

Original Article:**The epidemiology of microbial agents related to patients with multiple sclerosis (MS)**

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ABSTRACT

Multiple Sclerosis (MS) is one of the autoimmune diseases which affects the central nervous system and its etiology has not yet been identified. The disparity between youth and disability in reproductive ages is considered to be of particular importance for this disease and the need for research which illuminates various epidemiological, etiologic, clinical and therapeutic angles of multiple sclerosis is deeply felt. The purpose of this study is to consider the epidemiology of microbial agents related to patients with multiple sclerosis (MS). From 37 patients with multiple sclerosis according to the physician examination and McDonald criteria, serum samples were taken. Until testing, serum samples were stored in a freezer at -70 ° C. Subsequently, viral and bacterial agents were identified using specific primers and PCR method. In this study, the numbers of microbial agents were as the following: 7 retrovirus associated with MS (MRSV), 17 EBV, 8 HSV6, 11 JC virus, 10 CMV, 8 B19, 14 Corona virus, 1 *Helicobacter pylori*, 15 *Acinetobacter*, 9 *Borrelia burgdorferi*, and 19 *Chlamydia pneumoniae*. Identification of the relationship between different infectious agents in MS is necessary to prepare feasible data about tracing and treatment of MS related to these microorganisms that may be beneficial to clinicians to select a convenient empirical therapeutic diet in MS related to pathogens at the bedside and can open up a new path to new therapeutic approaches.

Key words: Multiple Sclerosis patients; Microbial Agents; PCR

INTRODUCTION

Multiple sclerosis (MS) is a chronic immune-mediated inflammatory disease of the central nervous system, (CNS) characterized pathologically by perivascular infiltrates of mononuclear cells, demyelination, axonal loss and gliosis with the formation of multiple plaques in the brain and spinal cord, and clinically by a variety of neurological signs and symptoms disseminated in time and space [1]. It is the second most common cause of neurological disability in young adults after trauma [2-4].

Recent advances in understanding the etiology and pathogenesis of MS has contributed to better diagnosis and a plethora of therapies which substantially affect disease activity and may have a long-term impact on the course and prognosis of MS. The routine use of magnetic

resonance imaging (MRI) as a diagnostic tool and a surrogate measure, the recognition of the need for early diagnosis and treatment of MS and the identification of clinical and imaging prognostic factors has resulted in several revisions of the diagnostic criteria for MS, aiming at as early and accurate as possible diagnosis of this weighty disease. MS affects mainly young people with onset usually at the age of 20–50 and a mean age of onset of 30, although the disease may develop also in childhood and after the age of 60, and is 3 times more common in females than in males. The cause of MS is still unknown. However, genetic, environmental and immunological factors have been implicated in the etiology of the disease. The total number of people living with MS worldwide is estimated to be 2.5 million, unevenly distributed throughout the world [1].

The prevalence of MS varies geographically. Areas of higher prevalence (60 cases per 100,000 people) include Europe, Russia, southern Canada, northern United States, New Zealand, and southeast Australia. In the United States, the prevalence is estimated to be 100 cases per 100,000 people [2]. Presentation of symptoms in MS usually begins between ages 20 and 40. When a patient is diagnosed with MS after the age of 50, the diagnosis is termed late-onset multiple sclerosis (LOMS). MS typically affects women more than men. In addition, it is more commonly seen in Caucasians of Northern European descent as compared with other ethnic groups, including African-Americans, Asians, and Hispanics/ Latinos. However, a recent study found that the risk of developing MS was higher in African-American women when compared with Caucasian American women and men [5]. Globally, the median estimated prevalence of MS is 112.0 per 100,000 and the median estimated incidence of MS is 5.2 per 100,000. Greatest prevalence observed in North America and Europe (140 and 108 per 100,000, respectively). Iran is considered as a country with high MS prevalence (51.52 per 100,000) in Middle East [6-8]. The prevalence of MS follows a latitudinal gradient in an incomplete distribution model, and varies from less than 5/100,000 in low-risk areas (e.g. most of Africa and Eastern Asia), to more than 100/100,000 in high-risk areas (e.g. northern and central Europe, North America and Southeastern Australia). Migration studies indicate that immigrants tend to develop a prevalence rate similar to that of the indigenous population, especially if they have migrated before puberty. Additionally, several clusters and "epidemics" of MS have been reported. Taken together, these observations support environmental factors in the etiology of MS, the most plausible ones being exposure to the Epstein-Barr virus (EBV) after early childhood and manifestations of infectious mononucleosis, reduced exposure to sunlight and ultraviolet radiation, vitamin D deficiency and cigarette smoking. Recent studies also suggest a role for dietary sodium in inducing pathogenic Th17 cells which may partially account for the observed worldwide increase in MS incidence. A wide variety of other environmental factors have also been suggested as triggers for MS, but their role is disputed [9-13]. Overall prevalence of familial MS in Iran was estimated as of 11.4% [95% confidence

interval (CI): 8.7-14.1]. Point prevalence varied between 3.3% and 26.7%. The familial prevalence of MS among Iranian people is relatively high. More studies are warranted to investigate the effect of familial history as a risk factor for MS [14]. Previously, Iran was considered a low prevalence area, but recent investigations have shown that the prevalence of MS in Iran has increased significantly [15].

This increasing pattern in the rate of MS may have several causes; however, changes in lifestyle and new and advanced diagnostic methods are regarded as the most important causes. Unfortunately, Iran does not have a national registry for patients with MS. Nevertheless, there is a national computerized registration system which holds the information of every patient with MS in the country who has registered and received beta interferon medication from the Ministry of Health and Medical Education (MOHME). The Iranian government covers a considerable percentage of the treatment costs for patients with MS receiving beta interferon according to this registry. Although studying the data on this group of patients does not yield precise and comprehensive information on all patients with MS in Iran, even an evaluation of these data demonstrates that the prevalence rate has increased significantly. In December 2011, the Iranian MOHME registry listed 34,605 MS patients in Iran. Seventy-seven percent were women. Given that Iran's population in 2011 was 75,600,000, the prevalence rate of MS was calculated as 45/100,000 of population [2]. Seventy percent of these patients were between 20-40 years of age. The maximum prevalence rate (80 per 100,000 populations) was seen in Isfahan province, located in the central part of Iran. The minimum prevalence rate (6 per 100,000 populations) was seen in Sistan-Baluchestan province, located in the southeast part of Iran. This province has a warm and dry climate [2]. Other environmental factors, such as smoking or viral and bacterial infections, have been shown to increase the risk of developing MS. So, in this study, the role of microbial agents in MS etiology and pathogenesis was studied.

MATERIALS AND METHODS

Patients

Thirty seven patients with multiple sclerosis were included in this study. The McDonald criteria was first established in 2001 as a standard means of diagnosing multiple sclerosis (MS) with sensitivity and speed by neurologist Ian McDonald and a team of researchers. (It is also known as the International Panel on MS Diagnosis, and the original and subsequent panels were, at least in part, organized by the National Multiple Sclerosis Society). The McDonald Criteria is distinguished by incorporating clinical evaluation with magnetic resonance imaging (MRI) scans in establishing MS. Nonetheless, like an earlier approach, it too requires: Evidence of damage to the central nervous system (CNS; the brain, spinal cord and optic nerves) that is “disseminated in time,” meaning damage that occurs on different dates; Evidence of damage “disseminated in space,” or found on two or more parts of the CNS. A 2010 revision to the McDonald Criteria (which followed a 2005 update) reflected better understanding of MS and improved MRI techniques and made possible a faster diagnosis — one that is, for a first time, potentially based on only one demyelinating relapse or attack provided certain criteria are met. These new criteria are dissemination in time, if two lesions are evident at a first attack and dissemination in both time and space if only one lesion is evident. This change was a key one because of the importance of moving patients with confirmed disease quickly onto disease-modifying therapies that may slow disease progression [16].

PCR conditions

The bacterial and viral genomic extraction was carried out using an extraction kit according to the manufacturer's protocol. The genes were then identified by PCR method. The sequences of primers HSV, EBV, JC virus, Retrovirus, B19, *Chlamydia pneumoniae*, *Borrelia*

burgdorferi and *Helicobacter pylori* were checked for nucleotide sequencing in the Gene Bank blast. By AccuPrep Genomic DNA extraction kit (cat.no.k-3032 lot no.1008J, BIONEER), DNA was extracted from all samples. PCR amplification profile comprised of a 300 nM concentration of each specific primer (Eurofins MWG Operon); 200 mM (each) deoxynucleoside triphosphates dCTP, dGTP, dATP, and dUTP; 0.125 U of Taq DNA polymerase; and 5.5 mM MgCl₂ (from GENET BIO, prime Taq TM DNA polymerase, URL:www.genetbio.com). The PCR products were analyzed by gel electrophoresis on 1.5% BIONEER agarose gels in 1X TBE buffer (890 mM of boric acid, 890 mM Tris, 40ml of 0.5 M EDTA, pH 8.0) at 100 V for 60 min. Green loading buffer with DNA stain (Jena Bioscience, Lot: 111.034) was used during loading the samples and ladder. The sizes of the PCR products were determined by comparison with the molecular size standard (50bp-1Kb linear scale); low range DNA ladder or 100bp-3Kb linear scale and mid-range DNA ladder, Jena Bioscience. All control strains and primer sequences were as described previously [17-24].

STATISTICS

Data analysis is performed using SPSS software version 22.

RESULTS

In this study, the numbers of microbial agents were as follows: 7 Retrovirus associated with MS (MRSV), 17 EBV, 8 HSV6, 11 JC, 10 CMV, 8 B19, 14 *Corona* virus, 1 *Helicobacter pylori*, 15 *Acinetobacter*, 9 *Borrelia burgdorferi*, and 19 *Chlamydia pneumoniae*. Based on figure 1, EBV virus was the highest and MRSV was the lowest in patients with MS. Based on figure 2 *Chlamydia pneumoniae* was the highest and *Helicobacter pylori* was the lowest in patients with MS.

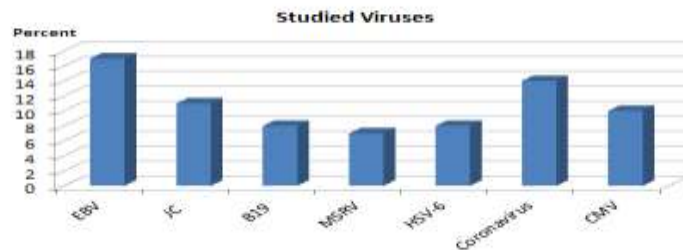


Figure 1. The numbers of viral agents were detected in patients with MS

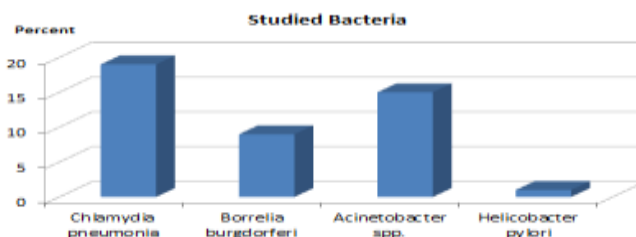


Figure 2. The numbers of bacterial agents were detected in patients with MS

DISCUSSION

MS disease is the most common cause of disability in young people. Until now, definitive treatment of this disease is not possible, but existing drugs can reduce the number of attacks and, as a result, disability due to the disease. More than 2 million people in the world suffer from the disease, and each patient's social and therapeutic cost is \$ 2.4 million. There is no accurate data on the disease and exact number of patients with MS has not been reported, but it seems that more than 40,000 people in our country suffer from the disease, most being young women. The disease has different steps in the affected population, as there are no signs and symptoms in some patients, but some of them suffer from significant disability in a short period of time. The prevalence of this disease in the world is increasing, with Iran being a part of it, but since the epidemiology of this disease in the country has not been made, the record is not precise [25]. According to a study done by Ramroodi N and et al, 74.2% of the case group (from 31 patients) and 2.34% of the control group (60 healthy individuals) were positive for antibodies antiviral HHV- 6. The presence of anti-HHV-6 was more than 5 times in control group. Indeed, based on this study, the human infection with HHV- 6 can be a factor in multiple sclerosis[26]. In 2013, a study by

Mohebbi et al. was conducted to investigate the effect of *Helicobacter pylori* infection on MS. In this study, 163 patients with MS were enrolled in the study, with a mean age of 32 years with a standard deviation of 8 years. In the control group, 150 patients were studied in 110 (73%) and 40 (27%) positive tests. Negative tests were completed in two groups of patients and controls for the amount of *Helicobacter pylori* infection. Based on the results of this study, infection with *Helicobacter pylori* is a protective factor against MS and the infection can reduce the amount of disability and brain damage [27]. In a study of 26 MS patients in England, antibodies against 5 strains of *Acinetobacter* strain were measured by ELISA technique. In this study, 20 patients were with cerebrovascular accidents and 25 healthy subjects were included. The study showed that the levels of IgA, IgG and IgM antibodies in patients with MS were significantly higher than the other two groups. Among the five strains studied of *Acinetobacter* strains, *Acinetobacter citacurus*, *Acinetobacter luffyia*, had the highest antibody titer, so that the level of antibodies synthesized was higher than 1: 6400 [28]. In the seroepidemiological studies conducted by Chemielewska-Badora et al., 10 out of 26 (38%) patients with MS had antibodies to *Borlia borgdorferrii*, while the result was only

149 out of 743 tons (20 %) of the patients with other neurological diseases ($P = 0.042$) [29]. In another study by Friedman et al., to understand the association between the virus and MS, the high IgG antibody titer synthesized against HHV-6 virus was seen in 21 out of 25 (80%) patients with MS while this high-titer was seen in only 3 out of 14 patients with other autoimmune diseases [30]. Wandinger K and ital., reported in their study that all patients with MS had anti-EBV antibodies, while only 86-95% of the control group had this characteristic. It was unknown whether the infection is a necessary condition for the development of MS or not.

In a study of 37 patients with MS who received cyclosporine, JC DNA was detected in urine samples of 30 (81%) patients by PCR technique. It was necessary to note that this virus was also present in 40% of the population in the urine. Genomic DNA of the JC virus in the cerebrospinal fluid was detected in 9% of patients with MS. DNA of the virus in none of the patients with other neurological diseases as well as the control group have been identified [31-33]. In another study by Stewart et al., which included 11 MS patients, 6 patients with other neurological diseases and 5 healthy subjects, *coronavirus* 229E was detected in 4 MS patients. However, in the other two groups no cases were found [34].

Anti-PVB19 antibodies from the IgG class were found in patients. The PCR was also used to detect the virus DNA in the cerebrospinal fluid of the patients was negative [35]. A further study by Garcia-Montojo et al. in 2012 was conducted to investigate the relationship between MSR/V and gender differences in clinical status in MS patients and control group. 178 patients (62.9% female) and 124 controls (56.5% female) were enrolled in this study. MSR/V was higher in patients with MS than in the control group. Women with MS had more MSR/V load than control women ($p = 0.009$) and had more load in men than controls ($p = 2.77-6$). In addition, women had a higher level of viral infection than patients ($p = 0.007$) and control group ($p = 1.24e-6$) [36]. In 2010, Handel and his colleagues conducted a meta-analysis to determine the risk of MS after infectious mononucleosis. In a study of 18 researches, 19390 MS patients and 16007 controls were examined. This study showed

that the risk of MS with a high power associated with infectious mononucleosis [37]. Another study by Sandelle et al., in 2011 investigated the prevalence of CMV and its role in general immunoglobulin pattern among Iranian patients with different MS subtypes. In this study, serum plasma, saliva and urine samples were collected from MS patients and healthy subjects. The presence of anti-CMV antibodies and anti-CMV-DNA antibodies in the samples were evaluated by nephelometric and PCR methods. The results showed an increase in anti-CMV-DNA antibodies in patients as compared to the control group ($P < 0.001$). In addition, systemic CMV infection was found in 25.5% of patients ($P < 0.001$). There was a high difference in IgG antibody titers against CMV-DNA and total IgE in patients and controls [38]. In 2001, a study was conducted by Hughes LE et al in which anti-*Acinetobacter* and *Pseudomonas aeruginosa* antibody responses were evaluated in MS patients. The study indicated that there was a similarity or molecular imitation between *Acinetobacter* antigens and human antigens could be a reason for the increased levels of antibodies against this bacterium in MS patients other than autoantibodies. Antibodies against 5 species of *Acinetobacter*, *Pseudomonas aeruginosa* and *Escherichia coli* were measured in 26 patients with MS. Significant increase in the IgA and IgG levels was observed for at least 3 *Acinetobacter* species in the MS patients compared to control group ($P < 0.0001$) [28]. In studies conducted on MS patients, *Chlamydia pneumoniae* was found to be significantly higher in CSF in MS patients than in the control group. In addition, a large percentage of MS patients had high levels of specific antibodies to *Chlamydia pneumoniae* antigens in their CSF as compared to the control group [39]. In another meta-analysis from 26 studies which investigated the association of *Chlamydia pneumoniae* with MS, a significant correlation was found between MS and the detection of *Chlamydia pneumoniae* DNA in CSF by PCR [40].

CONCLUSION

Considering that the disease of multiple sclerosis is increasing in Iran and the main causes of this disease are unknown. Recent studies have shown that infectious agents play

an important role in the development and development of the disease, the introduction of an agent as an agent. Identification of the relationship between different infectious agents in MS is necessary to prepare feasible data about tracing and treatment of MS related to these microorganisms that may be beneficial to clinicians for the purpose of selecting a convenient empirical therapeutic diet in MS related to pathogens at the bedhead and can open up a new path to new therapeutic approaches.

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AUTHORS' CONTRIBUTION

All authors cooperated in the all processes including sampling, testing, data analysis, and writing the paper.

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