High Prevalence of *Mycoplasma* infections among European Chronic Fatigue Syndrome patients

Examination of four *Mycoplasma* species in blood of Chronic Fatigue Syndrome patients

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Abstract

Prevalence of *Mycoplasma* species infections in Chronic Fatigue Syndrome (CFS) has been extensively reported in the scientific literature. However, all previous reports highlighted the presence of Mycoplasmas in American patients. In this prospective study, the presence of *Mycoplasma fermentans, Myc. penetrans, Myc. pneumoniae* and *Myc. hominis* in the blood of 261 European CFS patients and 36 healthy volunteers were examined using forensic polymerase chain reaction. One hundred and seventy-nine (68.6 %) patients were infected by at least one species of *Mycoplasma*, compared to 2 out of 36 (5.6 %) in the control sample ($P < .001$). Among *Mycoplasma*-infected patients, *Myc. hominis* was the most frequently observed infection (N = 96; 36.8 % of the overall sample), followed by *Myc. pneumoniae*- and *Myc. fermentans*-infections (equal frequencies; N = 67; 25.7 %). *Myc. penetrans* infections were not found. Multiple mycoplasmal infections were detected in 45 patients (17.2 %). Compared to American CFS patients (*Myc. pneumoniae > Myc. hominis > Myc. penetrans*), a slightly different pattern of mycoplasmal infections was found in European CFS patients (*Myc. hominis > Myc. pneumoniae, Myc. fermentans >> Myc. penetrans*).

Key words: Mycoplasma infection, prevalence, Chronic Fatigue Syndrome, forensic PCR

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Introduction

In addition to many other signs and symptoms, Chronic Fatigue Syndrome (CFS) is characterised by persistent or relapsing fatigue lasting six or more consecutive months [1,2]. The 1988 [1] and 1994 [2] Centre for Disease Control -criteria for diagnosis of CFS yielded a heterogeneous patient-group. No single underlying cause has been established for all CFS-patients, possibly due to the heterogeneity of this patient group. There is a growing international consensus to differentiate CFS into clinically relevant subcategories that may represent different disease states or potential co-morbid illnesses. One or more of these subgroups may include patients with chronic systemic infections that could potentially cause their illness, be a co-factor for the illness or cause an opportunistic infection that exacerbates the illness [3]. Therefore, identifying infections like *Chlamydia pneumoniae* [4] and *Mycoplasma spp* [5-10] in these patients may be of prime importance.

Mycoplasmal infections have been found at high incidence in American CFS patients [5-10]. These microorganisms are prokaryotes that lack a cell wall and certain cellular organelles. They contain circular

DNA and some ribosomes [11]. Mycoplasmas have been assigned taxonomically to the class of Mollicutes, and to the order Mycoplasmatales. The organisms capable of colonizing and infecting humans are situated in the family of Mycoplasmataceae [12]. Among healthy individuals, some species of *Mycoplasma* are part of the normal human flora in the mucosal surface of the oral cavity, stomach or urogenital tract. When certain species of *Mycoplasma* penetrate into tissues, blood and organs, they are capable of inducing complex systemic infections. Mycoplasmas are thought to contribute to patients' morbidity in rheumatoid arthritis [13], systemic lupus erythematosus [14], demyelinating and axonal neuropathies [15], and HIV-AIDS [12]. In contrast, systemic mycoplasmal infections are rarely seen in osteoarthritis [13] and acute respiratory infections [16-17].

The prevalence as well as clinical importance of mycoplasmal infections in CFS and related disorders (Gulf War Illness, Fibromyalgia Syndrome) has been extensively reported in the scientific literature [5-10]. These reports augment other evidence suggesting an organic basis of CFS. In this regard, mycoplasmas might function as important cofactors, or even as primary or secondary etiologic agents. However, all previous reports highlighted the presence of various *Mycoplasma* species in American CFS-patients [3, 5-10]. Epidemic peaks of certain infections, for example *Myc. pneumoniae* infections, [18] could prevent extrapolation of these results outside of the United States. We hypothesised that similar to North American CFS-patients European CFS patients have increased prevalence of systemic mycoplasmal infections compared to healthy volunteers. Therefore, the presence of various *Mycoplasma* species in the blood of 261 CFS-patients and 36 healthy volunteers were studied using forensic polymerase chain reaction (PCR) [7-9].

Materials and Methods

Patient recruitment

This prospective, comparative epidemiological study was conducted in Brussels, at a university-based outpatient clinic (Vrije Universiteit Brussel - VUB). Between the first of January and the end of June 1999, two hundred and sixty-one consecutive patients seeking care for prolonged fatigue as their major complaint were enrolled. Thirty-six age-matched healthy volunteers were recruited among college students, health care professionals and hospital employees and used as control subjects.

To fulfil the current Centre for Disease Control criteria for CFS, clinically evaluated, unexplained, persistent or relapsing chronic fatigue that is of new or definite onset, should result in a substantial reduction in previous levels of occupational, educational, social, or personal activities [2]. Additionally, at least four of the following symptoms must have persisted or recurred during 6 or more consecutive months and must have not predated the fatigue: impairment in short-term memory or concentration, tender cervical or axillary lymph nodes, muscle pain, multijoint pain, headache, unrefreshing sleep and postexertional malaise > 24 hours [2]. Any active medical condition that may explain the presence of chronic fatigue prohibits the diagnosis of CFS. Therefore, all subjects underwent an extensive medical evaluation, consisting of a standard physical examination, medical history, exercise capacity test and routine laboratory tests. The laboratory tests included a complete blood cell count, determination of the erythrocyte sedimentation rate, serum electrolyte panel, measures of renal, hepatic and thyroid function, as well as rheumatological and viral screens. When judged necessary, a structured psychiatric interview was performed. In a number of cases further neurological, gynaecological, endocrine, cardiac and / or gastrointestinal evaluations were performed. The 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 [2] exclusionary criteria, the patients were not included in the study. All subjects completed a questionnaire, which included demographic information, dates of illness onset and current health status. Additionally, all subjects were questioned about medication-use during the three months prior to the study. Patients and control subjects had to be free of antibiotic-treatment for two months prior to blood collection. Controls had to be free of disease at least three months prior to data collection. Subjects were excluded if they were < 18 or ≥ 65 years of age. All patients and controls were Caucasian. Demographic features of the data sample are shown in Table 1.

Collection of Blood

Subjects' blood was collected between 9.00 and 11.00 A.M. at the Fatigue Clinic, Academic Hospital, Vrije Universiteit Brussel. Blood was collected in EDTA-containing tubes and immediately brought to ice bath temperature and flash frozen as described previously [7,8]. Samples were coded and shipped with dry 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 minutes, 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 stored at –70°C until use. Multiple Mycoplasma tests were performed on all patients.

Amplification of Gene Sequences

Amplification of the target gene sequences (Table in Nasralla et al [8]) was performed in a total volume of 50 ul 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 (0.1 – 1 ng of DNA) was used as a positive control for amplification. The amplification was carried out for 40 cycles with denaturing at 94°C and annealing at 60°C (genus-specific primers and *Myc. penetrans*) or 55°C (*Myc. pneumoniae, Myc. hominis, Myc. 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 experimental run.

Southern Blot Confirmation

The amplified samples were run on a 1% agarose gel containing 5 ml / 100ml 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 hybridisation buffer consisting of 1x Denhardt's solution and 1 mg/ml salmon sperm DNA as blocking reagent. Membranes were then hybridized with ^{32}P-labeled internal probe (10^7 cpm per bag). After hybrization and washing to remove unbounded probe, the membranes were exposed to autoradiography film for 1- 2 days at –70°C.

Statistics

Subjects' demographic characteristics were assessed using descriptive statistics and students' t-tests (independent 2 samples t-test, 2-tailed). The significance level of the test was at 0.05. Pearson Chi-Square test was performed to compare prevalence data between patients and control subjects. Additionally, sex differences were investigated using Pearson Chi-Square test. Prevalence data were only analysed in a binary way (*Mycoplasma*-infected or no *Mycoplasma* infection), because subgroups lacked power. The data were processed using SPSS 7.5 © for Windows (Prentice Hall).

Results

Patients and controls were matched according to age characteristics (controls: mean age = 34; CFS-patients: mean age = 36; $P > 0.05$), but differed significantly according to sex distribution ($P < 0.05$). Thirteen of thirty-six (36 %) healthy volunteers were male, while 218 of 261 (84 %) patients were female. Demographic features of the sample are presented in Table 2.

All patients fulfilled current international Centre for Disease Control case definition for Chronic Fatigue Syndrome [2]. In 82 out of 261 Belgian CFS patients (31.4 %), mycoplasmal species infections could not be detected. One hundred and seventy-nine (68.6 %) patients were infected by at least one species of *Mycoplasma*. Two healthy volunteers (5.5 %) presented with a single mycoplasmal infection. Prevalence of

mycoplasmal infection differed significantly in CFS patients compared to healthy controls ($P < 0.001$) (Table 2).

Among *Mycoplasma*-infected European patients, *Myc. hominis* was observed most frequently (N = 96; 36.8 % of overall sample), followed by *Myc. pneumoniae* and *Myc. fermentans* (equal frequencies; N = 67; 25.7 %). *Myc. penetrans* was not detected in this patient group. Multiple infections were detected in 45 patients (17.2 %). With the absence of *Myc. penetrans* in mind, all possible combinations were present and equally distributed. These data are presented in Table 3 and Figure 1.

Among the CFS patients, no significant age differences between females and males were found (Table 1). Table V presents the sex differences of the prevalence of mycoplasmal infections among European CFS patients. No significantly different prevalence data were obtained between females and males.

Discussion

This sample of European CFS patients presented with increased prevalence of systemic mycoplasmal infections, compared to healthy volunteers. However, in contrast to American CFS patients, a slightly different pattern of mycoplasmal infection was observed. Only two earlier reports highlighted the presence of more than one different Mycoplasma species in the blood of CFS-patients [5,8]. Others were only interested in the presence of *Mycoplasma spp.* and *Myc. fermentans* infections [5-10]. Vojdani and co-authors identified *Myc. fermentans* as the most common type of *Mycoplasma* species in CFS-patients [5]. *Myc. hominis* and *Myc. penetrans* were rare (9 and 6 % of infected patients), whereas *Myc. pneumoniae*-detection was not performed. Nasralla and co-authors [8] reported *Mycoplasma pneumoniae* as the most common mycoplasmal infection in 54 of 91 *Mycoplasma*-positive patients and *Myc. fermentans* infections occurred in 44 of 91 patients. However, their subjects consisted out of a mixture of CFS and Fibromyalgia Syndrome patients, although most patients had both diagnoses. In European CFS patients, *Myc. hominis*-infections are more common compared to *Myc. pneumoniae* and *Myc. fermentans*. Even more striking was the absence of *Myc. penetrans* in our patient group. The observed pattern of multiple mycoplasmal infections, being *Myc. fermentans*- or *Myc. pneumoniae*-dependent, is in accordance with an earlier report [8].

Explanations for the observed difference between the prevalence of individual *Mycoplasma* species in the United States and Europe may depend on patient characteristics. Different inclusion criteria used (a combination of Fibromyalgia Syndrome and CFS-patients nor patients with both diagnoses in some reports) [7] and the existence of epidemic peaks [18] have been mentioned. In addition, differences in social structures might lead to different samples. Indeed, most European citizens (in particular Belgians) are all resigned to a uniform health system. However, the most obvious explanation is that European and American patients may be exposed to different infections based on their geographic separation. The prevalence of mycoplasmal infections among asymptomatic individuals varied in previous studies between 5 % and 15 % (reviewed in [9]). The small sample size might explain the extremely low prevalence of mycoplasmal infections observed in the healthy volunteers (2 of 36 (5.5 %) subjects infected).

Together with the reported prevalence of *Mycoplasma* infections, the dysregulation of the 2-5A-dependent RNase L-pathway [19-23] provides evidence for a biomedical pathogenesis in subsets of these patients. For example, there might be a co-morbid physiopathological mechanism between *Mycoplasma* species and the deregulation of the 2.5A synthetase / RNase L antiviral pathway in CFS. Indeed, *Mycoplasmas* are active in stimulating several components of the immune system. They can act as polyclonal T-cell and B-cell activators [24], and they can produce components that activate macrophages [25,26]. For example, *Myc. fermentans* produces a 2-kDa macrophage-activating lipopeptide that has been demonstrated to induce bone resorption [26]. Decreased bone mineral density has been suggested in Fibromyalgia Syndrome [27]. To bring about their phagocytic activity, monocytes produce the proteolytic enzyme elastase, which enables them to pass through connective tissues. Elastase is capable of cleaving 80 kDa RNase L, thus causing deregulation of the antiviral pathway. In addition, low molecular weight RNase L is capable of reducing T_H1 activity, automatically increasing susceptibility to infections. McGregor and colleagues (2000) have already stated that these interesting interactions deserve further investigation [28]. We are currently examining this in CFS patients.

We must be careful in extrapolating these results to the entire CFS patient population. First, this sample was not randomly selected. The aim of randomisation is to prevent selection-bias. All CFS patients visiting the Chronic Fatigue Clinic between January and June 1999 were included. Consequently, selection-bias can only be due to appointment-allocation or the unblinded nature of patients' screening. Our secretaries however, were blinded to patients' medical records when they allocated the appointments. Second, most of the patients were tertiary referrals (general physicians / rheumatologists / internists), resulting in selection of highly disabled patients. This might explain the high prevalence observed in this patient sample. Third, patients and controls differed significantly with respect to sex distribution. However, no major differences of *Mycoplasma*-prevalence were found between female and male patients in this and a similar study [8], suggesting sex characteristics have no prognostic value. Fourth, the validity of PCR techniques for *Mycoplasma* detection has been questioned. *Mycoplasma* detection based on antibody tests is characterised by a very high specificity, but its extremely low sensitivity renders it useless as a diagnostic tool. The *Mycoplasma* detection by PCR was performed in a College of American Pathologists certified laboratory, consequently they were obliged to perform an extensive test validation process. The sensitivity and specificity of the PCR method for *Mycoplasma* detection were determined by examining serial dilutions of purified DNA from *Myc. fermentans, Myc. pneumoniae, Myc. hominis* and *Myc. genitalium*. The primers produced the expected amplification product size in all test species, which was confirmed by hybridisation using the appropriate ^{32}P-labeled internal probe. Amounts as low as a few fg of purified DNA were detectable for all species with the specific internal probes. There was no cross-reactivity between the internal probes of one species and the PCR product from another species. In other experiments *Mycoplasma* species were added to whole blood at various concentrations. Specific PCR products down to 10 ccu / ml of blood could be detected [7,8]. Various sources of blood, including healthy laboratory animals, were tested and they were found to be free of PCR detectable *Mycoplasma*. Tests were always conducted in at least duplicate with complete concordance. Reproducibility of the PCR results has been checked extensively; multiple tests (as many as 10 aliquots from the same sample as well as multiple samples from the same patient) have shown the complete reproducibility of the testing procedures. Control studies with another laboratory found complete concordance with testing results using blinded samples sent by a third laboratory to both of these laboratories. In addition, because *Mycoplasma* detection was performed by blind assessors, these results contribute to the validity of forensic PCR for *Mycoplasma* detection.

To our knowledge, this is the first report concerning epidemiology of *Mycoplasma* species among European CFS patients. More than 2 out of 3 CFS-patients were infected with at least one type of *Mycoplasma*. *Myc. hominis* was the most frequently observed mycoplasmal infection. Multiple mycoplasmal infections were detected in more than seventeen percent of all patients. Finally, a novel mechanism for *Mycoplasma* effects on distinct biochemical pathways in cells may possibly explain the role of *Mycoplasma* species in causing metabolic disturbances in CFS patients.

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Table 1
Demographic data

	N	Mean age (SD)	Range	Number of males	Males (%)	Number of females	Females (%)	Fukuda et al [-]
Patients	261	36.4 (9.8)	[18-64]	43	16.5	218	83.5	272
Controls	36	34.6 (9.1)	[22-54]	13	36.1	23	63.9	0
Female patients	218	36.6 (9.8)	[18-60]	0	0.0	218	100.0	218
Male patients	43	35.3 (10.2)	[18-64]	43	100.0	0	0.0	43

Table 2
Comparison of *Mycoplasma* species detection between patients (N = 261) and healthy volunteers (N = 36)

	Free of mycoplasma (N ± SD)	Free of mycoplasma (%)	*Mycoplasma* infected (N ± SD)	*Mycoplasma* infected (%)	Pearson Chi-Square	Sign (2-sided)
CFS-patients	82 ± 9.1	31.4	179 ± 13.4	68.6	57.798	*P* < .001
Healthy controls	34 ± 5.8	94.4	2 ± 1.4	5.5		

Table 3
Prevalence of mycoplasmal infections among European CFS patients (N = 261)

Subgroup of Mycoplasma-genus	Number of patients (N ± SD)	%
None	82 ± 9.1	31.4
*Myc. fermentans**	67 ± 8.2	25.7
*Myc. hominis**	96 ± 9.8	36.8
*Myc. pneumoniae**	67 ± 8.2	25.7
*Myc. penetrans**	0 ± 0.0	0
Multiple infection	45 ± 6.7	17.2
Single infection	134 ± 11.6	51.3
Myc. fermentans + *Myc. Hominis*	12 ± 3.5	4.6
Myc. fermentans + *Myc. Pneumoniae*	13 ± 3.6	5.0
Myc. pneumoniae + *Myc. hominis*	14 ± 3.7	5.4
Myc. fermentans + *Myc. pneumoniae* + *Myc. Hominis*	6 ± 2.4	2.3

* alone or in combination with another type of Mycoplasma

Table 4

Prevalence of Mycoplasmal infections among European healthy volunteers (N = 36)

Subgroup of *Mycoplasma*-genus	Number of patients (N ± SD)	%
None	34 ± 5.8	94.4
Myc. fermentans	1 ± 1.0	2.8
Myc. hominis	1 ± 1.0	2.8
Myc. pneumoniae	0 ± 0.0	0.0
Myc. penetrans	0 ± 0.0	0.0
Multiple infection	0 ± 0.0	0.0
Single infection	2 ± 1.4	5.6

Table 5

Prevalence of Mycoplasmal infections among European CFS-patients (N = 261): sex differences

Subgroup of Mycoplasma-genus	Number of females (N = 218)	Females (%)	Number of males (N= 43)(%)	Males (%)	Pearson Chi-Square	*P* (2-sided)
None	71	32.6	11	25.6	.814	.367
Mycoplasma pos.	147	67.4	32	74.4	.814	.367
*Myc. fermentans**	61	28.0	6	14.0	†	†
*Myc. hominis**	77	35.3	19	44.2	†	†
*Myc. pneumoniae**	54	24.7	13	30.2	†	†
*Myc. penetrans**	0	0.0	0	0.0	†	†
Multiple infection	40	18.3	5	11.6	2.814	.245
Single infection	107	49.1	27	62.8	2.814	.245
Myc. fermentans + Myc. hominis	11	5.0	1	2.3	†	†
Myc. fermentans + Myc. pneumoniae	13	6.0	0	0.0	†	†
Myc. pneumoniae + Myc. hominis	11	5.0	3	7.0	†	†
Myc. fermentans + Myc. pneumoniae + Myc. hominis	5	2.3	1	2.3	†	†

* alone or in combination with another type of *Mycoplasma*

† not calculated due to lack of sample size

Figure 1: Prevalence of Mycoplasmas among European CFS-patients

100
90
80
70
60
50
40
30
20
10
0

no M spp | M pneumoniae | M hominis | M fermentans | M pn + ho | M fe + pn | M ho + fe | M ho + fe + pn

Type of infection

☐ frequencies ☐ %