International Journal Medicine Biology Environment 2000; 28(1):15-23.

EXAMINATION OF MYCOPLASMAS IN BLOOD OF 565 CHRONIC ILLNESS PATIENTS BY POLYMERASE CHAIN REACTION

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Summary

Mycoplasmal infections are associated with several acute and chronic illnesses. Patients with Chronic Fatigue Syndrome (CFS), myalgic encephalomyelitis (ME) and/or Fibromyalgia Syndrome (FMS) were examined for systemic mycoplasmal infections by analysis of blood specimens. Using an optimized protocol for forensic polymerase chain reaction (PCR) blood samples from 565 CFS or FMS patients (401 female, 164 male) and 71 healthy controls were investigated for presence of *Mycoplasma spp.* and *M. fermentans* infections. The *Mycoplasma spp.* sequence was amplified from the peripheral blood of 300/565 patients (53.1 %). Specific PCR products could not be detected in 265 patients (46.9 %). A significant difference ($p<0.001$) was found between mycoplasma-positive patients and healthy controls (7/71; 9.9%). The prevalence of *M. fermentans* infections (24.6%) was also significantly ($p<0.001$) higher in CFS/FMS patients than in controls (2/71; 2.8%). Moreover, the prevalence of mycoplasmal infections was similar in female and male CFS patients. The data indicate that mycoplasmas can be detected in blood specimens from a high proportion of CSF/FMS patients. *M. fermentans* can be an opportunistic infection, cofactor or causative agent resulting in morbidity in these patients.

Key words: chronic fatigue syndrome, myalgic encephalomyelitis, fibromyalgia syndrome, mycoplasmas, polymerase chain reaction, bacteria, chronic infection

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Introduction

Mycoplasmas are small, cell wall-lacking prokaryotes with diminutive genomic material and low G+C contents. They are free-living, self-replicating microorganisms[1, 2] and are often found as extracellular parasites attached to the external surfaces of host cells but some species reside and replicate inside eukaryotic cells.[3] There are more than 100 species of mycoplasmas, which are further subdivided into several strains, some of which could cause or more likely serve as cofactors or opportunistic infections in various chronic illnesses.[4]

Although mycoplasmas can exist on mucosal surfaces in the genitourinary tract, oral cavity and gut as commensal flora in humans, when certain mycoplasmas penetrate into the blood, organs and tissues they can cause acute and chronic signs and symptoms. Recent studies have shown that certain species of mycoplasmas, such as *M. fermentans* [5, 6, 7], *M. pneumoniae* [8, 9] or *M. hominis* [10, 11, 12, 13], may cause systemic infections and have been associated with acute and chronic illnesses including respiratory infections, rheumatoid arthritis, HIV-AIDS or non-gonococcal urethritis. Some species, such as *M. penetrans*, *M. fermentans* and *M. pirum* among others, can enter tissues and cells, resulting in complex systemic infections. Mycoplasmas have also been shown to share a complex relationship with the immune system, including B- and T-cell function.[3, 14]

Chronic fatigue is reported by 20% of all patients seeking medical care.[15, 16] It is associated with many well-known medical conditions[17] and may be an important secondary condition in several chronic illnesses. Although chronic fatigue is associated with many illnesses, Chronic Fatigue Syndrome (CFS) or myalgic encephalomyelitis (ME) and Fibromyalgia Syndrome (FMS) are distinguishable as separate syndromes based on established clinical criteria.[18, 19, 20] However, their clinical signs and symptoms strongly overlap. CFS/ME is characterized by medically unexplained persistent long-term disabling fatigue plus additional signs and symptoms, whereas patients with FMS suffer primarily from muscle pain, tenderness and soreness. In patients with either diagnoses other conditions that can explain their signs and symptoms are absent; thus in many patients a clear distinction between CFS/ME and FMS diagnoses is difficult. CFS/ME and FMS have been associated with immunological abnormalities and infectious illnesses, but investigations of infectious triggers have not revealed unifying results.

We have begun to examine patients with chronic illnesses for the presence of mycoplasmas in their blood. For example, our studies on Gulf War Illness (GWI) showed that 45% of gulf war veterans with chronic signs and symptoms similar to CFS/ME or FMS were positive for *M. fermentans* infections in their blood leukocytes.[21, 22, 23] Recently, using species-specific PCR we also demonstrated that about 50% of patients suffering from Rheumatoid Arthritis were positive for mycoplasmas in their blood.[24] Using synovial fluid specimens other studies showed similar findings in these patients.[25, 26]

In the present study we included 565 patients with a primary diagnosis of CFS/ME and/or FMS and 71 healthy controls, and using polymerase chain reaction (PCR) we investigated blood samples from these patients for the presence of all species of mycoplasmas and of *M. fermentans*. Results were confirmed by ^{32}P-labeled internal probe hybridization of PCR products.

Materials and Methods

Patients

From September 1996 to July 1999, blood samples from 565 adult patients (401 female, 164 male) diagnosed as CFS/ME and/or FMS were investigated. The female patients ranged in age from 18-91 years (mean 42±18 years), whereas male patients ranged from 18-69 years (mean 40±14 years).

Clinical diagnoses were obtained from referring physicians according to the latest case definition. The following criteria were used for patient's classification: (**a**) unexplained relapsing or persistent fatigue of new or definite onset which is not caused by ongoing exertion, not relieved by rest and that results in a substantial reduction of activity compared to levels prior to onset; (**b**) four or more of the following signs and symptoms persist for at least 6 month: (1) impaired memory or concentrations were enough to reduce levels or occupational, social, or personal activities; (2) sore throat; (3) tender cervical or axillary lymph nodes; (4) muscle pain; (5) multiple pain without swelling or redness; (6) new headaches; (7) unfreshened sleep; (8) post-exertion malaise lasting more than 24 h; (**c**) 11/18 site-specific tender points and body pain above and below the waist. Patients were selected based on a routine physical examination, their case history, and clinical signs and symptoms. Patients with substance abuse were excluded from the study. Other diagnostic tests were used to exclude other diagnoses that could explain patient's signs and symptoms.[19, 20] In most patients the signs and symptoms of CFS/ME and FMS were overlapping often resulting in both diagnoses; therefore, all patients were considered together. Patients were not treated with any antibiotics for at least two months before the tests were performed. The mean duration of illness was 145±140 months with a minimum of 12 months.

Voluntary healthy controls (n=71) were selected from comparable geographical areas without the clinical signs and symptoms described above. They were chosen after a routine clinical examination. Age (43±11 years) and gender (52 female, 19 male) of control subjects were comparable to patients' group. Their blood samples were taken freshly under the same conditions as patients' blood as described below. Control samples were run together with patient specimens. Mycoplasma tests were performed on all specimens in a blinded matter.

Illness Survey Forms were analyzed for the most common signs and symptoms at the time when the blood was drawn. Patients marked the intensity of signs and symptoms prior to and after onset of illness on a 10-point self-rating rank scale (0: none; 10: extreme). The data from 115 questions were arranged into 39 different categories and were considered positive if the average value in each category after onset of illness was

three or more points higher than prior to the illness. The most frequent signs and symptoms are shown in Table 1.

Specimens

Blood (10 cc) was collected in citrate-containing tubes under aseptic conditions, immediately cooled to ice temperature, shipped to our laboratory on ice using over night air courier and processed immediately for PCR. The following preparation of DNA from blood samples was performed under aseptic conditions as described previously.[24] Briefly, whole blood (50 μl) was used for preparation of DNA and 1.3 ml of nanopure water was added. After incubation with 200 μl of Chelex solution (Biorad) the samples were heated at 56°C for 15 min, vortexed for 10 sec and incubated at 100°C for 15 min. Aliquots from the supernatants were used immediately for PCR or stored at -70°C until use.

Amplification

Genus specific primers for mycoplasma were selected from 16S mRNA sequences. The universal probes GPO-1 and MGSO were used for the detection of mycoplasmas and the UNI- probe was used as an internal probe for hybridization confirmation of the PCR product.[27] Specific primers for *M. fermentans* (SB1: forward probe, SB2: reverse probe, SB3: internal probe) were selected from the *tuf* gene.[28] (Table 2) Amplification of the target sequence of 717 bp size (850 bp for *M. fermentans*) 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 μl each of dATP, dTTP, dGTP, dCTP, 100 pmol of each primer, and 0.5-1 μg of chromosomal DNA. *Mycoplasma fermentans* DNA (0.5-1 ng of DNA) was used as positive control for amplification. The amplification was carried out in a thermocycler (Perkin Elmer GeneAmp PCR System Model 9600). The reaction mixture was heated to 94°C for 5 min. Subsequently, 1.5 units of Taq DNA polymerase were added, and amplification consisted of 40 cycles of 60 sec denaturing at 94°C, 90 sec annealing at 60°C, and 90 sec extension at 72°C. Finally, product extension was allowed for 10 min at 72°C. Strict protocols were established to prevent contamination, including isolation of PCR reagent preparation, PCR product detection, amplification sites, aliquoting of reagents, and autoclaving and irradiation of reagents when possible. All glassware and pipette tips were decontaminated and positive displacement pipettes were used. Negative and positive controls were used in each experimental run.[29, 30]

Southern Blot Hybridization

Southern blot hybridization was performed as described.[24] Briefly, the amplified samples were run on a 1% Agarose gel containing 5 μl/100 ml of ethidium bromide in TAE buffer (pH 8.0). The band was visualized with a UV source, and a Polaroid picture was taken. For Southern blot hybridization the gel was denatured in 0.5 M NaOH for 30 min and neutralized in 1 M Tris-HCl (pH 8) for 30 min. DNA was transferred from the gel to a Nytran membrane (Schleicher&Schuell) using 10x SSC buffer. The membranes were washed and prehybridized for 24 h at 50°C with hybridization buffer consisting of 6x SSC, 0.2% SDS, 1x Denhardt's blocking solution and 1 mg/mL salmon sperm DNA.

Hybridization was performed with ^{32}P-labeled (T4-kinase method) UNI- or SB3 probe (10^7 cpm per bag) in 10 ml hybridization buffer (5 x SSC, 0.2% SDS, 1x Denhardt's blocking solution, 1 mg/ml salmon sperm) for 48 h at 50°C. After hybridization, the membranes were washed and then exposed to autoradiography film for 7 days at -70°C.

Statistical evaluation

Results between patients and healthy controls were compared using the StatMost program (DataMost Inc.). P-values were calculated using Students *t*-test and significant differences were accepted for $p<0.05$.

Results

Evaluation of results and test sensitivities

Using the genus-specific primers positive results were obtained if the PCR product was 717 base pair in size for the mycoplasma genus primer or 850 bp in size for *M. fermentans*-specific primers along with a negative and a positive control of the same size in the same gel. The results were confirmed by finding a visible band upon autoradiography after hybridization with the specific internal ^{32}P-labeled probe. In some cases where visible bands were not easily seen in the gel after ethidium bromide staining, a visible hybridization band of the appropriate size was found equal or more in intensity to hybridization product of 10 fg of positive control, and the result was then reported as positive. In the healthy control group (n=71) 7 samples (9.9%) were positive for *Mycoplasma spp.*, and 2 samples (2.8%) were positive for *M. fermentans*. Distilled water and buffers were also used as negative controls, and these showed no amplification product or hybridization signal.

In preliminary studies, the sensitivity and specificity of the PCR method were determined by examining serial dilutions of purified DNA from of *M. fermentans*, *M. pneumoniae, M. penetrans, M. hominis* and *M. genitalium*. The primers GPO 1 and MGSO produced the expected amplification product size in all tested species, which was confirmed by hybridization using the internal ^{32}P-labeled UNI- probe. Amounts as low as 1-10 fg of purified DNA were detectable for all species with the universal primers. (Figure 1) Only *M. fermentans* was detected with the *M. fermentans*-specific primers.

Preparation of DNA from blood samples

Fresh blood and immediate DNA preparation resulted in better results than blood that was processed after incubating for various periods of time at room temperature (Data not shown). Six positive blood samples were divided into 7 aliquots each and stored at room temperature for different time intervals (processed immediately or after 1, 2, 3, 4, 5 or 7 days). Over time the PCR signal decreased. In all samples that showed positive results in fresh DNA preparations, the PCR signal became weak after 2 days. After 4 days negative results were obtained in 4 cases, whereas the other two samples showed very weak bands. No specific PCR product was detectable after one week. Taken together sample

collection and immediate processing of blood specimen were of high relevance for reliable results.

Mycoplasma in blood samples

Mycoplasma tests were performed on patients as described above using Chelex purified blood preparations. The *Mycoplasma spp.* sequence was amplified from DNA extracted from the peripheral blood of 300/565 (53.1 %) patients. A specific PCR product could not be detected in the 265 negative patients (46.9 %). Southern blot results are shown in Figure 2. In 71 healthy controls without any clinical signs and symptoms positive results were shown in 7 cases for *Mycoplasma spp.* (Table 3). The difference between patients and control group was significant ($p<0.001$) and there was no significant difference between positive male and female patients (*Mycoplasma spp*., 55.3% vs. 52.3%).

The test for *M. fermentans* using the primer set SB1 and SB2 showed specific PCR products with the expected size, and confirmation with Southern hybridization was seen in 24.6 % of CFS/FMS patients. The difference between patients and healthy controls was significant ($p<0.001$). Two of the controls were positive for *M. fermentans* infections (2.8%). Although *M. fermentans* was more frequently detected in men than in women (32.6 % vs. 21.5 % positive), this difference was also not significant.

Discussion

Over the last decade mycoplasmas have been found at higher prevalence in blood and tissue specimens obtained from patients with various chronic illnesses compared to healthy controls.[31] Systemic mycoplasmal infections can cause chronic fatigue, muscle pain and many other signs and symptoms.[4] Since little is known about the possible involvement of mycoplasmas in the pathogenesis of chronic illnesses, it remains uncertain whether these findings indicate that some mycoplasmas are causal agents, cofactors, or opportunistic or superinfections.[4] Some mycoplasmas can invade virtually every human tissue and can suppress immune responses. For example, clinical and experimental studies have shown that mycoplasmas can activate or suppress B- and T-cell functions.[32] This may permit additional opportunistic infections by bacteria, viruses, and fungi. Mycoplasmas can also rapidly adapt to host microenvironments which is usually accompanied by rapid changes in cell surface adhesion receptors for more successful cell binding and entry as well as rapid changes in their antigenic structures to mimic host antigenic structures (antigen mimicry).[33, 34]

Several authors have described mycoplasma detection by PCR using the universal primers GPO1 and MGSO.[27, 35] Dussurget and Roulland-Dussoix[35] showed that these primers can cross-react with bacteria phylogenetically closely related to *Mollicutes* (*E. faecalis, C. innocuum*, *B. subtilis*) and some other bacteria and yeast species if less stringent conditions were used. However, the sensitivity of mycoplasma detection by the described method was assessed by the detection of control mycoplasma DNA and by internal hybridization using mycoplasma-specific probes. In addition, contamination during sample preparation is an important issue that needs to be considered. We used several procedures to confirm the specificity of our results. Samples obtained from

patients and healthy controls were possessed simultaneously, and positive and negative controls were used with each sample preparation. Using the described technique, blinded blood samples were investigated in a recent study sponsored by the U.S. Department of Defense. These samples contained live organisms from mycoplasmal cultures seeded in control, negative blood samples for independent tests run by four different laboratories. The results were the same in all laboratories (unpublished results). Using serial dilutions of mycoplasma DNA, the method was able to detect as low as 1-10 fg of DNA. Thus with the use of specific Southern hybridization this procedure can result in specific test results of high sensitivity. Some DNA bound to protein, however, might be precipitated during protein removal and not available for detection.[36]

Before systemic mycoplasmal infections can be considered important in causing morbidity of CFS/ME and FMS patients, certain criteria must be fulfilled.[27] The prevalence rate among diseased patients must be higher than in those without disease. *M. fermentans* was found at significantly higher prevalence in this study and by others.[37] Although this mycoplasma species has also been found in asymptomatic adults with comparable demographic characteristics, the prevalence in healthy controls is low (9-15%) compared to about 50% of CFS/ME/FMS patients. In addition, in a preliminary study on 91 patients with CFS/ME/FMS we found that *M. pneumoniae* and to a much lesser extent *M. hominis* and *M. penetrans* can be detected in their blood samples[38] which was confirmed by Voidjani et al.[37] In this study we also reported that patients positive for multiple mycoplasmal species showed a tendency of longer illness history and higher score values for the severity of signs and symptoms.[38]

Mycoplasmas are not easily detectable but can be identified by Nucleoprotein Gene Tracking[36] or forensic PCR. In previous studies using the Gene Tracking method we found mycoplasmal infections in about 45% of GWI patients with CFS/ME signs and symptoms.[21, 22] However, the detection of mycoplasmal DNA in these studies by various methods requires further confirmation by other techniques. Although accompanied by low sensitivity isolation of mycoplasmas in culture from CFS/ME and FMS patients should result in higher recovery of mycoplasmas from diseased patients than in those without these illnesses. An antibody response has been found but usually not until the disease has progressed. According to Lo et al.[39, 40] *M. fermentans* does not elicit a strong immune response in animal models until near death. In addition, although *M. fermentans* have been found in specimens from up to 50% of HIV-positive patients,[41] specific antibodies against this species were not found.[42] However, these data were obtained in small groups of patients. A clinical response should be accompanied by elimination of the mycoplasma. In our recent study on 87 mycoplasma-positive patients with GWI 69 patients recovered on up to 6 six-week cycles of antibiotic treatment and became negative for mycoplasmal infections. Only antibiotics that are effective against the pathogenic mycoplasmas resulted in recovery of GWI patients with mycoplasmal infections, and some antibiotics, such as penicillins, worsened the condition.[21, 22] Various animals have been used for the in-vivo investigation of mycoplasmal infections. For example, the injection of *M. fermentans* into monkeys resulted in development of fulminant disease that led to death. These animals displayed many of the chronic signs and symptoms found in CFS/ME patients.[40] *M. fermentans* also suppressed normal inflammatory or immune responses and caused a fatal systemic infection in these monkeys.[40]

Over the last years many studies have shown that microorganisms of otherwise unimpressive virulence, such as Mycoplasmas or Chlamydia, seem to be involved in slow progression of chronic diseases with a wide spectrum of clinical manifestations and disease outcomes.[43, 44] Our results suggest that mycoplasmas, such as *M. fermentans*, appear to be related to CFS/ME and FMS. However, it remains unclear whether they are causative, cofactors or opportunistic infections in these diseases. One may speculate that the ability of *M. fermentans* to invade into different types of human cells and disturb immune functions is responsible for its involvement in pathogenesis of different chronic illnesses. The identification of mycoplasmal infections in blood specimens of a rather large subset of rheumatoid arthritis, GWI, CFS/ME and FMS patients suggests that mycoplasmas, and probably other chronic infections as well, may be an important source of morbidity in these patients. If such infections are important in these disorders, then appropriate treatment with antibiotics may result in improvement and even recovery.[45] However, further investigations are necessary to establish the role of mycoplasmal infections in CFS/ME and FMS patients and their pathomechanisms.

References

Table 1:

Major signs and symptoms of CFS/ME and FMS patients. Severity of 114 signs and symptoms was assessed using a Patient Illness Survey Form. Their intensities were marked by patients on a 10-point scale (0: none; 10: extreme) prior to and after onset of illness. Average changes in scores were determined in each category (3-9 questions) and considered positive if the average value after onset of illness was three or more points higher than prior to the illness.

Signs/Symptoms	Percentage of patients with signs/symptoms
chronic fatigue	93
depression	91
paraesthesia	86
joint pain, reduced mobility	84
vision problems, light sensitivity	84
cognitive problems	84
muscle spasms, burning muscles	81
dizziness, balance disturbance	79
stuttering, difficulty speaking	78
breathing problems	76
flatulence, bloating	76
headache	74
lack of bladder control; frequent urination	71
chemical sensitivities	71
stomach cramps or pain	71
sinus pain, nasal congestion	69
changed alimentation	67
loss of libido, impotence	66
sore throat	62
skin rashes	62
nausea, vomiting, regurgitation	62
tinitus, hearing loss	62
cardiac problems	59
coughing frequently, frequent thick saliva clearing	59
night sweats	57
diarrhea	57
chest pain or pressure	57
frequent infections	55
frequent sores, yeast infection	55
allergies	53
dry or itchy eyes	52
menstrual or genital pain	50

Table 2:
Sequences of the primers used for PCR amplification and Southern hybridization

Primer	**Sequence** (5' - 3')	**Location**	**Gene**
GPO-1	ACTCCTACGGGAGGCAGCAGTA	338-359	16S rRNA
MGSO	TGCACCATCTGTCACTCTGTTAACCTC	1029-1055	16S rRNA
UNI-	TAATCCTGTTTGCTCCCCAC	763-782	16S rRNA
SB1	CAGTATTATCAAAGAAGGGTCTT	101-123	*tuf*
SB2	TCTTTGGTTAATACGTAAATTGCT	930-953	*tuf*
SB3	TTTTTCAGTTTCGTATTCGATG	201-222	*tuf*

Table 3:
Results of genius- and species-specific PCR: Percentage of positive detection

	n	Age (mean±SD)	*Mycoplasma spp.*	*M. fermentans*
CFS/ME/ FMS	565	42±15	53.1 %	24.6 %
Controls	71	43±11	9.9 %	2.8 %

Legends:

Figure 1

Result of PCR amplification for serial dilution of *M. fermentans* using mycoplasma-genius-specific primers GPO1/MGSO hybridized with ^{32}P-labeled UNI-probe. Lane 1: negative control; lane 2: 10 fg; lane 3: 100 fg; lane 4: 1 pg; lane 5: 10 pg; lane 6: 100 pg; lane 7: 1 ng; UV visualization of ethidium bromide stain in agarose electrophoresis gel (upper panel); hybridization with ^{32}P-labeled internal probe after Southern blot (lower panel).

Figure 2

PCR products from patient's blood sample using GPO1/MGSO primers hybridized with ^{32}P-labeled UNI-probe. Lanes 1+2: negative control in duplicate; lanes 3+4: positive patient in duplicate; lane 5: positive control (1fg *M. fermentans*); UV visualization of ethidium bromide stain in agarose electrophoresis gel (upper panel); hybridization with ^{32}P-labeled internal probe after Southern blot hybridization (lower panel).

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