Evaluation of Serological Tests on Different Disease Stages of Leptospirosis Infection in Humans

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Article

Evaluation of Serological Tests on Different Disease Stages of Leptospirosis Infection in Humans

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Abstract: Background/Objectives: Leptospirosis is a globally distributed zoonosis with symptomatology similar to other febrile syndromes in tropical countries, making clinical diagnosis difficult. This study aimed to analyze the performance and agreement between serological diagnostic tests for the detection of acute and convalescent human leptospirosis compared to the micro agglutination test (MAT) in an endemic region of the Colombian Caribbean. **Methods**: A prospective descriptive study was conducted on 275 patients with suspected leptospirosis. Paired serum samples were collected, and an epidemiological survey completed. Positive and negative predictive values, sensitivity, specificity, and kappa index were calculated using MAT as the gold standard. A Bayesian latent class model was used to compare the diagnostic tests. **Results**: In 223 paired serum samples, the sensitivity values for different stages of the disease varied between 10.8% to 54.1% in the acute and 6.1% to 66.7% in the convalescent phase when compared to MAT; and when using the Bayesian model 9.5% to 75.3% in the acute and 5.7% to 85.3% in the convalescent phase. The Kappa value, an indicator of agreement, was moderate for the IgM ELISA (0.553) in the acute phase, and substantial (0.692) in the convalescent phase. **Conclusions**: MAT was the best confirmatory test in both acute and convalescent phases. Despite the high specificity of ELISA, 21.62% of patients diagnosed as negative by IgM-ELISA in the acute and convalescent phases were confirmed as positive by MAT. It is necessary to re-evaluate diagnostic guidelines that do not employ MAT for confirmation, and to strengthen diagnostic and clinical identification of leptospirosis in healthcare institutions and public health laboratories, while providing a rapid and reliable test for its implementation.

Keywords: Colombia; diagnosis; serology; leptospirosis; seroprevalence; risk-factors; reemerging

1. Introduction

Leptospirosis is an emerging and re-emerging zoonosis with worldwide distribution with a rise in cases associated with increased rainfall and high temperatures. However, cases can occur at any time of the year [1], and it is traditionally considered an occupational exposure disease [2–4]. Leptospirosis is notifiable in some countries; however, in general it is under-reported due to lack of knowledge of the disease, similarity with other febrile diseases present in endemic areas, and difficulties in its clinical and laboratory diagnosis [5–7]. Laboratory diagnosis allows confirmation of leptospirosis where the disease is suspected based on clinical aspects, further determining the serovar that is causing the infection, the probable source of infection, and the potential reservoir and its location. This information contributes to the implementation of control strategies [8].

Clinical leptospirosis is a biphasic disease, with an acute phase that occurs between the fourth and tenth day of disease onset, followed by a convalescent (immune) phase that varies from 4 to 30

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days [9]. During the acute phase, bacteria are present in the blood, while in the convalescent phase they disappear from the blood with the appearance of IgM antibodies [10]. Laboratory diagnosis of leptospirosis is based on several methods: the microscopic agglutination test (MAT), detection of organism's DNA by polymerase chain reaction (PCR), isolation of microorganism by culture methods, or detection of antibodies against the microorganism [11]. For many years, serological diagnosis has been considered the cornerstone for identifying leptospiral infections. Typically, these studies are based on detection of specific antibodies against various leptospiral antigens [12]. The isolation of *Leptospira* spp. from clinical samples has low diagnostic sensitivity, requires experience personnel, and most importantly, culturing leptospires takes weeks. Therefore, diagnosis of leptospirosis relies on serological results [12].

During the acute phase of leptospirosis, timely confirmation is an important clinical priority to optimize both targeted treatment and supportive management [13]. Serological tests such as ELISA and rapid lateral flow assays have largely replaced the conventional MAT test due to their ease of implementation and performance with comparable sensitivity and specificity; in particularly during the acute stage of the disease [14]. Some of these serological tests have acquired the status of point-of-care rapid screening tests [15]. The detection of IgM by ELISA has been widely used but its specificity is affected by the antigen used in the test, the presence of antibodies from previous exposure (in endemic regions), and by the presence of other febrile diseases [1]. IgM detection tests have been developed in various rapid assay formats (dipstick, latex agglutination, lateral flow, and bidirectional platform) for implementation in the field or rural clinical laboratories [12]. However, there are important limitations for early diagnosis using any serological tests, and when performing them it should be mandatory to use paired sera [16]. Furthermore, it has been recommended that confirmation of results by rapid diagnostic tests be done using a reference test [15].

The MAT test, detects both IgM and IgG, is regarded as the gold standard for the diagnosis of leptospirosis, but requires a high level of technical experience, and the precise time of sample collection [5]. It also requires the maintenance of a diverse live panel of serovars from different serogroups of pathogenic leptospires [1]. The use of these live pathogens can create a risk of laboratory-acquired infections, which makes it poorly accessible to conventional clinical laboratories [17]. MAT can also produce many false negative results in the early stage of infection, since IgM antibodies detected by this test appear after day eight of the disease and reach their peak in the fourth week, with detectable serovar-specific antibody titers persisting for several months and even years [18,19]. Thus, cross reactions between serogroups occur mainly in the early stages of the disease [20]. Although, this test is highly specific, it has limited sensitivity in the acute phase because *Leptospira* antibodies are detectable around 7-10 days after the appearance of symptoms and commonly, a second serum sample is required for case confirmation, delaying diagnosis and treatment [21,22]. The World Health Organization (WHO) recommends using a locally optimized MAT panel that contains strains currently circulating in a particular region [8]. The basis for this is to improve the sensitivity of the test, since patients' sera are likely to react well with local strains [23]. However, knowledge about currently circulating strains is scarce in many highly endemic regions. Even the use of strains representative of a broad panel of serogroups for MAT is not feasible given the large resources needed for its implementation, and the cost of the procedure [23]. In Colombia, to date, native strains are not included in the diagnostic panels of the national surveillance system, which could improve the performance of MAT.

MAT, which is the serological reference test is valuable for epidemiologic studies, but it has limitations in the clinical context of acute disease [1,24]. MAT has been considered an imperfect standard for the evaluation for rapid diagnostic evaluation [25]. The Bayesian latent class model admits that all tests are imperfect and has been suggested as a more appropriate method to evaluate diagnostic tests, including immunodiagnostics for leptospirosis [25–27]. This study aimed to analyze the performance and agreement of four serological diagnostic tests available for the detection of acute and convalescent human leptospirosis in comparison with MAT in an endemic region of the Colombian Caribbean. Our results should provide rural health clinics and diagnostic laboratories

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where MAT is not possible to implement, a suitable assay for the detection of serum samples from suspected cases of leptospirosis.

2. Materials and Methods

Ethical considerations.

Patients were invited to participate in the project. They read the informed consent form approved by the ethics committee of the Faculty of Health Sciences of the University of Córdoba, who signed and completed the epidemiological survey once it was understood and accepted by the patient. The study was classified as risk-free according to the technical, scientific, and administrative standards for health research of the Colombian Ministry of Health (Resolution 008430, October 4, 1993) and the Declaration of Helsinki [28].

Study design.

We performed a cross-sectional study at the Department of Córdoba. A total of 275 patients who attended three healthcare institutions were recruited between December 2017 and March 2020 and met the operational case definition established by the Colombian National Institute of Health (INS) [29]. Residents of the Department of Córdoba were recruited through an alliance with the laboratory of the Department of Public Health. Patients who did not meet the operational case definition and those who received antibiotic therapy before sample collection were excluded. We applied a structured questionnaire to provide information on the individual characteristics of the participants, domestic and peri-domestic environmental characteristics, exposure to sources of environmental contamination, and the presence of potential animal reservoirs.

Collection and processing of samples

Eligible individuals were recruited after obtaining informed consent and completing an epidemiological survey. Blood samples were collected in tubes without additives to obtain the serum samples. All the samples were stored at room temperature and processed within 2 hrs. Whole blood samples were collected during the acute and convalescent phases, with differences of 10 and 15 days. Blood samples were transported at 4° C to the research laboratory of the Microbiological and Biomedical Research Group of Córdoba (GIMBIC), Bacteriology Program, University of Cordova.

Paired sera were analyzed using the commercial kit Panbio™ Leptospira IgM ELISA, immunochromatography (IgM) using two commercial kits, (SD Bioline Leptospira IgM™ and Leptocheck WB™) according to the manufacturer's instructions, and the microagglutination test (MAT) was performed. according to WHO specifications [8], using 14 serogroups and 19 serovars available in the GIMBIC laboratory with fourteen serogroups: serogroup: *Australis* serovar *Australis*, Bratislava; serogrupo *Autumnalis* serovar *Autumnalis*, serogrupo *Ballum* serovar *Ballum*, serogrupo *Bataviae* serovar *Bataviae*, serogrupo *Canicola* serovar *Canicola*, serogrupo *Celledoni* serovar *Celledoni*, serogrupo *Grippotyphosa* serovar *Grippotyphosa*, serogrupo *Hebdomadis* serovar *Hebdomadis*, serogrupo *Icterohaemorrhagiae*, *Copenhageni*, serovar *Icterohaemorrhagiae*, serogrupo *Louisiana* serovar *Louisiana*, serogrupo *Pomona* serovar *Pomona*, serogroup *Pyrogenes* serovar *Zanoni*, serogroup *Sejroe Hardjo* serovar *Balcanica*, *Saxkoebing*, *Sejroe*, and serogroup *Tarassovi* serovar *Tarassovi*.

Screening was performed using a 1:100 dilution of serum. Agglutination against a 1:100 dilution of serum was considered a positive result, and the sample was titrated by two-fold serial dilutions to determine the highest positive titer. The presumed infecting serogroup was the serogroup against which the highest agglutination titer was directed [8]. A sample with a high agglutination titer for several serogroups; was defined as mixed.

Criteria for defining confirmed leptospirosis.

A sample was considered positive for leptospirosis in the MAT test when a four-fold increase in titer to one or more serovars was present between the parallel-mounted acute and convalescent phase serum samples; or if the titers were equal to or greater than 1:800 with compatible symptoms [5].

Seroprevalent leptospirosis.

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In negative cases for human leptospirosis, a titer of ≥ 1:100 was found against one or more serovars in the MAT test, with no change in titer between the sample in the acute and convalescent phases. This indicated a previous exposure to *Leptospira* spp.

Information processing and statistical treatment of data.

The data were tabulated in a Microsoft Excel spreadsheet. Statistical analyses were performed using the IBM SPSS statistical software version 25.0. Kappa coefficients [30], positive and negative predictive values, sensitivities, and specificities were calculated. The online tool MICE (Modeling Center for Infectious Diseases, Mahidol-Oxford Research Unit, Thailand http://mice.tropmedres.ac/home.aspx) was used for Bayesian latent-class modeling [25]. This model made it possible to determine the probability that a specific patient was a carrier of leptospirosis, based on the persistence of the disease and the results of a serological test that determined its sensitivity and specificity.

3. Results

During the study period, 275 patients with clinical suspicion of leptospirosis were recruited: 180 males and 95 females; 18.1% were under 12 years of age, 10.18% between 13 and 18 years of age, 61.45% between 19 and 60 years of age, and 10.8% were over 60 years of age. Among the patients included in this study, 92.7% presented with fever, headache, and myalgia associated with jaundice and hepatomegaly (28% and 13.5%, respectively). Of these, 13.5% were confirmed positive for leptospirosis. The most frequent symptoms in the confirmed patients were myalgia (100%), headache (96.4%), fever (97.3%), jaundice (62.2%), nausea (45.9%), and abdominal pain (40.5%). Hepatomegaly (22.9%) was also associated with fever, headaches, and myalgia. The least frequent symptoms were conjunctival suffusion (4.4%), lymphadenopathy (3.6%), and hemoptysis (2.5%).

A total of 13.5% of the patients were confirmed as positive for leptospirosis. In these patients, the most common symptoms were myalgia (100%), fever (97.3%), headache (94.6%), jaundice (62.2%), nausea (45.9%) and abdominal pain (40.5%). In confirmed cases, fever, headache, and myalgia associated with jaundice and hepatomegaly occurred in 59.5% and 29.7%, respectively. Table 1 presents the results according to the diagnostic techniques implemented.

Table 1. Comparison of different screening test for detection of anti-*Leptospira* antibodies in human sera.

Screening Test	Frequency				n
	Positives	%	Negatives	%	
Acute Phase					
Leptocheck	16	5.81	259	94.19	275
ELISA	29	10.54	246	89.46	275
SD Leptospira	6	2.18	269	97.82	275
MAT	26	9.45	249	90.55	275
Convalescent Phase					
Leptocheck	13	5.82	210	94.18	223
ELISA	27	12.10	196	87.90	223
SD Leptospira	2	0.89	221	99.11	223
MAT	30	13.45	193	86.55	223

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This study included 223 paired serum samples. In 52 of the patients the second sample was unavailable (convalescence phase), 21 died before the second sample was collected, and the remaining 31 patients did not visit the medical institution providing health services for the second sample (Table 1). Table 2 lists the sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) of diagnostic tests implemented during the acute and convalescent phases using MAT as the gold standard and the Bayesian model.

Table 2. Comparison of the diagnostic accuracies of MAT, SD Leptospira, Leptocheck, and IgM ELISA using Bayesian latent class modeling.

Parameters	**MAT as serologic gold standard (%) ***		**Bayesian latent class model (%) ****	
	Acute phase	**Convalescent phase**	**Acute phase**	**Convalescent phase**
PATIENTS (%)	13.5 (9.8 – 18.2)	14.8 (10.5 - 20.3)	17.9 (12.8 – 23.8)	17.4 (12.7 - 23.0)
MAT				
Sensitivity	100	100	75.3 (57.8 - 91.9)	85.3 (69.9 - 95.1)
Specificity	100	100	100 (100 - 100)	100 (100 - 100)
PPV	100	100	100 (100 - 100)	100 (100 - 100)
NPV	100	100	94.9 (89.4 - 98.6)	97.0 (93.3 - 99.1)
SD Leptospira				
Sensitivity	10.8 (3.5 - 26.4)	6.1 (1.1 - 21.6)	9.5 (3.2 - 20.1)	5.7 (1.1 - 15.7)
Specificity	99.2 (96.7 - 99.9)	100 (97.5 - 100)	99.3 (97.5 - 100)	99.9 (98.6 - 100)
PPV	66.7 (24.1 - 94.0)	100 (19.8 - 100)	72.5 (32.3 - 99.5)	90.8 (33.2 - 100)
NPV	87.7 (83.1 - 91.3)	86.0 (80.5 - 90.1)	83.5 (77.7 - 88.4)	83.5 (77.9 - 88.1)
LEPTOCHECK				
Sensitivity	29.7 (16.4 - 47.2)	30.3 (16.2 - 48.9)	31.0 (18.7 - 45.7)	33.5 (20.1 - 49.5)
Specificity	97.9 (94.9 - 99.2)	98.4 (95.1 - 99.6)	99.6 (97.8 - 100)	99.9 (98.7 - 100)
PPV	68.8 (41.5 - 87.9)	76.9 (46.0 - 93.8)	94.8 (72.0 - 100)	98.2 (82.3 - 100)
NPV	90.0 (85.5 - 93.2)	89.0 (83.8 - 92.8)	87.0 (81.4 – 91.5)	87.7 (82.5 - 91.9)
ELISA				

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Sensitivity	54.1 (37.1 - 70.2)	66.7 (48.1 - 81.4)	55.0 (39.6 - 69.4)	68.3 (52.4 - 81.7)
Specificity	96.2 (92.7 - 98.1)	97.4 (93.6 - 99.0)	99.2 (96.2 - 100)	99.7 (97.6 - 100)
PPV	69.0 (49.0 - 84.0)	81.5 (61.3 - 93.0)	93.6 (71.8 - 100)	98.0 (84.7 - 100)
NPV	93.1 (89.0 - 95.8)	94.4 (89.9 - 97.0)	91.0 (85.9 - 94.8)	93.7 (89.3 - 96.7)

* The gold standard model assumed that MAT was a perfect test (100% sensitivity and 100% specificity; all patients with a positive gold standard test were diseased, and all patients with a negative gold standard test were not diseased). The values shown are the estimated means with 95% confidence intervals. ** The Bayesian latent class model assumed that all tests evaluated are imperfect. The values shown are the estimated median values with 95% confidence intervals.

Table 3 presents the Kappa values for each technique implemented in the acute and convalescent phases. Our results showed moderate agreement for the IgM ELISA in the acute phase and slight and fair agreement for SD Leptospira and Leptocheck, respectively. During the convalescent phase, we found substantial agreement for the IgM-ELISA and fair and slight agreements for Leptocheck and SD Leptospira, respectively.

Table 3. Kappa agreement values for each technique were calculated during the acute and convalescent phases**.**

SCREENING TEST	KAPPA COEFICIENT.	
	ACUTE PHASE	CONVALESCENT PHASE
IgM ELISA	0.553	0.692
SD Leptospira (IgM).	0.154	0.099
Leptocheck	0.363	0.383

4. Discussion

Various serological tests for diagnosing leptospirosis have been developed and implemented in recent years; however, their validation has yet to be performed in Colombia. An ideal diagnostic test should have a high sensitivity and specificity during the acute phase, be widely available at a reasonable cost, and offer rapid results. Different sensitivity and specificity values may be obtained depending on the evaluated population and the antigen used. Clinicians must understand these variations in the validation indices of diagnostic tests to determine their accuracy and reduce misdiagnosis [31]. In the present study, the sensitivity values for the different phases of the disease using the Bayesian model and MAT as the gold standard model varied between 6.1 to 68.3%. (Table 2). In studies on the Andaman and Nicobar Islands in India [32], Hawaii [24], Thailand [33], and in the USA [34], the sensitivity ranged from 25 to 92%. These findings differ from those studies conducted in Sri Lanka, which can be attributed to higher number of suspected and confirmed patients included in this study [14,15].

The results of this study suggest that MAT is the best immunological test for confirmation of cases in the convalescent phase. Analysis of the Bayesian model made it possible to determine true sick patients by confirming a greater number of them in the convalescence phase; nevertheless, it presented a lower sensitivity in the acute phase (Table 2). These findings are consistent with those of a Sri Lankan study that reported sensitivity values of 55.3% in the acute phase and 95% in the convalescence phase [14]. However, they contrast with those reported from Thailand, Palau, Hawaii,

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Illinois, and Puerto Rico; where higher sensitivity values were reported in the convalescence phase; findings attributable to the use of serogroups and serovars with greater circulation in the study regions [34].

Using native serovars in MAT has been reported to decrease the average cross-reactivity when local strains are used [35]. A study in Colombia which included a native strain in the MAT test panel, increased the percentage of positivity by 15% [7]. The antigen panel used in the MAT test should include all locally circulating serovars, and if these strains are unknown or subject to change, the panel should include serovars that represent all serogroups [1]. The sensitivity of the IgM ELISA validated and reported by the manufacturer in Australia and New Zealand was 96.5%, which is different from what was found in this study and what was previously reported [36]. These differences could be due to the unique eco-epidemiological characteristics of each region that determine the presentation and behavior of the disease. In addition, test values may vary depending on the evolution of the disease [8]. The IgM ELISA showed a higher performance than the immunochromatographic tests. Although the sensitivity values were not acceptable, we suggest their continuation as an initial screening test, and all results must be confirmed using MAT.

The ELISA test showed an increase in sensitivity in both phases of the infection when compared to MAT as the gold standard model and the Bayesian model, which can be attributed to the fact that there is a greater production of antibodies on day 15 of the disease [8]. Limmathurotsakul et al. [25] concluded that culture plus MAT was an imperfect gold standard when comparing diagnostic tests. They found that sensitivity and specificity of the diagnostic test increased when using the Bayesian latent class modelling. In leptospirosis the sensitivity of screening tests may be affected by the prevalence of different infectious serogroups, which affects their performance. In all screening tests for leptospirosis, the antigen must be broadly reactive with different infectious *Leptospira* serovars. The characteristics of the serovar panels may differ from one laboratory to another one. Screening tests must detect antibodies produced against site-specific leptospiral serovars. Laboratories must validate the performance of screening tests in the setting where they are to be implemented [37].

In contrast to IgM ELISA, the immunochromatographic test SD Leptospira that detects IgM had a low specificity in both, the acute and convalescent phases. Leptocheck WB performed better but with similar specificities in both phases. This could be explained by the persistence of IgM antibodies for months or years in confirmed leptospirosis cases [19,38,39]. It was possible to determine based on results from this study that immunochromatographic tests do not meet the parameters for screening tests in places with difficult geographic access and limited resources, because they did not detect all positive cases. The factors affecting the sensitivity and specificity of these tests may be due to the genus-specific nature of the antigen, and their inability to react to and recognize the infecting serovar [15].

The negative predictive values found in the current study for the different tests ranged from 83.5% (SD Leptospira) to 93.7% (IgM ELISA), indicating the probability of not having the disease if the test was negative, which is consistent with previous studies [14,37,40]. Regarding the percentage of concordance or Kappa value (Table 3), it was moderate for IgM ELISA in the acute phase and considerable in convalescence phase. These findings differ from a study conducted in Brazil [41]; where concordance has been reported for immunochromatographic tests in both phases of the disease; in the case of SD Leptospira, the immunochromatographic tests were moderate, and acceptable for Leptocheck. Furthermore, Bathia et al. [9] in India reported low concordance between IgM ELISA and LeptoCheck.

An ideal diagnostic test should have high sensitivity and specificity during the acute phase, be widely available at a reasonable cost, and provide rapid and accurate results. Our results showed that ELISA had a higher performance compared to the immunochromatographic tests. Although its sensitivity values were not acceptable, it can continue as a screening test, but all results must be confirmed by MAT. In the current study, we found a significant percentage (21.62%) of patients with negative IgM ELISA results in the acute and convalescent phases, which were confirmed by MAT. The INS leptospirosis surveillance and control protocol guidelines do not consider confirmation by MAT in cases in which the IgM ELISA results are negative. Therefore, these guidelines must be

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reconsidered. A greater antibody response is expected in MAT, considering that a greater number of serovars are tested; however, in IgM ELISA, the results may vary according to the antigen used. In Colombia, two imported tests are used: Panbio and Virion-Serión, both assays are used for detection of human IgM. The first includes serovars *Hardjo*, *Pomona*, *Copenhageni*, *Australis*, *Madanesis*, *Kremastos*, *Nokolaevo*, Celledoni, *Canicola, Grippotyphosa, Szwajizak, Djasiman*, and *Tarassovi*, and the second one includes non-pathogenic serovars. Using the Virion-Serión IgM ELISA, a 39% seropositivity was reported in Colombia, but only 0.3% by MAT [42]. *Leptospira* seroprevalence in humans in Colombia ranges from 6% to 35% depending on the geographical area [43].

Compared with other studies where the number of paired sera was lower [41,44], in our case having a large number of paired sera, allowed us greater confirmation of cases of disease. Despite the high specificity of ELISA, limitations were observed in the present study because the sensitivity values were not optimal, which led to the evaluation of other diagnostic methods, such as PCR for implementation in the acute phase. A study that compared MAT and PCR as a complement to MAT for the diagnosis of leptospirosis in the first days of the disease and in patients in whom paired serum was not obtained, allowed a more timely and accurate diagnosis of the evolution of the disease and reduced the index of indeterminate cases and false negatives that occur in many cases in which only MAT is performed [45].

In our study, the evaluated serological tests detect IgM except for MAT that detects both IgM and IgG. IgM antibodies are commonly associated with acute infection, but they can remain in circulation for a long period of time. This is what makes this study novel, because these serological tests compared immune responses in patients during both phases of infection. One requirement to improve the specificity of MAT is the isolation and characterization of circulating serovars in the region of study, to be included in the diagnostic panel to ensure better performance. Furthermore, antibodies cannot be detected when the causative serovar is not present in the test panel or low titers are present against the serovar that antigenically resembles the cause of the disease, which is not part of the diagnostic panels. Considering the presence of other febrile diseases in *Leptospira*-endemic areas of Colombia, it is imperative to strengthen diagnosis and clinical identification of leptospirosis at the level of institutions providing health services, and departmental public health laboratories of the Caribbean region to guarantee timely and accurate disease detection.

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