (1 kbp ladder, PB-L).

Results

Specificity of 16S primers pair

The detailed *in silico* analysis by comparison to the GenBank nr database confirmed that 16S primer pair is specific for the known intermediate leptospires species: *L. inadai*, *L. fainei*, *L. broomii*, *L. licerasiae*, *L. wolffii*, *L. venezuelensis*, *L. selangorensis*, *L. sarikeiensis*, *L. andrefontaineae*, *L. johnsonii*, *L. semungkisensis*, *L. langatensis*, *L. koniamboensis*, *L. fluminis*, *L. fletcheri*, *L. dzoumogneensis*, *L. saintgironisae*, *L. haakeii*, *L. hartskeerlii*, *L. neocaledonica* and *L. perolatii*. However, the preliminary raw analysis showed the primers would amplify also uncultured bacteria, unclassified *Leptospira* sp., and

Leptospira interrogans. Respect to the last one, all these *L. interrogans* submissions belonged to the same publication resource (Ganoza et al., 2006)³¹ (GenBank Access / original sequence name: DQ522181.1 / BEL020MA3_2; DQ522209.1 / PAD01_1; DQ522190.1 / BEL08-MA_1; DQ522219.1 / PAD55B_2; DQ522176.1 / BEL012LA_1; DQ522182.1 / BEL022LA2_1; DQ522183.1 / BEL022LA_1; DQ522188.1 / BEL044LA2_1; DQ522194.1 / CEH006_1; DQ522189.1 / BEL044LA2_2; DQ522197.1 / HAI029; DQ522216.1 / PAD08B_2; DQ522218.1 / PAD55A_2). In that publication, it was concluded that all these sequences belonged to intermediate leptospires or unclassified species and not to *L. interrogans* or other pathogenic leptospiral species. Therefore, unfortunately, these sequences seem to be submitted wrongly to the GenBank database.

On the other hand, the PCR performed to evaluate the specificity of the primers, demonstrated no amplification neither for pathogenic leptospires species nor for other bacteria species, as mentioned above. All these results together, confirmed that the 16S primer pair is specific for the identification of intermediate leptospires species.

Duplex PCR results

From the 114 bovine sera, 13 samples yielded a positive result to the PCR (11,4%) which indicates the presence of leptospiral DNA in the sera. From these samples, 9 were positive for the pathogenic clade (69,3%) and the others amplified for the intermediate clade (30,7%). The results of the MAT and the PCR for each PCR positive sample are shown in Table 1.

Table 1
– Comparison of MAT and triplex PCR results of the PCR positive samples.

Sample	Animal category	MAT result	Duplex PCR result		Province
			G1/G2	16SFw/ 16SRv	
B21	Cow	Serovar Wolffii 1/200	Negative	Positive	Buenos Aires
B22	Cow	Serovar Hardjo 1/200	Negative	Positive	Buenos Aires
B23	Cow	Serovar Hardjo 1/200	Negative	Positive	Buenos Aires
B25	Cow	Negative	Negative	Positive	Buenos Aires
B34	Cow	Serovar Pomona 1/200; Wolffii 1/200, Hardjo 1/200	Positive	Negative	San Luis
B47	Cow	Negative	Positive	Negative	Buenos Aires
B78	Bull	Serovar Grippotyphosa 1/400	Positive	Negative	Buenos Aires
B80	Bull	Serovar Grippotyphosa 1/400	Positive	Negative	Buenos Aires
B91	Cow	Negative	Positive	Negative	Buenos Aires
B95	Cow	Serovar Pomona 1/200; Wolffii 1/200; Hardjo 1/400	Positive	Negative	Buenos Aires
B97	Cow	Negative	Positive	Negative	Buenos Aires
B98	Cow	Negative	Positive	Negative	Córdoba
B100	Cow	Negative	Positive	Negative	Córdoba

MM: Molecular Marker. Rows 1 to 14: bovine samples (B7, B21, B22, B23, B25, B34, B44, B47, B48, B51, B52, B53, B64, B71). Row 15: *Leptospira interrogans* serovar Pomona. Row 16 and 17: *L. fainei* serovar Hurstbridge (from Pasteur Institute and the National Institute of Infectious Diseases respectively). Row 18: *Pseudomonas* spp. Row 19: water.

Conclusions And Discussion

Interestingly, B21, B22, B23 and B25 belonged all to the same dairy farm located in Lincoln City (Buenos Aires province) that had sent 27 samples because of an abortion outbreak. 16 of those samples yielded positive results to MAT (and thus reacted to pathogenic serovars), being 1/800 the highest titer obtained. Unfortunately, the farmer did not send paired sera of these animals to confirm diagnosis. The fact that all the 4 animals that showed amplification for intermediate leptospira had low MAT titrations is particularly interesting.

This study revealed several important findings. In the first place, although being the gold standard diagnostic test, the MAT is not always suitable for diagnosing the acute phase of the disease, as shown by the result of B34 (Table 2). As mentioned above, this technique detects antibodies, which are present from approximately one week after infection². This animal (adult cow) was sampled due to abortion and the PCR yielded a positive result for the pathogenic clade, but the MAT showed low and unspecific co-agglutination between different serovars which is common in early phases of the disease³.

Probably, it was sampled in an early stage of the disease, when there are still no detectable antibodies in the animal. This absence of antibodies explains the detection of circulating leptospiral DNA. On the other hand, although B34, B52 and B64 had a positive result to MAT, their titers were low and showed nonspecific co-agglutination between different serovars- Probably, if a second sample had been taken after 15 days, it would have thrown a negative seroconversion result to the serovars conventionally used in the MAT.

In the second place, the new duplex PCR developed in this study demonstrated to be extremely useful for the discrimination of intermediate cluster of *Leptospira* spp. The sequences of the 16S rRNA gene present high identity among *Leptospira* spp. However, the slight differences between the sequences of pathogenic and intermediate clusters were used to design the primer 16SRv (Fig. 2) which is specific to amplify intermediate cluster *in silico* and in animal sera as shown in this study. It is important to highlight that since the intermediate cluster is the newest group of *Leptospira* spp. reported, there are some sequences of *Leptospira* spp. submitted in the public database as uncultured bacteria or unclassified leptospiral species that could belong to leptospires intermedia group. Moreover, according with Guglielmini *et al.*, 2019¹⁷, as the taxonomy of leptospires became more complex over time and recently new species were discovered, we strongly recommend the revision of the crude database output to detect misclassification of strains and species (such as *L. interrogans* assigned specie).

In the third place, this is the first report in Argentina of the detection of intermediate leptospiral DNA in animal samples, and the second report of detection of intermediate clade leptospiral DNA in animal sera in the world¹⁸. These results, combined with those reported by Grune *et al.*, 2016³², highlight the importance of considering animal sera as a useful sample for detecting DNA of diverse pathogens for the diagnosis of diseases.

Another outstanding finding is that the prevalence of infection in these samples was higher for intermediate species than for pathogenic ones (78,57% vs. 21,43% respectively), which concurs with findings of other authors in humans²⁸.

It is difficult to assess with only these results the causality of intermediate leptospires in these abortions, because of the lack of additional information such as diagnosis of other abortive diseases in the same animals. Despite this, it is a very important finding that there are species of intermediate clade circulating in animals with clinical disease in our country, which highlights the importance of considering these new species in the diagnosis of reproductive failures in animals and the possibility of transmission to humans.

In conclusion, this is the first report of intermediate species circulating in livestock animals in Argentina and the second report in the world. The novel end-point duplex PCR developed in this study demonstrated to be of great use for the discrimination of pathogenic and intermediate cluster of *Leptospira* spp. in animal sera. Moreover, this methodology could also be a valuable tool to apply in human sera. Undoubtedly, it would be interesting to go on studying the role of intermediate species as etiological agents in clinical leptospirosis in animals and as pathogens of public health importance.

Abbreviations

PCR: polimerase chain reaction

qPCR:quantitative polimerase chain reaction

MAT: microscopic agglutination test

RFLP: Restriction fragment length polymorphism

INTA: National Institute of Agricultural Technology, Argentina

UV: ultraviolet

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: OW selected the samples, carried out all laboratory work, analyzed the results and wrote the main manuscript. FCH designed the primers and analyzed the results. VRS and MH carried out laboratory work. BFB and MLM guided the research. SGL designed the primers, analyzed the results, and guided the research. All authors have read and approved the manuscript.

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Figures

Figure 1

Agarose gel electrophoresis of duplex PCR amplified products generated from bovine sera samples. MM: Molecular Marker. Rows 1 to 14: bovine samples (B7, B21, B22, B23, B25, B34, B44, B47, B48, B51, B52,

B53, B64, B71). Row 15: *Leptospira interrogans* serovar Pomona. Row 16 and 17: *L. fainei* serovar Hurstbridge (from Pasteur Institute and the National Institute of Infectious Diseases respectively). Row 18: *Pseudomonas* spp. Row 19: water.

Figure 2

Partial 16S rRNA sequences alignment of *Leptospira* spp. The 16SFw primer hybridization sequence (440-459 bp) and 16SRv primer hybridization sequence (1055-1079 bp) are shown underlined.