Report on the unusual presence of latent microorganisms in animals: a risk to research and health of employees?

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ABSTRACT

We report the unusual finding of mobile spirochetal microorganisms with different morphologies and sizes, on dark-field microscopy of the blood of animals from the Vivarium of the Medical School of USP. The bacteria did not grow in common culture media, shows faint staining to Giemsa and silver-derived stains, and serologies and molecular tests were negative for *Borrelia* and *Leptospira*.

Electron microscopy revealed the presence of microorganisms with *Mycoplasma*-like morphology and, due to its mobility, it was suggested that they represented *Mollicutes* of the genus *Spiroplasma*. Microorganisms with the same morphology were also observed in 15 out of 26 employees (57.6%) of the Vivarium of FMUSP; however, clinical and laboratorial exams indicated that those individuals were healthy. Additional studies undertaken at the Rheumatology Department of FMUSP demonstrated the presence of the same structures identified at the Vivarium in approximately 94% of the patients with Baggio-Yoshinary syndrome (BYS) and 20% of healthy individuals. Electron microscopy of the blood of BYS patients showed bacteria that shared similarities with *Mycoplasma*, *Chlamydia*, and *Bacteroides*. Since serologies and molecular tests were negative for those contaminants, and based on publications in the medical literature, it was suggested that those latent infectious agents were L-form bacteria, defined as cell wall deficient bacteria, assuming, therefore, *Mycoplasma* morphology and they are, for the most part, harmless to the host. We concluded that spirochetal microorganisms visualized in animals and employees of the Vivarium were non-pathogenic L-form bacteria from contaminants in the environment, regular infections, or endogenous microorganism from the normal saprophytic flora. On the other hand, spirochetal organisms identified in BYS, by preserving the capacity to invade cells *in vitro*, are potentially pathogenic and related to the etiology of BYS. We consider BYS as a novel Brazilian zoonosis caused by spirochetes adapted to their latent form, possibly due to bacterial mutations in response to ecologic and geographic conditions unique to Brazil.

Keywords: spirochete, spirochete-like, L-form bacteria, *Mycoplasma*, Lyme-like disease, Baggio-Yoshinari syndrome, *Borrelia*, latent microorganism, Vivarium, laboratory animals, Brazil.

INTRODUCTION

On an administrative ruling published on 04/06/2002, the dean of USP, Professor Adolfo José Melfi, appointed Professors Natalino Hajime Yoshinari (Medical School of the Universidade de São Paulo – FMUSP), Silvio de Arruda Vasconcelos (Veterinary and Zootomy School of USP-FMZUSP), and Arary da Cruz Tiriba (Universidade Federal de São Paulo - UNIFESP) for a Commission created to investigate unusual bacteriological problems linked to Vivariums.

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The problem in the Vivarium of FMUSP began in the beginning of 2001 when Dr. Ismar Cestari detected *in vitro* the presence of spirochete-like microorganisms in culture of spleen cells of an animal from the Vivarium of the Medical School of USP (FMUSP).

To understand the extension of the problem, the Commission examined the blood of the animals on dark-field microscopy and, indeed, confirmed the presence of spirochete-like microorganisms in 15 out of 15 mice blood samples (BALB/c, A/SNELL1, SWISS, and C57BI/6), in two out of 5 rat blood samples (WISTAR), five out of five rabbit blood samples, and in none of the guinea pig blood samples.

Blood samples were examined again on the second semester of 2002 and 28 out of 40 (70%) blood samples of different species of mice and rats were positive. The same procedure was used in animals from the Veterinary and Zootomy School of USP (FMVZUP, from the Portuguese) and dark-field microscopy showed "spirochetal structures" in only two out of eight mice blood samples and in none of the hamsters, indicating a problem of Vivariums, although this finding was more common at FMUSP.

Clinically, animals from the USP Vivarium were apparently healthy. The veterinary Sueli Blanes Dani *et al*.[1] undertook preliminary biochemical analysis of serum pools of four female mice and five of WISTAR rats, showing increased levels of BUN, alkaline phosphatase, aspartate aminotransferase (AST), and alanine aminotransferase (ALT), suggesting the presence of some infectious or toxic factor responsible for the development of liver function-related biochemical disruption. In the same study, electron microscopy and immunohystochemical tests of the lungs, liver, spleen, heart, and kidneys of the animals, and they identified *Mycoplasma pulmonis* in almost 100% of the animals in conventional vivariums, and in 18% of those that maintained adequate sanitary barriers. They stated that studies in the literature reported liver diseases caused by *Mycoplasma* in sheep, doves, and goats, but they did not find reports on rats. To confirm those findings, we repeated the electron microscopy of the blood samples of rodents with spirochetal organisms (Figure 1).

Since optical microscopy revealed the presence of mobile microorganisms of different sizes, ranging from miniscule dots to elongated structures reaching up to 15-20 μm, in the blood sample of the animals, the Commission initially thought they could be spirochetes. Serology for *Leptospira* and *Borrelia*, hemocultures in aerobic and anaerobic media and BSK, as well as molecular testing (PCR) for *Leptospira* and *Borrelia*, performed at FMUSP, FMVZUSP, and Biological Institute were persistently negative. The uncommon size of the structures identified in the peripheral blood of the animals called the attention of the members of the Commission because they were large and apparently incompatible with *Mycoplasma* or *Chlamydia*. It was also interesting that it was difficult to cultivate those structures in common culture media and they were difficult to identify using stains like Giemsa, silver products, and vital stains, such as acridine orange. Those findings, associated with the absence of information in the medical literature, led many researchers to interpret those spirochetal structures as simple artifacts.

The major concerns were related to the anthropozoonotic aspects of this uncommon finding. Possible risks of this latent infectious process for employees who handled the animals, characterizing a work-related disease, as well as the interference of those contaminants on experimental studies, were hypothesized. It was interesting that animals underwent frequent bacteriological testing, but pathogenic microorganisms were never isolated. It is important to emphasize that the animals in the USP Vivarium were not isolated, nor were they "germ-free", besides being raised and kept in the Vivarium for approximately 20 years, except the dogs that are brought from outside the FMUSP complex.

The present study reports the problems and actions adopted by the Commission to answer the questions formulated by the Dean of USP regarding the risks to the employees and scientific investigations. The other objective was to discover the etiology and source of infection of the animals.

Due to the relevance of the subject, a complementary discussion resulting from the investigation of the Brazilian Lyme-like disease (Baggio-Yoshinari syndrome – BYS), which brought new understanding on the incidence of spirochetal organisms in laboratory animals and humans, was added. It was demonstrated that those structures are seen almost always in mammals, including humans (Figure 2A), and that they grow briefly in SP4 medium (Figure 2B).

When analyzed under electron microscopy, those structures are similar to *Mycoplasma*, which, in reality, represent microorganisms that lost the cellular wall (cell wall deficient bacteria or L-form) and are, for the most part, "harmless" to living beings. However, for unknown reasons, maybe due to ecologic and climatic factors inherent to the country, strong evidence indicate that the etiological factor of BYS is an atypical latent spirochete with characteristics suggestive of *Mycoplasma*, *Chlamydia*, and *Bacteroides* (Figures 3A, B, and C). However, since the structures isolated in BYS cases are capable of invading endothelial cells (Figure 4), it is believed that they are potentially pathogenic, justifying the clinical and laboratorial particularities of this emerging Brazilian zoonosis.

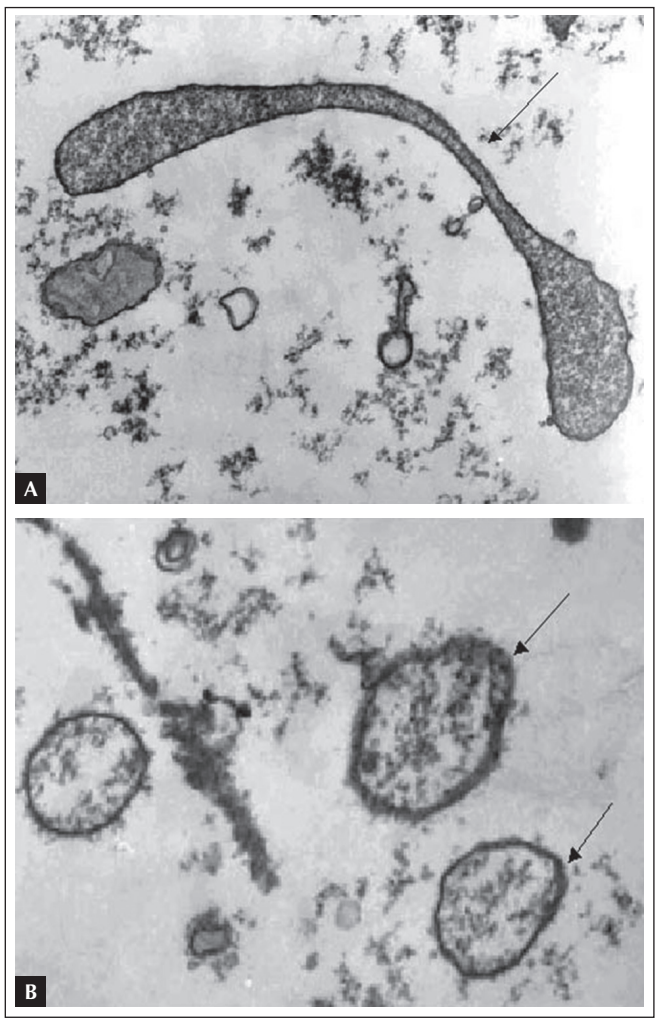

Figure 1. A) Electron photomicrography of the serum of Wistar rat from the Vivarium without treatment, showing microorganisms with *Mycoplasma* morphology. Collected on 11/06/2002. **B)** Presence of several structures with irregular shapes and sizes surrounded by a membrane, and with granular contents, compatible with *Mycoplasma*, in a rodent form the Vivarium.

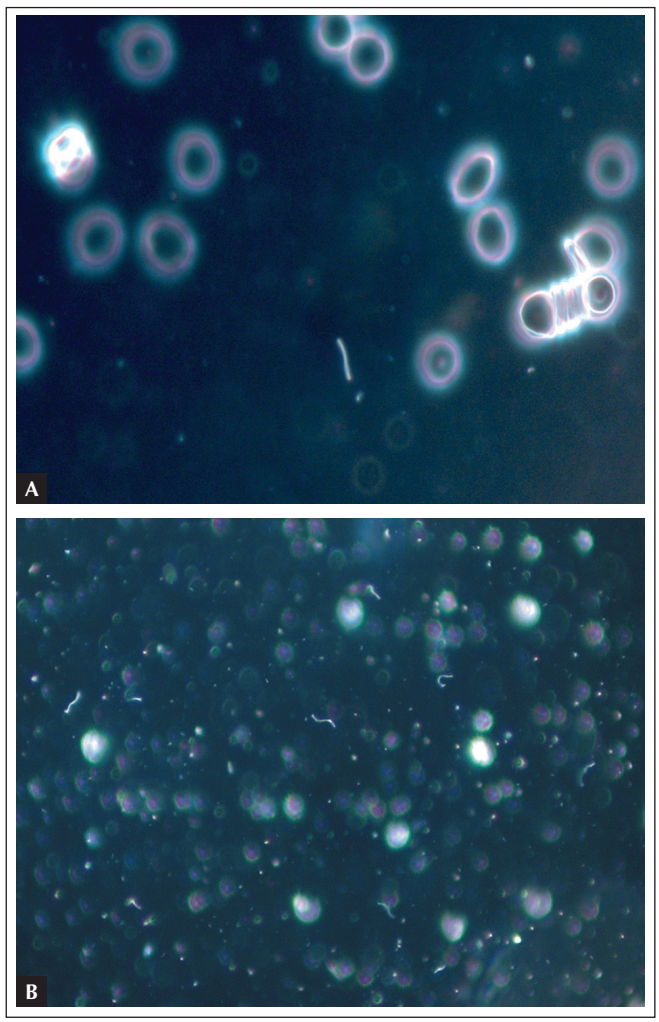

Figure 2. A) Dark-field microscopy showing spirochetal structures on the peripheral blood of a Vivarium employee; 1,000x. **B)** Blood culture of a patient with BYS in SP4 medium, which is adequate for the growth of Spiroplasmas, showing the growth of spirochetal structures; 400x.

The objective of this study was to demonstrate that the description of "harmless" L-form microorganisms in living beings is common. But, in some situations, as in the Baggio-Yoshinari syndrome, cell wall deficient spirochete, possibly bacteria of the genus *Borrelia*, would preserve their pathogenic properties, causing an extremely morbid disease distinct from Lyme disease seen in the northern hemisphere.

PATIENTS AND METHODS

All employees of the FMUSP Vivarium, evaluated according to the study protocol determined by the Commission instituted by the Dean of USP, were included in this study to evaluate the health of the employees who handled the animals or worked at the Vivarium.

This protocol included a clinical investigation with complete history and physical exam. As for complementary exams, the following were evaluated: 1; Blood: CBC (automated test, microscopy, and Panotic stain); 2. Biochemical: BUN (kinetic assay), creatinine (colorimetric kinetic assay), glucose (colorimetric enzymatic assay), total cholesterol (colorimetric enzymatic assay), triglycerides (colorimetric enzymatic assay), uric acid (colorimetric enzymatic assay), sodium (ion-selective electrode assay), potassium (ion-

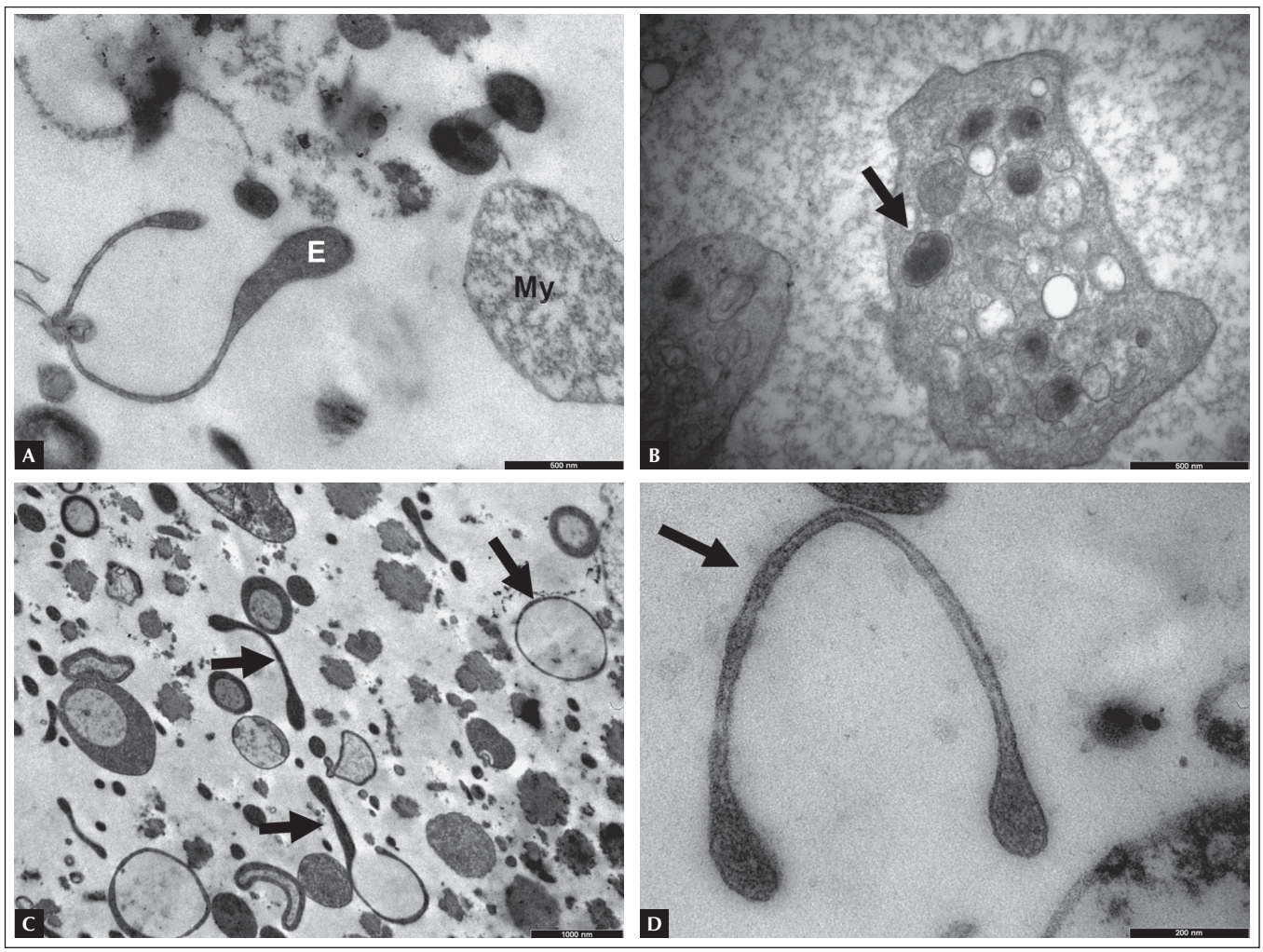

Figure 3. A) Electron microscopy of the blood of a patient with BYS on SP4 culture medium showing structures suggestive of spirochetes and *Mycoplasma*. E: Spirochetal organism; My: *Mycoplasma*. 24,000x; **B)** Electron microscopy of the peripheral blood of a patient with BYS showing a structure resembling *Chlamydia*. 24,000x; **C)** Electron microscopy of the peripheral blood of a patient with BYS cultured in SP4 medium showing structures resembling spirochetes. 6,200x; **D)** Electron microscopy of the peripheral blood of a patient with BYS cultured in SP4 medium showing structure similar to spirochetes. 65,000x.

selective electrode assay), calcium (colorimetric assay), serum iron (colorimetric assay), ALT (kinetic assay), AST (kinetic assay), gamma-glutamyl transferase (GGT – kinetic test), and creatine phosphokinase (CPK – kinetic test); 3. Urine analysis: urine type I; 4. Bacteriologic: hemoculture, coproculture (stool culture), and stool parasitologic (Leishman stain); 5. Immunologic and inflammatory: C-reactive protein (nephelometric assay), erythrocyte sedimentation rate (Westergren assay), C3 and C4 complement (nephelometric assay), protein electrophoresis, protein immunoelectrophoresis, rheumatoid factor (agglutination assay), antinuclear factor in Hep-2 cells (immunofluorescence assay), and anti-streptolysin O (nephelometric assay); 6. Serologies for infectious diseases: Lyme disease, at the Medical Investigation Laboratory (LIM-17, from the Portuguese) of HCFMUSP (immunoenzymatic assay – ELISA), leptospirosis, at the FMVZUSP (microscopic seroagglutination assay), and the remaining assays for syphilis (immunoenzymatic assay – ELISA), toxoplasmosis (microparticle immunoenzymatic assay – MEIA), hepatitis A and B (microparticle immunoenzymatic assay – MEIA), hepatitis C (chemiluminescence assay), and cytomegalovirus (immunoenzymatic assay – ELISA) at the Central Laboratory of HCFMUSP; 6. Imaging exams: chest X-ray and abdominal ultrasound (US) scan; and 7. Specialized medical evaluation when necessary.

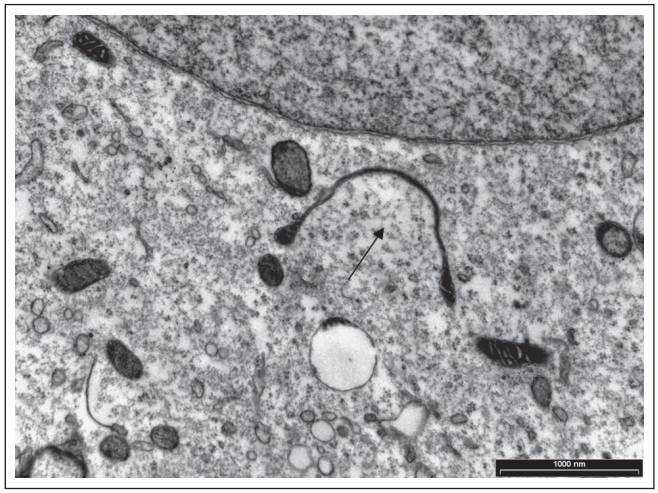

Figure 4. Electron microphotography of a spirochetal structure inside an endothelial cell after incubation in SP4 culture medium containing suspended spirochetal organisms, from a patient with BYS. 15,000x.

This study was approved by the Ethics Commission for the Analysis of Experimental Studies – CAAPPesq (from the Portuguese) of HCFMUSP (342/09).

RESULTS

Thirty-seven employees of the FMUSP Vivarium, five of which were administrative employees, were evaluated at the Internal Medicine Outpatient Clinic of the Hospital das Clínicas of FMUSP. Since this was a conventional Vivarium, without isolation areas, the employees were not discriminated according to their position. Twenty-five were males and 12 females; their age ranged from 21 to 54 years (36.6 ± 9.87).

HISTORY

Twelve out of 27 (32.4%) employees complained of some allergic manifestation, such as asthma, rhinitis, or skin eruption. The frequency of "social drinking" was 62.5%, *i.e.*, 15 out of 24 individuals who answered this question.

Out of 37 employees interviewed, ten complained of frequent fatigue and asthenia (27%), while three (8.1%) referred recurrent episodes of fever or chills.

As for the locomotor system, nine (24.5%) complained of constant back pain, eight (21.6%) had arthralgias, two had talalgia, and one had arthritis and myalgia. Sixteen employees (43.2%) did not have osteoarticular symptoms.

Regarding neurological manifestations, 12 (32.4%) employees had headaches regularly, nine (24.3%) reported being forgetful, seven (18.9%) had sleep problems, five (13.5%) were nervous or irritable, three (8.1%) had lack of concentration, three (8.1%) experienced episodes of dizziness, and one had facial paralysis. Nine employees (24.3%) denied having any neurological symptoms.

As for cardiovascular symptoms, five employees (13.5%) experienced frequent episodes of palpitations and three (8.1%) had atypical chest pain.

Review of the other systems showed that 12 (32.4%) complained of increased in the daily number of evacuations (more than two), six (16.2%) had sore throat frequently, and four reported constant episodes of coughing or upper airways infection. Five (13.5%) employees did not have any clinical complaints.

PHYSICAL EXAM

The physical exam did not show significant changes; cutaneous manifestations were observed in five patients (pustules, erythema macular, hyperhidrosis, pityriasis versicolor, and psoriasis). One employee had hepatomegaly and clubbing of the fingers, lung auscultation was compatible with bronchospasm in one employee, another had arthritis compatible with gout, and one had Heberden and Bouchard nodes (arthrosis of the hands).

LABORATORIAL TESTS

Complete blood count was normal, except in two cases in which it showed mild leukocytosis, three had leucopenia, and five had mild anemia. Changes in platelet count were not observed.

Table 1 shows the main results of blood biochemistry, and the number of employees with increased levels of liver and muscle enzymes is striking.

INFLAMMATORY ACTIVITY AND IMMUNOLOGIC TESTS

Table 1 shows the changes in inflammatory activity and immunologic tests of the employees of the FMUSP Vivarium. Note that 26% of the employees presented positive C-reactive protein and 36% had increased IgE.

SEROLOGIES FOR INFECTIOUS DISEASES

Table 2 shows the results of the serologies for the Brazilian Lyme-like disease, leptospirosis, syphilis, toxoplasmosis, hepatitis A, B, and C, and cytomegalovirus. The frequency

Table 1
Biochemical parameters, urine analysis, inflammatory activity tests, and immunologic tests of employees of the Vivarium of FMUSP

Biochemical parameters and urine analysis	Incidence of changes	Normal levels	Inflammatory activity tests and immunologic parameters	Incidence of changes	Normal levels
Elevated BUN	1/25 (4%)	10-45 mg/dL	Elevated α2 globulin	6/24 (25%)	0.4-0.7 g/dL
Elevated creatinine	0/25 (0%)	0.6-1.4 mg/dL	Elevated γ globulin	4/24 (16.6%)	0.7-1.6 g/dL
Elevated glucose	2/25 (8%)	70-110 mg/dL	Elevated C-reactive protein	6/23 (26%)	< 5 mcg/mL
Elevated cholesterol	8/28 (32%)	até 200 mg/dL	Elevated ESR	3/24 (12.5%)	até 15 mm
Elevated triglycerides	5/25 (20%)	até 200 mg/dL	Elevated α1 glycoprotein	2/24 (8.3%)	até 125 mg/dL
Elevated uric acid	4/24 (16.6%)	H:3.4-7.0 mg/ dL M:2.4-5.7	Elevated α2 globulin/ α1 glycoprotein/ESR or CRP	12/24 (50%)	–
Abnormal sodium	0/25 (0%)	135-145 mEq/L	≥ 2 of above parameters elevated	6/24 (25%)	–
Abnormal potassium	0/25 (0%)	3.5-5.0 mEq/L	Decreased C3 and C4	1/24 (4.1%)	C3 < 90 mg/dL e C4 < 10 mg/dL
Abnormal calcium	4/23 (17.5%)	8.8-10.5 mg/dL	Positive rheumatoid factor	2/23 (8.6%)	–
Abnormal iron	0/24 (0%)	acima 50 ug/dL	Positive ANF, speckled pattern	2/22 (9.0%)	IF acima de 1/40
Elevated AST	1/25(4%)	10-34 U/L	Elevated IgA	4/25 (16%)	70-400 mg/dL
Elevated ALT	4/25 (16%)	10-44 U/L	Elevated IgG	6/25 (24%)	700-1600 mg/dL
Elevated gamma GT	8/25 (32%)	11-50 U/L	Elevated IgM	4/25 (20%)	40-230 mg/dL
Elevated LDH	0/25 (0%)	240-480 U/L	Elevated IgE	9/25 (36%)	até 156 KU/L
Elevated CPK	9/25 (48%)	24-204 U/L	Elevated ASLO	2/24 (8.3%)	até 200 U/mL
Elevation of at least 1 enzyme*	16/25 (64%)	–			
Elevation of two or more enzymes*	5/25 (20%)	–			
Urine leukocytes	1/24 (4.1%)	até 5 por campo			
Urine erythrocytes	1/24 (4.1%)	até 5 por campo			
Proteinuria	0/24 (0%)	até 0.05 mg/L			

ALT: alanine aminotransferase; AST: aspartate aminotransferase; Gamma GT: gamma glutamyl transferase; LDH: lactate dehydrogenase; CPK: creatine phosphokinase; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; ANF: anti-nuclear factor; ASLO: anti-streptolysin O. *ALT, AST, gamma GT, CPK, or LDH.

of sera positive for *Borrelia burgdorferi* was similar to that observed in the normal control population. One patient tested positive for syphilis and two for toxoplasmosis, being referred to the Infectious Disease Department.

BACTERIOLOGY AND STOOL PARASITOLOGY

Table 2 describes the bacteriological and stool parasitologic tests, visualization of spirochetal structures on dark-field microscopy, and PCR for leptospirosis of the employees of FMUSP Vivarium. Blood cultures and coprocultures were negative; spirochetal structures were observed in 57.6% of the cases (Figure 2A), and the presence of *Blastocystis hominis* was demonstrated in 12% of the employees analyzed. Stool leukocytes were present in 36% of the cases.

IMAGING

Chest X-ray was normal in all employees who underwent this exam. Total abdominal US showed some changes in 10 out of 18 cases (55.5%), and the abnormalities observed included: hepatic steatosis in five cases, liver enlargement in four cases, peripancreatic lymph nodes in one case, hepatic calcifications in one case, dilated common hepatic duct in one, and hepatic nodules in one employee.

LIVER EVALUATION

Employees with abnormal abdominal US were referred to the gastroenterologist, but significant changes, deserving complementary investigation, were not observed.

Table 2
Column A: incidence of seropositivity for the Brazilian Lyme-like disease, leptospirosis, syphilis, toxoplasmosis, viral hepatitis, and cytomegalovirus. Column B: hemocultures, coprocultures, dark- and light-field microscopy, stool parasitology, and PCR for Leptospira spp. on employees of the Vivarium of FMUSP

Parameters (A)	Incidence	Parameters (B)	Incidence
Borrelia burgdorferi (IgG)	4/27 (14.8%)	Blood culture (aerobic) (n=25)	*S. epidermidis* (1 case)
Borrelia burgdorferi (IgM)	0/27 (0%)	Blood culture (anaerobic) (n=25)	Negative
Borrelia burgdorferi (IgG or IgM)	4/27 (14.8%)	Coproculture (*) (n=25)	Negative
Leptospirosis	0/27 (0%)	Coproculture for *Helicobacter sp.* (n= 5)(**)	Negative
Syphilis (serology)	1/25 (4%)	PCR for leptospirosis (n=5)(**)	Negative
Toxoplasmosis (IgM)	2/25 (8%)	Spirochetal structures on dark-field microscopy	15/26 (57.6%)
Toxoplasmosis (IgG)	8/25 (32%)	Light-field bacterioscopy (n= 5)(**)	Negative
Hepatitis A (IgM)	0/25 (0%)	Dark-field bacterioscopy (n= 5)(**)	Negative
Hepatitis A (IgG)	20/25 (80%)	Stool parasitology (*Blastocystis hominis*)	3/25 (12%)
HBs Ag	0/25 (0%)	Stool parasitology (*E. nana*)	1/25 (4%)
Anti-HBs Ag	1/25 (4%) (vaccinated)	Stool leukocytes	9/25 (36%)
Hepatitis C	0 (0%)		
Cytomegalovirus (IgM)	0/25 (0%)		
Cytomegalovirus (IgG)	21/25 (84%)		

(*) Coproculture for pathogenic enterobacteria: *Salmonella*, *Shigella*, pathogenic *E. coli*, and *Campylobacter*, performed at the Adolfo Lutz Institute. (**) Tests performed at the Biological Institute in suspicious samples of employees with diarrhea and spirochetal structures on dark-field microscopy at the LIM-17 of the Hospital das Clinicas of FMUSP. Light-field bacterioscopy of peripheral blood with Ryu and Giemsa stains. (***) The investigation for spirochetal structures was undertaken at the LIM-17 HCFMUSP.

DISCUSSION

A large number of professionals of different Research Institutions was mobilized to study the clinical condition of the employees of the USP Vivarium due to the uncommon finding of latent microorganisms in the animals of that institution, which might indicate an emerging zoonosis and a new work-related disease. Since references on microorganisms, with the characteristics described here, in the blood of animals and humans, were not found in the medical literature, it was very difficult to explain where they came from and what would be the pathogenic role of those microorganism in the host.

An explanation was urgently needed, not only to reassure the employees regarding the risks to their health, but also to inform the scientists of the institution who used animals from the Vivarium of any interferences of this infection on animal research.

The poor sanitary conditions of the Vivarium suggested that environmental factors could be influencing the development of those spirochetal structures, since the frequency of contaminants varied among the institutions investigated. It was surprising that the same structures present in animals were identified in 15 out of 26 employees (57.6%), indicating possible work-related transmission of those microorganisms.

The Commission also observed that the employees of the Vivarium had poor hygiene, since they ate and slept in the work place, sometimes manipulated animals without gloves, they did not wear boots when they were in direct contact with animal waste, and promiscuity among the different animal species was also observed. Therefore, educating the employees on proper hygiene and establishing strict rules regarding animal care were the first steps taken.

The medical exam of the employees demonstrated that they were in good clinical condition; however, the high frequency of allergic phenomena and diarrhea was striking. As for the laboratorial work up, some individuals had abnormal inflammatory activity assays, elevated liver and muscle enzymes, high levels of IgA and IgE, and leukocytes in the

stools. Initially, we thought we were seeing a higher incidence of liver, intestinal, and allergic complications. However, this impression was not confirmed due to the lack of a control group, with the same demographic characteristics and habits, but not working at the Vivarium.

Some aspects stood out, such as the high incidence of social drinking (62.5%), continuous exposure of the employees to animal waste, rations, and different biological products, besides the poor hygiene of those individuals. We thought those factors partly explained the clinical complaints and laboratorial changes seen, and that preventive measures would contribute to reduce the incidence of animal and human infection, in addition to the "eventual normalization" of the abnormal tests. We were reassured when employees whose abdominal US and laboratorial tests indicated hepatic changes were evaluated by the Gastroenterology Department of HCFMUSP and were considered normal, without the need of further procedures.

The next step of the study was to try to elucidate the etiology of the latent infection demonstrated by the finding of spirochetal structures in animals and employees of the Vivarium (Figure 2A). They were of different sizes and morphologies, mobile, nonculturable, did not stain by the Giemsa method and, although they resembled spirochetes, laboratorial tests were persistently negative for *Leptospira* and *Borrelia*. Serologies for the Brazilian Lyme-like disease, leptospirosis, and syphilis were negative, as well as molecular biology tests for *Leptospira spp.* and *Borrelia spp.* (data not presented).

As mentioned before, Damy SB *et al*.[1] identified *Mycoplasma pulmonis* in 100% of laboratory animals raised conventionally, and they reported that those microorganisms could cause hepatic damage, justifying the enzymatic changes seen in the animals of the FMUSP Vivarium.

Mycoplasmata are considered the smaller self-replicating organisms, require cholesterol for their survival, and do not have cellular wall. Similar to *Chlamydiae*, they are intracellular organisms that infect several cells, such as endothelial and epithelial cells, and macrophage, besides representing important co-factors of the increased virulence of infections caused by other microorganisms[2]. Higuchi *et al*.[3,4] reported that those microorganisms influence the development of unstable atheroma plaques. They were able to visualize, on electron microscopy, elliptical and cylindrical forms of *Mycoplasmata* of different sizes distributed in the extracellular matrix of affected human tissues.

However, we did not find any references in the medical literature to the possible role of *Mycoplasma* spp. and *Chlamydia* spp. as zoonotic agents. It was also intriguing that those microorganisms were minute and non-mobile, contradicting the findings of dark-field microscopy, which revealed mobile structures measuring up to 15 µm in length. Thus, the hypothesis that the microorganisms identified in the blood of rodents from the FMUSP Vivarium (Figure 1) were *Mycoplasmata* was not confirmed. According to the personnel of Professor J. Timenetsky of ICBUSP, a specialist in *Mycoplasmata*, the majority of rodents is infected by these bacteria and, therefore, the findings on electron microscopy could be incidental, not related to our findings.

But we investigated the possibility of finding mobile bacteria with greater dimensions and *Mycoplasma* morphology. It is known that the *Mollicutes* group of bacteria is composed by *Mycoplasma*, *Spiroplasma*, and *Acholeplasma*, cell wall deficient microorganisms surrounded by a cholesterol-rich cellular membrane. According to Shlomo T & Rami G[5], microorganisms of the genus *Spiroplasma* show circular and elliptical movement due to the presence of a cytoskeleton that works as a propeller, and they have chemotactic properties. They can reach up to 10 µm in length, are sensitive to erythromycin and tetracyclines, and some species are pathogenic for rats, mice, hamsters, and rabbits. They can also be identified in plants, bees, ticks, wasps, and mosquitoes[6]. It is curious that several *Spiroplasma* cells contain a virus (SpV)[7], whose pathogenic meaning is unknown.

Since the *Mollicutes* hypothesis might not be completely satisfactory, the Commission considered other agents with similar morphology to that of spirochetes on dark-field microscopy. Among other possibilities, we thought of mobile spiral microorganisms, such as *Helicobacter* spp[8]., *Serpuline* spirochetes[9], and *Anaerobiospirillum*[10], whose common trait includes difficulty growing in usual media and their role on the pathogenesis of human and animal infirmities. Spirochetes of the genus *Serpulina* live in the digestive tract of animals, causing diarrhea[11,12], and immunosuppressed patients may be equally infected by those difficult to diagnose spirochetes[12]. Additionally, it is known that several spirochetes are among the oral saprophytic flora of normal individuals[13]; however, we did not know whether those microorganisms would be able to invade the blood stream and express as spirochetal structures.

Despite different etiologic possibilities, none was satisfactory, except for the possibility that they might be *Spiroplasmas*, mobile microorganisms similar to *Mycoplasma*. The other microorganisms mentioned have extremely different morphology on electron microscopy, since they have cellular wall, some of them have flagella, and they are extremely small (except for the genus Serpulina/Brachyspira).

Based on the data collected and information available, we concluded that a latent infection, which has not been described

yet, caused by bacteria morphologically similar to *Mollicutes*, *i.e.*, cell wall deficient, was present in animals and employees of the Vivarium. Due to the electron microscopic morphology of the microorganisms, the possibility of infection by the *Spiroplasma* genus was suggested. The medical evaluation of the employees allowed the prediction that those latent bacteria have a low pathogenic potential, but extended follow up of those individuals is warranted. As for animal studies, the Commission suggested the continuation of the studies since animals from other vivariums were also contaminated and, apparently, they were all in good physical condition.

The commission also recommended the urgent improvement of hygiene conditions of the employees, modernization and reformulation of the Vivarium, and stricter sanitary conditions as useful preventive measures to reduce the severity and frequency of contaminations. Although animal studies were not formally contraindicated, the members of the Commission reminded investigators that specific studies that depend on the total lack of microorganisms could be influenced by this latent infection. However, due to the characteristics of this infection, *i.e.*, silent, occult, difficult to control, besides being disseminated among different vivariums, the Commission raised the possibility that this infection could be present in axenic or germ-free animals.

COMPLEMENTARY DISCUSSION ABOUT NEW KNOWLEDGE ON THE BAGGIO-YOSHINARI SYNDROME

The manuscript above, with some modifications, represents the Report sent to the Dean of USP and presented to the researchers of FMUSP, who use regularly animals from the Vivarium, and its employees.

Discovering the etiology of the Brazilian Lyme-like disease (BLLD), or Baggio-Yoshinari syndrome[14], has been a great challenge. There are no doubts that symptoms compatible with Lyme disease, including typical erythema migrans and the development of multiple systemic complications, are seen in Brazil[15,16]. Unlike LD, the etiological agent of BYS has never been identified by microbiological (cultures) and molecular (PCR) methods[17].

Thus, in our opinion, this marked difference in the etiology of both tick-transmitted zoonoses would justify the large number of clinical and laboratorial particularities between the diseases seen in Brazil and the northern hemisphere. Clinically, the Brazilian zoonosis has a high incidence of relapses, which is rare in LD. As for laboratory exams, patients with BYS have low immunological reactivity to *Borrelia burgdorferi sensu lato* antigens and high frequency of autoimmune disorders, such as the development of autoantibodies against neuronal elements[18].

We believe that, despite the differences, BYS is a zoonosis caused by spirochetes. We postulate that, due to the geographical, climatic, and ecological conditions seen in Brazil, such as the absence of the *Ixodes ricinus* tick, the main vector of LD in the northern hemisphere[19], conditions for the development of exotic spirochetes, maybe mutants, capable of surviving in vertebrate and invertebrate hosts in the country, do exist. Currently, we know that *Borrelia* organisms are capable of modifying their genome and proteome during their life cycle, which involves infection of ticks and animals[20,21,22,23,24,25].

To identify the etiological agent of BYS in the peripheral blood of affected patients on dark-field microscopy, we identified similar spirochetal structures in animals and employees of the Vivarium of FMUSP. Similarly, spirochetal organisms from patients with BYS did not grow in BSK medium or in any of several other culture media tested.

Initially, based on the conclusions of the report given to the Dean of USP, we believed those structures to be *Mollicutes* of the genus *Spiroplasma*. And reinforcing this hypothesis, we discovered that those latent bacteria were capable of growing and surviving for approximately 10 days in adequate medium for the development of *Spiroplasma*, known as SP4 (Figure 2B). On the other hand, seeding spirochete of the *Borrelia burgdorferi sensu lato* complex in SP4 medium caused their cellular degeneration to the point that they lost their typical helicoidal movement and became similar to those structures seen in BYS and in the animals of the Vivarium.

The presence of those latent microorganisms in the blood of normal individuals who were not employees of the Vivarium and who did not have a history of recent tick bite was a surprising finding. Upon investigating those structures in 52 patients with BYS and in 50 healthy individuals, we demonstrated the presence of those spirochetal structures in 49 of 52 (94.2%) samples of patients with BYS and in only 20% of healthy individuals (non-published data).

Analyzing the spirochetal structures seen in BYS on electron microscopy, Mantovani *et al*.[26] visualized microorganisms whose morphology was suggestive of *Mycoplasma*, *Chlamydia*, and spirochetes. This discovery led the authors to assume that the etiology of BIS would be linked to this diversity of latent microorganisms, characterizing a new tick-transmitted clinical entity. Additionally, the discovery that those latent microorganisms isolated in patients with BYS were capable of infecting endothelial cells *in vitro*, indicating that they are potentially pathogenic, was also very relevant (unpublished data) (Figure 4).

When we performed serologies and molecular biology testing (PCR) for *Mycoplasma* spp. and *Chlamydia* spp. in patients with the BLLD and healthy subjects, we noticed that the behavior in both groups was similar, indicating that those spirochetal structures were not the microorganisms imagined previously (unpublished data). At that moment, the hypothesis that those latent bacteria belonged to genera *Mycoplasma* and *Chlamydia* lost strength. By analogy, the hypotheses that animals and employees of the Vivarium were contaminated by *Mollicutes* of the genus *Spiroplasma* was also under suspicion.

Searching for answers, we discovered, after a deep review of the medical literature, that all bacteria can assume *Mycoplasma* morphology when they lose components of the cellular wall, which might happen in adverse conditions[27,28,29]. Those morphologically altered bacteria, structurally similar to *Mycoplasma*, are known as L-form, spheroplasts or cell wall deficient bacteria. This phenotypic change is also observed in spirochetes of the genera *Treponema* and *Borrelia*[30,31,32].

When spirochetes are cultivated under adverse conditions of pH, temperature, or in the presence of antibiotics, they undergo important morphological changes, giving rise to atypical structures of different sizes and shapes, ranging from miniscule dots and spores (known as blebs) to formations resembling elongated bacteria (spirochetal), dense corpuscles with a double membrane (similar to *Chlamydia*), and single-membrane cysts (suggestive of *Mycoplasma*) on electron microscopy[33]. Additionally, the presence of spirochetes with atypical morphology, such as those mentioned above, in the brain parenchyma of patients with neurological manifestations of syphilis and Lyme borreliosis, has been described[34,335]. Under favorable culture conditions, L-form spirochetes reassume the normal helicoidal morphology[36].

The medical literature considers most L-form bacteria non-pathogenic, with rare exceptions[27]. The aggregated knowledge of LIM-17 HCFMUSP led us to postulate that the presence of L-form bacteria in animals and humans would be relatively common. We considered that regular and transitory infections of the respiratory, digestive, and urinary tract would be the usual source of contamination. Places with improper sanitary conditions, such as those found in the FMUSP vivarium, would certainly present a high environmental bacterial proliferation, as well as contamination of humans and animals, leading to a high incidence of spirochetal structures (L-form bacteria) in the peripheral blood. The normal saprophytic flora would be another suggested source of spirochetal structures.

In most cases, L-form microorganisms are not pathogenic, as we mentioned on our report to the Dean of USP. However, in our opinion, the behavior of spirochetal structures found in BYS is different than normal since, by preserving the ability to invade endothelial cells, they reveal a high pathogenic potential. Since spirochetes in their helicoidal form were never cultivated and isolated in Brazil, we assumed that the etiology of BYS was linked to L-form spirochetes. We believe that the etiological agent of BYS adapted permanently to its atypical morphology due to the irreversible loss of genetic and cell wall lipoprotein contents (Osp) in order to survive in adverse conditions, such as the absence of *Iodes ricinus* ticks in Brazil. Recent publications demonstrated that the genetic diversity of different species of *Borrelia burgdorferi sensu lato* complex spirochetes is subjected to regional and continental influences[37,38,39].

Finally, by accepting that BYS is caused by atypical spirochetes, we are able to justify all clinical and laboratorial particularities of this Brazilian zoonosis. This theory explains the clinical relapses and evolution of BYS into the so called idiopathic chronic disorders; treatment difficulties, especially in chronic diseases; the interference of microorganisms on the immune system, leading to the development of immune-allergic reactions; why those bacteria are difficult to grow in different media and stain poorly by common staining methods; why those microorganisms cause low immunologic reactivity to *Borrelia burgdorferi*; and why molecular tests, such as PCR, are persistently negative, possibly due to the partial loss of plasmids.

The theory that, in Brazil, BYS is caused by a mutant spirochete, genetically modified, and devoid of most of the cellular wall (Osp) and periplasmic flagella, is supported by the medical literature, since a mutant form of *Borrelia burgdorferi* and deficient on Osp, A, B, C, and D, has been described[40]. Those surface proteins are important to distinguish the different species of spirochetes of the *Borrelia burgdorferi sensu lato* complex and they participate on the pathogenicity and triggering of immunologic host reaction to the microorganism. Additionally, the mobility and helicoidal form of *Borrelia* are dependent on the 7-11 periplasmic flagella[41,42], and when mutation of the *flab* gene (main flagellin gene) is present, the spirochete assumes a bacteroid morphology[43], resembling the electron microscopic shape visualized in Brazil. Thus, spirochetes that have lost their flagella and wall lipoproteins would assume a spirochetal aspect, on dark-field microscopy, and an aspect of *Mycoplasma* and *Chlamydia*, on electron microscopy.

Today, after 20 years of investigations, we dare to define BLLD, or BYS, as an original Brazilian disorder caused be latent bacteria with atypical L-form morphology, transmitted by ticks that do not belong to the *Ixodes ricinus* complex, that

produces clinical manifestations similar to those observed in LD, except for the high incidence of relapses, and a tendency for chronicity and immune-allergic reactions.

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