REPORT

Evidences of autochthonous infection by Borrelia burgdorferi *sensu lato in Cuba*

✍ Islay Rodríguez[1], Carmen Fernández[1], Omar Fuentes[1], Roberto A González[2], Bárbara Martínez[3], Eduardo Echevarría[1], Angel A Noda[1], Ana M Obregón[1]

[1]Instituto de Medicina Tropical Pedro Kourí, IPK
Autopista Novia del Mediodía, Km 6½, La Lisa, CP 11 400, La Habana, Cuba
[2] Unidad de Control de Vectores
[3] Centro de Asistencia Médica de Sierra del Rosario, Artemisa, Cuba
E-mail: islay@ipk.sld.cu

ABSTRACT

Borrelia burgdorferi sensu lato is the causative agent of Lyme disease, borreliosis not reported in Cuba but clinical and epidemiological suspicions exist since the 1980's, and there were no microbiological testing for confirmation. This led to perform a series of researches to provide scientific evidences about the presence of this agent in the country, including the evaluation and implementation of microbiological methods for the detection of this spirochete, confirmation of infection in clinical samples from patients with clinical and epidemiological suspicions, the estimated seroprevalence of antibodies against this agent in a population at risk and the molecular detection of borrelia in ticks of veterinary and medical importance. The microbiological tools evaluated (culture medium modified, methods of extracting genetic material from ticks for subsequent detection of *B. burgdorferi* sensu lato by molecular testing, and analytical sensitivity of two sets of primers) and implemented (specific serological tests) strengthened the IPK analytical capacity with novel methods for future researches. We found specific serological evidences of autochthonous infection by this organism in samples of individuals with clinical and epidemiological suspicion, and those exposed to tick bites. Genetic material of *B. burgdorferi* sensu lato was undetected in the analyzed ticks, what does not rule them out as potential carriers of the agent. In this paper we show evidence highly suggestive of infection with *B. burgdorferi* sensu lato and it is the first of its kind in the country.

Keywords: *Borrelia burgdorferi*, *Borrelia*, infection, tick, Cuba

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RESUMEN

Evidencias de infección autóctona por *Borrelia burgdorferi* sensu lato en Cuba. *Borrelia burgdorferi* sensu lato causa la enfermedad de Lyme, una borreliosis no reportada en Cuba. Desde la década de 1980 se tenían sospechas clínicas y epidemiológicas, pero no había pruebas microbiológicas que lo confirmaran. Las investigaciones científicas sobre su presencia en el país incluyeron la evaluación e implementación de métodos microbiológicos para su detección, la confirmación de la infección en muestras clínicas de pacientes con sospechas clínico-epidemiológicas de esta enfermedad, la estimación de la seroprevalencia de anticuerpos contra este agente en una población de riesgo y la detección molecular de borrelias en garrapatas de importancia médico-veterinaria. Las herramientas microbiológicas evaluadas (medio de cultivo modificado, métodos de extracción de material genético de garrapatas para la posterior detección de *B. burgdorferi* sensu lato por pruebas moleculares, y la sensibilidad analítica de dos juegos de cebadores) e implementadas (pruebas serológicas específicas) fortalecieron la capacidad analítica del Instituto de Medicina Tropical Pedro Kourí, en Cuba, con nuevos métodos para futuras investigaciones. En las muestras de individuos con sospechas clínico-epidemiológicas y en los expuestos a mordeduras por garrapatas, se encontraron evidencias serológicas específicas de la infección autóctona por este microorganismo. No se detectó material genético de *B. burgdorferi* sensu lato en las garrapatas analizadas, lo que no descarta que sean posibles vectores del agente. Las evidencias de este estudio son altamente sugestivas y constituye el primero de su tipo en Cuba.

Palabras clave: *Borrelia burgdorferi*, *Borrelia*, infección, garrapata, Cuba

*I*ntroduction

Borrelia burgdorferi sensu lato is a complex of spirochetes [1], which cause Lyme disease, zoonosis for which small and large mammals are the main reservoirs of *Borrelia*, which is spread mainly by hard ticks of the genus *Ixodes* [2]. By the late twentieth century, this entity was recognized as an important emerging disease by severe consequences to human health and the difficulties for its prevention and control [3]. This disease has only been described in the northern hemisphere and is the most common vector-borne illness in the United States of America and Eurasia regions [2].

In Cuba, since the 1980's, it is suspected the occurrence of infection with *B. burgdorferi* sensu lato in a community located in Sierra del Rosario, Candelaria, Artemisa province. The tick infestation in this place is high, mainly due to *Amblyomma cajennense*, and among the human population are reported historically idiopathic syndromes with a clinical and epidemiological background that mimics Lyme disease, but could not be confirmed by absence of laboratory diagnosis [4].

The first evidence of a probable case of infection with *B. burgdorferi* sensu lato also dates from the 1980s, an individual who was diagnosed retrospectively by a screening test as neuroborreliosis in a foreign country [5].

✍ Corresponding author

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This serological finding in a Cuban individual reinforced the suspicion of the existence of infection with *B. burgdorferi* sensu lato in Cuba; so that it became necessary to initiate microbiological investigations to provide scientific evidence about it.

For that, it was combined a group of studies with different epidemiological designs. Three experimental studies were conducted to evaluate microbiological methods of detection of *B. burgdorferi* sensu lato: a) evaluation of a modified culture medium for multiplication of *B. burgdorferi* sensu lato, b) evaluation of methods for extracting genetic material from ticks, for later use in the detection of this agent by PCR, and c) evaluation of the analytical sensitivity of PCR for detection of this spirochete. Two case-series studies were conducted using clinical samples of individuals with clinical and epidemiologic suspicion, divided into two periods (1998-2002 and 2003-2010) for confirmation of infection with *B. burgdorferi* sensu lato in humans. A cross-sectional observational study was performed from January to May 2006 to estimate the seroprevalence of antibodies against *B. burgdorferi* sensu stricto in a population at risk. In addition, two observational studies were conducted in different reservoirs: a) ticks collected from cattle and horses, and b) ticks collected from cattle, dogs and a house for the detection of *Borrelia* in ticks.

Results and discussion

Microbiological evaluation of methods for the detection of *B. burgdorferi* sensu lato

Evaluation of a modified culture medium for B. burgdorferi *sensu lato*

The modified medium was prepared according to the composition of BSK-II (commercial name: BSK-H) [6] replacing the medium CMRL 1066 by the medium 199 for not having it.

Strains NE4546 and NE83 of *B. garinii*, NE4502 of *B. burgdorferi* sensu stricto, NE4505 of *B. afzelii* and NE4555 of *B. valaisiana* were used to conduct a qualitative and quantitative evaluation of it. The commercial medium was used as control.

In the qualitative evaluation, we observed a color change from pink to orange in both media due to a decrease of pH as a consequence of the onset and increased the concentration of the final products of fermentation in media. Using the dark field microscopy, uniformly refractile and mobile spirochetes in both media were observed.

The quantitative evaluation yielded the growth of *B. burgdorferi* sensu stricto and *B. garinii* strains in both media. The multiplication of *B. afzelii* strain in the modified medium was not possible, only some borrelies with reduced motility were observed. It is reported that different strains of *Borrelia* species grow unevenly [7].

In addition, the analysis of the protein profile of the strain NE83 of *B. garinii* grown in these media and their antigenic properties of proteins was performed, which revealed the expression of the main proteins, both specific and nonspecific, and that a large number of these were antigenic, whatever the medium used. These results suggest that the modified medium evaluated can be used as an alternative to the cultivation of *Borrelia* strains in countries with few resources, taking into account its limitations for the growth of certain strains.

Evaluation of the efficacy of extraction methods for nucleic acids from hard ticks

From May to October 2007, the following protocols for DNA extraction were evaluated using the tick *Rhipicephalus (Boophilus) microplus*: the phenol-chloroform extraction proposed by Weng *et al*. [8], Halos *et al*. [9] and Fraga *et al*. [10] with modifications; the potassium acetate method reported by Gaillard and Strauss [11] with some modifications and the protocol of extraction using ammonium hydroxide and heat modified by Humair *et al*. [12].

Genomic DNA was only observed in extracts of adult female ticks obtained by Halos *et al*. [9] and Fraga *et al*. [10] procedures. RNA was confirmed in all of them, regardless of life stage and gender. PCR amplification of a gene fragment coding for the 16S subunit of mitochondrial RNA ticks allowed the identification of the DNA fragment in all cases except for the extracts obtained by the method of ammonium hydroxide and heat [10] in engorged female ticks. These results demonstrated the effectiveness of each procedure. This work constitutes the first report worldwide of the nucleic acid extraction using the potassium acetate method in ticks.

Evaluation of the analytical sensitivity of PCR to amplify DNA fragments of *B. burgdorferi* sensu lato

The evaluation by PCR of the 5S-23S intergenic region [13] and 16S subunit sequence [14] of *B. burgdorferi* sensu lato rRNA was done with reference DNA from *B. burgdorferi* sensu stricto (B31), *B. afzelii* (NE632), *B. garinii* (NE110 and *B. lusitaniae* (Poti-B1). The expected fragments for each of them were amplified. PCR of the 5S-23S intergenic region was able to detect 0.21 pg of DNA (84 copies of the genome), while PCR of the 16S sequence detected only 2.1 ng (8400 copies of the genome). Based on these results, it is proposed the PCR of 5S-23S for the detection of *B. burgdorferi* sensu lato.

Technology transfer of serological tests

The commercial system ELISA-C6 (IgM/IgG) (Immunetics, USA) and a Western blot-IgG (home-made) were established at the National Microbiology Laboratory of the Institute of Tropical Medicine Pedro Kourí (IPK) as screening and confirmatory tests, respectively.

Microbiological diagnosis of infection with *B. burgdorferi* sensu lato in clinical samples of individuals with clinical and epidemiological suspicion

Case-series study 1

During the period 1998-2002, 14 serum samples were studied, from an equal number of individuals with clinical and epidemiological suspicion of Lyme disease from a community of Sierra del Rosario, Artemisa. It was used Enzignost Borreliosis ELISA IgM/IgG (Behring, Margurg, Germany) as screening method and Western blot IgM and IgG as confirmatory test (University of Trieste, Italy).

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Five of the samples had positive values of IgM antibodies and one, limit values of both IgM and IgG antibodies by ELISA. Two of the six samples were positive to IgM by Western blot, and one of them showed a low titer of IgM antibodies to leptospirosis, which reaffirms the antigenic similarity between *Leptospira* spp. and the causative agent of infection in this patient.

One positive case was retrospectively investigated; being a male patient aged 16 with no previous history of travel outside the country, being suspected for Lyme disease from the age of five due to clinical manifestations and tick bite history.

Case-series study 2

It was conducted during 2003-2010 in collaboration with the Laboratory of Microbiology, ADMed, La-Chaux-de-Fonds, Switzerland. We studied 27 serum, 11 cerebrospinal fluids, one peripheral blood and one plasma samples from 30 individuals with clinical and epidemiological suspicions of this borreliosis from different provinces of Cuba. As serological screening tests were used the IgM/IgG ELISA VIDAS (BioMérieux, France), IgM-capture ELISA (IDEIA™ Borrelia IgM; Dako Cytomation, USA) and IgM/IgG ELISA-C6 (C6 Lyme ELISA™) (Immunetics, USA). As confirmatory tests were used in-house IgM (*B. garinii* (NE83) as antigen) and IgG (B. *burgdorferi* sensu stricto (B31) and *B. garinii* (NE83) as antigens) Western blot procedures, and also the Western blot-IgG (Bioline Diagnostic, Italy). The blood sample was studied by culture in BSK-H and BSK modified medium and by PCR 5S-23S.

Specific IgM antibodies were detected in 10% (3/30) of the individuals, confirming the presence of infection in early stages, while in 6.6% (2/30) of individuals the detection of specific IgG confirmed the presence of infection in disseminated or advanced stage. The positive samples were subjected to serological tests for leptospirosis and syphilis, being negative.

These results corroborated serological findings reported in the previous series, and confirmed the presence of this infection in Cuba based on serological evidence. The lack of previous history of travels abroad demonstrated the autochthonous nature of infection cases.

Prevalence of antibodies to *B. burgdorferi* sensu stricto in individuals from a population exposed to tick bites

This study was conducted from January to May 2006 in a community of Sierra del Rosario. The population at risk comprised 980 individuals, a representative sample of 247 of them selected through simple random sampling. Serum was collected for each individual and performed by Western blot-IgG analysis, (Ag: B31 strain of *B. burgdorferi* sensu stricto). For the interpretation, we used three different criteria: a) Criteria from the Microbiology Laboratory, ADMed of La-Chaux-de-Fonds, Switzerland: scoring system in accordance to band's specificity, b) Criteria of the Centers for Disease Control and Prevention (CDC, USA) [15] and c) Criteria of the CDC with the inclusion of OspA (31 kDa) and OspB (34 kDa) proteins [16].

The table below shows results on the prevalence of antibodies using different interpretation criteria. There is an overlap among the confidence intervals, so it was estimated a seroprevalence of 0.6-7.2% of specific antibodies against *B. burgdorferi* sensu stricto as a result of getting together the three intervals. In this study, there were no cross-reactivity with antibodies against *Leptospira* sp. and *Treponema* sp., and there are no other reports of *Borrelia* in the country.

The bands corresponding to 72, 60, 47 and 41 kDa proteins were revealed in more than 50% of the samples, although of weak intensity. In the samples identified as positive it was observed that 90/93, 72, 65/66, 60, 58, 56, 47 and 41 kDa proteins were significantly revealed and they corresponded to both highly specific and non-specific *B. burgdorferi* sensu lato proteins. Other revealed highly specific proteins were the 30 and 22/24 (OspC) kDa proteins, but few sera reacted with them.

Specific serological evidences of infection with *B. burgdorferi* sensu lato in samples of individuals from different provinces (Artemisa, Havana, Sancti Spíritus and Holguín) found in previous case-series studies and in this one latter suggest the circulation of the bacteria in the western, central and eastern regions of Cuba.

Molecular detection of *B. burgdorferi* sensu lato in ticks of medial and veterinary relevance

There were two observational studies, in which ticks were collected from different reservoirs (horses, cattle, dogs and a house) in the provinces of Artemisa and Havana by purposive sampling. The first was held in collaboration with the Laboratory of Ecoepidemiology of Parasites, Institute of Biology, University of Neuchâtel, Switzerland.

Taxonomic identification of ticks collected for the observational study 1 identified three species. In horses, 60% (57/95) of ticks were identified as *Dermacentor (Anocentor) nitens*, 37.9% (36/95) as *A. cajennense* and 2.1% (5/95) as *R. microplus*. This last specie corresponded the 100% (3/3) of the ticks captured in cattle. The observational study 2 showed that cattle were infected by *R. microplus* and dogs by *R. sanguineus* (100% in both cases). Ticks collected in the house were classified as *R. sanguineus*. Tick species identified in these studies did not correspond to known vectors for Lyme disease.

Genetic material of *B. burgdorferi* sensu lato was not detected in the extracts of any of them, by PCR amplification and reverse-line hybridization (study 1) [17] and PCR 5S-23S (study 2) [13].

Given that the number of ticks tested in this study is limited and not representative of the country, it is necessary to collect more ticks from pets, wild animals, migratory birds and vegetation in the area of

Table. Prevalence of *B. burgdorferi* sensu stricto antibodies in a population at risk

Interpretation criteria	Prevalence (%)	95% confidence interval
Microbiology Laboratory, ADMed, La-Chaux-de-Fonds, Switzerland	2.02	0.6-4.7
Centers for Disease Control and Prevention (CDC), USA	2.02	0.6-4.7
CDC with inclusion of OspA and OspB proteins	4.45	1.7-7.2

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Sierra del Rosario and to extend the research to other regions.

Relevance of the study

In this study, a modified culture medium was suggested as an alternative for the growth of *B. burgdorferi* sensu lato and a new internationally recognized methodology for the extraction of nucleic acids in ticks.

For the first time in Cuba, the microbiological tools required for the microbiological (bacteriological, serological and molecular) detection of infections with *B. burgdorferi* sensu lato have been implemented for human clinical specimens and the possible vectors, at the National Reference Laboratory of Microbiology of the IPK.

The first specific serological evidences of infection with *B. burgdorferi* sensu lato in individuals with clinical or epidemiological suspicion of Lyme disease in Cuba have been also shown. This investigation also demonstrated that the infection with *B. burgdorferi* sensu lato is not a local situation (western Cuba), having a wider geographical distribution.

The body of evidences about *B. burgdorferi* sensu lato infection suggests its autochthonous transmission in Cuba, which is an epidemiological alert for national authorities of veterinary and public health.

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