Journal of Chronic Fatigue Syndrome 2008; 14(4): 5-17.

Chronic Fatigue Syndrome Patients Subsequently Diagnosed with Lyme Disease *Borrelia burgdorferi*: Evidence for *Mycoplasma* species Co-Infections

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ABSTRACT. *Objective:* We examined the blood of 48 North American Chronic Fatigue Syndrome (CFS) patients subsequently diagnosed with Lyme Disease *Borrelia burgdorferi* and compared these to 50 North American CFS patients without evidence of *Borrelia burgdorferi* infections for presence of *Mycoplasma spp.* co-infections using forensic polymerase chain reaction. *Results:* We found that 68.75% of CFS/Lyme patients show evidence of mycoplasma co-infections (Odds Ratio=41.8, Confidence Limits=11.26-155.16, $p<0.001$) compared to controls, whereas 50% of CFS patients without a diagnosis of Lyme Disease *Borrelia burgdorferi* show mycoplasma co-infections (OR=19.0, CL=5.25-68.78, $p<0.001$ compared to controls). Since CFS patients without a diagnosis of Lyme Disease have a high prevalence of one of four *Mycoplasma* species and a majority show evidence of multiple infections, we examined CFS/Lyme patients' blood for various *Mycoplasma* species. We found that CFS patients with Lyme Disease *Borrelia burgdorferi* mostly had single species mycoplasma infections (OR=31.67, CL=8.63-116.16, $p<0.001$) with a preponderance of *M. fermentans* infections (50% of patients, OR=59.0, CL=7.55-460, $p<0.001$), whereas the most commonly found *Mycoplasma* spp. in CFS patients without Lyme Disease was *M. penumoniae* (34% of patients. OR=14.94. CL=3.25-68.73, $p<0.001$). *Conclusions:* The results indicate that a subset of CFS patients show evidence of infection with *Borrelia burgdorferi*, and a large fraction of these patients were also infected with *Mycoplasma fermentans* and to a lesser degree with other *Mycoplasma* species.

Keywords: Fatigue syndrome,chronic; Borrelia burgdorferi; Lyme disease; Mycoplasma;

INTRODUCTION

Chronic Fatigue Syndrome (CFS) patients can be subdivided into clinically relevant subcategories that may represent different disease states or co-morbid conditions or illnesses (1-5). An important subset of CFS patients is characterized by the presence of chronic bacterial and viral infections (3-16). Identifying systemic infections, such as those produced by *Mycoplasma* species (3-9), *Chlamydia pneumoniae* (9, 10), Human Herpes Virus-6 (HHV-6) (9, 11-13) and *Brucella* species (14), is likely to be important in determining the treatment strategies for many CFS patients. Although no single underlying cause has been established for CFS, there is growing awareness that CFS can have an infectious nature that is either causative, a cofactor for the illness or appears as an opportunistic infection(s) that cause or enhance patient morbidity (15, 16). There are several reasons for this (15), including the nonrandom or clustered appearance of CFS, sometimes in immediate family members (15-17), the presence of certain signs and symptoms associated with infection, the often cyclic course of the illness and its response to anti-microbial therapies (4, 15, 16).

Recently it has become apparent that some CFS patients have Lyme Disease (18). Lyme Disease is the most common tick-borne disease in North America and has been reported widely the USA and Eastern Canada. First described in Southeastern Connecticut in 1975, the infection is caused by a tick bite and the entry of the spiral-shaped spirochete *Borrelia burgdorferi* (19) and other co-infections, including *Mycoplasma fermentans* (20). Here we investigated the presence of mycoplasma infections in CFS patients who were also diagnosed with Lyme Disease *Borrelia burgdorferi* and compared them to CFS patients who tested negative for *Borrelia burgdorferi.*

MATERIALS AND METHODS

Patients and Borrelia burgdorferi Western Blot Analysis

All patients were from private practices in North America (Canada and the United States) and underwent a medical history, completed a sign/symptom illness survey and had routine laboratory tests. If necessary, medical records were also reviewed to determine if patients suffered from organic or psychiatric illnesses that could explain their symptoms. When results were found in any of the evaluations that did not met the Fukuda et al. (1) criteria, the patients were not included in the study. CFS patients were recruited who were previously tested for *Borrelia burgdorferi* using Western Blot analysis (21, 22). This was a retrospective study where laboratory results were examined for Lyme Disease testing, and the criteria for a positive Western blot for Lyme Disease was that at least two of the *Borrelia burgdorferi* genus-specific antigens (18K, 23K, 30K, 31K, 34K, 37K, 39K, 83K and 93K) were reactive in Western blots. Control subjects (N=60) were local volunteers and had to be non-symptomatic and free of a disease for at least three months prior to data collection.

Mycoplasma Testing by PCR

Blood samples were collected in EDTA-containing tubes and immediately brought to ice bath temperature as described previously (9, 14, 23-25). Samples were blinded and shipped with wet ice by air courier to the Institute for Molecular Medicine for analysis. Whole blood (50 μl) was used for preparation of DNA using Chelex (Biorad, Hercules, USA) as previously described (14, 23-25). Multiple aliquots were used for experiments on all patient samples.

Amplification of *Mycoplasma* species target gene sequences (14, 23-25) was performed in a total volume of 50 μl PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 9) containing 0.1% Triton X-100, 200 μm each of dATP, dTTP, dGTP, dCTP, 100 pmol of each primer, and 0.5-1 μg of chromosomal DNA. Purified *mycoplasma* DNA (0.5-1 ng of DNA) was used as a positive control for amplification. Additional primer sets were used to confirm the species specificity of the reaction (9, 14). The

amplification was carried out for 40 cycles with denaturing at 94°C and annealing at 60°C (genus-specific primers and *M. penetrans*) or 55°C (*M. pneumoniae*, *M. hominis, M. fermentans*). Extension temperature was 72°C in all cases. Finally, product extension was performed at 72°C for 10 min. Negative and positive controls were present in each experiment. The amplified samples were run on a 1% agarose gel containing 5 μl/100 ml of ethidium bromide in TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA, pH 8.0). After denaturing and neutralization, Southern blotting was performed as described below (9, 14, 23-25).

Southern Blot Confirmation

The amplified samples were run on a 1% agarose gel containing 5 ml/100 ml of ethidium bromide in TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA, pH 8.0). After denaturating and neutralization, Southern blotting was performed as follows. The PCR product was transferred to a Nytran membrane. After transfer, UV cross-linking was performed. Membranes were prehybridized with hybridization buffer consisting of 1x Denhardt's solution and 1 mg/ml salmon sperm DNA as blocking reagent. Membranes were then hybridized with digoxigenin–UTP or ^{32}P-labeled internal probe (10^7 cpm per bag). After hybrization and washing to remove unbounded probe, the membranes were examined (digoxigenin-UTP-labeled probe) or exposed to autoradiography film (^{32}P-labeled probe) for 0.5-2 days at –70°C (9, 14, 23-25).

Statistics

Subjects' demographic characteristics were assessed using descriptive statistics and students' t-tests (independent samples test, t-test for equality of means, 2-tailed). The 95% confidence limits (CL) was chosen for minimal significance. Odds Ratios (OR) were calculated using the maximum likelihood estimates (MLE) of the confidence intervals and the associated *p*-values by using the stat program "R" (Stat 571, http://cran.r-project.org).

RESULTS

Patients and Control Subjects

Ninety-eight patients and 60 controls (C) were recruited and their demographic data is shown in Table 1. Patients were from both rural and urban environments. All CFS patients fulfilled the revised international CDC case definition for Chronic Fatigue Syndrome (1).

There was a significant increase in the number of CFS patients who were positive for *Borrelia burgdorferi* (CFS=48 of 98, C=0 of 60; $p<0.001$ – chi square analysis). Similarly, there were increases in *Mycoplasma* spp. in the CFS patients compared with the controls (CFS=58 of 98, C=3 of 60; OR=27.5, 95%CL=8.0-95.1; $p<0.001$). Both *Mycoplasma fermentans* (CFS=38 of 98, C=1 of 60; OR=27.5, 95%CL=8.0-95.1; $p<0.001$) and *Mycoplasma pneumoniae* (CFS=28 of 98, C=2 of 60; OR=27.5, 95%CL=8.0-95.1; $p<0.001$) had increased numbers of positive subjects in the CFS group compared with the control subjects.

Mycoplasma Infections in CFS Patients with or without Borrelia burgdorferi infections

Using genus- and species-specific primers and PCR the incidence of various *Mycoplasma* species in the blood of *Borrelia burgdorferi*-positive and -negative CFS patients was examined (Table 2). Similar to previous reports (14, 23, 25), the majority of CFS patients had mycoplasma infections. Differences between CFS patients and control subjects were highly significant ($p<0.001$) compared to control subjects). However, we did find differences in CFS patients depending on whether they had tested positive or negative for *Borrelia burgdorferi* infections. The *Borrelia burgdorferi*-positive CFS

group had high overall rates of mycoplasma infections (68.75%) compared to controls (OR=41.8; CL=11.26-155.16, $p<0.0001$). Previously we found that approximately 51% of CFS patients had mycoplasma infections (9, 25, 26), and this was also found in the present study in *Borrelia burgdorferi*–negative CFS patients (Table 2). Next we examined the *Mycoplasma* species present in CFS patients and found that the *Borrelia burgdorferi*-positive CFS group had predominantly *M. fermentans* infections (50%; OR=59; CL=7.55-460.83, $p<0.0001$), followed by *M. pneumoniae* (22.9%; OR=8.62; CL=1.81-41.11, p=0.0069) and *M. hominis* infections, whereas the *Borrelia burgdorferi*-negative CFS group had more of a mixture of mycoplasma infections (*M. pneumoniae* 34%, *M. fermentans* 28%, *M. hominis* 16% and rarely *M. penetrans* 2%) (Table 2), similar to CFS patients in general (9, 25, 26). *Borrelia burgdorferi*-positive CFS patients tended to have single mycoplasma infections (62.5%; OR=31.67; CL=8.63-116, $p<0.0001$); whereas *Borrelia burgdorferi*-negative CFS patients tended to have multiple mycoplasma infections as found in other studies (9, 25, 26). Consistent with previous reports on chronic illness patients (9, 14, 23-25), control subjects rarely had mycoplasma infections. We found that only 5% of control subjects showed evidence of *Mycoplasma* species in their blood (2/50 *M. pneumoniae*, 1/50 *M. fermentans*), and the differences between CFS patients with and without *Borrelia burgdorferi* infections and controls were highly significant ($p<0.0001$ compared to compared to controls).

Multiple Mycoplasma Co-Infections in CFS Patients

Previous studies on the presence of mycoplasma infections in CFS patients indicated that a majority of patients had multiple *Mycoplasma* species in their blood, whereas the few control subjects that were positive for mycoplasma infections only had single species infections (9,14, 24). Similar results were found here for *Borrelia burgdorferi*–negative CFS patients; over one-half of the *Borrelia burgdorferi*–negative CFS patients had multiple *Mycoplasma* species in their blood. However, the *Borrelia burgdorferi*–positive CFS patients predominantly had single *Mycoplasma* species in their blood (OR=31.67, CL=8.63-116, p<0.0001). Most of the single *Mycoplasma* species infections in *Borrelia burgdorferi*–positive CFS patients were *M. fermentans* (OR=59, CL=7.55-460, $p<0.0001$, Table 2).

DISCUSSION

Previously we reported that chronic bacterial and viral infections appear to be a rather common feature of CFS, and most CFS patients examined had multiple infections (9, 14, 24). Since CFS patients often report that their CFS signs and symptoms slowly evolved after acute infections, this result is not unexpected (9, 16, 25). Also, the severity of CFS signs and symptoms appear to be related to the number of chronic infections but not their specific type (26).

CFS patients have also been diagnosed with Lyme Disease (18), and Eskow *et al.* (20) have found that the attachment of ticks and subsequent appearance of musculoskeletal signs and symptoms is associated with systemic *M. fermentans* infections (20). Thus it was not unexpected that CFS patients with evidence of *Borrelia burgdorferi* infections would also show evidence of *Mycoplasma* species in their blood. What is interesting is that the predominant presence of *M. fermentans* in the *Borrelia burgdorferi*-positive CFS patients is consistent with the finding of this species of *Mycoplasma* in ticks collected from the environment (20).

Previously we studied North American and European CFS patients and found that most showed evidence of mycoplasma infections (5, 9, 14, 24-26). Like *Borrelia burgdorferi*, *Mycoplasma* spp. are slow-growing, fastidious, intracellular infections that can invade a variety of tissues but they can also present as superficial infections (27-29). Others who studied CFS patients also found evidence of

widespread mycoplasma infections (6-8). When we examined the incidence of particular mycoplasma infections in North American CFS patients, we found that the most common species found was *M. pneumoniae* and most patients had multiple mycoplasma infections, which were for the most part combinations of *M. fermentans* and other mycoplasma species (9, 24, 26). However, in a study on European CFS patients a slightly different picture was found (5). The most common species found in Belgium and Dutch patients was *M. hominis,* and there was a lower overall rate of multiple mycoplasma co-infections in the European CFS patients (5). We also found that more than 50% of North American patients with rheumatoid arthritis had mycoplasma infections, and in the majority of these patients multiple mycoplasma co-infections were found (23, 27).

Patients with the Lyme Disease spirocyte *Borrelia burgdorferi* usually have multiple co-infections involving bacteria other than *Mycoplasma* spp., such as *Ehrlichia* spp. and *Bartonella* spp. as well as protozoan species of *Babesia* (30, 31). *Ehrlichia* species are small, gram-negative, pleomorphic, obligate intracellular infections similar to mycoplasmas in their structures, intracellular locations and resulting signs/symptoms (32). The other common bacterial co-infection is caused by *Bartonella* spp. (33), and this co-infection (along with *Mycoplasma* spp.) appears to be one of the most common tick-borne co-infections found with *Borrelia burgdorferi*. *Bartonella* spp., such as *Bartonella henselae*, which also causes cat-scratch disease (34), is often found in neurological cases of Lyme Disease (33, 35). A non-bacterial co-infection found with *Borrelia burgdorferi* is the intracellular protozoan *Babesia* spp. (36). There are over 100 species of the genus *Babesia*, but most Lyme Disease co-infections in humans in North America are caused by *Babesia microti* and in Europe by *Babesia divergens* and *Babesia bovis* (37, 38).

In CFS multiple infections are associated with more severe signs and symptoms (26), and similarly when multiple infections are present in Lyme Disease, the number of signs/symptoms and their severity and duration are usually greater in the early stages of disease (36). In Lyme Disease patients with multiple co-infections can present with high fever, chills, generalized weakness, gastrointestinal symptoms (anorexia, nausea, abdominal pain, vomiting, diarrhea, among others), anemia, muscle and joint pain, respiratory problems and dark urine. The combination of *Borrelia*, *Mycoplasma* and *Babesia* infections can be lethal in some patients (about 7% of patients can have disseminated intravascular coagulation, acute respiratory distress syndrome and heart failure), but the majority of patients with tend to have the chronic form of the disease. In *Babesia* infections patients can show mild to severe hemolytic anemia (probably correlating with the protozoan colonization of erythrocytes, which can be seen by experienced individuals in blood smears) and a normal to slightly depressed leukocyte count (36). However, these symptoms are usually not seen in patients who have progressed to the chronic phase of the disease, which can be similar in presentation to CFS (18).

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Table 1. Patient age and sex data.

	N	Mean age y (SD)	Range y	Females N (%)
All Patients	98	37.8 (9.5)	16-66	67 (68.4)
Borrelia burgdorferi-pos.	48	37.5 (9.3)	18-60	34 (70.8)
Borrelia burgdorferi-neg.	50	38.0 (10.0)	16-66	33 (66.0)
Controls	60	34.5 (9.3)	20-61	39 (65.0)

TABLE 2. Prevalence of Mycoplasma Infections in *Borrelia burgdorferi*-positive and -negative CFS Patients.

	CFS				C
	***Borrelia burgdorferi* +**		***Borrelia burgdorferi* -**		
N	48		50		60
Type of infection	**N(%)**	**OR (95% CL) *p***	**N(%)**	**OR (95% CL) *p***	
Mycoplasma spp.	33(68.8)	41.8 (11.3-155) <0.001	25(50)	19.0 (5.3-69) <0.001	3(5)
M. pneumoniae	11(22.9)	8.6 (1.8-41) 0.0069	17(34)	14.9 (3.3-69) <0.001	2(3.3)
M. fermentans	24(50)	59.0 (7.6-460) <0.001	14(28)	22.9 (2.9-182) 0.003	1(1.7)
M. hominis	4(8.3)	* Chi-Sq 0.023	8(16)	* Chi-Sq 0.0013	0
M. penetrans	0	*	1(2)	* Chi-Sq 0.27	0
Mycoplasma Infections					
Single	30(62.5)	31.7 (8.6-116) <0.001	12(24)	6.0 (1.6-23) 0.0083	3(5.0)
Multiple	3(6.3)	* Chi-Sq 0.05	13(26)	* Chi-Sq <0.001	0

C=Control; OR=Odds ratio; 95%CL=95% Confidence Levels. Chi-Sq=Chi-square probability. Statistical comparisons are between the Borrelia-positive and -negative subgroups with the control group.

*We obtained the maximum likelihood estimates (MLE) of the confidence intervals and the associated *p*-values by using the stat program "R". MLE assumes the parameter is not on a boundary. This becomes an issue with when some of the cells contain zero counts and we encounter the "zero-cells" problem. This causes computational issues and the algorithm will not converge due to a lack of a standard error. In these case Chi-square analysis was performed

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