Journal of Chronic Fatigue Syndrome 2005; 12(2): 5-17.

Evidence for *Brucella* spp. And *Mycoplasma* spp. Co-Infections in Blood of Chronic Fatigue Syndrome Patients

Garth L. Nicolson,[1] PhD, Robert Gan,[1] MB, PhD and Joerg Haier,[2] MD, PhD

[1]The Institute for Molecular Medicine, Huntington Beach, California, USA,[2]Department of Surgery, University Hospital, Munster, Germany

ABSTRACT. We examined the blood of 94 North American Chronic Fatigue Syndrome (CFS) patients using forensic polymerase chain reaction and found that a subset (10.6%) of CFS patients show evidence of *Brucella* spp. infections compared to one of 70 control subjects (Odds Ratio = 8.2, Confidence Limits 1-66, $P<0.01$). Rural patients showed a higher incidence of *Brucella* spp. infections over urban patients (OR = 5.5, CL 1.3-23.5, $P<0.02$). Since CFS patients also have a high prevalence of one of four *Mycoplasma* species and sometimes show evidence of infections with *Chlamydia pneumoniae*, we examined *Brucella*-positive patients for other bacterial infections. Previously we found that 8% of the CFS patients showed evidence of *C. pneumoniae* and about 50% show evidence of *Mycoplasma* spp. infections. Since the presence of one or more chronic systemic infections may predispose patients to other infections, we examined the prevalence of *C. pneumoniae* and *Mycoplasma* spp. infections in *Brucella*-positive patients. We found only one *Brucella*-positive patient with *C. pneumoniae* and four other patients with evidence of *Mycoplasma* spp., suggesting that such bacterial infections occur independently in CFS patients. Control subjects (N=70) had low rates of *Brucella* spp. (1.4%), *Mycoplasma* spp. (7.2%) or *C. pneumoniae* (1.4%) infections, and there were no co-infections in control subjects. The results indicate that a subset of CFS patients show evidence of infection with *Brucella* spp., and some of these patients also have other bacterial infections.

Correspondence: Prof. Garth L. Nicolson, Office of the President, The Institute for Molecular Medicine, 16371 Gothard Street H, Huntington Beach, California 92647. Tel: 714-596-6636; Fax: 714-596-3791; Email: gnicolson@immed.org; Website: www.immed.org

INTRODUCTION

Chronic Fatigue Syndrome (CFS) patients usually have multiple, nonspecific, overlapping signs and symptoms, which makes such illnesses difficult to diagnose and treat (1-4). CFS patients can be subdivided into clinically relevant subcategories that may represent different disease states or co-morbid conditions or illnesses (5). An important subset of CFS patients is characterized by the presence of chronic bacterial and viral infections (3-13). Identifying systemic infections, such as those produced by *Mycoplasma* species (3-9), *Chlamydia pneumoniae* (9,10) and Human Herpes Virus-6 (HHV-6) (9,11-13), 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 for the illness, a cofactor for the illness or appears as an opportunistic infection(s) that aggravate patient morbidity (14). There are several reasons for this (15), including the nonrandom or clustered appearance of CFS, sometimes in immediate family members (16,17), the presence of certain signs and symptoms associated with infection, the often cyclic course of the illness and its response to anti-microbial therapies (4,5,14).

Here we examined CFS patients to see if a subset of patients showed evidence of infection with *Brucella* species. Brucellosis is a nonspecific clinical condition, sometimes similar to CFS but characterized by confirmed *Brucella* spp. infection. Approximately 40% of patients with *Brucella* spp. infections have a systemic, multi-organ chronic form of brucellosis that is similar to CFS in its multi-organ signs and symptoms (18-20). We were particularly interested in assessing whether CFS patients with *Brucella* spp. infection were likely to show evidence of additional bacterial infections.

MATERIALS AND METHODS

Patients

All patients were from North America (Canada and the United States, N=94) 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 positive results were found in any of the evaluations that met the Fukuda et al. (1) exclusionary criteria, the patients were not included in the study. Additionally, all subjects were questioned about medication use during the three months prior to the study, and they had to be free of antibiotic treatment for two months prior to blood collection. Control subjects (N=70) had to be free of disease for at least three months prior to data collection, and they had to be free of antibiotic treatment for three months prior to blood collection.

Blood Collection

Blood was collected in EDTA-containing tubes and immediately brought to ice bath temperature as described previously (9,21-23). Samples were shipped with wet ice by air courier to the Institute for Molecular Medicine for analysis. All blood samples were blinded. Whole blood (50 μl) was used for preparation of DNA using Chelex (Biorad, Hercules, USA) as follows. Blood cells were lysed with nano-pure water (1.3 ml) at room temperature for 30 min. After centrifugation at 13 000 x g for 2 min, the supernatants were discarded. Chelex solution (200 μl) was added, and the samples were incubated at 56°C and at 100°C for 15 minutes each. Aliquots from the centrifuged samples were used immediately for PCR or flash frozen and stored at –70°C until use. Multiple aliquots were used for experiments on all patient samples.

Detection of Mycoplasma by Forensic PCR.

Amplification of the target gene sequences (9,21-23) 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 mycoplasmal 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). 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, 21-23).

Brucella spp. Detection by Forensic PCR.

PCR detection of *Brucella* spp. was done as described above for various *Mycoplasma* species, except that the conditions and primers differ. PCR was first carried out using the published *Brucella* spp.-specific primers consisting of amplification of a 223-bp fragment of of a 31 kDa membrane protein (24,25). In addition, we used the following primer set to confirm the primary results: 5'-AGTCGGGCGCTCTGGAGTCC-3' (upstream) and downstream 5'-TCCTTACGCGCAACGATATG-3'. The DNA was amplified for 40 cycles using standard cycle parameters, and the product evaluated by agarose-gel electrophoresis. The efficiency of the PCR process was monitored by amplification of β-actin mRNA. The presence of amplifications inhibitors was evaluated by spiking negative samples with 2 □l of DNA from stock. *Brucella* spp.-specific oligonucleotides in the PCR product were identified by Southern Blot and dot-blot hybridization using a 21-mer internal probe. The internal probe (5'-TCCGGGTAAAGCGTCGCCAG-3') was 3'-end-labelled with digoxigenin–UTP or ^{32}P-labeled probe.

Chlamydia pneumoniae Detection by Forensic PCR.

PCR detection of *Chlamydia pneumoniae* was done as described above for various *Mycoplasma* species, except that the conditions and primers differ. PCR was carried out using the *C. pneumoniae*-specific primers:

5'-TGACAACGTTAGAAATACAGC-3' (upstream) and downstream 5'-CGCCTCTCTCTCCTATAAAT-3'. Additional primer sets were used to confirm the species specificity of the reaction. The DNA was amplified for 30 cycles using standard cycle parameters, and the product evaluated by agarose-gel electrophoresis. The efficiency of the PCR process was monitored by amplification of β-actin mRNA. The presence of amplifications inhibitors will be evaluated by spiking negative samples with 2 □l of DNA from stock. *C. pneumoniae*-specific oligonucleotides in the PCR product were identified by Southern Blot and dot-blot hybridization using a 21-mer internal probe: (5'-CGTTGAGTCAACGACTTAAGG-3') 3' end-labelled with digoxigenin–UTP or ^{32}P-labeled probe.

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.

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 interval was chosen for minimal significance. Odds Ratios were calculated using logistic regression (Logit method) Statistica 5.5 (Statsoft, Tulsa, OK).

RESULTS

Patients and Control Subjects

Patients and control subjects were approximately similar in age characteristics (control subjects mean age = 34.4; CFS patients: mean age = 38.9). CFS patients differed significantly according to sex distribution ($p< 0.05$); 69.1% of the patients were female, while 30.9% of the patients were male. Similarly, 58.6% of control subjects were female, while 44.3% were male. Patients were from both rural and urban environments (Table 1). All CFS patients fulfilled the revised international CDC case definition for Chronic Fatigue Syndrome (1).

Brucella spp. Infections in CFS Patients

Brucella spp. infections were found in 10 of 94 CFS patients (10.6%) but in only one of 70 control subjects (1.4%) (Odds Ratio OR = 8.2, Confidence Limits CL 1-66, $P<0.01$). Of the ten positive patients five were male (17.2% of males) and five were female (7.7% of females). *Brucella* spp. infections are often found in rural areas where contact with domestic animals is more common than in urban environments (18). Therefore, we examined the incidence of *Brucella* spp. infections in rural inhabitants. The rural CFS patients living in rural environments had an increased Odds Ratio for a positive *Brucella* spp. Response compared with the urban CFS patients (OD = 5.5, CL 1.3-23.5, $P<0.02$). Males living in rural environments (37.9% of males) accounted for four of five *Brucella* spp. infections (80%), whereas females in rural environments (30.8% of females) accounted for three of five (60%) *Brucella* spp. infections (Table 2). The one positive control subject was from an urban environment, and there were no differences in the incidence of other infections between rural and urban patients.

Other Chronic Bacterial Infections in CFS Patients

When we examined CFS patients' blood for the presence of chronic bacterial infections other than *Brucella* spp. using forensic PCR, evidence for *Mycoplasma* species infections were found in 50% of CFS patients and 8.5% of control subjects (OR = 8.1). Evidence for *C. pneumoniae* infections were found in 8.3% of CFS patients and in 1.5% of control subjects (OR = 13.1). We did not find any multiple co-infections in control

subjects. The differences between chronic infections in CFS patients and control subjects were significant (Table 2).

Using species-specific primers and PCR the incidence of various *Mycoplasma* species in the blood of *Brucella* spp.-positive and -negative CFS patients was examined. Four of ten patients with *Brucella* spp. positive results (40%) were also positive for *Mycoplasma* infections, whereas 43 of 84 *Brucella* spp.-negative patients (51.2%) were also *Mycoplasma*-positive ($p< 0.001$ compared to control subjects). *M. pneumoniae* infections were observed in 27 of 43 *Brucella* spp.-positive CFS patients ($P<0.001$ compared to control subjects), *M. fermentans* infections occurred in 20 patients ($P<0.001$ compared to control subjects) and *M. hominis* in 14 patients ($p< 0.001$ compared to control subjects), whereas *M. penetrans* infections were found at lower (5 of 43 *Brucella* spp.-negative patients) incidence ($P<0.01$ compared to control subjects) (Table 2). We examined 70 control subjects who did not show clinical signs and symptoms and found that 6 were positive for a single species of Mycoplasma (Table 2). Differences between CFS patients and control subjects were highly significant ($P<0.001$ compared to control subjects).

Multiple Mycoplasmal Co-Infections in CFS Patients

Single infections with one of the four *Mycoplasma* spp. that were tested were observed in four of the *Brucella* spp.-positive patients, and interestingly we did not find multiple *Mycoplasma* spp. infections in *Brucella* spp.-positive patients (Table 2). In the seven control subjects that were positive for mycoplasmal infections we found two controls that were positive for *M. fermentans*, three for *M. pneumoniae* and one for *M. hominis* and none for *M. penetrans* but similar to previous studies (9,22,23) these were found only as single infections. Also similar to our previous studies (9,22,23), the most commonly observed infection was *M. pneumoniae* (29 of 94 CSF patients), followed by *M. fermentans* in 22 patients, *M. hominis* in 14 patients and *M. penetrans* in five patients. Multiple mycoplasmal infections were detected in 18 of the 43 *Brucella* spp.-negative patients (41.8%), whereas single infections were found in four of ten (40%) of the *Brucella* spp.-positive patients. The few control or healthy subjects that showed evidence for mycoplasmal infections only had single species infections. Similar to our previous results (9,22,23), we have not found patients positive for all four of the tested *Mycoplasma* species (Table 2).

Co-Infections with C. pneumoniae in CFS Patients

Chlamydia pneumoniae infections were found in 8 of 94 (8.5% of CFS patients and one control subject out of 70 (1.4%) that also did not have mycoplasmal infections ($p < 0.01$) (Table 2). This finding is similar but somewhat lower than previously reported for CFS patients (9,10). When we examined the incidence of *C. pneumoniae* infections in *Brucella* spp.-positive and –negative patients, we found that there was no preference for multiple infections, nor was there a preference for *C. pneumoniae* in *Brucella* spp. co-infections (Table 2). Thus there appeared to be no preference for particular combinations of co-infections in CFS patients.

DISCUSSION

We previously reported that chronic bacterial and viral infections appear to be a rather common feature of CFS, and many CFS patients have multiple infections (9,22,26). Since CFS patients often report that their CFS signs and symptoms slowly evolved after acute infections, this result is not unexpected (9,26). Also, the severity of CFS signs and symptoms appear to be related to the number of chronic infections but not their specific type (26). In addition to chronic infections, chemical exposures, multiple vaccinations, severe trauma or other conditions that are associated with immune suppression and opportunistic infections also may be related to results found here. Since about 40% of brucellosis patients have multiple organ signs and symptoms (19,20) that could be diagnosed as severe CFS in some patients, the finding that a subset of CFS patients have *Brucella* spp. infections and fit the diagnostic profile of CFS is not unreasonable. In some areas the incidence of *Brucella* spp. infections is fairly high, and in these patients multiple infections with other bacteria are not uncommon (24,27). We found that *Brucella* spp. infections were more prevalent in CFS patients from rural environments, but we did not have enough *Brucella* spp.-positive patients to statistically conclude that rural CFS patients were more likely to have *Brucella* spp. infections than CFS patients living in urban environments.

Brucella spp. cause slow-growing intracellular, gram-negative, facultative infections in animals and humans. These bacteria are zoonotic and capable of being transmitted from animals to humans. Although there are at least eight species of *Brucella* that are pathogenic, only *B. melitensis, B. abortus, B. suis* and *B. canis* have been reported to be pathogenic in humans (28). Differentiating between species of *Brucella* can be difficult because of morphological and antigenic similarities between species (26,27); however, use of PCR has enabled the differentiation of the most common *Brucella* species (28). PCR detection of *Brucella* spp. is also extremely sensitive and specific compared to other techniques (24,25) but it does have its limitations, especially if great care is not taken in the preparation of reagents and specimens. For our purposes the use of primers that can detect all of the pathogenic species in humans of the genus *Brucella* was sufficient. For treatment, it is not necessary to know the exact species of *Brucella*.

Previously we studied North American and European CFS patients and found that most showed evidence of mycoplasmal infections (5,9,22,23). Like *Brucella* spp., *Mycoplasma* spp. are slow-growing, fastidious, intracellular infections that can invade a variety of tissues but they can also present as superficial infections (29,33,34). Others who studied CFS patients also found evidence of widespread mycoplasmal infections (6-8). When we examined the incidence of particular mycoplasmal infections in North American CFS patients, we found that most patients had multiple mycoplasmal infections, which were for the most part combinations of *M. fermentans* and other mycoplasma species (9,22,29). However, in a study on European CFS patients a slightly different picture was found (5). The most common species found was *M. hominis,* and there was a lower overall rate of multiple mycoplasmal co-infections in the European CFS patients (5). We also found that more than 50% of North American patients with rheumatoid arthritis had mycoplasmal infections, and in the majority of these patients multiple mycoplasmal co-infections infections were found (21,32). Arthritis signs and symptoms are commonly found in focal forms of *Brucella* spp. infections (25,35)

In CFS patients we found that multiple co-infections involving *Mycoplasma* spp., *C. pneumoniae* and HHV-6 were common (9,29), and here we found that some *Mycoplasma* spp. co-infections also involve *Brucella* spp. Thus a common feature of CFS may be the presence of multiple, slow-growing, chronic, intracellular infections. The presence of multiple co-infections in CFS probably play an important role in determining the severity of systemic signs and symptoms found in CFS patients. In support of this we found that the severity of signs and symptoms in CFS patients was related to the presence of multiple chronic infections (29). Since CFS patients that previously tested positive for chronic infections have benefited from therapies directed at their chronic infections (4,34), we consider it important that *Brucella* spp. as well as *Mycoplasma* spp. and *C. pneumoniae* infections be carefully considered in the clinical management of CFS (4,34).

REFERENCES

1. Fukuda K, Strauss SE et al. The Chronic Fatigue Syndrome, a comprehensive approach to its definition and study. *Ann Intern Med* 1994; 121:953-959.
2. Hoffman C, Rice D, Sung H-Y. Persons with chronic conditions. Their prevalence and costs. *JAMA* 1996; 276:1473-1479.
3. Nicolson GL, Nasralla M, Haier J, et al. Diagnosis and treatment of chronic mycoplasmal infections in Fibromyalgia and Chronic Fatigue Syndromes: relationship to Gulf War Illness. *Biomed Ther* 1998; 16:266-271.
4. Nicolson GL, Nasralla M, Franco AR, et al. Diagnosis and Integrative Treatment of Intracellular Bacterial Infections in Chronic Fatigue and Fibromyalgia Syndromes, Gulf War Illness, Rheumatoid Arthritis and other Chronic Illnesses. *Clin Pract Alt Med* 2000; 1:92-102.
5. Nils J, Nicolson GL, De Becker P, et al. Prevalence of Mycoplasmal infections in European CFS patients. Examination of four *Mycoplasma* species. Submitted for publication.
6. Vojdani A, Choppa PC, Tagle C, et al. Detection of *Mycoplasma* genus and *Mycoplasma fermentans* by PCR in patients with Chronic Fatigue Syndrome. *FEMS Immunol Med Microbiol* 1998; 22: 355-365.
7. Huang W, See D, Tiles J. The prevalence of *Mycoplasma incognitus* in the peripheral blood mononuclear cells of normal controls or patients with AIDS or Chronic Fatigue Syndrome. *J Clin Microbiol* 1998; 231:457-467.
8. Choppa PC, Vojdani A, Tagle C, et al. Multiplex PCR for the detection of *Mycoplasma fermentans, M. hominis* and *M. penetrans* in cell cultures and blood samples of patients with Chronic Fatigue Syndrome. *Mol Cell Probes* 1998; 12: 301-308.
9. Nicolson GL, Nasralla M, Gan R, Haier J, De Meirleir K. Evidence for bacterial (mycoplasma, Chlamydia) and viral (HHV-6) co-infections in chronic fatigue syndrome patients. *J Chronic Fatigue Syndr* 2003; 11(2):7-20.

10. Chia JKS, Chia LY. Chronic Chlamydia pneumoniae infection: a treatable cause of Chronic Fatigue Syndrome. Clin Infect Dis 1999; 29:452-453.
11. Braun DK, Dominguez G, Pellett PE. Human herpesvirus-6. *Clin Microbiol Rev* 1997; 10:521-567.
12. Campadelli-Fiume G, Mirandela P, Menetti L. Human herpesvirus-6: an emerging pathogen. *Emerg Infect Dis* 1999; 5:353-366.
13. Patnaik M, Komaroff AL, Conley C, Orjin-Amaine EA, Peter JB. Prevalence of IgM antibodies to human herpesvirus-6 early antigen in patients with chronic fatigue syndrome. *J Infect Dis* 1995; 172:1164-1167.
14. Nicolson GL, Nasralla MY, Haier J et al. Mycoplasmal infections in chronic illnesses: Fibromyalgia and Chronic Fatigue Syndromes, Gulf War Illness, HIV-AIDS and Rheumatoid Arthritis. *Med Sentinel* 1999; 5:172-176.
15. Nicolson GL. Chronic infections as a common etiology for many patients with Chronic Fatigue Syndrome, Fibromyalgia Syndrome and Gulf War Illnesses. *Intern J Med* 1998; 1:42-46.
16. Walch CM, Zainal NZ, Middleton SJ, et al. A family history study of chronic fatigue syndrome. *Psych Genet* 2001; 11:123-128.
17. Nicolson GL, Nasralla MY, Nicolson NL, Haier J. High prevalence of mycoplasmal infections in symptomatic (Chronic Fatigue Syndrome) family members of mycoplasma-positive Gulf War Illness patients. *J Chronic Fatigue Syndr* 2002; 11(2):21-36.
18. Corbel MJ. Brucellosis: an overview. *Emerg Infect Dis* 1997; 3:313-321.
19. Young EJ. An overview of human brucellosis. *Clin Infect Dis* 1995; 21:283-290.
20. McLean DR, Russell N, Khan Y. Neurobrucellosis. Clinical and therapeutic features. *Clin Infect Dis* 1992; 15:582-590.
21. Haier J, Nasralla M, Franco RA, et al. Detection of mycoplasmal infections in blood of patients with rheumatoid arthritis. *Rheumatol* 1999; 38:504-509.
22. Nasralla M, Haier J, Nicolson GL. Multiple Mycoplasmal infections detected in blood of patients with Chronic Fatigue Syndrome and / or Fibromyalgia. *Eur J Clin Microbiol Infect Dis* 1999; 18:859-865.
23. Nasralla MY, Haier J, Nicolson NL, Nicolson GL. Examination of Mycoplasmas in blood of 565 chronic illness patients by polymerase chain reaction. *Intern J Med Biol Environ* 2000; 28(1):15-23.
24. Queipo-Ortuno MI, Morata P, Ocon P, Manchado P, Colmenero JD. Rapid diagnosis of human brucellosis by peripheral blood PCR assay. *J Clin Microbiol* 1997; 35:2927-2930.
25. Morata P, Queipo-Ortuno MI, Reguera JM, Miralles F, Lopez-Gonzalez JJ, Ccolmenero JD. Diagnostic yield of a PCR assay in focal complications of brucellosis. *J Clin Microbiol* 2001; 39:3743-3746.
26. Barham WP, Church P, Brown J, Paparello S. Misidentification of Brucella species with the use of rapid bacterial identification systems. *Clin Infect Dis* 1993; 17:1068-1069.
27. Picket M. Identification of *Brucella* species with a procedure for detecting acidification of glucose. *Clin Infect Dis* 1994; 19:976-977.
28. Da Costa M, Guillou J-P, Garin-Bastuji B, Thiebaud M, Dubray G. Specificity of six gene sequences for the detection of the genus *Brucella* by DNA amplification. *J Appl Bacteriol* 1996; 81:267-275.
29. Nicolson GL, Gan R, Haier J. Multiple co-infections (Mycoplasma, Chlamydia, human herpesvirus-6) in blood of chronic fatigue syndrome patients: association with signs and symptoms. *Acta Pathol Microbiol Immunol Scand* 2003; 111:557-566.
30. Giner P, El-Amrani A, Corrrales AJ, Guijarro R, Sanchez-Palencia JA, Jimenez-Alonso J. Simultaneous isolation of *Brucella melitensis* and *Mycobacterium tuberculosis* in pleural empyrema. *Enferm Infect Microbiol Clin* 1990; 8:505-506.
31. Fox KF, Fox A, Nagpal M, Steinberg P, Heroux K. Identification of Brucella by ribosomal-spacer-region PCR and differentiation of Brucella canis from other Brucella spp. pathogenic for humans by carbohydrate profiles. *J Clin Microbiol* 1998; 36:3217-3222.
32. Nicolson GL, Nasralla M, Franco AR, et al. Mycoplasmal infections in fatigue illnesses: Chronic Fatigue and Fibromyalgia Syndromes, Gulf War Illness and Rheumatoid Arthritis. *J Chronic Fatigue Syndr* 2000; 6(3/4):23-39.
33. Baseman J, Tully J. Mycoplasmas: sophisticated, reemerging, and burdened by their notoriety. *Emerg Infect Dis* 1997; 3:21-32.
34. Nicolson GL, Nasralla M, Nicolson NL. The pathogenesis and treatment of mycoplasmal infections. *Antimicrob Infect Dis Newsl* 1999; 17:81-88.
35. Colmenero JD, Reguera JM, Martos F, Sanchez-de Mora D, Delgado M, Causse M, Martin-farfan A, Juarez C. Complications associated with *Brucella melitensis* infection: a study of 530 cases. *Medicine* 1996; 75:195-211.

Table 1. Patient demographic data.

	N	Mean age (SD)	Range	Males (%)	Females (%)
Patients	94	38.9 (9.8)	19-69	29 (30.9)	65 (69.1)
Controls	70	34.4 (9.3)	20-61	31 (44.3)	41 (58.6)
Female patients	65	39.1 (9.8)	19-66	0 (0.0)	65 (100.0)
Male patients	29	38.2 (10.3)	21-69	29 (30.9) (100.0)	0 (0.0)
Rural female patients	20	40.3 (14.2)	22-61	0 (0.0)	20 (30.8)
Rural male patients	11	36.1 (13.2)	21-59	11 (37.9)	0 (0.0)

TABLE 2. Prevalence of Chronic Bacterial Infections in *Brucella* spp.-positive and *Brucella* spp.-negative CSF Patients.

Type of infection	***Brucella* spp.-positive CFS Patients N = 10 (%)**	***Brucella* spp.-negative CFS Patients N = 84 (%)**	***Brucella* spp.-positive Control Subjects N = 1**	***Brucella* spp.-negative Control Subjects N = 69**	**Odds ratio (95% CL) (CFS/Control)**
Brucella spp.	10 (100)	0 (0.0)	1 (100)	0 (0.0)	8.2 (1-66) $P<0.01$
C. Pneumoniae	1 (10.0)	7 (8.3)	0 (0.0)	1 (1.5)	13.1 (1.7-103) $P<0.001$
Mycoplasma spp.	4 (40.0)	43 (51.2)	0 (0.0)	6 (7.2)	8.1 (3.4-18.8) $P<0.001$
M. pneumoniae	2 (40.0)	27 (62.8)	0 (0.0)	3 (43.5)	9.9 (2.9-34.6) $P<0.001$
M. fermentans	2 (40.0)	20 (46.5)	0 (0.0)	2 (28.6)	10.4 (2.3-46.4) $P<0.001$
M. honinis	0 (0.0)	14 (32.6)	0 (0.0)	1 (14.3)	12.1 (1.5-95.7) $P<0.001$
M. penetrans	0 (0.0)	5 (11.6)	0 (0.0)	0 (0.0)	$x^2 = 0.19$
Single mycoplasmal infection	4 (100)	25 (58.1)	1 (100)	6 (100)	4.0 (1.6-9.9) $P<0.001$
Multiple mycoplasmal infections	0 (0.0)	18 (41.8)	0 (0.0)	0 (0.0)	$x^2 < 0.001$