IJID Regions 7 (2023) 233–236

Contents lists available at ScienceDirect

IJID Regions

journal homepage: www.elsevier.com/locate/ijregi

Evaluation of the prevalence of bovine leukemia virus DNA in peripheral blood mononuclear cells of multiple sclerosis patients

Ali Salimi Jeda [1,*], Anna Zarei [2], Soad Ghabeshi [3], Alireza Tabibzadeh [1], Mohsen Lotfi [4], Masoud Etemadifar [5], Mohammad Hadi Karbalaie Niya [6], Mehrdad Ghorbanlou [7], Maryam Esghaei [1,*]

[1] Department of Virology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[2] Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran
[3] Department of Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[4] Quality Control Department, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
[5] Department of Neurosurgery, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
[6] Gastrointestinal and Liver Diseases Research Center, Iran University of Medical Sciences, Tehran, Iran
[7] Anatomical Sciences, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Keywords:
bovine leukemia virus
multiple sclerosis
peripheral blood mononuclear cells
DNA

ABSTRACT

Multiple sclerosis (MS) is an immune system-mediated neurodegenerative disease. Recent studies suggest that viral agents, especially the Epstein Barr virus (EBV), are etiological agents for MS. The roles of other viruses in MS have been investigated. Studies have shown an increase in the level of antibodies against bovine leukemia virus (BLV) in patients with MS. In this regard, our study aimed to examine the presence of BLV DNA in peripheral blood mononuclear cells (PBMCs) of MS patients in Iran. In this cross-sectional study, the presence of BLV in 109 Iranian MS patients and 60 healthy controls was evaluated. The isolated PBMCs were used for DNA extraction and PCR, using specific primers for two distinct genes. The mean age of the participants was 39 ± 9.5 years, and 27 (24.77%) of them were male. Clinical evaluation of these patients showed the most frequent MS type to be relapsing-remitting MS (RRMS) (71; 65.14%). BLV evaluation did not show any BLV DNA presence in the PBMCs of individuals in either the MS or healthy control groups. Therefore, our study showed no evidence of BLV infection in Iranian MS patients.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) [1]. The onset of MS usually occurs between the ages of 20 and 40, and its prevalence in women is more than twice that found in men. The incidence of MS varies from 2–160 per 100 000 people, with around 2 million people affected worldwide. The pooled prevalence of MS in Iran is 100 in 100 000, with the disease rate in women 2.7 times higher than in men [2].

Although the causative agents and pathogenesis of MS are not fully understood, it is believed the disease is caused by an autoimmune response against myelin protein [3]. The pathology of MS includes demyelinated plaques, in which active mononuclear cells, including lymphocytes, macrophages, and microglia, affect the myelin and kill oligodendrocytes. Plaques mostly occur in periventricular white matter, optic nerves, and the spinal cord. The clinical signs of MS in different patients vary depending on the location of the nerve fibers involved and the severity of the injury. MS is usually characterized by symptoms such as vision problems, imbalance, confusion, fatigue, bladder problems, stiffness, and spasms [4].

Four main clinical subtypes of MS are known: relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), primary progressive MS (PPMS), and progressive-relapsing MS (PRMS). RRMS involves unpredictable recurrences, with the recurrence interval anything from several weeks to several years. This is the most common form of MS. SRMS occurs after the first stage, with increasing disability and fewer or no relapses. PPMS is a neurological disability with periods of exacerbation and recovery, in which relapse is not experienced. PRMS is a progressive disability with occasional recurrences [5].

Risk factors associated with MS include age, sex, genetic changes, and environmental factors, such as diet, smoking, vitamin D deficiency [6], and infectious agents, including Epstein–Barr virus (EBV), human herpes virus 6 (HHV6), and varicella-zoster virus (VZV) [7]. With regard to dietary factors, a relationship between infections in meat or milk and

* Corresponding authors: Department of Virology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
E-mail addresses: salimi.ali84@gmail.com (A.S. Jeda), maryam.esghaei@gmail.com (M. Esghaei).

https://doi.org/10.1016/j.ijregi.2023.04.002
Received 2 March 2023; Received in revised form 28 March 2023; Accepted 3 April 2023

Table 1
Specific primer pairs used this study.

Target gene	Primer	Sequence	Amplification size	Annealing temperature
β-actin	Forward Reverse	5′-TGGTTACAGGAAGTCCCTTGCC-3′ 5′-ATGCTATCACCTCCCCTGTGTG-3′	101 bp	60
BLV *Gag*	Forward Reverse	5′-TCTGTGCCAAGTCTCCCAGATA-3′ 5′-AACAACAACCTCTGGGAAGGGT-3′	598 bp	56
BLV *Tax*	Forward Reverse	5′-GGCCCCACTCTCTACATGC-3′ 5′-AGACATGCAGTCGAGGGAAC-3′	207 bp	56

MS has been reported, and suggested as a possible important element in the development of the disease [8].

Bovine leukemia virus (BLV) is a Deltaretrovirus in the Retroviridae family. The BLV genome contains *Gag, Pol*, and *Env* genes and *Rex* and *Tax* oncogenes, and has the potential to cause cancer in cows [9]. $CD5^+$ IgM^+ B-cells antibody in cattle can disrupt immunological regulation and thus reduce milk production and reproductive inefficiency in cows. The virus can also infect sheep and cause diseases such as B-cell lymphosarcoma, leukemia, and/or lymphoma [10].

Studies have shown that around 60% of the world's cows are infected with the BLV virus [11], and antibodies against BLV structural proteins have been found in humans who consumed BLV-infected dairy products [12]. Several studies have indicated an increase in the level of antibodies against BLV in patients with MS [13,14]. However, no study has reported the prevalence of this virus in the PBMCs of MS patients. Therefore, our study aimed to measure the prevalence of BLV in the PBMCs of MS patients, as determined through polymerase chain reaction (PCR).

Methods

In this cross-sectional study, 109 Iranian MS patients were evaluated for the possible presence of BLV infection. After obtaining written informed consent, 5 mL of peripheral blood was collected from each patient in EDTA anticoagulant tubes. In addition, 60 healthy controls were also evaluated for the possible presence of BLV in the normal population.

The obtained peripheral blood was used for PBMC isolation using Ficoll density gradient centrifugation. The PBMC samples were stored at −20°C before DNA extraction. The extraction was performed using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics), according to the manufacturer's protocols. The extracted DNA quality was evaluated using a NanoDrop spectrophotometer. The extracted samples were stored at −20°C before applying PCR.

FLK-BLV cells (Razi Vaccine and Serum Research Institute, Tehran, Iran) served as a positive control for optimizing PCR, and were confirmed by Sanger sequencing. Conventional PCR was performed for BLV detection in PBMC samples for two viral genes (*Gag* and *Tax*). The specific primer pairs for the *Gag* and *Tax* regions were designed using Primer3Plus software (version:3.2.6). The β-actin was used as the internal control for the PCR reactions. The list of the primers for the current study is provided in Table 1.

The mixture for β-actin, BLV *Gag*, and BLV *Tax* included 12 µL of the commercially available 2X Super MasterMix (Yekta Tajhiz Azma, Iran), 1 µL of each gene-specific forward, 1 µL of reverse primers, 6 µL of templet, and 5 µL of PCR-grade water. The PCR thermal program for all genes was similar, except for the annealing temperature. The annealing temperatures for each primer are listed in Table 1. The thermal programs included 10 minutes at 95°C for primary denaturation, followed by 45 cycles of 30 seconds at 95°C, 30 seconds at the primer-specific annealing temperature, and 40 seconds at 72°C. Each PCR cycle contained a final extension step for 10 minutes at 72°C. The PCR amplicons were visualized using 1% agarose gel electrophoresis.

Table 2
Patients' clinical and demographical data.

Variable		MS	Healthy control
Number of subjects		109	60
Age, years (mean ± SD)		39 ± 9.5	21 ± 8
Sex (male/female)		27/82	15/45
Type of MS	**RRMS**	71 (65.14%)	NA
	SPMS	21 (19.26)	NA
	PPMS	14 (12.84%)	NA
	PRMS	3 (2.75%)	NA
Duration of MS, years (mean)		3 ± 2	NA

All the statistical evaluations were performed using SPSS (version 24), and a *p*-value of less than 0.05 was considered a significant difference for every appropriate statistical test based on the variable.

Results

The study results represent that the mean age of the patients was 39±9.5, and 27 (24.77%) of them were male. The clinical evaluation of the evaluated patients represents the most frequent MS type is RRMS (n= 71, 65.14%) followed by SRMS (n= 21, 19.26%) (Table 2). There were no statistically significant differences between the MS type and patients' age or gender (P>0.05).

The BLV evaluation did not show any BLV DNA presence in the PBMC of MS or HC groups. (Figure 1***A***). All the PCRs include a positive control for both genes of BLV. The β-actin was evaluated in all samples as an internal control (Figure 1***B***).

Discussion

In 1978, Beverly et al. identified BLV as the causative agent of enzootic bovine leukosis (EBL). cDNA–DNA hybridization showed that the virus is an exogenous retrovirus. The proviral sequences in the lymphocytes and normal tissues of bovine and ovine samples with positive serology and tumor were reported to be 45% and 38%, respectively [15].

Hausen first mentioned the role of infectious agents in beef and milk, and listed these as risk factors for some cancers [16] and MS [17]. However, a study by Lee et al. on samples from 517 leukemia patients and 162 lung cancer cases, using PCR assay and primers for the BLV envelope gene, showed negative results for all samples [18]. In a study by Buehring et al. on 257 serum samples from healthy individuals, antibodies against BLV p24 antigen were found in 74% of the samples. However, this study stated that antibodies against this virus could have been caused by viral antigens consumed through food [12]. In a survey by Nikbakht et al., detection of anti-BLV antibodies by ELISA and of BLV provirus by PCR in 454 healthy human samples showed that 12.5% of people (57 samples) had antibodies against BLV, and that 12.3% of these antibody-positive samples (seven samples) (1.5%) were positive for BLV DNA [19]. However, the authors discussed the possibility that these BLV proviruses may be non-integrated elements that circulate in body fluids, and thus do not demonstrate that BLV DNA is integrated into human cells.

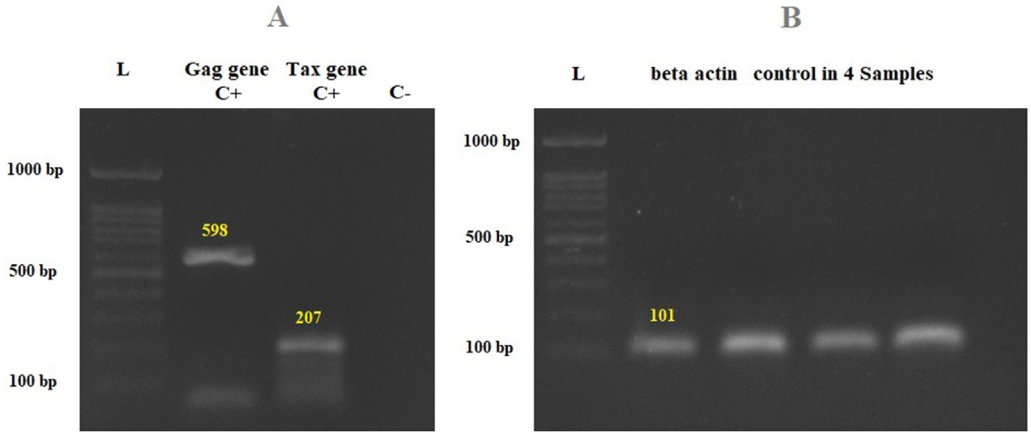

Figure 1. Gel electrophoresis of the PCR products. (A) The 598 bp band for *Gag* and the 207 bp band for *Tax* of BLV, compared with the negative control. (B) The β-actin 101 bp band as an internal control in four different samples.

There have been a few studies on the association between BLV and MS, most of which involved analyzing serum samples of patients. In a study by Koprowsky et al. on serum and spinal fluid samples of MS patients, antibodies were identified that reacted with the HTLV virus. Since antibodies against the HTLV virus also react with the BLV virus — there is an 11% homology between the genomic sequence of HTLV and BLV virus — this study suggested that the possibility of infection of these patients with BLV should be considered [14]. In another study by Clausen et al. on 27 serum samples of MS patients, it was found that the levels of antibodies against BLV increased in MS patients [13]. However, as mentioned above, this increase in antibody levels could be due to viral antigens consumed through meat or milk contaminated with the virus.

In our study, which was performed on 109 PBMC samples from patients with MS and 60 samples from healthy individuals, BLV DNA was evaluated by PCR with two pairs of primers for the *Gag* and *Tax* genes. No bands were obtained for the patient and healthy groups. Our results were consistent with the study by Lee et al. on cancer and healthy samples evaluated by PCR [18]. However, the small sample size of our study could considered a limitation. It is therefore suggested that more samples should be assessed for detection of BLV provirus by PCR, and that serum antibodies against BLV p24 antigen are evaluated simultaneously by ELISA to determine the role of BLV antigens that may be transmitted through the diet.

Conclusion

The results of our study represent no evidence of BLV in Iranian MS patients. Further evaluations of the possible association between BLV and MS are required for a clear conclusion.

Conflict of interests

The authors declare no conflicts of interest.

Declarations

Funding source

This study was supported by an Iran University of Medical Sciences grant (no. 15608-37-2-98).

Ethical approval statement

The ethical approval reference for this study is IR.IUMS.REC. 1398.850.

Author contributions

MS and ASJ conceived and designed the experiments. MF, SG, and AZ collected the samples. ML provided the FLK-BLV cells. MHK and AT performed the PCR and analyzed the data. ASJ, AZ, AT, and MG wrote the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgements

The authors would like to express their gratitude to the Virology Department of Iran University of Medical Sciences, the Neurosurgery Department of Isfahan University of Medical Sciences, and the Quality Control Department of Razi Vaccine and Serum Research Institute for their kind assistance.

References

[1] McGinley MP, Goldschmidt CH, Rae-Grant AD. Diagnosis and treatment of multiple sclerosis: a review. *Jama* 2021;**325**(8):765–79.
[2] Azami M, YektaKooshali MH, Shohani M, Khorshidi A, Mahmudi L. Epidemiology of multiple sclerosis in Iran: a systematic review and meta-analysis. *PLoS One* 2019;**14**(4) e0214738–e0214738.
[3] Karampoor S, Zahednasab H, Bokharaei-Salim F, Mirzaei R, Mojallal-Tabatabaei Z, Esghaei M, Keyvani H. HIV-1 Tat protein attenuates the clinical course of experimental autoimmune encephalomyelitis (EAE). *International Immunopharmacology* 2020;**78**:105943.
[4] Zimmermann M. *B cells and endogenous retroviruses in multiple sclerosis*. University of Basel; 2016.
[5] Lebrun C, Bensa C, Debouverie M, Wiertlevski S, Brassat D, de Seze J, Rumbach L, Pelletier J, Labauge P, Brochet B. Association between clinical conversion to multiple sclerosis in radiologically isolated syndrome and magnetic resonance imaging, cerebrospinal fluid, and visual evoked potential: follow-up of 70 patients. *Archives of Neurology* 2009;**66**(7):841–6.
[6] Ascherio A. Environmental factors in multiple sclerosis. *Expert Review of Neurotherapeutics* 2013;**13**(sup 2):3–9.
[7] Voumvourakis K, Fragkou P, Kitsos D, Foska K, Chondrogianni M, Tsiodras S. Human herpesvirus 6 infection as a trigger of multiple sclerosis: an update of recent literature. *BMC neurology* 2022;**22**(1):1–11.
[8] zur Hausen H, Bund T, Villiers E-Md. Infectious agents in bovine red meat and milk and their potential role in cancer and other chronic diseases. *Viruses, Genes, and Cancer* 2017:83–116.
[9] Polat M, Takeshima SN, Aida Y. Epidemiology and genetic diversity of bovine leukemia virus. *Virology Journal* 2017;**14**(1):209.
[10] Gillet N, Florins A, Boxus M, Burteau C, Nigro A, Vandermeers F, Balon H, Bouzar A-B, Defoiche J, Burny A. Mechanisms of leukemogenesis induced by bovine leukemia virus: prospects for novel anti-retroviral therapies in human. *Retrovirology* 2007;**4**(1):18.
[11] Moratorio G, Obal G, Dubra A, Correa A, Bianchi S, Buschiazzo A, Cristina J, Pritsch O. Phylogenetic analysis of bovine leukemia viruses isolated in South America reveals diversification in seven distinct genotypes. *Archives of Virology* 2010;**155**(4):481–9.
[12] Buehring GC, Philpott SM, Choi KY. Humans have antibodies reactive with bovine leukemia virus. *AIDS Research and Human Retroviruses* 2003;**19**(12):1105–13.
[13] Clausen J, Hoff-Jørgensen R, Rasmussen H. Antibody reactivity against animal retroviruses in multiple sclerosis. *Acta Neurologica Scandinavica* 1990;**81**(3):223–8.

[14] Cunningham AS. Bovine leukaemia virus and multiple sclerosis. *Nature* 1986;**320**(6059) 219–219.
[15] Kettmann R, Burny A, Cleuter Y, Ghysdael J, Mammerickx M. Distribution of bovine leukemia virus proviral sequences in tissues of bovine, ovine and human origin. *Annales de Recherches Vétérinaires* 1978:837–44.
[16] zur Hausen H. Red meat consumption and cancer: reasons to suspect involvement of bovine infectious factors in colorectal cancer. *Int J Cancer* 2012;**130**(11):2475–83.
[17] zur Hausen H. Risk factors: what do breast and CRC cancers and MS have in common? *Nature Reviews Clinical Oncology* 2015;**12**(10):569.
[18] Lee J, Kim Y, Kang CS, Cho DH, Shin DH, Yum YN, Oh JH, Kim SH, Hwang MS, Lim CJ, Yang KH, Han K. Investigation of the bovine leukemia virus proviral DNA in human leukemias and lung cancers in Korea. *JKMS* 2005;**20**(4):603–6.
[19] Nikbakht G, Rabani M, Emam M, Reza tofighi E. Serological and genomic detection of bovine leukemia virus in human and cattle samples. *Int J Vet Res* 2010;**4**(4):253–8.