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Multiple Mycoplasmal Infections Detected in Blood of Chronic Fatigue Syndrome and Fibromyalgia Syndrome Patients

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Abstract. The aim of the study is to investigate the presence of different mycoplasmal species in blood samples from patients with Chronic Fatigue Syndrome and/or Fibromyalgia Syndrome. We previously found that more than 60% of patients with Chronic Fatigue Syndrome/ Fibromyalgia Syndrome had mycoplasmal blood infections, such as *M. fermentans* . In this study, patients with chronic Fatigue Syndrome/Fibromyalgia syndrome were examined for multiple mycoplasmal infections in their blood. A total of 91 patients diagnosed with Chronic Fatigue Syndrome/ Fibromyalgia Syndrome and with a positive test for any mycoplasmal infection were investigated for the presence of *M. fermentans, M. pneumoniae, M. hominis* and *M. penetrans* infections using forensic polymerase chain reaction. Infections were detected with *M. pneumoniae* (54/91), *M. fermentans* (44/91), *M. hominis* (28/91) and *M. penetrans* (18/91) of mycoplasma-positive patients, respectively. Multiple mycoplasmal infections were found in 48 of 91 patients, with double infections being detected in 30.8% or triple infections in 22%, but only when one of the species was *M. pneumoniae* and/or *M. fermentans*. Patients infected with different *Mycoplasma* spp. generally had a longer history of illness, suggesting that patients may have contracted additional mycoplasmal infections with time.

Introduction

Chronic fatigue is reported by 20% of all patients seeking medical care [1, 2]. Many well-known medical conditions are associated with chronic fatigue [3], and it is often an important secondary condition in many diagnoses. Although chronic fatigue is associated with many illnesses, chronic fatigue syndrome (CFS) and fibromyalgia syndrome (FMS) are distinguishable as separate syndromes based on established clinical criteria [4, 5]. They are characterized by their complex multiorgan chronic signs and symptoms, including muscle pain, chronic fatigue, headaches, memory loss, nausea, gastrointestinal problems, joint pain and vision and breathing problems, among others. Many patients are diagnosed with both syndromes. Since physical and laboratory results do not usually find pathogenic agents or other causes, these conditions are often considered somatoforensic disorders. However, in many cases family members of these patients slowly display similar signs and symptoms, suggesting an infectious explanation for the illnesses [6].

Using forensic polymerase chain reaction (PCR) for detection of *Mycoplasma* spp. and *M. fermentans* in blood samples from 132 CSF/FMS patients, we previously found that 62.9% and 50% were positive for *Mycoplasma* spp. and *M. fermentans* infections, respectively [7]. In healthy controls without clinical signs and symptoms significantly fewer subjects were positive for *Mycoplasma* spp. (9.6%) or *M. fermentans* (0%) infections [7]. We also found that more than 50% of patients with Rheumatoid Arthritis had mycoplasmal infections and in 36% of these patients multiple infections with more than one mycoplasma species were detected [8]. The PCR tests that we used to identify mycoplasmal infections are very sensitive and highly specific. These tests are a dramatic improvement on the relatively insensitive serum antibody tests that are routinely used to assay for systemic mycoplasmal infections [9, 10].

Mycoplasmas are prokaryotes without cell walls of the class *Molecutes*. They are small, free-living, self-replicating organisms [11, 12]. Although mycoplasmas are found commonly in the oral cavity and as symbiotic gut flora, some species can cause acute and chronic illnesses when they penetrate into the blood vascular system and systemically colonize organs and tissues. For example, mycoplasmas, such as *M. penetrans*, *M. fermentans* and *M. pirum*, can enter a variety of tissues and cells and cause systemic signs and symptoms. Mycoplasmas have also been shown to have a complex relationship with the immune system. They are very effective at evading host immune responses, and synergism with other infectious agents has been seen [13].

The difference between the incidence of infection of any species of mycoplasma and *M. fermentans* supports the hypothesis that some patients have infections with mycoplasma species other than *M. fermentans*. In the present study, we extended our examination of mycoplasmal infections to include mycoplasma species other than *M. fermentans.* We found the presence of multiple mycoplasmal infection in many patients suffering from CFS and/or FMS.

Material and Methods

Patients and Specimens. Blood samples from 91 patients (67 female, 24 male) that were positive for mycoplasmal infections by PCR using the mycoplasma genus-specific primers and clinically diagnosed with CFS and/or FMS according to consensus criteria [4, 14, 15] were investigated for multiple mycoplasmal infections. The mean age of all patients was 43±14 years, while the illness history averaged 145±140 months. Voluntary healthy controls (n=32) were selected from comparable geographical areas without the clinical signs and symptoms described for patients. They were chosen after a routine clinical examination. Age (45±12 years) and gender (19 female, 13 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 at the same time. Mycoplasma tests were performed on all specimens in a blinded matter.

Blood was collected in citrate-containing tubes and immediately brought to ice bath temperature as described previously [7, 8]. Samples were shipped refrigerated or on wet ice by over night courier for analysis. Whole blood (50 µl) was used for preparation of DNA using Chelex (Biorad, Hercules, USA) as follows. Blood cells were lysed with nanopure water (1.3 ml) at room temperature for 30 min. After centrifugation at 13000 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 min 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.

Severity Score of Signs and Symptoms. Illness survey forms were given to each patient that analyzed the most common signs and symptoms of chronic illnesses at the time the blood sample was drawn, before and after onset of illness. Patients marked the intensity of 114 signs/symptoms prior to and after onset of illness on a ten-point self-rating rank scale (0: none; 10: extreme). The 114 questions were then grouped into 28 categories containing 3-9 questions each. An average score for each category was calculated as the average change of the intensity of all questions in the category (score = sum of differences between self-rating values prior to and after onset of illness / number of questions in the category). The data from prior to onset of illness and after onset as well as within the last week before the blood was drawn were compared. A significant difference was obtained if the score after onset/within the last week was three or more points higher than prior to the illness [9]. Additionally, the average score change with illness and the duration of illness were correlated with the results of different mycoplasma species. Surveys of 51 patients with negative mycoplasma test results were used to compare score values. Survey data were statistically analyzed using Spearman Rank correlation and Mann-Whitney tests (StatMost32, Dataxiom, USA).

Amplification of Gene Sequences. Amplification of the target gene sequences (Table 1) 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. 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 experimental run [8, 16-18].

Southern Blot Confirmation. 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 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 as blocking reagent. Membranes were then hybridized with ^{32}P-labeled internal probe (10^7 cpm per bag). After hybridization and washing to remove unbounded probe, the membranes were exposed to autoradiography film for 7 days at -70°C.

Results

M. pneumoniae, M. fermentans, M. penetrans and M. hominis Infections. For the determination of any mycoplasmal infections we used primer sets for the rRNA gene (GPO-1 and UNI-) [5]. Although the GPO-1 and UNI-sequences (*Mycoplasma* spp.) are capable of some possible cross-reactions with mycoplasma-related organisms, the conditions used yielded specific products for mycoplasmas as shown by van Kuppeveld et al. [5] and Dussurget et al. [19]. That the patients we examined had mycoplasmal infections was confirmed by species analysis. Using the *M. fermentans*-specific primers SB1 and SB2 from the *tuf* gene we found a single band of 850 bp size that hybridized only with the ^{32}P-labeled internal probe SB3. Similar results were obtained for the other mycoplasma species. To examine the reliability of the method we performed multiple assays (repeated 3-7 times) on 40 samples. All results were completely reproducible. In three cases, the sixth and seventh repeat of an initial positive result produced only a weak but positive signal due to degradation of DNA from repeated freezing and thawing.

The sensitivity of mycoplasma detection by the described method was assessed by the detection of control mycoplasma DNA and by internal Southern blot hybridization using mycoplasma-specific probes. Using serial dilutions of mycoplasma DNA the method was able to detect as low as 10 fg of DNA [7 and unpublished data]. In other experiments, *M. fermentans* was added to control blood samples at various concentrations. We were able to detect specific products down to 10 ccu/ml blood. Thus with the use of specific Southern blot hybridization the PCR procedure can result in specific test results of high sensitivity, down to the presence of a few microorganisms in a clinical sample. In our experience, conventional PCR yields similar results to forensic PCR with extracellular mycoplasma, but not with clinical samples that contain intracellular mycoplasmas. The reason for this is not known, but it could be due to inhibitors present in the clinical samples or to loss of mycoplasma DNA in the conventional extraction procedures due to protein complexing or degradation by cellular nucleases.

Using species-specific primers and forensic PCR the incidence of various mycoplasma species in CFS/FMS patients was examined. *M. pneumoniae* infections were observed in 54/91 patients with CFS/FMS. *M. fermentans* infections occurred in 44/91 patients, whereas *M. hominis* (28/91) and *M. penetrans* (18/91) were found at lower incidence (Figure 1a). In 28 cases, that were positive for the general mycoplasma test (*Mycoplasma* spp.), the four species examined were not found. Previously, some healthy controls without any clinical signs and symptoms were found to be positive (~9%) for mycoplasmal infections (*Mycoplasma* spp.). The difference between patients and control group was significant ($P<0.001$). Similarly, in a previous control study using the technique of nucleoprotein gene tracking we found 4 of 62 (~6%) normal healthy adults were positive for mycoplasmal infections [7, 9]. In the present study all 32 control subjects were negative for *M. fermentans*, *M. pneumoniae*, *M. hominis* and *M. penetrans* infections. Infections with *M. pneumoniae, M penetrans* or *M. hominis* were similar in female and male patients, whereas a significant difference ($P<0.01$) in the incidence of infections with *M. fermentans* was found between females (23/67) and males (12/24).

Multiple Mycoplasmal Infections. Single infections with one of the tested mycoplasmas were observed in 28 of the 91 of patients. The most commonly observed single infection was *M. pneumoniae* (18 of 91 patients). Single infections with the other species were detected in only a few cases (*M. fermentans* in 3 patients, *M. hominis* in 5 patients, *M. penetrans* in 2 patients). Multiple mycoplasmal infections were detected in 48 of the 91 of patients appearing as double infections in 28 patients or triple infections in 20 patients. We have not found patients positive for all four of the tested mycoplasma species. All patients with multiple infections showed combinations of *M. pneumoniae* and/or *M. fermentans* (with or without other species). The combination of *M. hominis* and *M. penetrans* was not seen (Figures 1A-D).

Mycoplasma Test Results and Signs and Symptoms Severity Score. We found that the severity of signs/symptoms was independent of the time of onset of illness, and the average of severity score was similar during the week the blood was drawn compared with the values after the onset of the disease. All categories for signs and symptoms showed an increase in score values (3 or more points) after onset of illness in more than half of the patients. Patients with negative test results for mycoplasmas had an illness history of 153±112 months, whereas patients with mycoplasmal infections had a duration of illness of 165±134 months. Although the difference was not significant ($P=0.11$), patients with three different mycoplasmal species infections

tended to have a longer illness history (211±235 months) than patients with one (126±144 months) or two (150±154 months) mycoplasma species in their blood. Differences in the duration of patients' illnesses between the different mycoplasma species were not found. (Table 2)

To evaluate the influence of specific species or multiple infections on the severity of illness we compared the average increase of the various scores prior to onset of illness with the values after onset of illness and during the week the blood was drawn. The highest increases in score values were found in fatigue/sleep problems, depression, memory loss, balance disturbances, muscle and joint pain or problems, head/neck aches, and night sweats/fevers. Differences between patients with infections with different mycoplasma species were not found. Tendencies for higher increases in the severity of signs and symptoms were found in patients with double infections compared to the other patients (Table 3).

Discussion

CFS and FMS patients suffer from complex overlapping chronic signs and symptoms. In these patients clinical conditions other than fatigue are absent that can explain the signs and symptoms, such as malignancies or autoimmune diseases are absent [4]. In contrast, FMS patients have muscle and overall pain as primary complaints, but they have most if not all of the commonly found signs and symptoms in CFS [15]. The major difference between these illnesses appears to be in the severity of specific signs and symptoms.

A majority of CFS and FMS patients possess systemic mycoplasmal infections that may explain most of their chronic signs and symptoms [6, 7]. Mycoplasmal infections are often associated with night sweats, intermittent fevers, chronic fatigue, skin rashes, increased dermal sensitivity, joint and muscle pain, swelling and reduced mobility of joints, heart palpitations, pain and arrhythmia, stomach cramps and regurgitation, loss of vision, double vision and other problems, depending on the organ or tissue system infected [20]. Although most mycoplasma species were previously considered as relatively benign microorganisms with a low pathogenic potential, recent studies have shown that mycoplasmal infections can cause a variety of illnesses, including a large percentage of pneumonia and asthma at high frequencies [21], and they can lead to fatal illness [22].

In our studies on Gulf War illness patients we found mycoplasmal infections in about one-half of over 200 patients, and these patients were found to have principally *M. fermentans* or *M. pneumoniae* infections [9, 10]. Similar results were recently reported by Choppa et al. [23]. Moreover, in over one-half of the civilians with CFS/FMS we have found a variety of pathogenic mycoplasma species in the leukocyte fractions of blood samples [7]. In the present study, we examined patients with CFS/FMS for the presence of multiple systemic mycoplasmal infections. CFS/FMS patients were tested for the cell-penetrating species *M. fermentans* and *M. penetrans,* as well as for *M. pneumoniae* and *M. hominis*. We found that the majority of the patients have multiple infections, with two or more mycoplasma species predominating. We detected mainly combinations of *M. fermentans* and *M. pneumoniae* infections in these patients.

The tests that we used to identify mycoplasmal infections, based on PCR, are very sensitive and highly specific [10]. 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. Using serial dilutions of mycoplasma DNA, the method was able to detect as low as 10 fg of DNA by specific Southern blot hybridization. The improved handling of blood samples, including DNA preparation, allowed specific detection of mycoplasmal infections in human blood with high sensitivity and reliability. Previously we found that blood samples deteriorate if stored or shipped at room temperature [7]. Thus, the rapid processing of blood samples is particularly important in obtaining reliable data.

Although we used genus primers to determine whether patients had mycoplasmal infections, the UNI- and GPO-1 primers (*Mycoplasma* spp.) are not totally genus-specific. To overcome the problems in specificity we confirmed the results for the *Mycoplasma* spp. assay with highly species-specific assays. We were able to identify at least one mycoplasma species in 73 of 91 patients where the general test was positive. In the remaining 28 patients it is likely that other mycoplasma species were responsible for the positive amplification signal, such as *M. arthritidis* or *M. pirum*, but cross-reactions with other closely related microorganisms are possible. The specificity of the general test cannot completely rule out such cross-reactivity. Future studies will examine additional mycoplasmal species using highly species-specific PCR primers. 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).

Infections with different mycoplasma species and possibly other infections may explain, in part, the complex signs and symptoms found in CFS/FMS patients [6]. In a previous study on Gulf War illness patients with mycoplasmal infections long-term antibiotic treatment using doxycycline, ciprofloxacin, azithromycin or clarithromycin led to significant decreases in the severity of signs and symptoms in about 70% of patients [7, 9, 10]. Multiple cycles were necessary, probably because of the intracellular locations of mycoplasmas like *M. fermentans* and *M. penetrans*, their inherent insensitivity to antibiotics and the slow-growing nature of these microorganisms [10]. After recovery, these patients were no longer positive for mycoplasmal blood infections [9, 10].

Although illnesses such as CFS and FMS may not be initially caused or triggered by chronic microorganism infections, our results suggest that chronic infections may be an appropriate explanation for much of the morbidity seen in many CFS/FMS patients. Although the differences were not significant, multiple infections with different mycoplasmal species appear to occur after onset of illness, suggesting that mycoplasma-positive patients contracted additional mycoplasmal infections with time. We also found that the severity of major signs and symptoms may be related to the type of mycoplasmal infection. Infections with more than one mycoplasma species were associated with greater increases in severity of signs and symptoms than in patients with single infections. Although significant differences were not found due to a high variance of the score values, there was a tendency for patients with mycoplasmal infections to have higher intensity scores of signs and symptoms than patients without such infections. Obviously, these chronic infections are not sufficient to explain all of the signs and symptoms found in every patient, and chemical exposures, psychological problems and other environmental toxic events may also be important sources of morbidity. It remains unclear whether mycoplasmal infections are causative, cofactors or opportunistic in CFS/FMS patients. The identification of these microorganisms in blood leukocytes does, however, offer an opportunity for more specific diagnosis and treatment of these chronic illnesses.

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Legends

Figure 1

Incidence of multiple infections with different mycoplasma species in mycoplasma-positive CFS/FMS patients. (A) overall incidence of detected mycoplasma species; incidence of (B) single, (C) double, or (D) triple infections with different mycoplasmal species; Data are shown as percent of all mycoplasma-positive patients (N=91); species were not identified in 30.8% of patients. (M.ferm.: *M. fermentans*; M.pneu.: *M. pneumoniae*; M.hom.: *M. hominis*; M.pen.: *M. penetrans*)

Table 1. Sequences, target genes and size of amplified products from mycoplasmal DNA used for mycoplasma genus -specific and species-specific PCR

Sequence name	Sequence	Target	Size [bp]	Source
GPO1 primer MGSO primer UNI- probe	**ACT CCT ACG GGA GGC AGC AGT A** **TGC ACC ATC TGT CAC TCT GTT** **A A C C T C** **TAA TCC TGT TTG CTC CCC AC**	16S mRNA genus	717	Van Kuppeveld 1992 [**Error! Bookmark not defined.**]
SB 1 primer SB 2 primer SB 3 probe	**CAG TAT TAT CAA AGA AGG GTC TT** **TCT TTG GTT ACG TAA ATT GCT TTT TTC AGT TTC GTA TTC GAT G**	*tuf* gene *M. fermentans*	850	Berg 1995 [**Error! Bookmark not defined.**]
MP5-1 primer MP5-2 primer MP5-4 probe	**GAA GCT TAT GGT ACA GGT TGG ATT ACC ATC CTT GTT GTA AGG CGT AAG CTA TCA GCT ACA TGG AGG**	unknown gene *M. pneumoniae*	144	Bernet 1989 [i]
Mhom1 primer Mhom2 primer GPO1 probe	**TGA AAG GCG CTG TAA GGC GC** **GTC TGC AAT CAT TTC CTA TTG** **C A A A** **ACT CCT ACG GGA GGC AGC AGT A**	16S mRNA *M. hominis*	281	Van Kuppeveld 1992 [**Error! Bookmark not defined.**]
MM-7 primer MM-5 primer MM-3 probe	**GGA AAC GGG AAT GGT GGA ACA GAT** **TTC TGC TAA TGT TAC AGC AGC AGG** **AGG GAA TCT GTG ATC TTA TTC**	P35 gene (lipoprotein) *M. penetrans*	704	Nasralla 1999

Table 2. Age and duration of illness compared with detection of mycoplasmas in blood specimens.

(Mean values)

	Mycoplasma spp.		**No. of different mycoplasmas species identified**				***Species of* Mycoplasma *identified***			
	Negative	**Positive**	**None**	**One**	**Two**	**Three**	***fermentans***	***pneumoniae***	***penetrans***	***hominis***
Age [years]	40.8	40.9	47.5	31.6	41.8	45.0	42.6	41.6	34.3	44.9
Illness duration [months]	153.3	167.5	199.5	127.6	150.3	211.7	183.7	170.0	121.1	197.5

Table 3. Changes in average severity of signs and symptoms in patients with different mycoplasma species in their blood after onset of illness. Severity of signs and symptoms was assessed using a Patient Illness Survey Form that included 114 signs and symptoms. The intensity of signs and symptoms were marked by patients on a 10-point scale (0: none; 10: extreme) prior to and after onset of illness. Scores were determined in each category (3-9 questions) as the sum of differences between values prior to and after onset of illness/number of questions in the category

	***Mycoplasma* spp.**		**No. of mycoplasma species identified**				**Mycoplasma species identified***			
	Neg	**Positive**	**None**	**Single**	**Double**	**Triple**	***M. ferm.***	***M. pneu.***	***M. pen.***	***M. hom.***
core values										
atigue/sleep problems	3.44	3.38	3.69	2.69	3.66	3.52	3.67	3.51	3.68	2.78
epression	3.59	4.15	3.47	4.00	4.70	4.04	4.40	4.08	4.50	4.21
Iemory problems	2.89	3.31	3.71	3.17	3.57	2.93	3.18	3.31	2.86	3.33
alance problems	2.86	3.81	4.06	3.65	4.00	3.61	3.79	3.80	3.29	4.03
Iuscle pain/ache	4.11	4.37	3.75	4.18	4.88	4.31	4.61	4.19	4.69	4.67
oint pain/ache	2.26	2.24	2.31	1.92	2.78	1.86	2.32	2.23	2.07	2.09
ıfections	4.34	3.22	4.34	3.10	3.53	2.32	2.96	3.10	2.73	2.53
kin disorders	1.96	2.32	1.90	2.51	2.53	2.13	2.24	2.40	2.13	2.56
air/Scalp disorders	1.36	1.59	1.88	1.22	1.94	1.39	1.64	1.24	2.24	1.42
kin rash/sensitivity	1.57	2.10	2.66	2.04	1.94	2.02	1.91	1.77	2.36	2.19
enital disorders	1.48	1.87	2.72	2.12	1.55	1.52	1.50	1.55	1.36	2.13
limentation	0.94	1.27	1.38	1.34	1.67	0.69	1.08	1.19	1.54	0.87
ense disorders	2.97	2.53	3.29	2.18	2.88	2.02	2.51	2.52	1.90	2.22
ead/neck ache	2.08	1.95	2.60	2.00	2.06	1.40	1.75	1.79	1.56	1.74
welling (tissue)	3.10	2.77	3.56	2.46	2.81	2.57	2.68	3.00	2.46	2.15
ight sweats/fever	2.70	2.25	2.53	2.29	2.26	2.04	2.11	2.10	2.04	2.45
astrointestinal problems	1.58	2.57	3.19	1.85	3.19	2.18	2.41	2.54	2.46	2.62
rinary problems	2.12	2.48	2.97	2.09	2.66	2.36	2.38	2.32	2.56	2.57
leeding	2.40	1.96	2.13	1.50	2.13	2.11	2.11	1.91	2.32	1.82
Iouth cavity problems	1.28	1.50	0.63	1.62	2.00	1.33	1.54	1.58	1.81	1.67
isual disorders	1.32	1.30	1.79	1.18	1.23	1.21	1.14	1.25	1.40	1.12
udial disorders	2.12	1.90	1.77	1.83	2.31	1.55	1.77	1.89	2.06	1.85
ye problems	2.28	1.96	2.69	2.31	1.63	1.61	1.66	1.64	1.71	1.94
aste/smell problems	2.26	2.46	3.00	2.44	2.81	1.76	2.36	2.11	2.43	2.16
asopharyngeal roblems	1.72	2.18	2.54	2.18	2.19	1.95	2.12	1.92	2.26	2.10
reathing problems	1.93	2.64	2.38	2.10	2.94	2.95	2.85	2.74	3.64	2.24
eart problems	1.97	2.26	2.18	1.86	2.64	2.24	2.35	2.09	2.73	2.38
hemical	2.65	2.29	2.58	1.97	2.52	2.17	2.29	2.29	2.81	1.76

*(M. ferm., *M. fermentans*; M. pneu., *M. pneumoniae*; M. hom., *M. hominis*; M. pen., *M. penetrans*; none, test for all four species negative)