

Coexistence of Antibodies to Tick-borne Agents of Babesiosis and Lyme Borreliosis in Patients from Cotia County, State of São Paulo, Brazil

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This paper reports a case of coinfection caused by pathogens of Lyme disease and babesiosis in brothers. This was the first case of borreliosis in Brazil, acquired in Cotia County, State of São Paulo, Brazil. Both children had tick bite history, presented erythema migrans, fever, arthralgia, myalgia, and developed positive serology (ELISA and Western-blotting) directed to Borrelia burgdorferi G 39/40 and Babesia bovis antigens, mainly of IgM class antibodies, suggestive of acute disease. Also, high frequencies of antibodies to B. bovis was observed in a group of 59 Brazilian patients with Lyme borreliosis (25.4%), when compared with that obtained in a normal control group (10.2%) (chi-square = 5.6; p < 0.05). Interestingly, both children presented the highest titers for IgM antibodies directed to both infective diseases, among all patients with Lyme borreliosis.

Key words: lyme borreliosis - lyme disease - spirochetosis - borreliosis - babesiosis - coinfection - tick-borne disease - Brazil

Babesiosis is a tick-borne disease distributed worldwide, caused by hemoprotozoans of the genus *Babesia*, which infects wild and domestic animals, promoting economic losses due to mortality, weight loss in cattle population, besides the costs involved in profilatic measures and treatment.

More than 70 species of babesias were described in animals: *Babesia bovis*, *Babesia divergens* and *Babesia bigemina* in cattle; *Babesia equi* and *Babesia caballi* in horses; *Babesia canis* in dogs; *Babesia microti* in rodents. Recently a new species very similar to *B. microti* named *Babesia WA-1* was described in USA (Quick et al. 1993).

The parasite was first discovered by Babes (1888), that reported intra-erythrocytic microorganisms in peripheral blood of cattle suffering of fever and hemoglobinuria. Smith and Kilbourne (1983) identified the tick as transmitter of disease, and this description was the first case of protozoan transmission by arthropods.

The first case of human babesiosis was discovered in Yugoslavia (Skrabalo & Deanovic 1957) in a 33 years old farmer, splenectomized, who died eight days later with fever, anemia, jaundice and hemoglobinuria, and *B. bovis* was identified in the blood and cattle. The beginning of knowledge on human babesiosis was dramatic, since three of four cases occurred in splenectomized patients followed by death.

The first case of babesiosis in a healthy person, with intact spleen, was reported in 1969 in a woman from Nantucket Island (Massachusetts, USA) (Wester et al. 1970), hospitalized with fever, headache and abdominal pain. Blood smear analysis revealed numerous ring-like structures suggesting *Plasmodium falciparum* infection, and the patient was treated with cloroquine. During the course of the disease, diagnosis of babesiosis was established, the treatment with cloroquine was introduced, and longed for two months. Blood from patient was inoculated in splenectomized rodent and monkey, with recovery of the etiological agent *B. microti*.

The only case reported in Brazil (Alecrim et al. 1983) was described from a patient from Tiama, State of Pernambuco, who was treated as having malaria, until the discovery of parasites identified as *Babesia* sp.

Ixodid ticks (Healey et al. 1976, Piesman & Piesman 1980) are responsible vectors for protozoan transmission, and belong to the genus *Ixodes* (*B. microti*, *B. divergens*), *Boophilus* (*B. bovis*), *Dermacentor* (*B. equi*), *Rhipicephalus* (*B. equi*, *B. canis*).

Humans are infected accidentally by tick bite or blood transfusion. The microorganisms invade the erythrocytes and present binary division, and differently from malaria, the parasite did not have sexual stage.

Human babesiosis or piroplasmosis is a zoonosis associated with protozoa of the family Piroplasmorida including *B. microti* and recently described babesia like piroplasm named *Babesia Wa-1* in the USA, and *B. divergens* in Europe. A few cases in humans have been described in China, Egypt, Mexico, South Africa and Taiwan (Gorenflot et al. 1998b).

The North American clinical manifestations of *B. microti* infection present incubation period that varies from one to four weeks, and first clinical manifestations

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include presence of fever, chills, myalgia, fatigue. Symptoms may remain for weeks until the correct diagnosis is done, sometimes with spleen enlargement and anemia. The disease may be asymptomatic or course as prolonged severe illness (Telford III et al. 1993). The clinical spectrum associated with *B. Wa-1* ranges from asymptomatic infection or influenza-like illness to fulminant fatal disease (Persing et al. 1995).

Between 1982 and 1993, 139 patients were hospitalized with babesiosis in the State of New York (White et al. 1998), nine patients died (6.5%), and the most common symptoms were fever, myalgia, headache, fatigue, malaise, weakness, hemolytic anemia, jaundice, hemoglobinuria. Among these patients, 12% had had previous history of Lyme disease, 12% had undergone splenectomy and 2% blood transfusion.

Osorno et al. (1976) performed an epidemiological survey at a risk area analysing 101 blood samples, and found antibodies to *Babesia* sp. in 38%, demonstrating that infection may be latent or asymptomatic.

The European presentation of infection in most of cases (76%) is caused by *B. divergens*, often it is a dramatic medical emergency, with lethality close to 50%, and it is clinically characterized by acute onset with appearance of high fever, chills, vomit, nausea, and important anemia, often followed by jaundice, hemolysis, hemoglobinuria, renal failure, pulmonary edema and death. In general, human cases were reported in splenectomized persons (Loutan 1995). Babesiosis must also be considered as differential diagnosis of febrile process in HIV positive patients in endemic areas (Falagas & Klempner 1996).

First case of human babesiosis in Poland (Humiczewska & Kuzna-Grygiel 1997) was diagnosed as imported from Brazil, and it was based on recovery of parasite after intraperitoneal inoculation of patient's blood in hamster, when characteristic forms of babesia were identified.

Diagnosis of human babesiosis includes history of visiting risk areas and development of clinical manifestations as fever, hemolytic anemia with normal leucogram, slight elevation of transaminases and alkaline fosfatase enzymes (Telford III et al. 1993).

Laboratorial confirmation includes analysis of peripheral blood smear stained by Giemsa or Wright methods, searching for protozoan of 1 to 3 μm diameter, located at erythrocyte cytoplasm, morphologically very similar with *Plasmodium* sp. parasites. Intravenous or intraperitoneal blood inoculation in hamster, gerbil or mice may be helpful to identify microorganisms, when parasitemia occurs after two to four weeks. Serologic assays (IF, ELISA) may reveal cross reactivity with malaria infection (Telford III et al. 1993). PCR is indicated when other methods had failed (Persing & Conrad 1995, Krause et al. 1998).

Treatment of human babesiosis is done using quinine 600 mg 3 times/day given orally, associated to clindamycin 600 mg 3 times/day intravenously during 10 days (Gorenflot et al. 1998a).

Lyme borreliosis has been diagnosed since 1992 in Brazil (Yoshinari et al. 1992a,b, 1993, 1997, Barros et al. 1993, Pirana et al. 1996, 1999, 2000), and nowadays it is considered an important differential diagnosis with interest to physicians of different specialities, because it pro-

duces systemic complications like cutaneous, rheumatic, neurologic and cardiac symptoms. The etiological agent is different in Brazil, and not isolated yet (Abel et al. 2000). The probable ticks transmitters of disease belong to the genus *Ixodes* and *Amblyomma*, the first implicated for keeping infection among wild animals, and the latter for promoting human disease (Barros-Battesti et al. 1995, 2000). Due to etiological, clinical and laboratorial differences when compared with American and European presentations, the infection has been named Lyme disease like illness in Brazil (Yoshinari et al. 1999, 2000).

The aim of the present paper is to report the presence of simultaneous infections caused by etiological agents of Lyme disease like illness and babesiosis in two brothers who acquired disease in Cotia county, State of São Paulo, in 1992. Interestingly, they were the first cases of borreliosis with cutaneous and systemic symptoms, with positive serology for *B. burgdorferi* G39/40 of American origin. Clinical suspicion was done by Dr Marcelo Mendonça when the boys were hospitalized at Instituto de Infectologia Emílio Ribas, and the laboratorial confirmation performed at Laboratório de Investigação em Reumatologia, Hospital das Clínicas, Faculdade de Medicina, Universidade de São Paulo (LIM-17). Serologic test for *B. bovis* was done only in 2000 at LIM-17, employing frozen sera kept at -70°C during eight years.

PATIENTS AND METHODS

Case 1 - EMDA, 10 years, male, white, resident in São Paulo city. The boy started with fever 19 days before hospitalization, followed by cutaneous lesions on elbows and knees, with latter dissemination on trunk and members, associated with headache, anorexia, myalgia, nausea, arthralgia of wrists. The skin lesions were multiple, expansive, and of different size, very suggestive of erythema migrans (EM).

The boy had visited an ownership of 125 hectares, named Transurb, located in Cotia, which is a region covered with Atlantic Forest, 20 days before the beginning of symptoms, and presented important epidemiological data like the history of contact with dog that died few days latter, and many episodes of tick bite, because parents were building a house in this ownership. Many species of wild animals were present in this geographic region.

The hemogram was normal, serum protein electrophoresis showed elevated alfa-2, beta and gamaglobulins with normal albumin. Sera biochemical results as transaminases, alkaline phosphatase, urea, creatinine, amylase and urinalysis were all normal. Antinuclear antibodies (ANA), rheumatoid factor, serology for spotted fever, leptospirosis, syphilis, mononucleosis, citomegalovirus, toxoplasmosis, mucoproteins, C-reactive proteins were negative.

Serologic tests (ELISA and Western blotting-WB) for Lyme disease were performed using American whole sonicated antigen of *B. burgdorferi* G39/40 strain. The first serum sample collected soon after hospitalization showed positive ELISA with IgM titer of 1/400, confirmed by WB that revealed several bands for IgM antibodies (Fig.1).

After laboratorial confirmation of Lyme borreliosis, treatment was introduced with 100 mg of doxycycline, twice a day, given for one month. Fever persisted for 14 days

after antibiotic introduction, and fatigue lasted for one month after the end of treatment.

Second blood sample collected 14 days after the beginning of treatment showed IgM titer of 1/100 (ELISA) (Fig. 1), and the last test done three months later revealed negative ELISA assay. However, few bands for IgG antibodies appeared at WB, revealing changes of immunoblotting pattern during the disease evolution.

Case 2 - BMDA, 12 years old, male, white, resident in São Paulo, brother of EMDA, and same epidemiological history.

The boy started with fever 30 days after tick bite history, appearance of skin lesions on members, and diarrhea 5 days after the beginning of fever. Physical examination showed cutaneous lesions suggestive of EM on elbows, arms and thighs.

Culture of feces was positive for *Salmonella* sp. Hemogram was normal, protein electrophoresis showed alpha 2 and gammaglobulin elevations, and others tests done to identify etiological agent were negative, except for Lyme disease serology. All the biochemical analysis done in the blood were normal.

ELISA and WB for *B. burgdorferi* were positive for IgM class antibodies in the beginning and 14 days after doxycycline introduction (Fig. 1). Serology repeated three months later was negative. The patient recovered completely after one month of antibiotic therapy.

Serology for *B. bovis*

Enzyme-linked immunosorbent assay (ELISA) - It was standardized at LIM-17 of Hospital das Clínicas, Universidade de São Paulo, employing *B. bovis* antigens offered by Dr CR Madruga from Embrapa Gado de Corte, Campo Grande, MS, Brazil. Briefly, according to Kessler et al. (1987), methodology, infected erythrocytes with a

parasitemia of 23% obtained from splenectomized calf, experimentally-inoculated with an isolate of *B. bovis*, were centrifuged three times with PBS pH 7.2, and stored at -72°C. The lysed erythrocytes were centrifuged three times at 10,000 g during 30 min at 4°C and washed with PBS pH 7.2. After the last centrifugation, the intermediate layer was collected and lysed with a buffer containing 100 mM Tris, 10 mM EDTA, 2% NP40, 0.2 mM N-alfa-ptosyl-L-lyl chloromethyl ketone (TLCK) and 2 mM phenylmethyl sulphonyl fluoride (PMSF), and ultrasonic disruption (100 W) for 10 min with interval of 2 min. The soluble material obtained after centrifugation for 60 min at 14,000 g and 4°C was used as antigen. Protein concentration was established by Folin method.

The ELISA was done by standart method (Voller et al. 1989) with modifications. Briefly, the microtitrations plate (Immulon) were coated with 200 µl of a 7.5 mg/ml solution of *B. bovis* antigens diluted in 0.05M sodium carbonate pH 9.6 and incubated overnight at 4°C. The plates were washed three times with PBS containing 0.05% Tween 20 and blocked with 1% rabbit serum diluted in PBS TW-20 0.05% pH 7.4. The washing procedure was repeated and test samples were diluted at 1/100 for IgM and 1/400 for IgG antibodies with blocking solution. Positive bovine serum diluted using serial two ratio dilution and sera of eight normal human beings were employed as negative control. After incubation at room temperature, the washing procedure was repeated and the conjugate diluted at 1/2000 in PBS was added (Sigma-goat anti human IgG and IgM alkaline phosphatase conjugated; except for the bovine positive control diluted serially that was incubated with rabbit anti bovine IgG alkaline phosphatase conjugated). Finally, it was added the substrate p-nitrophenyl sodium phosphate (Sigma) diluted at 1 mg/ml in glicine buffer pH 10.5. The plates were read at 405 nm in

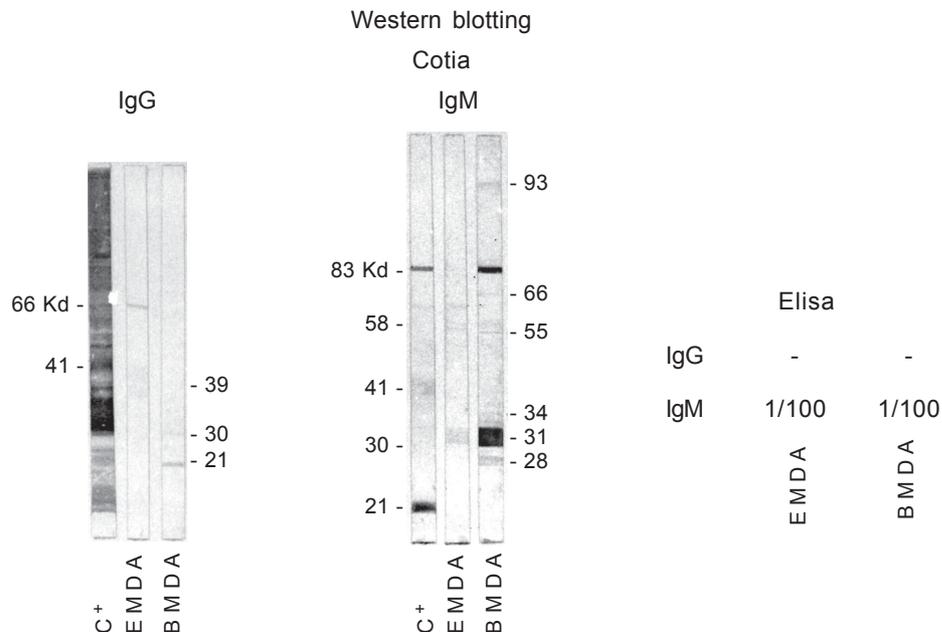


Fig.1: sequential serological analysis (Elisa and western blotting) for *Borrelia burgdorferi* in the sera of brothers with Lyme borreliosis (patients EMDA and BMDA).

microplate reader (Titertek Multiscan MCC/340, Flow Laboratories), and the assay stopped when the first dilution of the positive control serum reached the optical density value near 1.0.

Cut off value was obtained by considering the mean optical density plus three standard deviations of the eight control sera. Optical densities higher than cut off values were positive, and the titers were estimated by regression curve.

To date, in the laboratory (LIM-17), it is possible to substitute bovine positive control for human sera, because some sera obtained from patients with Lyme borreliosis, including sera of patients EMDA and BMDA, presented high titers of IgM and IgG antibodies to *B. bovis*.

Western blotting (WB) - Electrophoresis and immunoblotting were done as previously described with modifications (Grodzick & Steere 1988, Joppert et al. 2001). Briefly, 600 µg of *B. bovis* antigens reduced with SDS, were electrophoresed (Mini-Protean II System, Bio Rad) on a 10% acrylamide gel. After running, gel proteins were transferred to nitrocellulose paper over night (Mini-Trans Blot System, Bio Rad), and the paper cut in vertical strips, which were washed in distilled water. One strip containing *B. bovis* antigens and another with molecular weight markers were stained with a solution 1:1 of colloidal gold (Bio Rad). The others strips were blocked with 1% rabbit serum diluted in TBS Tween 20, 0.1% pH 7.4 for 1 h at room temperature, and then washed five times with TBS Tw-20, 0.1%. The test samples including positive and negative controls, were diluted 1:100 in blocking solution and added to incubate with strips for 1 h at room temperature. After washing, the strips were incubated with goat anti human IgG and IgM alkaline phosphatase conjugated (Sigma Chemical) diluted at 1:6000 for IgG and 1:3000 for IgM antibodies detection in blocking solution. Washing process was repeated and substrate consisting of NBT/BCIP diluted in carbonate buffer pH 9.8 was added. The reaction was stopped with distilled water when positive control developed color.

Note: bovine serum rich in IgG antibodies for *B. bovis* was used in the beginning until the discovery of a positive human control. Adequate conjugate was used, according to each standardized assay.

RESULTS

Sera of the patients presented high titers of IgM and IgG antibodies to *B. bovis* proteins, when compared with negative controls, and also reached optical density (OD) at same time of that observed with positive bovine serum at ELISA assay for IgG antibodies detection.

During the standardization stage of ELISA assay for babesiosis in humans, it was done a preliminary assay with 36 sera samples obtained from normal donors, aiming to calculate the mean value (\bar{x}) and standard deviation (σ) of the assays for detection IgG and IgM antibodies to *B. bovis*. ELISA for IgG antibodies in this normal group presented $\bar{x} = 0.022$ and $\sigma = 0.016$, and the cut off (mean value plus three standard deviations) obtained was equal to 0.070. ELISA for IgM antibodies revealed $\bar{x} = 0.077$ and $\sigma = 0.086$, with cut off of 0.335. Then, it was

possible to choose eight of these 36 sera samples which could represent the normal group.

After that, ELISA was performed in another group of 49 normal individuals, using the eight previously selected sera as control group, permitting to know the frequency of sero-positivity to babesiosis in a normal population. Eight of 46 sera (8.2%) were positive for IgG antibodies to *B. bovis*, and only two in 49 (2.04%) were positive for IgM antibodies. The study demonstrated that the frequency of antibodies (IgG or IgM) against babesiosis in a control group was of 10.2%.

ELISA tests for babesiosis were done in 59 patients with Brazilian Lyme borreliosis. It was found positivity in 10/49 patients (16.9%) for IgG antibodies and in 9/45 (15.25%) for IgM antibodies against *B. bovis*. Fifteen of 59 patients (25.42%) presented IgG or IgM antibodies to babesia antigens. Statistical analysis (chi-square test) demonstrated that patients with Lyme borreliosis presented higher frequency of isolated IgM (chi-square = 5.6; $p < 0.05$), and IgM or IgG antibodies (chi-square = 4.11; $p < 0.05$) when compared to control group. Surprisingly, the highest titers of IgG and IgM antibodies to *B. bovis* were observed with the sera of patients EMDA and BMDA, which were the first cases of Lyme borreliosis discovered in Brasil.

These results were confirmed by WB. Fig. 2 compares presence of IgG antibodies in the positive bovine serum with those found in both patients with Lyme borreliosis (EMDA and BMDA). Fig. 3 shows a sequential WB of patients, when the first sample was collected with 15 days of hospitalization (I), and the second after three months of follow up (II). The latter WB (II) revealed that number and intensity of IgM and IgG bands directed to babesia, increased during the clinical recovery of both children. IgM antibodies recognized *B. bovis* antigens of 24, 50, 66 and 97 KDa, while IgG reacted with proteins of 24, 45, 66, 85 and 106 KDa

In an attempt to discard hypothesis of cross-reactivity between antibodies to *B. burgdorferi* and *B. bovis*, diluted sera of patients were previously incubated "over night" with spirochetal antigens fixed in polystyrene EIA/RIA plate. The supernatant was used for repeat ELISA

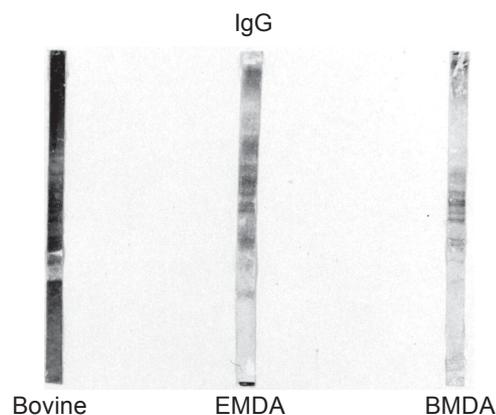


Fig. 2: western blotting (IgG) against *Babesia bovis* antigens using positive bovine control and sera of brothers with Lyme borreliosis (patients EMDA and BMDA).

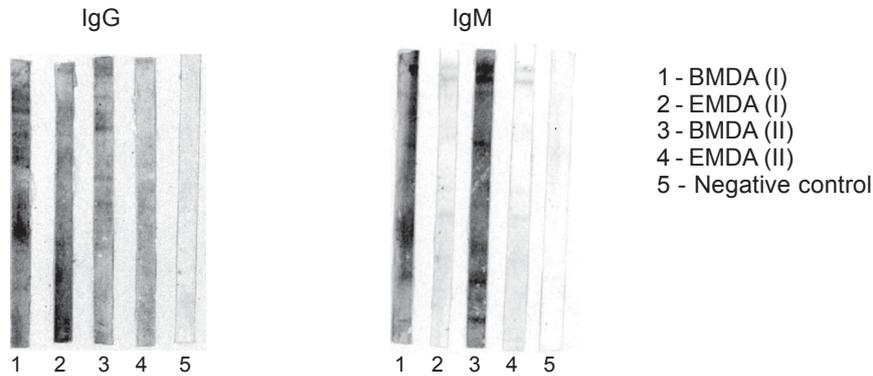


Fig. 3: sequential western blotting (IgM and IgG) against *Babesia bovis* in brothers with Lyme borreliosis (patients EMDA and BMDA); (I) serology done at hospitalization date; (II) serology done three months latter.

and WB against babesia antigens, wich revealed similar results with that obtained with not adsorved sera (data not shown). Otherwise, repeated serology (ELISA) using these adsorved sera against *B. burgdorferi* showed decreased reactivity

The demonstration of parasites in peripheral blood of patients was impossible, because only frozen sera kept frozen at -80°C since 1992, were available for serological tests.

DISCUSSION

Lyme borreliosis is an emerging zoonosis in Brazil, and identification of new clinical suspected patients is growing fast, because physicians are concerned about the epidemiological and clinical aspects of the disease.

The present manuscript demonstrates existence of coinfection with etiological agents of Lyme borreliosis and babesiosis in Brazilian population, which is an original and important conclusion. Surprisingly, this discovery was more evident studying the sera of brothers, who were the first cases of Lyme borreliosis in our country. This coinfection was confirmed when ELISA and WB were performed in 59 patients with Brazilian Lyme borreliosis, when it was proved that the frequencies of antibodies directed to *B. bovis* were significantly higher in this group with borreliosis, when compared with normal population.

We have not identified babesias in the peripheral blood smears stained by Giemsa, neither hematologic or serum biochemical abnormalities in patients with Lyme borreliosis. For these reasons, we believe that babesia infection, in most of the cases is probably asymptomatic. Otherwise, the interaction between infective parasite and human must be different in immunossupressed individuals.

Many publications report coinfection in humans and animals caused by different tick-borne pathogens as borrelia, rickettsia, babesia, ehrlichia, and virus (Steere 2001). Physicians must think about coinfections, when the clinical picture is different from that seen with isolate infection. In such cases, is required additional laboratorial analysis, and eventual association of drugs to control the coinfection.

Patient EMDA presented prolonged fatigue that lastened one month after the end of antibiotic treatment. This kind of feature eventually is part of Lyme borreliosis, but is also a common complaint described in association with babesiosis (Krause et al. 1996)

Patients coinfectod with borrelias and babesias can experience a lot of complains for a longer period of time. The main clinical manifestations found in coinfectod individuals included fatigue, headache, sweats, chills, anorexia, emotional lability, conjuntivitis and splenomegaly. Serological survey done in 1156 subjects from Rhode Islands (USA) showed that 97 (8.4%) were sero reactive against Lyme disease spirochete antigens, of whom 14 (14%) also reacted against babesia antigens. In this community, in a group of 240 patients diagnosed with Lyme disease, 26 (11%) were coinfectod with babesiosis, and 50% of these patients were symptomatic for three months or longer, concluding that patients whith both infections experienced additional manifestations and persistence of illness (Krause et al. 1996).

Hilton et al. (1999) studied sero prevalence and seroconversion for tick borne diseases in a high-risk group in Long Island, NY, searching for antibodies to Rocky Mountain spotted fever, Lyme disease, ehrlichiosis and babesiosis. In the population of 671 individuals, 88 (13.2%) presented antibodies to one or more tick borne agents, including 34 (5%) with antibodies to *B. burgdorferi*. Seven participants seroconverted during one year follow up, five of whom were symptomatic for Lyme disease. Antibodies to spotted fever group rickettsiae were observed in 28 participants (4.1%), six had seroconversion during the study, and none had symptoms of the disease. Twenty four individuals (3.5%) had serologic evidence of exposure to *Ehrlichia* sp.; five seroconverted (0.7%), and three subjects were asymptomatic. Antibodies to *B. microti* were seen in seven participants (1%) including one asymptomatic seroconversion during the period of the study. The authors concluded that in a high-risk population, there was evidence of seroconversion, however many infections were asymptomatic, and coinfection were rare.

Magnarelli et al. (1995), studied coexistence of antibodies to tick-borne pathogens of babesiosis, ehrlichiosis, and Lyme disease in human sera obtained in Connecticut and Minnesota, USA. There was serologic evidence of exposure to multiple infectious agents in 15 of 227 (6.6%) samples. Of these, 10 samples were from patients who had had previous Lyme disease. The authors did not find cross-reactivity among the antisera and antigens tested for the four tick-borne pathogens, and recommended to search for coinfection when clinical picture was unclear.

Coinfection with agents of human granulocytic ehrlichiosis, Lyme disease and babesiosis was observed in wild white-footed mice (*Peromyscus leucopus*) in Connecticut (Stafford et al. 1999). Twenty three of 48 (47.9%) captured animals, presented antibodies to *Ehrlichia* sp. strains determined by indirect immunofluorescence method, and the majority (83.3%) of mice had antibodies to *B. burgdorferi* identified by ELISA. Moreover, 20 of 26 (76.9%) animals presented antibodies to *B. microti* detected by indirect IF method.

The present paper demonstrates the existence of coinfection of Lyme borreliosis and babesiosis pathogens in Brazil, identified by serological methods. Also, it was not observed serological cross-reactivity between these agents. Otherwise, it is possible to assume that human babesiosis is misdiagnosed and perhaps confused with malaria, which is a very common disease in our country. Serological discernment is not easy, because there are reports of cross-reactivity among protozoal infections of the phylum Apicomplexa, including organisms of the genera *Plasmodium*, *Babesia*, *Toxoplasma* and *Cryptosporidium*, which are closely related organisms (Kemper 1997).

Babesia and *plasmodium* infections present similar clinical manifestations, both having symptoms like headache, fever, chills, disseminated intravascular coagulation, anemia with diserythropoiesis, hypotension, respiratory distress, and renal insufficiency (Clark & Jacobson 1998). Due to these reasons, it is reasonable to accept that it is difficult to establish differential diagnosis between malaria and *babesia* infections at risk areas where both diseases are endemic.

We have done serology for *B. bovis* in other groups of diseases with the aim to discharge possibility of serological cross-reactivity with others clinical entities. Abrão (2001) performed ELISA test in the sera of 30 patients with diffuse connective disease (10 systemic lupus erythematosus, 10 scleroderma and 10 rheumatoid arthritis), 26 with syphilis, 102 with facial palsy, 28 with septicemia caused by different bacteria, 38 with rheumatic fever, and the final conclusion was that only the group of patients with Lyme borreliosis presented high frequencies of antibodies against *babesia* antigens, when compared with control group.

Ishikawa et al. (1997), standardized serology for *B. burgdorferi* in cattle in Brazil, performing ELISA assay to detect IgG class antibodies in two bovines, one of them immunized with heat-inactivated suspension of borrelias, and the other inoculated with sterile PBS. The study permitted to analyse the sequential synthesis of antibodies in sensitized and testimony animals. Ishikawa (2000), showed that when the mentioned bovines, were submit-

ted to different challenges, like vaccinations (carbuncle, brucellosis and leptospirosis), natural infections (*B. bigemina*, *B. bovis*, *Anaplasma marginale*) and spontaneous infestations by *Boophilus microplus*, presented in same instances, modifications of *B. burgdorferi* IgG antibodies titer. The main conclusions related to our research were that natural infections due to mentioned protozoans, confirmed by demonstration of parasites in the blood and serologic diagnosis, did not modify the antibody production against *B. burgdorferi* in cattle, while vaccination against leptospirosis and natural ticks infestation changed antibody synthesis. These data confirm the absence of humoral cross reactivity between borreliosis and babesiosis.

Some questions on Lyme borreliosis remain unsolved. Previous epidemiological studies performed at risk areas for Lyme borreliosis in Cotia, State of São Paulo (Barrros-Battesti et al. 1995, 2000), and in Campo Grande, State of Mato Grosso do Sul (Costa et al. 2002) demonstrated the presence of Ixodid ticks of genus *Amblyomma* and *Ixodes* (not found in Campo Grande), considered as potential vectors of Lyme borreliosis in Brazil. Otherwise, babesiosis in our country is a tick-borne disease transmitted by other species of ticks (*B. microplus*, *Rhipicephalus sanguineus*, *Dermacentor* sp.), which becomes difficult to understand how coinfection happened. It is possible to admit the existence of another species of vector, or presence of a different strain of *babesia* present among wild or domestic animals, with particular biological and epidemiological features, undiscovered yet.

Also, in the present study we employed *B. bovis* antigens to do ELISA and WB assays, but it is possible to admit existence of cross-reactivity between different species of *babesias*. Particularly important, was the epidemiological history that the boys had presented contact with dog that died a few days later. In the ownership, others domestic animals like bovines and horses were identified.

The research on Lyme borreliosis in Brazil is a challenge, since etiologic agent(s), vector transmitter(s) and laboratorial diagnosis are quite different from that observed in USA or Europe. Etiological agent does not grow in BSK medium, and PCR done with use of known primers, is not capable to identify borrelias in ticks, humans, and reservoirs. The existence of disease is based on identification of patients with classical clinical features of disease, including the finding of characteristic erythema migrans. *Borrelia*-like microorganisms has been demonstrated by dark field microscope in blood and cerebrospinal fluid of patients. Due to these particularities, the disease has been named Lyme disease like illness in our country.

Despite many questions not completely understood concerning Lyme borreliosis in Brazil, identification of new cases by physicians of different specialties, is growing fast, often with discovery of unusual clinical aspects of this emerging zoonosis of great medical interest. The good therapeutic response and follow up, mainly of early diagnosed patients, are strong stimulus to keep looking for solutions of unsolved questions. Description of coexistence of borreliosis with another tick borne-disease is

an important medical report, since physicians may be prepared to diagnose Lyme borreliosis with uncommon clinical evolution, making possible to introduce therapeutic changes, and making easy the patient recovery.

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