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## Original Article

# Detection of CMV and EBV DNA in PBMCs of MS Patients and Healthy Individuals in Gorgan, Iran

**Tazarghi SA [1], Tabarraei A [2], Naeimi MH [3], Moradi A [1], Ahmadi A [4], Javid N [1]**

1 Department of Microbiology, Golestan University of Medical Sciences, Gorgan, Iran.
2 Infectious Diseases Research Center, Golestan University of Medical Sciences, Gorgan, Iran.
3 Department of Neurology, Sayyad Hospital, Golestan University of Medical Sciences, Gorgan, Iran.
4 Department of Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

## Abstract

**Background and Aims:** Multiple Sclerosis is a chronic and autoimmune disease, which causes inflammation and demyelination of the axons in CNS. Studies have indicated that Herpesviruses might be one of the potential environmental elements that can trigger the initiation of the inflammatory cascade in MS. The purpose of this study was to determine whether the genome of CMV and (or) EBV is present in PBMCs of the MS patients and healthy individuals and if so, consider the sequencing process for DNA-positive samples.

**Materials and Methods:** Blood samples from 50 MS patients and healthy people were collected and extraction of DNA was done on PBMCs of the mentioned samples. PCR was used for conserved and variable regions of the genome to seek the presence of EBV and (or) CMV DNA in extraction products.

**Results:** Neither in PBMCs samples of the MS patients (n=50) nor in those of healthy individuals (n=50) could we find any evidence to prove the presence of the two mentioned viral DNAs in DNA extraction products of the PBMCs samples. Genotyping of mentioned viruses was consequently omitted from the study purposes.

**Conclusions:** Based on the findings in this study, there seems to be no significant relation between CMV/EBV infection and the risk of MS development.

**Keywords:** CMV, EBV, Multiple Sclerosis, PCR.

## Introduction

Multiple Sclerosis (MS), known as the most common autoimmune inflammatory demyelinating disease of the CNS, has a vague etiology. It seems that genetic and environmental elements can collaborate together to cause the onset of the disease invasion mostly in 20 to 40 year old (1). Based on Etemadifar M et al study, Golestan province in north of Iran with 1,777,014 citizens has a rate of 18 MS patients per 100,000 individuals (2).

Herpesviruses are able to aim for different types of cells and establish an infection which might remain in latency phase or turn into an active, lytic infection over time. This alternation in infection phases may happen periodically and suggests a pathological pattern that resembles the relapsing and remitting periods of the disease in multiple sclerosis (3). It's suggested that EBV and CMV, two members of herpesviridae family, belonging to subfamilies Gamma-herpesvirinae and Beta-herpesvirinae respectively, might have a role in pathogenesis of MS by demolishing the endothelial cells in blood-brain barrier (BBB) and facilitating the autoimmune leukocytes infiltration into the brain parenchyma (4, 5). It's also indicated that gp350 from EBV

*Corresponding author: Alijan Tabarraei, PhD; Infectious Diseases Research Center, Department of microbiology, Golestan University of Medical Sciences, Gorgan, Iran.
Tel: (+98) 17 32422652, Fax: (+98) 17 32440225
Email: alijant@yahoo.com.

increases the expression of MSRV env (Multiple Sclerosis Associated Retroviruses envelope protein) and Syncytin-1 in infected cells. These two belong to a family of human genes named HERV W (Human Endogenous Retrovirus W) and own an inflammatory function which might contribute to the neurodegeneration process in MS (4, 6). There are also epidemiological studies that support the hypothesis of a contribution between MS and EBV or CMV infection (7, 8, 9, 10, 11, 12, 13, 14).

In this study, we were going to look for CMV and EBV genomic DNA in peripheral blood mononuclear cells (PBMCs) of patients with MS and healthy individuals, and compare the results in order to clarify the probable presence of a significant relation between these two viral infections and the risk of developing MS.

## Methods

**Sample collection.** Fifty blood samples (38 women, 12 men) were collected from patients who were diagnosed suffering from RRMS in Mahestan Clinical Center in Gorgan, Iran. Sampling from mentioned patients was done when they were in remitting phase of the disease. Simultaneously, fifty blood samples were also collected from healthy individuals with no sign of MS (42 women, 8 men) as control group (Fig 1). The range of the age in both groups was between 20 to 50 year old, but mostly concentrated around 30 ± 5 year old.

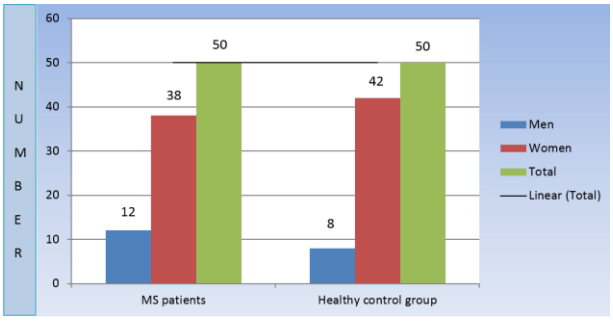


**Fig. 1.** Gender distribution in MS patients and healthy control group.

5ml of whole blood with EDTA was used to separate PBMCs and they were frozen in -80°C for further examinations, which were done in Golestan University of Medical Sciences, Gorgan, Iran.

**DNA extraction.** DNA was extracted from PBMCs according to Shahbazi et al (15). To this purpose, erythrocytes were lysed with R buffer (ammonium carbonate, potassium hydrogen carbonate, EDTA 0.5 M), and some extra components including 700 µl salt EDTA, 25µl proteinase K (25 mg/ml) and 30 µl SDS 10% were added to the pellet, which was then incubated at 65°C for 1 hour. Afterwards, a mixture of phenol-chloroform-isoamyl alcohol was added to the incubated pellet and the sample tubes were centrifuged to create the two organic (below) and aquatic layers (atop). Then isopropanol and acetate sodium were added to the supernatant to precipitate the DNA and make it visible. The sample tubes were centrifuged again and DNA was dissolved in 100µl sterile distilled water after removing the isopropanol and acetate sodium and letting the alcohol droplets dry. Extraction products were stored in -20°C for future examinations.

**Polymerase Chain Reaction (PCR).** To verify the presence of DNA in extraction products, PCR was performed by targeting a 225bp sequence of GAPDH gene and analyzing 5µl of the amplification products with gel electrophoresis (1.5% agarose) containing SYBR Green. Then three pairs of primers selected to identify the presence of CMV and EBV genome were used for PCR and the results were analyzed similarly. These pairs of primers were designed to recognize the two sides of a 257bp and 702bp sequences of RL11 and UL27 genes from CMV respectively, and an 183bp sequence of LMP-2A gene from EBV (table 1).

Besides, the concentration of materials used to prepare master mix, and the protocol of amplification temperatures for each primers pair are shown in tables 2 and 3. By the way, sterile distilled water was used as PCR negative control in all the tests.

**Gel Electrophoresis.** 4µl of PCR products were mixed with 1µl of loading dye and the mixture was conveyed to 1.5% agarose gel containing SYBR Green. Then the electrophoresis started and continued with a

**Table 1.** Sequence and the product size of the primers used in PCR amplification to recognize the presence of genomic DN CMV, EBV and human PBMCs

| Primers target | Virus | Sequence | Amplicon size (bp) |
|---|---|---|---|
| **RL11** | CMV | 5'- CTA CAC GCA CGC TGG TTA CC -3'<br>5'- GTA GAA AGC CTC GAC ATC GC- 3' | 257 |
| **UL27** | CMV | 5'- AGA AGA CCA TGA CGA CGG- 3'<br>5'- TGC GTC CGG TAC TTG ACG- 3' | 702 |
| **LMP-2A** | EBV | 5'- AAC ATT GGC AGC AGG TAA GC- 3'<br>5'- ACT TAC CAA GTG TCC ATA GGA GC- 3' | 183 |
| **GAPDH** | ------- (internal control) | 5'- GAA GGT GAA GGT CGG AGT- 3'<br>5'- GAA GAT GGT GAT GGG ATT TC- 3' | 225 |

**Table 2.** Concentration and volume of materials in master mix preparation

| Materials | Stock Con* | Master Mix Con* | | | | Volume(µl) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | RL11 | UL27 | LMP2A | GAPDH | RL11 | UL27 | LMP2A | GAPDH |
| **PCR Buffer** | 10X | 1X | 1X | 1X | 1X | 2.5 | 2.5 | 5 | 2.5 |
| **$MgCl_2$** | 25 mM | 2mM | 2mM | 2mM | 2mM | 2 | 2 | 4 | 2 |
| **dNTP** | 10 mM | 0.2mM | 0.2mM | 0.2mM | 0.2mM | 0.5 | 0.5 | 1 | 0.7 |
| **Primers (F and R)** | 100 Pmol/µl | 10 Pmol/µl | 10 Pmol/µl | 10 Pmol/µl | 10 Pmol/µl | F: 1<br>R: 1 | F: 1<br>R: 1 | F: 1<br>R: 1 | F: 1<br>R: 1 |
| **Taq Pol** | 5 U/µl | 1.5 U/µl | 2 U/µl | 2.5 U/µl | 1 U/µl | 0.3 | 0.4 | 0.5 | 0.2 |
| **D.W.** | | | | | | 12.7 | 12.6 | 32.5 | 14.6 |
| **Template** | | | | | | 5 | 5 | 5 | 3 |
| **Total** | | | | | | 25 | 25 | 50 | 25 |

*Con: Concentration

**Table 3.** Temperature protocol for PCR amplification

| Primers | Step | | | | | | | | Cycle |
|---|---|---|---|---|---|---|---|---|---|
| | Denaturation | | Annealing | | Extension | | Final Extension | | |
| | Temp* | Time | Temp* | Time | Temp* | Time | Temp* | Time | |
| **RL11** | 94 | 1 min | 56 | 1 min | 72 | 1 min | 72 | 5 min | 35 |
| **UL27** | 94 | 1 min | 55 | 1 min | 72 | 1 min | 72 | 7 min | 35 |
| **LMP-2A** | 94 | 30 sec | 58 | 40 sec | 72 | 50 sec | 72 | 7 min | 40 |
| **GAPDH** | 95 | 30 sec | 60 | 30 sec | 72 | 30 sec | 72 | 7 min | 35 |

* Temp: Temperature

voltage of 100v for 40 to 50 minutes and finally the result was observed by means of gel documentation system.

## Results

PCR results for GAPDH gene proved the presence of DNA in extraction products. According to figure 2, five samples from MS patients (4 to 8) and 5 samples belonging to healthy individuals (9 to 13) were analyzed with gel electrophoresis (agarose 1.5%) along with a positive control (1) and a negative control (2) in the left side of the 50bp marker (3).

PCR results yet for both RL11 and UL27 genes indicated that there was no CMV genomic DNA in both MS patients' PBMCs and healthy individuals' (figs 3, 4 and tables 4, 5). Furthermore, the result for LMP-2A from EBV was the same and no positive samples were found in MS patients or healthy individuals subsequently (fig 5, table 6).

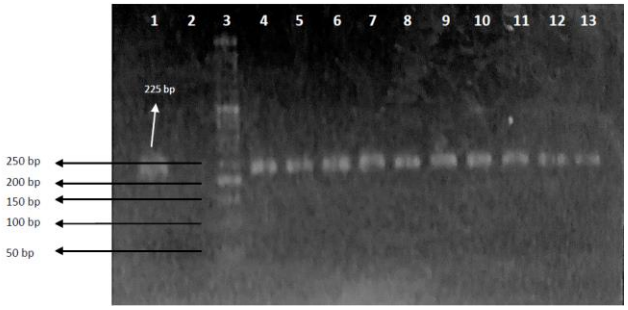


**Fig. 2.** PCR amplification result for GAPDH on agarose 1.5%. Column 1: Positive control, 2: Negative control (without band), 3: 50bp marker, 4 to 8: MS patients' samples, 9 to 13: Healthy individuals' samples.

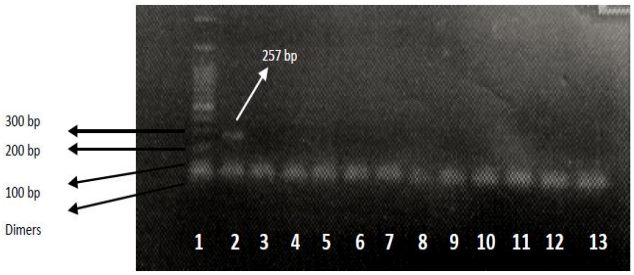


**Fig. 3.** PCR amplification result for RL11 on agarose 1.5%. Column 1: 100bp marker, 2: Positive control (with 257bp band), 3: Negative control, 4 to 13: MS patients' samples.

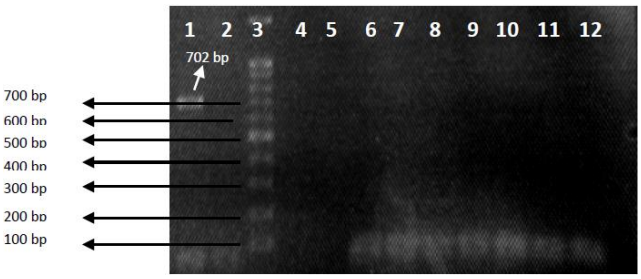


**Fig. 4.** PCR amplification result for UL27 on agarose 1.5%. Column 1: Positive control (with 702bp band), 2: Negative control, 3: 100bp marker, 4 to 12: MS patients' samples.

**Table 4.** PCR result for RL11 gene.

| | CMV DNA positive | | | CMV DNA negative | | |
|---|---|---|---|---|---|---|
| | **Male** | **Female** | **Total** | **Male** | **Female** | **Total** |
| **MS patients** | 0 | 0 | 0 | 12 | 38 | 50 |
| **Healthy control group** | 0 | 0 | 0 | 8 | 42 | 50 |

**Table 4.** PCR result for UL27 gene.

| | CMV DNA positive | | | CMV DNA negative | | |
|---|---|---|---|---|---|---|
| | **Male** | **Female** | **Total** | **Male** | **Female** | **Total** |
| **MS patients** | 0 | 0 | 0 | 12 | 38 | 50 |
| **Healthy control group** | 0 | 0 | 0 | 8 | 42 | 50 |

**Table 5.** PCR result for LMP-2A gene.

| | CMV DNA positive | | | CMV DNA negative | | |
|---|---|---|---|---|---|---|
| | **Male** | **Female** | **Total** | **Male** | **Female** | **Total** |
| **MS patients** | 0 | 0 | 0 | 12 | 38 | 50 |
| **Healthy control group** | 0 | 0 | 0 | 8 | 42 | 50 |

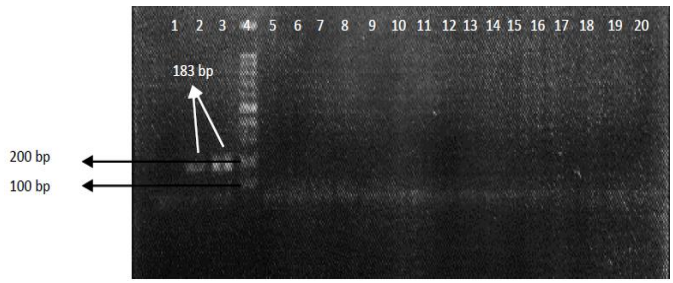


**Fig. 5.** PCR amplification result for LMP-2A on agarose 1.5%. Column 1: Negative control, 2 and 3: Positive control (with 183bp band), 4: 100bp marker, 5 to 20: MS patients' samples.

## Discussion

Although there are studies that indicate the presence of an association between being infected with members of herpesviridae and a higher risk of developing multiple sclerosis or causing the deterioration of this disease (1), our study results show some contrast. According to our findings, there seems to be no significant relation between CMV/EBV infection and a higher or lower risk of developing MS. These results are opposite to some studies which were done in other cities of Iran or even abroad in different countries (7, 8, 9, 10, 11, 12, 13, 14). On the other hand, our findings are in favor of the results of some other studies, which have not found such correlation between CMV/EBV infection and MS development (16, 17, 18, 19, 20). These controversies in different studies point out that we need to consider more experimental elements like various environmental or genetic factors to attain a vaster overlook upon the role of EBV, CMV and other viruses in the mechanism of MS pathogenesis.

According to Camille Rose et al (21), Lielian Zuo et al (22), Kazuhiro Kondo et al (23) and

Barry Slobedman's studies (24), the number of CMV and EBV genome copies in latency phase can be 1 to less than 100. It is worth to mention that the low number of CMV and EBV genome copies in cells that carry the latent episomes may be a reason for our Snegative results since our qualitative PCR tests might have lacked enough sensitivity to identify and amplify this low copy number. We assume that primers designed to recognize the sequences of latency specific RNAs (like EBERs) may render more accurate and reliable results since these latency associated transcripts (LATs) are much more abundant in infected cells containing CMV or EBV episomal genomes.

Also we recommend that further studies are better to consider some other variables and elements that can help us scrutinize the details about how viruses may associate with MS pathogenesis and its progression. It can be the type of the molecular techniques and their protocols, primer designation and extraction methods, demographic information of the population under study, and people's precedence of infection with lifelong viral elements like HIV, which could suppress the host's immunity system and might have a role in decreasing the autoimmune responses in multiple sclerosis.

## Conclusion

Our findings suggest that there's still a long way to claim that EBV and CMV can be two etiological factors associated with development of multiple sclerosis. This correlation is still controversial and further studies are needed to make an established theory.

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## Conflict of interest

None declared.

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