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No evidence of XMRV provirus sequences in patients with myalgic encephalomyelitis/chronic fatigue syndrome and individuals with unspecified encephalopathy

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SUMMARY

Xenotropic murine leukemia virus-related virus (XMRV) has been considered a possible trigger of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) and could also be linked with unspecified encephalopathy.
The aim of this study was to analyse the frequency of XMRV proviral sequences in peripheral blood leukocyte (PBL) DNA from 150 patients with ME/CFS and 30 apparently healthy individuals, as well as in PBL and brain tissue DNA from 61 individuals with/without unspecified encephalopathy.
Targeting the XMRV proviral *gag* gene sequence by nested polymerase chain reaction (nPCR) with previously reported primer sets, provirus was not detected either in DNA from patients with ME/CFS and individuals with unspecified encephalopathy, or in apparently healthy individuals. Only the positive control gave the amplimer of 410 base pairs (bp) after the second round that corresponds to the expected XMRV *gag* gene fragment. In addition, DNA was found to be negative in nPCR assays, targeting XMRV specific *env* gene sequence, using previously described primer sets. Also only positive control gave the amplimer of 218 bp after the second round, corresponding to the expected XMRV *env* gene fragment.
Using nPCR we found no evidence of XMRV infection either in apparently healthy individuals or in patients with ME/CFS and individuals with unspecified encephalopathy.

KEY WORDS: XMRV, ME/CFS, Encephalopathy, nPCR.

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INTRODUCTION

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is defined as a complex disease involving profound dysregulation of the central nervous system and immune system, dysfunction of cellular energy metabolism and ion transport, and cardiovascular abnormalities (Carruthers *et al.,* 2011). The main clinical sign is severe, unexplained, persistent chronic fatigue lasting more than six months, accompanied by at least four of the following eight symptoms: sore throat, tender cervical or axillary lymph nodes, muscle pain, impaired memory or concentration, un-refreshing sleep, post-exertional malaise and headache of new type (Fukuda *et al.,* 1994). CFS was first defined in 1988. Later, in 1994 this definition was revised and various other definitions were created. Recently the International Consensus Panel developed criteria and suggested also using the term "myalgic encephalomyelitis" (ME), due to widespread inflammation and the multisystem-

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ic neuropathology of the disease (Carruthers *et al.,* 2011). The primer for clinical practitioners, published in 2012, gives advice on how to diagnose ME/CFS and which therapies can be used (Friedberg, 2012).

According to population-based studies, already reported 15 years ago, the estimated worldwide prevalence of ME/CFS is from 0.2 up to 2.6%. According to the latest publication reports, it affects 0.4-1% people. The disease is six times more common in women than in men (Bansal *et al.,* 2012). The diagnosis of ME/CFS is based on clinical symptoms and differential diagnosis therefore it is important to identify specific parameters, which can be used as diagnostic markers. Still effective standardized and reproducible diagnostic tests, medical treatment and prevention strategies for ME/CFS are being sought (Albright *et al.*, 2011). Viral infection, immune dysfunction, neurotic hypotension and depression are considered possible etiological factors for ME/CFS (Bansal *et al.*, 2012). ME/CFS is diagnosed only for of wwpart those people suffering from this illness, affecting not only individuals, but also national economics. ME/CFS can affect everyone, irrespective of age, gender and social-economic status, therefore it is important to clarify initiation, progression and maintaining process mechanisms of the disease, as well as develop biomarkers for earlier diagnostics.

Encephalopathy is an abnormal structure or condition of the brain tissues, which can occur at any age. It can be caused by viral or bacterial infection, liver disease, metabolic or nutritional diseases, high blood pressure and hereditary diseases. Viruses are implicated in neurological diseases and neurological disorders, such as Alzheimer's, Parkinson's, multiple sclerosis, encephalitis and mesial temporal lobe epilepsy. It is known that encephalopathy is a common problem accompanied by such viral infections as influenza, human herpesvirus-6 and rotavirus (Hoshino *et al.,* 2012). Still, there is a possibility that unspecified encephalopathy could be caused by xenotropic murine leukemia virus-related virus (XMRV) infection.

XMRV belongs to the *Retroviridae* family, *Orthoretrovirinae* subfamily, *Gammaretrovirus* genus. It was first identified in 2006, investigating the lack of ribonuclease L-coding antiviral gene RNASEL function in patients with prostate cancer (Urisman *et al.,* 2006). The results of studies on XMRV in patients with prostate cancer in Europe and America are controversial. Some researchers report on finding this virus in prostate cancer tissue by PCR, immunohistochemistry, fluorescence *in situ* hybridization and/or serological assays, but others failed to detect XMRV in patients with prostate cancer (Schlaberg *et al.,* 2009; Switzer *et al.,* 2011).

Observation of RNase L proteolysis in peripheral blood mononuclear cells (PBMCs) in patients with ME/CFS and viral infection-like chronic immune system activation prompted the search for XMRV in patients with these disorders. XMRV has been considered a possible trigger of ME/CFS since October 2009, when Lombardi *et al.* (2009) reported finding XMRV in 68/101 (67%) persons with ME/CFS and 8/218 (3.7%) healthy controls (Lombardi *et al.,* 2009). Many subsequent studies found no evidence of XMRV in patients with ME/CFS (Groom *et al.*, 2010; Hohn *et al.,* 2010; Hong *et al.,* 2010; van Kuppeveld *et al.,* 2010). Lo and co-authors reported on murine leukemia virus (MLV)-related virus *gag* gene sequence in 32/37 (86.5%) patients with ME/CFS and 3/44 (6.8%) healthy donors, whereas the *env* gene sequence was amplified only from one patient and one donor. These sequences are more closely related to polytropic and modified polytropic MLV viruses (Lo *et al.,* 2010). Subsequently XMRV was not detected either in patients with ME/CFS, or in apparently healthy individuals (Ali *et al.,* 2011; Cool *et al.,* 2011; Alter *et al.,* 2012). No signs of XMRV were found testing not only samples from patients with ME/CFS and a control group, but also from patients with rheumatoid arthritis and transplant recipients (Henrich *et al.,* 2010). Patients with spondyloarthritis, multiple sclerosis, as well as pediatric patients with idiopathic and respiratory diseases have been shown to be XMRV negative (Jeziorski *et al.,* 2010; Maric *et al.,* 2010). Likewise, XMRV has not been found in human immunodeficiency virus (HIV)-positive patients (Henrich *et al.,* 2010).

Independent studies show that XMRV could be detected due to contamination of commercially available reagents for PCR or human DNA samples contaminated with mouse DNA that

contains MLV-related virus genomic sequences (Sato *et al.,* 2010; Oakes *et al.,* 2010). The finding of XMRV could also emerge from the infected prostate cancer cell line 22Rv1 and cloned or amplified XMRV DNA (Hue *et al.*, 2010; Smith, 2010; Kearney *et al.,* 2012).

After several years of efforts to replicate the finding of XMRV in patients with ME/CFS, previous publications by Lombardi *et al.* (2009) and Lo *et al.* (2010) were retracted because of insufficient quality of experiments and the subsequent inability to detect XMRV in ME/CFS cases (Alberts, 2011; Lo *et al.,* 2012).

We investigated the involvement of β-herpesviruses human herpesvirus (HHV)-6 and HHV-7 and parvovirus B19 infection in the development of ME/CFS, post-infection and unspecified origin encephalopathy. Active viral infection including concurrent infection is found in 64.8% (70/108) of patients and in 13.3% (12/90) of practically healthy persons (Chapenko *et al.,* 2012). It is known that gammaretrovirus-like sequences occur in most vertebrate genomes and murine leukemia virus-like retroviruses (MLLVs) are a subset, which may be pathogenic and spread across species (Blomberg *et al.,* 2011). Taking into account the immunomodulating properties of human β-herpesviruses HHV-6 and HHV-7 and parvovirus B19 as well as the fact that activation of one virus could lead to activation of another we examined XMRV in Latvian ME/CFS patients and individuals with unspecified origin encephalopathy. The aim of this study was to analyse the frequency of XMRV proviral sequences in DNA extracted from peripheral blood leukocyte (PBL) samples of patients with ME/CFS and apparently healthy individuals, as well as in PBL and brain tissue DNA isolated from individuals with and without unspecified encephalopathy in Latvia.

MATERIAL AND METHODS

One hundred and fifty patients [52 (35%) male and 98 (65%) female, mean age 37.8±9.5] with clinically diagnosed ME/CFS according to the Centers for Disease Control and Prevention diagnostic criteria and 30 age and gender-matched apparently healthy individuals, as well as 61 [41 (67%) male and 20 (33%) female, mean age 56.6±10.5] individuals post-mortem (30 with and 31 without unspecified encephalopathy) were involved in this study. The cohort was established with the approval of the Ethics Committee of Riga Stradins University. DNA from PBL and brain tissue samples was isolated using a phenol-chloroform extraction method, DNA quality assured by β-globin gene polymerase chain reaction (PCR). Potential carry-over contamination was prevented, processing samples in separate rooms and multiple negative controls were included in each assay. The presence of XMRV provirus *gag* and *env* sequences was detected by nested PCR (nPCR) using previously described primer sets. The sensitivity of the nPCR was five copies per reaction (Lombardi *et al.*, 2009; Lo *et al.,* 2010). XMRV VP62 plasmid (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: XMRV VP62 cDNA from Drs Robert H. Silverman and Beihua Dong) was used as a positive control (Urisman *et al.,* 2006; Dong *et al.,* 2007). Electrophoretic analysis of amplification products was carried out in 2% agarose gel.

RESULTS

Using a PCR gradient method positive control DNA was tested by nPCR assays with modified oligonucleotide primer melting temperatures for hybridisation. An optimal result was obtained at temperatures 59.8ºC, 65.6ºC, 66.6ºC and 67ºC, while a weaker band was visible at 57ºC and 64.2ºC. By contrast, a result was practically impossible to detect using 57.4ºC, 58.4ºC, 61.3ºC and 62.7ºC hybridisation temperatures (Figure 1).

Targeting the XMRV specific *gag* gene sequence by nPCR with previously reported primer sets 419F, 1154R for the first round and GAG-I-F, GAG-I-R for the second round, XMRV proviral *gag* gene sequence was not detected either in DNA isolated from 150 patients with ME/CFS PBL or DNA extracted from PBL and brain samples of 61 individuals post-mortem (30 with and 31 without unspecified encephalopathy), or in 30 apparently healthy individuals' DNA. Only the positive control gave amplimers of 735 bp after the first round and 410 bp after the

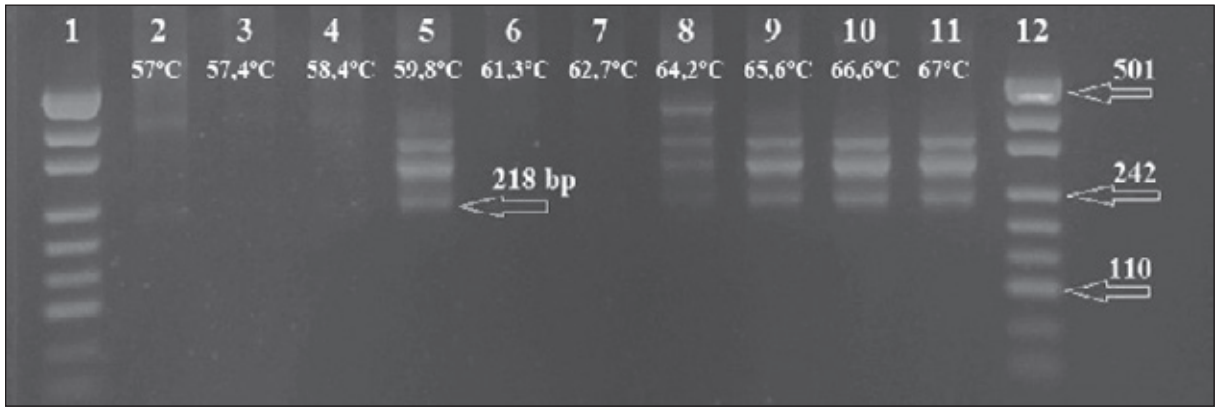

FIGURE 1 - *Gradient method-amplified XMRV specific env gene sequence product in 2% agarose gel. 1., 12. Marker [pUC19DNA/MspI (HpaII)]; 2. - 11. Positive control (XMRV VP62 plasmid).*

second round, corresponding to the expected XMRV *gag* gene fragment (Figure 2).

Moreover, patients with ME/CFS, individuals with and without unspecified encephalopathy, and apparently healthy individuals' DNA was found to be negative in nPCR assay, targeting the XMRV provirus specific *env* gene sequence with previously described primer pairs 5922F, 6273R for the first round and 5942F, 6159R for the second round. In addition, only the positive control gave amplimers of 351 bp after the first round and 218 bp after the second round, corresponding to the expected XMRV *env* gene fragment (Figure 3).

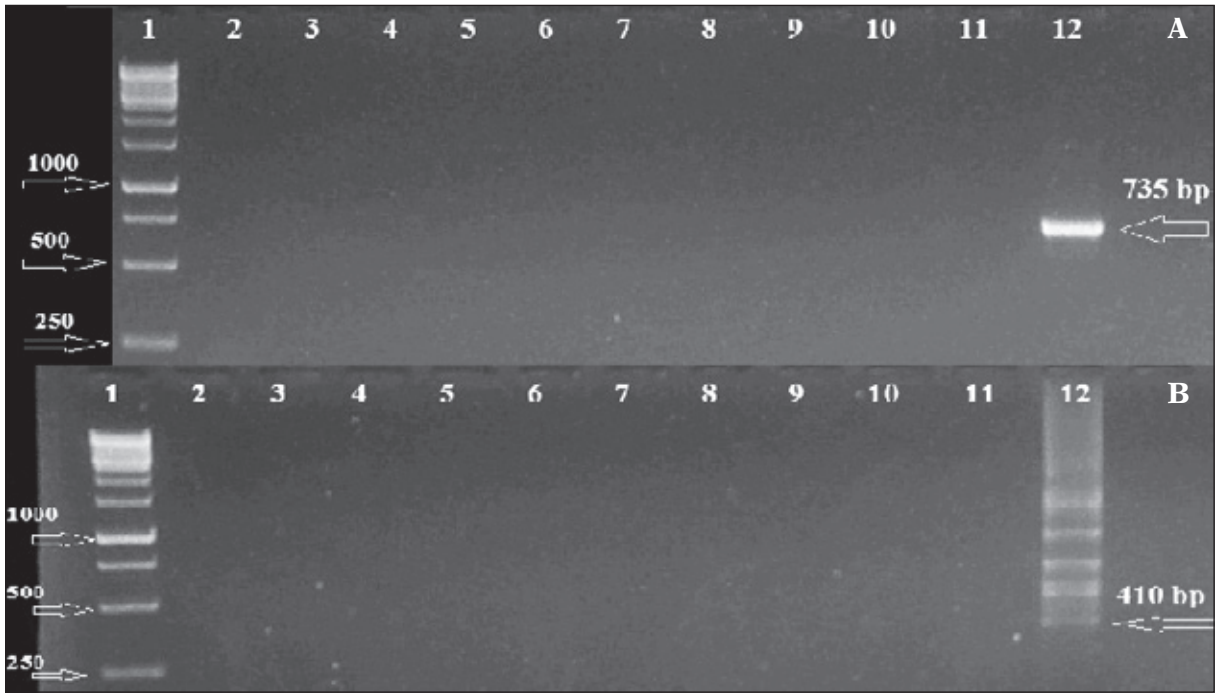

FIGURE 2 - *Electrophoretic analysis of XMRV specific gag gene sequence amplification product in 2% agarose gel after the first round (A) and after the second round (B). 1. Marker (GeneRuler 1 kb DNA Ladder); 2. Reagent water control; 3-11. Samples without viral sequence; 12. Positive control (XMRV VP62 plasmid).*

FIGURE 3 - *Electrophoretic analysis of XMRV specific env gene sequence amplification product in 2% agarose gel after the first round (A) and after the second round (B). 1. Marker [pUC19DNA/MspI (HpaII)]; 2. Reagent water control; 3-11. Samples without viral sequence; 12. Positive control (XMRV VP62 plasmid).*

DISCUSSION

ME/CFS is a heterogeneous disease accompanied by a group of symptoms, often followed by viral infection or long-lasting stress. Despite intensive research, there is no consensus on the presence, form or level of immune dysfunction in this condition (Bansal *et al.,* 2012). The disease is characterized by severe fatigue making people unable to work, and lacking a pathophysiological explanation. Different studies on ME/CFS use various diagnostic criteria for the disease, causing heterogeneity between patient groups and uncertainty in results. Therefore, it is necessary to use one common diagnostic criterion. Most patients report a sudden start of the illness with flu-like symptoms, therefore viral infections are considered as one of the possible ME/CFS causative agents. Some viruses may cause fatigue after infection and many patients with ME/CFS have immunological disturbances that could be the result of viral infection or promoted by the infection. Still possible involvement of viral infections in the etiopathogenesis of ME/CFS is discrepant (Morinet and Corruble, 2012). It is not clear whether viral infection causes ME/CFS or follows after the disease.

In 2009, Lombardi and colleagues reported on the prevalence of XMRV in 67% patients with ME/CFS and 3.7% of healthy donors (Lombardi *et al.,* 2009). In 2010, Lo *et al.* also found MLV-related virus *gag* gene sequence in 86.5% patients with ME/CFS and 6.8% healthy donors, still the *env* gene sequence had one patient and one donor (Lo *et al.,* 2010). However, the findings of the current study do not support the previous research. In this study XMRV specific *gag* and *env* gene sequences were not detected either in DNA from 150 patients with ME/CFS or 61 individuals post-mortem (30 with and 31 without unspecified encephalopathy), or in the DNA of 30 apparently healthy donors. Obtained results are in accordance with studies in Sweden, China, Netherland, Germany, United States of America, United Kingdom, Japan and Canada which found no evidence of XMRV in patients with ME/CFS or donors (Blomberg *et al.,* 2011; Hong *et al.,* 2010; van Kuppeveld *et al.,* 2010; Hohn *et al.,* 2010; Satterfield *et al.,* 2011; Erlwein *et al.,* 2011; Furuta *et al.,* 2011; Steffen *et al.,* 2011). In addition, XMRV or MLV-related sequences, infectious virus or antibodies were not detected in tested groups of patients with ME/CFS, previously tested as XMRV positive, including part of patient samples from the original study by Lombardi *et al.* (2009) (Shin *et al.,* 2011; Knox *et al.,* 2011). This retrovirus was also absent in ME/CFS patients' cerebrospinal fluid (Schutzer *et al.,* 2011). The presence of XMRV

infection was sought in post-mortem brains of autistic individuals, but no signs of XMRV or MLV-related viruses were found (Lintas *et al.*, 2011). Studies published later consistently showed no association between XMRV and prostate cancer (Furuta *et al.,* 2011; Lee *et al.,* 2012). Furthermore, researchers were not able to detect any signs of XMRV infection markers in HIV-positive patients and patients with fibromyalgia (Arredondo *et al.,* 2011; Gingaras *et al.,* 2012). XMRV was not found in patients with such chronic inflammatory diseases as rheumatoid arthritis, systemic lupus erythematosus and Behcet's disease (Ali *et al.,* 2011). In addition, chronic hepatitis B and C, lymphoid malignancies and lymphadenopathy are proven not to be associated with XMRV (Arredondo *et al.,* 2011; Waugh *et al.,* 2011).

The findings of the current study are consistent with other research concluding after more than two years of intensive research on XMRV that this virus is not associated with human diseases and the positive results may be due to contamination (Groom and Bishop, 2012). However, a group of researchers in Italy recently reported the detection of a *gag* gene sequence in two out of 12 (16.6%) patients with ME/CFS. One was closely related to polytropic MLV-related virus and other was similar to XMRV. Although the amount of XMRV proviral DNA is very low, the role of XMRV in human diseases remains obscure (Paolucci *et al.,* 2012).

This study using nPCR found no evidence of XMRV infection either in patients with ME/CFS or individuals with unspecified encephalopathy, or in apparently healthy individuals.

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