UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CÂMPUS DE JABOTICABAL

BORRELIOSIS IN HORSES: EPIDEMIOLOGY, EXPERIMENTAL INFECTION AND THERAPEUTIC

Roberta Carvalho Basile

Bachelor in Veterinary Medicine

2016

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Roberta Carvalho Basile
Advisor: Prof. Antonio de Queiroz Neto, BVM, PhD
Co-Advisor: Prof. Delphim da Graça Macoris, BVM, PhD

Thesis presented to the Faculdade de Ciências Agrárias e Veterinárias – Unesp, Campus of Jaboticabal, as partial fulfillment of requirements for the obtention of PhD Degree in Veterinary Medicine (Clinical Medicine).

2016

Basile, Roberta Carvalho
B311c Borreliosis in horses: epidemiology, experimental infection and therapeutic, Jaboticabal, SP / Roberta Carvalho Basile. – – Jaboticabal, 2016
xvii, 131 p. : il. ; 28 cm

Tese (doutorado) – Universidade Estadual Paulista, Faculdade de Ciências Agrárias e Veterinárias, 2016
Orientador: Antonio de Queiroz Neto
Coorientador: Delphim da Graça Macoris
Banca examinadora: Jairo Jaramillo Cardenas, Adolorata Aparecida Bianco Carvalho, Estevam Guilherme Lux Hoppe, Natalino Hajime Yoshinari
Bibliografia

1.Borrelia burgdorferi . 2. Carrapatos . 3. Infecção. 4. ELISA. 5. PCR. I. Título. II. Jaboticabal - Faculdade de Ciências Agrárias e Veterinárias.

CDU 619:616.98:636.1

Ficha catalográfica elaborada pela Seção Técnica de Aquisição e Tratamento da Informação – Serviço Técnico de Biblioteca e Documentação - UNESP, Câmpus de Jaboticabal.

CURRICULUM OF THE AUTHOR

Roberta Carvalho Basile - Born in São Paulo, Brazil, on February 20, 1978, daughter of Lucio Godinho de Carvalho and Dolores de Carvalho, married to Marcelo Toledo Basile since January 2005 and mother of Matheus Carvalho Basile, born in March 2012. In December of 2012, she graduated in Veterinary Medicine at the Univ. Estadual Paulista, Jaboticabal, SP. She attended specialization in Clinical and Equine Surgery at the Jaguariúna University, SP, between 2013 and 2014, and Veterinary Acupuncture in this same University between 2011 and 2012. She graduated also in civil engineering at the University of São Paulo, School of Engineering of São Carlos, SP, in 2001 and completed his Master of Aeronautics and Mechanical Engineering at the Technological Institute of Aeronautics in Sao Jose dos Campos, Brazil, in 2003. She has held the Engineer profession as flight test engineer at Embraer between 2003 and 2008. She joined at the Graduate Program in Veterinary Medicine at the Univ. Estadual Paulista, Campus of Jaboticabal, SP, in March 2013 as a PhD student. She has been working as Assistant Professor at Camilo Castelo Branco University since 2015, being responsible for the Large Animals courses and attendances.

“Sometimes, the most interesting and unexpected results come from unlikely jobs.”

M.L.

DEDICATE

To my family, my fortress and greater wealth.

ACKNOWLEDGEMENTS

I am very grateful for my parents' love and its everlasting support, for my husband complicity, love and partnership, and for my son's comprehension and patience for the non played moments.

I am thankful to Prof. Queiroz, which was much more than an Advisor, a real partner whichever I needed during this journey. You are not from here!

To Prof. Delphim Macoris, my tutor which welcomed and sheltered me since the first day and I would say till actual days.

To Dear Virginia Bonoldi and Elenice Mantovani, which masterfully carried out all serological and molecular trials. Without that, nothing of this would be possible.

To my cherished Mariana Vieira and Luiz Felipe, loyal warriors always by my side.

To my Unesp colleagues: Lara, Talissa, Gabriel, Luciana, Aline, Renatha, Mateus, Raquel, Flora, Julia, Tamires, Buca, Walter, Mayara, Jessica, Bel, Gabriela, Vanessa, Yuri, Márcia Ajusi, each one of you had an essential contribution for this project.

To Dear Goido e Deco, my eternal acknowledgment for each drop of sweat and effort spent in these pages.

To the Professors Marcos R. André, Canola, Paulo Aléscio, Guilherme Ferraz, Rosângela Zacarias, each one of you helped guiding myself to overcome very hard times.

To the Veterinarians Rodolfo Lauricella, Cassiano Ricardo Rios, Paulo H. Machado, Renata Calegari, Reinaldo de Campos, Mariana Invernezzi, Claudia Leschonsky, Roberto Macedo, Alexandre Bloem, Rafael Daia which were somehow available to contribute in this project.

To the properties Sociedade Hípica Araraquara, Fazenda São Sebastião, Haras Vila Colonial, Manege Steel Horse, Haras das Mangueiras, Haras Interagro, Haras Raízes, Haras Las Vegas, Haras Santa Fé, Joquey Clube de SP, Haras TH, Haras Mont Serrat, Haras Vassoural, Universidade do Cavalo, Haras Lagoinha, Haras

Tango, Equinocultura Unesp Jaboticabal, which opened their gates and made their horses available allowing this study possible.

To the horses Pérsico, David Off, Ross, Duque d'Alba, Trufa, Alegria, Mara, Minie, Carriele, Angélica, Tigresa, Seif Alah, Cindy (*In Memoriam*), there is no words to describe, only my feelings.

To the Professors who attended my examining board, dedicating their times for revising and contributing towards the improvement of this work, as my personal and professional development.

For the Faculdade de Ciencias Agrárias e Veterinárias (UNESP Jaboticabal), their teachers and staff, for sheltering me throughout my graduation and Doctor degree.

To the agency FAPESP and São Paulo's State Government, which funded this project.

To God, who never abandoned me and has slowly and wisely shown that nothing was in vain. For my Guardian Angel, who often blows in my ear the right paths to follow.

SUMMARY

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CERTIFICADO

Certificamos que o Protocolo nº 001968/13 do trabalho de pesquisa intitulado **"Caracterização clínica, laboratorial e terapêutica de equinos infectados pela Borreliose de Lyme Símile (brasileira)"**, sob a responsabilidade do Prof. Dr. Antonio de Queiroz Neto está de acordo com os Princípios Éticos na Experimentação Animal, adotado pelo Colégio Brasileiro de Experimentação (COBEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 05 de fevereiro de 2013.

Jaboticabal, 05 de fevereiro de 2013.

Prof. Dr. Andrigo Barboza De Nardi
Coordenador - CEUA

Faculdade de Ciências Agrárias e Veterinárias
Via de Acesso Prof. Paulo Donato Castellane, s/n CEP 14884-900 - Jaboticabal - SP - Brasil
Tel. 16 3209 2600 - fax 16 3202 4275 www.fcav.unesp.br

BORRELIOSIS IN HORSES: EPIDEMIOLOGY, EXPERIMENTAL INFECTION AND THERAPEUTIC

Abstract – Lyme borreliosis is a disease caused by the spirochete *Borrelia burgdorferi* sensu lato, cosmopolitan and transmitted by the bite of ticks which remain adhered to the host for more than 24 hours. In humans, it can cause articular, cardiac and neurological diseases. In horses, so far the disease had been described by means of case reports and extrapolations of its pathogenesis in humans. This study aimed to investigate the clinical signs and hematological changes of Lyme disease in horses. Furthermore, it is also assessed the feasibility of treating infected horses with sodium ceftriaxone. To this end, the experiment consisted of three main phases. The first phase consisted of an epidemiological survey of the disease in São Paulo State, specifically in cities with suspected cases of Lyme borreliosis in humans. It was collected blood samples and clinical history of 760 horses that resulted in an average of 21% seropositivity in the state. In this stage, it was concluded that there was a high relationship between seropositivity, *Amblyomma sculptum* tick presence, the presence of capybaras in the property, lymphopenia, abortion and retained placenta. The second phase consisted of an experimental infection of two adult horses with *B. burgdorferi* strain G39 / 40. The horses were evaluated for 90 days of infection and we found that the animals showed nonspecific clinical signs and hematologic changes only in the first 11 days of infection. It was noted the presence of mild hypochromic normocytic anemia, muscle pain, pale mucous membranes, lethargy and swollen lymph nodes, signs that can easily be confused with chronic piroplasmosis. During phase 3 of the experiment, the two horses experimentally infected underwent treatment with intravenous sodium ceftriaxone. Already during the first application, both developed an anaphylactoid reaction moderate to severe with colic syndrome as consequence for one horse and laminitis to the other. Both recovered and were finally treated with oxytetracycline.

Keywords: *Borrelia burgdorferi*, tick, ELISA, PCR, sodium ceftriaxone.

BORRELIOSE EM CAVALOS: EPIDEMIOLOGIA, INFECÇÃO EXPERIMENTAL E TERAPÊUTICA

Resumo – A Borreliose de Lyme é uma doença causada pela espiroqueta *Borrelia burgdorferi* sensu lato, cosmopolita, transmitida por meio da picada de carrapatos que permanecem aderidos ao hospedeiro por mais de 24 horas. Em humanos, pode provocar doenças articulares, cardíacas e neurológicas. Nos equinos, até o presente momento a doença havia sido descrita por meio de relatos de caso e extrapolações de sua patogenia nos humanos. Por meio do presente estudo, pretende-se pesquisar os sinais clínicos e alterações hematológicas da borreliose de Lyme nos equinos. Além disso, avaliou-se também a viabilidade de se tratar os equinos infectados com ceftriaxona sódica. Para tanto, o experimento foi composto por três principais fases. A primeira fase foi composta por um levantamento epidemiológico da doença no Estado de São Paulo, especificamente nas cidades com casos suspeitos de borreliose de Lyme em humanos. Coletou-se amostras de sangue e histórico clínico de 760 equinos e obteve-se média de 21% de soropositividade no estado. Desta fase, concluiu-se que existe grande relação entre a soropositividade, presença de carrapatos *Amblyomma sculptum*, presença de capivaras na propriedade, linfopenia, abortamento e retenção de placenta. A segunda fase foi composta por uma infecção experimental de dois equinos adultos com *B. burgdorferi* cepa G39/40. Os equinos foram avaliados durante 90 dias de infecção e foi possível verificar que os animais apresentaram sinais clínicos e alterações hematológicas inespecíficas somente nos primeiros 11 dias de infecção. Notou-se a presença de anemia normocítica hipocrômica discreta, dores musculares, palidez de mucosas, letargia e aumento de linfonodos, sinais que podem facilmente ser confundidos com a piroplasmose crônica. Durante a fase 3 do experimento, os dois equinos infectados experimentalmente foram submetidos ao tratamento com ceftriaxona sódica por via intravenosa. Já durante a primeira aplicação, ambos desenvolveram uma reação anafilactóide de moderada à severa, com consequência de síndrome cólica para um deles e laminite para o outro. Ambos se recuperaram e foram finalmente tratados com oxitetraciclina.

Palavras-chave: *Borrelia burgdorferi,* carrapato, ELISA, PCR, ceftriaxona sódica.

1. INITIAL CONSIDERATIONS

1.1 INTRODUCTION

Lyme borreliosis is a multisystemic disease discovered in the mid-1970s in the United States, in children of Connecticut with polyarthritis without determined causes. It is a long term studied and diagnosed condition in the Northern Hemisphere, caused by the spirochete *Borrelia burgdorferi* present in Ixodidae family of ticks. This pathogen is able to infect mammals including humans and each ones have different clinical responses upon the infection. The most important reservoirs in Brazil should be the capybaras, which also could serve as carriers and disseminators of ticks in pastures and near local lakes and rivers.

My history with borreliosis began in 2004, while still working as an engineer I fell in love with the horses. I began to enjoy horseback riding in a training center in Sao Jose dos Campos in the end of my working days, which came up to contribute immensely to this PhD project. Everything was doing well until the day I started to feel pain in the carpal joints. The pain only regressed with anti-inflammatory drugs and started get around other joints such as elbows and knees. In a few months, I could not sleep soundly because the pain progressively increased. At this time I did not remember having found any tick, actually I did not even know what it was.

My joints were swollen, purplish and painful. In the morning, I did have no strength to turn the door handle, it became a common routine pushing the toothpaste tube with my elbow, and to step down the stairs of my house seated. I have visited many physicians as clinical, orthopedic, homeopaths, rheumatologists, and numerous tests were carried out without any clear diagnosis. In their view, I had rheumatoid arthritis, lupus, fibromyalgia, stress, I was eating wrong, etc.… Everything

seemed to be a cause, unless an infectious disease. I have achieved a slight improvement with a physician who told me, despite my inconclusive tests, I should treat me to lupus with steroids and chemotherapy.

Since then, four years have passed and I started a degree in veterinary medicine. In the third year, I remember having attended a zoonosis class where the teacher introduced me to this illness for the first time. I was thoughtful, remembering what I had passed a few years ago. The information was stored, waiting to be used.

When I was in fourth year of my under-graduation, we worked with some horses in the university. This site was known to be full of ticks and, one day, I found one stuck in my body, I did not know exactly how long. I removed it immediately, and in the following days it was formed a generous inflammatory circle around the bite.

A few days later, I started feeling extremely tired; it looked like building a house every day. I generally arrived at home early evening always feverish, around 38^{o}C. I felt weak, discouraged, with headaches, depression and pain in the throat that does not regressed with painkillers. I went to an endocrinologist, because we really thought I could have some thyroid problem. It was when, during the attendance, my husband asked me to comment with him that I had recently been bitten by tick. When he said this, two seconds became six years in my head. I looked at his face and thought: "Eureka!". And the doctor looked at my face thinking "Is your husband crazy?"

Without explaining the reasons, immediately I asked the doctor if he could request a serology for Lyme. And he spoke with doubt "Laaaaiiiiiiiiiimiiiiiiiiiii is the spotted fever?". I replied: "No doctor, also transmitted by ticks, but the spotted fever

is transmitted by a rickettsia and Lyme, borrelia." And then the doctor added: "OK, how it is spelled?" And finally asked for IgG and IgM serology for Lyme.

My disease seemed to be finally diagnosed and I start seeking for a medical advice in this field. In my brief research I found Dr. Natalino Yoshinari, the pioneer physician in the study of the disease in Brazil and member of the research group of this project. I was treated with antibiotics and anti-inflammatory drugs for a period of 60 days, when we later found out that I was pregnant. But the treatment was a successful and the pregnancy. Since 2012, I have had mild annual recurrences, which also was controlled with chemotherapeutic agents.

1.2 OBJECTIVES

General objectives

To characterize the Lyme disease in horses describing their clinical, serological and hematological parameters during natural and experimental infection.

Specific objectives

Horses naturally infected (Chapter 3):

i. To evaluate the serological IgG anti-*Borrelia burgdorferi* s.l. antibodies profile of horses from regions of high frequency of seropositivity in humans in the Sao Paulo State,
ii. To assess the clinical signs history and management factors associated with seropositive horses,
iii. To compare the clinical and hematological alterations of seropositive horses in a case-control study.

Horses experimentally infected (Chapter 4):

i. To standardize the experimental infection technique in horses using *B. burgdorferi* stricto sensu strain G39/40 via subcutaneous and intradermal applications,
ii. To describe the serological profile of IgG immunoglobulins during the infection and treatment,
iii. To verify the presence of *B. burgdorferi* stricto sensu strain G39/40 in equine blood and tissues using the polymerase chain reaction (PCR) test with gene primers of flagellar hook (*flgE*) and flagellar filament (*FlaB*),
iv. To describe the clinical manifestations and hematological alterations during ninety days of the infection.

Horses experimentally infected and treated with sodium ceftriaxone (Chapter 5):

i. To assess the applicability of the sodium ceftriaxone antibiotic in the treatment of borreliosis in horses,
ii. To describe the consequences of the treatment applied in the two horses experimental infected by *B. burgdorferi* stricto sensu.

1.3 RATIONALE

National studies restrict their researches in the serological evaluation of horses and other mammals and in the demand to isolate the pathogen, without describing the pattern of the disease and the possible characteristics restrict to the Brazilian borreliosis in horses, as has been described for humans in Baggio-Yoshinari

Syndrome. Note also that there is little dissemination and knowledge of the disease in national veterinary events and technical publications.

Even with regard to the disease caused by *Borrelia burgdorferi* stricto sensu, the few studies of experimental infection reported to date directed their reviews for molecular detections of the agent before and after the treatment, without describing the clinical course of the disease in horses. In addition, these experimental infections did not describe the amount of inoculated pathogen, remaining doubts about their own methodology.

Six chapters compose this Thesis. The Chapter 1 introduces my personal experience with borreliosis, announcing the objectives and rationale of our project. Chapter 2 presents the literature review of the history and current status of the researches of borreliosis in humans and domestic animals. Chapter 3 presents an Editorial published in the *Immunogenetics: Open Access* that questions whether in fact the scientific community knows the course of this disease in horses, given the context of the papers published so far. Chapter 4 describes the epidemiology of the disease in horses from Sao Paulo and Chapter 5 reveals the course of the disease in two adult horses experimentally infected. In Chapter 6, we present the report of the consequences of treating those infected horses with sodium ceftriaxone and, finally in the Chapter 7 we conclude the thesis suggesting some topics for future research.

2. LITERATURE REVIEW

Paper submitted to *BRAZILIAN JOURNAL OF MICROBIOLOGY* in 24/09/2015.

Brazilian borreliosis with special reference to humans and horses

Roberta Carvalho Basile, Natalino Hajime Yoshinari, Elenice Mantovani, Virgínia Nazário Bonoldi, Delphim da Graça Macoris, Antonio de Queiroz-Neto

ABSTRACT

Borreliosis is a cosmopolitan zoonosis studied worldwide, called Lyme disease in many countries of North Hemisphere and Baggio-Yoshinari Syndrome in brazilian humans. However, in Brazil, despite the increasing number of suspect cases, this disease is still neglected by the medical and veterinary community. Brazilian Borreliosis (BB) is caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex, which should use capybaras as reservoirs. Domestic animals are key carriers for pathogen dissemination. This zoonosis has been studied very little in horses in Brazil, and the first survey was performed in the state of Rio de Janeiro. The Brazilian Borreliosis has many differences from the disease widely described in the Northern Hemisphere. The etiological agent has different morphological and genetic characteristics, the disease has a higher recurrence rate after treatment with antibiotics, and the pathogen stimulates intense reactive symptoms like a broader immune response in the human host. Additionally, brazilian zoonosis are not transmitted by I*xodes ricinus* complex. Due to these divergences, the borreliosis present in Brazil was named Lyme-like disease or Baggio-Yoshinari syndrome (BYS). With respect to clinical manifestations, BYS has been reported to cause neurological, cardiac, ophthalmic, muscle and joint alterations in humans. These symptoms can

possibly occur in horses. The present study shows a current panel of studies involving the disease in human and equine medicine, especially in Brazil.

KEYWORDS: *Borrelia burgdorferi*, zoonosis, ticks, equine, Lyme-like, Baggio-Yoshinari Syndrome.

Lyme disease (LD) or Lyme borreliosis (LB) is the most common tick-borne disease in temperate regions of the Northern Hemisphere and is caused by the spirochete *Borrelia burgdorferi* sensu lato. It is a multistage disease that can affect multiple organs but manifests predominantly in the skin, joints and nervous system humans (Koedel et al., 2015).

In 1976, in a geographical region of the United States, specifically near the town of Lyme, Connecticut, children were affected by a mysterious syndrome (Mast and Burrows, 1976) that was initially diagnosed as juvenile rheumatoid arthritis (Steere et al., 1977). In 1981, the entomologist and physician Willy Burgdorfer, along with Alan Barbour and Jorge Benach, found a spirochete in the midgut of ticks of the genus *Ixodes* in an area of the state of New York, a known endemic focus of Lyme disease. The researchers cultivated samples from ticks in culture media developed for the relapsing fever spirochete (*B. hermisii*) and found a new species of *Borrelia*, subsequently named *B. burgdorferi* (Burgdorfer et al., 1982). Later, the same bacterium was isolated from the blood of patients with Lyme disease and cultivated.

The diseases termed Lyme borreliosis are known to be caused by a large number of species related to *B. burgdorferi*, which are called *B. burgdorferi* sensu lato (Samules and Radolf, 2010). Of the 34 existing *Borrelia* spp., 20 are called *Borrelia burgdorferi* sensu lato and cause Lyme disease, which is transmitted by ticks

of the genus *Ixodes*. Of these 20 species, only nine have been isolated from humans in the Northern Hemisphere (*B. afzelii*, *B. bavariensis, B. bissetti, B. burgdorferi* stricto sensu, *B. garinii, B. kurtenbachii, B. lusitaniae, B. spielmanii,* and *B. valaisiana*) (Rudenko et al., 2011).

The first isolation of *B. burgdorferi* sensu lato in the Southern Hemisphere was performed by Barbieri et al. (2013) in Uruguay from *Ixodes pararicinus* ticks. Afterwards, the bacterium was also identified in Argentina (Nava et al., 2014) and Chile (Ivanova et al., 2014; Huang et al., 2015), where it was named *B. chilensis*. All three isolations revealed the bacterium in the ticks of the *I. ricinus* complex and used the 16S ribosomal gene, 5S-23S intergenic spacer and flagellin gene (*fla*) during amplification for species identification.

Borrelia burgdorferi sensu lato is a highly invasive gram-negative spirochete. Its pathogenicity depends on mobility, cytotoxicity, antigenic variability, lymphocyte stimulation and resistance to complement activation in the absence of specific antibodies (Wasiluk et al., 2011).

The pathogen is mainly transmitted by ticks of the *I. ricinus* complex (Radolf et al., 2012). However, there are reports of *B. burgdorferi* s.l. transmission by *Amblyomma americanum* in the states of Florida and Georgia in the United States (Clark et al., 2013), and it has been identified in *Dermacentor nitens* in the state of Paraná, Brazil (Gonçalves, et al., 2013).

The bacteria infect the tick if and if it feeds on an infected reservoir host (Radolf et al., 2012). After molting to the nymph stage, ticks are able to transmit the pathogen to the animal that provides its next blood meal. Transtadial transmission

may not always be successful; therefore, transmission of the bacteria is ensured by an enzootic cycle in which the tick feeds on various vertebrate hosts (Kurtenbach et al., 2006).

The spirochetes are deposited into the bite wound along with the tick saliva. For infection to succeed, the tick must feed for at least 24 hours adhered to the host, a period after which there is reduced expression of the Outer Surface Proteins A and B (*OspA* and *OspB*) and increased expression of the Outer Surface Protein C (*OspC*). The *OspA*s and *OspB*s are lipoproteins essential for *Borrelia* spp. survival in the tick midgut. The *OspC*s are crucial for the establishment of infection in the invertebrate host because they allow the bacteria to migrate from the tick midgut to the salivary glands, where they will be carried along with the saliva to the vertebrate host (Kenedy et al., 2012). In the vertebrate host, *OspC*s also have an important role because they induce immunosuppression, favoring infection (Kurtenbach et al., 2006). Tilly et al. (2006) found that bacteria lacking *OspC* do not establish infection in mice by either bacteria inoculation via injection or by tick bite.

In the vertebrate host, the *Borrelia* spp. is recognized by several mechanisms of immune response, including the complement system and diverse innate immune cells (Mason et al., 2014). Despite being classified as gram-negative, *B. burgdorferi* does not produce lipopolysaccharide (LPS) but expresses *OspC* in vertebrates. The recognition of *Borrelia* spp. by dendritic cells leads to their maturation and triggers the transcription of a large set of genes, such as chemokine genes, apoptosis inhibitors, matrix metalloproteases and a large subset of cytokines, including

proinflammatory mediators, neutrophils attractants and immunomodulatory cytokines (Berende et al., 2010).

Following antigen presentation by dendritic cells, T_H1 and T_H2 lymphocyte helper T cells start an adaptive response, promoting the release of interferon IFN-γ and interleukyn IL-4, which are directly related to the severity of acute symptoms (Mason et al., 2014). Subsequently, the cytokines released by T_H cells induce the proliferation of B-lymphocytes and, consequently, immunoglobulin production (Berende et al., 2010).

Although the immune system tries to prevent *Borrelia* infection, the spirochete has its own mechanisms to avoid the host defense system. Components of the tick's saliva (as *Salp*15) are known to be able to suppress the dendritic cell response, increasing the pathogenic virulence of *Borrelia* (Mason et al., 2014). The spirochetes can also inactivate the host complement system by binding to host complement regulatory proteins, thereby inactivating the C3b mechanism. Another mechanism employed by *Borrelia* to escape the immune response is antigenic variation. The variable major protein-like sequence gene locus (*vls*E) on plasmid 28-1 undergoes extensive variation, which is stimulated by tick feeding (Berende et al., 2010).

Acute infection is typically manifested by an expanding erythematous skin lesion. Late manifestations may include arthritis, acrodermatitis chronica atrophicans, lymphocytoma, myocarditis, conjunctivitis, uveitis and neurological signs (Stanek et al, 2012).

The existence of borreliosis in Brazil was first suggested in humans by Yoshinari et al. (1989); however, the first case in the country was only diagnosed in

1992. The increasing number of cases identified in Brazil showed differences between the disease that occurs in the Northern Hemisphere and the disease that occurs in Brazil (Yoshinari et al., 1997; Yoshinari et al., 1999; Yoshinari et al., 1999b; Costa et al., 2001). Regarding the epidemiological aspects, in Brazil, the occurrence of *Ixodes* ticks is insufficient; therefore, they cannot be considered the preferred vectors. Clinically, despite the occurrence of signs such as erythema migrans and the usual systemic complications, the Brazilian disease progresses with recurrences, especially if antibiotic treatment is initiated later than three months after the infection. Brazilian patients have been reported to have a high frequency of antibodies against different autologous cell components. Therefore, the Brazilian Borreliosis (BB) was initially called Lyme-like disease, Lyme-like borreliosis or Baggio-Yoshinari syndrome (BYS) to distinguish it from the classical disease (Mantovani et al., 2007).

In addition, studies conducted in the Laboratory of Rheumatology of the Clinical Hospital of the School of Medicine, University of São Paulo (LIM-17 Hospital das Clínicas – Faculdade de Medicina, Universidade de São Paulo - FMUSP) showed the occurrence of microorganisms with morphological structures similar to *Mycoplasma spp.*, *Chlamydia spp.* and non-flagellated spirochetes in the peripheral blood of patients with BYS. However, those patients had negative serology for *Mycoplasma spp.* and *Chlamydia spp.*, suggesting a morphological difference between Brazilian *B. burgdorferi* sensu lato and the microorganism identified as the possible causative agent of SBY (Yoshinari et al., 2010). Once, motile and spiral spirochetes were never isolated or cultured in the country, researchers from LIM-17 assumed that etiological agent in Brazil was present at cystic form.

Due to these reasons, Brazilian Borreliosis is defined as an emerging tick bone disease, different from Lyme Disease (LD), caused by *Borrelia burgdorferi* sensu lato found at atypical morphologies and transmitted by ticks not belongin to Ixodes ticks. Possibly, borrelia passage through ticks from genera *Amblyomma, Rhipicephalus. Dermacentor*, causes morphologic and genetic modifications on spirochetes in both vertebrate and invertebrate hosts, originating a new disease similar to LD.

BYS differs from LD because the disease has higher morbidity due to presence of symptoms recurrence; severe reactive manifestations like autoimmunity and need of prolonged treatment. The laboratorial diagnosis of BYS is difficult, because serological tests (ELISA - enzyme immunosorbent assay or Western Blot) to *B. burgdorferi* show low sensitivity and specificity (Yoshinari et al., 2010; Gouveia et al., 2010; Santos et al, 2010a; Mantovani et al., 2007; Fonseca et al., 2005, Shinjo et al 2009) because it is used antigens from *B. burgdorferi* stricto sensu from north hemisphere to evaluate immunoglobulins of brazilian *B. burgdorferi* sensu lato.

BYS can causes some similar symptoms observed in LD, including erythema migrans in approximately 50%, arthritis in 35%, neurological symptoms in 35% and cardiac disease in nearly 5 % of patients. The disease is often unrecognized, especially at secondary or tertiary stages, when patients do not remember of facts occurred months or years before the current disease. Certainly, many of patients with unrecognized chronic neurological or articular diseases are in fact cases of BYS not identified and treated at early stages of the disease (Yoshinari et al., 2010).

The first studies in Brazil to report the occurrence of *B. burgdorferi* sensu lato were published in 2010 when dermatologists identified the spirochete in skin biopsies of the erythema migrans of four patients using immunohistochemistry (Santos et al., 2010b). In a subsequent study that used immunohistochemistry and focus floating microscopy, Talhari et al. (2010) also identified the bacteria in skin lesions of 22 patients. Despite conducting PCR (Polimerase Chain Reaction) using a set of four primers, none of the 22 samples was positive; therefore, the challenge to standardize a PCR technique for the identification of the *Borrelia* that occurs in Brazil remained.

Mantovani et al. (2012) demonstrated the presence of spirochetes of genus *Borrelia* in the blood of human patients with Brazilian borreliosis doing amplification of the *flgE* genes, and also in ticks from genus *Rhipicephalus*. Similarly, Gonçalves et al, 2013 identified *B. burgdorferi* s.l. strain B31 in *Dermacentor nitens* ticks collected on horse in Paraná State (Nested PCR targeting the 5S (rrf) 23S (rrl) intergenic spacer region, 99,9% of BLAST similarity with *B. burgdorferi* s.s.). Additionally, the same research group, identified *Borrelia burgdorferi* sensu lato in blood human from rural area in Paraná State (Nested PCR targeting the 5S (rrf) 23S (rrl) intergenic spacer region, 100% of BLAST similarity with *B. burgdorferi* s.s.) (Gonçalves et al., 2015).

Recent studies show the possibility of ticks of the genera *Amblyomma* and *Rhipicephalus* being directly related to the dissemination of the disease. Rezende et al. (2012) reported that embryonic cells of *Rhipicephalus microplus* and *A. cajennense* could serve as substrate for the growth of *B. burgdorferi* sensu stricto strain G39/40. Clark et al. (2013) identified *B. burgdorferi s*ensu lato in *A.*

americanum ticks collected from patients with Lyme disease diagnosed by ELISA and PCR.

Borrelia burgdorferi sensu lato can be capable of infecting wild and domestic animals. Domestic animals, such as dogs, cattle and horses, can be carriers of the disease. Unlike the unapparent disease that is observed in wild animals, this etiological agent is capable of causing clinical symptoms in domestic animals (Fonseca et al., 1996; Magnarelli and Anderson, 1989; Magnarelli et al., 1987; Skarda, 2005).

Salles et al. (2002) observed that according to indirect ELISA and Western Blot results, horses exposed to ticks have a higher frequency of seropositivity for *B. burgdorferi* sensu lato than horses subjected to strict tick control. Viable *Borrelia* have been found in the urine of healthy horses in an endemic region of the United States (Manion et al., 1998), warning of the possibility of transmission of the agent by contact routes in addition to tick bites. Chang et al. (2005) validated an equine Lyme disease model by exposing ponies to ticks harboring *B. burgdorferi* for seven days. They evaluated the antibody response and the treatment efficacy 120 days after infection and concluded that the antibody levels of treated animals returned to negative levels in 10 months.

In Brazil, Salles et al. (2002) detected an average ELISA seropositivity of 9.8% in horses in the state of Rio de Janeiro, and in the municipality of Seropédica, the frequency was 42.8%. Madureira et al. (2007) observed a frequency of 28.4% of anti-*Borrelia* homologous ELISA antibodies in horses in the municipalities of Três Rios and Vassouras, Rio de Janeiro state, whereas in the municipality of Belém, state of

Pará, the frequency was 26.7% (Galo et al., 2009). Guedes Junior et al. (2008) identified 54.9% of cattle as being seropositive in state of Paraná. In dogs, the rate of seropositivity was 48.25% in Rio de Janeiro city (Alves et al., 2004). Corradi et al. (2006) investigated the occurrence of LB in wild animal veterinarians and observed 6.4% seropositivity for *B. burgdorferi* s.l. in São Paulo city.

Borreliosis in horses is still underdiagnosed and poorly known by veterinarians. According to a survey conducted in Germany of 118 veterinarians, only 56% of the professionals believe that Lyme borreliosis can affect horses. When asked about the number of animals diagnosed in Germany, 45% answered that no animals are being diagnosed. Regarding control of the parasite, 46.5% stated that owners rarely perform ectoparasite control measures. A relatively large percentage of the veterinarians (30.5%) said that they would confirm the diagnosis only by serology and would not perform serological monitoring over time. They would treat positive animals with antibiotics and anti-inflammatory medication (54%) and stated that 71% of the horse owners are unaware of the disease (Gall and Pfister, 2006).

Studies indicate that Lyme Borreliosis in horses has pathogenicity similar to the human disease (Butler et al., 2005) and can cause clinical manifestations in horses such as fever and lethargy (Magnarelli et al., 1988), arthritis (Burgess et al., 1987; Hahn et al., 1996), polysynovitis (Passamonti et al., 2015), lameness, muscle stiffness (Divers et al., 2003), abortion, meningitis, cranial neuritis, radiculoneuritis and encephalitis (Burgess and Mattison, 1987; James et al., 2010), uveitis (Hahn et al., 1996; Imai et al., 2011; Priest et al., 2012) and premature death of foals.

To study spirochete distribution in horses, Chang et al. (2000) experimentally infected eight ponies using *I. scapularis* ticks infected with *B. burgdorferi*. The ponies were euthanized approximately nine months after infection, and samples from several tissues, such as lymph nodes, skin, muscles, synovial capsule and meninges, were subjected to molecular tests using primers targeting the membrane surface protein A (*Osp*A) gene and the 23S ribosomal portion and to cell culture. *B. burgdorferi* was mainly isolated from the skin, fascia and muscle.

According to Divers et al. (2012), Lyme Disease in horses must be diagnosed based on 1) the possibility of exposure to ticks infected with *Borrelia burgdorferi*, 2) clinical signs compatible with Lyme Disease, 3) absence of other causes for the clinical signs, and 4) a high titer of anti-*B. burgdorferi* antibodies. The most common technique used for the diagnosis of *B. burgdorferi* in humans and horses is antibody detection. The most frequently used techniques are the immunofluorescent antibody test (IFA), Western Blot and ELISA, with the latter having greater applicability for horses because it is faster and cheaper (Divers et al., 2001). Specificity tests for IFA and ELISA showed minimal cross-reactivity with anti-*Leptospira* antibodies (Salles, 2001; Magnarelli and Fikrig, 2005). In general, due to the slow multiplication of the spirochete in the host, immunoglobulin G (IgG) titers may take three to six weeks to be detected and eight to sixteen weeks to reach their maximum concentration (Chang et al., 2000; Salles et al., 2002; Chang et al., 2005). However, because the specificity of IFA and ELISA tests is still questionable, a positive result must be confirmed by a second diagnostic method. Western Blot is typically used to detect antibodies against *Borrelia*-specific antigens (Trevejo et al., 1999); alternatively,

researchers have been used PCR, which has the highest sensitivity and specificity (Divers et al., 2001; Chang et al., 2005).

Chandrashekar et al. (2008) evaluated the feasibility of using a commercial enzyme immunoassay kit developed for dogs (SNAP® 4Dx) for the detection of anti-C6 peptide antibodies in 160 horses infected by *B. burgdorferi* and previously tested by western blotting (QualiCode™ IgG/IgM Western Blot Kits, Immunetics Inc., Boston, MA). The kit showed 100% specificity and 95% sensitivity compared to the gold standard, and the authors indicated that this kit is a quick and safe test for the diagnosis of Lyme Borreliosis in horses in the field.

A subsequent study found that the serological tests using an anti-C6 commercial kit (SNAP® 4Dx) could identify most of the infected animals; however, it also produced false positive and false negative results. In addition, serological tests for the detection of anti-C6 peptide antibodies and Outer Surface Proteins C and F (*OspC* and *OspF*) associated with clinical signs were found to consistently support the diagnosis of borreliosis in horses (Wagner et al., 2013).

Treatment with oxytetracycline (6.6 mg/kg, IV, every 12 h) for three weeks was more effective than the use of doxycycline (10 mg/kg, VO, every 12 h) or ceftiofur (2.2 mg/kg, IM, every 12 h) in experimentally infected ponies (Divers et al., 2003). Oxytetracycline was the only antibiotic that led to negative results both in culture and in tissue PCR (lymph nodes, skin, muscle fascia, synovial membranes, pericardium and meninges) at the end of the treatment. Oxytetracycline can also be administered (5.0 mg/kg, IV, every 24 h) for four weeks, having high efficacy against *B. burgdorferi* in experimentally infected ponies (Chang et al., 2005). Divers et al. (2012) found that

the treatment of LB in naturally infected horses is effective when using tetracycline (6.6 mg/kg, IV, every 24 h).

Lyme disease is a condition of extreme importance because it is a zoonosis that causes physical and psychological sequelae in affected individuals. It is still poorly investigated in Brazil, especially in the field of veterinary medicine. Therefore, studies on the description of the particularities of the disease and the etiological agent found in the country are needed.

Acknowledgements

The authors acknowledge the financial support of FAPESP (Process 2013/05871-0 and 2013/03732-0).

2.1 REFERENCES

Alves AL, Madureira RC, Silva RA, Correa FN, Botteon RCCM. (2004) Frequência de anticorpos contra *Borrelia burgdorferi* em cães na região metropolitana do Rio de Janeiro. Pesquisa Veterinária Brasileira 24(4): 203-206.

Barbieri AM, Venzal JM, Marcilia, Almeida AP, Gonzales EM, Labruna MB. (2013) *Borrelia burgdorferi* sensu lato infecting ticks of the *Ixodes ricinus* complex in Uruguay: First report for the Southern Hemisphere. Vector-Borne and Zoonotic Diseases 13(3): 147-153.

Berende A, Oosting M, Kullberg BJ, Netea MG, Joosten LAB. (2010) Activation of innate host defense mechanisms by *Borrelia*. European Cytokine Network 21(1): 7-18.

Burgdorfer W, Barbour AG, Benach JL, Grunwaldt E, Davis JP. (1982) Lyme Disease – A tick-borne spirochetosis? Science 18: 1317-1319.

Burgess EC, Mattison M. (1987) Encephalitis associated with *Borrelia burgdorferi* infection in a horse. Journal of American Veterinary Medicine Association 191: 1457-1458.

Butler CM, Howers DJ, Jongejan F, Van Der Kolk JH. (2005) *Borrelia burgdorferi* infections with special references to horses. A review. Veterinary Quartely 27(4): 146-156.

Chang YF, Novosol V, Mcdonough SP, Chang CF, Jacobson RH, Divers T, Quimby FW, Shun S, Lein DH. (2000) Experimental infection of ponies with *Borrelia burgdorferi* by exposure to Ixodid ticks. Veterinary Pathology 37: 68-76.

Chang YF, Ku YW, Chang CF, Chang CD, Mcdonough SPP, Divers T, Pough M, Torres A. (2005) Antibiotic treatment of experimentally *Borrelia burgdorferi*-infected ponies. Veterinary Microbiology 107: 285-294.

Chandrashekar R, Daniluk D, Moffit S, Lorentzen L, Williams J. (2008) Serologic diagnosis of equine borreliosis: evaluation of an in-clinic enzyme-linked immunosorbent assay (SNAP 4Dx). Internal Journal of Applied Veterinary Medicine 6(3): 145-150.

Clark K L, Leydet B, Hartman S. (2013) Lyme borreliosis in human patients in Florida and Giorgia, USA. International Journal of Medical Sciences 10(7): 915-931.

Corradi DA, Carvalho VM, Coutinho SD. (2006) Anticorpos para *Borrelia burgdorferi* em indivíduos que trabalham com animais silvestres. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 58(5): 966-968.

Costa IP, Bonoldi VLN, Yoshinari NH. (2001) Perfil clínico e laboratorial da Doença de Lyme-símile no estado do Mato Grosso do Sul: análise de 16 pacientes. Revista Brasileira de Reumatologia 41: 142-150.

Divers TJ, Chang YF, Jacobson RH, Mcdonough SP. (2001) Lyme disease in horses. Compendium for Continuim Education for the Practising Veterinary 23: 375-380.

Divers T, Chang YF, Mcdonough PL. (2003) Equine Lyme disease: a review of experimental disease production, treatment efficacy and vaccine protection. Anais eterônicos... 49th *Annual Convention of the American Association of Equine Practioners*. http://www.ivis.org/proceedings/aaep/2003/divers2/ivis.pdf

Divers TJ, Grice AL, Mohamed HO, Glaser AL, Wagner B. (2012) Changes in *Borrelia burgdorferi* ELISA antibody over time in both antibiotic treated and untreated horses. Acta Veterinaria Hungarica 60(4): 421-429.

Fonseca AH, Ishikawa MM, Soares C. (1996) Lyme borreliosis serology in cattle in Brasil. Revista da Univesidade Rural do Rio de Janeiro 18: 85-89.

Fonseca AH, Salles RS, Salles SAN, Madureira RC, Yoshinari NH. (2005) Borreliose de Lyme símile: uma doença emergente e relevante para a dermatologia no Brasil. Anuário Brasileiro de Dermatologia 80(2): 171-178.

Gall Y, Pfister K. (2006) Survey on the subject of Lyme borrelioisis. International Journal of Medical Microbiology 296(s1): 274-279.

Galo KR, Fonseca AH, Madureira RC, Barboza Neto J. (2009) Frequência de anticorpos homólogos anti-*Borrelia burgdorferi* em equinos na mesorregião metropolitana de Belém, Estado do Pará. Pesquisa Veterinária Brasileira 29(3): 229-232.

Gonçalves DD, Carreira T, Nunes M, Benitez A, Lopes-Mori FMR, Vidotto O, Freitas JC, Vieira ML. (2013) First record of *Borrelia burgdorferi* B31 strain Dermacentor nitens ticks in the northern region of Parana (Brazil). Brazilian Journal of Microbiology 44(3): 883-887.

Goncalves DD, Moura RA, Nunes M, Carreira T, Vidotto O, Freitas JC, Vieira ML. (2015) *Borrelia burgdorferi* sensu lato in humans in a rural área of Paraná State, Brazil. Brazilian Journal of Microbiology 46(2): 571-575.

Gouveia EA, Alves MF, Mantovani E, Oyafuso LK, Bonoldi VN, Yoshinari NH. (2010) Profile of patients with Baggio-Yoshinari syndrome admitted at “Instituto de Infectologia Emilio Ribas”. Revista do instituto de Medicina Tropical de São Paulo 52(6): 297-303.

Guedes Junior DS, Araujo FR, Silva FJM, Rangel CP, Barbosa Neto JD, Fonseca AH. (2008) Frequency of antibodies to *Babesia bigemina*, *Anaplasma marginale*, *Trypanosoma vivax* and *Borrelia burgdorferi* in cattle from the northeastern region of the state of Pará, Brazil. Revista Brasileira de Parasitologia Veterinária 17(2): 105-109.

Hahn CN, Mayhew IG, Whitwell KE, Smith KC, Carey D, Carter SD, Read RA. (1996) A possible case of Lyme borreliosis in a horse in the UK. Equine Veterinary Journal 28: 84-88.

Huang W, Ojami C, Fallon JT, Travisany D, Maass A, Ivanova L, Tomova A, Gonzales-Acuña D, Godfrey HP, Cabello F. (2015) Genome sequence of *Borrelia chilensis* VA1, a South American member of the Lyme Borreliosis group. Genome Announcements 3(1): 1-2.

Imai DM, Barr BC, Daft B, Bertone JJ, Feng S, Hodzic E, Johnston JM, Olsen KJ, Barthold SW. (2011) Lyme neuroborreliosis in 2 horses. Veterinary Pathology 48(6): 1151-1157.

Ivanova LB, Tomova A, Gonzales-Axuña D, Murúa R, Moreno CX, Hernández C, Cabello J, Cabello C, Daniels TJ, Godfrey HP, Cabello FC. (2014) *Borrelia chilensis*, a new member of the *Borrelia burgdorferi* sensu lato complex that extends the range of this genospecies in the Southern Hemiesphere. Environmental Microbiology 16(4): 1069-1080.

James FM, Engiles JB, Beech J. (2010) Meningitis, cranial neuritis and radiculoneuritis associated with *Borrelia burgdorferi* infection in a horse. Jounal of American Veterinary Medicine Association 237(10): 1180-1185.

Kenedy MR, Lenhart TR, Akins DR. (2012) The role of *Borrelia burgdorferi* outer surface proteins. Immunology & Medical Microbiology 66: 1-19.

Koedel U, Fingerie V, Pfister HW. (2015) Lyme neuroborreliosis – epidemiology, diagnosis and management. Nature Reviews: Neurology, advance online publication: 1-11. http://dx.doi.org/10.1038/nrneurol.2015.121.

Kurtenbach K, Hanicová K, Tsao JI, Margos G, Fish D, Ogden NH. (2006) Fundamnetal process in the evolutionary ecology of Lyme borreliosis. Nature Reviews: Microbiology 4: 660-669.

Madureira RC, Correa FN, Cunha NC, Guedes Junior DS, Fonseca AH. (2007) Ocorrência de anticorpos homólogos anti-*Borrelia burgdorferi* em equinos em propriedades dos municípios de Três Rios e Vassouras, estado do Rio de Janeiro. Revista Brasileira de Ciência Veterinária 14(1): 43-46.

Magnarelli LA, Anderson JF, Schreier AB, Ficke CM. (1987) Clinical and serologic studies of canine borreliosis. Journal of American Veterinary Medicine Association 191: 1089-1094.

Magnarelli LA, Anderson JF, Shaw E, Post JE, Palka FC. (1988) Borreliosis in equids in northeastern United States. American Journal of Veterinary Research 49: 359-362.

Magnarelli LA, Anderson JF. (1989) Class-specific and polyvalent enzyme-linked immunobsorbent assays for detection of antibodies to *Borrelia burgdorferi* in equids. Journal of American Veterinary Medicine Association, 195: 1365-1368.

Magnarelli LA, Fikrig E. (2005) Detection of antibodies to *Borrelia burgdorferi* in naturally infected horses in the USA by enzyme-linked immunosorbent assay using whole-cell and recombinant antigens. Research in Veterinary Science 79: 99-103.

Manion TB, Khan MI, Dinger J, Bushmich SL. (1998) Viable *Borrelia burgdorferi* in the urine of two clinically normal horses. Journal of Veterinary Diagnostic Investigation 10: 196-199.

Mantovani E, Costa IP, Gauditano G, Bonoldi VL, Higuchi ML, Yoshinari NH. (2007) Description of Lyme-like disease in Brazil. It is a new tick borne disease or Lyme disease variation. Brazilian Journal of Medicine and Biology Research 40: 443-456.

Mantovani E, Maragoni RG, Gauditano G, Bonoldi VLN, Yoshinari NH. (2012) Amplification of the flgE gene provides evidece for the existence of a brazilian borreliosis. Revista do Instituto de Medicina Tropical 54(3): 153-157.

Mason LMK, Veerman CC, Geitenbeek TBH, Hovius JWR. (2014) Ménege à trois: *Borrelia*, dendritic cells and tick saliva interactions. Trends in Parasitology 30(2): 95-103.

Mast WE, Burrows WM. (1976) Erythema chronicum migrans and “lyme arthritis”. Journal of American Medical Association 236(21): 2392.

Nava S, Barbieri AM, Maya L, Colina R, Mangold AJ, Labruna MB, Venzal JM. (2014) *Borrelia* infection in *Ixodes pararicinus* ticks (Acari: Ixodidae) from northwestern Argentina. Acta Tropica 139:1-4.

Passamonti F, Veronesi FV, Cappeli K, Capomaccio S, Reginato A, Miglio A, Vardi DM, Steffanetti V, Coletti M, Bazicca C, Pepe M. (2015) Polysynovitis in a horse due to *Borrelia burgdorferi* sensu lato infection – Case study. Annals of Agricultural and Environmental Medicine 22(5): 247-250.

Priest HL, Irby NL, Schlafer DH, Divers TJ, Wagner B, Glaser AL, Chang YF, Smith MC. (2012) Diagnosis of *Borrelia*-associated uveitis in two horses. Veterinary Ophtalmology 15(6): 398-405.

Radof JD, Caimano MJ, Stevenson B, Hu LT. (2012) Of ticks, mice and men: understanding the dual host lifestyle of Lyme disease spirochaetes. Nature Reviews: Microbiology 10: 87-99.

Rezende J, Rangel CP, Cunha NC, Fonseca AH. (2012) Primary embrionic cells of *Rhipicephalus microplus* and *Amblyomma cajennense* ticks as a substrate for the development of *Borrelia burgdorferi* (strain G39/40). Brazilian Journal of Biology 72(3): 577-582.

Rostami A, Zaeemi M, Piazhak N, Fard RMN. (2011) A case report of atypical borreliosis in a dog. Iranian Journal of Veterinary Science and Technology 3(2): 49-56.

Rudenko N, Golovchenko M, Grubhoffer L, Oliver Jr JH. (2011) Updates on *Borrelia burgdorferi* senso lato complex with respect to public health. Ticks and Tick Borne Diseases 2(3):123-128.

Salles RS. (2001) Borreliose de Lyme em equinos no estado do Rio de Janeiro. 104 f. Thesis - Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ.

Salles RS, Fonseca AH, Scofield A, Madureira RC, Yoshinari NH. (2002) Sorologia para *Borrelia burgdorferi* lato sensu em equinos no estado do Rio de Janeiro. A Hora Veterinária 22: 46-49.

Samuels DD, Radolf JD. (2010) Borrelia: Molecular biology, host interaction and pathogenesis. Caister Academic Press.

Santos M, Haddad Jr V, Ribeiro-Rodrigues R, Talhari S. (2010a) Borreliose de Lyme. Anuario Brasileiro de Dermatologia 85(6): 930-938.

Santos M, Ribeiro-Rodrigues R, Talhari C, Ferreira LCL, Zelger B, Talhari S. (2010b) Presence of *Borrelia burgdorferi* sensu lato in patients with morphea from the Amazonic region in Brazil. Tropical Medicine Rounds 50: 1373-1378.

Shinjo SK, Gauditano G, Marchiori PE, Bonoldi VLN, Costa IP, Mantovani E, Yoshinari NH. (2009) Manifestação neurológica na síndrome de Baggio-Yoshinari (Síndrome brasileira semelhante à doença de Lyme). Revista Brasileira de Reumatologia 49(5): 492-505.

Skarda J. (2005) Lyme borreliosis, the present diagnostic criteria. Folia Veterinaria 49: s33-s34.

Stanek G, Wormser GP, Gray J, Strele F. (2012) Lyme borreliosis. The Lancet 379: 461-473.

Steere AC, Malawista SE, Snydman DR, Shope RE, Andiman WA, Ross MR, Steele FM. (1977) Lyme arthritis: and epidemic oligoarticular arthritis in children and adults in three connecticut communities. Arthritis Rheummatoids 20(1): 7-17.

Talhari S, Santos MNS, Talhari C, Ferreira LCL, Silva JR RM, Zelger B, Massone C, Ribeiro-Rodrigues RR. (2010) *Borrelia burgdorferi* sensu lato in Brazil: Occurrence confirmed by immunohistochemistry and focus floating microscopy. Acta tropica 115: 200-204.

Tilly K, Krum JG, Bestor A, Jewett MW, Grimm D, Bueschell D, Byram R, Doward D, Vanraden M, Stewart P, Rosa P. (2006) *Borrelia burgdorferi* OspC protein required exclusively in a crucial early stage of mammalian infection. Infection and Immunity 4(6): 3554-3564.

Trevejo RT, Krause PJ, Sikand VK, Schriefer ME, Ryan R, Lepore T, Porter W, Dennis DT. (1999) Evaluation of two-test serodiagnostic method for early Lyme disease in clinical practice. Journal of Infectious Diseases 179: 931-938.

Yoshinari NH, Steere AC, Cossermelli W. (1989) Revisão da borreliose de Lyme. Revista da Associação Medica Brasileira 35: 34-38.

Yoshinari NH, Barros PJL, Bonoldi VLN. (1997) Perfil da borreliose de Lyme no Brasil. Revista do Hospital das Clinicas da Faculdade de Medicina de São Paulo 52: 111-117.

Yoshinari NH, Barros PJL, Gaurditano G, Fonseca AH. (1999) Reporto of 57 cases of Lyme-like disease in Brazil. Arthitis Rheumatoids 43: 1888.

Yoshinari NH, Bonoldi VLN, Barros-Battesti DM, Schumacher TTS. (1999b) Doença de Lyme-símile no Brasil. Revista Brasileira de Reumatologia 39: 57-58.

Yoshinari NH, Mantovani E, Bonoldi VLN, Maragoni RG, Gauditano G. (2010) Doença de Lyme-símile brasileira ou síndrome Baggio-Yoshinari: zoonose exótica e emergente transmitida por carrapatos. Revista da Associação Médica Brasileira 56(3) 363-369.

Wagner B, Goodman LB, Rollins A, Freer HS. (2013) Antibodies to OspC, OspF and C6 antigens as indicators for infection with *Borrelia burgdorferi* in horses. Equine Veterinary Journal 45: 533-537.

Wasiluk A, Zalewsksa-Szajda B, Waszkiewicz N, Kepka A, Szajda DS, Wojewódzka-Zelezniakowicz M, Ladny JR, Pancewicz S, Zwiersz ZW, Zwierz K. (2011) Lyme disease: etiology, pathogenesis, clinical courses, diagnostics and treatment. Progress in Health Sciences 1(2): 179-186.

3. ARE THE CLINICAL SIGNS OF LYME BORRELIOSIS REALLY UNDERSTOOD IN HORSES?

Paper published as Editorial in *IMMUNOGENETICS: OPEN ACCESS* in October 21, 2015. http://dx.doi.org/IGOA-1000e101

Are the Clinical Signs of Lyme Borreliosis Really Understood in Horses?

Roberta Carvalho Basile

3.1 EDITORIAL

Lyme disease is the most common zoonosis transmitted by ticks in North America and Europe, which is caused by *Borrelia burgdorferi* sensu lato [1]. It has also diagnosed in Asia [2, 3], Africa [4] and South America [5, 6, 7, 8]. This kind of borreliosis is transmitted to mammals by ticks exposure to nymph or adult stage as far as a 24 hours adhesion in the host is established, which ensures the regulation of outer surface proteins that are responsible for their survival against the host immune system [9].

The disease was first described in horses by Van Heerden and Reyers in 1984 [10] and since then many studies have been reported the occurrence of clinical signs associated with seropositive horses. These signs are often nonspecific and attributed to horses by analogy of the disease in humans, such as stiffness, lameness, myopathies, back soreness, lethargy, fever, swelling of limbs, encephalitis and behavioral changes [9]. There are also some reports of concomitant uveitis and

presence of *Borrelia* in the ocular chamber [11] and pseudolymphoma responsive to treatment with doxycycline [12].

However, there is only one work experimentally dedicated to the description of the disease in horses [13]. In this study, ponies were experimentally infected by exposure to ticks containing *Borrelia burgdorferi* and they were observed for 9 months. Afterwards, they were carried out to euthanasia for molecular detection of the agent in various tissues. Moreover, ponies had showed detectable antibodies from five to six weeks followed the exposure, although they have not presented relevant clinical signs at any moment.

In this uncertain scenario, questions begin to be risen through the scientific community regarding the significance of Lyme disease in horses and the real importance of horses in the dissemination of the etiologic agent [14]. The set of scientific papers in this area is mostly composed by reports of individual cases, serological surveys and agent molecular researches.

The Lyme borreliosis will be only better understood in horses after presentation of clinical epidemiologic studies and their correlated risk factors, in addition to controlled experimental infections aiming a more precise description on how the disease evolves in horses.

3.2 REFERENCES

[1] Koedel U, Fingerie V, Pfister HW. (2015) Lyme neuroborreliosis – epidemiology, diagnosis and management. Nature Reviews: Neurology, advance online publication: 1-11. http://dx.doi.org/10.1038/nrneurol.2015.121.

[2] Hou X, Xu J, Hao Q, Xu G, Geng Z, Zhang L. (2014) Prevalence of *Borrelia burgdorferi* sensu lato in rodents from Jiangxi, southeasterb China region. Internal Journal of Clinical Experimental Medicine 7: 5563-5567.

[3] Fukunaga M, Takahashi Y, Tsuruta Y, Matsushita O, Ralph D, McClelland M, Nakao M. (1995) Genetic and phenotypic analysis of *Borrelia miyamotoi* sp isolated from Ixodid ticks *Ixodes persulcatus*, the vector of Lyme disease in Japan. International Journal of Systematic Bacteriology, 804-810.

[4] Mediannikov O, Abdissa A, Socolovschi C, Diatta G, Trape JF, Raoult D. (2013) detection of a new *Borrelia* species in ticks taken from cattle in Southwest Ethiopia. Vector-Borne and Zoonotic Diseases 13: 266-269.

[5] Gonçalves DD, Moura RA, Nunes M, Carreira T, Vidotto O, Freitas JC, Vieira ML. (2015) *Borrelia burgdorferi* sensu lato in humans in a rural área of Paraná State, Brazil. Brazilian Journal of Microbiology 46(2): 571-575.

[6] Ivanova LB, Tomova A, Gonzales-Axuña D, Murúa R, Moreno CX, Hernández C, Cabello J, Cabello C, Daniels TJ, Godfrey HP, Cabello FC. (2014) *Borrelia chilensis*, a new member of the *Borrelia burgdorferi* sensu lato complex that extends the range of this genospecies in the Southern Hemiesphere. Environmental Microbiology 16(4): 1069-1080.

[7] Nava S, Barbieri AM, Maya L, Colina R, Mangold AJ, Labruna MB, Venzal JM. (2014) *Borrelia* infection in *Ixodes pararicinus* ticks (Acari: Ixodidae) from northwestern Argentina. Acta Tropica 139:1-4.

[8] Barbieri AM, Venzal JM, Marcilia, Almeida AP, Gonzales EM, Labruna MB. (2013) *Borrelia burgdorferi* sensu lato infecting ticks of the *Ixodes ricinus* complex in

Uruguay: First report for the Southern Hemisphere. Vector-Borne and Zoonotic Diseases 13(3): 147-153.

[9] Divers TJ. (2013) Equine Lyme disease. Journal of Equine Veterinary Science 33:488-492.

[10] Van Heerden J, Reyers F. (1984) *Borrelia* sp. infection in a horse. Journal of South African Veterinary Association 55: 41-43.

[11] Priest H, Irby N, Schlafer D. (2012) Diagnosis of *Borrelia*-associated uveitis in two horses. Veterinary Ophtalmology 15: 398-405.

[12] Sears KP, Divers TJ, Neff RT. (2012) A case of *Borrelia*-associated cutaneous pseudolymphoma in a horse. Veterinary Dermatology 23: 153-156.

[13] Chang YF, Novosol V, McDonough SP, Chang CF, Jacobson RH, Divers T, Quimby FW, Shin S, Lein DH. (2000) Experimental infection of ponies with *Borrelia burgdorferi* by exposure to Ixodid ticks. Veterinary Pathology 37: 68-76.

[14] Bartol J. (2013) Is Lyme disease overdiagnosed in horses? Equine Veterinary Journal 45: 529-530.

4. CLINICAL, HEMATOLOGICAL AND RISK FACTORS ASSOCIATED WITH BORRELIOSIS IN HORSES FROM SAO PAULO, BRAZIL

Paper submitted to *ACTA TROPICA* in 04/12/2015.

Clinical, hematological and risk factors associated with borreliosis in horses from Sao Paulo, Brazil

Roberta Carvalho Basile, Mariana Rodrigues Vieira, Lara Antoniassi Del Rio, Talissa Camargo de Bonis, Gabriel Paiva Domingues do Amaral, Ana Paula Reiff Janini, Elenice Mantovani, Virgínia Nazario Bonoldi, Natalino Hajime Yoshinari, Vando Edésio Soares, Delphim da Graça Macoris, Antonio de Queiroz Neto

ABSTRACT

Lyme borreliosis is caused by *Borrelia burgdorferi* sensu lato and affects humans and many other mammals, including horses. This disease is poorly studied and reported in horses, and epidemiological surveys are required to provide more precise information about the course of the disease. The aims of the present study were to determine the prevalence of seropositive horses for *Borrelia burgdorferi* sensu lato in São Paulo State, Brazil and to collect data on possible risk factors associated with the disease along with clinical and hematological changes in seropositive horses. It was verified that there was a high correlation between the occurrence of seropositive horses infested with *Amblyomma sculptum* ticks and the presence of capybaras on the property as well as the occurrence of abortion and retained placenta in mares. Hematological changes include increase in neutrophils, lymphopenia and increased creatine phosphokinase. Borreliosis in horses from São Paulo, Brazil can be

associated with presence of *Amblyomma scupltum* ticks, proximity with capybaras and can be manifested as alterations in reproduction of mares.

KEYWORDS: *Borrelia burgdorferi*, *Amblyomma sculptum*, capybaras, reproduction, lymphopenia.

4.1 INTRODUCTION

Lyme disease is the most common infectious disease transmitted by ticks to humans and is caused by *Borrelia burgdorferi* spirochetes (Koedel et al., 2015). The group of *B. burgdorferi* sensu lato (s.l.) comprises at least 20 genospecies of related bacteria, including *B. burgdorferi* stricto sensu (s.s.), *B. afzelii, B. garinii, B. bavariensis, B. bissetti, B. kurtenbachii, B. lusitaniae, B. chilensis, B. japonica, B. sinica, B. americana* and others (Nava et al., 2014). Most *B. burgdorferi* s.l. species are related to hard ticks of the genus *Ixodes*, especially the *Ixodes ricinus* complex. However, there are reports supporting the occurrence of that *Borrelia spp.* in *Amblyomma americanum* (Clark et al., 2013) and *Dermacentor nitens* (Gonçalves et al., 2013).

Lyme borreliae are carried in the midgut of unfed ticks. When an infected tick acquires a blood meal over at least 18 hours of attachment, the number of spirochetes increases, and the spirochetes express outer surface proteins that support their survival in the vertebrate host. These bacteria migrate from the midgut to salivary glands and are carried into the animal (Stanek et al., 2012).

In humans, acute infection generally causes skin inflammation at the site of a tick bite, referred to as erythema migrans, and may be accompanied by systemic symptoms such as fever, muscle and joint pain, headache, enlargement of the lymph nodes and neurological symptoms (Wasiluk et al., 2011). In horses, a broad

spectrum of clinical manifestations have been attributed to *B. burgdorferi* infections including arthritis, lameness, muscle tenderness, anterior uveitis, encephalitis, abortion, foal mortality, low-grade fever and lethargy (Butler et al., 2005).

The first report of suspected borreliosis in humans from Brazil came from Yoshinari et al. (1989). However, the first case in the country was diagnosed in 1992. An increasing number of identified cases have demonstrated differences between the symptoms described for the Northern Hemisphere disease versus that occurring in Brazil (Yoshinari et al., 1997; Costa et al., 2001). Concerning epidemiology, the occurrence of *Ixodes* ticks is insufficient to allow them to be classified as main vectors in Brazil. Clinically, in addition to the identification of patients with erythema migrans and the usual systemic complications, the Brazilian disease presents many recurrences, autoimmune pathogenesis and challenges in treatment (Mantovani et al., 2007). The spirochete *B. burgdorferi* (s.l.) has been identified in skin biopsies from 22 human patients from Amazonas via immunohistochemistry and focus floating microscopy (Talhari et al., 2010), showing a cystic form without flagelae. Due to those differences, this borreliosis is called Baggio-Yoshinari Syndrome in Brazil (Yoshinari et al., 1997).

Equine borreliosis is still poorly diagnosed in horses from Brazil, and only three studies have been conducted to describe the disease in native horses. Salles et al. (2002) found that an average of 9.8% of horses from Rio de Janeiro State exhibited anti-*B. burgdorferi* (s.l.) antibodies. Madureira et al. (2007) reported that 28.4% of horses showed seropositivity in Minas Gerais State, and Galo et al. (2009) diagnosed the disease in 26.7% of horses in Pará State.

The aims of the present study are to present the prevalence of seropositive horses in the cities of São Paulo state with the most registered suspected cases of Lyme disease in humans and to present the main clinical, hematological and risk factors associated with seropositive horses.

4.2 MATERIAL AND METHODS

This study was approved by the Univ. Estadual Paulista Ethics and Welfare Committee (CEUA) in document no. 001968/13.

4.2.1 Epidemiologic assessment

Animal samples

The minimum sample size for the survey of serological prevalence was calculated using the methodology proposed by Thrusfield (2007) to determine the number of horses in groups in two stages (geographic regions and equine properties) with a confidence level of 95%.

The calculation of the minimum number of animals (Ts) followed the model:

$$Ts = \frac{1{,}96^2 . g . P_{esp}(1 - P_{esp})}{g . d^2 - 1{,}96^2 . V_c} \qquad (1)$$

where

$$V_c = c[\frac{c . V . K_1}{T^2 (c - 1)} - \frac{K_2 . \hat{P}(1 - \hat{P})}{T}] \qquad (2)$$

$$V = (\hat{P}^2 . n^2)\Sigma\{(2 . \hat{P}) . \Sigma nm\} + \Sigma m^2 \qquad (3)$$

following

Ts: minimum animal sample

g: number of groups (cities x properties)

P_{esp}: constant, which is 0.10 for a confidence level of 95%

d: constant error of 0.05

c: amount of properties from a previous survey (Salles et al., 2002)

K_1: constant value of 1.0 because the number of sampled groups is far below the number of population groups

K_2: constant value of 1.0 because the number of animals sampled is much lower than the population of the animals

n: number of animals per property (Salles et al., 2002)

m: number of seropositive animals (Salles et al., 2002)

T: sum of n

$\hat{P}$: m/n

The geographic regions to be assessed in the equine survey were determined based on the occurrence of recent (less than 10 years) suspected cases of human Lyme disease diagnosed by the Rheumatology Laboratory of the Medicine Faculty of the University of Sao Paulo. Eleven cities were chosen in Sao Paulo State, and two properties were randomly chosen per city. The number of horses that was distributed in each city was proportional to the local population of horses and the number of humans suspected of Lyme disease (presence of anti-*B. burgdorferi* immunoglobulins, specific clinical signs and a recent report of a tick bite). Only adult horses were selected for this survey, with their ages varying between 3 and 20 years.

Serology: Indirect ELISA

Immunoglobulins, specifically IgG, against *B. burgdorferi* sensu lato strain G39/40, were detected in accordance with ELISA procedures previously described [20, 21]. Briefly, the preparation of the antigen included sonicating a whole spirochete suspension, which was made from a culture of *B. burgdorferi* organisms grown in a

500 ml bottle containing Kelly's medium at 33ºC at higher-phase growth. The medium was centrifuged at 10,000 g for 20 min at 4ºC, and the pellets were washed 3 times with cold 0.01 M phosphate-buffered saline (PBS) with 5 mM magnesium chloride (pH 7.4). The suspension of spirochetes was sonicated on ice with a cell sonicator, and the supernatant was filtered (45 µm membrane). The protein content was determined by Folin's method, and the antigen preparations were stored in aliquots at -70ºC until further analysis.

Polystyrene plates with 96 holes[1] were coated with antigen at a concentration of 20 mg/mL, incubated in a humidified chamber overnight at 4°C, washed with PBS Tween 20 buffer and then blocked with 1% rabbit serum. The positive control sera were obtained from horses administered 4 serial immunizations over 15 days, and the negative control serum was obtained from 8 healthy horses without history of exposure to ticks. The test and control sera (8 negative and one positive) were diluted 1:800 in PBS Tween 20, incubated and washed. Conjugated rabbit anti-horse IgG linked to alkaline phosphatase[2] was added, incubated and washed. Next, the solution of PNPP[3] diluted in glycine buffer with a pH 10.5 was added, and the samples were read using a spectrophotometer[4] at a wavelength of 405 nm. The cutoff line was established at a confidence level of 99.99%, according to the mean plus three standard deviations of the optical density of the negative controls.

Horse management survey

Concerning the epidemiologic survey, the following questionnaire was presented to the owners:

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[1]Bio-Rad Laboratories
[2]Sigma Chemical
[3]Sigma Chemical
[4]BioRad Laboratories, model 550 Microplate Reader

- Type of stabling: stall (score 0), paddock (score 1) or pasture (score 2),

- Presence of *Amblyomma sculptum* ticks on the horses: present (score 1) or absent (score 0),

- Presence of capybaras (*Hydrochoerus hydrochaeris*) on the property: present (score 1) or absent (score 0).

Medical history assessment

In the same questionnaire, the owners were encouraged to provide information concerning the last 5 years of medical history only for well-known horses. The medical history inquiries included arthritis, myositis, lameness, ataxia, uveitis, abortion, placental retention, back pain and recurrent hemoparasitosis, adopting a score of 1 for presence of the clinical sign and a score of 0 for its absence.

4.2.2 Case-Control clinical trial

Animals

Twenty seropositive horses (Case group) and twenty seronegatives (Control) were chosen for this evaluation. The Case group was composed by adult horses (mares, geldings and stallions aged between 3 and 20 years), in activities of sport or reproduction, from 3 distinct horse farms, that exhibited ELISA titers between 1/400 and 1/3,200 and had been exposed to *Amblyomma sculptum* (Nava et al., 2014) and/or *Dermacentor nitens* ticks. The negative control group was composed by 20 adult horses (mares, geldings and stallions), aged in the same range of Case group, retired or in sport activities, from a single horse farm in which all horses were seronegative due to a severe control of ticks in horses and installations since 2005.

Clinical assessment

All 40 horses were clinically evaluated by a veterinarian. The physical examination included assessment of the color of mucosa, cardiac rhythm, capillary perfusion, respiratory sounds, rectal temperature and dermatological alterations. Qualitative parameters were classified as 0 for normal and 1 for abnormal.

Hematology

Blood samples were collected to evaluate erythrocytes, white blood cells, platelets, bilirubin, creatine phosphokinase, creatinine, gamma glutamyl transferase, total protein, albumin, globulins, aspartate aminotransferase and blood urea nitrogen. The hematological tests were performed with a Poch®-100 IV DIFF automatic analyzer, and biochemical tests were performed with a Roche®COBAS MIRA Plus using Labtest® kits.

4.2.3 Statistical analysis

Nonparametric epidemiological data were verified by Kolmogorov-Smirnov test, analyzed by Chi-Square test at 95% significance and by Cluster Analysis multivariate test using the complete linkage rule and measurement based on Euclidean distances. The case-control parametric data were verified by ANOVA and analyzed with a t-Test with 95% significance and through a multivariate principal component analysis using information on the two major factors. All data were processed using the Statistica® v.12 software (StatSoft Inc., DELL Software).

4.3 RESULTS

4.3.1 Epidemiologic assessment

The methodology proposed by Thrusfield (2007) resulted in a minimum sample of 651 animals to provide sufficient data to estimate the prevalence of seropositivity among the horses in the 11 cities with registered cases of suspected

Lyme disease in humans. In this study, blood samples for serological ELISA tests were collected from 760 horses, distributed among 22 training centers or horse farms, which were randomly chosen. The macro-region (radius of 50 km from the city) formed by the cities of Ribeirão Preto, Jaboticabal and Analândia, located in the center of São Paulo State, presented the highest prevalence rates of seropositivity among horses (Table 1).

Table 1 – Distribution of horses according to their antibody titers against *B. burgdorferi* sensu lato, obtained through ELISA, in the 11 cities of São Paulo state, Brazil, with the most reported cases of suspected Lyme disease in humans.

		Antibody titer (ELISA)						
City	N	0	1/400	1/800	1/1600	1/3200	Horse prevalence	Human cases
São Paulo	10	10	0	0	0	0	0%	5
Sorocaba	30	30	0	0	0	0	0%	9
Bauru	30	28	1	1	0	0	7%	2
Campinas	96	89	4	2	1	0	7%	4
São José do Rio Preto	28	24	3	0	1	0	14%	44
Araraquara	30	25	2	3	0	0	17%	1
São José dos Campos	246	199	36	8	2	1	19%	30
Colina	98	72	18	6	2	0	27%	1
Ribeirão Preto	129	89	27	10	2	1	31%	29
Analândia	17	10	5	2	0	0	41%	1
Jaboticabal	46	24	15	6	1	0	48%	1
Total	760	600	111	38	9	2	21%	127

Considering the total of 760 horses, data about risk factors were obtained regarding the stabling type, presence of ticks in horses and capybaras in the properties, and recent clinical history (past 5 years) of a subgroup of 124 animals, representing approximately 15% of all tested horses. It should be noted that seropositivity for *B. burgdorferi* was directly related to infestation by ticks and the presence of capybaras on the property (Table 2).

Table 2 – Scoring points for the risk and clinical history (5 years) parameters of a subgroup of 124 adult horses (59 seronegatives and 65 seropositives for *B. burgdorferi*), stabled on 22 properties from 11 cities of São Paulo State.

	Seronegatives (n=59)		Seropositives (n=65)		p-value
	Mean	Std. Dev.	Mean	Std. Dev.	
Presence of ticks	**1.90**	**0.80**	**2.45**	**0.59**	**p < .003**
Presence of capybaras	**0.39**	**0.49**	**0.83**	**0.38**	**p < .001**
Idiopathic arthritis	0.08	0.28	0.05	0.21	p > .10
Recurrent myositis	0.08	0.28	0.00	0.00	p > .10
Idiopathic lameness	0.15	0.36	0.03	0.17	p > .10
Ataxia	0.08	0.28	0.02	0.12	p > .10
Recurrent uveitis	0.03	0.18	0.03	0.17	p > .10
Abortion	0.09	0.29	0.15	0.36	p > .10
Retained placenta	0.00	0.00	0.05	0.21	p > .10
Recurrent hemoparasitosis	0.00	0.00	0.03	0.17	p > .10
Back pain	0.03	0.18	0.00	0.00	p > .10

Lines in bold show differences between seronegative and seropositive horses according to the Chi-Square test compared to ELISA, with 95% significance.

Multivariate cluster analysis revealed parameters that pointed to a close inter-relationship with the presence of seropositivity, which included the following: ticks, capybaras, abortion and retained placenta, in which constituted Group A. Group B was composed of the type of stabling (stall, paddock or pasture), idiopathic lameness, back pain, arthritis, myositis, ataxia, uveitis and recurrent hemoparasitosis, which showed a weak inter-relationship with seropositive horses (Figure 1).

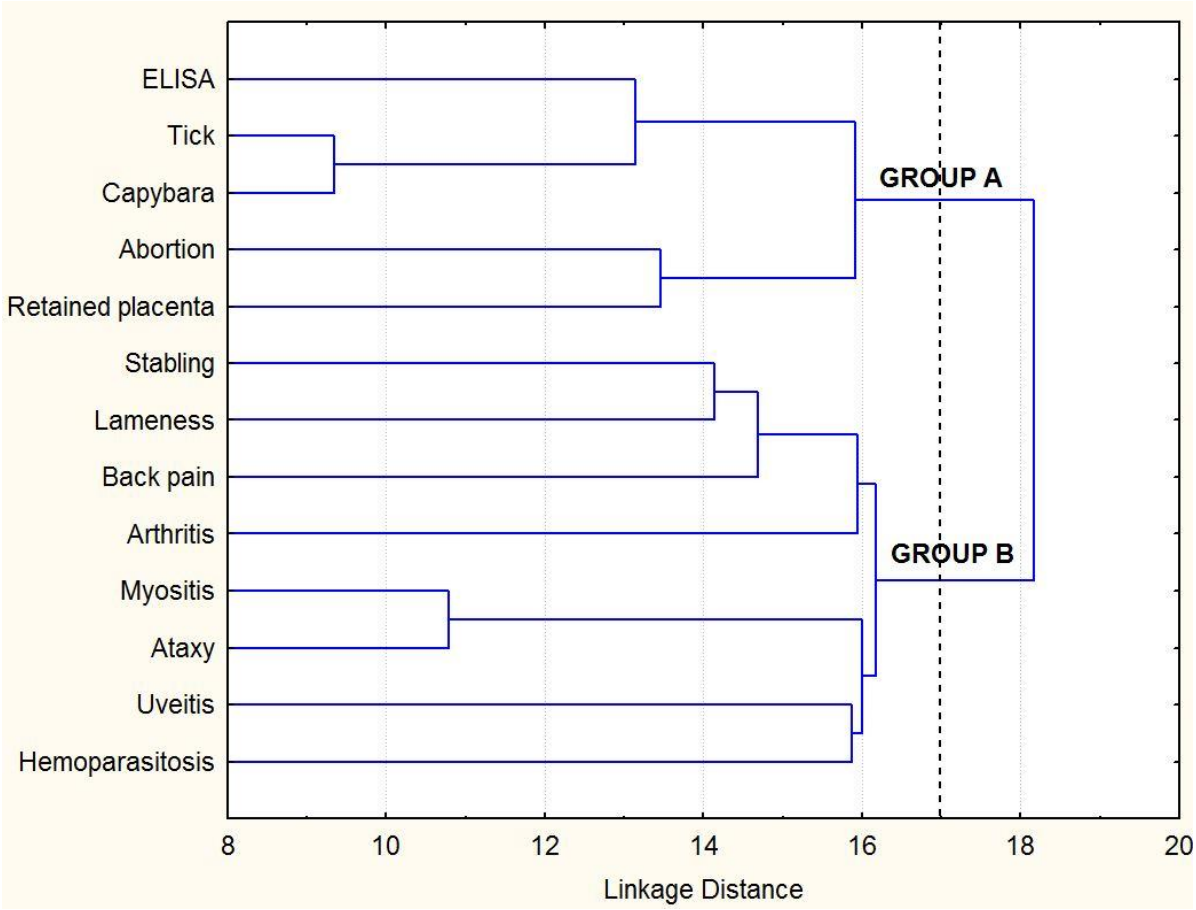

Figure 1 – Cluster analysis of the risk factors and clinical parameters of 124 adult horses (59 seronegatives and 65 seropositives for *B. burgdorferi*) stabled in 22 properties from 11 cities of São Paulo state.

Table 3 presents the difference of occurrence of seropositivity according the type of stabling of horses. The major cases of seropositive horses was stabled in stalls, when compared with paddocks or pasture. Additionaly, in stalls, it was verified more cases of seropositivity than seronegativity.

Table 3 – Number of cases of seropositivity and seronegativity for *B. burgdorferi* sensu lato in a subgroup of 291 horses from Sao Paulo, Brazil, according to the type of stabling.

ELISA	Stall	Paddock	Pasture	Total
Seronegative	$28^{A,a}$	$59^{B,a}$	$89^{C,a}$	176
Seropositive	$53^{A,b}$	$30^{B,b}$	$32^{B,b}$	115
Total	81	89	121	291

Values in columns followed by capital letters differs each other by Tukey's test with 95% of significance. Values in lines followed by lower case letters differs each other by Tukey's test with 95% of significance.

4.3.2 Case-control clinical trial

No changes in the color of mucous membranes, cardiac rhythm, capillary perfusion, respiratory sounds or rectal temperature or dermatological alterations were recorded in either group of animals. Hematological parameters showed a difference between the groups in terms of the packed cell volume, white blood cells, neutrophils (band and mature), lymphocytes, creatine phosphokinase, creatinine and aspartate aminotransferase, but all of these parameters were within the normal range (Table 4).

Table 4 – Hematological parameters of 20 seronegative and 20 seropositive horses in a case-control clinical trial, from São Paulo, Brazil.

Hematological parameters	Seronegatives (n=20)		Seropositives (n=20)		p-value	Reference values (Orsini and Divers, 2013)	
	Mean	Std. Dev.	Mean	Std. Dev.		Min	Max
Red blood cells, $x10^9$/mL	7.5	0.94	7.9	1.24	0.2134	6.8	12.9
Hemoglobin, g/dL	11.3	1.49	12.2	1.58	0.0638	11	19
Packed cell volume, %	**34.1**	**4.39**	**37.0**	**4.65**	**0.0497**	**32**	**53**
Mean corpuscular volume, fl	45.5	3.02	45.5	7.57	0.9935	37	58.5
Mean corpuscular hemoglobin, pg	15.1	1.06	15.5	1.38	0.2745	12.3	19.9
Mean corpuscular hemoglobin concentration, g/dl	33.1	0.76	33.0	0.44	0.6667	31	38.6
White blood cells, per mL	**8730.0**	**1095.49**	**9780.0**	**2029.42**	**0.0488**	**5400**	**14300**
Neutrophils (band), per mL	**211.7**	**59.61**	**396.7**	**320.85**	**0.0155**	**0**	**100**
Neutrophils (mature), per mL	**5223.8**	**859.18**	**6569.1**	**1428.74**	**0.0009**	**2300**	**8600**
Eosinophils, per µL	210.0	229.58	449.8	496.91	0.0574	0	1000
Lymphocytes, per mL	**2880.3**	**1102.36**	**2134.5**	**745.43**	**0.0166**	**1500**	**7700**
Monocytes, per mL	204.3	55.55	230.0	76.83	0.2338	0	1000
Basophils, per mL	0.0	0.00	0.0	0.00	1.0000	0	290
Platelets, per mL	168550.0	34841.71	161850.0	32472.30	0.5330	100000	600000
Bilirubin conjugated, mg/dL	0.2	0.05	0.3	0.10	0.0604	0	0.4
Bilirubin unconjugated, mg/dL	0.6	0.22	0.6	0.19	0.9939	0.2	2
Creatine phosphokinase, IU/L	**161.2**	**50.14**	**259.4**	**145.31**	**0.0069**	**119**	**287**
Creatinine, mg/dL	**1.3**	**0.14**	**1.1**	**0.27**	**0.0168**	**0.9**	**1.9**
Gamma-glutamyl transferase, IU/L	9.9	2.75	10.1	4.56	0.8676	4	44
Protein (total), g/dL	5.4	0.74	5.7	1.25	0.3730	5.8	8.7
Albumin, g/dL	2.2	0.31	2.1	0.58	0.3461	2.6	3.7
Globulin, g/L	3.2	0.62	3.7	0.82	0.0701	2.6	4
Aspartate aminotransferase, IU/L	**290.5**	**32.01**	**234.8**	**64.22**	**0.0013**	**226**	**336**
Urea, mg/dL	30.1	6.40	32.8	8.84	0.2757	21	51

Lines in bold show a difference between seronegative and seropositive horses based on a t-Test at 95% significance.

Multivariate analysis of principal components assembled in the same data quadrant, including serology ELISA, creatine phosphokinase, neutrophils, white blood cells, platelets, lymphocytes, monocytes, hematocrit, hemoglobin and erythrocytes (Figure 2), indicating a high degree of inter-relationship between these variables.

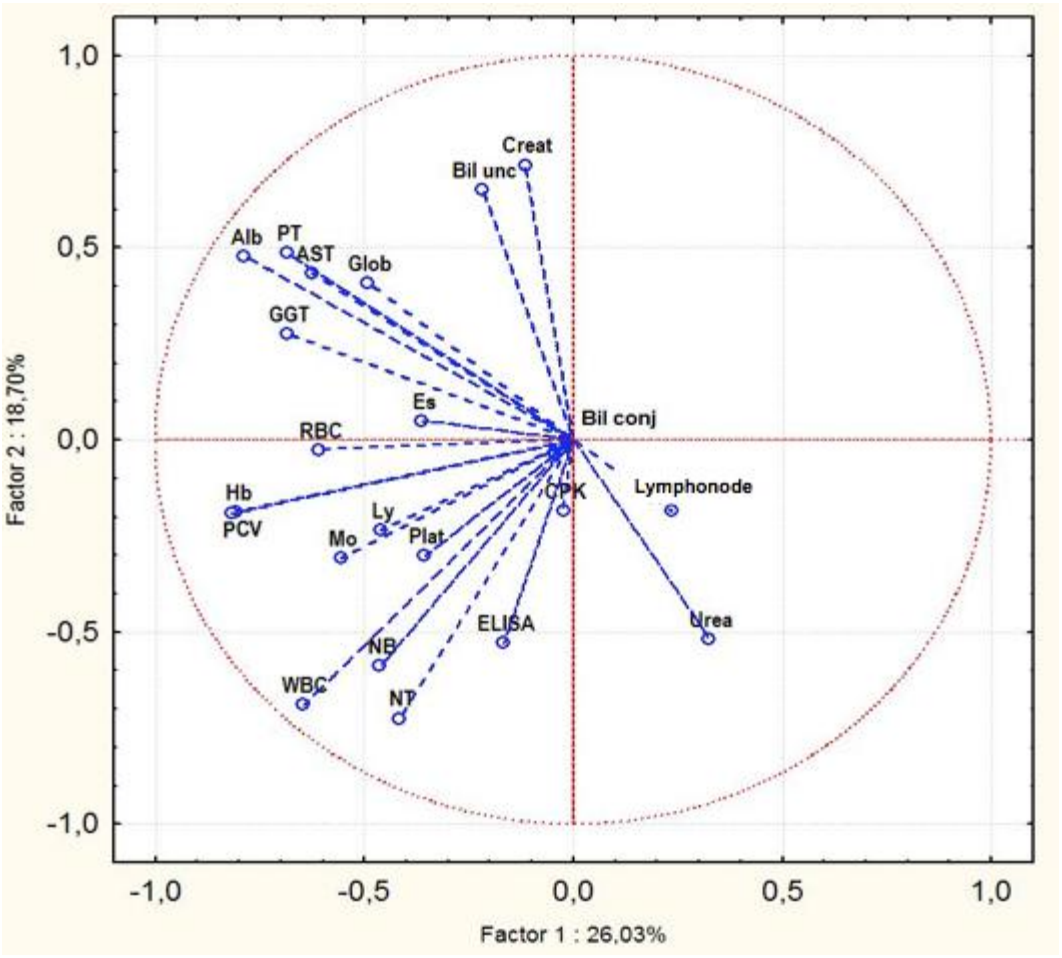

Figure 2 - Principal component analysis of the clinical and hematological parameters of 20 seropositive and 20 seronegative horses (immunoglobulins anti-*Borrelia burgdorferi*). Creat: creatinine, Bil unc: bilirubin unconjugated, Glob: globulins, AST: aspartate aminotransferase, PT: total protein, Alb: albumin, GGT: gamma glutamyl-transferase, Es: eosinophils, RBC: red blood cells, Hb: hemoglobin, PCV: packed cell volume, Mo: monocytes, Ly: lymphocytes, Plat: platelets, WBC: white blood cells, (cont.) (continued) NB:

neutrophils (band), NT: neutrophils (mature), ELISA: enzyme-linked immunosorbent assay, CPK: creatine phosphokinase.

4.4 DISCUSSION

The findings presented in this study challenge the findings about borreliois in horses published to date. Because of the scarcity of epidemiological studies that have clinically described the disease and its risk factors (Manion et al., 2001) and the fact that there are few studies involving experimental infection of horses (Chang et al., 2000; Chang et al, 2005), the clinical signs and risk factors attributed to horses are mostly supported by case reports (Manion et al., 1998; James et al., 2010; Imai et al., 2011; Sears et al., 2011; Priest et al., 2012; Passamonti et al., 2015), resulting in extrapolation of the clinical signs that have been detected in humans (Divers, 2013). These uncertainties may indicate that Lyme borreliosis could be overdiagnosed in horses (Bartol, 2013).

We note an intrinsic relationship between the presence of *Amblyomma sculptum* ticks, proximity to capybaras (*Hydrochoerus hydrochaeris*) and the incidence of seropositivity in the horses on the surveyed properties. Capybaras are the most important group of hosts for all parasitic stages of *Amblyomma sculptum* (Nava et al., 2014; Krawczak et al., 2014) in Brazil, and the southeastern portion of the country comprises an endemic area for the occurrence of both species (Szabó et al., 2013). Although there are no reports of the isolation of *Borrelia burgdorferi* from *Amblyomma sculptum*, a study identified the presence of this pathogen in *Amblyomma americanum* specimens obtained from human patients in the United States (Clark et al., 2013).

In Brazil, DNA fragments of *Borrelia burgdorferi* sensu lato strain B31 have been detected through molecular analysis of *Dermacentor nitens* ticks sampled from

equine ears (Gonçalves et al., 2013) in the southern region (Nested Polimerase Chain Reaction – PCR, targeting the 5S (rrf) 23S (rrl) intergenic spacer region, 99,9% of BLAST similarity with *B. burgdorferi* s.s.). Additionally, molecular detection of *Borrelia burgdorferi* sensu lato has been recorded in the blood of human patients from the same area (Nested PCR targeting the 5S (rrf) 23S (rrl) intergenic spacer region, 100% of BLAST similarity with *B. burgdorferi* s.s.) (Gonçalves et al., 2015).

Multivariate analysis of epidemiological data also showed a relationship between seropositivity and the occurrence of abortion and retained placentas in mares, besides it was not verified the occurrence of another most common diseases like herpes virus (EHV-1) and leptospirosis. This association of *B. burgdorferi* seropositivity and abortion shall be better evaluated in mares. There are reports of abortion associated with borreliosis in cattle (Parker and White, 1992) and humans infected by *Borrelia burgdorferi* sensu lato (Carlomagno et al., 1988; Markowitz et al., 1986; Shapiro, 2014). The exact pathogenesis of the *B. burgdorferi* acquired during pregnancy is undefined. There are doubts about if the bacteria colonize fetuses or placenta causing the negative pregnancy outcomes (Lakos and Solymosi, 2010).

In this study, the major seropositivity was identified in horses stabled in stalls, which contradicts the results reported by Manion et al. (2001), who observed a higher incidence of Borrelia-seropositive horses in pastures. This result is intriguing and suggests the possibility of existence of another vectors in Brazil besides ticks, like arthropods as *Stomoxys calcitrans*, very present in stalls and less frequent in pastures. *Borrelia afzelii* was isolated from *Aedes vexans* (Halouzka et al., 1998) and from *Culex pipiens* (Zakovska et al., 2006) in Czech Republic. The presence of the pathogen in the mosquitoes does not prove their participation in the epidemiological

cycle because they are hematophagous, but these results suggest the need of more researches in this field.

Clinical signs that are usually attributed to Lyme disease in horses, such as arthritis, uveitis, ataxia, lameness, myositis and recurrent hemoparasitosis (Divers, 2013) showed no relationship with seropositivity in our survey.

In the case-control study, despite all of the normal hematological parameters, a difference between leukocyte values was observed, characterized by light neutrophilia and lymphopenia in seropositive horses, in addition to hemoconcentration and a slight increase of creatine phosphokinase.

The increase in neutrophils can be explained by the innate immune response of the host system in contact with *Borrelia*. Although these bacteria are Gram negative, *B. burgdorferi* do not produce endotoxins (lipopolysaccharides) but express alternative proteins known as outer surface proteins (Osp's). These proteins act as pathogen-associated molecular patterns (PAMPs) that activate the innate immune system via toll-like receptors (TLRs). The recognition mediated by TLRs promotes an immune signaling cascade, involving chemokines, matrix metalloproteinases and a large subset of cytokines, including proinflammatory mediators and neutrophil attractants (Berende et al., 2010; Mason et al., 2014.).

Lymphopenia in *B. burgdorferi* infections is directly associated with the cytopathic mechanisms of these bacteria in T and B-lymphocytes. In an *in vitro* study using *Borrelia burgdorferi* sensu lato and human lymphocytes, it was observed that these spirochetes invade leukocytes approximately 1-2 hours after their first contact. Spirochetes adhere to more than 90% of lymphocytes and form invaginations in their membranes, similar to those formed in the pinocytosis mechanism. Then, the cells

are penetrated, and vacuoles were formed. Researchers have observed high mobility of bacteria in these vacuoles, indicating an absence of lysosomal action. Subsequent cell membrane rupture in numerous lymphocytes has been previously reported (Dorward et al., 1997).

An elevation of creatine phosphokinase has also been reported in humans (Holmgren and Matteson, 2006), and in most cases, only one group of muscles is affected by myositis. This inflammation and sarcomere rupture may be associated with extensive migration of borrelias to the connective tissue, the presence of high plasmocellular infiltration and degeneration of fibers (Reimers et al., 1993).

Epidemiological surveys can contribute to existing knowledge of the risk factors, clinical signs and pathogenesis of diseases. For equine Lyme borreliosis in particular, many clinical signs that have been associated with horses due to their occurrence in humans can be misinterpreted. Brazilian borreliosis still presents an obscure pathogenesis in horses, but this study shows that *Borrelia* should have an impact on the reproductive tract of mares. Capybaras and *Amblyomma sculptum* can play an important role in the biological cycle of *Borrelia burgdorferi* in São Paulo State, Brazil and their transmission to horses can be associated to another vectors besides ticks.

Acknowledgements

The authors acknowledge financial support from FAPESP (Process 2013/05871-0 and 2013/03732-2).

4.5 REFERENCES

Barros, P.S.L., 2000. Caracterização clínica e laboratorial da doença de Lyme no Brasil, através de métodos imunológicos e reação da cadeia de polimerase. [Thesis] São Paulo, Faculdade de Medicina da Universidade de São Paulo.

Bartol., J., 2013. Is Lyme disease overdiagnosed in horses? Equine Vet. J. 45, 529-530.

Berende, A., Oosting, M., Kullberg, B.-J., Netea, M.G., Joosten, L.A.B., 2010. Activation of innate host defense mechanisms by borrelia. Eur. Cytokine Netw. 21, 7-18.

Butler, C.M., Howers, D.J., Jongejan, F., Van der Kolk, J.H., 2005. *Borrelia burgdorferi* infections with special reference to horses. A review. Vet. Q. 27, 146-156.

Carlomagno, G., Luksa, V., Candussi, G., Rizzi, G.M., Trevisan, G., 1988. Lyme borrelia positive serology associated with spontaneous abortion in endemic Italian área. Acta Eur. Fertil. 19, 279-281.

Chang YF, Novosol V, Mcdonough SP, Chang CF, Jacobson RH, Divers T, Quimby FW, Shun S, Lein DH., 2000. Experimental infection of ponies with *Borrelia burgdorferi* by exposure to Ixodid ticks. Veterinary Pathology 37: 68-76.

Chang YF, Ku YW, Chang CF, Chang CD, Mcdonough SPP, Divers T, Pough M, Torres A., 2005. Antibiotic treatment of experimentally *Borrelia burgdorferi*-infected ponies. Veterinary Microbiology 107: 285-294.

Clark, K.L., Leydet, B., Hartman, S., 2013. Lyme borreliosis in human patients in Florida and Georgia, USA. Int. J. Med. Sci. 10, 915-931.

Costa, I.P., Bonoldi, V.L.N., Yoshinari, N.H., 2001. Perfil clínico e laboratorial da Doença de lyme-símile no estado do Mato Grosso do Sul: análise de 16 pacientes. Rev. Bras. Reumatol. 41, 142-150.

Divers, T.J., 2013. Equine Lyme disease. J. Eq. Vet. Sci. 33, 488-492.

Dorward, D.W., Fischer, E.R., Brooks, D.M., 1997. Invasion and cytopathic killing of human lymphocytes by spirochetes causing Lyme disease.Clin. Infect. Dis. 25, S2-S8.

Galo, K.R., Fonseca, A.H., Madureira, R.C., Barbosa Neto, J., 2009. Frequência de anticorpos homólogos anti-*Borrelia burgdorferi* em equinos na mesorregião metropolitana de Belém, Estado do Pará. Pesq. Vet. Bras. 29, 229-232.

Gonçalves, D.D., Carreira, T., Nunes, M., Benitez, A., Lopes-Mori, F.M.R., Vidotto, O., Freitas, J.C.d, Vieira, M.L., 2013. First record of *Borrelia burgdorferi* B31 strain in *Dermacentor nitens* ticks in the northern region of Parana (Brazil). Braz. J. Microbiol. 44, 883-887.

Gonçalves, D.D., Moura, R.A., Nunes, M., Carreira, T., Vidotto, O., Freitas, J.C., Vieira, M.L., 2015. *Borrelia burgdorferi* sensu lato in humans in a rural area of Paraná state, Brazil. Braz. J. Microbiol. 46, 571-575.

Halouzka, j., Postic, D., Hubalek, Z., 1998. Isolatiojn of the spirochaete Borrelia afzelii from the mosquito Aedes vexans in the Czech Republic. Medical and Veterinary Entomology 12, 103-105.

Holmgren, A.R., Matteson, E.L., 2006. Lyme myositis. Arthritis Rheum. 54, 2697-2700.

Imai, D.M., Barr, B.C., Daft, B., Bertone, J.J., Feng, S., Hodzic, E., Johnston, J.M., Olsen, K.J., Barthold, S.W., 2011. Lyme neuroborreliosis in 2 horses. Vet. Pathol. 48, 1151-1157.

James, F.M., Engiles, J.B., Beech, J., 2010. Meningitis, cranial neuritis, and radiculoneuritis associated with *Borrelia burgdorferi* infection in a horse. JAVMA 237, 1180-1185.

Koedel, U., Fingerle, V., Pfister, H., 2015. Lyme neuroborreliosis – epidemiology, diagnosis and management. Nat. Rev. Neurol. 11, 446-56.

Krawczak, F.S., Nieri-Bastos, F.A., Nunes, F.P., Soares, J.F., Moraes-Filho, J., Labruna, M.B., 2014. Rickettsial infection in *Amblyomma cajennense* ticks and capybaras (*Hydrochoerus hydrochaeris*) in a Brazilian spotted fever-endemic area. Parasit. Vectors 7, 1-7.

Lakos, A., Solymosi, N., 2010. Maternal Lyme borreliosis and pregnancy outcome. Int. J. Infect. Dis., 14, 494-498.

Madureira, R.C., Correa, F.N., Cunha, N.C., Junior, G., Fonseca, A.H., 2007. Ocorrência de anticorpos homólogos anti-*Borrelia burgdorferi* em equinos em propriedades dos municípios de Três Rios e Vassouras, estado do Rio de Janeiro. Revista Brasileira de Ciência Veterinária 4, 43-46.

Manion, T.B., Bushmich, S.L., Khan, M.I., Dinger, J., Werner, H., Mittel, L., Laurendeau, M., Reilly, M., 2001. Suspected clinical lyme disease in horses: serological and antigen testing differences between clinically ill and clinically normal horses from an endemic region. J. Eq. Vet. Sci. 21, 229-234.

Manion, T.B., Khan, M.I., Dinger, J., Bushmich, S.L., 1998. Viable *Borrelia burgdorferi* in the urine of two clinically normal horses. J. Vet. Diagn. Invest. 10, 196-199.

Mantovani, E., Costa, I.P., Gauditano, G., Bonoldi, V.L.N., Higuchi, M.L., Yoshinari, N.H., 2007. Description of Lyme disease-like syndrome in Brazil: Is it a new tick borne disease or Lyme disease variation? Braz. J. Med. Biol. Res., 40, 443-456.

Markowitz, L.E., Steere, A.C., Benach, J.L., Slade, J.D., Broome, C.V., 1986. Lyme disease during pregnancy. JAMA-J. Am. Med. Assoc. 255, 3394–3396.

Mason, L.M.K., Veerman, C.C., Geijtenbeek, T.B.H., Hovius, J.W.R., 2014. Menage a trois: borrelia, dendritic cells, and tick saliva interactions. Trends Parasitol. 30, 95-103.

Nava, S., Barbieri, A.M., Maya, L., Colina, R., Mangold, A.J., Labruna, M.B., Venzal, J.M., 2014. Borrelia infection in ixodes parariciNus ticks (acari: Ixodidae) from northwestern Argentina. Acta Trop. 139, 1-4.

Nava, S., beati, L., Labruna, M.B., Cáceres, A.G., mangold, A.J., Guglielmone, A.A., 2014. Reassessment of the taxonomic status of *Amblyomma cajennense* (Fabricius, 1787) with the description of three new species, *Amblyomma tonellinae* n. Sp., *Amblyomma interandinum* n. sp. and *Amblyomma patinoi* n. sp., and reinstatement of *Amblyomma mixtum Koch*, 1844, and *Amblyomma sculptum* Berrlese, 1888 (Ixodida: Ixodidae). Ticks Tick Borne Dis. 5, 252-76.

Orsini, J.A., Divers, T.J., 2013. Equine Emergencies: Treatment and Procedures, fourth ed. Saunders Elsevier, Linn, Missouri, USA.

Parker, J.L., White, K.K., 1992. Lyme borreliosis in cattle and horses: a review of the literature. Cornell Vet. 82, 253-274.

Passamonti, F., Veronesi, F., Cappelli, K., Capomaccio, S., Reginato, A., Miglio, A., Vardi, D., Stefanetti, V., Coletti, M., Bazzica, C., Pepe, M., 2015. Polysynovitis in a

horse due to *Borrelia burgdorferi* sensu lato infection - Case study. Ann. Agric. Environ. Med. 22, 247-250.

Priest, H.L., Irby, N.L., Schlafer, D.H., Divers, T.J., Wagner, B., Glaser, A.L., Chang, Y., Smith, M.C., 2012. Diagnosis of borrelia-associated uveitis in two horses. Vet. Ophthalmol. 15, 398-405.

Reimers, C.D., Konig, J., Neubert, U.. Preac-Mursic, V., Koster, J.G., Müller-Felber, W., Pongratz, D.E., Duray, P.H., 1993. *Borrelia burgdorferi* myositis: report of eight patients. J. Neurol. 240, 278-283.

Salles, R.S., Fonseca, A.H., Scofield, A., Madureira, R.C., Yoshinari, N.H., 2002. Sorologia para *Borrelia burgdorferi* lato sensu em equinos no estado do Rio de Janeiro. A Hora Veterinária 22, 46-49.

Sears, K.P., Divers, T.j., Neff, R.T., Miller, W.H., McDonough, P., 2011. A case of borrelia-associated cutaneous pseudolymphoma in a horse. Vet. Dermatol. 23, 153-156.

Shapiro, E.D., 2014. Lyme disease. N. Engl. J. Med. 371, 684.

Stanek, G., Wormser, G.P., Gray, J., Strle, F., 2012. Lyme borreliosis. Lancet 379, 461-473.

Szabó, M.P.J., Pinter, A., Labruna, M.B., 2013. Ecology, biology and distribution of spotted-fever tick vectors in Brazil. Front. Cell. Infect. Microbiol. 3, 1-9.

Talhari, S., de Souza Santos, M.N., Talhari, C., de Lima Ferreira, L.C., Silva Jr, R.M., Zelger, B., Massone, C., Ribeiro-Rodrigues, R., 2010. *Borrelia burgdorferi* “sensu lato” in Brazil: occurrence confirmed by immunohistochemistry and focus floating microscopy. Acta Trop. 115, 200-204.

Thrusfield, M., 2007. Veterinary Epidemiology, third ed. Blackwell Publishing, Hoboken, NJ. 234-238.

Wasiluk, A., Zalewska-Szajda, B., Waszkiwicz, N., Kepka, A., Szajda, D.S., Wojewodzka-Zelezniakowicz, M., Ladny, J.R., Pancewicz, S., Zwierz, Z.W., Zwierz, K., 2011. Lyme disease: etiology, pathogenesis, clinical courses, diagnostics and treatment. Prog. Health Sci. 1, 179-186.

Yoshinari, N.H., Barros, P.J.L., Bonoldi, V.L.N., 1997. Perfil da borreliose de Lyme no Brasil. Revista Hospital das Clinicas da Faculdade de Medicina de São Paulo 52, 111-117.

Yoshinari, N.H., Steere, A.C., Cossermelli, W., 1989. Revisão da borreliose de Lyme. Rev. Associação Medica Brasileira 35, 34-38.

Zakovska, A., Capkova, L., Sery, O., Halouzka, J., Dendis, M., 2006. Isolation of Borrelia afzelii from overwintering Culex pipens biotype molestus mosquitoes. Ann Agric. Environ. Med. 13, 345-348.

5. EXPERIMENTAL LYME BORRELIOSIS IN TWO ADULT HORSES

Paper submitted to *EQUINE VETERINARY JOURNAL* in 07/12/2015.

Experimental Lyme borreliosis in two adult horses

Roberta Carvalho Basile, Mariana Rodrigues Vieira, Lara Antoniassi Del Rio, Talissa Camargo de Bonis, Gabriel Paiva Domingues do Amaral, Flora Helena de Freitas D'Angelis, Luciana Soares Leão, Elenice Mantovani, Virgínia Nazario Bonoldi, Natalino Hajime Yoshinari, Delphim da Graça Macoris, Antonio de Queiroz Neto

SUMMARY

Reasons for performing this study: There is only one study of an equine experimental infection with *Borrelia burgdorferi* to date. Thus, the clinical signs and hematological findings in horses are still based on case reports and extrapolations of those observed in humans.

Objectives: This study sought to establish an experimental infection in adult horses with standardised, repeatable techniques and to provide data regarding the clinical course of the disease.

Study design: Experimental infection of two adult horses was initiated by subcutaneous and intradermal inoculation of *Borrelia burgdorferi* strain G39/40.

Methods: The infected horses were evaluated for 90 consecutive days with respect to clinical signs, hematologic parameters, serum biochemistry, antibody titer, and molecular tests of blood, muscle, liver and spleen samples.

Results: The infected horses showed mild clinical changes (enlarged lymph nodes, lethargy and myalgia) within the first seven days after infection and hematologic (mild anemia) and blood biochemistry changes (increase in creatine phosphokinase) until the 11th day after infection. The maximum antibody titers of infected horses detected by ELISA (enzyme-linked immunosorbent assay) reached 1/1,600 after the seventh

day and regressed to zero near 90th day, without treatment in none of the horses. Bacteria were not detected by PCR analysis in blood samples and in biopsies from the triceps muscle, spleen and liver.

Conclusion: Acute borreliosis in horses promotes mild clinical and hematological changes within the first two weeks of infection, which are very nonspecific and can be easily confounded with other tick-borne diseases.

Keywords: Borrelia burgdorferi, tick, infection, clinical signs, ELISA, PCR.

5.1 INTRODUCTION

Horses innately carry a high probability of being infested by ticks, especially those stabled in pastures or paddocks. At least once in their lives, horses have contact with ticks, even if it is only during the foal phase. These ectoparasites can carry numerous pathogens, including hemoparasites and bacteria capable of infecting and promoting diseases in horses [1, 2].

Borrelia burgdorferi sensu lato complex is formed by gram-negative spirochetes that causes Lyme syndrome in humans and horses [3]. These bacteria replicate in the gut of the tick, and upon the presence of blood during the adhesion of these ectoparasites on animals, they migrate to the salivary glands and become pathogenic. *Borrelia* spp. require at least 24 hours of adhesion of ticks to the host to regulate their membrane surface proteins (Osp's) and ensure survival in the vertebrate organism [4]. To be carried by saliva to the site of the bite, ticks promote a local inflammatory reaction, whereby the bacteria are spread to the lymphonodes, synovial membranes, muscle tissue or nervous system [3]. Clinical signs attributed to the infection in horses include low-grade fever, weakness, lameness, myalgia,

soreness, joint swelling, lethargy, behavioral changes [3], uveitis, encephalitis, dermatitis, chronic fatigue and weight loss [2].

The first report of borreliosis in horses was published by Van Heerden and Reyes [5]. Since then, only isolated reports of diagnoses exist for *B. burgdorferi* sensu lato isolated from the urine of healthy horses [6], cerebrospinal fluid [7], aqueous humor of horses with severe uveitis and blindness [8], duramater and leptomeninges of horses with neurological signs and neuromuscular clinical signs [9] and synovial fluid of lame horses [10].

The only known equine experimental protocol of infection by *B. burgdorferi* was published by Chang and co-workers [11, 12]. These authors performed an infection of twelve ponies through exposure by *Ixodidae* ticks from an endemic forest in the United States. Clinical and serological analysis was carried out for 9 months, and after euthanasia, various tissues were examined for the presence of the etiologic agent. No clinical signs were reported during the 9 months of infection, and PCR (polymerase chain reaction) - amplified DNA of *B. burgdorferi* was detected in samples of skin, lymph node, muscle, synovial membrane, myocardium, pericardium, kidneys, renal capsule, urinary bladder, and dura mater.

In this study, we standardised an experimental infection of two adult horses with *B. burgdorferi* sensu lato strain B31 and followed the course of the disease for 90 days, to report the occurrence of acute and chronic clinical signs, hematological changes and the evolution of the antibody titer in infected animals over the time.

5.2 MATERIAL AND METHODS

Animals

Seven healthy Arabian horses, weighing 425.85 ± 57.58 kg, aged between 5 and 10 years, and including three males and four females, were used in this experiment. The horses have been submitted to conventional protocols of vaccination and deworming. The negative control group (C-) was composed by two seronegative horses that were serologically evaluated 3 times during the previous 6 months and maintained this seronegativity. The positive control group (C+) was composed of 2 naturally seropositive horses with antibody titers of 1/800 in the last 6 months, and the infected group (INF) was composed of two horses initially seronegative (last 6 months) that were submitted to experimental infection with *B. burgdorferi* sensu lato strain G39/40. One horse was immunized (Immun) with *B. borgdorferi* stricto sensu strain G39/40 at the same dose as the infected horses; however, the samples were inactivated by exposure to high temperature prior to immunization. The immunized horse provided hyperimmune serum for a positive control of the serological ELISA (enzyme-linked immunosorbent assay) test.

Throughout the study, the infected horses were kept in isolation stalls, and horses in the negative, positive and immunized controls were maintained in separate pickets. The horses were fed daily with 2.0 kg of commercial feed containing 12% protein, coast-cross hay, water and mineral salt ad libitum. Preventive control of ticks was performed weekly by means of baths, brushing and application of deltamethrin[5], according to the manufacturer's instructions. This study received approval from the

[5]Butox, MSD Saúde Animal

Univ. Estadual Paulista Ethics and Welfare Committee (CEBEA) in document no. 001968/13.

Inoculate preparation

Live spirochetes of *B. burgdorferi* sensu lato strain G39/40 were obtained from a culture maintained in the Rheumatology Laboratory of the Faculty of Medicine, University of São Paulo (LIM17-USP). Two liters of culture medium Barbour-Stoenner-Kelly (BSK) were used to cultivate approximately 10^9 bacteria; the cultures were grown for 60 days and maintained in an incubator at 36°C. The final volume after centrifugation of the culture medium was 20 mL, i.e., the inoculum had a concentration of 5 x 10^7 bacteria/mL. The immunization samples were inactivated by high-temperature (56°C) exposure for 60 minutes.

Horse inoculation

The bacterial inoculum concentration was based on a study of experimental infection in *Rhesus* monkeys infected with *B. burgdorferi* [13] and was also performed in accordance with the doses reported on experimental infections of horses using another pathogens [14, 15, 16]. On day 0, a total of 10 mL of bacteria in BSK medium supplemented with 5 mL of venous blood was divided between the subcutaneous and intradermal routes in the caudal third of the neck above the chain of cervical lymph nodes, totaling 4,7 x 10^8 bacteria per horse. Prior to inoculation, the bacteria were counted by dark field microscopy using an improved Neubauer counting chamber [17]. The negative control horses were inoculated at the same site but with 10 mL of BSK medium supplemented with 5 mL of venous blood. The immunized horse received the same dose of inactivated *Borrelia* on days 0, 14, 28 and 49, to provide hyperimmune serum.

Clinical examination

The horses were clinically assessed by two examiners before inoculation (day 0) and on days 2, 7, 11, 14, 21, 28, 49, 63 and 90 post-inoculation. The following parameters were evaluated:

- Behavior: 0 (normal), 1 (abnormal, lethargic or excited);
- Mucous membranes: 0 (pink), 1 (abnormal – pale, icteric, congested);
- Lymph nodes: 0 (normal), 1 (increased);
- Capillary refill time (CRT): 0 (normal = 2 s), 1 (abnormal >3 s);
- Cardiac rhythm: 0 (sinus), 1 (arrhythmia);
- Intestinal motility: 0 (normal), 1 (abnormal: hypomotility, hypermotility);
- Rectal temperature: in degrees Celsius;
- Feces: 0 (normal), 1 (abnormal);
- Myalgia: 0 (absent), 1 (present);
- Lameness: 0 (absent), 1 (present);
- Uveitis: 0 (absent), 1 (present);
- Joint swelling: 0 (absent), 1 (present);
- Ataxia: 0 (absent), 1 (present).

Hematological tests

Blood samples were collected for the hematological and biochemical evaluation of bilirubin, creatine phosphokinase, gamma glutamyltransferase, albumin, immunoglobulins, aspartate aminotransferase, urea and creatinine. The

hematological and biochemical tests were performed in automatic analyzers[6] using commercial reagents[7].

Tissue samples

Samples of the triceps muscle [18], liver and spleen [19] were obtained by means of ultrasound-guided[8] biopsies and stored in a -80°C freezer until DNA extraction. Samples were collected on days 0, 28, 49 and 63.

Serology: Indirect ELISA

Immunoglobulins, specifically IgG, against *B. burgdorferi* sensu lato strain G39/40, were detected in accordance with ELISA procedures previously described [20, 21]. Briefly, the preparation of the antigen included sonicating a whole spirochete suspension, which was made from a culture of *B. burgdorferi* organisms grown in a 500 ml bottle containing Kelly's medium at 33ºC at higher-phase growth. The medium was centrifuged at 10,000 g for 20 min at 4ºC, and the pellets were washed 3 times with cold 0.01 M phosphate-buffered saline (PBS) with 5 mM magnesium chloride (pH 7.4). The suspension of spirochetes was sonicated on ice with a cell sonicator, and the supernatant was filtered (45 µm membrane). The protein content was determined by Folin's method, and the antigen preparations were stored in aliquots at -70ºC until further analysis.

Polystyrene plates with 96 holes[9] were coated with antigen at a concentration of 20 mg/ml, incubated in a humidified chamber overnight at 4°C, washed with PBS Tween 20 buffer and then blocked with 1% rabbit serum. The positive control sera were obtained from one horse that was administered 4 serial immunizations over 15

[6]Poch®-100 IV DIFF and COBAS MIRA Plus Roche®
[7]Labtest®
[8]MyLab 30 - Esaote®, abdominal transducer CA541
[9]Bio-Rad Laboratories

days, and the negative control serum was obtained from 8 healthy horses without history of exposure to ticks. The test and control sera (8 negative and one positive) were diluted 1:800 in PBS Tween 20, incubated and washed. Conjugated rabbit anti-horse IgG linked to alkaline phosphatase[10] was added, incubated and washed. Next, the solution of PNPP[11] diluted in glycine buffer with a pH 10.5 was added, and the samples were read using a spectrophotometer[12] at a wavelength of 405 nm. The cutoff line was established at a confidence level of 99.99%, according to the mean plus three standard deviations of the optical density of the negative controls.

DNA extraction

To facilitate and expedite the DNA extraction from blood samples and tissues, a commercial DNeasy® Blood & Tissue Kit (Qiagen GmbH) was used according to the procedures recommended by the manufacturer.

PCR (LIM17-FMUSP)

DNA amplification was performed according to previously described procedures [22] using the following primers: for the FlaB gene (flagellin of *Borrelia spp*.), FLA LL (5' - ACA TAT TCA GAT GCA GAC AGA GGT - 3') and FLA RL (5' - GCA ATC ATA GCC ATT GCA GAT TGT - 3')[23]; and for the flgE gene (flagellar hook of *Borrelia spp*.), flgE 470 FW (5' - CGCCTATTCTAACTTGACCTGAAT - 3') and flgE 470 REV (5' - CAACTCTAAGTCCAAGAACACCAA - 3') [22]. Briefly, the amplification was performed using a 50 mL final reaction volume containing 5 pmoles of each primer, 10 mM of Tris-HCl, 1.5 mM of $MgCl_2$, 1.25 mM of dNTPs, 1.5 U of Taq DNA polymerase in DNAse/RNAse-free H_2O and 5 ml of the DNA sample.

[10]Sigma Chemical
[11]Sigma Chemical
[12]BioRad Laboratories, model 550 Microplate Reader

DMSO (1.5 mL) was added immediately before the start of the first cycle (final concentration 3%). The PCR cycles were composed of initial denaturation for 3 min at 95°C; 40 repeated cycles of 45 s at 95°C, 45 s at 64°C, and 45 s at 72°C; and a final extension for 7 min at 72°C. Good laboratory practices were adopted to avoid contamination of the samples, and in each reaction, negative controls were included to check for possible contamination. The positive control consisted of *B. garinii*. The amplified products were fractionated by electrophoresis in 1.5% agarose gel, stained with SYBR[13] and examined by ultraviolet transillumination.

Statistical analysis

Nonparametric variables were evaluated with Kruskall-Wallis tests using Roy's test with 95% significance. Parametric variables were evaluated using multivariate analysis of variance (MANOVA) in terms of days and groups and tested using Dunnett's test with 95% significance comparing with negative control group.

5.3 RESULTS

Physical examination of the horses showed little changes over the 90 days of evaluation. Clinical signs presented by infected horses were restricted to the initial 7 days following inoculation and were unspecific. Swelling below the site of the application was noted, suggesting subcutaneous edema one day after inoculation, but only in infected horses (Figure 3).

[13]Invitrogen

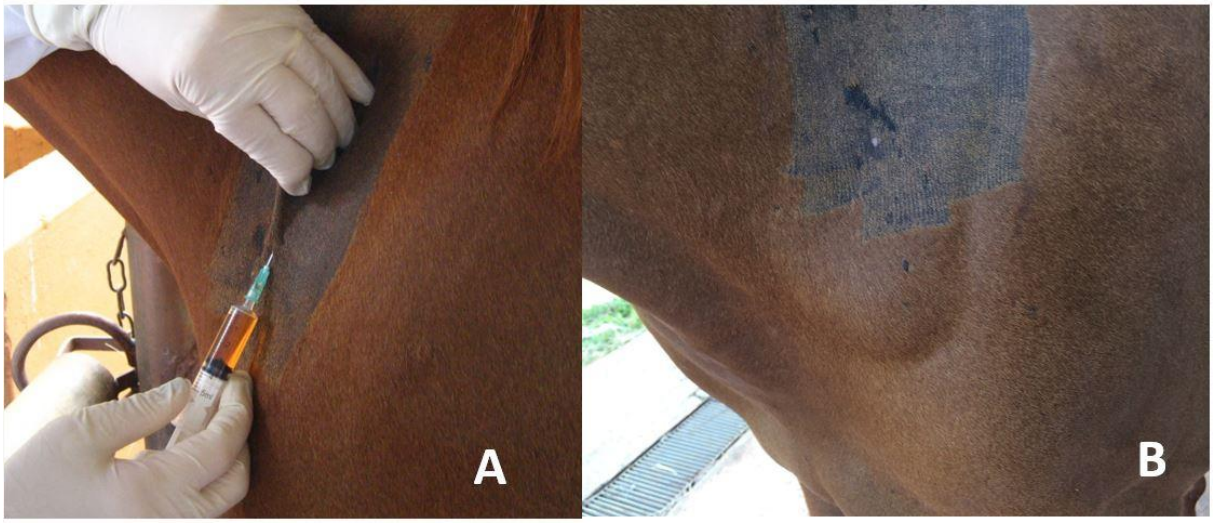

Figure 3– A – Subcutaneous application of *Borrelia burgdorferi* diluted in BSK medium near the cervical chain of lymph nodes in a mare. B – Increased volume below the injection site one day after application of the inoculum.

Between the second and seventh day post-inoculation, infected horses presented increased mandibular and sublingual lymph node swelling, apathetic behavior and pale mucous membranes. On day seven, muscle pain in the infected horses was verified, particularly along the back. The myalgia resolved without any medical intervention but relapsed at day 28 in the infected horses (Table 5). The clinical parameters not presented in Table 1 did not show any variation during the 90 days of evaluation.

Table 5 – Qualitative parameters for the clinical evaluation of seven horses during 90 days of experimental infection. Groups: C-: negative control (n=2), INF: experimentally infected horses (n=2), Immun: experimentally immunized horse (n=1), C+: naturally immunized horses (n=2).

Day	Group	ELISA 1/x		Behavior score		Mucosa score		Limphonode score		Myalgia score		Lameness score	
		Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD
0	C-	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	INF	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Immun	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	C+	**800**	**0**	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5	1.0	0.0
2	C-	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	INF	0	0	**1.0**	**0.0**	**1.0**	**0.0**	**1.0**	**0.0**	0.5	1.0	0.0	0.0
	Immun	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	C+	**800**	**0**	0.0	0.0	0.0	0.0	0.0	0.0	0.5	1.0	0.5	1.0
7	C-	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	INF	**1200**	**566**	**1.0**	**0.0**	**1.0**	**0.0**	**1.0**	**0.0**	**1.0**	**0.0**	0.0	0.0
	Immun	**1600**	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	C+	**800**	**0**	0.0	0.0	0.0	0.0	0.0	0.0	0.5	1.0	0.5	1.0
11	C-	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	INF	**1200**	**566**	0.0	0.0	0.5	1.0	0.5	1.0	0.5	1.0	0.0	0.0
	Immun	**3200**	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	C+	**600**	**283**	0.0	0.0	0.0	0.0	0.0	0.0	0.5	1.0	0.5	1.0
14	C-	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	INF	**800**	**0**	0.0	0.0	0.0	0.0	0.5	1.0	0.5	1.0	0.0	0.0
	Immun	**3200**	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	C+	400	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	1.0
21	C-	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	INF	**800**	**0**	0.0	0.0	0.0	0.0	0.0	0.0	**1.0**	**0.0**	0.0	0.0
	Immun	**6400**	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	C+	200	283	0.0	0.0	0.0	0.0	0.0	0.0	0.5	1.0	0.0	0.0
28	C-	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	INF	**800**	**0**	0.0	0.0	0.0	0.0	0.0	0.0	0.5	1.0	0.0	0.0
	Immun	**6400**	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	C+	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
49	C-	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	INF	**600**	**283**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Immun	**12800**	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	C+	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
63	C-	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	INF	200	283	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Immun	**12800**	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	C+	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
90	C-	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	INF	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Immun	**25600**	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	C+	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Nonparametric values in bold differed from the negative control (C-) based on Roy's test with 95% significance.

Hematological tests revealed mild anemia in infected animals until the tenth day of infection, represented by the decreases in packed cell volume and hemoglobin concentration (Table 6). Infected animals also showed an isolated event of eosinophilia on the seventh day after infection. The positive control group began the experiment with mild neutrophilia, which quickly returned to normal.

Table 6 – Hematological test results of seven horses during 90 days of experimental infection with *Borrelia burgdorferi* strain G39/40. Groups: C-: negative control (n=2), INF: experimentally infected horses (n=2), Immun: experimentally immunized horse (n=1), C+: naturally immunized horses (n=2).

		ELISA		TR		RBC		Hb		PCV		WBC		NB		NT		Eos		Lynf		Mono		Platelet	
Day	Group	1/x		oC		x103/ul		g/dl		%		per ul		per ul		per ul		per ul		per ul		per ul		per ul	
		Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD
0	C-	0	0	37.9	0.0	8.950	1.344	13.35	1.63	41.5	3.5	8400.00	1697.056	204	17	4236	424	264	170	3480	1867	216	102	195000	5657
	INF	0	0	37.0	0.1	8.100	0.424	12.75	0.49	37.5	0.7	9350.00	212.132	235	71	6498	81	140	63	2246	183	233	61	228000	50912
	Immun	0	0	37.5	0.0	8.000	0.000	11.00	0.00	35.0	0.0	8600.00	0.000	258	0	4644	0	110	0	2567	0	212	0	170000	0
	C+	**800**	**0**	37.2	0.1	7.700	0.566	10.90	0.71	36.5	2.1	10450.00	1202.082	266	104	**7228**	**1125**	266	104	2491	7	201	124	149000	50912
2	C-	0	0	37.6	0.2	8.750	0.495	12.25	1.06	36.5	3.5	7400.00	1131.371	148	23	4869	72	263	92	1939	969	181	24	179500	20506
	INF	0	0	37.4	0.1	7.050	0.212	10.95	0.21	**32.0**	**0.0**	8750.00	70.711	219	60	5684	820	219	64	2454	886	175	1	240000	63640
	Immun	0	0	37.6	0.0	7.890	0.000	10.90	0.00	35.0	0.0	7800.00	0.000	220	0	4567	0	156	0	2435	0	198	0	178000	0
	C+	**800**	**0**	37.8	0.1	7.500	0.990	11.55	1.06	34.5	3.5	9150.00	494.975	275	15	5517	412	491	426	2641	1370	227	52	124000	48083
7	C-	0	0	37.8	0.1	8.950	0.354	12.75	1.06	37.0	5.7	7350.00	777.817	226	127	4980	1561	108	40	1856	837	181	33	197000	38184
	INF	**1200**	**566**	37.1	0.1	6.450	0.495	**9.70**	**0.42**	**29.5**	**0.7**	8600.00	707.107	299	36	5565	148	**466**	**144**	2054	957	218	78	164000	36770
	Immun	**1600**	**0**	37.3	0.0	7.200	0.000	10.50	0.00	32.0	0.0	9200.00	0.000	278	0	4988	0	156	0	2356	0	198	0	125000	0
	C+	**800**	**0**	37.1	0.6	6.450	0.636	10.40	0.85	**31.5**	2.1	7900.00	1131.371	237	34	4898	701	166	134	2406	289	194	28	123500	47376
11	C-	0	0	37.8	0.3	8.650	0.636	12.10	1.56	37.0	4.2	7400.00	424.264	222	13	5293	356	151	113	1587	66	148	8	188000	12728
	INF	**1200**	**566**	37.2	0.1	6.550	0.071	**9.90**	**0.14**	**29.5**	**0.7**	8000.00	565.685	240	17	5466	8	180	6	2016	594	198	42	172500	30406
	Immun	**3200**	**0**	37.7	0.0	7.400	0.000	11.20	0.00	33.0	0.0	8700.00	0.000	266	0	5100	0	234	0	2211	0	151	0	165000	0
	C+	**600**	**283**	37.5	0.5	7.400	0.707	11.60	0.99	34.5	3.5	9800.00	141.421	344	74	6666	373	344	74	2251	383	196	3	132000	59397
14	C-	**0**	**0**	37.0	0.5	8.150	0.636	11.50	0.99	34.5	3.5	7950.00	1202.082	283	98	5301	468	452	265	1721	927	195	26	185000	15556
	INF	**800**	**0**	37.3	0.4	7.350	0.212	10.90	1.27	33.0	2.8	8450.00	494.975	257	134	4746	755	210	47	3069	357	169	10	166000	7071
	Immun	**3200**	**0**	37.1	0.0	7.400	0.000	9.80	0.00	30.0	0.0	7700.00	0.000	154	0	4851	0	273	0	2569	0	233	0	294000	0
	C+	400	0	37.3	0.3	6.750	0.636	10.25	1.06	30.5	2.1	10150.00	1767.767	590	456	7383	2134	191	108	1740	687	248	28	212000	22627
21	C-	0	0	36.9	0.5	9.000	0.990	13.00	1.41	39.0	4.2	7800.00	707.107	193	37	4650	238	536	171	2266	1139	156	14	174500	33234
	INF	**800**	**0**	37.5	0.1	7.450	0.636	11.25	0.35	34.0	1.4	8600.00	848.528	258	25	5452	356	672	611	1960	170	258	25	195500	47376
	Immun	**6400**	**0**	36.0	0.0	7.700	0.000	10.30	0.00	31.0	0.0	9400.00	0.000	289	0	5358	0	334	0	3239	0	224	0	150000	0
	C+	200	283	37.2	0.4	7.300	0.424	10.85	0.92	33.5	2.1	10350.00	919.239	256	50	6375	785	256	50	3154	207	311	28	141500	4950
28	C-	0	0	36.6	0.8	8.150	0.354	11.80	0.28	35.0	1.4	8050.00	1202.082	206	87	5491	1045	449	383	1744	429	161	24	155500	27577
	INF	**800**	**0**	37.1	0.2	8.100	0.141	12.15	0.21	36.5	0.7	8600.00	141.421	172	3	5589	30	771	352	1854	578	215	57	153500	27577
	Immun	**6400**	**0**	37.0	0.0	7.000	0.000	9.20	0.00	28.0	0.0	7600.00	0.000	152	0	5624	0	312	0	1292	0	468	0	292000	0
	C+	0	0	37.1	0.2	7.350	0.919	11.00	1.41	33.5	3.5	9300.00	141.421	326	71	6652	430	514	337	1669	632	139	64	157500	21920
49	C-	0	0	37.6	0.1	8.450	1.202	12.75	0.78	39.0	1.4	7950.00	1626.346	205	97	5497	1235	114	32	1942	342	193	16	157000	28284
	INF	**600**	**283**	37.9	0.4	8.750	0.071	13.20	0.28	39.5	2.1	9550.00	494.975	290	150	6406	602	191	10	2427	211	237	55	129500	31820
	Immun	**12800**	**0**	37.1	0.0	7.900	0.000	10.40	0.00	38.0	0.0	7000.00	0.000	140	0	4340	0	140	0	2240	0	70	0	166000	0
	C+	0	0	37.7	0.1	8.750	0.778	13.35	0.21	40.5	0.7	8100.00	1272.792	198	25	4725	445	126	76	2889	1642	162	25	186000	22627
63	C-	0	0	37.2	0.6	8.350	0.071	12.75	0.35	38.5	0.7	9350.00	1060.660	230	40	5481	819	576	328	2826	139	238	93	172500	30406
	INF	200	283	36.8	0.1	7.500	0.990	11.25	1.48	35.0	4.2	9850.00	919.239	296	28	5681	163	519	437	3152	1334	204	158	148000	41012
	Immun	**12800**	**0**	36.2	0.0	7.800	0.000	10.20	0.00	32.0	0.0	10700.00	0.000	285	0	8560	0	107	0	4173	0	214	0	281000	0
	C+	0	0	36.2	0.1	7.550	0.495	10.50	0.71	31.5	2.1	9150.00	636.396	275	19	4569	1167	179	117	3993	1956	135	55	141000	22627
90	C-	0	0	37.4	0.1	7.050	0.354	9.00	1.13	28.0	2.8	9300.00	707.107	235	83	6382	433	401	429	2047	1401	235	83	134000	62225
	INF	0	0	37.6	0.0	8.350	0.071	12.75	0.35	38.5	0.7	9350.00	1060.660	230	40	5481	819	576	328	2826	139	238	93	172500	30406
	Immun	**25600**	**0**	37.1	0.0	7.900	0.000	10.30	0.00	31.8	0.0	9800.00	0.000	259	0	7899	0	98	0	3987	0	199	0	250000	0
	C+	0	0	37.3	0.3	7.550	0.636	10.50	0.71	31.0	2.8	9555.00	784.889	242	87	4722	959	146	79	4220	1895	141	56	140000	28284

Values in bold differed from the negative control (C-) based on Dunnet's test with 95% significance.

During the period of evaluation, the infected horses presented mildly increased levels of muscular creatine phosphokinase (CPK) and globulins on the 28th day of infection. Other hematologic and biochemical parameters did not change for all three groups (Table 7).

Table 7 – Biochemical serum analysis of seven horses during 90 days of experimental infection with *Borrelia burgdorferi* strain G39/40. Groups: C-: negative control (n=2), INF: experimentally infected horses (n=2), Immun: experimentally immunized horse (n=1), C+: naturally immunized horses (n=2).

Day	Group	ELISA 1/x		Dir Bil mg/dl		Ind Bil mg/dl		CPK IU/l		Creat mg/dl		GGT IU/l		Pt g/dl		Alb g/dl		Glob g/dl		AST IU/l		Urea mg/dl	
		Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD	Aver.	SD
0	C-	0	0	0.22	0.03	0.67	0.02	202	30	1.2	0.1	29.5	27.6	6.0	0.0	2.6	0.1	3.5	0.1	337	4	35	5
	INF	0	0	0.17	0.01	1.40	0.28	183	32	1.3	0.2	21.5	0.7	6.7	0.0	2.6	0.1	4.1	0.0	380	18	28	1
	Immun	0	0	0.25	0.00	0.75	0.00	102	0	1.0	0.0	13.0	0.0	5.8	0.0	2.3	0.0	3.5	0.0	285	0	24	0
	C+	**800**	**0**	0.43	0.01	0.69	0.44	165	7	1.3	0.1	12.0	1.4	6.6	0.1	2.3	0.2	4.4	0.4	264	40	24	1
2	C-	0	0	0.23	0.04	0.40	0.28	145	50	1.2	0.1	26.5	21.9	5.4	0.2	2.3	0.2	3.2	0.0	319	25	26	7
	INF	0	0	0.19	0.04	0.17	0.08	**700**	**54**	1.1	0.1	19.5	0.7	6.5	0.3	2.5	0.1	4.1	0.2	357	33	25	1
	Immun	0	0	0.15	0.00	0.09	0.00	328	0	1.1	0.0	14.0	0.0	5.6	0.0	2.7	0.0	2.9	0.0	494	0	33	0
	C+	**800**	**0**	0.22	0.06	0.22	0.10	187	10	1.1	0.1	11.5	2.1	6.6	0.1	2.6	0.4	4.0	0.3	275	45	32	6
7	C-	0	0	0.26	0.06	0.36	0.13	223	42	1.2	0.1	22.0	15.6	6.0	0.2	2.7	0.0	3.4	0.2	332	13	22	6
	INF	**1200**	**566**	0.15	0.01	0.16	0.01	**751**	**90**	1.3	0.1	19.5	2.1	6.2	0.2	2.6	0.1	3.7	0.1	324	39	20	4
	Immun	**1600**	**0**	0.13	0.00	0.15	0.00	198	0	1.0	0.0	9.0	0.0	5.4	0.0	2.5	0.0	2.9	0.0	449	0	34	0
	C+	**800**	**0**	0.27	0.23	0.38	0.43	206	42	1.3	0.1	8.0	2.8	6.3	0.2	2.7	0.2	3.7	0.4	298	23	28	2
11	C-	0	0	0.13	0.01	0.82	0.96	176	23	1.2	0.0	21.5	16.3	6.3	0.0	2.8	0.1	3.6	0.1	262	9	23	1
	INF	**1200**	**566**	0.11	0.01	0.76	0.91	495	91	1.2	0.2	16.5	2.1	6.2	0.5	2.7	0.4	3.6	0.1	257	27	24	6
	Immun	**3200**	**0**	0.13	0.00	0.12	0.00	110	0	1.1	0.0	10.0	0.0	6.3	0.0	2.8	0.0	3.5	0.0	323	0	30	0
	C+	**600**	**283**	0.17	0.03	0.12	0.10	158	4	1.3	0.1	10.5	2.1	6.7	0.1	2.8	0.2	4.0	0.2	250	18	23	1
14	C-	0	0	0.10	0.01	0.13	0.03	207	139	1.1	0.1	21.0	15.6	5.6	0.1	3.4	0.0	2.2	0.1	317	6	18	1
	INF	**800**	**0**	0.10	0.01	0.09	0.04	199	100	1.1	0.0	18.5	4.9	5.4	0.1	2.5	0.0	2.9	0.1	272	40	14	1
	Immun	**3200**	**0**	0.08	0.00	0.11	0.00	118	0	1.2	0.0	10.0	0.0	4.7	0.0	2.5	0.0	2.2	0.0	346	0	18	0
	C+	400	0	0.08	0.01	0.07	0.00	331	288	1.3	0.1	10.5	3.5	5.9	0.5	2.5	0.2	3.4	0.7	296	68	15	1
21	C-	0	0	0.54	0.08	0.30	0.20	188	42	1.5	0.1	19.5	13.4	6.4	0.5	2.5	0.4	3.9	0.8	310	54	27	6
	INF	**800**	**0**	0.25	0.10	0.40	0.13	226	36	1.1	0.1	13.5	3.5	5.5	0.6	1.9	0.1	3.6	0.4	291	86	25	1
	Immun	**6400**	**0**	0.26	0.00	0.73	0.00	109	0	1.3	0.0	11.0	0.0	7.2	0.0	2.2	0.0	5.0	0.0	492	0	33	0
	C+	200	283	0.24	0.05	0.54	0.11	168	16	1.3	0.0	9.0	2.8	6.9	0.1	2.2	0.0	4.7	0.1	302	57	28	6
28	C-	0	0	0.24	0.01	0.55	0.13	191	11	1.2	0.1	15.5	7.8	6.1	0.7	2.0	0.1	4.1	0.8	344	145	19	6
	INF	**800**	**0**	0.26	0.04	0.65	0.04	284	192	1.8	0.6	18.5	2.1	**7.3**	**0.1**	2.5	0.0	**4.8**	**0.1**	312	62	31	9
	Immun	**6400**	**0**	0.25	0.00	0.55	0.00	107	0	1.3	0.0	10.0	0.0	6.5	0.0	2.2	0.0	4.3	0.0	268	0	34	0
	C+	0	0	0.21	0.01	0.46	0.14	164	60	1.5	0.0	8.5	3.5	6.3	1.2	2.2	0.4	4.2	0.8	477	417	16	4
49	C-	0	0	0.19	0.01	0.65	0.10	269	71	1.5	0.4	13.5	6.4	5.8	0.2	2.2	0.4	3.7	0.1	292	25	17	4
	INF	**600**	**283**	0.26	0.02	1.20	0.74	287	157	1.4	0.2	24.0	8.5	6.7	0.2	2.3	0.1	4.5	0.2	465	185	21	5
	Immun	**12800**	**0**	0.19	0.00	0.70	0.00	133	0	1.3	0.0	9.0	0.0	6.2	0.0	2.0	0.0	4.2	0.0	232	0	20	0
	C+	0	0	0.29	0.05	0.11	0.06	183	33	1.5	0.3	9.5	4.9	5.6	0.1	2.1	0.4	3.5	0.3	279	12	14	4
63	C-	0	0	0.21	0.11	0.08	0.03	456	438	1.2	0.1	25.5	10.6	6.5	0.1	2.4	0.0	4.1	0.1	363	86	14	1
	INF	200	283	0.33	0.11	0.22	0.21	209	15	1.4	0.1	11.0	0.0	6.3	0.4	2.3	0.4	4.1	0.0	257	35	19	7
	Immun	**12800**	**0**	0.22	0.00	0.11	0.00	124	0	1.3	0.0	10.0	0.0	6.6	0.0	2.6	0.0	4.0	0.0	266	0	21	0
	C+	0	0	0.28	0.21	0.42	0.37	187	16	1.2	0.2	7.5	2.1	5.8	0.5	2.7	0.2	3.2	0.4	382	95	30	6
90	C-	0	0	0.13	0.04	0.08	0.01	432	277	1.2	0.1	12.0	2.8	6.0	0.6	2.6	0.1	3.4	0.7	388	151	31	3
	INF	0	0	0.21	0.11	0.08	0.03	156	14	1.2	0.1	15.5	3.5	6.5	0.1	2.4	0.0	4.1	0.1	363	86	14	1
	Immun	**25600**	**0**	0.17	0.00	0.21	0.00	198	0	1.3	0.0	11.0	0.0	6.5	0.0	2.5	0.0	4.0	0.0	290	0	20	0
	C+	0	0	0.30	0.22	0.28	0.31	212	3	1.2	0.1	11.5	0.7	5.8	0.4	2.6	0.2	3.2	0.1	375	121	29	8

Values in bold differed from the negative control (C-) based on Dunnet's test with 95% significance. (Cont.)

(Continued) Parameters: Dir Bil – direct bilirubin, Ind Bil – indirect bilirubin, CPK – creatine phosphokinase, Creat – creatinine, GGT – gamma glutamyl transferase, Pt – total protein, Alb – albumin, Glob – globuline, AST – aspartate amino transferase.

Figure 4 shows the change in antibody titer over the 90-day period for the seven horses. Note that in both groups, the naturally immunized and experimentally infected horses showed a decline in antibody titers over time without treatment. As the immunized horses received four antigen applications, their antibody titers steadily increased over time.

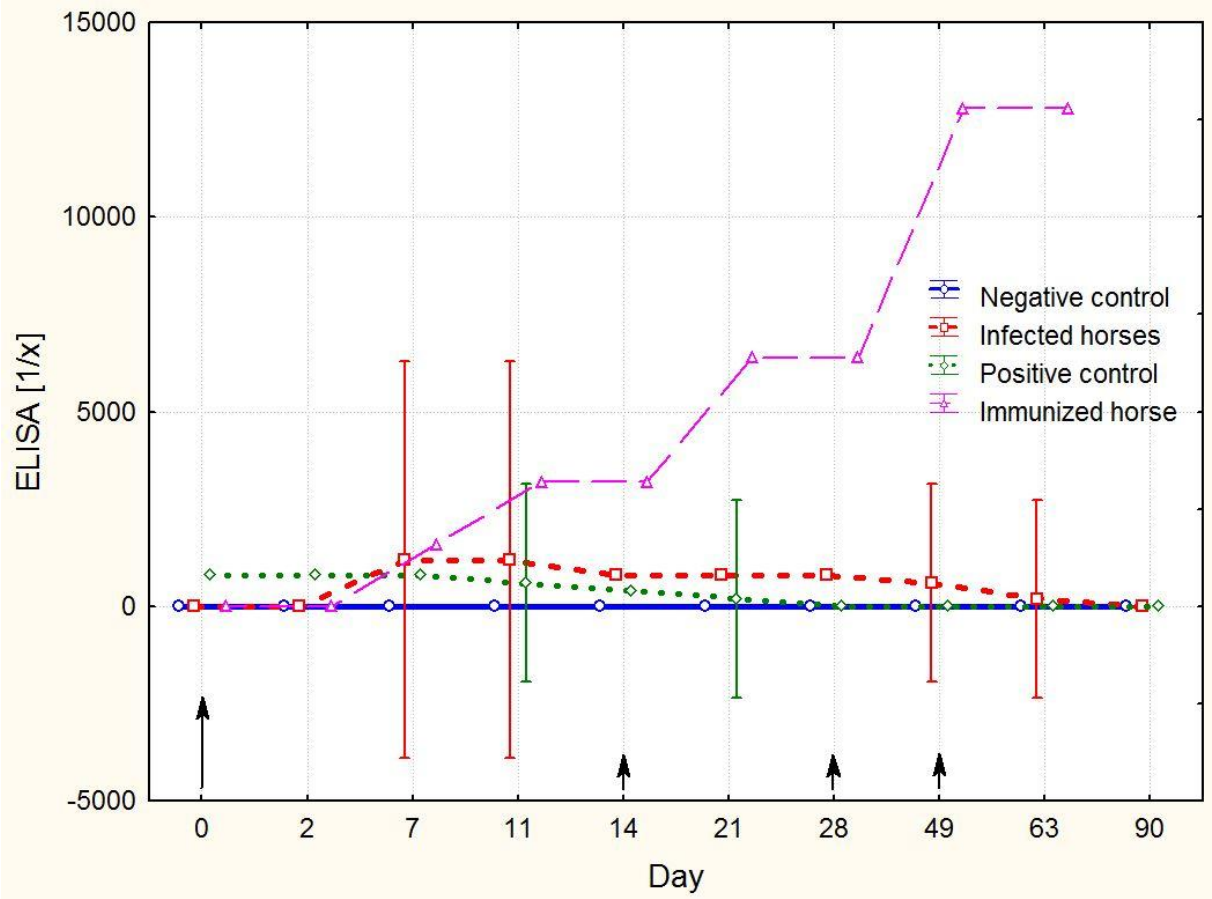

Figure 4 – Antibody titers evaluated by indirect ELISA for seven horses, two of which received experimental infection. The large arrow indicates the day of infection with *Borrelia burgdorferi* strain G39/40 in two horses (Infected horses) and the first immunization of another horse (immunized horse), (cont.)

(continued) with temperature-inactivated bacteria. The negative control group included two seronegative horses, and the positive control group included two naturally seropositive horses.

The molecular tests did not amplify any *Borrelia* fragment in the blood, muscle, liver or spleen samples.

5.4 DISCUSSION

To date, few studies of equine experimental infection with *Borrelia spp*. have been conducted [11, 12]. Therefore, the clinical signs of Lyme disease in this animal species are routinely based on case reports [6, 7, 8, 9, 10] and extrapolations of those observed in humans [13].

The experimental infection protocol used in this study was feasible, reproducible and safe, enabling monitoring of clinical signs and hematological changes during the infection period. It is known that *B. burgdorferi* expression of Osp's, in particular OspC, leads to a higher probability of survival in the host organism. OspC is a protein that binds to Salp15 (tick salivary protein), and together they form a structure that protects the spirochetes against antibodies and the complement system [24], thereby protecting the bacteria from phagocytosis by macrophages [25]. Expression of OspC is governed by pH and temperature changes [26] that occur when the bacteria is transferred from the salivary glands of the tick to the bite site in the host. These pH and temperature changes are provided by contact of the bacteria with the blood of the host during the tick’s engorgement [27]. The blood promotes an increase in temperature and reduction in pH, thereby stimulating the bacteria to express OspC but not Salp15, which is also important in the early infection in the vertebrate host. Therefore, in this study, the application of bacteria in

BSK medium followed by the application of venous whole blood provided the microenvironment necessary for the establishment of infection.

In our study, nonspecific clinical signs and hematological changes during the acute phase of disease were observed. An increase in lymph node swelling near the site of inoculation, lethargy and back pain between the second and seventh day after infection, with a recurrence of muscle pain on the 21st day post-infection, were observed. It is known that *Borrelia* can spread hematogenously, through lymphatics or between connective tissue [28], promoting inflammation during their migration that usually starts after the second day of infection [24]. The inflammation and muscle pain promoted by Lyme borreliosis is very well reported in humans [29, 30]. Muscle pain in horses was accompanied by moderate increases in the serum level of CPK, reinforcing the hypothesis of muscle catabolism due to inflammation and infection.

Slight reduction in hemoglobin and packed cell volum was observed in horses between the second and eleventh day after infection, and this observation was confirmed by the pale appearance of the oral and ocular mucosa. *B. burgdorferi* can promotes hemolysis when the bacteria make contact with red blood cells of horses [31]. In other species, such as in cattle, sheep and rabbits, hemolysis is not as pronounced. The hemolysin promoted by *Borrelia* binds to specific receptors present on the membranes of erythrocytes. It is assumed that the membranes of horse erythrocytes contain a greater amount of these receptors than in other species, thus favoring the onset of hemolytic anemia during infection [31]. The nonspecific changes associated with acute borreliosis can be easily confused with chronic equine piroplasmosis. Moreover, untreated infection caused by *Babesia caballi* or *Theileria*

equi also presents signs of lethargy and mild anemia associated with weight loss and poor performance in horses [32].

The dynamics of the serum concentration of immunoglobulins against *Borrelia* showed that within approximately 90 days (in the case of no re-infection and no treatment during this period), the immune system of the horses stopped producing these antibodies. This dynamic was observed both in naturally immunized horses and in experimentally infected horses. This scenario reinforces the theory of host immune evasion by the bacteria or the intense antigenic variation [24]. Spirochetes are able to stimulate immune suppression mechanisms, thereby reducing tissue damage and prolonging their presence in the mammalian host [24].

The absence of *B. burgdorferi* detection in Polymerase Chain Reactions (PCR's) could be explained by the low bacteremia promoted by these bacteria in the blood and tissues surveyed associated to low sensibility of PCR protocol used in this experiment. It is known, for example, that relapsing fever borreliae can be found in the blood in amounts as high as 10^8 per mL, while *B. burgdorferi* rarely achieves densities greater than 100 per mL. The dissemination of the bacteria occurs mainly in the lymphatic system [33]. Unfortunately, in this study there was no analysis of lymphonodes and muscle fascia. In a study of experimental infection, the authors amplified DNA of *B. burgdorferi* sensu lato strain B31 in various tissues such as lymphonodes, muscle, fascias, synovial membranes, myocardium, pericardium, kidneys, urinary bladder and dura mater [11].

Information that reinforces the hypothesis of experimental infection has been successful, despite the negative results in the PCR's, was an anaphylactoid reaction occurred during the application of ceftriaxone sodium on both horses in the first day

of treatment [34]. The sodium ceftriaxone was chosen for the treatment due to the fact that horses have been reduced to zero their antibody titers 90 days after inoculation, coming to doubt whether the bacteria had escaped to the central nervous system. It is known that, among the antibiotics used for borreliosis treatment, sodium ceftriaxone is the one with greater penetration in the blood-brain barrier [35] The horses experimentally infected were submitted to the start of treatment on 95th day and, a few moments after the application, both horses showed signs of cardiorespiratory changes. As a result of hemodynamic implications, both equines evolved to colic syndrome between 48 to 72 hours post anaphylactoid reaction. Both horses were treated for colic and survived in the end of the experiment.

The anaphylactoid reaction in patients infected with *Borrelia* spp at the initial moments of the treatment is described as Jarish-Herxheimer Reaction [36]. In this reaction, the massive death of spirochetes results in release of high doses of toxins present in borreliae, which induce systemic vasoconstriction immediately followed by profound vasodilatation. These hemodynamic changes may promote tachycardia, dyspnea, pulmonary edema, edema of the intestinal mucosa and tissue hypo perfusion. The changes on both horses during the treatment with ceftriaxone [34] strongly indicate the possibility of they had developed Jarish-Herxheimer Reaction, as the first report in the equine species. Subsequently, two horses were treated with oxytetracicline (5.0 mg / kg, intravenous, each 24 hours, per 21 days), [3] without any adverse reaction.

Acute Lyme borreliosis in horses is a disease with nonspecific clinical signs and hematological changes. This infection causes alterations only in the first days following infection and can be easily confused with chronic piroplasmosis in horses.

Even without treatment, approximately 90 days after infection, horses stop producing anti-*Borrelia* immunoglobulins, which makes the disease even more difficult to detect. Treatment with potent antibiotics may induce undesirable anaphylactoid reactions due to the massive death of bacteria, suggesting the treatment for 21 days using oxytetracicline in horses.

Acknowledgements

The authors acknowledge the financial support of FAPESP (Process 2013/05871-0 and 2013/03732-2).

5.5 REFERENCES

1. Magnarelli, L.A., Ijdo, J.W., van Andel, A.E.V., Wu, C., Padula, S.J. and Fikrig, E. (2000) Serologic confirmation of Ehrlichia equi and Borrelia burgdorferi infections in horses from the northeastern United States. *JAVMA* **217**, 1045-1050. doi:10.2460/javma.2000.217.1045.
2. Butler, C.M., Houwers, D.J., Jongejan, F. and Van der Kolk, J.H. (2005) *Borrelia burgdorferi* infections with special reference to horses. A review. *Vet Q* **27**, 146-156. doi:10.1080/01652176.2002.9695196.
3. Divers, T.J. (2013) Equine Lyme disease. *J Eq Vet Sci* **33**, 488-492. doi:10.1016/j.jevs.2013.03.187.
4. Kraiczy, P., Skerka, C., Kirschfink, M., Zipfel, P.F. and Brade, V. (2002) Immune evasion of *Borrelia burgdorferi*: insufficient kill of the pathogens by complement and antibody. *Int J Med Microbiol* **291**, 141-146.
5. Van Heerden, J. and Reyers, F. (1984) *Borrelia* sp. infection in a horse. *J S Afr Vet Assoc* **55**, 41-43.

6. Manion, T.B., Khan, M.I., Dinger, J. and Bushmich, S.L. (1998) Viable *Borrelia burgdorferi* in the urine of two clinically normal horses. *J Vet Diagn Invest* **10**, 196-199. doi:10.1177/104063879801000219.
7. James, F.M., Engiles, J.B. and Beech, J. (2010) Meningitis, cranial neuritis, and radiculoneuritis associated with *Borrelia burgdorferi* infection in a horse. *JAVMA* **10**, 1180-1185.
8. Priest, H.L., Irby, N.L., Schlafer, D.H., Divers, T.J., Wagner, B., Glaser, A.L., Chang, Y. and Smith, M.C. (2012) Diagnosis of borrelia-associated uveitis in two horses. *Vet Ophtalmol* **15**, 398-405. doi:10.1111/j.1463-5224.2012.01000.x.
9. Imai, D.M., Barr, B.C., Daft, B., Bertone, J.J., Feng, S., Hodzic, E., Johnston, J.M., Olsen, K.J. and Barthold, S.W. (2011) Lyme neuroborreliosis in 2 horses. *Vet Pathol* **48**, 1151-1157. doi:10.1177/0300985811398246.
10. Passamonti, F., Veronesi, F., Cappelli, K., Capomaccio, S., Reginato, A., Miglio, A., Vardi, D., Stefanetti, V., Coletti, M., Bazzica, C. and Pepe, M. (2015) Polysynovitis in a horse due to *Borrelia burgdorferi* sensu lato infection – Case study. *Ann Agric Environ Med* **22**, 247-250. doi:10.5604/12321966.1152074.
11. Chang, Y.F., Novosol, V., McDonough, S.P., Chang, C.F., Jacobson, R.H., Divers, T., Quimby, F.W., Shin, S. and Lein, D.H. (2000) Experimental infection of ponies with Borrelia burgdorferi by exposure to ixodid ticks. *Vet Pathol* **37**, 68-76. doi:10.1354/vp.37-1-68.
12. Chang, Y., Ku, Y., Chang, C., Chang, C., McDonough, S.P., Divers, T., Pough, M. and Torres, A. (2005) Antibiotic treatment of experimentally Borrelia burgdorferi-infected ponies. *Vet Microbiol* **107**, 285-294. doi:10.1016/j.vetmic.2005.02.006.

13. Embers, M.E., Hasenkampf, N.R., Jacobs, M.B. and Philipp, M.T. (2012) Dynamic longitudinal antibody responses during *Borrelia burgdorferi* infection and antibiotic treatment of *rhesus* macaques. *Clin Vaccine Immunol* **19**, 1218-1226. doi:10.1128/CVI.00228-12.
14. Palmero, J., Pusterla, N., Cherry, N.A., Kasten, R.W., Mapes, S., Boulouis, H.J., Breitschwerdt, E.B. and Chomel, B.B. (2012) Experimental infection of horses with Bartonella henselae and bartonella bovis. *J Vet Intern Med* **26**, 377-383. doi:10.1111/j.1939-1676.2012.00890.x.
15. Grause, J.F., Ueti, M.W., Nelson, J.T., Knowles, D.P., Kappmeyer, L.S. and Bunn, T.O. (2013) Efficacy of imidocarb dipropionate in eliminating Theileria equi from experimentally infected horses. *Vet J* **196**, 541-546. doi:10.1016/j.tvjl.2012.10.025.
16. Wagnerová, P., Sak, B., Kvetonová, D., Marsálek, M., Langrová, I. and Kvác, M. (2013) Humoral immune response and spreading of *Encephalitozoon cuniculi* infection in experimentally infected ponies. *Vet Parasitol* **197**, 1-6. doi:10.1016/j.vetpar.2013.05.007.
17. Abaracón, D. (1987) Programa de adiestramento em salud animal para America Latina. Organización Paramericana de Salud, Terra Nova AS, Washington, D.C., USA.
18. D'Angelis, F.H.F., Ferraz, G.C., Boleli, I.C., Lacerda-Neto, J.C. and Queiroz-Neto, A. (2005) Aerobic training, but not creatine supplementation, alters the gluteus medius muscle. *J Anim Sci* **83**, 579-585.

19. Queiroz, D.J. (2014) *Alterações clínicas e laboratoriais de equinos submetidos à biópsia hepática com agulha tru-cut guiada por ultrassom*. Master's Dissertation, Univ. Estadual Paulista, Jaboticabal, Brazil.
20. Joppert, A.M., Hagiwara, M.K. and Yoshinari, N.H. (2001) *Borrelia burgdorferi* antibodies in dogs from cotia county, São Paulo State, Brasil. *Rev Inst Med Trop S Paulo* **34**, 251-255.
21. Salles, R.S., Fonseca, A.H., Scofield, A., Madureira, R.C. and Yoshinari, N.H. (2002) Sorologia para *Borrelia burgdorferi* latu sensu em equinos no Estado do Rio de Janeiro. *A Hora Veteriária* **22**, 46-49.
22. Mantovani, E., Marangoni, R.G., Gauditano, G., Bonoldi, V.L.N. and Yoshinari, N.H. (2012) Amplification of the *flg*E gene provides evidence for the existence of a Brazilian borreliosis. *Rev Inst Med Trop S Paulo* **54**, 153-157. doi:10.1590/S0036-46652012000300007.
23. Sal, M.S., Li, C., Motalab, M.A., Shibata, S., Aizawa, S.I. and Charon, N.W. (2008) *Borrelia burgdorferi* uniquely regulates its motility genes and has an intricate flagellar hook-basal body structure. *J Bacteriol* **190**, 1912-1921. doi:10.1128/JB.01421-07.
24. Radolf, J.D., Caimano, M.J., Stevenson, B. and Hu, L.T. (2012) Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes. *Nat Rev Micro* **10**, 87-99. doi:10.1038/nrmicro2714.
25. Carrasco, S.E., Troxell, B., Yang, Y., Brandt, S.L., Li, H., Sandusky, G.E., Condon, K.W., Serezani, C.H. and Yang, X.F. (2015) Outer surface protein OspC is an antiphagocytic factor that protects Borrelia burgdorferi from phagocytosis by macrophages. *Infect Immun* **83**, 4848-4860.

26. Berende, A., Oosting, M., Kullberg, B.J., Netea, M.G. and Joosten, L.A.B. (2010) Activation of innate host defense mechanisms by *Borrelia*. *Eur Cytokine Netw* **21**, 7-18.
27. Tokarz, R., Anderton, J.M., Katona, L.I. and Benach, J.L. (2004) Combined effects of blood and temperature shift on *Borrelia burgdorferi* gene expression as determined by whole genome DNA array. *Infect Immun* **72**, 5419-5432. doi:10.1128/IAI.72.9.5419-5432.2004.
28. Wasiluk, A., Zalewska-Szajda, B., Waszkiwicz, N., Kepka, A., Szajda, D.S., Wojewodzka-Zelezniakowicz, M., Ladny, J.R., Pancewicz, S., Zwierz, Z.W. and Zwierz, K. (2011) Lyme disease: etiology, pathogenesis, clinical courses, diagnostics and treatment. Prog. *Health Sci* **1**, 179-186.
29. Reimers, C.D., Koning, J., Neubert, U., Preac-Mursic, V., Koster, J.G., Müller-Felber, W., Pongratz, D.E. and Duray, P.H. (1993) Borrelia burgdorferi myositis: report of eight patients. *J Neurol* **240**, 278-283. doi:10.1007/BF00838161.
30. Holmgren, A.R. and Matteson, E.L. (2006) Lyme myositis. *Arthritis Rheum* **54**, 2697-2700. doi:10.1002/art.22007.
31. Williams, L.R. and Austin, F.E. (1992) Hemolytic activity of *Borrelia burgdorferi*. *Infect Immun* **60**, 3224-3230.
32. Wise, L.N., Pelzel-McCluskey, A.M., Mealey, R.H. and Knowles, D.P. (2014) Equine piroplasmosis. *Vet Clin North Am Eq Pract* **30**, 677-693. doi:10.1016/j.cveq.2014.08.008.
33. Samuels, D.S., Radolf, J.D. (2010) Borrelia: Molecular biology, host interaction and pathogenesis. Caister Academic Press, Norfolk, UK, 547 p.

34. Basile, R.C., Rivera, G.G., Del Rio, L.L., de Bonis, T.C.M., Amaral, G.D.P., Giangrecco, E., Ferraz, G., Yoshinary, N.H., Canola, P.A., Queiroz-Neto, A. (2015) Anaphylactoid reaction induced by sodium ceftriaxone in two horses experimentally infected by *Borrelia burgdorferi*. *BMC Veterinary Reasearch* **11**, 197.

35. Halperin J.J. (2014) Nervous system Lyme disease. *Handbook of Clinical Neurology* **121,** 1473-1483.

36. Bryceson, A.D.M., Cooper, K.E., Warrell, D.A., Perine, P.L., Parry, H.O. (1972) Studies of the mechanism of the Jarish-Herxheimer reaction in louse-borne relapsing fever: evidence for the presence of circulating *Borrelia* endotoxin. *Clinical Science* **43**, 343-354.

6. ANAPHYLACTOID REACTION INDUCED BY SODIUM CEFTRIAXONE IN TWO HORSES INFECTED BY BORRELIA BURGDORFERI

Paper published in *BIOMED CENTRAL VETERINARY RESEARCH* (2015) 11: 197. http://dx.doi.org/10.1186/s12917-015-0478-6

Anaphylactoid reaction induced by sodium ceftriaxone in two horses experimentally infected by *Borrelia burgdorferi*

Roberta Carvalho Basile, Gabriela Gomes Rivera, Lara Antoniassi Del Rio, Talissa Camargo Mantovani de Bonis, Gabriel Paiva Domingues do Amaral, Edson Giangrecco, Guilherme de Camargo Ferraz, Natalino Hajime Yoshinari, Paulo Aléscio Canola, Antonio de Queiroz Neto

ABSTRACT

Background: Lyme borreliosis is a disease transmitted by ticks to mammals, especially in horses and humans. Caused by a spirochete *Borrelia burgdorferi*, it can result in lameness, arthritis, carditis, dermatitis and neurological signs. Anaphylactoid reactions are severe responses caused by direct action of substances (drugs, toxins), which can pose risks to life. Still poorly documented in horses, these reactions are caused by the effects of inflammatory mediators such as histamine, kinins and arachidonic acid metabolites. The last two are the most clinically relevant for the species. **Case Presentation:** The simultaneous occurrence of anaphylactoid reaction in two horses experimentally infected by *Borrelia burgdorferi* undergoing intravenous treatment with ceftriaxone sodium is reported. It was administered 4.7 x 10^8 spirochetes intradermal and subcutaneous applications in both horses to evaluate clinical aspects of the Lyme disease, ninety-five days before the application of sodium ceftriaxone. During the administration, one horse (a gelding) showed immediate and severe anaphylactoid symptoms such

as urticaria, dyspnea, tachycardia, and eyelid edema, which were controlled by injecting dexamethasone. After one day, it expressed signs of abdominal discomfort, caused by severe bloat, which was treated surgically via celiotomy. Subsequently, this gelding had piroplasmosis and severe anemia, requiring treatment with an antimicrobial and blood transfusion. Second horse (a mare) showed signs of hypotension during the application of the antibiotic, which disappeared only when the application was interrupted. Days after the event, the mare developed moderate large colon bloat, which was treated with medication only. Subsequently the mare was evolved into the prodromal phase of laminitis in one of the forelimbs, which was treated for 10 days with non-steroidal anti-inflammatory and rheology modifying drugs and cryotherapy. **Conclusions:** From the two cases presented here, it does appear that sodium ceftriaxone can induce anaphylactoid reactions in horses infected by *Borrelia burgdorferi*, which may evolve into colic syndrome, laminitis and the occurrence of opportunistic infections. However, further evidence should be collected in order to draw definite conclusions.

Keywords: hypersensitivity, Lyme, colic, laminitis.

6.1 BACKGROUND

Lyme borreliosis is a multisystemic disease that affects humans, domestic and wild animals, transmitted by ticks and caused by spirochete *Borrelia burgdorferi* sensu lato. In horses, were necessary at least 18 h of tick attachment to transmission of the bacteria to the host, which can present lameness, arthritis, uveitis, encephalitis, abortion, foal mortality and recurrent hemoparasites infections. The treatment can be conducted using tetracycline, doxycycline or ceftiofur [1].

Conducting sodium ceftriaxone therapy for borreliosis has been reported only in humans [2].

Anaphylaxis is the most severe form of allergic reaction called Type 1 hypersensitivity, and occurs in horses mainly in response to toxins from venomous animals, drugs and food allergens [3]. The classical anaphylactic reaction is caused by the IgE antibodies binding onto the surface of mast cells and basophils with subsequent release of inflammatory mediators [3, 4, 5, 6]. Anaphylactoid reactions are symptomatically similar, but they are caused by direct action of toxic substances (e.g. endotoxins, certain drugs and chemicals) with the subject has not necessarily been previously “sensitized” [5]. They do not involve IgE participation and occur through a direct nonimmune-mediated release of the same mediators [7].

The main mediators of a reaction are biogenic amines (histamine, serotonin, catecholamines), vasoactive polypeptides (kinins, cationic proteins, complement system anaphylaxins C_{3A}, C_{5A}, C_{567}), lysosomal enzymes, vasoactive lipids (prostaglandins, endoperoxides, thromboxanes), phospholipids (platelet activating factor) and chemotactic substances [5, 6].

Anaphylactoid reactions are difficult to be reproduced experimentally, however there are reports of anaphylaxis induction experiments in horses by sensitization with bovine serum [8] that were conducted on 20 adult ponies under anesthesia. Cardiovascular, respiratory, hematological, electrolyte and biochemical responses were evaluated during the acute phase. The results contributed significantly to describing the cascade of events that occur during anaphylaxis in the species. The authors reported that the first event occurred about two minutes after contact with the antigen. Blood pressure (common carotid artery) decreased significantly while

pulmonary artery pressure increased and the pressure of the abdominal vena cava decreased slightly. During anaphylaxis, there was hemoconcentration, leukopenia, thrombocytopenia, and hyperkalemia. Initially, plasma concentrations of histamine and bradykinin increased significantly (more than four times). Clinically, tachypnea and tachycardia were also observed, which returned to normal in about 12 minutes.

The therapeutic response of 16 ponies with induced anaphylaxis to antagonistic drugs of the main anaphylactic mediators [9] has also been evaluated. The author compared the antagonist effects of burimamide and tripelennamine on histamine and also evaluated methysergide as serotonin blocker. The efficacy of meclofenamate sodium, and acetylsalicylic acid as non-steroidal anti-inflammatory drugs was evaluated, as well. The results show that meclofenamate sodium and acetylsalicylic acid effectively inhibited and managed the anaphylactic cardiovascular and respiratory symptoms, suggesting that in horses, prostaglandins, thromboxanes and kinins have greater clinical relevance to immediate hypersensitivity [6]. On the other hand, histamine is primarily responsible for most adverse events in humans and dogs [4].

There are few case reports in the literature about anaphylaxis and anaphylactoid reactions in horses. There is a case description of a gelding that received intravenous ivermectin and developed a lethal anaphylactic reaction [10]. More recently, there is an unusual case of anaphylaxis in a neonatal foal caused by inadvertent intravenous administration of breast milk [11], which was reversed with epinephrine, and dexamethasone, and intensive outpatient treatment for nine days. The most recent publications on IgE-mediated hypersensitivity in horses emphasize

only dermatological aspects as signs of atopy, allergy to insect stings, food and parasitism associated allergies [12, 13].

Sodium Ceftriaxone, classified as third-generation cephalosporin, is a broad spectrum antibiotic with a beta lactam ring in its structure and capable of bypassing the blood-brain barrier [14]. Its pharmacokinetics has been evaluated in mares [15], adult horses [16] and foals [17]. These studies were performed by intravenously injecting a single dose between 25 and 50 mg/kg, diluted in saline solution. No adverse drug reactions have been reported in these studies.

To date, there are no reports regarding the occurrence of adverse reactions to ceftriaxone sodium in horses, as has already been observed in humans [16]. Therefore, this case report describes the occurrence of non-fatal anaphylactic reaction in two horses after intravenous administration of ceftriaxone sodium, the immediate clinical outcome, consequences and subsequent therapeutic strategies adopted.

6.2 CASE PRESENTATION

Two sound horses, a gelding and a mare chosen from the group of experimental horses of the University, approximately eight years old, were experimentally infected with *Borrelia burgdorferi,* using a dose of 4.7×10^8 bacteria per horse, divided in four simultaneous applications (2 subcutaneous and 2 intradermal), in the region of the superficial cervical lymph nodes, with the approval of the Ethics Committee on Animal Use - CEUA of UNESP (protocol 001968/13). The objective of the experiment was to compare the clinical and laboratorial differences between the infection of *Borrelia burgdorferi* strain G39/40 in horses versus the

Borreliosis diagnosed in Brazil in addition to their treatment viability with sodium ceftriaxone, since it is an effective antibiotic to control borreliosis, including its neurological forms [13]. There were no previous reports of sodium ceftriaxone adverse reactions in horses.

Both horses had only mild acute clinical alterations in the first 14 days after infection, characterized by augmented submandibular lymph nodes, pale mucosa, dorsal sensibility and hyporexia.

Ninety five days after experimental infection, both animals were injected intravenously (4 drops/s) ceftriaxone sodium at the dose of 25 mg/kg, diluted in 500 ml of sterile 0.9% sodium chloride saline solution, by catheterization[b] of the left jugular vein. The clinical and hematological parameters of both horses were checked before administration and were within normal range. However, the clinical and hematological parameters changed approximately two minutes after the drug was administered. Both horses that received sodium ceftriaxone developed an anaphylactoid reaction. The entire experiment was conducted on only these two animals.

Case 1: Gelding

A male purebred Arabian horse weighing 410 kg, submitted to 6h of food fasting had a heart rate (HR) of 40 bpm, respiratory rate (RR) of 22 mrm, rosy mucous membranes, capillary refill time (CRT) of 2 s, rectal temperature (RT) of 37.1°C and hematological parameters within the reference range for the species (Table 8, Basal) before the administration of the antibiotic. About seventeen minutes into the administration of ceftriaxone sodium (Day 1), after 205 mL of the solution had

dripped, the horse showed tachycardia (heart rate = 96 bpm), dyspnea and urticaria on the neck left side (the same side where the drug was being injected). The drug dripping was discontinued immediately, but the animal still presented severe dyspnea. The gelding respiratory function normalized a minute after 0.05 mg/kg dexamethasone was injected. A noticeable swelling of eyelids was observed an hour after the allergic event. At this time the patient had a HR of 48 bpm, RR of 20 mrm, pale mucosa, RT of 37.7°C and a slight reduction of intestinal motility. The horse behavior remained apathetic for the rest of the day, with no significant changes upon physical examination and was fed only hay.

Table 8 – Gelding red blood cell count before and after anaphylactic reaction to sodium ceftriaxone, which occurred on day 1.

	Basal	Day 2	Day 3	Day 6	Day 10	Day 20	Day 21	Day 28	Reference[1]
Erythrocytes x 10^6 [cells/ml]	8.40	6.72	6.10	6.32	6.30	*3.10*	6.20	6.50	6.0-9.7
Hemoglobin [g/dL]	12.5	10.6	9.8	9.8	9.2	*4.2*	8.6	10.0	8.3-14.4
Hematocrit [%]	38.0	*29.5*	*26.7*	*27.4*	*28.0*	*13.0*	*27.0*	30.0	30-44
MCV [fL]	45.2	-	-	-	44.4	41.9	43.5	46.2	36-52.1
MCH [pg]	14.9	-	-	-	14.6	13.5	13.9	15.4	11.5-18.2
MCHC [g/dL]	32.9	-	-	-	32.9	32.3	31.9	33.4	31.2-34.9
Leucocytes [cells/uL]	10100	9800	12900	5700	*11200*	*10800*	*14800*	9500	6400-10600
Basophiles [cells/uL]	0	0	0	0	0	0	0	0	0
Eosinophils [cells/uL]	808	0	0	100	112	216	148	285	0-320
Segmented Neutrophils [cells/uL]	6060	*7800*	*9100*	6600	8736	6804	*11692*	5700	2775-7530
Rod Neutrophils [cells/uL]	202	0	100	0	*448*	216	*1480*	285	94-420
Lymphocytes [cells/uL]	2727	2100	*800*	2900	1680	3348	1184	3135	1088-5096
Monocytes [cells/uL]	303	100	0	*400*	224	216	296	190	90-318
Platelets [cells/uL]	194000	246000	242000	279000	*323000*	90000	96000	*368000*	90000-322000

[1] Reference ranges obtained for total blood cell count for 33 domestic horses of different breeds, serum negative for *Borrelia burgdorferi*, kept under the same feeding and management conditions.

MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration.

On day 2 in the morning, the gelding was fed 1.0 kg of commercial feed, but due to abdominal discomfort it positioned itself on lateral recumbency. Upon examination, the horse had severe bilateral abdominal distention, a HR of 37 bpm, RR 20 mrm, RT 37.2 °C, pale mucosa, intestinal hypomotility and yellowish and turbid peritoneal fluid. In the laboratory, the peritoneal fluid was characterized by total protein of 96 g/dL; pH 7.6; density 1.010 g/dL and 1250 cells/mL (71% segmented neutrophils, 22% lymphocytes and 7% macrophages). Hematological and blood gas evaluation of venous blood showed anemia and hemoconcentration (Table 8, Day 2), metabolic and respiratory acidosis, hyponatremia, hypokalemia, hypochloremia, hyperglycemia, and a blood lactate concentration (Table 9, Day 2). The abdominal ultrasonography showed severe gaseous distension, *ileus*, along with wall thickening of small intestine (Figure 5).

Table 9 – Gelding blood gas analysis after anaphylactic reaction to sodium ceftriaxone, which occurred on day 1.

	Day 2	**Day 3**	**Day 28**	**Reference[1]**
pH	*7.271*	*7.467*	7.374	7.384-7.408
pCO2 [mmHg]	*69.6*	42.3	*45.1*	35-45
pO2 [mmHg]	*55.4*	38.5	35	35-45
HCO_3^- [mmol/L]	*17.7*	27.8	26.4	21-53
Na^+ [mmol/L]	*116.4*	*130.1*	138	135-148
K^+ [mmol/L]	*2.96*	*2.51*	4.0	3.5-4.5
Cl^- [mmol/L]	*88.5*	100.5	102.2	98-107
Glucose [mmol/L]	*7.3*	*8.1*	4.0	4.1-5.9
Lactate [mmol/L]	*5.6*	1.1	1.2	1.0-1.7

[1] Reference ranges obtained with the iStat EG7+ device.

pCO_2: partial pressure of carbon dioxide; pO_2: partial pressure of oxygen; HCO_3^-: bicarbonate ion; Na^+: sodium ion; K^+: potassium ion; Cl^-: chloride ion

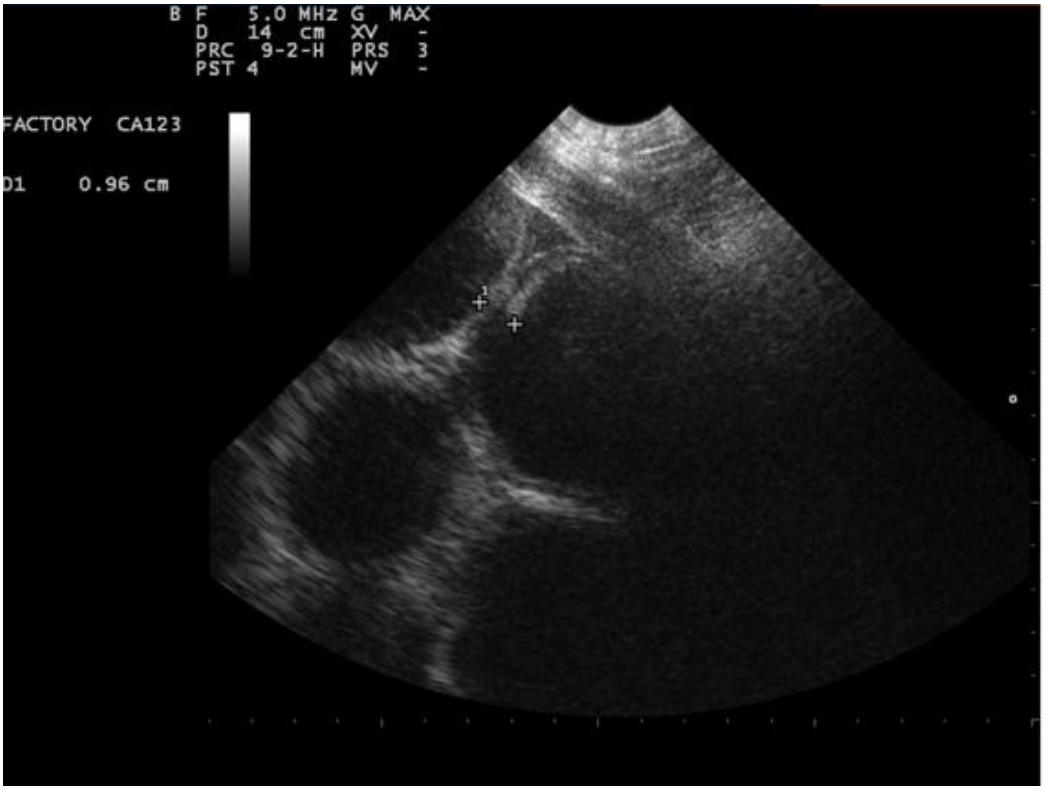

Figure 5 - Abdominal ultrasound of the gelding with tympanic colic as a result of anaphylactic reaction to sodium ceftriaxone. Image obtained by ventral-abdominal positioning of the probe, caudal to xiphoid. Note the thickening of intestinal wall, distention, atony and *ileus* in segments of the small intestine. Diagnostic Imaging Sector - FCAV - UNESP - Jaboticabal.

The ultrasonography results were consistent with volvulus of the small intestine; therefore, the gelding was referred to the surgical center for exploratory celitomy. Before the surgery, an intravenous antibiotic therapy with 20.000U/kg potassium penicillin[c] was initiated. During surgery, were observed gas distention throughout the small bowel, wall edema of the small and large colon, cecum, jejunum and *ileus* (Figure 6). There were also retroflexion of the pelvic flexure and displacement of cecum. The small intestine milking was followed by gas aspiration of

the cecum, colon repositioning and washing of the abdominal cavity with neomycin diluted in 10% Sodium lactate Ringer's solution.

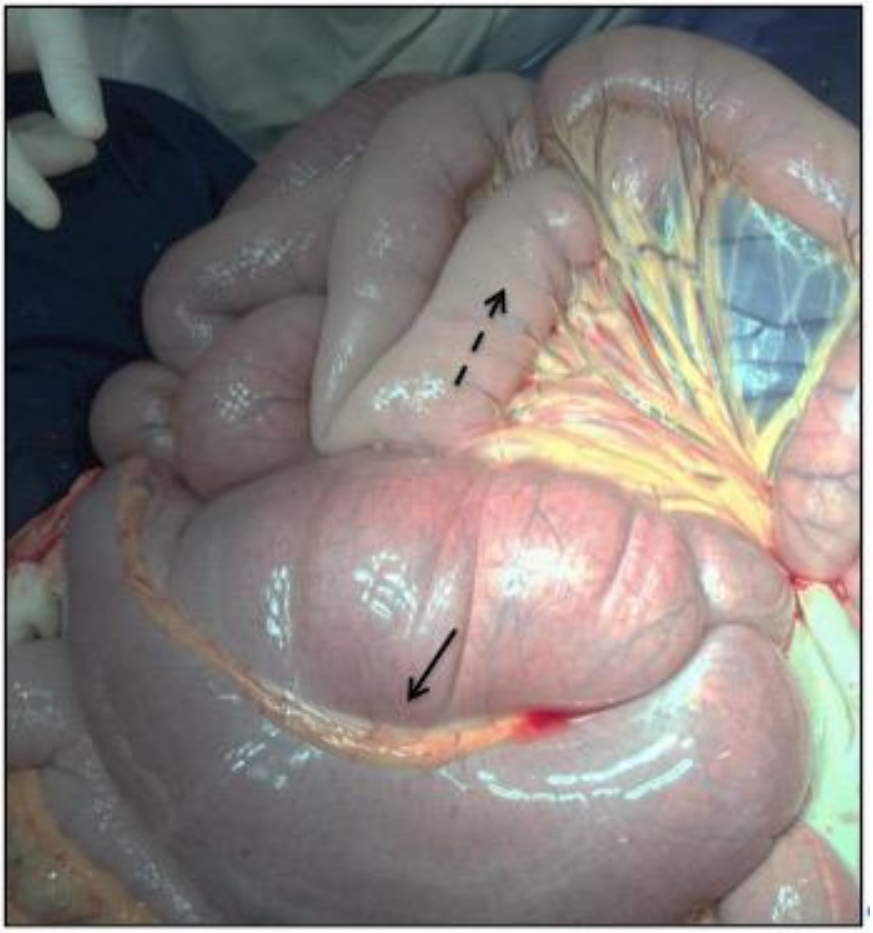

Figure 6 - Exposure of the gelding cecum and jejunum during exploratory celiotomy. Full line arrow: cecum. Dashed line arrow: jejunal serosa showing edema due to anaphylaxis caused by sodium ceftriaxone, in lighter color compared to other intestinal loops.

After surgery, the horse recovered uneventful from anesthesia and defecated upon rising. The antibiotic therapy was continued post operatively with penicillin potassium (20,000U/kg), every 8 hours, for three consecutive days. The patient was also treated with a single dose of both furosemide (0.5 mg/kg) and dexamethasone

(0.05 mg/kg) to reduce the intestinal edema; 30 mL of Potassium Chloride (150 mg/mL) diluted in 1 L of Sodium Lactate Ringer's solution to correct the hypokalemia; three liters of solution of 10% DMSO solution (100 mL/L in Ringer's lactate solution), once daily for two days; and, flunixin meglumine (0.25 mg/kg) once daily for three days. The abdominal incision was cleaned using aqueous chlorhexidine solution while repellent was applied around the surgical wound twice a day, for 10 days.

This patient progressed steadily until the eighteenth day after anaphylaxis, when it showed apathy, loss of appetite, fever of 40.1 °C, pale mucosa, 3s CRT, HR 72 bpm, RR 44 mrm and severe back pain. A blood sample was collected using EDTA tube and sent to the Parasitology Laboratory for Polymerase Chain Reaction *(*Nested-PCR) to detect the presence of *Babesia caballi* and *Theileria equi,* as well as Real-Time PCR for *Anaplasma phagocytophilum*. The test revealed *Theileria equi* infection. Therefore, intramuscular treatment with imidocarb[d] (4.0 mg/kg) was instituted, divided into two daily doses for two days, along with Dipyrone (25 mg/kg), every 12 hours for two days.

Subsequent to treating piroplasmosis, the gelding also revealed high serum concentrations of indirect bilirubin, creatine kinase, gamma glutamyl transferase, globulin and fibrinogen (Table 10, Day 20).

Table 10 - Gelding serum biochemical parameters before and after anaphylactic reaction to sodium ceftriaxone, which occurred on day 1.

	Basal	Day6	Day10	Day20	Day21	Day28	Reference[1]
Direct bilirubin [mg/dL]	0.28	-	*1.10*	0.29	*0.35*	0.27	0.12-0.31
Indirect bilirubin [mg/dL]	0.06	-	0.40	*2.25*	*4.12*	0.51	0.07-1.07
Creatine phosphokinase [U/L]	146	-	*80*	*448*	*1162*	120	84-368
Urea [mg/dL]	15	21	26	26	20	41	14-41
Creatinine [mg/dL]	1.3	1.6	*0.9*	*0.9*	*0.7*	1.3	1.1-1.7
Aspartate Amino Transferase [U/L]	302	220	173	296	426	271	232-447
Gamma Glutamyl Transferase [U/L]	18	*68*	*21*	16	18	17	2.0-19.0
Total Protein [g/dL]	6.4	6.4	5.4	6.9	*7.6*	6.7	4.0-7.7
Albumin [g/dL]	2.4	-	2.9	2.1	2.3	1.7	1.7-3.2
Globulin[g/dL]	4.0	-	2.5	4.8	*5.3*	*5.0*	2.3-4.8
Fibrinogen [g/dL]	0.1	0.4	0.5	*1.2*	*0.9*	0.2	0.1-0.4

[1] References ranges obtained from the hematological analysis of 33 domestic horses of several breeds, serum negative for *Borrelia burgdorferi*, kept under the same management and feeding conditions.

On the twentieth day after anaphylactoid reaction, the gelding showed marked hypochromic normocytic anemia and neutrophilia (Table 8, day 20) and the patient was transfused with 6 L of whole blood. The following day, the blood parameters improved (Table 8, Table 10, Day 21). However, the horse showed signs of abdominal discomfort though sternal recumbence and anorexia with normal intestinal motility, fecal output and consistency, mild tachycardia and depression, suggestive of gastritis. A twenty-day course of omeprazole was instituted and a satisfactory improvement of its clinical and hematological status occurred 28 days after the anaphylaxis (Table 8, Table 9, Table 10, Day 28).

Case 2: Mare

The Anglo-Arab mare weighing 420 kg, submitted to food fasting for 6 h, had a basal heart rate (HR) of 38 bpm, respiratory rate (RR) of 16 mrm, rosy mucous membranes, capillary refill time (CRT) of 2 s, rectal temperature (RT) 36.6ºC and

hematological parameters within the reference range (Table 4, Basal) before starting the antibiotic treatment. After 95 mL of the ceftriaxone sodium (Day 1) solution had dripped, the mare presented paresis in the pelvic limbs, suggestive of circulatory hypotension. The antibiotic solution drip was suspended immediately, and within a few minutes, the mare was back to normal with no apparent need for pharmacological intervention. Two days after the anaphylactoid reaction (Day 3), the mare started showing signs of discomfort and slight abdominal distention. The physical examination revealed a HR of 52 bpm, RR of 34 mrm, RT 37.5°C, CRT 3 s, pale mucosa and a severe intestinal hypomotility. No abnormalities were found upon rectal palpation, except for the presence of gas in the bowel loops and wall edema of the rectum. The colic event did not alter the hematological parameters or serum biochemistry of the patient (Table10, Table 11, Day 3). The support treatment consisted of administering 3L of 10% DMSO solution, 10 ml intravenous dexamethasone, gastric lavage and100 mL of 30% oral silicon solution to reduce bloating and flunixin meglumine (0.5 mg/kg), intravenously. The patient showed improvement in the intestinal motility six hours after the drug treatment had started.

Table 11 – Mare red blood cell count before and after anaphylactic reaction to sodium ceftriaxone, which occurred on day 1.

	Basal	Day 3	Day 10	Day 28	Reference[1]
Erythrocytes x 10^6 [cells/ml]	8.3	7.1	8.4	7.4	6.0-9.7
Hemoglobin [g/dL]	13	11.2	13	11.5	8.3-14.4
Hematocrit [%]	39	31	39	35.0	30-44
MCV [fL]	47	-	46.4	47.3	36-52.1
MCH [pg]	15.7	-	15.5	15.5	11.5-18.2
MCHC [g/dL]	33.3	-	33.3	32.9	31.2-34.9
Leucocytes [cells/uL]	8600	6600	10000	6900	6400-10600
Basophiles [cells/uL]	0	0	0	0	0
Eosinophils [cells/uL]	*344*	66	200	138	0-320
Segmented Neutrophils [cells/uL]	4902	4224	7300	3588	2775-7530
Rod Neutrophils [cells/uL]	258	0	*800*	207	94-420
Lymphocytes [cells/uL]	2924	2244	1500	2898	1088-5096
Monocytes [cells/uL]	172	66	200	69	90-318
Platelets [cells/uL]	151000	154000	191000	165000	90000-322000

[1] Reference ranges obtained for total blood cell count for 33 domestic horses of different breeds, serum negative for *Borrelia burgdorferi*, kept under the same feeding and management conditions.

Table 12 - Mare serum biochemical parameters before and after anaphylactic reaction to sodium ceftriaxone, which occurred on day 1.

	Basal	Day3	Day10	Day28	Reference[2]
Direct bilirubin [mg/dL]	0.13	0.39	*1.1*	0.18	0.12-0.31
Indirect bilirubin [mg/dL]	0.10	*1.66*	0.2	0.63	0.07-1.07
Creatine phosphokinase [U/L]	166	-	276	223	84-368
Urea [mg/dL]	13	25	18	35	14-41
Creatinine [mg/dL]	1.1	1.6	1.1	1.0	1.1-1.7
Aspartate Amino Transferase [U/L]	424	199	263	368	232-447
Gamma Glutamyl Transferase [U/L]	*33*	*76*	*36*	*23*	2.0-19.0
Total Protein [g/dL]	6.5	6.4	6.6	7.2	4.0-7.7
Albumin [g/dL]	2.4	2.44	3.4	2.3	1.7-3.2
Globulin[g/dL]	4.1	-	3.2	*4.9*	2.3-4.8
Fibrinogen [g/dL]	0.1	0.5	*0.9*	0.2	0.1-0.4

[1] References ranges obtained from the hematological analysis of 33 domestic horses of several breeds, serum negative for *Borrelia burgdorferi*, kept under the same management and feeding conditions.

Three days after the tympanic colic episode (Day 6), the temperature of the dorsal hoof wall of the left forelimb (LF) increased and the mare revealed strong pulse in the palmar digital arteries (Figure 7, Day 6). Cryotherapy was performed in the region between the hoof and carpus for one hour, three times a day, and intravenous flunixin meglumine, anti-endotoxemic dose (0.25 mg/kg), every 12 hours.

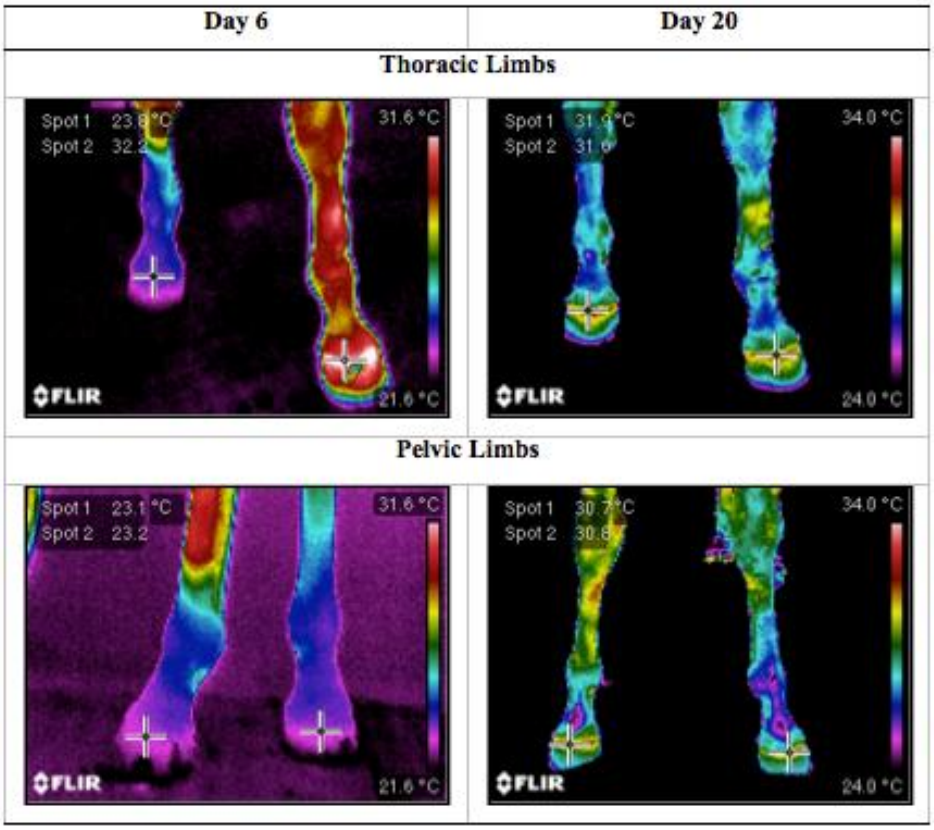

Figure 7 - Infrared images to monitor the evolution of the laminitis treatment in the mare that had colic secondary to anaphylactic reactions to ceftriaxone sodium. Day 6 was the second day of temperature rise in the hoof. The crosses indicate the temperature of the hoof crown for each member. Temperature profile of the hooves was back to normal on Day 20. Spot 1: right limb, Spot 2: left limb.

Two days after initiating the cryotherapy (Day 8), no improvement was noted in the LF temperature profile (Figure 8). Therefore, along with cryotherapy, firocoxib was administered orally (0.1 mg/kg), every 24 hours, in combination with pentoxifylline (8.4 mg/kg), every 12 hours, for 10 days. At the end of the 10th day, the temperature profile of the left forelimb returned to normal values (Figure 5.3, Day 20). The mare showed no signs of pain or lameness in the affected limb at any time; thus, suggesting the development of laminitis was treated within the prodromal phase. Therefore, no hoof radiographic assessment was performed.

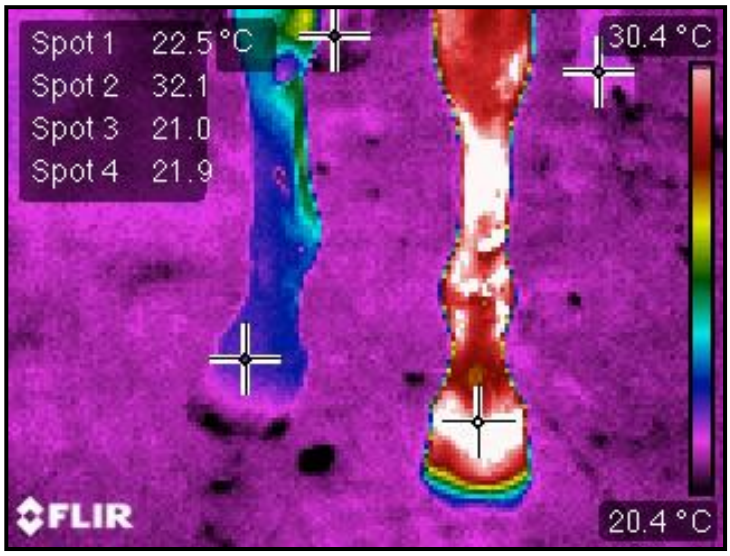

Figure 8 – Infrared image of the four limbs of the mare two days after treatment started (Day 8). The crosses indicate the temperature of the hoof crown of each member. Spot 1: right forelimb, Spot 2: left forelimb, Spot 3: right hindlimb, Spot 4: left hindlimb.

There were no alterations in the environment or feeding throughout ceftriaxone administration at the beginning of the colic period that would explain the abdominal discomfort occurrence and the resultant laminitis. Therefore, the authors suggest that

the possible hypotension was associated with the liberation of inflammatory mediators which caused the subsequent laminitis.

Table 13 summarizes the events that occurred with the two animals over time.

Table 13 – Timetable of the events described for the anaphylactic reaction to sodium ceftriaxone of a horse and a mare.

Chronology	Gelding	Mare
Basal	Total blood cell count before treatment started	Total blood cell count before treatment started
Day 1	Administration of ceftriaxone sodium and anaphylaxis	Administration of ceftriaxone sodium and anaphylaxis
Day 2	Tympanic colic and laparotomy	Stable
Day 3	Stable	Tympanic colic resolved clinically
Day 6	Stable	Prodromal laminitis and treatment
Day 8	Stable	Change in the laminitis treatment
Day 18	Theileriosis treated with imidocarb	Stable
Day 20	6L of whole blood transfusion	Stabilization of the clinical symptoms
Day 21	Gastritis	Stable
Day 28	Clinical symptoms back to normal	Stable

Penicillins are the most important antibiotic class followed by cephalosporins, which contains a beta-lactam ring in their molecular structure. Both groups can induce hypersensitivity reactions mediated by IgE [18]. *In vitro* studies demonstrated that IgE antibodies are reactive to both terminals of cephalosporin molecules. This hybridoma formation has shown that cephalosporins can generate unique structures capable of inducing specific allergic reactions, which may exhibit cross-reactivity with penicillin in 5 to 15% of the cases [19, 20]. In this study, both animals had been previously treated with penicillin without any signs of an allergic reaction, which suggests an anaphylactoid reaction.

To date, there are no reports regarding the manifestation of hypersensitivity to cephalosporin in horses. This was one of the reasons why a drug of this group (ceftriaxone sodium) was chosen to treat the experimental infection with *Borrelia burgdorferi.* In addition to the cephalosporin pharmacokinetic studies conducted in horses [15, 16, 17], clinical trials were also conducted in camels [21], dogs [22], calves [23] and lactating goats [24] without any reports regarding the occurrence of hypersensitivity reactions.

The evolution of the anaphylactoid symptoms in all of the tried horses was consistent with a severe hypersensitivity reaction in the gelding (without reaching shock parameters) and moderate reaction in the mare, which showed only signs of circulatory hypotension. Typically the symptoms of mild anaphylactoid reactions and hypersensitivity in horses are limited to local and dermatological [25, 26] changes.

During a classical anaphylaxis, it is necessary a first substance exposure to immunologic sensitization and a cascade of events associated with the IgE molecule binding to basophils and mast cells occurs, triggering the release of histamine and other vasoactive substances [3, 27], such as serotonin, catecholamines, kinins, products of arachidonic acid and platelet activating factor [5]. The anaphylactoid reaction is a non-IgE mediated hypersensitivity response, caused by immune aggregates, complement activation, coagulation activation or autoimmune mechanisms, with the same clinical appearance of a classical anaphylaxis [27]. Both horses had never been exposed previously to sodium ceftriaxone and did not have any reaction to penicillin, resulting in an anaphylactoid reaction. Besides, it was not possible to know whether the massive death of spirochetes during antibiotic application also had correlation with clinical signs presented by both horses. Further

evidences shall be collected in order to define if the anaphylactoid reaction was caused only by the sodium ceftriaxone or the interaction between the drug and the *B. burgdorferi* infection in horses.

The pharmacological treatment with dexamethasone reversed the acute symptoms of the anaphylactoid reaction in the gelding, corroborating published studies [9, 28, 29]. It has been reported that anti-inflammatory non-steroidal drugs (NSAID's) showed greater efficacy in reversing the cardiovascular and respiratory effects of experimentally induced anaphylaxis in horses compared to antihistamines. Together with corticoids, the animals may also have benefited from epinephrine for controlling acute signs of hypotension and edema [25].

The vascular and hemodynamic changes that occurred during reaction in the gelding resulted in a mural edema of various intestinal segments, in addition to *ileus*, with signs of abdominal discomfort. During celiotomy, mural edemas were observed on the colon and small intestine, as well as a large amount of liquid and gaseous content and petechiations promoted by fragility and alteration of vascular permeability. While recovering from the surgical procedure, the gelding erythrogram displayed parameters on the lower limit of normality, neutrophilia with a regenerative left shift or leukopenia (Day 3), which may have been caused by the steroid therapy or anaphylactoid reaction.

Along the scenario of metabolic acidosis, there were also electrolyte abnormalities on the day following the anaphylactoid reaction, with decreased plasma concentrations of sodium, potassium and chlorine. The anaphylaxis report where a reaction was induced by intravenous administration of breast milk on a foal lists the same pattern of changes on the following day [11]. Such changes can be

directly associated to the systemic inflammatory response syndrome induced by the beta lactam compound [30].

During an anaphylactoid reaction, large amounts of fluids are sequestered to tissue compartments richer in mast cells and basophils due to antigen-antibody bonds that promote the release of vasoactive amines and hence induce the local vasodilatation. It is known that tissue concentrations (muscle and viscera) of sodium, potassium and chloride increase during anaphylaxis, resulting in a homeostatic imbalance of these serum electrolytes [31].

A significant increase in the concentrations of plasma glucose and lactate was also observed, which may be linked to an increased anaerobic metabolism due to difficult tissue gas exchange resulting from the edema. This fact can be explained by the concomitant increase of venous partial pressures of oxygen and carbon dioxide, shown a day after the anaphylactoid reaction in the gelding.

Hematologic and metabolic changes may have predisposed the gelding to the appearance of Theileriosis on Day 18. However, the enzymatic biochemical profile of the gelding did not change significantly as a result of anaphylaxis but only after the hemoparasitosis.

In the mare, it could be that the anaphylactoid mediators caused the possible hypotension and this may be responsible for starting the systemic inflammatory response that resulted in abdominal discomfort and laminitis (day 6). The inflammatory response was detected by the 10^{th} day, through laboratory confirmation of neutrophilia and increased plasma fibrinogen. The infrared images show apparent temperature increase in the hoof of the left forelimb (day 6), which responded

adequately to the non-steroidal anti-inflammatory therapy, cryotherapy and peripheral vasodilator, returning to normal on day 20 post infection.

Both horses survived and were treated with oxytetracycline (5mg/kg, intravenous diluted in saline 0.9% solution of 500 ml, s.i.d, during 21 days) to resolve the infection, without any adverse effects.

Conclusions

An anaphylactoid reaction is a potential risk to the lives of horses. It is characterized by hypotension and potential cardiovascular collapse, associated with redistribution of the blood volume in the lung and gastrointestinal tract, and may progress to tissue edema and hypertension. Horses that survive these reactions may develop a number of complications, including colic syndrome, laminitis and manifestation of latent infectious diseases.

Endnotes

[a]Celltriaxon®, Agila Especialidades Farmacêuticas, Campos dos Goytacazes – RJ – Brazil. Batch: 7402209. Manufacture: 10/2013. Validity: 10/2015.

[b]Angiocath® 14G, BD, São Paulo – Brazil.

[c]Novapen® – Hertape Calier, Juatuba – MG.

[d]Imizol® – MSD Saúde Animal, São Paulo – SP.

[e]Gastrozol® 2.28g – Hertape Calier, Juatuba – MG.

Competing interests

The authors declare no competing interests.

Authors' contributions

RCB and AQN designed and planned the experiment. RCB, GGR, THIEF, TCMB, GPDA, EG, GCF, AQN and PAC executed the experimental and clinical procedures. RCB and AQN drafted the manuscript. All authors read and approved the manuscript.

Acknowledgements

The authors acknowledge the financial support of FAPESP (Process 2013/2013 and 05871-0/03732-0).

The authors also thank the valuable contribution to the therapeutic monitoring of both animals during the reported event: Aline Silva Tempesta, Luciana Leon Scrivano, Flora D'Angelis de Freitas, Helena Cristina Brito Delgado, Jessica Lage, Julia Garcia de Carvalho, Yuri da Silva Bonaccin and Vanessa Barroco de Paula.

Abbreviations

C3A	Complement component 3A
C567	Complement components 5, 6 and 7
C5A	Complement component 5A
CEUA	Comitê de Ética e Uso Animal
CRT	Capillary refill time
dL	Deciliter
DMSO	Dimetil sulfoxide
FAPESP	Fundação de Ampoaro e Apoio à Pesquisa do Estado de SP
g	Gram
HR	Heart hate
IgE	Immunoglobulin E
kg	Kilogram

L	Liter
LF	Left forelimb
LH	Left hindlimb
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
mg	milligram
MG	Minas Gerais
NSAID	Non steroidal anti-inflammatory drugs
PCR	Polymerase Chain Reaction
RF	Right forelimb
RH	Right hindlimb
RJ	Rio de Janeiro
RR	Respiratory rate
RT	Retal temperature
s	Second
SP	São Paulo
U	Units
UNESP	Universidade Estadual Paulista

6.3 REFERENCES

[1] Butler CM, Houwers, DJ, Jongejan F, Van der Kolk JH: ***Borrelia burgdorferi* infections with special reference to horses. A review.** *Veterinary Quartely* 2005, **27**: 146-156.

[2] Stanek G, Wormser GP, Gray J, Strle F: **Lyme borreliosis**. *The Lancet* 2012, **379**: 461-473.

[3] Voehringer D: **Protective and pathological roles of mast cells and basophils**. *Nature Reviews – Immunology* 2013*,* **13**: 362-375.

[4] Estelle F, Simons R: **Anaphylaxis.** *Journal of Allergy Clinical and Immunology* 2010, **125:** s161-s181.

[5] Eyre P: **Pharmacological aspects of hypersensitivity in domestic animals: A review**. *Veterinary Research Communications* 1980, **4**: 83-98.

[6] Chand N, Eyre P: **Nonsteroidal anti-inflammatory drugs: A review. New applications in hypersensitivity reactions in cattle and horses.** *Canadian Journal of Comparative Medicine* 1977, **41:** 233-240.

[7] Lagopoulos V, Gigi E: **Anaphylactic and anaphylactoid reactions during perioperative period**. *Hippokratia* 2011, **15**: 138-140.

[8] Eyre P, Lewis AJ: **Acute systemic anaphylaxis in the horse**. *British Journal of Pharmacology* 1973, **48:** 426-437.

[9] Eyre P: **Preliminary studies of pharmacological antagonism of anaphylaxis in the horse.** *Canadian Journal of Comparative Medicine* 1976, **40:** 149-152.

[10] Breukink HJ, Eysker M: **Intravenous administration of Ivomec in horses**. *Tijdschr Diergeneeskd* 1995, **120:** 113-114.

[11] Alcott CJ, Wong DM: **Anaphylaxis and systemic inflammatory response syndrome induced by inadvertent intravenous administration of mare's milk in an neonatal foal**. *Journal of Veterinary Emergency and Critical Care* 2010, **20:** 616-622.

[12] Fadok VA: **Update on equine allergies**. *Veterinary Clinics of North America – Equine Practice* 2013, **29:** 541-550.

[13] Wagner B: **IgE in horses: Occurrence in health and disease.** *Veterinary Immunology and Immunopatology* 2009, **132:** 21-30.

[14] Halperin JJ: **Nervous system Lyme disease**. *Handbook of Clinical Neurology* 2014, **121:** 1473-1483.

[15] Gardner SY, Aucoin DP: **Pharmacokinetics of ceftriaxone in mares**. *Journal of Veterinary Pharmacology and Therapeutics* 1994, **17:** 155-156.

[16] Ringger NC, Pearson EG, Gronwall R, Kolepp SJ: **Pharmacokinetics of ceftriaxone in healthy horses**. *Equine Veterinary Journal* 1996, **28**: 476-479.

[17] Ringger NC, Brown MP, Kohlepp SJ, Gronwall RR, Merritt K: **Pharmacokinetics of ceftriaxone in neonatal foals.** *Equine Veterinary Journal* 1998, **30:** 163-165.

[18] Romano A, Gaeta F, Valluzi RL, Caruso C, Rumi G, Bousquet, PJ: **IgE-mediated hypersensitivity to cephalosporins: Cross-reactivity and tolerability of penicilins, monobactams and carbapenems.** *Journal of Allergy and Clinical Immunology* 2010, **126:** 994-999.

[19] Pham NH, Baldo BA: **Beta-lactam drug allergens. Fine structural recognition patterns of cephalosporin-reactive IgE antibodies**. *Journal of Molecular Recognition* 1996, **9:** 287-296.

[20] Baldo BA: **Penicillins and cephalosporins as allergens – structural aspects of recognition and cross-reactions**. *Clinical Experimental Allergy* 1999, **29:** 744-749.

[21] Goudah A: **Pharmacokinetic parameters of ceftriaxone after single intravenous and intramuscular administration in camels *(Camelus Dromedarius).*** *Research in Veterinary Science* 2008, **84:** 483-489.

[22] Rebuelto M, Albarellos G, Ambros L, Kreil V, Montoya L, Bonafine R, Otero P, Hallu R: **Pharmacokinetics of ceftriaxone administered by intravenous, intramuscular or subcutaneous routes in dogs.** *Journal of Veterinary Pharmacology and Therapies* 2002, **25:** 73-76.

[23] Maradiya JJ, Goriya HV, Bhavsar SK, Patel UD, Thaker AM: **Pharmacokinetics of ceftriaxone in calves.** *Veterinarski Arhiv* 2010, **80:** 1-9.

[24] Ismail MM: **Pharmacokinetics, urinary and mammary excretion of ceftriaxone in lactating goats**. *Journal of Veterinary Medicine Series A* 2005, **52:** 354-358.

[25] Orsini JA, Divers TJ: *Equine emergencies: Treatment and Procedures – Third Edition.* Elseviers Saunders: St. Louis, EUA; 2013.

[26] Swiderski CE: **Hypersensitivity disorders in horses.** *Veterinary Clinics of North America – Equine Practice* 2000, **16:** 131-151.

[27] Peters LJ, Kovacic JP: **Histamine: metabolism, phisiology, and pathophysiology with applications in veterinary medicine**. *Journal of Emergency and Critical Care* 2009, **19:** 311-328.

[28] Eyre P, Hanna CJ, Wells PW. Mcbeat DG: **Equine immunology 3: Immunopharmacology – Anti-inflammatory and antihypersensitivity drugs.** *Equine Veterinary Journal* 1982, **14:** 277-281.

[29] Hanna CJ, Eyre P, Wells PW, Mcbeat DG: **Equine immunology 2: Immunopharmacology – Biochemical basis of hypersensitivity.** *Equine Veterinary Journal* 1982, **14:** 16-24.

[30] Hoorn EJ, Zietse R: **Severe hypokalaemia caused by flucloxacillin**. *Journal of Antimicrobial Chemotherapy - Research Letters* 2008, 1396-1398.

[31] Einbinder JM, Nelson CT, Fox Jr CL: **Tissue electrolyte changes in anaphylactic shock.** *American Journal of Physiology* 1954, **179:** 347-352.

7. FINAL CONSIDERATIONS

7.1 GENERAL CONCLUSIONS

This experiment provided more information about possible clinical signs of Lyme borreliosis in horses, besides the disease remains obscure. In its early phase, horses can present non-specific clinical signs such as lethargy, pale mucous, muscular back pain and enlarged lymphonodes. The hemolytic activity of *Borrelia burgdorferi* front of the red blood cells of horses can promote a mild anemia in the first week of infection. This mild alterations can induce veterinarians to misdiagnose piroplasmosis in horses infected by *Borrelia burgdorferi*. It is also possible to verify the occurrence of abortion and retained placenta associated with laminitis in mares. Even without the horses are treated, after 90 days of infection, it is no longer possible to detect antibodies in blood. The seropositivity of horses can be related to the presence of *Amblyomma sculptum* and capybaras in the property. The treatment of the infection with ceftriaxone can cause an anaphylactoid reaction in horses that may be associated with massive bacterial death, with consequences like colic syndrome. The horses treated with oxytetracycline for 21 days tolerated the therapy with no side effects presentation.

7.2 FUTURE RESEARCH

- To evaluate acute phase proteins from natural and experimentally infected horses;

- To evaluate the blood samples and biopsies from liver, muscle, spleen, limph nodes, uterus and arthritic synovial liquid using other primers (5S and 23S) to confirm the absence/presence of *B. burgdorferi* in these tissues of naturally and experimentally infected in horses;

- To evaluate the vector competence of ticks *Amblyomma sculptum* to transmit *Borrelia burgdorferi* to horses, providing artificial feeding;

- To evaluate the epidemiology of abortion and retained placenta of *Borrelia* seropositive mares, verifying the possibility of presence of another pathogens as Equine Herpes Virus – 1*, Leptospira spp., Klebisciella spp., Theileria equi, Babesia caballi,* entre outros.

- To evaluate samples of blood and *Amblyomma sculptum* from capybaras, searching for evidences of presence of *Borrelia burgdorferi* using ELISA and PCR tests;

- To search the prevalence of seropositive imported horses and infected with *Borrelia burgdorferi* sensu lato, allowing to check the actual transfer status of the etiologic agent from other countries to Brazil;

- To verify the presence of B. burgdorferi sensu lato and vetorial competence of *Stomoxys calcitrans* present in horses properties from Brazil.