ORIGINAL ARTICLE

Characterization of *Leptospira* isolates from humans and the environment in Uruguay

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ABSTRACT

Laboratory diagnosis of human leptospirosis usually relies on indirect methods exploring specific immune response. Isolation and identification of the involved strains are cumbersome, but can provide biological resources for pathogenic studies and relevant information for guiding prevention and control measures. The aim of the research we are hereby reporting was the characterization of *Leptospira* isolates obtained from humans and the environment in Uruguay. Blood cultures were performed from early samples of 302 Uruguayan patients, mainly rural workers, and from 36 water samples taken from their living or working environments. Eight human isolates and seven environmental isolates were obtained and analyzed by end point Polymerase Chain Reaction (PCR), Multilocus Variable Number of Tandem Repeat Analysis (MLVA) and other molecular methods. Human isolates corresponded to several serogroups and serovars of *Leptospira interrogans* and *Leptospira kirschneri* species, probably reflecting the infection with similar involved *Leptospira* species and serovars of an extended animal reservoir in rural settings of the country, mostly dedicated to meat and dairy production. Culture-positive patients were older than usually affected workers, and presented signs and symptoms of severe illness. A high organic and circulating bacterial burden may explain an easier positive result from these workers' samples. Environmental isolates were mainly identified as *Leptospira biflexa* strains, with a single *L. meyeri* isolate of uncertain significance.

KEYWORDS: *Leptospira*. Human isolates. MLVA-VNTR-PCR. Leptospirosis. Environment *Leptospira*. *Leptospira interrogans*. *Leptospira kirschneri*. *L. meyeri*.

INTRODUCTION

Some estimated 500 to 1,000 human leptospiral infections occur annually in Uruguay, mainly as mild or subclinical illnesses, but sometimes with a severe course or a lethal outcome[1].

Human leptospirosis presents in our country as sporadic cases or limited outbreaks occurring mainly in rainy periods or during floods, and usually affecting young males involved in cattle or dairy farming, rural work, abattoirs or animal transport. Breeding animals largely outnumber the Uruguayan population of 3.3 million people[2]; a variable proportion of the 6.6 million ovine and porcine livestock, and of more than 12 million bovine cattle inhabiting the country make part of the huge reservoir of this zoonosis[3].

Diagnosis of leptospirosis has relied for many years on the investigation of specific antibodies through various procedures such as immunofluorescence, ELISA,

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immune-chromatographic methods for early detection of IgM and IgG, or Micro Agglutination Technique (MAT) as the reference indirect test revealing immune response to *Leptospira* in serum samples[4,5].

MAT applied to serum samples of infected patients explores the antibody reactivity to several strains, using bacterial antigens, usually yielding positive results, but even higher titers do not reliably point out to the actual infecting species or serovar. This information can only be obtained through culture and identification of isolates.

Leptospira do not readily develop in usual microbiological media and are easily overgrown by contaminating bacteria or fungi. Nevertheless, they can be cleanly recovered from blood cultures, but only during the first days of illness. Urine cultures can possibly be performed for some further weeks because bacteria are eliminated in this fluid for longer periods; nevertheless, isolation from this source is not easy due to difficulties for obtaining and transporting samples without contamination, and because of the short survival of *Leptospira* in acid urine samples.

Anyway, isolation of *Leptospira* from human infections, although cumbersome and difficult[6], must be attempted for epidemiological reasons, aiming to identifying involved species and serovars, comparing isolates with those obtained from animal reservoir to study infection sources and routes, and to select important strains for animal and human vaccine development.

PCR assays, and qPCR, since 2000, have also been applied as useful diagnostic tools, but their performance is satisfactory on blood samples only during the initial days of illness, when blood cultures are potentially positive[7-9].

Molecular identification methods such as Multilocus Variable number tandem-repeat Analysis, (MLVA), Multilocus Sequence typing (MLST), Pulsed-Field Gel Electrophoresis (PFGE), and partial or whole-genome sequencing (P-WGS) are increasingly employed for further characterization of isolates. When combined with serotyping, they enable to define species, serovars and strain variants, providing information of great value for epidemiological, pathogenic and preventive studies of *Leptospira* infections[10,11].

No previous work has been carried out in our country regarding the characterization of human and environmental *Leptospira* isolates. As part of our program of individual diagnosis and epidemiological study of leptospirosis in Uruguay, we decided to regularly perform blood cultures from presumptively infected persons that are routinely examined by MAT. Urine, organs or blood cultures from animal reservoir, and environmental cultures from water collections or resources in sites where human or animal infections have been reported were also included.

The aim of the research that we are hereby reporting was the recovery and molecular identification of isolates from human and environmental origin, as performed in our laboratories (Bacteriology and Virology Department, Hygiene Institute, *Universidad de la República*, Uruguay) with co-workers from the Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.

MATERIAL AND METHODS

Sample collection and culture conditions

This study was performed from January 2010 to December 2016. In this period, serum antibodies from 4,200 patients and blood cultures from 302 of them were studied. Samples were obtained and sent by Health workers for diagnosis of human patients with suspected leptospirosis, mainly living in rural areas. Thirty six samples were additionaly obtained and cultured by our workgroup from urban water collections close to human slum houses, or from water collections found in cattle raising and dairy farms where positive human cases had been confirmed, and which harboured at risk population groups holding extended contact with the animal reservoir.

Blood cultures were seeded from blood collected in EDTA tubes which were usually accompanied by the first serum sample of the same patient and by a record form providing relevant clinical and epidemiological information (personal data, onset date of symptoms and of blood extraction, type of patient's work and others).

When blood had been extracted no more than ten days after the illness onset, it was inoculated into two culture media: Ellinghausen-McCullough-Johnson-Harris EMJH broth (Difco-BD®) and Fletcher semisolid medium (Difco-BD®) supplemented with membrane-filtered inactivated rabbit serum. Two drops (50-100 uL) of blood were included into five mL of media prepared in screw-capped tubes.

Water samples were collected in sterile plastic containers, slowly filtered through 0.22-uM pore membranes and inoculated (0.5 mL) into the same both media and tubes.

Culture tubes were placed in 28 °C incubators, and examined periodically under dark field microscopy, with 600x (40x15) magnification, in a Nikon Eclipse Ci-L® equipment. The first observation was performed one or two weeks after inoculation, and then weekly until six to eight months' incubation[5,12].

Visually suspected positive cultures were subcultured in the above-mentioned media, and if a mixed flora was found or presumed, membrane filtration or inoculation into EMJH with 200 µg/mL 5-Fluorouracil (or both) were performed for selecting *Leptospira*.

Bacterial DNA extraction for PCR assays

DNA of suggestive cultures with turbidity equivalent to 0.5 in the McFarland scale (1.5 x 10^8 bacteria/mL) was extracted through silica gel column with DNeasy Blood & Tissue® kit (Qiagen®, Germany), following the manufacturer instructions. The DNA was kept frozen at -20 °C until use.

PCR (Polymerase Chain Reaction)

The extracted nucleic acid was analyzed by end point PCR. *LipL32*-45F and *LipL32*-286R primers were used to amplify gene sequences coding for the *LipL32* membrane lipoprotein which is only found in pathogenic *Leptospira*[13]. To amplify *Leptospira 16S* ribosomal RNA coding sequences, primers were LeptoA F and LeptoB R[14]. Saprophytic *Leptospira* can yield positive *16S* results, but not *LipL32* amplicons[15].

Both amplifications were performed in a total volume of 25 uL, including 1X Buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.64 uM of each *LipL32* primer (SBS Genetech Co. Ltd.) and 0.5 uM for *16S* primers, 1 ug/uL BSA (Bovine Serum Albumin, fraction V, Sigma®), 1.5U DNA Taq polymerase (Thermofisher Scientific inc.®) and 1 uL DNA template.

Conditions were the same for both reactions, consisting of an initial denaturation step of 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 57 °C, and 30 s at 72 °C. The final extension step was set at 72 °C for 10 min.

Amplification reactions were carried out in a Gene Amp PCR System 2700 thermocycler (Applied Biosystems®, California, USA).

PCR products were loaded in 2% agarose gels prepared in 0.5X Tris-Borate-EDTA (TBE) buffer and separated by electrophoresis at 100 V for 45 min. DNA bands were revealed by ethidium bromide staining and visualized with UV light in the FOTO/Analyst Investigator Eclipse FOTODYNE® system (Thermo Fisher Scientific, Waltham, MA, USA).

An additional PCR primer pair, *23S*-P1 and *23S*-P2[16], was applied to water isolates yielding positive *16S* and negative *LipL32* results for differentiating *L. biflexa* isolates from those of other saprophytic *Leptospira* species. In amplifications, 0.8 uM of *23S*-P1 and *23S*-P2 primers were used with the same reaction conditions as those of *16S* PCR, except for the annealing temperature, which was set at 54 °C.

MLVA (Multilocus Variable-Number of Tandem Repeat (VNTR) Analysis)

Human isolates showing positive *16S* and *LipL32* PCR results were kept in Fletcher or EMJH semisolid media, subcultured bimonthly and later studied through the MLVA molecular typing procedure, seeking for individual VNTR profiles that may contribute to a more precise identification of strains in terms of species and serovar. Three primer pairs were used to amplify *VNTR 4, VNTR 7* and *VNTR 10* loci according to methods published by Salaün *et al.*[17]. They allow characterizing strains of *L. interrogans, L. borgpetersenii* and *L. kirschneri.*

PCR reactions were carried out in a total volume of 25 uL, with 1X Buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl), 1.4 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 uM primers, 1.3 U Taq DNA polymerase (Thermofisher®) and 2.5 uL of template DNA. The amplification program included an initial 5 min denaturation step at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min 30 s at 72 °C. The final extension step was set at 72 °C for 10 min. Agarose gel electrophoresis and examination of DNA bands were performed as described above.

PFGE (Pulsed-Field Gel Electrophoresis)

Two of the six available isolates, obtained from patients with negative MAT results, were selected for MLST and PFGE analysis.

PFGE was performed following a modified technique published by Galloway and Levett[11]. Bacterial suspensions were adjusted to an optical density of 1.4 at 610 nm, mixed with equal volume of 1.2% pulse-field grade certified agarose solution preheated to 55 ºC (Bio-Rad, Hercules, CA, USA), and placed into suitable molds. Plugs were washed six times with wash solution, cut into ca. 2 mm height pieces and digested for 2 h at 37 ºC with 25U of *Not*I restriction enzyme (New England Biolabs Inc., Ipswich, MA, USA).

Separation of DNA fragments was performed using a CHEF DR-II system (Bio-Rad). *Salmonella enterica* serotype Braenderup (CDCH9812) was used as a DNA standard pattern[18].

Gels were stained with ethidium bromide, washed three times with distilled water, and examined by an image capture system, as mentioned.

MLST (Multilocus Sequence Typing)

MLST was performed on both human isolates that were also examined through PFGE. According to the procedure previously described by Boonsilp *et al.*[19], amplification of internal sequences from seven house-keeping genes (*glmU, pntA, pfkB, caiB, mreA, sucA* and *tpiA*) was followed by sequencing of the obtained DNA products. Five uL of

each endpoint PCR product were run in 2% agarose gel electrophoresis to determine if the amplification had been effective. The remaining volume was sequenced in RPT01A-PDTIS/FIOCRUZ DNA sequencing equipment (http://plataformas.cdts.fiocruz.br/). Sequences were then entered into the database (http://leptospira.mlst.net/; https://pubmlst.org/leptospira/) and matched with known allelic sequences using the MAFFT program for multiple sequences alignment (www.ebi.ac.uk/Tools/msa/mafft/)[20] to determine the allelic profile and Sequence Type (ST) of the examined strains.

Partial *16S* rDNA sequencing

Lepto A and Lepto B primers[14] were used to amplify a 330 bp size segment from the *16S* rDNA gene of each strain. Amplicon sequencing was performed in both directions at the IPMont (Institut Pasteur, Montevideo) using primers LeptoC 5´ CAAGTCAAGCGGAGTAGCA-3´ and S4-5´ TCTTAACTGCTGCCTCCCGT-3´. The obtained sequences were compared with those of strains deposited on GenBank using the BLAST tool (https://blast.ncbi.nlm. nih.gov/Blast.cgi). Results are shown in Table 1.

Micro-Agglutination Technique for serum antibodies investigation

MAT was performed in all serum samples according to the standard technique with a two-step procedure[1,4,21]. Each serum was initially diluted 1:25 in saline and mixed with equal volumes of each of the 20 live cultures of *Leptospira* serovars that yield reactive results very frequently in the region and are able to promote cross-reactions. The serogroups and serovars of the employed strains were: Australis Australis; Australis Bratislava; Autumnalis Autumnalis; Autumnalis Butembo; Ballum Castellonis; Bataviae Bataviae; Canicola Canicola; Cynopteri Cynopteri; Grippotyphosa Gryppotyphosa; Hebdomadis Hebdomadis; Icterohaemorrhagiae Copenhageni; Mini Mini; Pomona Pomona; Pomona Kennewicki; Pyrogenes Pyrogenes; Sejroe Hardjo; Sejroe Sejroe; Sejroe Wolffii; Semaranga Patoc; Tarassovi Tarassovi.

In a second step, agglutinating serovars were tested against serial dilutions of the patient's serum. Titers equal or higher than 400 in a single serum sample against at least one serovar were considered a confirmed case of leptospirosis. In addition, a fourfold increase in titers from the acute to the convalescent sample was interpreted as a confirmed result.

Table 1 - Identification of human and environment isolates

	ISOLATE ID	MAT RESULT	PCR *Lip*L32/*16Sr*RNA[12,13]	*23S* PCR[15]	MLVA[16] ANALYSIS	PFGE[11] ANALYSIS	MLST[18]	Sequencing*	Identification
HUMAN ISOLATES(X)	AH1	1st pos 2nd pos	pos/pos	ND	ND	ND	ND	*L. interrogans*	*L. interrogans* Pomona Pomona
	AH2 (2ˣ)	only one neg sample	pos/pos	ND	5-0-10	*L.interrogans* Pomona Kennewicki	ST 140	*L. interrogans*	*L. interrogans* Pomona Kennewicki
	AH3 (3ˣ)	only one neg sample	pos/pos	ND	1-10-3	*L.interrogans* Canicola Canicola/ Portlandvere	ST 37	*L. interrogans*	*L. interrogans* Canicola Canic/Portlandvere
	AH4 (6ˣ)	1st neg 2nd pos	pos/pos	ND	3-2-11	ND	ND	*L. interrogans*	*L. interrogans* Sejroe Wolffi/Romanica
	AH5 (10ˣ)	only one pos sample	pos/pos	ND	1-5-4	ND	ND	*L. kirschneri*	*L. kirschneri* Australis Ramisi
	AH6 (4ˣ)	1st neg 2nd pos	pos/pos	ND	ND	ND	ND	*L. kirschneri*	*L. kirschneri* serov. Mozdok?
ENVIRONM. ISOLATES	AA1#	ND**	neg/pos	neg	ND	ND	ND	*L. meyeri*	*Leptospira meyeri*
	AA2#	ND	neg/pos	pos	ND	ND	ND	ND	*Leptospira biflexa*
	AA3##	ND	neg/pos	pos	ND	ND	ND	ND	*Leptospira biflexa*
	AA4##	ND	neg/pos	pos	ND	ND	ND	ND	*Leptospira biflexa*
	AA5##	ND	neg/pos	pos	ND	ND	ND	ND	*Leptospira biflexa*
	AA6##	ND	neg/pos	pos	ND	ND	ND	ND	*Leptospira biflexa*
	AA7+	ND	neg/pos	pos	ND	ND	ND	ND	*Leptospira biflexa*

*: Partial *16S* sequence; **: not done; ˣ: time (days) elapsed between onset of symptoms and blood extraction for culture; #: water from slums; ##: water from rural cattle or dairy farms; +: water from a small island.

RESULTS

Human isolates

Eight blood cultures were identified as positive in the considered period through a presumptive microscopic observation and molecular confirmation methods. They were positive through both *16S* and *LipL32* PCR, indicating they are pathogenic isolates, as expected of bacteria recovered from symptomatic patients with signs compatible with leptospirosis.

Positive blood cultures were usually detected in both inoculated media (Fletcher and EMJH), although estimated initial bacterial populations (through dark field microscopy) were not equal. AH4 was only recovered from EMJH, and AH6 solely from Fletcher. All positive cultures were obtained from rural workers, most of them laboring in dairy farms, located in Southern departments of the country. Their age varied from 32 to 63 years old, and evolution of illness was frequently severe, with liver involvement or other organic disorders, added to general signs and symptoms of fever, headache, asthenia, myalgia and arthralgia. MAT tests were negative in three cases, because no second sample was obtained to study seroconversion. When MAT was positive in the first or second serum sample, the isolated serovar did not correspond to the serovar of the reference strain yielding the highest antibody titer.

Six isolates could be further characterized. The first one, AH1, was identified by serotyping as *Leptospira interrogans* serogroup Pomona, serovar Pomona in Leptospirosis Reference Laboratory of Queensland, Australia. Four additional human isolates could be studied through MLVA with primers to amplify VNTR 4, 7 and 10. The number of copies of the corresponding VNTR fragments, as shown in Table 1, could be calculated from the amplicon molecular weights leading to advanced identification when comparing problem strain profiles with those published from reference strains. A sixth recent isolate, AH6, is still being examined.

Partial sequencing of *16S* rDNA afforded solid data identifying species (Table 1), but did not allow to assert clear serovar results for all strains. However, combining those data with obtained VNTR profiles by the MLVA technique, and based on reference data published by Salaün *et al.*[17], we can state that the AH2 isolate belongs to *Leptospira interrogans* species, serogroup Pomona, serovar Kennewicki (Figure 1); the AH3 isolate to *Leptospira interrogans* serogroup Canicola serovar Canicola or serovar Portlandvere; the AH4 isolate to *Leptospira interrogans* serogroup Sejroe, serovar Wolffii or serovar Romanica, and the AH5 isolate to *Leptospira kirschneri* serogroup Australis, serovar Ramisi.

For AH3 and AH4 cultures, it was not possible to differentiate between 2 serovars with the employed primers and the target VNTRs.

The BLAST analysis of sequences amplified from the AH6 isolate showed 100% identity of *16S* rRNA with *L. kirschneri* serovar Mozdok (GenBank access N° KP 125531.1).

PFGE was applied to AH2 and AH3 strains. Clearcut results were obtained with the AH2 isolate: its PFGE pattern only matched with that of the reference strain *L. interrogans* serogroup Pomona, serovar Kennewicki. Conversely, the AH3 pattern matched both Canicola and Portlandvere serovars of *L. interrogans* serogroup Canicola (Figure 2), hindering further differentiation.

AH2 and AH3 were also studied by using MLST assays. PCR image revealed that in both cases amplification was positive for all seven gene fragments, enabling further sequencing. The analysis of amplicon sequences from all seven genes revealed that the AH2 isolate could be assigned to ST 140 and AH3 to ST 37 (Table 1).
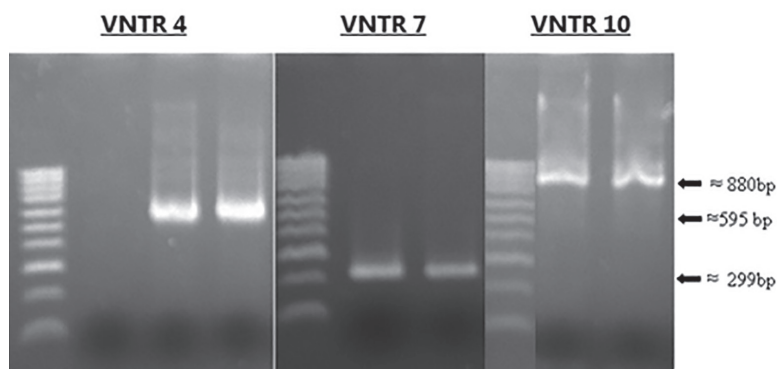
In PUBMLST *Leptospira* database, serogroup Pomona

Figure 1 – Results obtained by VNTR assays with the AH2 human isolate corresponding to *Leptospira interrogans* Pomona Kennewicki. **VNTR 4**: lane 1, DNA ladder 100 bp (Bioline, Meridian®; lane 2, negative control; lane 3, isolate #AH2; lane 4, strain IH23 *L. interrogans* Pomona Kennewicki. **VTR7**: lane 1, DNA ladder 100 bp; lane 2, isolate #AH2; lane 3, strain *L. interrogans* Pomona Kennewicki. **VNTR 10**: lane 1, DNA ladder 100 bp; lane 2, isolate #AH2; lane 3, strain *L. interrogans* Pomona Kennewicki.
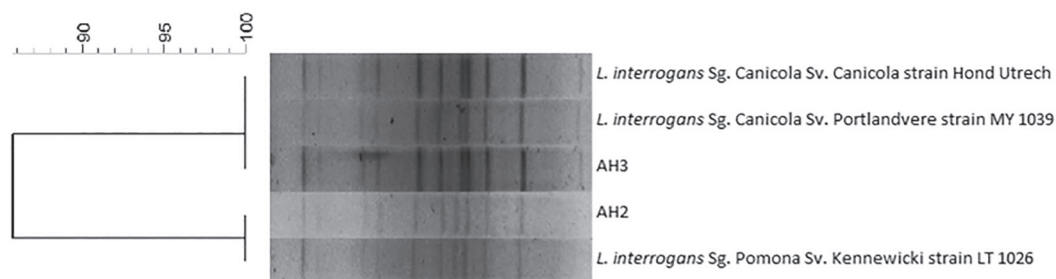
Figure 2 - PFGE image and dendrogram showing that the AH2 pattern matches that of the reference strain *L. interrogans* Pomona Kennewicki (P.K) and the AH3 pattern corresponds to those of *L. interrogans* Canicola Canicola (C.C) and Canicola Portlandvere (C.P). Dice (Opt: 1.50%) (Tol 1.5%) (H>0.0% S>0.0%) [0.0%-100.0%] NotI

serovar Pomona and serovars Grippothyphosa and Pyrogenes are included in the same 140 ST. Similarly, serogroup Canicola serovar Canicola, and serogroup Pyrogenes serovar Pyrogenes share the same ST 37 sequence type.

Environmental isolates

Seven environmental positive cultures have also been recovered. Two of them (AA1 and AA2) were isolated from water collections sampled in slums surrounding Montevideo, one (AA7) from a water supply well of a small island, and four (AA3-AA6) from rural cattle or dairy farms where human leptospirosis cases had been identified.

Five of seven isolates were identified as *Leptospira biflexa* according to *16S* and *23S* PCR results. A sixth isolate yielded a positive *16S* PCR, but sequencing revealed it belongs to the *Leptonema illini* species. AA1 yielded negative results in both *23S* and *LipL32* PCR, and positive *16S* reaction, indicating it was a saprophytic or partially pathogenic *Leptospira* of a different species. Sequencing of amplified *16S* fragment enabled to conclude that it is a *Leptospira meyeri* isolate.

DISCUSSION

Leptospira isolation has only recently been included in diagnostic and epidemiologic studies about leptospirosis in Uruguay, after an extended period in which knowledge was only based on clinical approach and indirect laboratory methods. This paper reports the first results obtained in our country with culture and identification procedures, which require prolonged and careful work to build a solid contribution to the control of this important zoonosis.

Blood culture performance data from either the Fletcher or EMJH media confirm that benefits are obtained by employing both culture media. Positive cultures of these bacteria must be thoroughly purified and periodically subcultured, or preserved by freezing to keep viable bacterial populations, enabling their full identification.

Isolates can otherwise be lost due to contaminants that overgrow *Leptospira* or to progressive irreversible cell damage. Thus, six of our eight initially recovered isolates could be successfully maintained, and five of them have already been extensively identified.

In the considered period, the number of inoculated blood cultures was slightly higher than 300, selecting samples usually taken no further than 10 days after the onset of illness. Eight positive results represent little more than 2% of the analyzed samples (CI 95% [0.8-4.4]). This proportion is not satisfactory and can probably be improved with frequent, careful microscopic observation and subcultures[22-24].

In future studies, a more strict selection of early blood samples for culture may improve the rate of positive results though reducing the total number of obtained isolates required for broadening the bacterial identification scope.

Most patients yielding positive cultures were older than those composing the general group of leptospirosis cases diagnosed through MAT (usually 20-40 years old in a previous study)[1]. Their illness appeared to be particularly severe, showing jaundice and organ dysfunction and requiring prolonged hospitalization. We had described in a 10-year follow-up that patients aged 40 years old or more developed jaundice more frequently than younger workers[1]. A high organic and circulating bacterial burden may explain an easier positive result from severely affected patients.

In many patients, a convalescent-phase serum sample is not collected for several reasons (uninformed health personnel, communication difficulties, illness improvement that leads to the belief that repeated studies are unnecessary). In this series, three patients with initial negative MAT result and without second MAT assays showed positive blood cultures, stressing the need for a complete diagnosis of leptospirosis. Positive culture results are usually delayed and not useful for guiding the patient care, but anyway may contribute to the epidemiological knowledge.

When MAT positive results were available together with positive blood cultures, differences were observed between

isolated serovar and serovar yielding maximum serum titer, reminding us that cross-reactions are frequent and MAT results should not be taken as a source of information about the infecting serovar in humans or animals[25].

Twenty known *Leptospira* species have been differentiated through DNA sequencing studies[5]. Nine of these are human or animal pathogenic species: *L. interrogans*, *L. borgpetersenii, L. kirschneri, L. santarosai, L. kmetyi, L. weilii, L. alexanderi, L. alstoni* and *L. noguchii.* There is a defined set of saprophytic species of different evolutionary history; an additional group of partially pathogenic species can be found both in environmental and clinical settings, and they require more studies to understand their role in infection[26,27].

Human isolates were diverse in our series, corresponding to known pathogenic species (four *L. interrogans* isolates, two *L. kirschneri*) and included two different variants of *Leptospira interrogans,* Pomona serogroup. In two additional cases, it was not possible to maintain viable cultures to determine the bacterial species or to complete its molecular study. A recently confirmed isolate is still being examined.

Serovar definition of three studied isolates requires further analysis, especially for the AH5 isolate, whose VNTR profile does not enable fully solid conclusions.

Combination of several molecular techniques has proven to be useful for identifying isolates, and serologic studies could allow completing the characterization of pending cases, though requiring expensive and hardly available resources.

Identification of human infecting *Leptospira* has not been frequently published in reports from neighboring countries. In urban settings, *L. interrogans* serovar Icterohaemorrhagiae has been usually identified in Brazil[28]. Other species and serovars are probably common in rural and other areas, including *L. noguchii*[29]. and *L. kirschneri* [30]. *L. santarosai* has been reported in Northwestern Colombia and nearby countries[31]. *Leptospira* isolates reported from Argentina have been generally obtained from animals; Pomona serogroup is prevalent in bovine cattle population[32]. *Leptospira interrogans* serogroups Canicola, Sejroe, Icterohaemorrhagiae and others have been isolated from humans[33].

Given the suggestive epidemiologic data, identified Uruguayan human cultures probably reflect infection of animal reservoir in rural settings with similar involvement of *Leptospira* species and serovars. Serovar Icterohaemorrhagiae, a common isolate from urban samples in the region, has not been identified in our small series. It is usually considered the origin of severe human cases, which in this study were found to be caused by other serovars. A collaborative project is presently ongoing for isolating, identifying and comparing bovine isolates with

human infecting variants. These data will be important for guiding vaccine development and prevention of animal and human illness considering health and economic reasons.

Rural workers in contact with bovine cattle, especially in dairy farms, are the most frequently infected persons in our country, and such is the case of the patients with positive blood cultures. Prevention measures are strongly required for these human groups at risk, through personal protection equipment (gloves, boots), education and cattle immunization. Saprophytic, intermediate or pathogenic species of *Leptospira* can all survive for extended periods in aquatic environments with poor nutrient content[34]. Water isolates examined in this study were taken from environments where humans and animals of diverse species (bovine cattle, synanthropic and wild rodents, otters, horses, dogs) are potential hosts of pathogenic strains which could thus, be expected to be found in cultures. Among other studies, a Chilean report has recently confirmed this chance[35,36]. However, nearly all identified species in our series were saprophytic, mainly of *Leptospira biflexa* species, as defined with primers *23S*-P1 and *23S*-P2, that allow to amplify specific *23S* rRNA coding sequences from *L. biflexa*, differentiating this species from other saprophytic *Leptospira*. There was a single exception: one *L. meyeri* isolate, which is difficult to be taxonomically classified because this species includes pathogenic serovars, such as Sophia, and saprophytic serovars such as Semaranga; other *L. meyeri* serovars, e.g. Perameles and Ranarum, can be found in both pathogenic and saprophytic subgroups[31]. The presence of this species in environmental water sources may be important, due to its potential pathogenicity, and requires further characterization through serovar specific antisera or other procedures.

Following this brief report, *Leptospira* isolation and identification in Uruguay from human, animal and environmental sources must be increasingly performed and technically improved through scientific cooperation, for obtaining a reliable picture of bacterial variants' distribution and spread that may guide valuable preventive actions.

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