

**Detection of *Chlamydia Pneumoniae* in the  
Cerebrospinal Fluid of Multiple Sclerosis Patients  
and other Neurological Disorders**

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**Abstract**

**Background:** Multiple Sclerosis (MS) is a chronic demyelinating and neurodegenerative disease of the central nervous system (CNS). Although the etiology of MS is not completely understood, there is evidence suggesting a role for infectious agents in the pathophysiology of MS. There are evidences of a possible association between MS and *Chlamydia pneumoniae* (CPn), however a consensus on this issue has not been reached. The aim of this study was to shed light on the possible role of CPn in MS by analyzing cerebrospinal fluid (CSF) of MS patients and a control group of patients with other neurological disease (OND) for CPn DNA and anti CPn IgG. We studied 71 CSF samples, including 35 specimen from MS patients and 36 from patients with OND. Collected samples underwent PCR designed to amplify a 207 base pairs (bps) fragment of CPn DNA using *omp1* primers specific for *omp1* gene and also were analyzed for the presence of anti-CPn IgG by a commercially available Enzyme-linked immunosorbent assay (ELISA) kit. Successful amplification of a 207 bps fragment, was considered as positive results by PCR. Out of 35 MS patients, 9 (25.7%) samples were positive for CPn while only one sample (2.77%) from OND group showed positive result. The P value for this  $\chi^2$  test with 1 degree of

freedom is 0.016. That is, the non-homogeneity of positive response between these two groups of experiment and control was significant at the level of less than 0.016 ( $\chi^2=5.94 > \chi^2_{0.016,1}=3.84$ ). The mean optical density of samples from patients and control group was  $0.17 \pm 0.170$  and  $0.18 \pm 0.213$  respectively (P value=0.85). Our results are in line with the results reached by the meta-analysis of other studies in this field, and may suggest that CPn probably plays a bystander role in the pathology of MS. Past exposure to CPn is nearly equal in MS and controls, but owing to infecting immune cells, CPn is more frequently detected in the site of inflammation (CSF) as evidenced by PCR. This view is also supported by studies which show that use of antibiotics active against CPn is not associated with a decreased risk of MS and that MS patients do not benefit from antibiotic treatment against CPn.

**Keywords:** Multiple Sclerosis, *Chlamydia pneumoniae*, cerebrospinal fluid, Polymerase Chain Reaction, Isfahan, Iran

## Introduction

Multiple Sclerosis (MS) is an autoimmune, inflammatory and demyelinating disease of the central nervous system which mostly affects young adults. Environmental and genetic influences have been proposed to contribute to the susceptibility to MS. (8,13)

Infectious agents have long been considered to be involved in the pathophysiology of MS, however, conclusive evidence for a causal relationship between any infectious agent and MS is currently lacking. Among bacteria, *Chlamydia pneumonia* (CPn), an obligate intracellular and gram-negative pathogen, has been linked to various neurological diseases such as Alzheimer disease and MS.(22) The studies in MS, however, have yielded conflicting results. After the first demonstration of CPn in the CSF samples of MS patients by Sriram and colleagues in 1999,(21) the association between MS and *Chlamydial* infection was studied by several groups which have yielded conflicting results.(2,7,11,12,13,14,15,17,19,20,23)

The aim of this study provide more data on the possible role of CPn in MS by Measuring simultaneously the presence of DNA and IgG synthesis against CPn in cerebrospinal fluid (CSF) of MS patients and a control group of patients suffering from other neurological disease (OND).

## Materials and Methods

### *Participants*

Patients were recruited from Isfahan MS Clinic and neurology out-patient clinics at Al-Zahra hospital in Isfahan, Iran.(4,18) Totally 35 patients (18 women and 17 men, aged from 20-45 years) with a diagnosis of MS according to the revised McDonald's criteria, (16) were collected. Out of 35 samples, collected from MS patients, 14 samples were taken from those suffering from Relapsing – Remitting MS (RR-MS). 7 samples were obtained from patients with Secondary-Progressive MS (SP-MS) and 3 CSF samples were in Primary-Progressive Group. 2 samples were also taken from Progressive –Relapsing MS (PR-MS) patients. Disease course was not available for 9 patients. 36 patients (19 women and 17 men, aged from 18-53 years) with other neurological disease [Pseudo-tumor cerberi (n=33), normal pressure hydrocephaly (n=2), epilepsy (n=1)] were collected. The CSF specimens were obtained through a lumbar puncture following a standard protocol, and stored at -80° C until analysis. Clinical and demographic characteristic of patients were recorded in a specially designed questionnaire.

The study protocol was approved by the local ethics committee and all patients signed an inform consent form before inclusion in the study.

### *DNA extraction*

DNA was extracted from 2-4 ml of CSF specimens using a QIAmp DNA mini kit (Qiagen, USA). In brief, each specimen was centrifuged for 30 minutes at 1000 round per minute (rpm). The pellet was re-suspended in 180µl of Buffer ATL (Qiagen USA) with 20µl of proteinase-K and then incubated at 56°C with occasional vortexing until the pellet was completely lysed, which usually took 30 minutes. After lysis of the sample, 200µl of buffer AL (Qiagen, USA) was added to the sample and the mixture was incubated with 200µl of absolute ethanol and mixed by pulse-vortexing for 15 seconds. The mixture was applied to a spin column, which holds a silica gel membrane, and spun for 1 minute at 8000 rpm. The spin column was washed with 500µl of buffer AW1 and then with 500µl of buffer AW2 by centrifugation at 14000 rpm for 3min. The DNA bound on membrane was eluted by centrifugation with 50µl of buffer AE (Qiagen, USA) after 5-minute incubation at room temperature. The DNA extracts were stored at -20°C until polymerase chain reaction (PCR) assessment.

*Detection of Chlamydia pneumoniae by PCR*

To detect the CPn DNA in CSF samples, the extracted DNAs were subjected to PCR with primers specific for CPn *omp1* gene and a 207 base pairs (bps) fragment was amplified. The sequences of the primers were 5'-TTA TTA ATT GAT GGT ACA ATA-3' and 5'- ATC TAC GGC AGT AGT ATA GTT-3'.(9) Briefly, amplification was carried out in a Minicycler. The first cycle, consisting of 5-min pre-denaturation at 94°C, was followed by 40 cycles each of 30 s at 94°C, 45 s at 50°C and 60 s at 72°C, with a final extension for 10 min at 72°C. PCR was performed on 3µl of the extracted DNA in a final reaction mixture of 25µl. The final reaction mix contained 4 mM MgCl<sub>2</sub>, 0.8µl deoxynucleotide triphosphate, 10 pM of each primers and 1U of Taq polymerase. The PCR products were analyzed by agarose gel (1.5%) electrophoresis. In this study, purified DNA of CPn (Vircell, Spain) was used as positive control and distilled water was used as the negative control.

In order to demonstrate the presence of DNA in negative samples, specific primers for GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) gene which is detectable in all eukaryotic cells, were used. The sequence of GAPDH primers used were:

5'-ATGGGGAAGGTGAAGGTCG-3' and 5'GGGGGTCATTGATGGCAACA-3'. (25)

*Detection of IgG against Chlamydia pneumonia*

A commercially available Enzyme-linked immunosorbent assay (ELISA) kit (Euroimmune, Germany) specifically detecting IgG reactive with CPn was used to detect the level of IgG in the samples. According to the kit manufacturer, a 1:100 dilution should be used for serum or plasma samples. However the concentration of most proteins is lower in CSF, thus CSF samples were diluted 1:2 to prevent false negative responses. Instructions provided by the manufacturer accompanied by the kit were then followed. The optical density of each sample was transferred to positive or negative by Z-Score in SPSS software.

*Statistical analyses*

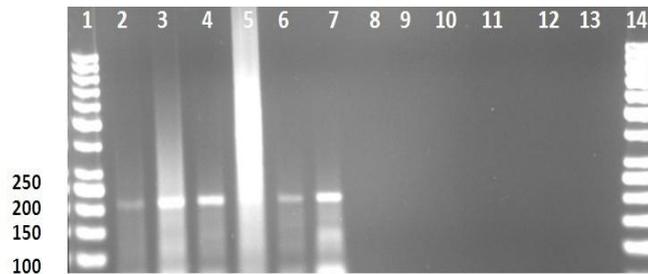
Data analysis was carried out based on calculated results. Homogeneity test was used to compare the rate of presence of CPn between two groups. To compare the means of optical density between two mentioned groups, the Mann Whitney test was used due to not having normality, based on the results from the Kolmogorov-Smirnov.

## Results

Successful amplification of a 207 bps fragment was considered as positive results by PCR (Fig1). Out of 35 MS patients, 9 (25.7%) samples were positive for *C.pneumoniae* while only one individual from 36 samples (2.77%) from OND group showed positive result. The P value for this  $\chi^2$  test with 1 degree of freedom is 0.016 ( $\chi^2=5.94 > \chi^2_{0.016,1}=3.84$ ).

Out of 35 MS patients, 12 (34.2%) were IgG positive and among those suffering from other neurological disease 11 (31.4%) were positive for IgG against CPn.

The mean optical density of CSF samples from patients and control group was  $0.17 \pm 0.170$  and  $0.18 \pm 0.213$  respectively (P value=0.85).



**Fig1:** Analysis by agarose gel (1.5%) electrophoresis of the PCR products: The 207 bp DNA corresponds to Specific *Chlamydia pneumoniae* DNA sequence. Lane 1 and lane 14: **50bp** ladder. Lane 2: Positive control (*C.pneumoniae* DNA). Lane 3,4, 5, 6 and 7: Products of positive samples. . Lane 8 to12: Negative CSF samples. Lane 13: Negative control.

## Discussion

Our results showed that past infection with *Chlamydia pneumoniae* as evidenced by the humoral IgG immune response in the CSF, did not differ between MS patients and controls. However, as measured by PCR detection of DNA, CPn was more frequently present in the CSF of MS patients than in controls.

In our study, 25.7% of CSF samples of MS patients were positive for CPn DNA. The presence of CPn DNA varies widely amongst the studies ranging from 0-97% (2,5,6,12,19,20). These inconsistencies stem from methodological differences

including DNA extraction method, primers sequences or PCR protocols.(10) It has been shown that PCR conditions must be optimized in order to obtain a signal for CPn in the presence of low copy number of organism(9). DNA extraction by Qiagen QIAmp DNA mini kit with bacterial extraction (as used in this study) rather than Qiagen QIAmp Blood mini kit has been shown to result in increased PCR sensitivity (20 copies versus 200 copies, respectively)(9). It has also been demonstrated that primers for MOMP DNA gene which were utilized in this study, had a 10-fold increase in sensitivity in comparison with primers for the species-specific regions 16SrRNA gene.(9) Thus our method accordingly had an acceptable sensitivity for detecting CPn, reducing the chances of false negative results.

The difference for presence rate of CPn DNA in MS patients and OND controls was significant in our study. However, different studies used different control groups and also some included patients with inflammatory CNS disease and non-inflammatory CNS diseases, which might have caused the conflicting results.(6) However, a recent meta-analysis suggests statistically significant association between MS and the PCR detection of Cpn DNA in the CSF, which is confirmatory to the results of this study.(2) Taken together, the increased rate of the presence of CPn DNA in the CSF of MS patients implies that CPn traffics into the CNS or simply to the site of pathology/inflammation. Given that CPn can potentially infect macrophages, monocytes, and lymphocytes (22). It could be hypothesized that CPn could be transported to site of inflammation by infected immune cells. There are evidences that CPn DNA is more present in the CSF of relapsing-remitting MS where inflammation is a major component compared to secondary progressive and primary progressive MS which are less inflammatory and more degenerative. In addition, CPn DNA could be detected more frequently in clinically and radiologically active than stable MS(3). These evidences suggest that the rate of presence of CPn DNA in the CSF of patients is dependent on the degree of inflammation present in the CNS.

Nearly one third of both the MS group and control group were positive for IgG against CPn in their CSF. In line with our results, a recent meta-analysis showed that CSF IgGs against CPn have no association with MS.(2) The equal humoral IgG response indicates that both groups have been equally exposed to CPn in the past. The fact that both groups are exposed equally to CPn is also shown in a prospective study which serum antibodies to CPn did not predicted the risk of MS (14).

In conclusion, our results are in line with the results reached by the meta-analysis of other studies in this field, and may suggest that CPn probably plays a bystander

role in the pathology of MS. Past exposure to CPn is nearly equal in MS and controls, but owing to infecting immune cells, CPn is more frequently detected in the site of inflammation (CSF) as evidenced by PCR. This view is also supported by studies which show that use of antibiotics active against CPn is not associated with a decreased risk of MS and that MS patients do not benefit from antibiotic treatment against CPn. (1,24)

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