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PCR-Based Method for Isolation and Detection of *Chlamydia pneumoniae* DNA in Cerebrospinal Fluids

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Since current studies indicate the possible involvement of *Chlamydia pneumoniae* in the pathogenesis of multiple sclerosis (MS), demonstration of *C. pneumoniae* in the cerebrospinal fluid (CSF) of patients with MS is highly desirable. However, there is controversy concerning the detection of *C. pneumoniae* in CSFs from MS patients due to the lack of a standard protocol for extraction and detection of *C. pneumoniae* DNA. In this regard, we attempted to establish a highly effective extraction protocol for *C. pneumoniae* DNA from CSFs utilizing a commercial kit and a PCR detection method. The extraction and PCR detection protocol established in this study succeeded in detecting as few as 20 *C. pneumoniae* organisms in 200 μl of mock CSF. The use of this protocol to detect *C. pneumoniae* DNA in CSFs revealed that 68% of CSF samples obtained from patients with MS were positive (11 out of 16 samples) for chlamydia DNA. Thus, the protocol established here is sensitive enough to detect chlamydia DNA from CSFs and can be used by other laboratories for evaluation of the presence of chlamydiae in CSFs because the protocol is based on the use of a commercial kit.

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) characterized by focal areas of demyelination. Although the exact etiology of MS is unknown, it is generally accepted that autoimmunity is involved and that the autoantigen(s) probably resides in CNS myelin, the target of the immune response (1). In this regard, current studies argue for an infectious agent as an initiating or enhancing factor for MS with immunological mechanisms (5). To identify a specific causative agent for MS, many groups have attempted to detect microbes in cerebrospinal fluid (CSF) as well as in CNS lesions obtained from MS patients. However, no consistent results have been obtained with any given pathogen. Recent studies conducted by Sriram et al. (12) highlighted the possible involvement of a bacterium in MS, with the finding of *Chlamydia pneumoniae* in the CSF of almost all patients with MS but in only a small proportion of CSF samples from control subjects without MS. That study has shown the highest association of any organism with MS to date. However, other research groups either could not detect *C. pneumoniae* in CSFs from MS patients or detected it only in a small proportion of specimens (2, 8, 14). This may be due to the lack of a standard method for *C. pneumoniae* detection in CSFs. For study of the involvement of *C. pneumoniae* in the pathogenesis of MS, a reliable standard evaluation protocol for *C. pneumoniae* in clinical specimens is essential. Therefore, in the present study, we attempted to establish an efficient extraction protocol for *C. pneumoniae* DNA in CSFs by use of a commercial kit followed by PCR specific for *C. pneumoniae*. Furthermore, the extraction and detection system established for *C. pneumoniae* DNA was applied to demonstration of the presence of *C. pneumoniae* in CSFs obtained from patients with MS. The results indicate that the protocol established was sufficient to detect *C. pneumoniae* DNA in CSFs of patients with MS.

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MATERIALS AND METHODS

CSF. Sixteen CSF samples from nine patients with MS were collected at the University Hospital of St. Marianna University School of Medicine, Kawasaki, Japan, and stored at −80°C until they were used for an assessment. All MS patients were diagnosed clinically, with six diagnosed, as probable MS patients and three as definite MS patients. The study protocol was approved by the University Ethics Committee.

Bacterial DNA. Formalin-fixed *C. pneumoniae* (strain TWAR) organisms were obtained from the Washington Research Foundation, Seattle, Wash. The chlamydia organisms were spiked into phosphate-buffered saline (PBS) with 2% fetal calf serum (FCS) at concentrations from 10^6 to 10^1 bacterial particles/ml.

DNA extraction. Bacterial DNA was extracted from 200 μl of PBS–2% FCS spiked with *C. pneumoniae* or from CSFs from MS patients using either a QIAmp Blood Mini Kit (QIAGEN Inc., Valencia, Calif.) or a QIAmp DNA Mini Kit with a bacterial DNA extraction protocol. When the QIAmp DNA Mini Kit with bacterial DNA extraction was used, 200 μl of sample 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 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 after a 5-min incubation at room temperature. The resulting DNA extracts were stored at −20°C until PCR assessment. When the QIAmp Blood Mini Kit was used, 200 μl of sample was combined with 20 μl of protease and 200 μl of buffer AL and then incubated at 56°C for 10 min. After incubation, the mixture was combined with 200 μl of absolute ethanol and mixed by pulse-vortexing for 15 s; then the protocol described, for the QIAmp DNA Mini Kit was followed. All reagents and spin columns were supplied in the kit (QIAGEN).

TABLE 1. Primer sequences used for PCR

Primer	Sequence	Size of fragment (bp)
omp1		
Sense	5′-TTA TTA ATT GAT GGT ACA ATA-3′	207
Antisense	5′-ATC TAC GGC AGT AGT ATA GTT-3′	
16S rRNA		
Sense	5′-TCT AAC GAG ACT GCC TGG GT-3′	231
Antisense	5′-GTA TTC ACG GCG TTA TGG CT-3′	

PCR. The extracted DNAs were subjected to PCR with primers specific for *C. pneumoniae omp1* (7) or the 16S rRNA gene (4). In brief, 2 μl of DNA extracts was processed in a 25-μl reaction volume containing PCR buffer (10 mM Tris [pH 9.0], 50 mM KCl, 0.01% gelatin), 200 μM deoxynucleoside triphosphates, 3.5 mM $MgCl_2$, 0.5 μM each primer, and 1 U of *Taq* polymerase (Promega, Madison, Wis.). The sequences of the primers are shown in Table 1. Amplifications were carried out in a Minicycler (MJ Research, Watertown, Mass.). The first cycle, consisting of a 5-min denaturation at 94°C, was followed by 50 cycles each of 30 s at 94°C, 45 s at 50°C, and 1 min, 30 s, at 72°C, with a final extension for 10 min at 72°C. In the case of the temperature gradient experiment, a range of annealing temperatures from 45 to 58°C was carried out in a Mastercycler gradient (Eppendorf Scientific Inc., Westbury, N.Y.).

Optimized conditions for PCR, such as pH and concentration of Mg^{2+}, were determined using a PCR Optimizer Kit (Invitrogen, Carlsbad, Calif.). The PCR products were visualized in 2% agarose gels containing 0.5 μg of ethidium bromide/ml. The specificity of the PCR products for *omp1* was confirmed by Southern blot analysis with a probe of *omp1* cDNA provided by S. Sriram, Vanderbilt Stallworth Rehabilitation Hospital, Nashville, Tenn.

RESULTS

***C. pneumoniae* DNA extraction.** In order to determine whether commercial DNA extraction kits can be used for extraction of *C. pneumoniae* DNA from CSF, two commercial kits, the QIAmp Blood Mini Kit and the QIAmp DNA Mini Kit, were used for this purpose. The QIAmp Blood Mini Kit is designed to extract mammalian DNA from biological specimens, including whole blood and body fluids, and has been utilized for extraction of *C. pneumoniae* DNA from CSF (2, 8). The QIAmp DNA Mini Kit is designed for extraction of DNA from solid tissues and bacteria. The *C. pneumoniae*-spiked PBS-FCS was used as a mock CSF with bacteria for evaluation of the efficacies of extraction of *C. pneumoniae* DNA by the two kits. Two hundred microliters of the mock CSF, which contained various concentrations of *C. pneumoniae*, 10^6 to 10^1 organisms/ml was processed with each of the two kits, and the resulting DNA was dissolved into 50 μl of the buffer. The extracted DNA was further subjected to PCR with primers specific for *C. pneumoniae omp1*. The results are shown in Fig. 1. The QIAmp DNA Mini Kit with a bacterial DNA extraction protocol showed high efficiency in extracting *C. pneumoniae* DNA from mock CSF compared with the QIAmp Blood Mini Kit. As few as 20 bacteria in 200 μl of mock CSF (100 bacteria per ml of sample) were consistently detected in the experiments by the protocol of the DNA Mini Kit, whereas the lower limit of detection for the Blood Mini Kit was 200 bacteria.

PCR conditions for *C. pneumoniae* DNA. Since PCR conditions, such as the annealing temperature, concentrations of Mg^{2+}, and pH, are known to affect the final products of PCR, the optimized PCR conditions for primers specific for *C. pneumoniae omp1* and the 16S rRNA gene were determined. As shown in Fig. 2, the optimized annealing temperature and the optimized concentration of Mg^{2+} for PCR with primers for *omp1* were 50°C and 3.5 mM, respectively. The optimized pH for the PCR was 9.0. The optimized PCR conditions for the 16S rRNA gene were also determined. It was found that the conditions for 16S rRNA gene PCR were the same as those for the *omp1* PCR (data not shown).

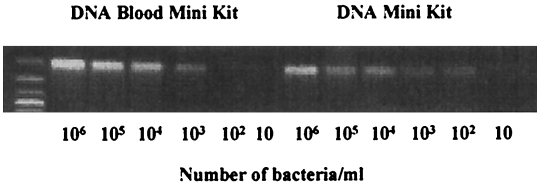

FIG. 1. Comparison of extraction efficacy of *C. pneumoniae* DNA from mock CSFs using two DNA extraction kits. The mock CSFs (200 μl) spiked with serially diluted bacteria were extracted either with the QIAmp DNA Mini Kit with a bacterial DNA extraction protocol or with the QIAmp DNA Blood Mini Kit. Two microliters of the extracted DNA (50 μl) was subjected to PCR with primers for *omp1*. Results are representative of three experiments.

Sensitivity of detection of *C. pneumoniae* DNA by PCR. Since it is known that the sensitivity of PCR for detecting chlamydia antigen in clinical specimens is dependent on the primers used (9), we examined the sensitivities of two PCRs, one with primers for *omp1* and one with primers for the 16S rRNA gene. As shown in Fig. 3, the PCR specific for *omp1* was at least 10 times more sensitive than the PCR for the 16S rRNA gene. The DNA obtained from more than 0.8 chlamydia organism in 2 μl was detected by the PCR for *omp1*. In contrast, the PCR for the 16S rRNA gene detected DNA extracted from more than 8 chlamydia organisms.

Detection of *C. pneumoniae* DNA in CSFs. In order to determine how the established system is sensitive enough to detect *C. pneumoniae* DNA in CSFs, CSFs obtained from patients with MS were utilized. The DNAs extracted from 16 CSFs from nine MS patients were evaluated by PCR with primers specific for either *omp1* or the 16S rRNA gene. Figure 4 shows a representative PCR result of three experiments, which indicates clearly that the system utilizing the PCR with

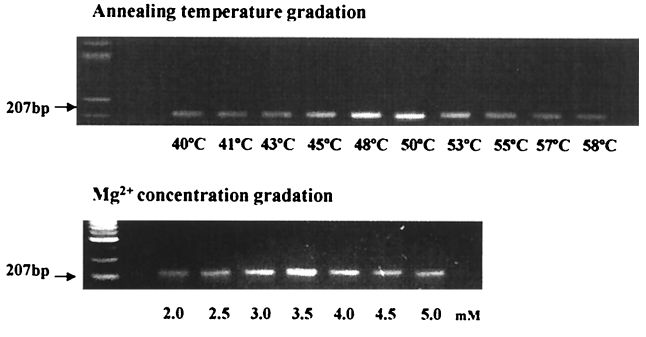

FIG. 2. Optimization of annealing temperature and Mg^{2+} concentration for PCR with primers for *omp1*. The annealing temperature was optimized in a Mastercycler gradient (Eppendorf). The Mg concentration for PCR specific for *omp1* was optimized with a PCR optimization kit (Invitrogen). The target DNA for PCR was extracted from *C. pneumoniae* with the QIAmp DNA Mini Kit.

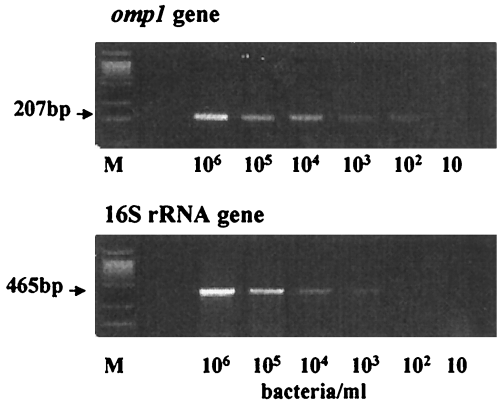

FIG. 3. Detection sensitivity of PCR for *omp1* versus the 16S rRNA gene. Two hundred microliters of the mock CSF containing a specific number of *C. pneumoniae* organisms (as indicated), was used for DNA extraction with the QIAmp DNA Mini Kit. A 2-μl portion of the 50-μl volume of DNA extracts was subjected to PCR. The optimized PCRs for *omp1* and 16S rRNA gene were conducted (see Materials and Methods).

primers specific for *omp1* was sensitive enough to detect *C. pneumoniae* DNA in the CSF of patients. The proportion of CSFs positive for *C. pneumoniae* DNA was 68% (11 out of 16 CSF samples), but the proportion of MS patients among the CSF donors was 100% (9 out of 9 patients). It is noteworthy that CSF samples obtained at different clinical stages from the same patients were not all PCR positive. In contrast with the PCR for *omp1*, when the PCR with primers for the 16S rRNA gene was utilized for detection of *C. pneumoniae* DNA in CSFs, no positive result was obtained, even though positive controls showed a strong band for PCR-specific product, indicating that the PCR worked correctly.

DISCUSSION

Since bacteria have a rigid cell wall, which may resist an ordinary digestion protocol for DNA extraction, the extraction protocol for bacterial DNA in clinical specimens should be considered. *C. pneumoniae* is a gram-negative bacterium and has peptidoglycan, lipopolysaccharide, and other outer membrane components in its cell wall (6, 11), which contribute to osmotic stability as well as to rigidness, particularly of elementary bodies, an infectious form that resists physical and chemical pressures in the extracellular environment. Therefore, the procedure for extraction of *C. pneumoniae* DNA from clinical specimens must be designed for bacterial DNA extraction, particularly for specimens which may have few bacteria, such as CSF of MS patients. In fact, when two extraction protocols, one designed for extraction of mammalian DNA from blood samples and one designed for extraction of bacterial DNA, were examined in this study, the extraction protocol for bacterial DNA (QIAmp DNA Mini Kit with a bacterial DNA extraction protocol) extracted *C. pneumoniae* DNA more efficiently.

The PCR protocol and selection of target genes for PCR are also critical for the overall sensitivity of detection by PCR. In the present study, we attempted to establish the optimized PCR conditions for two different chlamydia target genes, i.e., species-specific *omp1* and the 16S rRNA gene. Even though the sequences of the primers for *omp1* and the 16S rRNA gene were different, the optimized PCR conditions, such as annealing temperature, concentration of Mg^{2+}, and pH, were the same for the two PCRs. The detection sensitivities of the two PCRs with *omp1* versus 16S rRNA gene primers were compared under the same PCR conditions, which were optimal for both primers. It was found that the PCR for *omp1* was at least 10 times more sensitive than that for the 16S rRNA gene. Furthermore, when both PCRs were used for detection of *C. pneumoniae* in CSFs obtained from MS patients, the PCR for the 16S rRNA gene could not detect any *C. pneumoniae* DNA, even though the PCR for *omp1* detected *C. pneumoniae* DNA in the same CSF samples. These results indicate that the number of *C. pneumoniae* organisms in CSFs from MS patients was low and was not sufficient to be detected by the PCR for the 16S rRNA gene. Therefore, the sensitivity of detection by PCR as well as the DNA extraction efficacy is critical for detection

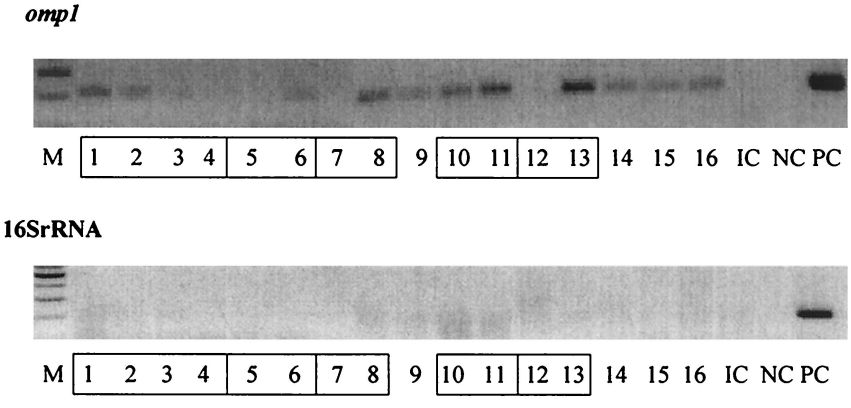

FIG. 4. Detection of *C. pneumoniae* DNA by PCR in CSFs obtained from patients with MS. Two hundred microliters of CSFs obtained from MS patients was processed for extraction of DNA utilizing the QIAmp DNA Mini Kit, and 2 μl of the resulting 50-μl DNA solution was subjected to PCR with primers for either *omp1* or the 16S rRNA gene. Data are representative of three PCR experiments. Boxed numbers indicate CSFs obtained from the same patient at different time points. Each number is the sample number. M, molecular marker; PC, positive control for PCR; NC, negative control for PCR; IC, negative control for DNA extraction.

of *C. pneumoniae* in clinical specimens of patients with neurological disease, such as MS.

The recent study by Mahony et al. showed similar findings, i.e., there are differences in sensitivity among PCRs with three different primers for *C. pneumoniae* DNA (9). The reason for these differences in sensitivity is not known. The target copy numbers of the two genes in *C. pneumoniae* are not much different, since only a single *omp1* gene and only two rRNA operons are present in the *C. pneumoniae* genome (13). Therefore, differences in sensitivity between the primers specific for *omp1* and those specific for the 16S rRNA gene may be due to the nature of each primer sequence, which may affect the affinity of the primer for the target DNA.

The sensitivity of the *omp1* PCR (DNA obtained from more than 0.8 *C. pneumoniae* organism per PCR) was not comparable with previous reports of *C. pneumoniae* DNA detection by PCR, which showed a sensitivity as low as 0.004 to 0.4 inclusion-forming unit (IFU; activity of chlamydial inclusion formation in epithelial cells) of *C. pneumoniae* (3, 4, 9). However, these reports used sequentially diluted DNAs as test samples, which are different from the individually extracted DNAs from different concentrations of *C. pneumoniae*. Furthermore, the number of IFU utilized in previous studies may contain more bacterial particles due to the counting of infectious bacteria only.

The application of the extraction and detection protocol established in this study to clinical specimens, such as CSFs obtained from patients with MS, succeeded in detecting *C. pneumoniae* DNA. The proportion of PCR-positive CSFs among those tested was as high as 68% (11 out of 16 samples). In previous studies by other investigators, the percentage of CSFs from MS patients that tested positive for *C. pneumoniae* DNA by PCR was variable, from 97 to 0% (2, 8, 12). Sriram et al. employed their own DNA extraction protocol for isolation of *C. pneumoniae* DNA from CSF and obtained a high positive rate for CSFs from MS patients (12). However, their DNA extraction protocol requires many sensitive steps with house reagents and a tiny DNA precipitate, which necessitated guesswork. In contrast, both Boman et al. (2) and Layh-Schmitt et al. (8) utilized a commercial kit (QIAmp Blood Mini Kit) for isolation of *C. pneumoniae* DNA, but they failed to detect bacterial DNA in CSFs of MS patients or found only a low positive rate. As shown in this study, the QIAmp Blood Mini Kit is not designed for isolation of bacterial DNA and was less effective for extracting bacterial DNA from CSFs, particularly when specimens contained few bacteria. The reasons for the variation in the *C. pneumoniae* DNA positive rate between the reports are not clear. However, from the findings in this study, it can be speculated that the extraction protocol used may be one of the reasons.

The percentage of CSFs in this study that tested positive for *C. pneumoniae* DNA does not permit any conclusion regarding the possible involvement of *C. pneumoniae* in the pathogenesis of MS, since only a limited number of CSFs were tested, and these included no control CSFs obtained from non-MS patients. Although the aim of this study was not to evaluate a possible involvement of *C. pneumoniae* in MS, it is noteworthy that both positive and negative PCR results were observed in CSFs obtained from the same patients at different clinical stages. These results may indicate a possible relation between a recrudescence of *C. pneumoniae* in the CSF and clinical symptoms or treatments, because these patients were treated with steroids for a certain period between CSF samplings (data not shown). Nevertheless, the extraction and detection protocol established for *C. pneumoniae* DNA was demonstrated to be sufficiently sensitive to detect only a few *C. pneumoniae* organisms in CSFs. Since the protocol established was based on a commercial kit, this can be used by other laboratories to assess the presence of *C. pneumoniae* in CSFs.

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