Detection of Chlamydia *pneumoniae* in Multiple Sclerosis Patients

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Abstract

Introduction: Multiple Sclerosis (MS) is a demyelinating neurological disease. Although it is considered an autoimmune, the exact etiology of MS has yet to be identified. MS is hypothesized to be caused by infectious agents that initiate an autoimmune reaction and/or death of oligodendrocytes. These latter events result in gradual disappearance of the myelin sheath of nerve fibers causing multiple symptoms and, ultimately, neurological deficit. Among the infectious agents linked to MS is Chlamydia *pneumoniae*. This agent has been found in various studies to be prevalent in MS patients compared to control individuals.

Objectives: In this study, we investigated the presence of C. *pneumoniae* in sera and cerebrospinal fluids (CSF) of MS patients.

Methods: Serum and CSF were obtained form 36 MS patients and 37 control donors, including healthy donors and patients with other neurological diseases. In order to increase the sensitivity of detection, nested polymerase chain reaction (PCR) was utilized.

Results: Although multiple protocols of DNA extraction and PCR procedures were utilized, C. *pneumoniae* DNA was not evident in all samples of MS patients. On the other hand, amplified DNA of C. *pneumoniae* was inconsistently detected in serum samples in only three control individuals.

Conclusions: These results suggest lack of apparent association of C. *pneumoniae* to MS.

Keywords: Multiple sclerosis, Chlamydia *pneumoniae*, nested PCR.

Abbreviations: MS, multiple sclerosis; CSF, cerebrospinal fluid; MOMP, major outer membrane protein.

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Introduction

Multiple Sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system. [1]

MS is thought to be an autoimmune disease where the immune system attacks the myelin protein. The disease may also be caused by death of oligodendrocytes, the cells that produce myelin.

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The clinical manifestations of the disease are highly variable and can include impaired vision as well as abnormalities in the motor, sensory, and coordination systems. About 80% of patients initially present with a relapsing-remitting disease, which transforms into a secondary progressive course. A smaller subgroup of patients presents with a primary progressive form, which usually results in a more rapid accumulation of disability than the other forms of the disease.

Although the etiology of MS is unknown, several causative factors have been implicated. The contribution of genetic factors is clear from previous studies reporting significantly higher risk of MS in monozygotic twins than in dizygotic twins [2] and in first-degree relatives. [3]

However, incomplete concordance indicates the involvement of nongenetic factors. A possible etiological factor of MS is infectious agents as indicated by the geographical distribution of the disease [4] as well as migration trends from low-to high-susceptible regions. [5, 6] The increase in IgG levels in cerebrospinal fluid (CSF) manifested as oligoclonal bands in patients with MS may also suggest an infectious cause. [7]

Numerous infectious agents have been considered as potential causes. An agent that has been associated with MS is Chlamydia *pneumoniae*. This biological agent is an obligate intracellular bacterium often associated with various respiratory illnesses. Using a nested polymerase chain reaction (PCR) technique, Sriram et al. [8] reported a remarkable 97% of the CSF samples from MS patients to be positive for C. *pneumoniae*, compared to only 18% of the samples from controls. These authors also isolated the organism from CSF of 64% of MS patients compared to 11% of patients with other neurological diseases. In support of these results, Treib et al. [9] and Layh-Schmitt et al. [10] identified this organism, also by PCR, in the CSF of approximately 50% of their MS patients relative to none of patients with other neurological diseases.

In a recent study, the overall prevalence of MS in Jordan was found to be 38–39/100,000. [11, 12] According to the classification of Kurtzke, [4] the distribution of MS in Jordan makes the disease highly prevalent and, hence, critical to be studied. Based on the interesting pathogenic link to multiple sclerosis, we have decided to analyze serum and cerebrospinal fluid of Jordanian MS patients in comparison to control individuals for the presence of DNA of C. *pneumoniae*. Due to the effectiveness and sensitivity of nested PCR, we have used this technique for DNA detection.

Materials and Methods

Patients: Thirty six MS patients (25 females and 11 males) with clinically definite MS were randomly selected from those attending the Neurology Clinic at Al-Bashir Hospital and the Neurology Clinic at the University Hospital of Jordan University during the period of April 2007 and May 2008. These patients suffered from either relapsing- remitting MS, secondary-progressive MS, or primary-progressive MS with various symptoms and clinical manifestations. MS patients included in the study were selected to be 18 years and older, having had an exacerbation or progression of MS disease within the preceding 3 years, and having had a complete neurological assessment within 12 months.

The control patients included 34 healthy individuals randomly selected from the Al-Karak Municipal Hospital, as well as a group of four patients with Other Neurological Diseases (ONDs) who were attending the Neurology Clinic at Al-Bashir Hospital. The OND patients were diagnosed with common neurological disorders including idiopathic intracranial hypertension, chronic inflammatory demyelinating polyneuropathy, peripheral neuropathy and Guillian-Barre syndrome. The MS patients and controls were of similar age and sex distribution.

This project received the approval of the scientific committees at the Faculty of Medicine and the Faculty of Academic Research of Mu'tah University. Participants, whether MS patient or control, were excluded if the individual had a known or possible active Chlamydial-based

disease within the preceding 12 months, or had received treatment with antibiotic agents within the preceding 30 days.

Patient Samples: Approximately, 2 ml of serum and/or cerebrospinal fluid (CSF) were collected, fractionated into aliquots, and frozen. Serum samples included 34 samples from MS patients, 34 from healthy individuals, and 3 from OND patients. CSF samples included 5 from MS patients and 4 from OND patients.

DNA Extraction: Total DNA extraction from serum and CSF samples was performed using Genomic Column DNA Express from Sacace Biotechnologies (Caserta, Italy) according to manufacturer's protocol starting with 200 µl. DNA was eluted in 50 µl of elution buffer. Total DNA from CSF samples was also extracted using DNA-Sorb-B DNA Extraction Kit (Sacace Biotechnologies, Caserta, Italy).

Polymerase chain reaction (PCR) **of bacterial DNA**

Bacterial Major Outer Membrane Protein (MOMP) gene was the amplification target using nested PCR. The first reaction was performed using outer primers that defined a 704-bp region (Table 1). This was followed by a second reaction using inner primers amplifying a 238-bp region. The PCR reaction was run using two methods that differed at the primer annealing temperature of the outer primers. In the first method, a touchdown PCR reaction was used as described by Sriram et al. [13] whereby the annealing temperature for the outer primers was reduced by 0.5°C for every cycle from 60°C down to 50°C. The amplification reaction was continued for an additional 20 cycles at 50°C. In the other method, the annealing temperature for the same set of primers was fixed at 45ºC for all cycles. The annealing temperature for the inner primers in both methods was set at 58ºC. Both PCR reaction methods started at 95ºC for 10 minutes, followed by 40 cycles of a denaturation step at 95ºC for 1 min, the set annealing temperature for 1 min, and a DNA polymerization step at 72ºC for 1 min. These cycles were followed by a last step at 72ºC for 10 min for final extension of amplified DNA. All PCR reactions were carried out using Thermal Cycler (MJ Research, Waltham, MA, USA). Amplification of DNA was tested by gel electrophoresis (Promega, Madison, WI, USA).

Polymerase chain reaction (PCR) **of beta-globin**

The quality of samples was examined by amplification of a 268- bp beta- globin DNA using KM29 primer (5'-GGTTGGCCAATCTACTCCCAGG-3') and RS42 primer (5'-GCTCACTCAGTGTGGCAAAG-3'). The PCR reaction was run at 94ºC for 10 minutes as an initial step, then 32 cycles of 94ºC for 30 seconds, 58ºC for 30 seconds, and 72ºC for 30 seconds. These cycles were followed by a last step of 10 min at 72ºC for final extension of amplified DNA.

Results

Patients and Samples: Serum and/or CSF samples were collected from 36 MS patients with a mean age of 29.7 ± 7.5 yrs. and a median age of 30 yrs. Twenty five of these patients were females (Table 2). Thirty patients suffered from relapsing-remitting MS, five of them had secondary-progressive MS, and one had primary-progressive MS with various symptoms and clinical manifestations with more than half of them having abnormalities in motor, vision, and sensory systems (Table 3). The majority of the collected MS samples were serum (34 samples), in contrast to only 5 CSF samples with 3 patients donating both serum and CSF samples. The low number of CSF samples obtained was due to the fact that patients enrolled in this study had been diagnosed prior to this study and had previously been subjected to a lumbar puncture. The majority of samples (30 samples) were obtained from relapsing-remitting MS patients representing 83.3% of the different types of samples collected, whereas five samples (13.8%) were taken from patients with secondary progressive and only 1 sample (2.7%) from a patient with primary progressive MS (Table 2).

In order to confirm the suitability of the samples for PCR reactions, beta-globin DNA was amplified in all samples. Based on the presence of 268-bp DNA fragment, the availability of amplifiable DNA was determined in 30 serum samples from MS patients, 33 serum samples from controls (30 from healthy individuals and 3 OND donors), in addition to 3 CSF samples from OND donors, and 4 from MS patients.

Detection of C. *pneumoniae DNA in MS patients*

In order to correlate the presence of C. *pneumoniae* to MS, nested PCR was also performed on CSF and serum samples of MS and control donors. Upon amplifying the MOMP gene of C. *pneumoniae* in the CSF samples, there was no evidence for the presence of any DNA fragment corresponding to 238 bp.

Amplification of bacterial DNA was also investigated in the serum samples of both MS patients and controls. However, no bacterial DNA was detected in serum samples of MS patients. On the other hand, only one control sample showed inconclusive positive results using the method described by Sriram et al. (13). We also attempted a modified PCR method using a lower annealing temperature (45ºC). This temperature was calculated using different formulas to determine the appropriate annealing temperature of the primers used. Although, at this condition, two control serum samples revealed faint DNA fragment, these two samples were not the same as the sample regarded positive for bacterial DNA using the former method.

Table (1): Sequence of oligonucleotide primers used in PCR reactions of *C. pneumoniae* ***MOMP gene.***

PCR primers	***Sequence***
Ex-sense	*5′-AACTATACTACTGCCGTAGA-3′*
Ex-antisense	*5′-GTAGTAGACAATGCTGTGG-3′*
IN-sense	*5′-ACACCTCTTTCTCTTGGAGCGT-3′*
IN-antisense	*5′-TTGATGGTCGCAGACTTTGTTC-3′*

Table (2): Summary of patient information.

Total number patients =	*36*
Average age (± SD) =	*29.7 ± 7.5 yrs.*
Median age =	*30 yrs*
Number of samples (types) =	*36 (34 serum; 5 CSF)**
Type of disease:	
RRMS§	*30 (83.3%)*
SPMS§	*5 (13.8%)*
PRMS§	*1 (2.7%)*

****3 patients with both serum and CSF samples and 2 patients with CSF samples only***

§RRMS = Relapsing-remitting MS; PRMS= Primary progressive MS; SPMS= Secondary progressive MS.

Table (3): Distribution of major clinical manifestations among MS patients.

Symptoms	***Total cases (%)***
Motor system	*27 (75%)*
Vision	*22 (61.1%)*
Sensory system	*23 (63.9%)*
Coordination	*7 (19.4%)*

Discussion

Although the etiology of MS is not known, a number of hypothetical mechanisms may explain how infectious agents can cause MS. One of these mechanisms entails a direct injury to the blood brain barrier resulting in exposure of immunogenic neuroantigens such as myelin to systemic immune cells and a consequent autoimmune reaction. Another theory is based on the concept of molecular mimicry where a pathogen's antigen is similar in sequence and/or structure to a host's antigen eliciting a subsequent autoimmune reaction. Similarities in sequences between pathogens' protein and human's encephalitogenic antigens have previously been illustrated. [14, 15] However, the process seems to be more intricate than a simple sequence or structural homology between peptides to elicit an autoimmune reaction. For example, Lang et al.[16] have illustrated the importance of a structural complex of antigens and major-histocompatibility complex HLA proteins. This may partially explain the genetic association, in particular of MHC molecules, to MS. [17] In addition, it has been found that demyelination is associated with the death of oligodendroglial cells and prior to any inflammatory reaction. [18] A link between a bacterial agent and MS is supported by the efficacy of antibiotic treatment in reducing the number of active brain lesions indicative of inflammatory reactions. [19, 20]

Several reports have illustrated a higher prevalence of *C. pneumoniae* in MS patients. Interestingly, a 20-mer peptide from a protein specific to C. *pneumoniae* and shares a 7-aa motif with a critical epitope of myelin basic protein, a major CNS antigen targeted by the autoimmune response in MS, was identified. This bacterial peptide induces a Th1-cell response accompanied by severe clinical and histological experimental autoimmune encephalomyelitis in rats; a condition closely reflective of many aspects of MS. [21] In addition, development of MS-like symptoms in animals injected with *C. pneumoniae* antigens provides strong evidence for a causative effect. [21, 22] However, conflicting results have been reported where either C. pneumoniae was not detected or it was detected equally in MS patients and control individuals. [23, 24]

In this study, we analyzed serum and CSF samples from MS patients for detection of C. *pneumoniae* DNA. The types of samples collected reflect the distribution of these types of MS among Jordanian populations. [12] For example, relapsing-remitting MS has been identified in approximately 90% of MS patients, similar to what was collected for this study. In regards to the clinical manifestations, patients studied had clinical manifestations covering motor dysfunctions (75%), vision impairment (63%), sensory symptoms (61%), and coordination dysfunction (19.6%). These figures are also similar in terms of the ratio of the clinical manifestations found in Jordanian MS patients. [12]

Well-validated PCR assays can detect chlamydial DNA from various genes of C. *pneumoniae* at the level of sensitivity of one inclusion-forming unit per experimental sample. [13] In addition to the PCR protocol previously reported for the detection of *C. pneumoniae* DNA, [13] we also employed another approach with a lower annealing temperature. Such an approach was previously found to be valid. [25] However, neither approach resulted in the amplification of bacterial DNA in samples of MS patients. In contrast, only 1 control sample revealed the presence of bacterial DNA using the previously published protocol, and two different samples using the lower annealing temperature. These results may suggest nonspecific amplification of DNA as argued by Tondella et al. [26] It is also possible that C. *pneumoniae* were present in patients, but at undetectable levels. Otherwise, bacterial cells may specifically be restricted to brain lesions as has been reported earlier. [27]

In conclusion, we were unable to detect any bacterial DNA in our MS samples. These results do not support an association between incidence of MS and infection with C. *pneumoniae*. This may indicate an insignificant role of this pathogen in the initiation of MS. However, it is also possible that various pathogens are responsible for the onset of the disease since the

samples are obtained from a geographical population different from those of published reports and given the considerable heterogeneity of MS. [28-30] This heterogeneity has been partially illustrated in differences of expression profiling between responders to a common MS treatment and non-responders. [31] This observation has prompted investigators to propose individualized treatment of MS. [32]

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تقصي وجود بكتيريا كلاميديا نيومونيا عند المصابين بمرض التصلب اللويحي المتعدد

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الملخص

يعتقد أن مرض التصلب اللويحي المتعدد مرض عصبي ينتج من إزالة مادة الميلين. وعلى الرغم من الإعتقاد السائد بمهاجمة جهاز المناعة الخلايا الذاتية، إلا أن السبب المحفز لذلك غير معروف. تذهب إحدى النظريات الى أن من مسببات هذا المرض الإصابة بعوامل بيولوجية معدية والتي تحفز جهاز المناعة ليهاجم الجسم ذاته و/أو تتسبب بموت الخلايا الدبقية قليلة التغصن مسببا بذلك أعراضا مختلفة ومنتهياً بعجز عصبي. وأحد هذه العوامل البيولوجية المعدية التي يعتقد بارتباطها بمرض التصلب اللويحي المتعدد بكتيريا كلاميديا نيومونيا. وجدت عدة تقارير هذه البكتيريا شائعة عند مرضى التصلب اللويحي المتعدد مقارنة بعينات ضابطة من أشخاص أصحاء أو من غير المصابين بهذا المرض.

هدف البحث: في هذه الدراسة، تقصينا وجود بكتيريا كلاميديا نيومونيا في عينات دم وعينات السائل النخاعي المخي لمرضى التصلب اللويحي المتعدد.

طريقة البحث: تم الحصول على عينات دم وعينات السائل النخاعي المخي لستة وثلاثين مريضا بالتصلب اللويحي المتعدد بالإضافة لسبعة وثلاثين عينة من أشخاص أصحاء أو مصابين بأمراض عصبية أخرى. لزيادة فعالية اكتشاف المادة الوراثية للبكتريا، تم استخدام طريقة التفاعل السلسلي البلمري المحتوي.

النتائج: على الرغم من استخدام طريقتين لاستخلاص المادة الوراثية للبكتيريا ولعملية التفاعل السلسلي البلمري، إلا أنه لم يتم اكتشاف المادة الوراثية للبكتيريا في أي من عينات مرضى التصلب اللويحي المتعدد وإنما تم اكتشافه فقط في ثلاث عينات الدم الضابطة بشكل متباين.

الخلاصة: هذه النتائج تبين عدم وجود علاقة ظاهرة بين مرض التصلب اللويحي المتعدد وبكتيريا كلاميديا نيومونيا.

الكلمات الدالة: التصلب اللويحي، بكتيريا كلاميديا نيومونيا، السائل النخاعي المخي، طريقة التفاعل السلسي البلمري المحتوى.