Biomédica 2015;35:557-62
doi: http://dx.doi.org/10.7705/biomedica.v35i4.2504

BRIEF COMMUNICATION

Serological evidence of leptospirosis in patients with a clinical suspicion of dengue in the State of Ceará, Brazil

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Introduction: Dengue and leptospirosis are two febrile illnesses of great clinical and epidemiological importance in Brazil. Their significant degree of symptomatic similarity makes clinical diagnosis difficult.
Objective: To diagnose leptospirosis differentially in patients with clinically suspected dengue.
Materials and methods: In this study, 86 patients with clinically suspected dengue underwent virological and serological diagnostic evaluations for dengue (reverse transcriptase polymerase chain reaction, NS1 immunochromatographic test, and NS1 enzyme-linked immunosorbent assay, ELISA), as well as tests to detect immunoglobulin M (IgM; IgM/IgG Rapid Test and IgM ELISA). The same patients were subsequently evaluated for leptospirosis using Rapid Test IgG/IgM (Bioeasy®) and Leptospira IgM ELISA (PanBio®).
Results: Of the 86 patients, 48 (55.8%) had positive results for dengue in at least one of the tests and five (7.35%) showed positive reactions for leptospirosis.
Conclusion: During dengue epidemics, this disease may be misdiagnosed as other infections, including leptospirosis, when diagnosis is based on nonspecific clinical and laboratory criteria alone.

Key words: Leptospirosis; dengue; differential diagnosis; reverse transcriptase polymerase chain reaction; enzyme-linked immunosorbent assay.

doi: http://dx.doi.org/10.7705/biomedica.v35i4.2504

Evidencia serológica de leptospirosis en pacientes con sospecha clínica de dengue en el estado de Ceará, Brasil

Introducción. El dengue y la leptospirosis son dos enfermedades febriles de gran importancia clínica y epidemiológica en Brasil, y presentan una similitud sintomatológica significativa, lo cual dificulta el diagnóstico clínico.
Objetivos. Hacer el diagnóstico diferencial para leptospirosis en pacientes con sospecha clínica de dengue.
Materiales y métodos. En este estudio, 86 pacientes con sospecha clínica de dengue fueron sometidos a examen para el diagnóstico de dengue mediante pruebas de virología y serología (RT-PCR, prueba de inmunocromatografía NS1 y ELISA-NS1) y pruebas para la detección de IgM (prueba rápida IgM/IgG y ELISA-IgM). En los mismos pacientes también se evaluó la presencia de leptospirosis mediante la prueba rápida IgG/IgM (Bioeasy®) y ELISA IgM-Leptospira (PanBio®).
Resultados. De los 86 pacientes, 48 (55,8 %) fueron positivos para dengue en, por lo menos, una de las pruebas y 5 (7,35 %) presentaron reacciones positivas para leptospirosis.
Conclusión. Durante las epidemias de dengue, otras infecciones y entre ellas la leptospirosis, pueden dar lugar a confusiones cuando se el diagnóstico se hace únicamente con base en criterios inespecíficos clínicos y de laboratorio.

Palabras clave: leptospirosis, dengue, diagnóstico diferencial, reacción en cadena de la polimerasa con transcriptasa inversa, ensayo de inmunoadsorción enzimática.

doi: http://dx.doi.org/10.7705/biomedica.v35i4.2504

Author's contributions:
Laiane Fernanda de Melo Bezerra, Raissa Matos Fontes and Danielle Malta Lima wrote most of the manuscript and were responsible for data collection, analysis and interpretation.
Danielle Malta Lima was also involved in conception and design of the study, as well as fieldwork.
Almira Maria Monteiro Gomes participated in data collection.
Dyana Alves da Silva participated in the realization of diagnostic tests.
Jeová Keny Baima Colares participated in the interpretation of results and writing of the manuscript.

Dengue fever is the most prevalent arboviral disease; it has a widespread global distribution and occurs in both tropical and subtropical areas. This disease most often occurs during the rainy season and may cause various clinical manifestations that range from asymptomatic infections to more severe conditions. The initial phase of dengue is generally characterized by an acute febrile syndrome (fever, adynamia, headache, retro-orbital pain, myalgia and arthralgia), without symptoms or focal signs (1-3).

Brazil has a high number of dengue fever cases and has already experienced several epidemics. The state of Ceará, located in the northeastern region, reports thousands of cases of the disease each year, the largest epidemics having occurred in 1994, 2008, 2011 and 2012 (4,5). Dengue has a wide clinical spectrum and, in some cases, it may be difficult to differentiate it from other infections based solely on clinical-epidemiological criteria. Specific laboratory techniques are therefore needed for diagnosis. In dengue-endemic regions, it is essential to perform a differential diagnosis to differentiate it from other febrile syndromes such as leptospirosis (1,6).

Leptospirosis is an infectious febrile disease caused by pathogenic spirochetes of the genus *Leptospira.* Infections may be asymptomatic, mild or severe and acute or chronic (7-9). Notification of cases of leptospirosis has been mandatory in Ceará since 1995 (10). Although this disease is one of the most widespread global zoonoses, reliable data on its incidence and prevalence in humans are scarce, mainly because it is difficult to diagnose. Although the microagglutination test (MAT) is the laboratory test of choice and is considered to be the standard test for leptospirosis, it is time-consuming and requires expensive equipment, specialized training and use of live *Leptospira* spp. cultures (11).

Due to the high number of cases of dengue in particular and the similarity of its symptoms to those of leptospirosis, some studies have shown that diagnostic confusion between these diseases may occur in routine clinical practice in many areas, including Ceará (12). A method for differential diagnosis of these infections is needed since the two pathologies require different, specific treatments. The objective of the present study was, therefore, to perform differential diagnosis of leptospirosis in patients with clinical suspicion of dengue.

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Recibido: 23/08/14; aceptado: 14/05/15

Materials and methods

We conducted a prospective observational study between February and December, 2010. The source population consisted of patients aged at least 18 years who had sought treatment in the emergency ward of a local hospital. The inclusion criteria for the study were: Presence of up to five days of fever with no obvious focal source associated with two of the following symptoms: adynamia, headache, retro-orbital pain, arthralgia, myalgia or exanthema. A suspected dengue case is defined by the Brazilian Ministry of Health as follows: Acute febrile illness accompanied by at least two of the following symptoms: headache, retro-orbital pain, myalgia, arthralgia, prostration and/or rash.

Each of the patients selected underwent an initial clinical evaluation and blood samples were collected for serological and virological tests. The blood samples were collected in two steps; the first sample was obtained during the first five days of fever for use in a specific dengue test (virological test; One Step RT-PCR, Qiagen®, USA), dengue virus NS1 glycoprotein detection (Dengue NS1 Ag strip rapid test, Bio-Rad®, France) and enzyme-linked immunosorbent assay (ELISA) NS1 (Bioeasy®, Brazil). Genetic material was extracted according to the manufacturer's instructions (Qiagen®) and genomic amplification performed using the primers described by Lanciotti, *et al.* (1992) (13). The RT-PCR was carried out using a Mastercycler® personal thermocycler (Eppendorf ®). To visualize the amplified fragments, 10 µl of reaction product, 3 µl of running buffer and 2 µl of Gel Red™ dye were loaded onto an agarose gel (Sigma, St. Louis, USA) and subjected to horizontal electrophoresis (60 V/h), with gels being visualized under UV illumination.

The second blood sample was collected when the patient returned to the hospital during the recovery phase, defined as the period following the fifth day of symptoms, to undergo serological tests (Dengue duo test; Bioeasy®), a dengue ELISA immunoglobulin M assay (IgM; Bioeasy®), anti-*Leptospira* (IgG/IgM rapid test for leptospirosis; Bioeasy®) and *Leptospira* IgM ELISA (PanBio®, Australia).

All patients were initially subjected to tests to identify dengue infections. Given the high prevalence of this infection in the region, several techniques were employed for virological and serological detection. After being tested for dengue, samples were also analyzed for leptospirosis. Epidemiological, clinical, and nonspecific laboratory data were collected from all patients in the study and entered onto a standardized datasheet. Patients were defined as dengue cases based on clinical, virological and serological criteria; thus, all cases positive for reverse transcriptase polymerase chain reaction (RT-PCR) and/or IgM and/or NS1 expression were considered to have dengue. The present study had a descriptive cross-sectional design and was approved by the Research Ethics Committee of the São José Hospital of Infectious Diseases, Fortaleza (protocol No. 064/2009).

Results

Of the 86 patients evaluated with clinical suspicion of dengue, 48 (55.8%) were positively confirmed as having dengue in at least one of the laboratory tests performed. Among these patients, 23 (47.9%) were positive for dengue by the ELISA-NS1, 14 (29.1%) by the NS1 rapid test (Dengue NS1 Ag Strip), 16 (33.3%) by RT-PCR, 38 (79.1%) by the ELISA-IgM and 36 (75%) by the IgM rapid test (Dengue Duo Test). Five patients (5.8%) with a clinical suspicion of dengue had positive results in the leptospirosis test and all showed positive results for ELISA-IgM showing titers ≥1:100, whereas only one patient (1.16%) was diagnosed with dengue through the rapid test. Thirty-five patients (40.6%) remained without a specific laboratory diagnosis and were classified as having undifferentiated febrile syndrome.

The main clinical findings among the patients with leptospirosis were fever (5/5; 100%), prostration (5/5; 100%), headache (4/5; 80%), myalgia (4/5; 80%), arthralgia (3/5; 60%), retro-orbital pain (2/5; 40%) and exanthema (1/5; 20%). In addition to these common symptoms of dengue, the patients also reported anorexia (5/5; 100%), nausea (3/5, 60%), vomiting (2/5, 40%), diarrhea (2/5, 40%) and coughing (2/5, 40%). The following abnormalities were found among the nonspecific laboratory test findings: low hemoglobin (<11.6 g/dl) and hematocrit levels (<36%) in three patients (60%), thrombocytopenia (<150,000/mm^3) in two patients (40%), lymphopenia (<20%) in three patients (60%), elevated aspartate aminotransferase (>40 U/L) and alanine aminotransferase levels (>40 U/L) in three patients (60%) and mild leukocytosis (>11,000/mm^3) in one patient (20%) (table 1).

Among the five patients positive for leptospirosis, two showed specific laboratory diagnoses for both this and dengue (table 2). The two patients with positive results for both infections were female outpatients with fever, myalgia, prostration and arthralgia. We used a numerical code to preserve anonymity. Patient one had no abnormalities in the nonspecific tests, whereas patient three showed low hemoglobin (<11.6 g/dl) and hematocrit levels (<36%).

Discussion

The clinical features of leptospirosis can be confused with those of dengue, especially during epidemic periods of the latter, when diagnosis can only be confirmed by clinical and epidemiological criteria (6,14). Brown, *et al.*, assessed 590 patients suspected of dengue infection of whom 314 showed positive results for serological tests for dengue and 27 were positive for leptospirosis (14). In this study, five (5.8%) of the 86 patients with clinical suspicion of dengue were positive for leptospirosis. Although the number of patients with leptospirosis was small, our results corroborate those of Brown, *et al.*'s study. The leptospirosis-positive results of the five patients with initial suspicion of dengue suggested that, in the state of Ceará, confusion in clinical diagnosis might be occurring due to the similarity

Table 1. Nonspecific laboratory test results of the patients with leptospirosis

Nonspecific laboratory tests	Case 1	Case 2	Case 3	Case 4	Case 5
Hemoglobin (11.5-16.4 g/dl)	12.8	10.9*	9.6*	12.3	9.2*
Hematocrit (36.0-48.0%)	38.4	32.3*	29.4*	36.7	31.5*
Platelets (150,000-450,000/mm^3)	215,000	76,000*	201,000	196,000	66,000*
Leukocytes (3,600-11,000/mm^3)	3,630	7,160	6,760	11,250*	9,900
Lymphocytes (20-50%)	27	11*	32	8*	30
AST (<40 U/L)	14	100*	20	129*	154*
ALT (<40 U/L)	14	81*	15	57*	84*

AST: Aspartate amino-transaminase; ALT: Alanine amino-transaminase
*: Altered results

Table 2. Patients positive for dengue and leptospirosis concomitantly

Specific diagnosis			
Case	**RT-PCR Dengue**	**ELISA-IgM Dengue**	**ELISA-IgM Leptospirosis**
1	Positive	Negative	Positive
3	Negative	Positive	Positive

ELISA: Enzyme-linked immunosorbent assay
RT-PCR: Reverse transcriptase-polymerase chain reaction
IgM: Immunoglobulin M

of the symptoms of these two diseases. Our data support the findings of Oliveira, *et al.*, who reported cases of leptospirosis during a dengue epidemic in Ceará. In the present study, although no other diagnostic tests for leptospirosis were performed, we considered patients who were positive for the ELISA-IGM to be probable cases of leptospirosis. We wish to emphasize that it is not possible to categorically state that this result confirms an active infection. Unfortunately, additional collections were not carried out to evaluate seroconversion and other diagnostic tests were not performed because standardized PCR and MAT execution were lacking, representing a limitation of our study. However, published articles suggest that patients can test positive for leptospirosis using only IgM detection (12,14).

The tests most commonly used for the laboratory diagnosis of leptospirosis in Brazil are ELISA-IgM and MAT (Brazil, 2010) (15). Recently, the use of ELISA tests has increased because of the various difficulties in conducting the MAT. In addition, immunoenzymatic assays can detect IgM at an early stage and have high degrees of sensitivity and specificity. New tests are also being developed, such as immunochromatographic assays, which have provided good results for IgM detection (16-18).

IgM antibodies are present from the third day of leptospirosis infection and may persist for up to five months (18). All of our samples were collected between the fifth and tenth days after onset of symptoms and were thus within the period during which IgM antibodies are present, allowing both tests to be used for detection. The ELISA showed higher positivity than the immunochromatographic tests, detecting anti-*Leptospira* antibodies in five patients. Sehgal, *et al*., reported that the immunochromatographic test showed a positivity rate similar to that of the immunoenzymatic assay, in contrast to the results obtained in the present study (19). However, Blacksell, *et al*., indicated that the rapid test showed low positivity when compared with the ELISA, corroborating our data (19). Cohen, *et al*., suggested that immunochromatographic tests showed good positivity only when conducted during the convalescence phase. These authors also recommended re-testing patients with continued symptoms if the first test result was negative (20).

The symptomatology of patients positive for leptospirosis corroborates the findings of Daher, *et al.*, although jaundice was not observed in the present study. These authors reported that patients with leptospirosis presented fever (96.5%), headache (74.6%), myalgia (92.5%), jaundice (94.5%), vomiting (71.6%), dehydration (63.5%) and chills (62.2%) (21). It is worth emphasizing that of the five patients who were positive for leptospirosis, three were hospitalized, where they probably developed some of the most severe forms of leptospirosis. The most frequent laboratory abnormalities in the patients with leptospirosis were thrombocytopenia, leukocytosis and transaminase elevation, as well as low hematocrit and hemoglobin levels (21). These abnormalities were also observed in the present study.

The simultaneous occurrence of two or more infections is not rare and presents challenging clinical features. The therapeutic options and prognoses of patients can be similarly complex (22). Some studies have shown that co-infection with dengue and leptospirosis is possible, which re-emphasizes the need for specific diagnostics for both diseases (23-25). In our study, patients 1 and 3 tested positive for both dengue and leptospirosis. However, detection of one of the etiologies using a specific test was only observed in patient 1 by confirming the presence of the dengue antigen through RT-PCR, although anti-dengue antibodies were not detected. Thus, because both patients were positive for serological tests alone and these antibodies may remain in circulation for a prolonged period, we could not differentiate between potential co-infections and sequential infections. The IgM antibodies for dengue and leptospirosis generally remain in circulation for prolonged periods of three or five months respectively, hindering the classification of disease as recent or current (26). However, Behera, *et al.,* considered a patient who was positive for the ELISA to be a case of co-infection, showing anti-dengue and anti-*Leptospira* antibodies (27). Thirty-five patients remained without a specific laboratory diagnosis, suggesting that they had been infected by other

infectious agents causing clinical features similar to those of dengue; they may also have been infected by the agents studied here, although these were not detectable with the techniques used. Several studies have reported diagnostic confusion between dengue and other pathologies such as hantavirus, rubella, hepatitis, influenza A virus, and melioidosis (28-30).

In this study, we identified patients with a clinical suspicion of dengue who were positive for leptospirosis, demonstrating that diagnostic confusion may occur between these diseases and underlining the need for specific laboratory techniques for the diagnosis of these pathologies. We recognize, however, that this study presents some limitations, such as the absence of paired samples. Anti-*Leptospira* IgM antibodies can be detected for months after initial contact with the antigen and we therefore cannot confirm that the patients had active infections. Nevertheless, this study provides a strong indication that cases of leptospirosis are likely to be confused with dengue infections.

The results reported here demonstrate the need for differential diagnosis of dengue, in particular in regions that report dengue epidemics; due to similarity of symptoms with other pathologies, cases of other diseases may be underreported. Thus, it is essential to perform additional studies on more efficacious tests for the laboratory diagnosis of both infections. Accurate and early diagnosis is critical in clinical practice, as the appropriate treatments for patients with dengue and leptospirosis are quite different. In addition, epidemiological surveillance and control measures can only be performed correctly when the real infectious agent is known. More efficient diagnostic tests for these infections are therefore needed.

Acknowledgments

We would like to thank the *Laboratório de Biologia Molecular e do Desenvolvimento, Universidade de Fortaleza* (UNIFOR), as well as the physicians and other staff members of the *Hospital Maternidade Nossa Senhora da Conceição*, *Hospital São José de Doenças Infecciosas* (Fortaleza), and the *Setor de Parasitologia do Departamento de Patologia e Medicina Legal* (UFC), and the *Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico/ Centro Nacional de Desenvolvimento Científico e Tecnológico* (FUNCAP/CNPq) for financial support.

Conflicts of interest

All the authors declare that they have no conflicts of interest in the present study.

Financial support

We received support from FUNCAP/CNPQ (PPP-No. 011-00139.01.00/09). Grant holder: Laiane Fernanda de Melo Bezerra (FUNCAP-BMD-0008-0123.01.07/10)

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