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Genotyping of *Leptospira interrogans* strains from Argentina by Multiple-Locus Variable-number tandem repeat Analysis (MLVA)

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ABSTRACT

Leptospirosis outbreaks occur regularly in Argentina and other South American countries, but little is known about their epidemiological relationships. Application of new molecular tools, such as the Multiple-Locus Variable-number tandem repeat Analysis (MLVA) is limited by scant available data on regional strains. We have analyzed the genetic diversity of a collection of 31 strains of *Leptospira interrogans* isolated in Argentina during the past five decades from humans and animals, including a strain from an environmental source and another isolated from an opossum. Genotyping was performed by MLVA using the loci VNTR4, VNTR7, VNTR9, VNTR10, VNTR19, VNTR23 and VNTR31, as described by Majed et al. [Identification of variable-number tandem-repeat loci in *Leptospira interrogans* sensu stricto. J Clin Microbiol 2005;43:539–45 [1]]. Clustering analysis revealed eight distinct MLVA genotypes, with a dominant one, genotype A. Strains with this genotype were consistently isolated since 1960, representing 55% of the total strains and spanning an extensive geographical distribution. Other seven genotypes were less frequent, and only genotypes A and Hond Utrecht IV were isolated during the last decade. Different kinds of repeat blocks for each VNTR locus were identified by sequence analysis. VNTR copy number differences among genotypes always involved only one of these blocks. MLVA patterns obtained reveal the genetic diversity and relationships between strains, and constitute the framework for the genotyping of leptospires in the region.

1. Introduction

In recent years leptospirosis, a zoonotic disease, has emerged as a globally important infectious disease occurring not only in rural regions worldwide but also in urban environments of both developing and industrialized countries [2,3]. Leptospirosis control has proven to be difficult because leptospires have the ability to survive for a long time in a wide range of environmental reservoirs, and both domestic and wild animals act as important disseminators of the disease. Leptospirosis can cause substantial economic losses as this disease affects reproductive performance in cattle and pigs, leading to reduced pregnancy rates, fetal mortality or the birth of weak animals [4–9].

Outbreaks of animal and human leptospirosis have been documented to occur fairly regularly in Argentina and other South American countries, such as Brazil and Peru [2,8–26]. Exposures that occur during flooding events and rural activities are the main risk factors of human leptospiro-

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Table 1
Strains of *Leptospira interrogans* used in this study.

MLVA genotype	Number of isolates	Strains	Source	Province and date of isolation	Genotype reference
A (serovar Pomona)	17	Marcos Juarez	Cow	Córdoba, 1960	[30]
		Fulton	Cow	Buenos Aires, 1976	[30]
		Longchamps	Human	Buenos Aires, 1977	[30]
		Pujato	Cow	Buenos Aires, 1981	[30]
		Marcos Paz	Pig	Buenos Aires, 1982	[30]
		Cañuelas	Pig	Buenos Aires, 1982	[30]
		San Alfredo	Pig	Santa Fe, 1984	[30]
		San Alfredo II	Pig	Santa Fe, 1985	[30]
		Rojas	Cow	Buenos Aires, 1985	[30]
		Cañuelas II	Pig	Buenos Aires, 1986	[30]
		Las Heras	Cow	Buenos Aires, 1987	This study
		Bayur	Cow	Buenos Aires, 1989	[30]
		Pig Ranch	Pig	Buenos Aires, 1995	[31], this study
		Patógena	Cow	Buenos Aires, 2004	[30]
		Pomona JCA	Cow	Buenos Aires, 2005	This study
		Draghi III	Cow	Corrientes, 2008	This study
		Lujan I	Cow	Buenos Aires, 2009	This study
B (serovar Pomona)	1	Macedo Balcarce	Cow	Buenos Aires 1981	[30]
C (serovar Pomona)	1	417	Human	Buenos Aires, 1999	[30]
D (serovar Pomona)	2	Draghi I	Cow	Corrientes, 1983	[30]
		Draghi II	Sheep	Corrientes, 1992	[30]
Baires	1	Baires	Dog	Buenos Aires, 1983	This study
"Ictero"	3	Entre Ríos I	Human	Entre Ríos, 1981	This study
		Reconquista II	River	Buenos Aires, 1996	This study
		Cañuelas III	Pig	Buenos Aires, 1996	This study
Hond Utrecht IV	4	M.4	Human	Unknown, 2004	This study
		M.5	Cow	Buenos Aires, 2004	This study
		Comadreja	Opossum	Buenos Aires, 2005	This study
		E3	Human	Buenos Aires, 2005	This study
MY 1039	2	Corrientes 266	Cow	Corrientes, 1985	This study
		Corrientes 289	Cow	Corrientes, 1985	This study
Total	31				

sis in Argentina [26], in accordance with transmission dynamics observed in other regions of the world [27]. An analysis of human serum samples from nearly all over the country showed that the most prevalent serogroups infecting humans are Icterohaemorrhagiae, Pomona, Ballum and Canicola, with the majority of cases occurring during the warm and rainy months [26].

New molecular methods like genotyping by Multiple-Locus Variable-number tandem repeat Analysis (MLVA) have been recently developed for typing leptospires [1,28,29], but their application is limited by the scarce available data on regional strains. In Argentina and in South America in general, there is scant data on molecular epidemiology of leptospirosis, in spite of the importance of this disease.

The genotyping of a collection of 16 strains of *Leptospira interrogans* serovar Pomona isolated from animals and humans from a wide region of Argentina has been recently performed in our laboratory in order to examine their genetic diversity and epidemiological relationships [30]. In that work, analysis of the loci VNTR4, VNTR7, VNTR9, VNTR10, VNTR19, VNTR23 and VNTR31 of the MLVA proposed by Majed et al. [1], revealed four new distinct genotypes within the serovar Pomona, denominated A, B, C and D, being genotype A numerically dominant. Later, five strains of serogroup Pomona isolated in Brazil from pigs were found to have a genetic profile identical to genotype A described in the Argentine strains [8]. These results indicated that most strains isolated in the region had genetic profiles different from those of other parts of the world, so it was necessary to characterize the genotypes of leptospires prevalent in the region in depth in order to obtain a suitable database to be used in epidemiological tracing analysis.

The present study describes the MLVA patterns of strains of *L. interrogans* isolated during different outbreaks occurring in humans, cattle, pigs and wild animals throughout Argentina in the last 50 years. These widely representative data reveal the genetic diversity and relationships between regional strains, and constitute the framework for the genotyping of leptospires in the region.

2. Materials and methods

2.1. *Leptospira interrogans* strains and geographical references

The strains of *L. interrogans* used in this study are part of the collection of the Leptospirosis Laboratory, INTA (Instituto Nacional de Tecnología Agropecuaria) and OIE — World Organization for Animal Health reference laboratory. Sources, dates and areas of isolation are listed in Table 1. Strains Pomona (serovar Pomona, serogroup Pomona), M20 (serovar Copenhageni, serogroup Icterohaemorrhagiae), RGA and Ictero No. I (serovar Icterohaemorrhagiae, serogroup Icterohaemorrhagiae) and Hond Utrecht IV (serovar Canicola, serogroup

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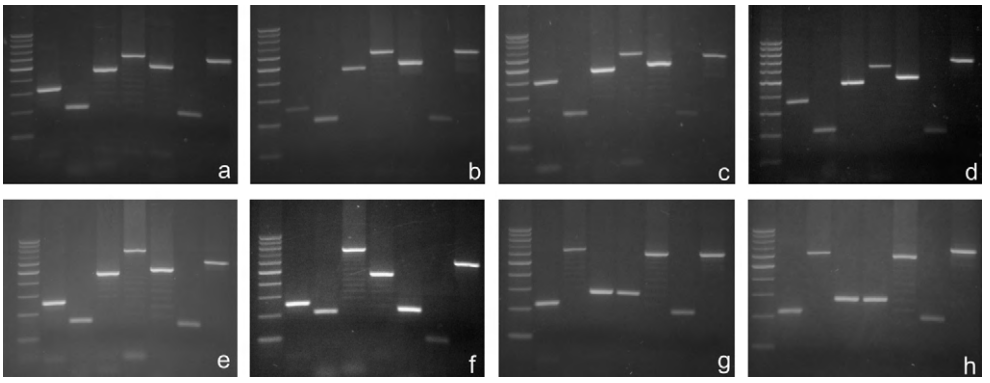

Fig. 1. MLVA patterns for the following *L. interrogans* genotypes: (a) genotype A, serovar Pomona (strain Pig Ranch); (b) genotype B, serovar Pomona (strain Macedo Balcarce); (c) genotype C, serovar Pomona (strain 417); (d) genotype D, serovar Pomona (strain Draghi II); (e) genotype Baires (strain Baires); (f) genotype "Ictero" (strain Entre Ríos I); (g) genotype Hond Utrecht IV (strain E3); and (h) genotype MY 1039 (strain Corrientes 266). The VNTR loci evaluated were: VNTR4 (lane 2), VNTR7 (lane 3), VNTR9 (lane 4), VNTR10 (lane 5), VNTR19 (lane 6), VNTR23 (lane 7) and VNTR31 (lane 8). Lane 1: CienMarker.

Canicola) were used as reference strains in this work. MLVA data of MY 1039 (serovar Portlandvere, serogroup Canicola) was obtained from Majed et al. [1].

2.2. Strain genotyping

Each *L. interrogans* strain was grown in EMJH or Fletcher media (Difco Laboratories) at 28 °C. Samples of cultures (100 μl) were incubated at 100 °C for 10 min and used directly as DNA template in a MLVA strain typing procedure performed with the primers described by Majed et al. [1] flanking VNTR4, VNTR7, VNTR9, VNTR10, VNTR19, VNTR23 and VNTR31 loci. The final volume (50 μl) of each reaction mixture contained PCR buffer (20 mM Tris–HCl, pH 8.4; 50 mM KCl), 200 μM deoxinucleoside triphosphates, 2 μM each corresponding primer, 2 mM $MgCl_2$, 1.25 U of Taq DNA polymerase (Invitrogen) and 5 μl of DNA template. PCRs were carried out in a MiniCycler PTC-150 (MJ Research) as follows: 94 °C for 5 min, followed by 35 cycles of denaturalization at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 90 s, with a final cycle at 72 °C during 10 min. Each amplified sample (15 μl) was subjected to electrophoresis in a 2.2% agarose gel in buffer TAE (40 mM Tris–acetate, 1 mM EDTA) with 0.2 μg/ml of ethidium bromide at 100 V for 50 min. Amplified DNA bands were visualized upon UV light exposure (DynaLight, Labnet). Amplicon sizes were estimated using CienMarker (Biodynamics) and were then converted into repeat copy number using the formula Number of repeats (bp) = [Fragment size (bp) – Flanking regions (bp)]/Repeat size (bp). Repeat copy numbers were rounded down to the closest whole numbers. If the copy number was less than one it was rounded down to zero.

2.3. Sequencing of PCR products

Amplicons of Marcos Juarez, Macedo Balcarce, 417, Draghi I, Baires and Pomona strains were purified and sequenced in an Applied Biosystems 3730xl Automatic DNA Sequencer by Macrogen (Korea) using amplification primers. The partial sequences of the VNTR loci of the field strains of this study and the Pomona reference strain have been deposited in GenBank, under the Accession Nos. GU362888–GU362928.

2.4. Data analysis

DNA sequences were analyzed using the GenBank database of the National Center for Biotechnology Information BLAST network service. Tandem Repeats Finder programme was used to define exactly the copy number of each VNTR locus [32]. UPGMA (unweighted pair group method with arithmetic mean) clustering analysis was performed using the Sequence Type Analysis and Recombinational Tests (STAR) software [33] on genotype scores from a combined data set constituted by the Argentine genotypes and the genotypes of the strains used as reference in this work.

3. Results

A collection of 31 strains of *L. interrogans* isolated from animal and human samples obtained from outbreaks occurring in several regions of Argentina during the past 50 years were analyzed for genetic diversity (Table 1). Most strains were isolated from cows, but the analysis also included strains from humans and domestic and wild species like pig, sheep, dog and white-eared opposum. The collection also includes an environmental strain isolated from a water sample obtained from the river Reconquista, in a densely urbanized zone (Gran Buenos Aires) nearly 20 km from the city of Buenos Aires.

Seven-locus MLVA of the 31 strains showed a total of eight distinct MLVA genotypes, four of them corresponding to the new genotypes we had previously described for Argentine strains restricted to the serovar Pomona [30], and four additional genotypes. Genotype A, within the serovar Pomona, was the dominant one found in 17 isolates, and the rest of the genotypes were poorly represented, with a number of strains for each of them ranging between one and four. The eight patterns obtained are shown in Fig. 1.

Prevalent genotype A (Fig. 1a) was observed in strains isolated during outbreaks occurring mostly in Buenos Aires (13 out of 17), Santa Fe (2 out of 17), Córdoba (1 out of 17)

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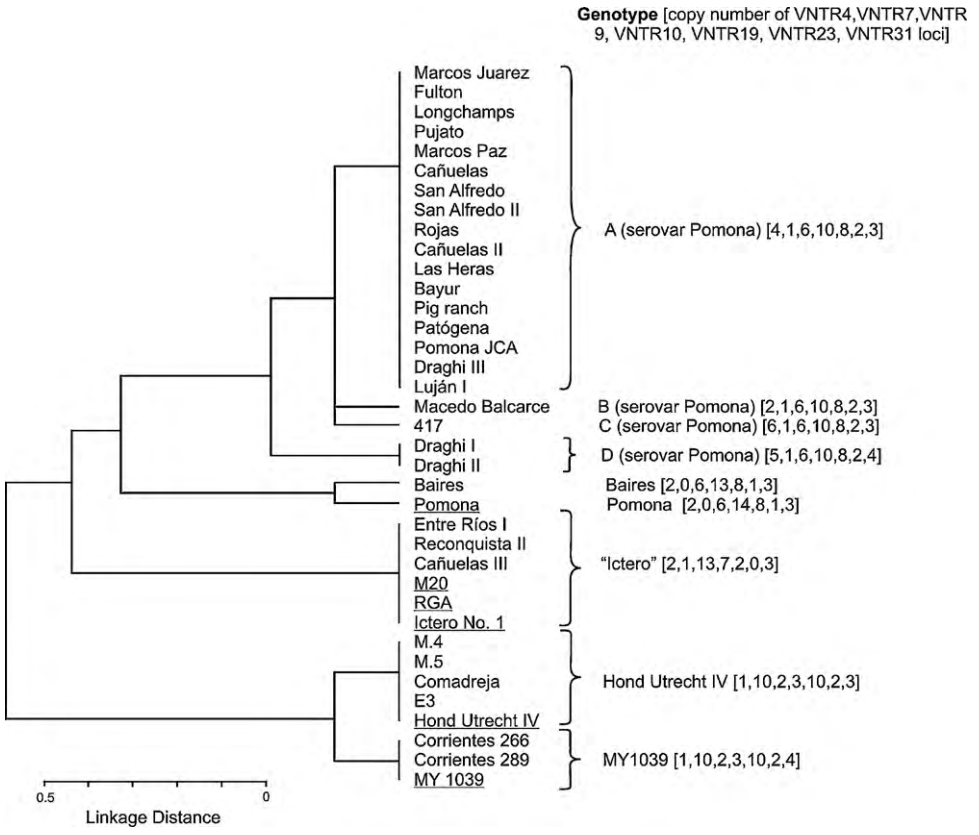

Fig. 2. Genetic relationships among Argentine strains of *L. interrogans*. Seven VNTR marker loci were analyzed to estimate the genetic relationships among all of the strains examined in this study, using UPGMA clustering analysis. In this analysis we included previous data [1] of the genotypes of reference strains indicated in materials and methods. Names of strains used as reference are underlined.

and Corrientes (1 out of 17), between 1960 and 2009, all of them involving cows and pigs except for one human isolate (Table 1). This genotype forms a cluster (Fig. 2) the previously reported genotypes B, C and D, within the serovar Pomona, and all them show a distant genetical relationship to the genotype of strain Pomona (serovar Pomona, serogroup Pomona) [30]. None of the strains isolated in Argentina during the last 10 years had the rare genotypes B, C and D (Table 1).

Strain Baires, isolated from a dog in Buenos Aires in 1983, and typed as belonging to the new serovar Buenos Aires (serogroup Djasiman) using serological and molecular methods [34], showed the same MLVA pattern as the Pomona strain in agarose gels (Fig. 1e). However, sequence analysis revealed 13 copies of the repeat unit in the VNTR10 locus, instead of the 14 copies published for the Pomona strain [1]. According to the clustering analysis, the new Baires genotype appears to be closely related to the Pomona genotype and more distantly related to the four recently reported genotypes for Argentine strains [30] (Fig. 2).

The genotype of three strains isolated from very diverse sources (human, pig and river water), serologically typed as belonging to the serogroup Icterohaemorrhagiae, was identical to a genotype common to three strains of serogroup Icterohaemorrhagiae, M20 (serovar Copenhageni) and RGA (serovar Icterohaemorrhagiae) described in a previous work [1], and Ictero No. I (serovar Icterohaemorrhagiae) analyzed in our laboratory. Consequently, this genotype was generically named as "Ictero" (Figs. 1f and 2). One of the strains was isolated from pig urine and the second from the superficial water of a river close to the place where the pigs were raised.

The genotype of four of the strains analyzed, isolated in 2004 and 2005 also from very varied sources (human, cow and the white-eared opossum *Didelphis albiventris*), matched exactly the genotype previously described [1] for strain Hond Utrecht IV (serovar Canicola, serogroup Canicola), and the genotype of two strains isolated in 1985 from cows showed an exact match to the genotype described [1] for strain MY 1039 (serovar Portlandvere, serogroup Canicola) (Figs. 1g and h and 2).

Considering all the strains in the collection, the locus VNTR4 presented the highest variability among the seven loci analyzed (Figs. 1 and 2), with five different alleles, one shared by three genotypes (B, Baires and "Ictero"), another by two (Hond Utrecht IV and MY 1039), and the rest unique to the remaining genotypes (A, C and D). Four different alleles were found for VNTR10, the first shared by genotypes A–D, the second by genotypes Hond Utrecht IV and MY 1039, and the other two represented by genotypes Baires and "Ictero" respectively. VNTR7, VNTR9, VNTR19 and VNTR23 showed three alleles each one. For VNTR7, genotypes A–D and "Ictero" shared the most common allele, while genotypes Hond Utrecht IV and MY 1039

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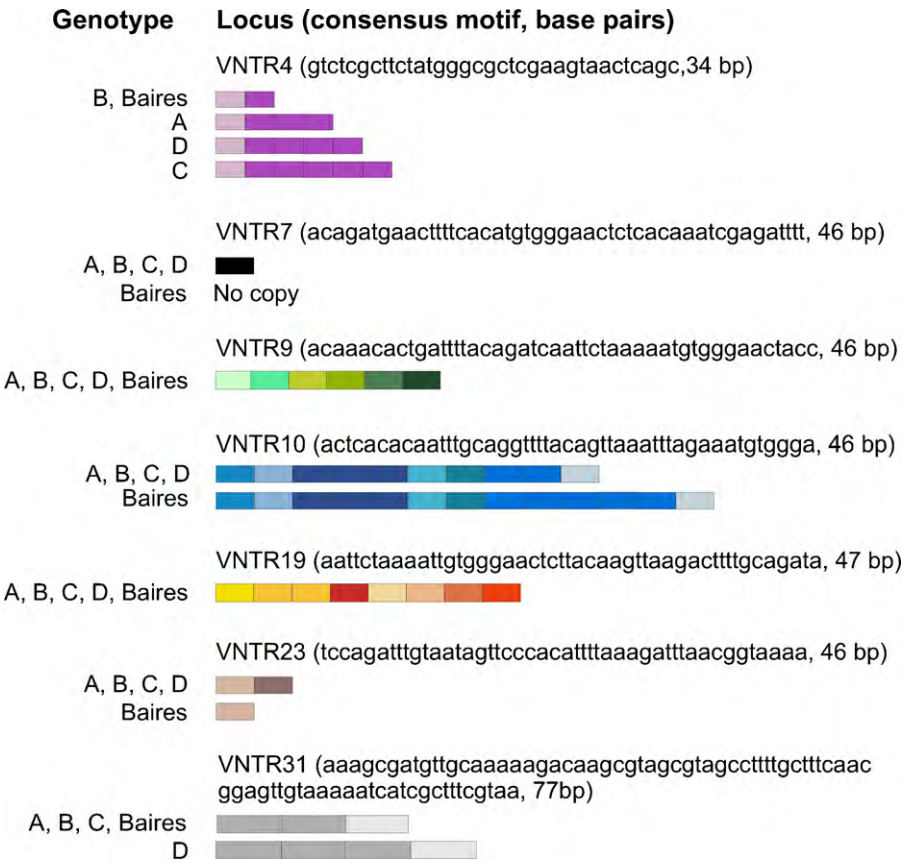

Fig. 3. Structure of the VNTR loci corresponding to the genotypes A–D and Baires. For each VNTR locus, rectangles represent different kinds of blocks. Each kind of block, shown in different color shades, is defined by specific small sequence changes (single-base mismatches or indels) compared to the corresponding consensus pattern, indicated for each VNTR between brackets.

shared the second and Baires was the only genotype with no repeat copies. In the case of VNTR9 and VNTR19, genotypes A–D and Baires shared one allele, Hond Utrecht IV and MY 1039 the second, and "Ictero" had the last allele. For VNTR23, genotypes Baires and "Ictero" presented unique alleles, while the rest of the genotypes shared the predominant allele. VNTR31 was the less informative locus, with only two alleles, the first shared by genotypes A–C, Baires, "Ictero" and Hond Utrecht IV, and the second by genotypes D and MY 1039.

To further characterize the Argentine strains, all VNTR loci from representatives of the genotypes unique to the region (genotypes A of serovar Pomona found in Argentina and Brazil; and genotypes B, C and D of serovar Pomona and genotype Baires found in Argentina) were sequenced and analyzed using the Tandem Repeats Finder program. Sequence data corroborated the number of repeats for each locus deduced by agarose gel electrophoresis visualization, except for VNTR10 of genotype Baires, mentioned above. In this case, the correct copy number could only be determined after sequence analysis.

Small sequence divergence between the repeat units of each VNTR locus was observed when compared to the corresponding consensus pattern obtained with the Tandem Repeats Finder program. This repeat degeneracy could be due to single-base mismatches or single-base indels. These variations enabled the identification within each locus of different kinds of "blocks", corresponding to variants of a given repeat unit compared to the consensus sequence (Fig. 3). Variations in the copy number of each VNTR locus always involved differences in the number of copies for only one of the different blocks, while the rest of the blocks showed the same number of copies in all genotypes.

4. Discussion

Recently developed MLVA assays for typing leptospires have demonstrated to be innovative, easy, rapid and highly discriminatory methods to achieve this purpose [1,28,29]. Besides their accessibility, they are convenient for the exchange of information between different laboratories implicated in diagnosis of leptospirosis or molecular epidemiology studies [1,30]. Other new methods proposed for typing *L. interrogans* [35,36], based on multilocus sequence typing, require extensive sequencing, which makes them difficult to implement as routine techniques, while MLVA approaches only involve simple electrophoretic techniques once DNA amplification has been performed.

The analysis of 31 strains of *L. interrogans* by seven-locus MLVA revealed a great genetic diversity in the most populated and main farming region of Argentina, comprising the provinces of Buenos Aires, Córdoba, Santa Fe, Corrientes

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and Entre Ríos. Eight MLVA genotypes were recognized, five of them unique to the region, including one previously undescribed genotype (genotype Baires) corresponding to a strain isolated from a dog. Genotype A is the numerically dominant genotype, described in isolates from Argentina and Brazil [8,30], and it is genetically related to the recently reported genotypes B–D, corresponding to strains isolated in Argentina [30]. These four genotypes are closer to each other than to any other MLVA genotype previously defined, and all of them correspond to strains included in serovar Pomona [30]. One of the eight MLVA genotypes showed a perfect match with the common genotype of several strains (M20, RGA and Ictero No. I) of serogroup Icterohaemorrhagiae. The last two MLVA genotypes found in the local isolates matched exactly those already described for strains Hond Utrecht IV and MY 1039 from serogroup Canicola [1]. Data from a recent report describing leptospires isolated from sea lions and several other animals could unfortunately not be compared with our data due to the usage of different sets of VNTR loci and copy number calculation criteria [37].

Strains with genotype A were consistently isolated during the last 50 years, all of them from cows and pigs, with the exception of one strain that was isolated from a human sample. They represented 55% of the strains in the collection, and had an extensive geographical distribution. Strains with genotype Hond Utrecht IV, isolated in two consecutive years, represented 13% of the total collection and were restricted to Buenos Aires, except for one strain of unknown origin. Nearly 10% of the strains of the collection, including samples from Buenos Aires and Entre Ríos, presented genotype "Ictero". The less abundant genotypes were genotypes B and C, found only in one isolate each, both from Buenos Aires; genotype D, found only in two isolates from Corrientes; and genotype MY 1039, found in two strains, both isolated the same year in Corrientes. The isolates from the last decade presented only genotypes A (4/8) and Hond Utrecht IV (4/8) and no new isolates showed genotypes B, C or D, nor genotypes Baires, MY1039 or "Ictero".

The greatest diversity corresponded to Buenos Aires, represented by two thirds of the samples (21/31), that gathered genotypes A–C, Baires, "Ictero" and Hond Utrecht IV. This diversity is probably related to the high density of cattle population in this province and the higher availability of sanitary resources compared to other regions of the country.

No prevalent genotype was detected in *L. interrogans* strains isolated from humans, as strains obtained during different outbreaks presented genotypes A (1/5), C (1/5), "Ictero" (1/5) and Hond Utrecht IV (2/5).

Leptospires isolated from cows, corresponding to half of the strains in the collection, showed the most varied genotypes with genotype A being prevalent: A (10/15), B (1/15), D (1/15), Hond Utrecht IV (1/15) and MY 1039 (2/15). Genotype A was also prevalent in pigs (6/7), with only one pig isolate presenting genotype "Ictero". This last result was in accordance with a recent survey carried out in Brazil [8], where serovar Pomona and genotype A were identified as the prevalent serovar and genotype associated to leptospirosis in swine. Among the strains in our collection found to have the genotype "Ictero", one was isolated from pig urine and another could be traced back to the superficial water of a river close to the place where the pigs were raised. *L. interrogans* strains were also isolated from sheep (one isolate with genotype D) and a dog (one isolate with genotype Baires). Interestingly, one strain isolated from the South American white-eared opossum *D. albiventris*, representing the first leptospire isolated from this marsupial, presented genotype Hond Utrecht IV. Previous studies have reported the isolation of strains of *L. interrogans*, *L. borgpetersenii, L. santarosai* and *L. noguchii* in other opossum species from North and South America, such as *D. marsupialis* and *Philander opossum* [38–41] but, to the best of our knowledge, leptospires of *L. interrogans* serogroup Canicola have not been isolated from opossums up to date.

Both the strains obtained from river water and the opossum reinforce the known importance of environmental water and wild animals as sources of infection and dissemination of leptospires [3]. More than 30 years ago several isolates of *L. interrogans* and *L. noguchii* were obtained in Argentina from another wild animal, the armadillo (*Chaetophractus villosus*), and at that time it was suggested that the armadillo was an important natural reservoir for pathogenic leptospires [42]. Unfortunately, as none of the strains isolated from armadillos have survived, they could not be analyzed in this study.

All available isolates in Argentina from the past 50 years, spanning different geographical regions, have been used in this study. However, all epidemiological interpretations must be considered carefully, as a possible sampling bias cannot be ruled out due to the lack of a systematic sampling.

Considering the eight genotypes analyzed, locus VNTR4 presented the highest variability among the seven loci studied, with five different alleles, followed by VNTR10 with four alleles. Loci VNTR7, VNTR9, VNTR19 and VNTR23 showed three alleles each, being VNTR31 the less informative locus with only two alleles.

Analysis of the sequences of all VNTR loci corresponding to the five regional genotypes (A–D and Baires) showed sequence divergence between the repeats, so that different kinds of repeat blocks could be distinguished by distinctive sequence tags. These tags made it possible to determine the number of copies of each block within each VNTR locus and also to identify which blocks showed copy number variations among the genotypes. Differences in the number of copies for each VNTR locus corresponding to each genotype were always observed to involve only one of the blocks. This suggests that the variation in the number of repeats occurs by slipped strand mispairing as suggested for other short sequence repeats [43–45]. It is probable that this same organization will be observed in other genotypes when sequence information becomes available.

It is interesting that only in Argentina, five novel genotypes have been described, four in a previous study [30] and the new genotype Baires described in the present work, reinforcing the idea that the real diversity of the leptospires remains yet to be discovered. This work sets the groundwork for a regional *Leptospira* genotype databank, and provides essential information for future molecular diagnosis and epidemiological tracing analysis for this important disease.

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