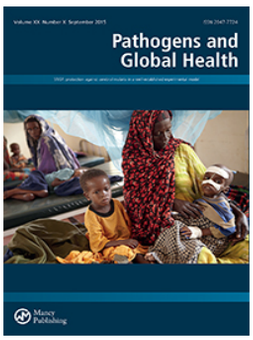

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Simplified MLST scheme for direct typing of *Leptospira* human clinical samples

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ABSTRACT

Leptospirosis is a globally distributed zoonosis. Epidemiological data are scarce and present major challenge because of the varied clinical presentations. Multilocus Sequence Typing has already proven to be a robust molecular typing method providing accurate results for strain characterization. We have adapted our MLST scheme by reducing the set of *loci to facilitate Leptospira* typing directly from human clinical samples. The application of this 3-locus scheme provides *Leptospira* species and allelic profiles of the samples retaining the power of discrimination of the whole scheme. Moreover, an approach to the serogroups was also achieved. Our results contribute to the epidemiological study of Leptospirosis, since the direct typing on clinical specimens could detect and update allelic variants and serogroups present in a region. The simplified scheme allowed at the same time to take advantage of limited genetic material available in clinical samples that may increase the sources of information for epidemiological monitoring.

KEYWORDS

Leptospira; MLST; clinical samples; serogroup

Introduction

Leptospirosis is probably the most widely distributed zoonosis in the world [1]. The infection is transmitted through direct or indirect contact with the urine of infected animals [2]. The disease is (re-) emerging globally and numerous outbreaks have occurred during the past decade [3]. Epidemiological investigations of leptospirosis outbreaks are a major challenge in most settings because of varied clinical presentation, difficult diagnosis, and the complex transmission cycle of the disease. These challenges are particularly noticeable in areas where the disease is highly endemic and resources and technical capacity are limited. In Argentina, the main risk factor for humans has been the extended contact with floods that occurred in recent years [4]. The definitive results of *Leptospira* infection by isolating the pathogen in culture is difficult to achieveand may take several months, given the low sensitivity of this method. Therefore, the characterization of the infecting strainis problematic, especially in outbreak investigations [5].

Culture and microscopic agglutination test (MAT) are the most widely used diagnostic tools for leptospirosis. Culture is time consuming and does not permit the identification of the infecting strain. On the other hand, MAT is laborious but allows the identification of the serogroup; however, serogroup is not exclusive of a single species.

Recently MLST have emerged as alternative molecular diagnostic and typing method of choice for *Leptospira*spp [6–8]. In a previous work we have reevaluated the different MLST schemes in order to find a combination of the best *loci* that, in a single scheme, allow the maximum discrimination between non-epidemiological related strains. This improved combination was successful both in resolving species in a global strain collection asin providing a higher level of intra-species discrimination. Furthermore, we described a unidirectional correlation between allelic profiles and serogroupsthat supports the implementation of MLST as a complementary approach to serological methods [9,10].

Molecular typing is usually performed on DNA obtained from isolates. Nonetheless, the isolation of *Leptospira* entails several difficulties regardingstarting material, expertise, contamination, and differences in growing requirements fordifferent species. Altogether, these factors result in the low sensitivity for the achievement of the isolates. However, clinical samples (whole blood, urine, serum) from animals and humans are readily available in different laboratories, but they present other problems, such as obtaining good quality and quantity of bacterial DNA, which is desirable for amplification of a standard 7-locus MLST scheme. Chiani and colleagues applied a 7-locus scheme to clinical samples, serum and whole blood from patients suffering from leptospirosis. The results

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Supplementary data can be accessed here

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showed that 21.6% of the samples provided interpretable data for MLST typing, confirming that MLST is a suitable technique to be applied on clinical samples [11]. Despite these results, is still difficult to complete an MLST scheme based on a 7-locus in numerous samples. Recently, Weiss and colleagues established an extended MLST based in nested PCRfor typing of clinical samples. The strategy allowed the determination of the 7-locus allelic profile 23% of the samples [12]. Although this strategy showed an improvement over other studies on clinical samples,the application of nested PCR could not be a practical routine technique to implement in clinical settings, due to possible cross contamination among samples during handling.

In this context, we propose an adaptation of our MLST scheme, by reducing the set of locito facilitate *Leptospira* typing directly from clinical samples, in order to increase the number of typable samples. The development of a simplified scheme that allows at the same time to reduce handling procedures and costs will permit to take advantage of the limited genetic material available in clinical sampleswithout losing the power of discrimination achieved by the whole scheme. Thus, providing additional information on the allelic variants and even serogroups circulating in a region and increasing the sources of information for epidemiological monitoring.

Materials and methods

Selection of loci for the reduced scheme

The selection of a minimum set of loci was performed using MLSTest v1.0.1.23 program(http://mlstest.codeplex.com/) [13] using as input the sequences of the 116 isolates generated during our previous published 7-locus MLST optimization [Supplementary Table S1]. The 7-locus sequences are available in PubMLST database (http://pubmlst.org/leptospira/scheme #2). The number of Sequence Types (STs) generated using differentnumber of loci in every possible combinations (from 1 to 7 loci) was calculated (Table 1).

Spiking experiment

In order to quantify the limit of detection (LoD) of leptospiral DNA in a clinical sample, a spiking assay was performed in which a known amount of leptospires was added to samples of leptospires-free human blood. The blood samples were extracted from healthy donors with no history of leptospirosis and were collected into Venosafe tubes containing EDTA (Terumo) and BD Vacutainer tubes (BD Diagnostics). The samples were processed a few hours after collection from volunteers. The study was approved by the National Institute of Respiratory Diseases (Ministry of Health, resolution 1470/2011).

For the spiking assay, *L. interrogans* serovar Canicolastrain Hond Utrech IV was grown at 30°C until the optical density reached 0.3 at 420 nm. Exponential-phase bacteria were collected by centrifugation at room temperature and washed by resuspension in phosphate buffered saline (PBS) 1X, pH 7.6. Suspensions of live leptospires in PBS were counted in a PetroffHausser counting chamber (Fisher Scientific) and adjusted to 2×10^8 bacteria/ml. Ten fold serial dilutions from 2×10^8 to 2×10^1 bacteria/ml were then performed in PBS 1X. Bacterial suspensions were spiked into 225 µl of whole blood so that the final concentrations were 2×10^1, 2×10^2, 2×10^3, 2×10^4, 2×10^5, 2×10^6, 2×10^7, 2×10^8 bacteria/ml. After a 2 h incubation at room temperature, total DNA was extracted from 200 µl of whole blood using QIAamp DNA Blood Mini Kit(Qiagen, Valencia, CA)according to the manufacturer's directions, and used for testing the sensibility by PCR amplification of MLST loci selected.

Clinical samples

The samples used in this study consisted of serafrom patients clinically suspected of leptospirosis, provided by the National Leptospirosis Reference Laboratory of Santa Fe (NLRL), where clinical specimens are remitted periodically. We selected 34 samples that had been diagnosed positive in the NLRL using *lip*L32 as the target for quantitative PCR (qPCR) [11] The concentration of leptospires per ml according to qPCR for each sample are showed in the Table 2.

DNA extraction

DNA was extracted from 200 µl of serum using QIAamp DNA Blood Mini Kit (Qiagen, USA) according to the manufacturer's directions, and used for amplification of 16S rRNA and MLST loci.

PCR conditions and sequencing

PCR reactions were performed in a final volume of 50 µL. Species identification was determined using the set of primers described by Mérien and colleagues for 16S rRNA amplification: (LA: 5´-GGCGGCGCGTCTTAAACATG-3´ and LB: 5´-TTCCCCCCATTGAGCAAGATT-3´) [14]. The PCR cycling conditions consisted of an initial denaturation step of 94°C for 3 min followed by one cycle of annealing at 54°C for 90 secand an extension step of 72°C for 2 min (1 cycle); 29 cycles of denaturation step at 94°C for 1 min, annealing at 54°C for 90 sec, and extension at 72°C for 2 min and a final extension step at 72°C for 10 min.

The reduced-MLST PCR conditions were also performed in a final volume of 50 µl, containing

0.5 pmol/µl of each primer, 0.8 mM of dNTPs, 2.5 mM of $MgCl_2$ and 1.25 U of Taq DNA polymerase (GoTaq, Promega, USA). The cycling conditions consisted of a touchdown scheme with an initial denaturation step at 95°C for 5 min followed by 10 cycles of further denaturation at 94°C for 1 min, annealing at 59°C decreasing one grade by cycle for 1 min 30 sec, and extension at 72°C for 2min; finally 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C 1 min 30 sec, and extension at 72°C 2min; the final extension step was at 72°C for 10 min.

The PCR products were analyzed on 1% agarose gels and purified using ethylene diamine tetra acetic acid disodium salt (EDTA)-ethanol precipitation.

Sequencing was performed in the Genotyping and Sequencing facility of the Biotechnology Institute (INTA). Both strands were sequenced on a Big Dye Terminator v3.1 kit from Applied Biosystems and analyzed on an ABI 3130XL genetic analyzer from the same supplier. The STADEN Package software was used to obtain a consensus sequence between the both strands (MRC-LMB, UK) [15]. The 16 S rRNA consensus sequences were analyzed using the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

MLST allele assignment and phylogenetic analysis

MLST alleles were assigned using the *Leptospira* MLST scheme #2 from the PubMLST database (https://pubmlst.org/leptospira/).

MLST sequences from the selected loci were concatenated using MLSTest into a single, longer sequence, which was used as input for the phylogeny. The best model of nucleotide substitution was determined using JModelTest2 software [16,17]. Subsequently, phylogenetic relationships among concatenated sequences were inferred by maximum likelihood (ML) [18] testedwith 1000 bootstrap replications, using MEGA 6.0 software [19]. The parameters of the phylogenetic analysis were adjusted according to the resulting model.

Results

Selection of loci for the reduced MLST scheme

Based on the 7-locus scheme that we hadpreviously optimized, (*adk, glm*U, *icd*A, *lip*L32, *lip*L41, *mre*A, *pnt*A) [10], we selected a minimum set of *loci* that retains the discrimination power of the original scheme. The aim was to simplify the amplification of leptospiral DNA from clinical samples considering the scarce amount of DNA that these samples usually have. By means of the software MLSTest, different combinations of loci were evaluated, with each combination producing a different number of STs in the collection of 116 strains. After analyzing 35 possible combinations, the software MLSTest showed 3 optimized 4-locus schemes and one optimized 3-locus scheme that described 93 STs and 90 STs respectively (Table 1). We focused on the combinations of 4 and 3 loci, because the final number of STs was near to the obtained for the 7- loci scheme (96 STs), despite the reduction. For these combinations, we calculated the *number of STs/number of strains* ratio, as a measure of the genetic diversity described by each scheme (Table 1). Accordingly, the 3-locus scheme was chosen to be further tested, becauseit fulfilled the criteria of describing a high proportion of the diversity in the collection byusing a minimum number of loci. The combination of 3 loci optimized by the software was *adk, lip*L41 and *mre*A.

Table 1. Optimized schemes derived from MLSTest software, number of STs found and index of diversity described in each scheme.

Number of loci included (n° of combinations)	Maximum number of STs found	No of STs/No of strains (116)
1 (7)	54	0.46
2 (21)	78	0.67
3 (35)	**90**	**0.77**
4 (35)	93	0.80
5 (21)	95	0.81
6 (7)	96	0.82
7 (1)	96	0.82

The strain collection (116 isolates, Table S1) previously typed by the whole scheme was used to reevaluate the phylogenetic relationships established by a3-locus genotype. For this purpose, we performed a maximum likelihood analysis using the concatenated sequences of the 3 loci (*adk* – *lip*L41 -*mre*A). The resulting tree showed that strains belonging to the same species were grouped in clusters and intraspecificclusteringamong strains of the major serogroups (Icterohaemorrhagiae, Pomona, Canicola) was observed.

Application of the simplified scheme on clinical samples

After *in silico* validation of the simplified scheme, we tested the LoD for the three pair of primers in a spiking procedure using whole blood from healthy donors. The LoD reached was 1×10^4 leptospires/ml for *adk* and 1×10^5 leptopsires/ml for both *lip*L41 and *mre*A.

A total of thirty-four samples consisting of DNA extracted from serum were analyzed. As a first step, the determination of species was performed by 16 S rRNA amplification [14]. All samples were positively amplified. The distribution of species among the 34 positive samples was as follows: 30 belonged to the species *L. interrogans*, 1 to *L. borgpetersenii* and 1 to *L. kirschneri*. The remaining 2 rendered low quality sequences, so they were excluded from further analysis.

Table 2. Clinical samples used in this study. Grey indicates clinical samples positives for the three *loci*.

No	ID	sample	No x 10^4 Leptospires/ml	16 S PCR	Species determination	*adk*	*lip*L41	*mre*A
1	136	Serum DNA	1.92	+	ND	ND	ND	ND
2	196	Serum DNA	1.92	+	ND	ND	ND	ND
3	182	Serum DNA	1.93	+	*L. interrogans*	-	-	-
4	477	Serum DNA	2.03	+	*L. interrogans*	-	-	-
5	130	Serum DNA	2.08	+	*L. interrogans*	-	-	-
6	64	Serum DNA	2.13	+	*L. interrogans*	-	-	-
7	464	Serum DNA	2.18	+	*L. interrogans*	-	-	-
8	131	Serum DNA	2.27	+	*L. interrogans*	-	-	-
9	180	Serum DNA	2.37	+	*L. interrogans*	-	-	-
10	27	Serum DNA	2.39	+	*L. interrogans*	-	-	-
11	59	Serum DNA	2.42	+	*L. interrogans*	-	-	-
12	143	Serum DNA	2.47	+	*L. interrogans*	-	-	-
13	129	Serum DNA	2.51	+	*L. interrogans*	-	-	-
14	133	Serum DNA	2.59	+	*L. interrogans*	-	-	-
15	174	Serum DNA	2.60	+	*L. interrogans*	-	-	-
16	73	Serum DNA	2.66	+	*L. interrogans*	-	-	-
17	21	Serum DNA	2.68	+	*L. interrogans*	-	-	-
18	28	Serum DNA	2.68	+	*L. interrogans*	-	-	-
19	431	Serum DNA	3.26	+	*L. interrogans*	+	-	-
20	178	Serum DNA	3.27	+	*L. interrogans*	-	-	-
21	197	Serum DNA	3.52	+	*L. interrogans*	-	-	-
22	29	Serum DNA	3.55	+	*L. interrogans*	-	-	-
23	39	Serum DNA	3.55	+	*L. interrogans*	-	-	-
24	471	Serum DNA	3.59	+	*L. interrogans*	+	-	-
25	466	Serum DNA	4.17	+	*L. interrogans*	+	+	-
26	157	Serum DNA	4.25	+	*L. interrogans*	-	-	-
27	172	Serum DNA	4.30	+	*L. interrogans*	-	-	-
28	452	Serum DNA	4.34	+	*L. interrogans*	+	+	+
29	120	Serum DNA	4.42	+	*L. interrogans*	-	-	-
30	99	Serum DNA	4.51	+	*L. kirschneri*	-	-	-
31	439	Serum DNA	4.54	+	*L. interrogans*	+	+	+
32	37	Serum DNA	4.71	+	*L. interrogans*	+	+	+
33	179	Serum DNA	5.95	+	*L. interrogans*	+	+	+
34	31	Serum DNA	6.46	+	*L. borgpetersenii*	-	+	+

± indicates positive/negative amplification.

The 32 pathogenic positive samples were further explored using the simplified MLST scheme proposed here. The results showed that 8 samples (23.5%) were positive for one, two or the three loci. (Table 2), whereas 24 samples (70%) were negative for all loci. Four samples (11.7%) produced positive results for the three loci, thus allowing a deeper characterization by phylogenetic analysis. The remaining four samples yielded positive amplification for one or two loci. The efficiency of the MLST amplification was directly related to the amount of leptospires per ml in the samples (Table 2). We found that all the samples with positive amplification for the three loci had a concentration above 4×10^4 leptospires/ml. Even though, the amplification of one or two loci also occurred in samples less concentrated, the higher frequency of two loci amplification increased near that concentration.

MLST results

MLST results were obtained using the database generated for the 7-locus scheme (http://pubmlst.org/leptospira/scheme#2). Although there is not a sequence type (ST) associated to the simplified scheme, the 3-locus allelic profile or even 1 or 2 locus can be linked to one or more STs generated by the 7-locus scheme. We selected a 3-locus combination proposed for the simplified scheme using the database. There were no new alleles for any of the locus used in the simplified scheme. The alleles and linked STs are shown in Table 3. Using these data, we were able to infer the probable serogroup or serogroups of each sample. That was performed by loading the sequences in the database, which allows the determination of the numeric code for each locus. The output includes a list of strains that share these alleles, which is accompanied by the corresponding STs and serogroups. (Table 3).

The results showed that the samples 179 and 452 present the ST3, which is based on the combination of the alleles for the 3-loci obtained. According to the database, the strains belonging to this ST also belong to two possible serogroups: Canicola or Pomona. The remaining two positive samples for the 3- locus (37 and 439) rendered two possible STs: ST8/ST47 for sample 37 and ST5/ST6 for sample 439. These STs are in both cases, according to the database related to serogroups Icterohaemorraghiae and Sejroe respectively. The sample 466, which was positive for two loci (*adk* and *lip*L41), showed two possible STs: ST5 or ST6. Similar to sample 439, those STs are related just to serogroup Sejroe. The samples positive for only one locus were 431, 471 (locus *adk*) and 31 (locus *lip*l41). The sample 431 presented the allele 4 for *adk*. This allele is part of three possible STs: ST5,

Table 3. MLST results. Alleles assignation, possible STs (scheme #2) and inference of possible serogroup.

No	Sample ID	*adk*	*lip*L41	*mre*A	Possible STs (2)	Possible serogroup
19	431	4	-	-	5, 6 or 35	Sejroe/Sejroe/Icterohamorraghiae
24	471	54	-	-	96	Autumnalis
25	466	4	2		5 or 6	Sejroe/Sejroe
28	452	3	3	1	3	Pomona or Canicola
31	439	4	2	9	5 or 6	Sejroe/Sejroe
32	37	5	5	4	8 or 47	Icterohamorraghiae/Icterohamorraghiae
33	179	3	3	1	3	Pomona or Canicola
34	31	-	29	-	62, 74, 88, 90, 91, 92 or 93	Javanica/MIni

-: indicates no amplification

ST6 andST35, which in turn were related to two serogroups, Sejroe and Icterohameorraghiae. The sample 471 presents the allele adk-54 and, according to the available data, this locus only appears in the ST 96, which is related solely to serogroup Autumnalis. The sample 31 contains the allele 29 for the locus*lip*L41. This allele appears in seven different STs, however those STs are onlyrelated to serogroups Javanica and Mini.

These results are directly associated to the data currently available in the database and for that reason they are limited. Nevertheless, the application of the 3-loci scheme or even two or one locus, allow us to narrow the possible serogroups of the samples, even in some cases where the presence of only one allele yielded one ST which was related to only one serogroup. Moreover, although one allele was present in many different STs, the serogroups related to those STs were one or two, circumscribing the multiple serogroups that could be present in the sample, which makes this methodology a useful tool to complement MAT.

MLST results from clinical samples were also supported applying the same phylogenetic approach used to test the selected reduced loci. The analysis was repeated by adding to the collection of 116 strains the concatenated sequences of the three loci amplified in the four positive samples for the simplified scheme. The samples clustered in the main clade of the phylogenetic tree, which in turn were associated to the main serogroups: Icterohaemorrhagiae, Pomona and Canicola (Supplementary Figure 1)

Discussion

We have developed a simplified MLST scheme for direct detection of pathogenic Leptospira from human sera samples. The *in silico* validation of the scheme using our previous strains collection rendered a phylogenetic tree consistent with that obtained using the 7-loci scheme allowing the accurate species assignment without losing power of discrimination and therefore making possible its direct application on clinical samples.

Different LoDs have been reported in many leptospirosis diagnostic research studies using sera samples with different amplification methods and targets [14,20,21], but the aim in those cases was only the diagnosis of the disease. The LoD reached here for each primer was similar to that obtained by Agampodi and colleagues where clinical samples were tested in a direct MLST method using an end-point PCR method [5], that together with our previous work [11], constitutes the only studies using this kind of approach.

As mentioned before higher rates of detection was reached in Weiss and colleagues' work, but the scheme proposed here reached an improved rate of detection (23.5% of the samples with one, two or three loci) over our previous work where the 7-loci MLST scheme was applied and 21% of clinical samples tested rendered interpretable data [11]. Although in our previous work the number of samples tested was larger than here and the sensitivity of the reduced scheme will be an issue to improve using a larger set of samples, the possibility of using less number of loci to obtain the same results make this approachdesirable in terms of time, costs and simplicity.

Notably, there was no new alleles for the three locus applied which is possibly associated to the number of samples tested. The allelic profiles obtained are directly associated to the data currently available in the database and for that reason are limited. Nevertheless, the application of the 3-loci scheme or the two and also the one locus, allowed us to narrow the possible serogroups of the samples, even in some cases where the presence of only one allele yielded a unique ST which was related to only one serogroup. Moreover, although one allele was present in many different STs, the serogroups related to those STs were one or two, circumscribing the multiple serogroups that could be present in the sample, which makes this methodology a useful tool for MAT complementation. Although, we have recently described that phylogenetic analysis is not always appropriate for MLST concatenated sequences due to recombination, gene conversion or lateral gene transfer present in Leptospira genus [23], in this work the possible serogroups from samples with the 3-locus

profile were confirmed by adding their concatenated sequences to the collection of 116 strains supporting in that way the robustness of MLST for narrowing and inferring possible serogroup of a given sample by using genetic material.

In this work we have only included human samples with clinically confirmed leptospirosis and no treatment at the time of obtaining the sample, which increased the likelihood of the sample to have a higher bacterial load. Although this task is not easy when working with livestock or domestic animal samples where the disease often presents as a chronic infection and its detection is more complex, this method is still applicable to any biological sample, and could be usedas a complementary technique for surveys in domestic or wild animals, providing a fast source of epidemiological information.This simplified MLST scheme allows the enrichment of epidemiological information from samples readily accessible even when the sensitivity of DNA amplification from clinical samples may be low. The information generated from the sequences of each locus is useful to determine new alleles, which thus could in turn enable the identification of new circulating genotypes. This would be particularly useful during an outbreak, where rapid typing of the samples is required, allowing a better monitoring in order to establish its origin and other epidemiological connections. In these cases, culture is not feasible however, clinical samples can be used directly. Furthermore, although this technique does not intend to replace the serological determination methods, it may be useful for approximating a serogroup associated to that genotype, which otherwise would be unknown. As mentioned in our previous work, the possibility of inferring or providing an approach to the possible serogroups of an isolate or clinical sample through MLST, may bring an alternative to serological methods when a quick approach is required. The ultimate aim wasto strengthen the epidemiological knowledge, through a straightforward methodology that may be available to most laboratories.

The application of this simplified MLST scheme led to the following achievements in different levels, the increasing in *Leptospira* detection fromclinical samples, since isolation is avoided and more samples can be exploited together with the possibility of updating the information on currently circulating genotypes, thus bringing useful dataforimproving diagnosis and epidemiological control of this disease.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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References

[1] Abdollahpour G, Diguimbaye C, Hartskeerl R, et al. Report of the second meeting of the Leptospirosis Burden Epidemiology Reference Group(LERG). Ginebra, Suiza, 2010: World Health Organization; 2011.
[2] Bharti A, Nally J, Ricaldi J, et al. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis. 2003;3:757–771.
[3] Hartskeerl R, Collares-Pereira M, Ellis W, et al. control and re-emerging leptospirosis: dynamics of infection in the changing world. Clin Microbiol Infect. 2011;17:494–501.
[4] Vanasco NB, Schmeling MF, Lottersberg J, et al. Clinical characteristics and risk factors of humans leptospirosis in Argentina (1999–2005). Acta Trop. 2008;107:255–258.
[5] Agampodi SB, Moreno AC, Vinetz J, et al. Short report: utility and limitations of direct multi-locus sequence typing on qPCR-positive blood to determine infecting *leptospira* strain. Am J Trop Med Hyg. 2013;88:184–185.
[6] Boonsilp S, Thaipadungpanit J, Amornchai P, et al. A single multilocus sequence typing (MLST) scheme for seven pathogenic leptospira species. PLoS Negl Trop Dis. 2013;7(e1954).
[7] Thaipadungpanit J, Wuthiekanun V, Chierakul W, et al. A dominant clone of *leptospira interrogans* associated with human leptospirosis in Thailand. PLoS Negl Trop Dis. 2007;1:e56. other authors.
[8] Ahmed N, Devi SM, Valverde M, et al. Multilocus sequence typing method for identification and genotypic classification of pathogenic Leptospira species. Ann Clin Microbiol Antimicrob. 2006;5(28). DOI:10.1186/1476-0711-5-28
[9] Goarant C. Leptospirosis: time to move to molecular epidemiology: comments on "Reassessment of MLST schemes for Leptospira spp. typing worldwide" by Varni and colleagues. Infect Gen Evol. 2014;21:484–485.
[10] Varni V, Ruybal P, Lauthier JJ, et al. Reassessment of MLST schemes for *Leptospira* spp. typing worldwide. Infect Gen Evol. 2014;22:216–222.
[11] Chiani Y, Jacob P, Varni V, et al. Isolation and clinical sample typing of human leptospirosis cases in Argentina. Infect Gen Evol. 2016;37:245–251.
[12] Weiss S, Menezes A, Woods K, et al. An extended multilocus sequence typing (MLST) scheme for rapid direct typing of *Leptospira* from clinical samples. PLoS Negl Trop Dis. 2016;10:e0004996.
[13] Tomasini N, Lauthier JJ, Ayala FJ, et al. How often do they have sex? A comparative analysis of the population structure of seven eukaryotic microbial pathogens. PLoSOne. 2014;9:e103131.
[14] Mérien F, Amouriaux P, Perolat P, et al. chain reaction for detection of *Leptospira* spp. in clinical samples. J Clin Microbiol. 1992;30:2219–2224.
[15] Staden R, Beal KF, Bonfield JK. Computer methods in molecular biology bioinformatics methods and

protocols. In: Misener S, Krawetz SA, Eds. The staden package. Vol. 132. Totowa, NJ 07512: The Humana Press Inc.; 1998. p. 115–130.

[16] Darriba D, Darriba D, Taboada GL, et al. jModelTest 2: more models, new heuristics and parallel computing. NatMethods. 2012;9:772.

[17] Guindon S, Gascuel OA. simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. Syst Biol. 2003;52:696–704.

[18] Caimi K, Varni V, Melendez Y, et al. A combined approach of VNTR and MLST analysis: improving molecular typing of Argentinean isolates of *Leptospira interrogans*. Mem Inst Oswaldo Cruz. 2012;107:644–651.

[19] Tamura K, Peterson D, Peterson N, et al. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molec Biol Evol. 2011;28:2731–2739.

[20] Bourhy P, Bremont S, Zinini F, et al. Comparison of real-time PCR assays for detection of pathogenic *Leptospira* spp. in blood andidentification ofvariations in target sequences. J Clin Microbiol. 2011;49(2154). DOI:10.1128/JCM.02452-10

[21] Stoddard RA, Gee JE, Wilkins PP, et al. Detection of pathogenic Leptospira sppthrough TaqMan polymerase chain reaction targeting the LipL32 gene. Diag Microbiol Infect Dis. 2009;64:247–255.

[22] Caimi K, Repetto SA, Varni V, et al. species molecular epidemiology in the genomic era. Infect Genet Evol. 2017;54:478–485.