

PCR in Diagnosis of Infection: Detection of Bacteria in Cerebrospinal Fluids

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The PCR is the most sensitive of the existing rapid methods to detect microbial pathogens in clinical specimens. In particular, when specific pathogens that are difficult to culture in vitro or require a long cultivation period are expected to be present in specimens, the diagnostic value of PCR is known to be significant. However, the application of PCR to clinical specimens has many potential pitfalls due to the susceptibility of PCR to inhibitors, contamination and experimental conditions. For instance, it is known that the sensitivity and specificity of a PCR assay is dependent on target genes, primer sequences, PCR techniques, DNA extraction procedures, and PCR product detection methods. Even though there are many publications concerning basic protocols of a PCR assay, including DNA extraction and preparation as well as the amplification and detection of amplicons, PCR detection of bacteria in clinical specimens such as cerebrospinal fluid (CSF) has not yet been reviewed. Since a variety of clinical specimens, such as blood, urine, sputum, CSF and others, vary in regard to the nature of the content and amount available, careful design of the PCR assay for each specific specimen before a PCR application is conducted is essential. In particular, a diagnosis based on detection of a few bacteria in clinical specimens by using PCR must be carefully evaluated technically as well as microbiologically. In this regard, current studies concerning detection of *Chlamydia pneumoniae* in CSF obtained from patients with multiple sclerosis (MS) by using PCR provide a good example for discussion of use of the PCR assay in diagnosis. Because *C. pneumoniae* is difficult to culture in vitro, often low numbers of bacteria may be detected in the CSF of patients with chronic neurological diseases by PCR. Therefore, in this review general PCR protocols for detection of bacteria in clinical specimens, as well as a specific example of using PCR for detection of *C. pneumoniae* in CSF, will be discussed.

METHODOLOGICAL ASPECTS

The PCR assay in diagnosis involves several critical steps, such as DNA extraction from specimens, PCR amplification, and detection of amplicons. In particular, when specific clinical specimens, such as CSF, with only a few bacteria present are tested by PCR, each procedure must be carefully designed and performed.

CSF. CSF is widely utilized for diagnosis of diseases of the central nervous system (CNS). Because CSF has important functions, including cushioning the brain, maintaining a constant intracranial pressure, providing nutrients, and removing toxic metabolites from the CNS, an indirect assessment of brain status can be obtained from the CSF. Since CSF is considered germfree, detection of microbes in CSF, even in low numbers, provides valuable information about possible infection. However, it must be noted that detection of microbes in the CSF does not always indicate a CNS infection, since impairment of the blood-brain barrier may permit transit of microbes. Nevertheless, detection, identification, and quantitation of microorganisms in CSF is important in diagnosis of meningitis and other CNS infections. In particular, recent studies indicate possible involvement of microorganisms in specific diseases of the CNS, including Alzheimer's disease and MS (3, 30, 45, 56). Therefore, detection of even a few microorganisms in CSF by a standardized protocol is a critical matter for diagnosis of such diseases. The normal adult produces approximately 500 ml of CSF per day, with approximately 150 ml of CSF in the CNS at any given time (34). Thus, the available amount of CSF and numbers of samplings for diagnosis are limited. Therefore, performing PCR using a CSF specimen will become the first-line diagnostic test for CNS infections (11, 33), due to a sensitivity requiring only a limited amount of CSF, the specificity of the assay, and speed. In fact, a number of trials using PCR for detection of a broad range of bacteria in CSF specimens have been reported (37, 38, 42, 66). However, the sensitivity and specificity of PCR assay for detection of pathogens may not be better than those

of culture assay, which has been standardized and validated for most pathogens, due to the dependability of PCR sensitivity on the assay process. Therefore, a negative PCR result can be used with moderate confidence to rule out a diagnosis of infection (33).

The stability of target bacterial DNA during CSF storage is an important practical matter in clinical laboratories. However, only limited information on the effects of various handling and storage conditions on the stability of bacterial DNA in CSF is available. Exposure of CSF to various environmental conditions, such as room temperature versus 4°C for up to 96 h and freeze–thawing up to three times, does not affect the ability of a highly sensitive PCR assay to detect bacterial DNA in CSF samples (60). That report, however, tested only limited environmental conditions. Therefore, proper storage and handling of CSF are still essential for detection of microbes after PCR amplification.

Contamination. Since PCR is based on DNA amplification, false–positive or –negative outcomes may easily occur. In particular, a single PCR cycle results in very large numbers of amplifiable molecules that can potentially contaminate subsequent amplifications of the same target sequence (39). In fact, a primary source of false–positive reactions has been identified as carryover of amplified product from previous reactions (41). Carryover contamination of reagents, pipetting devices, laboratory surfaces, or even the skin of workers (35) can yield false–positive results. To control such carryover contamination, one must prevent physical transfer of DNA between amplified samples, and between positive and negative experimental controls. For this purpose, preparation of samples for PCR assay must be in a room or biosafety hood separate from that in which the reactions are performed. Using a pipette tip with an aerosol barrier is essential for avoiding cross contamination as well as carryover contamination. UV exposure is also able to destroy contaminating amplicons but is efficient only on surfaces and with amplicons greater than 300 bp size (19). Using uracil *N*–glycosylase (UNG) to cleave the dUTP incorporated in PCR products is considered a powerful protocol to prevent carryover amplicon contamination enzymatically (41), particularly in a clinical laboratory that is performing PCR extensively. This is performed by substituting dUTP for dTTP and adding UNG to the master mixture. To protect the dUTP–containing product, UNG must be inactivated chemically or by heat before the PCR product can be analyzed further. Therefore, the dUTP protocol requires only two changes in a standard PCR protocol: the substitution of dUTP for dTTP in all reactions and the incubation of all PCR mixtures with UNG prior to temperature cycling. In fact, this protocol has been applied successfully to detection of *Toxoplasma gondii* in CSF as well as other clinical specimens (46).

DNA extraction. Since clinical specimens have PCR inhibitors, such as hemin, which binds to *Taq* polymerase and inhibits its activity (10), DNA purification is important to avoid such effects. In fact, inhibitors are detected frequently in CSF specimens (14), and boiling of CSF is not sufficient for removal of inhibitors which affect the detection of microbes by PCR (9). The extraction yield of target DNA is also a critical factor in the PCR detection of bacteria in clinical specimens, particularly when only a few bacteria are expected to be in specimens. 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 an additional consideration for sample preparation.

The classical DNA extraction protocol is based on purification with organic solvents like phenol–chloroform, followed by precipitation with ethanol. The precipitates obtained from CSF containing only a few bacteria may contain too little material and may not be visible. Therefore, handling of these precipitates may require guesswork, particularly during the washing of the precipitates. Thus, it seems likely that the resulting yield of bacterial DNA from CSF with only a few bacteria may not be consistent. In this regard, a recent study developed a new protocol for purification of DNA by using solid–phase carriers, which selectively absorb nucleic acids (7). This protocol is based on the nature of nucleic acids, which can bind to silica or glass particles in the presence of chaotropic agents such as NaI or NaClO₄ (44, 62, 65). A chaotropic extraction–glass fiber filter DNA purification (GFX; Pharmacia Biotech, Milwaukee, Wis.) is such a protocol and utilizes a glass fiber matrix for DNA isolation. A DNA isolation kit based on the guanidinium isothiocyanate–silica bead method (7) is also commercially available (NucliSens isolation kit; Organon Teknika, Durham, N.C.). The NucliSens isolation kit results in sufficient DNA yield and a highly reproducible PCR for β–globin on fixed cells (16). However, there is no report regarding application of such kits to

the isolation of bacterial DNA from clinical specimens. Fahle and Fischer (22) examined the efficacy of viral DNA isolation from clinical specimens, including CSF, using six different commercial DNA extraction kits. It was concluded in the report that the NucliSens isolation kit and the Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, Minn.) were the most sensitive among the kits tested, including the Generation capture column kit (Gentra Systems), MasterPure DNA purification kit (Epicentre Technologies, Madison, Wis.), IsoQuick nucleic acid extraction kit (MicroProbe Corp., Bothell, Wash.), and QIAamp blood kit (Qiagen, Valencia, Calif.), for extracting cytomegalovirus DNA from clinical specimens, based on DNA recovery with the broad range of specimen types evaluated. Similar evaluations of DNA extractions with commercial kits were also performed by three other groups (13, 36, 61). From these studies, the QIAamp kit was found to be more suitable than other commercial and noncommercial methods evaluated for the extraction of DNA for PCR. Some commercially available extraction and purification kits based on a solid-phase purification protocol are listed in Table 1. These kits eliminate not only guesswork but also labor-intensive phenol-chloroform extraction steps. However, there is only limited information regarding bacterial DNA isolation from clinical specimens, particularly CSF, with these commercial kits.

View this table: In this window In a new window	TABLE 1. Commercial DNA extraction kits based on the solid-phase purification protocol
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Target genes. The choice of target genes and the design of oligonucleotide primers are critical elements in determining the sensitivity of PCR (29, 53). Even when the same gene is selected as a target, PCR with different primer sets shows a 100- to 1,000-fold sensitivity difference between primer sets (29). Therefore, the sequence of primers is important in the sensitivity and specificity of PCR. The sensitivity of PCR is also dependent on the target gene selected, because copy numbers of genes or operons per bacterium vary. In this regard, if only the sensitivity of PCR is considered, reverse transcription-PCR is another selection method due to the multiple copy numbers of mRNAs per bacterium. However, the practical value of reverse transcription-PCR in diagnosis is limited due to the short life span and the vulnerability of bacterial mRNAs.

Sequence polymorphism of a target gene is another concern in regard to PCR specificity. Some bacterial genes, such as the *C. trachomatis* outer membrane protein gene, have hypervariable regions within the gene (23). Therefore, PCR products of different sizes as well as different sequences may occur between clinical isolates of the bacterium when such a gene is selected as a target for PCR.

A universal PCR that amplifies conserved regions in various bacteria is ideal to detect any pathogen in screening of clinical specimens (8, 40, 42, 49, 63). For this purpose, the conserved region of the 16S rRNA gene has been selected as a target gene for the universal PCR due to the fact that almost all common bacterial pathogens found in body fluids have been sequenced (27, 42, 49). Utilizing this universal PCR, a high detection sensitivity of PCR for 10 gram-negative and 250 gram-positive bacteria in CSF has been reported (42). However, since the universal PCR can detect almost all bacteria, including normal flora such as staphylococci on the skin, discrimination for contaminants is difficult, particularly when specimens contain few bacteria.

PCR protocols. There are several PCR protocols to enhance sensitivity, especially when dealing with small numbers of bacteria as the target. Nested PCR is one of these protocols for detection of only a few bacteria in clinical specimens. The process utilizes two consecutive PCRs. The first PCR contains an external pair of primers, while the second contains either two nested primers that are internal to the first primer pair or one of the first primers and a single nested primer. The larger fragment produced by the first reaction is used as the template for the second PCR. The sensitivity and specificity of DNA amplification can be considerably improved by using such nested PCR, sometimes with 1,000 times more sensitivity than a standard PCR. However, in the case of detection of *C. pneumoniae* by nested PCR in a standard solution spiked with bacteria, sensitivity was not always improved compared with that of a standard single PCR. For example, nested and single PCRs with primers specific for the *C. pneumoniae* *omp-1* gene showed the same sensitivity (0.005 inclusion or 2.5 elementary

bodies per PCR) (2).

A frequently encountered problem in PCR amplification of target gene sequences is the appearance of spurious smaller bands in the product spectrum (17). This is usually interpreted to be due to mispriming by one or both of the oligonucleotide primers to the target template. Touchdown PCR is designed to avoid such problems and provides a clearly specific PCR band. The touchdown PCR utilizes a protocol with decreasing annealing temperatures at every cycle from above to below the expected annealing temperature (17). The application of this technique to detection of *C. pneumoniae* provided an improved analytical sensitivity (0.004 to 0.063 inclusion-forming unit per PCR) (43).

Detection of PCR products. There are several different detection protocols reported for PCR products besides the traditional electrophoresis method on an ethidium bromide-containing agarose gel. Southern hybridization with a specific probe labeled with a radioisotope or nonradioisotope marker to PCR amplicons has been widely utilized for the study of PCR specificity. This detection protocol also provides a higher sensitivity than the ethidium bromide detection method but requires extra blotting and hybridization steps. The digoxigenin (DIG)-PCR-enzyme-linked immunosorbent assay (ELISA) is one of the PCR amplicon detection methods utilizing a microtiter plate and is now commercially available (Roche Molecular Biochemicals, Indianapolis, Ind.). This method involves capture amplicons labeled with DIG by the probe immobilized onto the surface of a streptavidin-coated ELISA plate. The bound hybrid is detected with an anti-DIG-peroxidase conjugate and the colorimetric substrate. This ELISA system has been shown to be 10 to 100 times more sensitive than the traditional electrophoresis method (48). The PCR-immunoassay detection method utilizing a special small device (Clearview Immunoassay Detection Device; Oxoid Inc., Ogdensburg, N.Y.), which holds a membrane and a sample application pad containing latex beads labeled with an anti-2,4-dinitrophenol antibody, is another type of detection method for amplicons designed to detect specific bacteria in clinical isolates (12, 59). The membrane utilized in this system is coated with lines of antibiotin antibody and anti-DIG antibody. Therefore, an evaluation of PCR results as positive or negative by utilizing this detection kit in clinical laboratories which do not have electrophoresis equipment is relatively easy. The application of this kit for detection of *Neisseria meningitidis* in CSF showed a detection limit of one to three organisms per PCR, which is 10 times more sensitive than detection of PCR products on traditional electrophoresis with agarose gels (54). Fluorescent probe-based assays with labeled primers or specific probes labeled with a fluorescent dye have been developed with the advantages of a closed system that avoids carryover contamination during the PCR and increased detection sensitivity for amplicons. There are two types of assays, using real-time and end point readings. Particularly the real-time PCR, which provides quick and accurate information regarding target genes, has been increasingly utilized. This approach has the advantage of quantitating the PCR in the exponential phase rather than using the end point accumulation of PCR product or trying to capture the PCR in the exponential phase, as was done previously in many quantitative PCRs (52). This non-gel-based technique has several other advantages over ordinary agarose gel-based techniques. For instance, this system allows a large increase in throughput. The fluorescent-probe assay is run in a 96-well format, and many of the steps in the assay are automated. The assay is a closed system in which the reaction tube is never opened after amplification. In addition, it uses an automated detection system that quantitates and calculates the degree of fluorescence over that for the control at each cycle and hence accurately defines the cycle number and linear range for a positive result (52). Even though presently there are few reports on detection and quantitation of bacteria in CSF by real-time PCR, this technique has excellent potential as a major protocol for PCR detection of bacteria in clinical specimens, including CSF, due to these advantages.

DETECTION OF *C. PNEUMONIAE* IN CSF BY PCR

C. pneumoniae is an obligate intracellular bacterium responsible for a variety of respiratory illnesses, including 10% of community-acquired pneumonias, bronchitis, pharyngitis, and sinusitis. Seroepidemiologically, 50 to 80% of the adult population has been shown to have prior exposure to this pathogen (4, 26, 57). Furthermore, recent studies have revealed that this bacterium may be involved in some chronic inflammatory diseases, such as asthma (28), arthritis (25), atherosclerosis (47), Alzheimer's disease (3), and MS (56). Since the culture of *C. pneumoniae* is difficult in most clinical laboratories, determination of this bacterium in clinical specimens has been widely performed using the PCR

technique even though there is no standardized PCR method for detection of this organism (2). Therefore, PCR results with clinical specimens to detect this bacterium vary widely (2).

MS is a chronic demyelinating disease of the 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 any immunological mechanisms (24). To identify a specific causative agent for MS, many groups have attempted to detect microbes in CSF as well as lesions of CNS obtained from MS patients. However, there have been no consistent results with specific pathogens. Recent studies by Sriram et al. (56) highlighted a possible involvement of a bacterium in MS with the finding of *C. pneumoniae* in the CSF of nearly all patients with MS but in only a small proportion of CSF samples from control subjects without MS by utilizing PCR and culture methods. That study has shown the highest association with MS of any organism to date. However, other research groups were not successful in detecting this bacterium or found only a low rate of detection of *C. pneumoniae* in CSF from MS patients (Table 2).

View this table: In this window In a new window	TABLE 2. Detection of <i>C. pneumoniae</i> DNA in CSF by PCR
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To date, there have been 10 reports concerning detection of *C. pneumoniae* in CSF from MS patients by PCR (Table 2). The results of studies concerning the presence of *C. pneumoniae* in CSF of MS patients as determined by PCR have shown a very wide variation in the positive rate, ranging from 0% to almost 100%. Such variation of the *C. pneumoniae* positive rate in CSF may be dependent on the source of CSF and/or the PCR protocol utilized. Since there is no standard PCR protocol for *C. pneumoniae* detection and no consistent pattern of positive results among the various laboratories determined by a multicenter comparison trial of PCR assays for detection of *C. pneumoniae* (2), each step of the PCR protocol utilized in each study should be carefully reviewed.

***C. pneumoniae* DNA extraction.** Five of the 10 published papers mentioned above were letters; therefore, details of DNA extraction and the PCR protocols utilized in these studies were not fully described. For instance, Numazaki and Chiba (K. Numazaki and S. Chiba, Letter, Neurology 57:746, 2001) and Pucci et al. (E. Pucci, C. Taus, E. Cartechini, M. Morelli, G. Giuliani, M. Clementi, and S. Menzo, Letter, Ann. Neurol. 48:399-400, 2000) did not provide information as to the amount of CSF tested. The amount of starting material as well as the final volume of DNA solution appears to affect the sensitivity of overall detection. Therefore, whether the protocol utilized is sensitive enough or not cannot be evaluated due to the lack of information in such reports. Only two reports, which were full papers, described the amount of CSF tested, the DNA extraction protocol, the final volume of the DNA solution, and the final concentration of CSF used for PCR. For instance, Ikejima et al. (31) extracted DNA in 50 µl from 200 µl of CSF, which means a fourfold concentration of the original CSF volume. In the work of Sriram et al. (56), DNA was extracted in 20 µl from 300 µl, a 15-fold concentration of CSF. It is apparent that these different concentrations of CSF may affect the final sensitivity of the PCR assay. None of the reports except those of Ikejima et al. (31) and Sriram et al. (56) provided the final volume of DNA extracted.

Most studies utilized the DNA extraction protocol with solid-phase carriers, such as Qiagen columns that hold a silica gel membrane. In only two studies, those of Sriram et al. (56) and Pucci et al. (letter), was DNA extraction performed by a standard extraction protocol such as phenol-chloroform and ethanol precipitation. As mentioned above, the use of solid-phase carriers for DNA isolation may contribute to a consistent DNA extraction yield, particularly with CSF, which may not contain many leukocytes or microbes in a sample if the patients do not have meningitis.

It is notable that only one report (31) considered the extraction protocol for bacterial DNA. *C. pneumoniae* is a gram-negative bacterium and has lipopolysaccharide and other outer membrane components in its cell wall, which

contribute to osmotic stabilities as well as to rigidity, particularly of elementary bodies, the 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 that may have only a few bacteria, such as CSF from MS patients. In fact, the study showed that when two extraction protocols were examined, one designed for extraction of mammalian DNA from blood samples and one designed for extraction of bacterial DNA, the protocol for bacterial DNA extracted the microbial DNA more efficiently (31).

PCR method. The major outer membrane protein (MOMP) genes, such as *omp-1* (*ompA*) (32), of *C. pneumoniae* have been utilized widely in PCR as a target gene for detection of this bacterium. *C. pneumoniae* has many outer membrane proteins, including cysteine-rich proteins OmcA and OmcB (20, 21) as well as the MOMP encoded by *omp-1*. It is known that *C. trachomatis* MOMP has genetic variation, including in amino acid sequences (58), but not much information regarding *C. pneumoniae* MOMP is available. Therefore, the design of primers for MOMP genes must be undertaken with special care. The species-specific region of the 16S rRNA gene is also frequently utilized as a target gene in PCR for detection of *C. pneumoniae* (15, 18; S. A. Morre, C. J. De Groot, J. Killestein, C. J. Meifer, C. H. Polman, P. Van der Valk, and A. J. Van den Brule, Letter, Ann. Neurol. 48:399, 2000). In this regard, it is noteworthy that the detection sensitivities of the two PCRs with *omp-1* versus 16S rRNA gene primers under each set of optimal conditions were different (31). The PCR for *omp-1* 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 CSF obtained from MS patients, the PCR for the 16S rRNA gene could not detect any *C. pneumoniae* DNA, even though the PCR for *omp-1* detected *C. pneumoniae* DNA in the same CSF samples. Whether the design of primer sequences or the selected target gene is responsible for the different sensitivities of these PCRs is unknown, but it is apparent that both primer design and choice of target gene for PCR for detection of *C. pneumoniae* in CSF are important in the detection of *C. pneumoniae* by PCR.

It is generally accepted that nested PCR may be more sensitive than single PCR due to the utilization of two consecutive PCRs. However, in practice, nested PCR does not always give a higher sensitivity than single PCR (2). In addition, nested PCR is much more prone to contamination. Therefore, detection of *C. pneumoniae* in CSF which may not contain many bacteria by nested PCR must be carefully performed; otherwise, no other method presently can confirm positive PCR results.

Detection of *C. pneumoniae* in CSF. Controversy surrounds the detection of *C. pneumoniae* in CSF obtained from MS patients, primarily because of the lack of a definitive test for detecting the small numbers of *C. pneumoniae* present. Culture is always considered the "gold standard" in microbiology but is difficult to perform for certain fastidious bacteria such as *C. pneumoniae* in specific clinical specimens. For instance, this bacterium has not been successfully cultured from blood samples, although its DNA can be detected in blood and the organism has been recovered in limited numbers from vascular tissue specimens (18). Even though PCR enables the detection of low concentrations of bacteria in clinical specimens, great variability of detection is usually found in CSF from MS patients (Table 2), atherosclerotic tissue samples, and peripheral blood mononuclear cells, ranging from a 0 to 100% detection rate between studies (5, 6, 64). In this regard, a recent study conducted by Smieja et al. (55) demonstrated the relationship between target concentrations and PCR detection rate; that is, lower concentrations of *C. pneumoniae* were only intermittently PCR positive, and this relationship was predictable from a statistical viewpoint. From this point of view, theoretically a larger number of replicates of a PCR assay may result in a better chance for detecting low numbers of bacteria. In other words, the negative PCR results obtained from a single PCR test may not be a true negative due to the low validity of detection with a lower concentration of target. Since none of the papers reporting results of *C. pneumoniae* detection by PCR in CSF from MS patients provided any replicate number of PCR tests, the negative results reported may possibly not be true negatives but could indicate that there were few bacteria, if any, present.

CONCLUSIONS

It has been well documented that specific infectious agents may be involved in

autoimmune diseases, such as *Trypanosoma cruzi* as the causative agent of Chagas' disease and *Streptococcus pyogenes* and measles virus for encephalomyelitis (51). Some chronic inflammatory diseases which are not yet definitely classified as autoimmune diseases are also considered to be linked to some microbial infections. However, in contrast to infectious diseases, causative or contributing microbes for such chronic inflammatory diseases, including autoimmune diseases, may not be readily detected in specimens obtained from the specific lesion. Since chronic inflammatory diseases as well as autoimmune diseases, including atherosclerosis and MS, may be attributed to the immune response through molecular mimicry and/or the possible adjuvant effect of infectious agents (50), the presence of microbes in a lesion may not always be necessary. Even under such circumstances, a consistent detection of bacteria in specimens should be critical in diagnosis and future therapy. In this regard, PCR is the most reliable assay for detection of microbes in clinical specimens. Careful design and protocol for a PCR assay to detect, measure, and identify microbes in clinical specimens are essential. Analysis of PCR results is also a critical issue in diagnosis, particularly for chronic inflammatory diseases. Application of a clinically relevant PCR assay to these issues in monitoring bacterial presence in CSF may reveal a role for bacteria, such as *C. pneumoniae*, in chronic inflammatory or autoimmune diseases, such as MS.

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FOOTNOTES

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