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In-house isolation protocol from human serum samples demonstrates the circulating of a broad diversity of *Leptospira* serogroups in Costa Rica

Diana Chinchilla[1]✉, Inés Sánchez[1], Diego Montero[1], Mathieu Picardeau[2] & Ricardo Gutiérrez[1,3]✉

The isolation of pathogenic *Leptospira* is fundamental for a comprehensive characterization of circulating strains in endemic regions. Unfortunately, culture methods of *Leptospira* spp. are laborious and challenging. Here, we present a method for the isolation of these pathogenic bacteria from non-fresh serum samples, previously stored at 4–8 °C for several days. Briefly, 730 serum samples collected from leptospirosis-suspected patients (presenting acute signs) were screened for *Leptospira* DNA by real-time PCR. Thirty-one PCR-positive sera were then assessed for *Leptospira* isolation on specialized media for up to 6 months. Using this methodology, 11 *Leptospira* isolates were obtained, resulting in an isolation rate of 35.4% (11/31). Through whole-genome analysis, ten strains were identified as *Leptospira santarosai* and one strain as *Leptospira borgpertersenii*. The isolates were classified into six different serogroups, namely Hebdomadis, Shermani, Tarassovi, Pyrogenes, Ballum, and Grippotyphosa, demonstrating a wide diversity of *Leptospira* strains circulating in Costa Rica. This study reveals that serum is a suitable sample for *Leptospira* isolation in patients with positive PCR results, even after maintenance at cold conditions, promoting the use of serum for *Leptospira* isolation in reference laboratories around the world.

Keywords *Leptospira*, Culture, Isolation, Leptospirosis, Serum, Costa Rica

Leptospirosis is a widespread zoonosis caused by pathogenic bacteria of the genus *Leptospira*[1,2]. The highest incidences of *Leptospira* infection occur in tropical and subtropical countries, where abundant rainfall and frequent flooding favors its transmission[3]. Over one million cases of leptospirosis are estimated annually, responsible of approximately 60,000 deaths worldwide[4]. In humans, the disease manifests as an acute febrile illness with non-specific symptoms such as fever, malaise, joint pain, headache. Remarkably, this disease can evolve into severe multiorgan failure, involving renal failure and jaundice, a condition known as "Weil Syndrome", associated with a mortality rate that ranges from 5 to 50%[5,6]. In the tropics, the disease is frequently underdiagnosed, since the symptoms overlap with other circulating febrile diseases, such as dengue fever, rickettsioses, ehrlichiosis, brucellosis, malaria, chikungunya, and zika[3,7].

The main reservoirs of *Leptospira* are wild and domestic mammals, with rodents being the most common hosts[8]. In humans, infection occurs through direct contact with the urine or body fluids of infected animals, or indirectly through the exposure of environments, such as soil or water, contaminated with the urine of reservoir infected animals[9,10].

Costa Rica is an endemic country for leptospirosis[11,12]. This country has a great variety of microclimates and experiences a prolonged rainy season from May to the end of November. These conditions often result in flooding in both urban and rural areas, creating a favorable environment for the permanence of *Leptospira*.

[1]Laboratory of Zoonotic Febrile Diseases, National Reference Center of Bacteriology, Costa Rican Institute for Research and Education in Nutrition and Health (INCIENSA), Cartago, Costa Rica. [2]Institut Pasteur, Biology of Spirochetes Unit, French National Reference Center for Leptospirosis, WHO Collaborating Center for Reference and Research on Leptospirosis, Paris, France. [3]One Health Center for Zoonoses and Tropical Infectious Diseases, Ross University School of Veterinary Medicine, P.O. Box 334, Basseterre, Saint Kitts, Saint Kitts and Nevis. ✉email: dchinchilla@inciensa.sa.cr; rgutierrez@rossvet.edu.kn

Notably, in 1997, an outbreak of leptospirosis in Costa Rica was linked to water rafting in flooded rivers[13]. Between 2001 and 2005, the Costa Rican National Reference for Leptospirosis group isolated *Leptospira santarosai*, *Leptospira interrogans*, and *Leptospira noguchii* from febrile patients (laboratory records, unpublished data). Among these isolates, three strains of *L. santarosai* corresponded to serovars not previously described. These were named serovar Arenal (belonging to serogroup Javanica[14]), serovar Corredores (from the serogroup Tarassovi) and serovar Costa Rica (belonging to serogroup Pyrogenes[12]). These findings confirm the endemic nature of leptospirosis in Costa Rica and suggest a widespread diversity of *Leptospira* in the region.

The isolation and proper identification of *Leptospira* spp. is crucial for comprehending the local epidemiology and transmission cycles of circulating strains in an endemic region. Such insights can subsequently improve serological panels used for diagnosing leptospirosis in humans and animals[15]. However, the culture of *Leptospira* spp. is a laborious technique that has historically presented many challenges for reference laboratories. The slow growth rate of *Leptospira* makes then highly susceptible to contamination, and their isolation requires specialized media[16].

In human diagnosis, whole blood has traditionally been the specimen of choice for *Leptospira* isolation, whereas other samples, such as urine and cerebrospinal fluid, are also used[1]. Although serum samples are commonly submitted to clinical laboratories for serological diagnosis of febrile illnesses, they are not typically used for molecular diagnosis or isolation of *Leptospira* spp. Here, we present an *in-house* culture protocol for the isolation of *Leptospira* from serum samples previously maintained in refrigeration (i.e., 4–8 °C) for up to ten days. The implementation of this protocol allowed the culture of 11 *Leptospira* isolates from serum samples collected from 2020 to 2021[17]. Through molecular analyses, ten isolates were confirmed as *Leptospira santarosai* and one as *L. borgepetersenii*, classified into six distinct serogroups. The results present here demonstrated the wide biodiversity of *Leptospira* strains circulating in Costa Rica. We are certain that this method will promote the isolation of *Leptospira* in other laboratories worldwide.

Materials and methods
Clinical samples
In Costa Rica, all the clinical samples from patients suspected of leptospirosis are referred to the Laboratory of Zoonotic Febrile Diseases of the National Reference Center of Bacteriology (previously known as Costa Rican National Reference for Leptospirosis), at the Costa Rican Institute of Research and Education in Nutrition and Health (INCIENSA), for diagnosis and confirmation. Accordingly, from November 2020 to December 2021, 730 serum samples from patients in the acute phase of the disease (1–6 days since onset of symptoms) were received. All the samples were analyzed by real-time PCR and serology, without excluding samples in which the patient had previously received treatment with antibiotics. The clinical samples were stored at 4 °C from one to ten days before further analyses (Fig. 1).

Clinical and epidemiological information of the patients
The clinical and epidemiological information of the patients was obtained from the specimen submission forms that are referred together with the clinical samples to the INCIENSA for diagnosis of leptospirosis. The information included geographic distribution, sex, age, symptoms, of each patient, as well as risk factors of exposure.

Serological diagnosis of *Leptospira*
The 730 serum samples were analyzed by the Serion ELISA Classic *Leptospira* IgM (Virion/Serion, Germany) kit, according to manufacturer´s instructions. In addition, the samples were evaluated using the microagglutination test (MAT)[18] with a panel of 35 reference *Leptospira* serovars (Supplementary Table 1).

DNA extraction and real-time PCR amplification
Genomic DNA was extracted from 200 μl of serum using the StarMag 96 extraction kit and the automatized platform SEEPREP 32 (Seegene, Korea) according to manufacturer's instructions. Pathogenic *Leptospira* DNA was amplified using the real-time PCR assay targeting the *secY* gene[19] in a 7500 Fast Real-Time PCR system (Applied Biosystems). The following conditions were applied: reactions were performed in a total volume of 25 μl, consisting of 1x Platinum SYBR Green qPCR SuperMix-UDG (Fischer Scientific, USA), forward (SecYIVF, 5´-GCGATTCAGTTTAATCCTGC-3´) and reverse primers (SecYIV, 5´-GAGTTAGAGCTCAAATCTA-AG-3´) were added at a final concentration of 400 nM each, 0.5 μl of a solution of ROX 1X, freshly prepared for in each run, and 10 μl of extracted DNA. The amplification protocol consisted of 4 min at 95 °C, followed by 45 cycles of amplification (95 °C for 10 s, 54 °C for 5 s, 72 °C for 15 s). Subsequently, the reaction was stopped at 95 °C for 2 min, cooled (20 °C for 1 min), followed by a melting analysis from 70 to 95 °C, with plate readings set at 0.5 °C. The samples were analyzed by triplicates. A sample was considered positive with a Ct value < 42, and only if the melting temperature (Tm) of two or more replicates meet the *Leptospira-secY* profile (single peak of 78.5–84.5°C), not varying by more than 0.5 °C.

Serum *in-house* isolation protocol for *Leptospira* spp.
Serum samples *secY*-PCR-positive were processed for *Leptospira* isolation by an *in-house* culture protocol (Fig. 1). Briefly, the refrigerated serum samples and culture reagents were tempered at room temperature before inoculation. After brief mixing by vortex, five drops of each serum were inoculated in 6 ml of liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco) supplemented with albumin-fatty-acid-supplement (AFAS, University Medical Centers AMC, Netherlands), hereinafter referred as "EMJH", and in 6 ml of EMJH supplemented with "sulfamethoxazole-trimethoprim-amphotericin-fosfomycin-5-fluorouracil" media (STAFF)[20], hereinafter referred as "EMJH-STAFF". Then, culture tubes were incubated at 30°C for up to
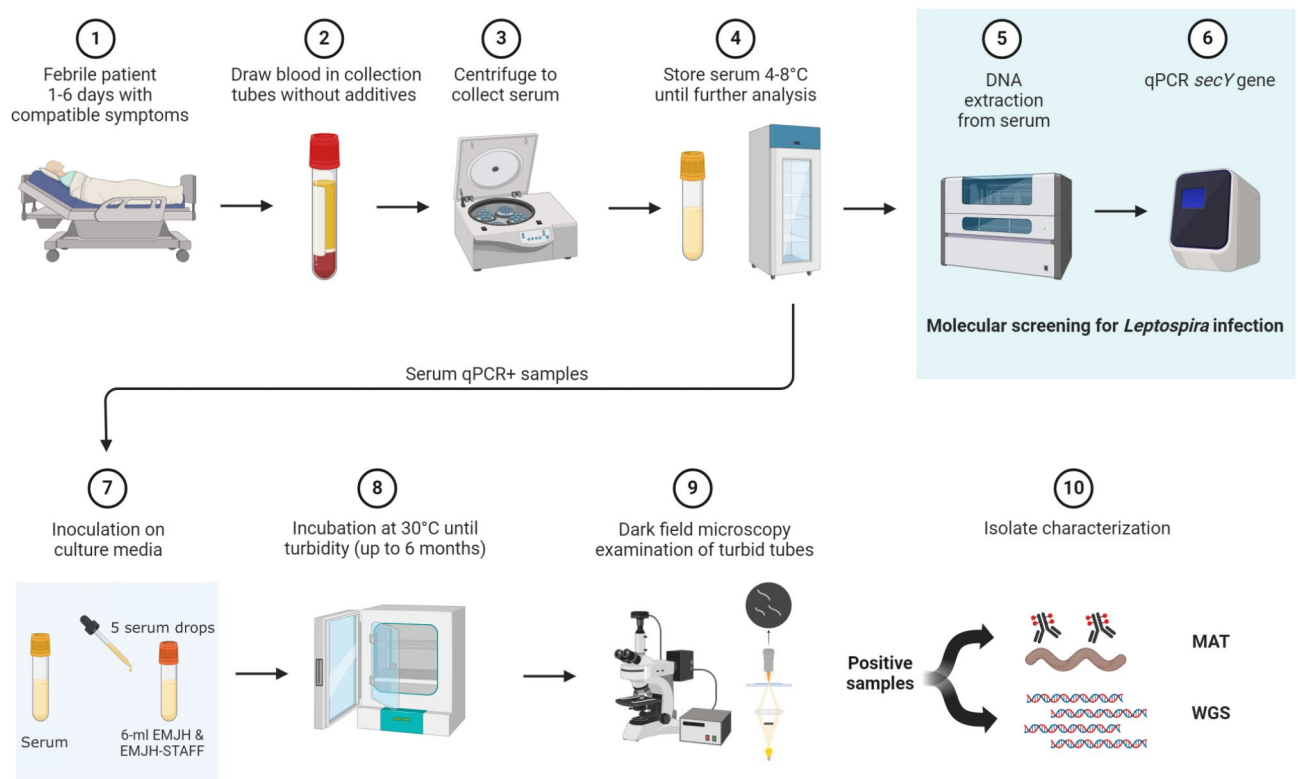
Fig. 1. Stepwise protocol for the isolation of *Leptospira* from serum human samples. (**1**) Patient with febrile symptoms compatible with leptospirosis and with 1–6 days from the onset of symptoms. (**2**). Draw blood into collection tubes without additives. (**3**) Centrifugation of blood sample in a bench centrifuge at 3500 rpm for 10 min to obtain serum. (**4**) Storage of serum samples at 4–8 °C until further analysis. (**5**) Extraction of genomic DNA from 200 μl of serum samples. (**6**) Real time PCR targeting the *Leptospira-secY* gene (a Ct of 40 was considered positive when the Tm value of the amplicon from 2 or more replicates did not vary by more than 0.5 °C). (**7**) Five drops of each of the qPCR-positive serum samples were inoculated in 6 ml of Ellinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with albumin-fatty-acid-supplement (AFAS) and in EMJH medium supplemented with sulfamethoxazole-trimethoprim-amphotericin-fosfomycin-5-fluorouracil (STAFF) and AFAS. (**8**) The culture tubes were incubated at 30 °C for 6 months until turbidity was observed. (**9**) Once a tube becomes turbid, the culture was examined by dark field microscopy to determine the presence of leptospires. (**10**) Cultures confirmed positive by dark-field microscopy were further characterized using microagglutination test (MAT) and whole-genome sequencing (WGS).

6 months or until turbidity was observed (weekly checked). Once turbid, a 5 μl culture aliquot was examined by dark field microscopy to determine the presence of leptospires. To avoid contamination, culture tubes were only opened in a biosafety cabinet and only once turbidity was visually detected.

Serotyping of isolates

The serotyping of the isolated strains to serogroup level was performed by the microagglutination test (MAT) using a panel of 45 polyclonal rabbit antisera against reference serovars of *Leptospira* comprising 23 pathogenic and one saprophytic serogroup (OIE Reference Laboratory for Leptospirosis, AMC, Netherlands) (Supplementary Table 2). High rates of agglutination (cut-off titer ≥ 1:320) of the isolate with the antisera of a specific serogroup were indicative of the presumptive serogroup. Isolates were further analyzed by MAT using sets of specific monoclonal antibodies (Supplementary Table 3; OIE Reference Laboratory for Leptospirosis, AMC, Netherlands) to determine isolate affiliation between serogroups, for those cases where high titers were found to different serogroups.

Whole-genome characterization

The libraries and whole-genome characterization of the isolates was performed at the Biology of Spirochetes Unit, Institut Pasteur, Paris, France, as previously described[17]. Genomic analyses to determine the species of the strains and the core-genome multilocus sequence typing (cgMLST) profiles were performed using the Bacterial

Isolate Genome Sequence Database (BIGSdb) hosted at the Institut Pasteur (https://bigsdb.pasteur.fr/leptospira/). Moreover, genomic assemblies were compared to the deposited *Leptospira* genomes in the BIGSdb. For this, 61 additional genomes of *L. santarosai* and 287 genomes of *L. borgpetersenii* were downloaded. Prokka[21] was used for genome annotation and core genome was obtained using Panaroo protocol[22]. A maximum likelihood phylogenetic tree was constructed using the core gene alignment output from Panaroo on IQ-TREE[23]. The resulting ML tree was uploaded in GrapeTree[24]. The presence and absence output table obtained from Panaroo was converted into a binary table and imported to R[25] to calculate Jaccard distances using vegan package[26] and ggpubr package[27] to calculate Spearman's rank correlation tests. The average nucleotide identities (ANI) were calculated with FastANI[28].

Data accessibility
Genome assemblies were deposited in the BIGSdb (https://bigsdb.pasteur.fr/leptospira/) under the identification (id) numbers 1251–1261.

Ethical considerations
According to decree number 40556-s of the General Health Law of Costa Rica, epidemiological studies that incorporate the review of clinical records do not require the approval of an ethics-scientific committee. Human samples were anonymized, and collection of the samples was conducted according to the Declaration of Helsinki.

Consent statement
The need to obtain informed consent was waived by the Costa Rican Institute for Research and Education in Nutrition and Health (INCIENSA) Scientific Committee.

Results
Serological diagnosis of *Leptospira* in study samples
Out of 730 sera evaluated, 41 (5.6%, 41/730) tested reactive for *Leptospira* IgM ELISA. However, all 730 samples were negative when tested by the microscopic agglutination test (MAT).

Molecular detection of *Leptospira* in serum samples
From the 730 sera analyzed by real-time PCR targeting the *secY* gene, amplification of pathogenic *Leptospira* DNA was detected in 42 (5.7%) samples (Supplementary Table 4). Seven (16%) of these PCR-positive patients were also ELISA *Leptospira* IgM reactive (Supplementary Table 4).

Isolation of *Leptospira* strains from serum samples
Thirty-one of the PCR positive serum samples stored at 4 °C were cultured in EMJH and EMJH-STAFF. Due to insufficient volume, culture could not be performed on 11 PCR-positive sera. A total of 11 *Leptospira* strains were isolated, both in the EMJH and the EMJH-STAFF media, yielding a 35.4% (11/31) isolation rate. Previously to the isolation protocol, the storage time at 4 °C of the positive samples ranged 3 to 9 days (Supplementary Table 4). The average time for culture positivity was 7.45 weeks (5 to 10 weeks). Notably, the average (± standard deviation) Ct value of the serum samples that were positive by culture was 37.49 ± 2.62. These amplicons presented an average Tm value of 81.36 ± 0.45 °C (Supplementary Table 4). Average Ct and Tm values of the samples in which *Leptospira* was not isolated were 37.41 ± 2.80 and 81.16 ± 0.69, respectively, suggesting that the number of bacteria in the original sample was not crucial for a successful isolation.

Clinical and epidemiological information of the patients
We collected the clinical and epidemiological information of the 11 patients from which *Leptospira* was isolated (Table 1). The samples were referred from the north, central and south regions of the country (Fig. 2). Ten out

Patient	Isolate	Age	Sex	Location	Risk factor/exposure	Symptoms	Outcome
1	CR2020	17	M	San Carlos	Agricultural work, contact with fresh water	Fever, arthralgia, myalgia, headache, abdominal pain, vomit	Non fatal
2	CR2120	63	M	Pérez Zeledón	Agricultural work, contact with fresh water	Fever, arthralgia, myalgia, chills, headache, vomit	Non fatal
3	CR0421	19	M	San Carlos	Not indicated	Fever, myalgia, headache, vomit, diarrhea	Non fatal
4	CR0521	22	M	Coto Brus	Not indicated	Fever	Non fatal
5	CR0821	18	M	Guatuso	Not indicated	Fever, myalgia, headache, chills, dizziness	Non fatal
6	CR1421	27	M	San Carlos	Not indicated	Fever, arthralgia, myalgia, chills, jaundice, abdominal pain, vomit	Non fatal
7	CR1821	47	M	Acosta	Agricultural work	Fever, arthralgia, myalgia, headache, chills, vomit	Non fatal
8	CR2021	53	M	Oreamuno	Agricultural work, pest control	Fever, arthralgia, myalgia, chills, jaundice, convulsions, liver failure. ICU ward	Non fatal
9	CR2621	21	F	Guatuso	Agricultural work	Fever, arthralgia, myalgia, headache, chills, abdominal pain	Non fatal
10	CR2821	21	M	Guatuso	Livestock and dairy	Fever, arthralgia, myalgia, headache, chills, abdominal pain, vomit	Non fatal
11	CR2921	68	M	Coto Brus	Agricultural work, coffee plantation	Fever, headache, chills	Non fatal

Table 1. Clinical epidemiological data of the patients in which *Leptospir*a was isolated in Costa Rica from 2020 to 2021.
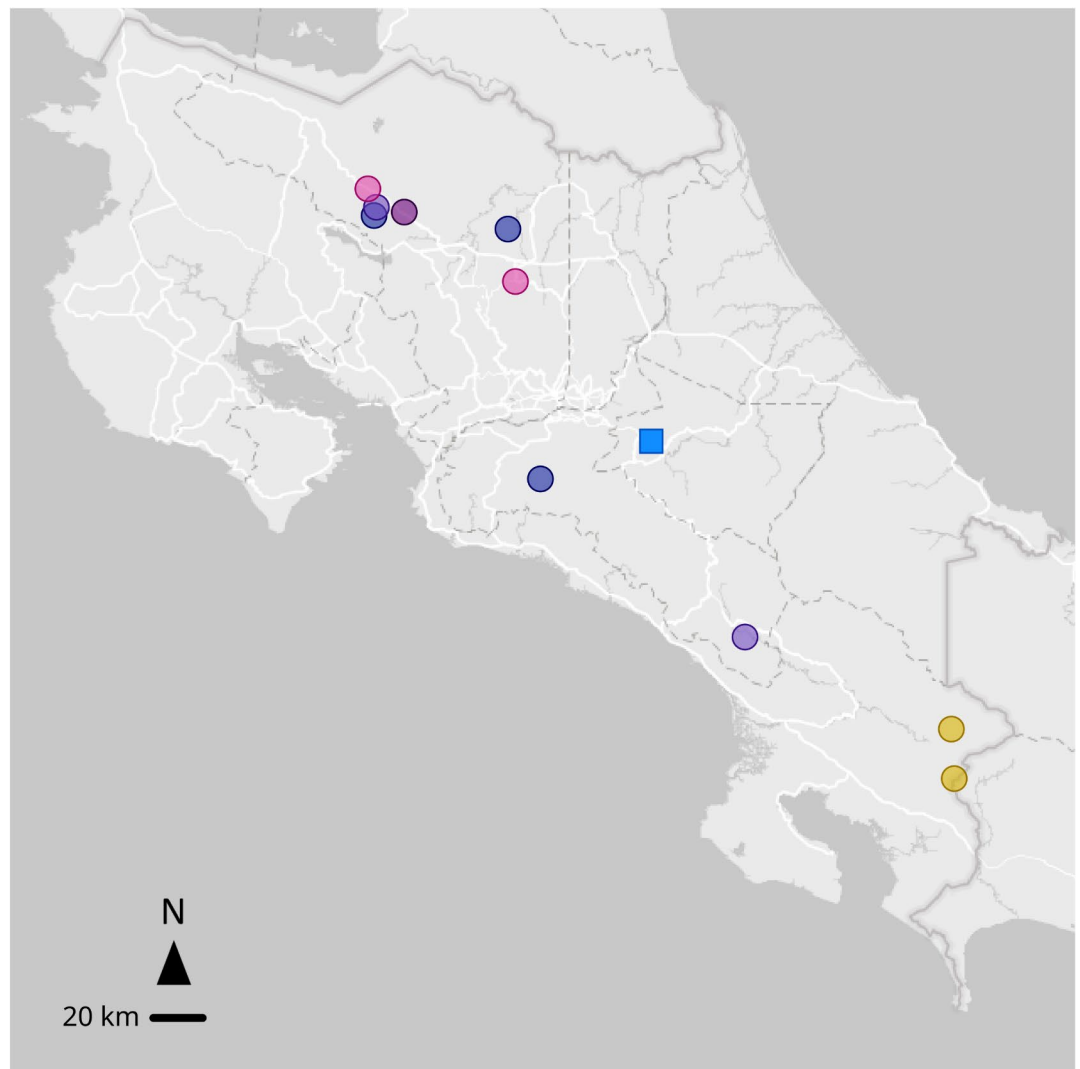
Fig. 2. Map of Costa Rica indicating the geographic location of the residence of the patients from which *Leptospira* spp. strains were isolated. North arrow and scale are indicated at the bottom left of the map.

of the 11 patients were male. The age of the patients ranged from 17 to 68 years old, being seven of the patients under 28 years old.

Most of the patients presented mild symptoms at the time of sample collection, including fever, arthralgia, myalgia, headache, abdominal pain, vomit, diarrhea, and chills, except for patient #8 (Table 1), who presented a severe disease with jaundice, liver failure, seizures, and had to be admitted to the intensive care unit. None of the patients died from the infection.

Seven patients reported a risk factor for infection with *Leptospira*, including agricultural work, livestock occupation and exposure to water bodies. In four patients, risk factor data was not included in their report.

Serotyping of the *Leptospira* isolates

Serotyping of the 11 *Leptospira* isolates identified six distinct serogroups, namely: Hebdomadis, Shermani, Tarassovi, Pyrogenes, Grippotyphosa and Ballum (Table 2). Notably, the polyclonal antisera exhibited strong cross-reactivity between the serogroups Manhao-Pyrogenes, Hebdomadis-Mini-Sejroe, Shermani-Tarassovi, Autumnalis-Ballum, Grippotyphosa-Autumnalis, respectively. In ten isolates, the monoclonal antibodies determined the final affiliation to the specific serogroup. Isolate CR0421 (Table 2), resulted in strong reactions with the polyclonal antisera against serogroups Grippotyphosa (>1:20480), Djasiman (1:2560), Louisiana (1:1280) and Cynopteri (1:640). The monoclonal antibodies F71C9 (Grippotyphosa), F69C11, F69C15 (Cynopteri) yielded negative results against this isolate.

Whole-genome characterization of the isolates

Whole genome sequencing analyses identified 10 isolates as *L. santarosai* and one isolate as *L. borgpetersenii* (Table 2). Genomic analyses and comparison with the BIGSdb database showed that all isolates correspond to novel and different cgMLST sequence types (cgST), except for the *L. borgpetersenii* that matched its cgST with

Isolate	BIGSdb ID	Species	Serogroup	cgST	Genome length (bp)	%G + C
CR2020	1251	*L. santarosai*	Hebdomadis	1025	4,091,227	41.8
CR2120	1252	*L. santarosai*	Shermani	1026, 1098	4,026,198	41.8
CR0421	1253	*L. santarosai*	Grippothyphosa*	1027	4,299,466	41.6
CR0521	1254	*L. santarosai*	Tarassovi	1028, 1099	3,917,764	41.8
CR0821	1255	*L. santarosai*	Shermani	1029, 1100	4,191,464	41.7
CR1421	1256	*L. santarosai*	Pyrogenes	1030	3,882,339	41.9
CR1821	1257	*L. santarosai*	Grippothyphosa	1034	3,921,283	41.8
CR2621	1259	*L. santarosai*	Grippothyphosa	1031	3,834,577	41.9
CR2821	1260	*L. santarosai*	Pyrogenes	1032	3,971,537	41.8
CR2921	1261	*L. santarosai*	Tarassovi	1033	4,006,350	41.7
CR2021	1258	*L. borgpetersenii*	Ballum	578,1043	3,923,244	40.0

Table 2. Molecular and serological characterization of *Leptospira* isolates from serum samples. *Strain also shows strong cross reactions against serogroups Djasiman and Cynopteri.

isolates from Uruguay (*n* = 1, from human source), Cuba (*n* = 1, from human source), and Saint Kitts and Nevis (*n* = 3, from rodent sources) (Supplementary Figs. 1 and 2).

The phylogenetic analyses between *L. santarosai* strains from Costa Rica showed no identical pair of genomes (Fig. 3). Accordingly, whole-genome ANI values ranged from 98.57 to 99.88%, being the farthest related pair of genomes BIGSdb-id 1255 (Shermani strain from this study) and BIGSdb-id 1215 (Inciensa-04 strain, Pyrogenes serogroup collected in 2004), whereas the closest pair was BIGSdb-id 1254 and BIGSdb-id 1257 (a Tarassovi and a Grippotyphossa strains, respectively). Despite the nucleotide similarities revealed, marked differences were evident among their genetic content (i.e., shared genes), with diversities ranging from 64.5 to 94.1% of shared genes (i.e., Jaccard distance ranging from 0.059 to 0.355; (Fig. 3).

Discussion

In this study we demonstrated that serum is a suitable sample for isolation of *Leptospira* in patients confirmed of leptospirosis by real-time PCR. Using this method, we could isolate 11 *Leptospira* non-clonally related strains, belonging to six serogroups. The ability to isolate different serogroups and species, suggested that this method presents no bias to certain group of leptospires.

Although whole blood has traditionally been the sample of choice for the molecular detection and isolation of *Leptospira*, it presents several challenges due to the low bacterial loads commonly found in such samples[29,30]. Firstly, blood contains PCR-inhibitory substances, such as hemoglobin, IgG immunoglobulins and lactoferrin, as well as anticoagulants commonly used in blood collection tubes, including sodium heparin and EDTA[31]. Secondly, additional factors such as antibiotics administered in cases where therapy has begun can further reduce the success of *Leptospira* isolation. Consequently, using blood for *Leptospira* diagnosis often lead to reduced sensitivity in both real-time PCR systems and isolation protocols[16,32]. While serum samples may also contain inhibitory substances, their concentrations are much lower than those found in whole blood[31]. For instance, in healthy individuals, normal hemoglobin levels range from 12 to 18 g per deciliter (g/dl) in whole blood, while in plasma or serum ranges below 5 mg/dL[33]. Therefore, serum samples are proposed as a more suitable matrix for *Leptospira* molecular diagnosis and isolation[34].

It has been acknowledged that the successful isolation of *Leptospira* depends significantly on processing freshly obtained clinical samples[15]. This means that the time elapsed between sample collection and inoculation into leptospiral culture medium is crucial for achieving successful isolation[15]. However, in our study, four out of the 11 isolates were obtained from specimens with six or more days of storage at 4 °C, demonstrating that *Leptospira* remains viable in non-fresh clinical serum samples, even in samples with low *Leptospira* loads, as evidenced by their high real-time PCR Ct values. Similarly, the isolation of *Leptospira weilii* from a separator tube was previously reported after stored at 4 °C for a week[35]. These findings will facilitate and promote the isolation of *Leptospira* in samples commonly received in reference laboratories for other diagnostic methodologies such as serology.

In this study, 16% of the patients with a positive PCR result were also positive for anti-*Leptospira* IgM antibodies, demonstrating that detectable leptospires can be found in patients concomitantly with an early IgM antibody response. Remarkably, none of the patients were positive by MAT, evidencing the lower sensitivity of this technique for the initial stages of the disease[15].

Six different serogroups were identified among the patients, namely Hebdomadis, Shermani, Grippotyphosa, Tarassovi, Pyrogenes and Ballum. Since different animal hosts are preferentially infected with different *Leptospira* serovars[36], the serological identification of the isolates allows the identification of the potential sources of the infection in humans[15]. Accordingly, raccoons, marsupials, and armadillos have been described as reservoirs for Hebdomadis serogroup[37,38]; Shermani has been associated with livestock[39]; Grippotyphosa serogroup has been associated with a wide range of mammals such as opossums[40], rodents, bats[37], horses[41], dogs[42], among others; while swine is the main reservoir for Tarassovi serogroup[36,43,44]; Pyrogenes serogroup has been associated mainly to dairy cattle[45]; and, the reservoirs for Ballum serogroup are mainly rodents, such as mice and rats[46–48]. Remarkably, Costa Rica is one of the most biodiverse places on the planet, hosting the 4.5%
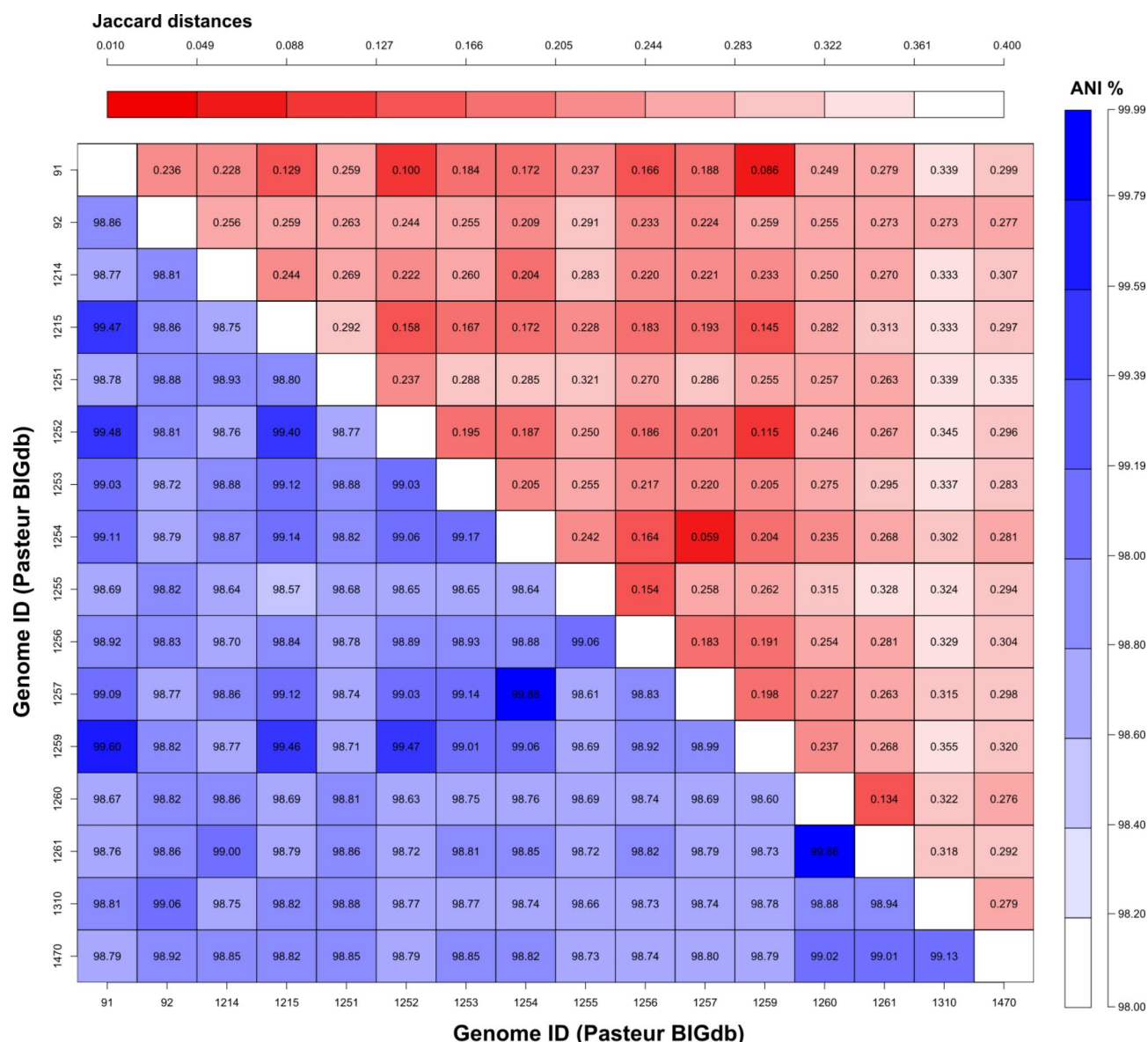
Fig. 3. Genomic comparison of *L. santarosai* genomes isolated from Costa Rica. Pairwise comparison matrix based on the average nucleotide identity (ANI) percentages (lower triangle) and the Jaccard distances according to the presence and absence of genes (upper triangle).

of the world's ecological diversity[49] and has a wide variety of microenvironments in which *Leptospira* could thrive and maintain in nature. Thus, the diversity of *Leptospira* serogroups detected in this study correlates with this potential reservoir diversity. Serovars Arenal, Corredores and Costa Rica, belonging to serogroups Javanica, Tarassovi and Pyrogenes respectively, were described for the first time in Costa Rica from human blood samples[12,14], suggesting that more serovars not yet described may be actively circulating in this territory. Therefore, exploring environmental and animal samples in Costa Rica is essential to gaining a comprehensive understanding of the presence and distribution of both saprophytic and pathogenic *Leptospira* in the region.

Genomic analyses of the isolates in this study classified ten strains as *L. santarosai* and one isolate (CR2021 Ballum) corresponds to *L. borgpertersenii*. The genomic characterization of the sequences from *L. santarosai* strains demonstrated a close phylogenetic but not clonal relationships between the isolates. In fact, none of the strains were identical in their nucleotide identities (i.e., ANI comparison). Moreover, their gene content was diverse within this species, reflecting its open pangenome[17]. Interestingly, we found only a moderate correlation between the gene content and the nucleotide similarities among all sequenced *L. santarosai* genomes. Altogether, these results demonstrated the broad diversity of *Leptospira* spp. not only at the nucleotide level but also in the genes carried within their genomes.

From the confirmed leptospirosis cases in which *Leptospira* was isolated, 10 common non-specific signs were recorded: fever, arthralgia, myalgia, headache, abdominal pain, vomit, chills, diarrhea, dizziness, and jaundice. Only one patient showed severe symptomatology, presenting convulsions and liver failure. Interestingly, this

patient carried the only *L. borgpertersenii* isolated. Notably, *L.santarosai* and *L. borgpetersenii* belong to the subclade P1 of pathogenic *Leptospira* and are grouped together in subgroup 2[50,51]. At the time of the drawn of the samples, ten of the patients infected with *L. santarosai* showed mild to moderate symptomatology and none of the patients had a fatal outcome. It will be interesting to further explore potential pathogenicity levels between the species of the subclade P1 and to analyze the gene content of clinical isolates in patients showing a wide variety of symptoms and outcomes, from mild to severe.

A limitation of this study arises from the use of *secY* as the target for detection of acute leptospirosis. PCR assays directed to *secY* and other genes, such as *lipL32* [52,53], present low sensitivity for *Leptospira* spp. with intermediate pathogenicity, hence limiting their diagnosis scope. Thus, it is important the exploration of new targets that may allow the detection of both highly and intermediate pathogenic leptospires to better understand the etiology of leptospirosis in the country.

In conclusion, this study presents a method that facilitates the isolation of *Leptospira* from the serum of ill-patients. We demonstrated that a great diversity of *Leptospira* serogroups belonging to two pathogenic species were successfully isolated from the serum of patients in a year period, even after the maintenance of the serum samples at 4 °C, evidencing that the methodology is not biased towards a certain group of strains. We believe that our results will serve other reference laboratories in their diagnosis of Leptospirosis.

Data availability
Genome assemblies were deposited in the BIGSdb (https://bigsdb.pasteur.fr/leptospira/) under the identification (id) numbers 1251-1261.

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Author contributions

Conceptualization: DC, IS, DM. Data curation: DC, MP, RG. Formal analysis: DC, MP, RG. Methodology: DC, IS, DM. Resources: DC, MP. Supervision: DC, MP, RG. Writing - original draft: DC, RG.Writing - review & editing: DC, MP, RG.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to D.C. or R.G.

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