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Comparative Study of the Presence of *Chlamydia pneumoniae* in Cerebrospinal Fluid of Patients with Clinically Definite and Monosymptomatic Multiple Sclerosis

Subramaniam Sriram,[1]* Song-yi Yao,[1] Charles Stratton,[2] Peter Calabresi,[3] William Mitchell,[2] Hideaki Ikejima,[4] and Yoshimasa Yamamoto[4]

Department of Neurology[1] and Department of Pathology,[2] Vanderbilt School of Medicine, Nashville, Tennessee; Department of Neurology, University of Maryland, Baltimore, Maryland[3]; and Department of Medical Microbiology and Immunology, University of South Florida, College of Medicine, Tampa, Florida[4]

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There is considerable controversy concerning the evidence for the presence of *Chlamydia pneumoniae* in the cerebrospinal fluid (CSF) of both multiple sclerosis (MS) patients and patients with other neurological diseases (OND). In order to clarify this issue, the laboratories at Vanderbilt University Medical Center (VUMC) and the University of South Florida (USF) examined the reproducibility of their respective PCR assays for the detection of *C. pneumoniae* DNA in the CSF of a common group of MS patients and OND controls. The two laboratories used different DNA extraction and PCR techniques in order to determine the prevalence of the *C. pneumoniae* genome in both monosymptomatic and clinically definite MS patients as well as in OND controls. In clinically definite MS patients, the VUMC and USF detection rates were 72 and 61%, respectively, and in patients with monosymptomatic MS, the VUMC and USF detection rates were 41 and 54%, respectively. The PCR signal was positive for 7% of the OND controls at VUMC and for 16% at USF. These studies confirm our previous reports concerning the high prevalence of *C. pneumoniae* in the CSF of MS patients. The presence of *C. pneumoniae* in patients with monosymptomatic MS would also suggest that infection with the organism occurs early in the course of the disease.

Although the etiology of multiple sclerosis (MS) is not known, clinical and pathological observations suggest that interplay occurs between an infectious agent(s) and an autoimmune response to myelin antigens in the development of the disease (10). Epidemiologic studies have implicated environmental factors and most likely infectious agents as necessary elements in the development of MS (7).

The diagnosis of MS is made by establishing the presence of inflammatory demyelinating lesions in the white matter of the central nervous system (CNS) that are disseminated in time and space (9, 12). MS affects young adults and most often presents as a relapsing remitting disease. The initial presentation of a clinical attack usually involves the optic nerve brain stem or spinal cord. Patients presenting with an initial clinical attack in these anatomical regions are at high risk for the development of MS; however, the progression to clinically definite MS is not absolute (2).

We have previously reported the high prevalence of *C. pneumoniae* CNS infection in MS patients but not in patients with other neurological disease (OND controls) (16). Some investigators have confirmed the presence of *C. pneumoniae* DNA in the cerebrospinal fluid (CSF) of MS patients, although usually in a smaller percentage of patients than we reported (4, 5, 8; C. Contini and E. Fainardi, Abstr. 41st Intersci. Conf. Antimicrob. Agents and Chemother., abstr. L-2171, 2001). Others have been unable to detect any chlamydial DNA in MS patients or controls (1, 13; M. A. Marcos, A. Sanz, J. Vidal, F. Graus, and M. T. Jimenez, presented at the International *Chlamydia* Meeting, Helsinki, Finland, 2000). The inconsistencies among these results from different laboratories strongly suggest that methodological problems are present in the PCR detection of *C. pneumoniae* from CSF and that these problems might, in part, account for the differences in published results. This is not surprising, as there are no well-accepted methods and techniques for demonstrating the presence of *C. pneumoniae* in CSF (3). In order to reconcile these differences, the laboratories at both the Vanderbilt University Medical Center (VUMC), Nashville, Tenn., and the University of South Florida (USF) compared a common set of CSF samples. In the past, these two laboratories have shown the presence of *C. pneumoniae* in the CSF of a majority of MS patients (5, 16, 18). We therefore predicted that techniques developed independently in the two laboratories might be useful in comparing PCR techniques for detection of the organism in the CSF. We believed that a comparative analysis for the detection of *C. pneumoniae* that used a common set of CSF samples would validate the earlier observations from these two laboratories.

The presence of *C. pneumoniae* in the CSF does not necessarily ascribe a causal relationship between *C. pneumoniae* infection and the development of MS. However, the presence of chlamydial CNS infection early in the course of MS would be more suggestive of a causal link between *C. pneumoniae* infection of the CNS and the development of MS. Thus, we also examined the prevalence of *C. pneumoniae* in the CSF of

* Corresponding author. Mailing address: Multiple Sclerosis Research Laboratory, 1222H Vanderbilt Stallworth Rehabilitation Hospital, 2201 Capers Ave., Nashville, TN 37212. Phone: (615) 963-4042. Fax: (615) 321-5247. E-mail: Srirams@ctrvax.vanderbilt.edu.

TABLE 1. Sequence and position of primers on the MOMP (*ompA*) gene of *C. pneumoniae*

Primer	Sequence	Position	PCR product size (bp)
VU-MOMP			
External			
Sense	5′ AAC TAT ACT ACT GCC GTA GA 3′	286–305	
Antisense	5′ GTA GTA GAC AAT GCT GTG G 3′	971–989	704
Nested			
Sense	5′ ACA CCT CTT TCT CTT GGA GCG T 3′	554–575	
Antisense	5′ TTG ATG GTC GCA GAC TTT GTT C 3′	779–791	238
Tong and Sillis			
External			
Sense	5′ TTA CAA GCC TTG CCT GTA GG 3′	61–80	
Antisense	5′ GCG ATC CCA AAT GTT TAA GGC 3′	373–393	333
Nested			
Sense	5′ TTA TTA ATT GAT GCT ACA ATA 3′	100–120	
Antisense	5′ ATC TAC GGC AGT AGT ATA GTT 3′	286–306	207
USF-MOMP			
Sense	5′ AGG AGA TCC TTG CGA TCC T 3′	135–153	
Antisense	5′ TAG GCT TGG CTC CCA TAG AA 3′	250–269	134

patients with clinically isolated syndromes that are harbingers of MS.

MATERIALS AND METHODS

Patients and patient selection. Patients with clinically isolated demyelinating syndromes, clinically definite MS patients, and OND controls were prospectively recruited from VUMC and the Brown University MS Clinic at Rhode Island Hospital, Providence. None of the patients in this study have been used in studies reported previously. CSF was immediately aliquoted into 0.5-ml vials and kept frozen at −70°C. Blinded samples of CSF were sent to the laboratory of Y. Yamamoto, USF, for analysis. Sufficient CSF samples from six monosymptomatic patients seen at the Rhode Island MS clinic and two OND controls were not available for analysis at both centers and were, therefore, examined only at VUMC. The time interval between fluid collection and performance of the studies ranged from 3 months at VUMC to 24 months at USF. All samples, both those sent from the MS centerin Rhode Island and those shipped to USF, were packaged on dry ice and sent via overnight delivery.

PCR amplification of the major outer membrane protein (MOMP) gene of *C. pneumoniae* at VUMC. The DNA extraction protocol used was modified from previously published protocols (16). Briefly, 300 μl of CSF sample was centrifuged at 30,000 rpm (40,000 × *g*) in a TL-100 ultracentrifuge (Beckman) for 30 min. Two hundred fifty microliters of supernatant was discarded, and the remaining 50 μl was resuspended and mixed with 450 μl of lysate buffer containing 20 mM Tris-HCl (pH 7.5), 0.5% sodium dodecyl sulfate, 1% NP-40, 0.2 M NaCl, 10 mM EDTA, and 10 mM dithiothreitol. The mixture was vortexed and incubated for 30 min at room temperature. Twenty-five microliters of proteinase K (10-mg/ml stock solution; Worthington Biochemical Corporation, Lakewood, N.J.) was added, and the sample was then vortexed for 30 s and incubated overnight at 37°C. The DNA was extracted by the phenol chloroform method and dissolved in 30 μl of Tris-EDTA buffer. The DNA extraction protocol was also performed on fluid containing the extraction buffer alone, normal CSF, and normal CSF spiked with 5 inclusion-forming units (IFU) of *C. pneumoniae* elementary bodies (EB). These were used as internal positive and negative controls.

The presence of *C. pneumoniae* was examined by PCR with two different sets of internal and external primers that are specific for the *C. pneumoniae* MOMP gene (also known as the *ompA* and *omp1* gene) (Table 1). For PCR amplification, 5 μl of DNA in Tris-EDTA buffer was mixed with 50 mM KCl, 1.5 mM $MgCl_2$, 25 pmol of each primer, 200 μM each deoxynucleoside triphosphate, and 1 U of Ampli*Taq* polymerase (Roche, Indianapolis, Ind.) in a volume of 25 μl. DNA amplification of the *C. pneumoniae* MOMP gene was performed by using a touchdown PCR or nested-PCR technique. The first amplification of the outside product was done by using the touchdown technique, in which the annealing temperature was reduced by 0.5°C for every cycle from 60 down to 50°C. The amplification was performed for an additional 20 cycles at 50°C. The denaturation and extension temperatures were 94 and 74°C, respectively. At each point, the temperature was held for 1 min and a final extension was done at 74°C for 5 min. Two microliters of the first product was then subjected to a second amplification with internal primers. The PCR mixture was the same as before except that the $MgCl_2$ concentration was increased to 2 mM along with the specified internal primers (Table 1). The nested-PCR amplification was run for 35 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 74°C. The PCR product was run on a 1.5% agarose gel and stained with ethidium bromide. All CSF samples were tested in duplicate assays. In all experiments, reaction buffer alone plus DNA from normal CSF or water spiked with 0.4 IFU of *C. pneumoniae* EB were used as positive and negative internal controls, respectively.

PCR amplification of the MOMP gene of *C. pneumoniae* at USF. Bacterial DNA was extracted from 200-μl specimens of CSF by using a QIAmp DNA mini kit (Qiagen, Valencia, Calif.) as described previously. In brief, each specimen was centrifuged for 30 min at 20,000 × *g*. The pellet was resuspended in 180 μl of buffer ATL (Qiagen) with 20 μl of proteinase K and then incubated at 56°C with occasional vortexing until the pellet was completely lysed, which usually took 30 min. After lysis of the sample, 200 μl of buffer AL (Qiagen) was added to the sample and the mixture was incubated for 10 min at 70°C. The mixture was then combined with 200 μl of absolute ethanol and mixed by pulse-vortexing for 15 s. The mixture was applied to a spin column, which holds a silica gel membrane, and spun for 1 min at 6,000 × *g*. The spin column was washed with 500 μl of buffer AW2 by centrifugation at 20,000 × *g* for 3 min. The DNA bound on a membrane was eluted by centrifugation with 50 μl of buffer AE (Qiagen) after a 5-min incubation at room temperature. The resulting DNA extracts were stored at −20°C until PCR assessment.

The extracted DNA samples were subjected to PCR with primers specific for *C. pneumoniae* MOMP (Table 1). In brief, 2 μl of DNA extracts was processed in a 25-μl reaction volume containing PCR buffer (10 mM Tris [pH 8.3], 50 mM KCl), 200 μM deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 0.5 μM each primer, and 1.25 U of *Taq* polymerase (AmpliTaq Gold; Applied Biosystems, Foster City, Calif.). The PCR cycle consisted of an initial denaturation for 5 min at 94°C, which was followed by 60 cycles of denaturation at 94°C for 45 s, annealing beginning at 62°C and ending at 52°C for 45 s and extension at 72°C for 1 min. The annealing temperature was reduced by 1°C every four cycles until it reached 52°C, with six more cycles at the last annealing temperature. The final extension was for 10 min at 72°C. The PCR products were visualized by 2% agarose gel electrophoresis staining with ethidium bromide. The specificity of the PCR product was confirmed by performing Southern blot hybridization with a MOMP-labeled probe (5). For quality control for each PCR, a mock DNA

TABLE 2. PCR for *C. pneumoniae* in the CSF of patients with clinically definite MS

Patient	Diagnosis[a]	Age (yr)	Gender	CSF characteristics			IgG index[b]	OC[c] bands	PCR assay results		
				Cell count (no. of cells/μl)	Protein concn (mg/dl)	Glucose concn (mg/dl)			VUMC[d]		USF with MOMP primer[e]
									VU-MOMP	Tong and Sillis	
1	Sec MS	45	F	ND[f]	ND	ND	ND	ND	+	+	+
2	RR MS	47	M	4	44	58	1.2	+	+	−	+
3	RR MS	26	F	ND	ND	ND	ND	ND	+	+	+
4	RR MS	40	M	1	34	68	0.6	−	+	+	+
5	RR MS	51	M	4	62	54	1.27	+	+	+	+
6	RR MS	59	F	2	51	60	0.6	+	−	−	+
7	RR MS	48	F	2	48	59	0.7	+	+	−	+
8	Sec MS	58	M	2	67	100	1.09	−	+	+	+
9	Sec MS	44	F	1	66	63	1.15	+	+	+	+
10	Sec MS	55	M	0	35	69	0.75	+	−	−	−
11	Sec MS	42	F	1	31	54	0.84	+	−	+	−
12	RR MS	55	F	1	28	48	0.73	+	−	−	−
13	RR MS	55	F	2	37	57	0.49	−	+	+	−
14	Sec MS	61	F	ND	ND	ND	ND	ND	+	+	−
15	Sec MS	66	F	1	64	61	0.58	−	+	+	−
16	RR MS	32	F	5	17	61	0.72	−	−	−	−
17	Sec MS	57	M	4	82	64	0.57	+	+	+	+
18	RR MS	62	M	3	43	55	0.07	+	+	+	+

[a] Abbreviations: Sec MS, secondary progressive MS; RR MS, relapsing remitting MS.
[b] IgG, immunoglobulin G.
[c] OC, oligoclonal.
[d] The total numbers of samples positive were 13 and 12 for the Vu-MOMP and Tong and Sillis primers, respectively.
[e] The total number of samples positive was 11.
[f] ND, not done.

extraction sample from water, PCR buffer without a sample, and *C. pneumoniae* DNA were used as negative and positive controls. The PCR was sensitive in detecting EB at a level of 1 IFU per PCR.

RESULTS

Patient demographics. All 18 patients in the clinically definite group met the criteria for MS described by Poser et al. (12), which are shown in Table 2. None of these patients have been used in previous studies. Of the 18 patients in the clinically definite group, 8 patients were diagnosed with secondary progressive disease and the remaining 10 were diagnosed with relapsing remitting disease. In the OND group, five patients had either an elevated level of CSF protein or a cell count suggesting increased permeability of either the blood-brain or

TABLE 3. Prevalence of *C. pneumoniae* in the CSF of OND controls

Patient	Age (yr)	Gender	CSF characteristics			Diagnosis	PCR assay results		
			Cell count (no. of cells/μl)	Protein concn (mg/dl)	Glucose concn (mg/dl)		VUMC[a]		USF with MOMP primer[b]
							VU-MOMP	Tong and Sillis	
1	72	M	2	65	121	Pneumonia	+	−	+
2	30	M	4	39	77	Metabolic encephalopathy	−	−	−
3	21	F	5	54	79	Secundum septum aneurysm and fever	−	−	−
4	48	F	0	41	52	Delirium	−	−	−
5	25	F	1	33	62	Headache	−	−	−
6	36	F	1	40	58	R/O[c] vasculitis	−	−	−
7	77	F	100	63	88	Meningitis	−	−	−
8	24	F	0	18	56	Pseudotumor cerebri	−	−	−
9	30	F	1	36	69	R/O MS	−	−	−
10	30	F	0	120	37	Acute leukemia	−	−	ND[d]
11	39	F	66	135	33	CNS sarcoidosis	−	−	ND
12	45	F	1	71	75	Delirium following cardiac transplant	−	−	+
13	46	F	0	36	49	Myelopathy	−	−	−
14	48	F	0	44	66	Myelopathy	−	−	−

[a] The total numbers of samples positive were 1 and 0 for the VU-MOMP and Tong and Sillis primers, respectively.
[b] The total number of samples positive was two.
[c] R/O, rule out.
[d] ND, not done.

TABLE 4. Prevalence of *C. pneumoniae* in monosymptomatic MS patients

Patient	Age (yr)	Gender	CSF characteristics			Initial event[a]	IgG index[b]	OC[c] bands	Brain MRI results[d]	Cervical cord MRI results	PCR assay results		
			Cell count (no. of cells/μl)	Protein concn (mg/dl)	Glucose concn (mg/dl)						VUMC[e]		USF with MOMP primer[f]
											VU-MOMP	Tong and Sillis	
1	27	M	21	59	53	SC	0.55	−	Normal	Single T2 lesion	+	−	−
2	32	M	10	38	58	SC	1.23	−	Normal	Two T2 lesions	+	+	+
3	42	M	3	86	70	SC	0.51	−	Normal	Enhancing Lesion	−	−	−
4	27	F	0	13	51	ON	0.46	−	Normal	Normal	+	−	+
5	38	F	1	24	64	BS	0.5	−	Two T2 PV lesions	Normal	−	−	−
6	45	F	0	23	50	SC	0.99	+	Normal	Enhancing Lesion	+	+	+
7	52	M	7	Unknown	71	ON	0.61	−	Three PV lesions	ND[g]	−	−	−
8	34	F	1	50	55	ON	0.6	−	Multiple PV lesions	ND	−	−	+
9	39	F	1	43	60	BS	0.39	−	Multiple PV lesions	ND	−	+	ND
10	33	F	1	56	57	BS	0.45	+	Multiple PV lesions	Normal	−	−	−
11	31	F	9	49	62	ON	1.41	+	Single enhancing lesions	Single T2 lesion	+	+	+
12	28	F	2	32	54	ON	0.74	+	Normal	Single T2 lesion	−	−	+
13	57	F	1	15	53	SC	ND	+	Brian Normal	ND	−	ND	ND
14	47	F	2	52	52	BS	ND	+	Single T2 lesion Pons	ND	−	ND	ND
15	42	F	21	26	65	SC	ND	−	NSWM abnormal	ND	−	ND	ND
16	41	F	1	21	65	BS	ND	+	3 NSWM lesions	ND	+	ND	ND
17	25	F	0	27	59	BS	ND	+	4 NSWM lesions	ND	+	ND	ND

[a] Abbreviation for locations of initial events: SC, spinal cord; ON, optic nerve; BS, brain stem.
[b] IgG, immunoglobulin G.
[c] OC, oligoclonal.
[d] PV, periventricular; NSWM, nonspecific white matter.
[e] The total numbers of samples positive were 7 and 4 with the VU-MOMP and Tong and Sillis primers, respectively.
[f] The total number of samples positive was 2.
[g] ND, not done.

blood-CSF barrier (Table 3). Patient 11 was diagnosed with CNS sarcoidosis and demonstrated oligoclonal bands. Of the 17 patients with monosymptomatic MS, 6 presented with spinal cord syndromes, 6 presented with brain stem syndromes, and 5 presented with optic neuritis (Table 4).

PCR results. The VUMC laboratory used two different sets of external and internal PCR primers of the MOMP gene (Table 1) and employed a nested-PCR or touchdown PCR assay for the detection of *C. pneumoniae*. One of the sets was the Tong and Sillis primer set for the MOMP gene, which has been used previously for detecting *C. pneumoniae* in clinical samples, including CSF samples from MS patients (8). In addition, a new set of primers was designed for the amplification of the MOMP gene (VU-MOMP); this primer set was, in preliminary studies, sufficiently sensitive to detect *C. pneumoniae* EB at a concentration of 0.04/IFU. The internal primer set of Tong and Sillis produced a 207-bp product, while the internal primer of the VU-MOMP primers produced a 238-bp product (Table 1).

The overall DNA extraction, which is designed to extract bacterial DNA, and the PCR protocols utilized at USF showed remarkable detection sensitivity, with approximately 125 organisms detected per ml of CSF (1 IFU/PCR performed with 2 μl of a 50-μl volume of DNA extracted from 200 μl of CSF). The results of these protocols did not show any smear, a condition which often makes the PCR products difficult to read.

We were able to detect the *C. pneumoniae* signal in samples from 13 of 18 (72%) of the clinically definite MS patients with the VU-MOMP primers and in samples from 12 of 18 (67%) of the same patients with the Tong and Sillis primers (Table 2). In samples from 11 patients, the PCR signal was present with both the VU-MOMP and the Tong and Sillis primers. In the sample from patient 2, a signal was seen only with the VU-MOMP primers, while in the sample from patient 11, the signal was seen only with the Tong and Sillis primers. When the positive assay results with both primers were combined, the overall detection rate was 78% (14 of 18) in CSF samples analyzed at VUMC. Although the Tong and Sillis primers were as sensitive as the VU-MOMP primers for the detection of *C. pneumoniae*, there was often a smear on the agarose gel above the PCR product of the Tong and Sillis primers. The smear was less prominent with the VU-MOMP primers.

For the CSF samples studied at USF with a touchdown enzyme time release PCR specific for the MOMP gene, those for 11 of 18 (61%) patients in the clinically definite MS group were positive (Table 2). The sample from patient 6 was positive at USF and negative at VUMC. Samples from patients 11, 13, 14, and 15 were positive at VUMC (with the Tong and Sillis primers for the patient 11 sample and the VU-MOMP primers for patient samples 13, 14, and 16) and were negative at USF. Samples from 8 of 18 (44%) patients were positive with all three sets of primers, and 10 patient samples (56%) were positive with the VU-MOMP and USF MOMP primer sets. When the results of all three primers were combined, the overall detection rate was 83% (15 of 18 patients). Only 3 of 18 MS patients (17%) were negative with all three sets of primers. These studies show significant correlations between the differ-

ent laboratories in the detection of *C. pneumoniae* in the CSF of MS patients.

For the OND controls, VUMC and USF compared 12 samples (Table 3). The sample from patient 1 was positive at both centers while that from an additional patient, patient 10, was positive at USF. None of the samples from the OND controls was positive with the Tong and Sillis primers. The detection rate in OND controls was significantly less than that in MS patients with all three MOMP primers ($P < 0.01$).

We next studied the presence of *C. pneumoniae* in patients with monosymptomatic MS. With the VU-MOMP primers, 7 of 17 (41%) samples were positive, while with the Tong and Sillis primers, 3 of 11 (27%) were positive. The detection rate with the USF primers was 55% overall (6 of 11 patients), with four patients sharing a positive signal with both VU-MOMP and USF primers. Three samples (from patients 2, 6, and 11) were positive with all three sets of primers. The overall detection rate in patients with monosymptomatic MS was 57% (10 of 17) when all three primers were used, which was higher than that seen in the OND controls ($P < 0.01$) and lower than that in the clinically definite group ($P < 0.05$).

DISCUSSION

The results of this study suggest a strong degree of correlation between the abilities of two different laboratories to detect the presence of *C. pneumoniae* in the CSF from MS patients but not from OND controls. These results also show that these two laboratories were able to detect *C. pneumoniae* at levels that were significantly higher in MS patients than in OND controls. The two laboratories used different techniques for DNA extraction and for PCR testing. The VUMC laboratory modified the lysis buffer and used phenol-chloroform for DNA extraction. A nested-PCR assay with two different sets of primers, both of which are specific for the MOMP gene of *C. pneumoniae*, were used. While the Tong and Sillis primers have been used before for the detection of *C. pneumoniae* in clinical specimens, the running conditions for the present study were modified to detect *C. pneumoniae* in CSF at concentrations of 0.05 IFU/μl (17). Similar strategies were also employed prior to the use of the VU-MOMP primers.

The USF laboratory performed DNA extraction with the Qiagen DNA mini kit with bacterial DNA extraction protocols. The primer set that the USF laboratory used was developed and previously reported to be sensitive for the detection of chlamydial DNA at 1 IFU per PCR mixture. Since the IFU number shows only the number of infective EB, which may be affected by the protocols of EB isolation and culture conditions, the absolute PCR sensitivities for protocols based on IFU numbers prepared in different institutes are difficult to compare. The results of this comparative study make it unlikely that the PCR product detected in CSF from MS patients was an artifact of DNA amplification.

Since our initial report on the association between the presence of *C. pneumoniae* in the CSF of MS patients and the development of MS, there have been a number of studies that have attempted to reproduce these observations. Most of these studies have met with limited success. Layh-Schmitt et al. (8) was able to show the presence of the organism in 22% of MS patients, and this number increased when phenol-chloroform extraction techniques were used (17). More recently, Contini and Fainardi (41st ICAAC) have reported the presence of *C. pneumoniae* in the CSF of 58% of MS patients, and another report (15) noted the presence in 10% of MS patients. It should be noted that in all these studies, the presence of the *C. pneumoniae* genome was statistically higher in MS patients than in control patients. In a report by Gieffers et al., the presence of *C. pneumoniae* in the CSF was demonstrated in a high number of OND patients by PCR (4). This OND group included a number of individuals with inflammatory disease, including optic neuritis. Therefore, these patients might represent patients with early symptoms of MS. Investigators from the University Hospital of Umeå could not document the presence of *C. pneumoniae* by PCR in any CSF samples previously collected from 48 MS patients as well as in 51 OND patients (1). Other groups also have failed to identify *C. pneumoniae* by PCR in CSF samples and the tissues of patients with MS (13, 18; Marcos et al., presented at the International *Chlamydia* Meeting, Helsinki, Finland, 2000).

In a recent collaborative study involving four different laboratories, the presence of *C. pneumoniae* was examined in 52 blinded CSF samples (6). VUMC was able to detect *C. pneumoniae* in 73% (22 of 30) of MS patients compared to 22% (5 of 22) of controls. Three other laboratories (C. Gaydos, Johns Hopkins University, Baltimore, Md.; J. Boman, Umeå, Sweden; and L. Tondilla, Centers for Disease Control and Prevention, Atlanta, Ga.) failed to show the presence of *C. pneumoniae* in any of the CSF samples. This study also suggests that the presence of the organism is not due to any regional differences in the patient population, since all samples were obtained from Carolinas Medical Center, Charlotte, N.C. The procedures used by these four laboratories were different, and since the samples were blinded with regard to the underlying diagnosis, technical differences could very likely account for the differences in the outcomes of the PCR studies. The ability of the laboratory at USF to validate results obtained from VUMC suggests that technical problems in the PCR procedure (which may include sample collection and storage as well as extraction of DNA and PCR testing) might account for the discrepancies among results from different laboratories.

The preliminary study conducted at USF and published earlier had shown that optimization of the PCR conditions is necessary for obtaining a signal for *C. pneumoniae* in the presence of low copy numbers of the organism (5). These issues were extensively reviewed by us, and the importance of DNA extraction techniques, primer selection, and optimization or running conditions have been previously discussed (Zuzak et al., presented at the International *Chlamydia* Meeting, Helsinki, Finland, 2000). It is clear, for example, that primers for MOMP are more sensitive than the 16S RNA gene for the detection of *C. pneumoniae*. This may explain the inability of the C. Gaydos laboratory to detect *C. pneumoniae* in the CSF of MS patients (6).

In our previous studies, we had amplified the entire 1.1-kb MOMP gene. In the present study, we used the Tong and Sillis primers, since they have been used extensively to document the presence of *C. pneumoniae*, in addition to new primers that produced less background signal. The laboratory of J. Boman has also used the Tong and Sillis MOMP primers but has relied on the Qiagen blood mini kit for DNA extraction (6). We were

unable to detect a signal for *C. pneumoniae* in the CSF of MS patients with the Tong and Sillis primers following DNA extraction with the Qiagen blood mini kit. We therefore believe that DNA extraction may prove more efficient when performed with phenol-chloroform or with the QIAmp bacterial DNA extraction kit.

Although we have not quantitated the number of copies of the *C. pneumoniae* gene that is present in the CSF of MS patients, it is very likely that this copy number is low. Others have shown that performance of PCR in triplicate or more, as well as performance of probit analysis, may be necessary to exclude the problems of dilution and statistical variability in the presence of low copy numbers (14).

Importantly, our study also suggests that CNS infection with *C. pneumoniae* occurs early in the course of the disease, since the organism was identified in 50% of MS patients who presented heralding signs of MS but who had not yet shown dissemination of the disease. In situations wherein patients present with clinically isolated syndromes that are highly characteristic of MS (e.g., optic neuritis, internuclear ophthalmoplegia, or partial transverse myelopathy), the definitiveness of the diagnosis is uncertain, since the essential criteria of the dissemination of lesions over time has not yet been satisfied. Long-term studies of patients with isolated clinical syndromes suggest that the initial abnormalities on magnetic resonance images (MRI) and the presence of oligoclonal bands are likely to predict conversion to clinically definite MS (2, 11). These prospective studies suggest that at 15 years, 88% of patients presenting with clinically isolated syndromes suggestive of MS progress to clinically definite MS (2). Although the number of patients in our study was small, three patients (patients 1, 2, and 4) who were positive by PCR for the presence of the *C. pneumoniae* gene and one patient (patient 3) who was negative by PCR for *C. pneumoniae* have subsequently progressed into clinically definite MS.

In conclusion, our study further substantiates our initial observation regarding the increased presence of *C. pneumoniae* genes in the CSF of MS patients relative to that of controls. Also, the presence of *C. pneumoniae* in patients with signs of MS indicates that infection by this microorganism in MS occurs early in the course of the disease. The significance of this observation in relation to the role of *C. pneumoniae* in the pathogenesis of MS remains uncertain. We believe that a clinical trial that monitors the course of MS during and after eradication of the organism is most likely to explain conclusively the causal nature of this association.

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