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Issue by screening 47 pairs of monozygotic twins discordant for CFS using a sensitive PCR assay

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Abstract

The recent debate regarding the possible involvement of xenotropic murine leukemia virus-related virus (XMRV) in chronic fatigue syndrome (CFS) in humans has been intense due to the conflicting results from different studies and the unclear origin of the XMRV virus. In this study, we have addressed this issue by screening 47 pairs of monozygotic twins discordant for CFS using a sensitive PCR assay. The results are clearly negative for all samples. This is consistent with studies showing no evidence for the involvement of XMRV in CFS and supports the possibility that previous positive studies could be caused by laboratory artifacts.

Keywords: Chronic fatigue syndrome (CFS), idiopathic chronic fatigue, infectious disease, xenotropic murine leukemia virus-related virus (XMRV), monozygotic twins.

INTRODUCTION

Chronic fatigue syndrome (CFS) is a severe syndrome characterized by prolonged and impairing fatigue and the absence of other disorders (Fukuda et al., 1994; Reeves et al., 2003). The initial symptoms are often similar to an infection. Immune dysfunction, which could result from a chronic infection or an inappropriate response to an initial infection (Komaroff and Buchwald, 1998; Mihrshahi and Beirman, 2005; Devanur and Kerr, 2006; Hempel et al., 2008; Lorusso et al., 2009), is believed to be a major causative factor. The possible role of a range of specific viruses in CFS has been investigated by searching for case-control differences in past or current viral infection (e.g., cytomegalovirus, Epstein-Barr virus, GBV-C, human herpes virus-6 and parvovirus B19) (Devanur and Kerr, 2006). Several reports of positive associations with a particular virus have been published, but it has not been possible to replicate the findings.

This is exemplified by the recent series of publications and intense debate on the role of the recently discovered xenotropic murine leukemia virus-related virus (XMRV) in CFS. XMRV was claimed to be present in 67% of cases with CFS and 3.7% of controls in a study from 2009 (Lombardi et al., 2009); however, these findings did not replicate in multiple independent samples (McClure and Wessely, 2010) but an NIH study (Lo et al., 2010) also found a significant increase in the frequency of retroviral infection in CSF patients. More recent studies have suggested that the positive XMRV findings were likely to be due to a sample contamination (Hue et al., 2010; Smith, 2010; Sato et al., 2010; Oakes et al., 2010; Robinson et al., 2010; Shin et al., 2011; Knox et al., 2011), which explained the conflicting results, and it is even possible that XMRV in itself was created as a laboratory artifact (Paprotka et al., 2011).

In order to further clarify the issue of XMRV and CFS further, it is useful to search for the virus in well-selected sets of patients and controls, as inconsistent findings

across case-control studies could be due to bias if controls are inappropriate to cases. For example, in the initial XMRV study, apart from the contamination issue, cases were highly selected (chronically ill patients treated in medical practices specializing in CFS) and controls were described only as “healthy” (Lombardi et al., 2009). The study of discordant monozygotic twins offers substantially improved experimental control (that is, an individual affected with CFS contrasted with their well monozygotic twin) (Byrnes et al., 2009). In this study, 47 pairs of monozygotic twins discordant for chronic fatigue, which have been used for CFS studies, including virus screening in our laboratory (Byrnes et al., 2009; Sullivan et al., 2011), were screened for XMRV using a sensitive PCR assay.

METHODS

The protocol was approved in advance by the ethical review board at UNC-CH and the Karolinska Institutet and all subjects provided written informed consent. The selection and diagnosis of patients was described exhaustively in Byrnes et al. (2009) and Sullivan et al. (2011). Briefly, 61,000 twin pairs from the Swedish twin registry was screened and 47 out of 140 pairs that were initially showed disconcordance for chronic fatigue were found to meet the rigorous inclusion criteria, regarding monozygosity, demographics, other medical conditions and other factors that could possibly influence the study (Byrnes et al., 2009). A discordant twin pair was defined as one twin meeting criteria for either CFS or idiopathic chronic fatigue (ICF) and the other twin was required never to have experienced impairing unusual fatigue or tiredness lasting more than one month.

Several rounds of PCR of XMRV from cellular DNA from white blood cells from patients and controls were performed using conditions described by Lombardi et al. (2009). The primers used were: X419F ATCAGTTAACCTACCCGAGTCGGAC; X1154R GCCGCCTCTTCTTCATTGTTCTC; XGAGOF CGCGTCTGATTTGTTTTGTT; and XGAGOR CCGCCTCTTCTTCATTGTTC. The first PCR was carried out in a mixture containing 1xPCR buffer II (Applied Biosystems) with 2.5 mM MgCl2 , 200 µM dNTPs, 0.4 µM of the forward and reverse primers, and 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 5 µl of the clinical sample in a total volume of 50 µl. The reactions were incubated 10 min at 94°C followed by (94°C for 1 min, 52°C for 1 min, 72°C for 2 min) x 35 cycles, followed by 72°C for ten minutes, and they were subsequently held at 4°C. The second PCR was carried out using 2 µl from the first PCR in a mixture containing 1xPCR buffer II (Applied Biosystems) with 2.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM of the forward and reverse primers, and 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 5 µl of the clinical sample in a total volume of 50 µl. The reactions were incubated 10 min at 94°C followed by (94°C for one minute, 52°C for one minute, 72°C for two minutes) × 35 cycles, followed by 72°C for five minutes, and they were subsequently held at 4°C.

A synthesized and cloned XMRV fragment (Blue Heron Bio) was used as a positive control. The sequence of the synthesized fragment was:

GCCCATTCTGTATCAGTTAACCTACCCGAGTCGGACTTTTTGG
AGTGGCTTTGTTGGGGGACGAGAGACAGAGACACTTCCCGCC
CCCGTCTGAATTTTTGCTTTCGGTTTTACGCCGAAACCGCGCC
GCGCGTCTGATTTGTTTTGTTGTTCTTCTGTTCTTCGTTAGTTTT
CTTCTGTCTTTAAGTGTTCTCGAGATCATGGGACAGACCGTAA
CTACCCCTCTGAGTCTAACCTTGCAGCACTGGGGAGATGTCCA
GCGCATTGCATCCAACCAGTCTGTGGATGTCAAGAAGAGGCG
CTGGGTTACCTTCTGTTCCGCCGAATGGCCAACTTTCAATGTA
GGATGGCCTCAGGATGGTACTTTTAATTTAGGTGTTATCTCTCA
GGTCAAGTCTAGAGTGTTTTGTCCTGGTCCCCACGGACACCC
GGATCAGGTCCCATATATCGTCACCTGGGAGGCACTTGCCTAT
GACCCCCCTCCGTGGGTCAAACCGTTTGTCTCTCCTAAACCCC
CTCCTTTACCGACAGCTCCCGTCCTCCCGCCCGGTCCTTCTGC
GCAACCTCCGTCCCGATCTGCCCTTTACCCTGCCCTTACCCTC
TCTATAAAGTCCAAACCTCCTAAGCCCCAGGTTCTCCCTGATA
GCGGCGGACCTCTCATTGACCTTCTCACAGAGGATCCCCCGC
CGTACGGAGCACAACCTTCCTCCTCTGCCAGGGAGAACAATG
AAGAAGAGGCGGCCACCACCTCCGT.

This fragment was also used in a titration experiment using serial dilution. We found that the detection limit of the nested PCR was between 1 to 5 copies of the fragment/PCR reaction. The PCR products were analyzed by 1% agarose gel electrophoresis.

RESULTS

PCR for XMRV was carried out for each patient and control DNA sample using the conditions described in the foregoing. The same samples have previously been used for detection of other viruses via PCR, including hepatitis C virus and GB virus C, and they have been found to be suitable for PCR analysis (Sullivan et al., 2011). We carried out nested PCR to detect provirus in the human DNA samples. Despite a detection level of 1-5 copies per PCR, and repeated PCRs, none of the twins suffering from CFS or their healthy siblings were found to be positive for XMRV (Figure 1). A non-reproducible band close to the correct size was seen for one of the unaffected twins in one PCR reaction (Figure 1c), and the affected twins showed no such products. The PCR conditions were closely modeled on those used in the original XMRV study (Lombardi et al., 2009), where XMRV was first detected in CSF patients. The results thus conflict the initial reports by Lombardi et al. (2009) and Lo et al. (2010) and are consistent with the hypothesis that the positive findings were due to an artifact. These results are in agreement with other studies performed in Europe (McClure and Wessely, 2010).

DISCUSSION

We did not detect XMRV in our CFS cases and controls. The high detection level of the PCR assay and the quality of the DNA samples used in this study makes it highly unlikely that the negative result is caused by low-level infections. There are recent indications that there is some variation among the XMRV and XMRV-like viruses found in CFS (Lo et al., 2010). We therefore cannot completely rule out the possibility that a related virus could have been present in our samples but which was undetected. This possibility would require the presence of specific geographical sequence variation in XMRV and no data currently exists to address this issue. In addition, no such

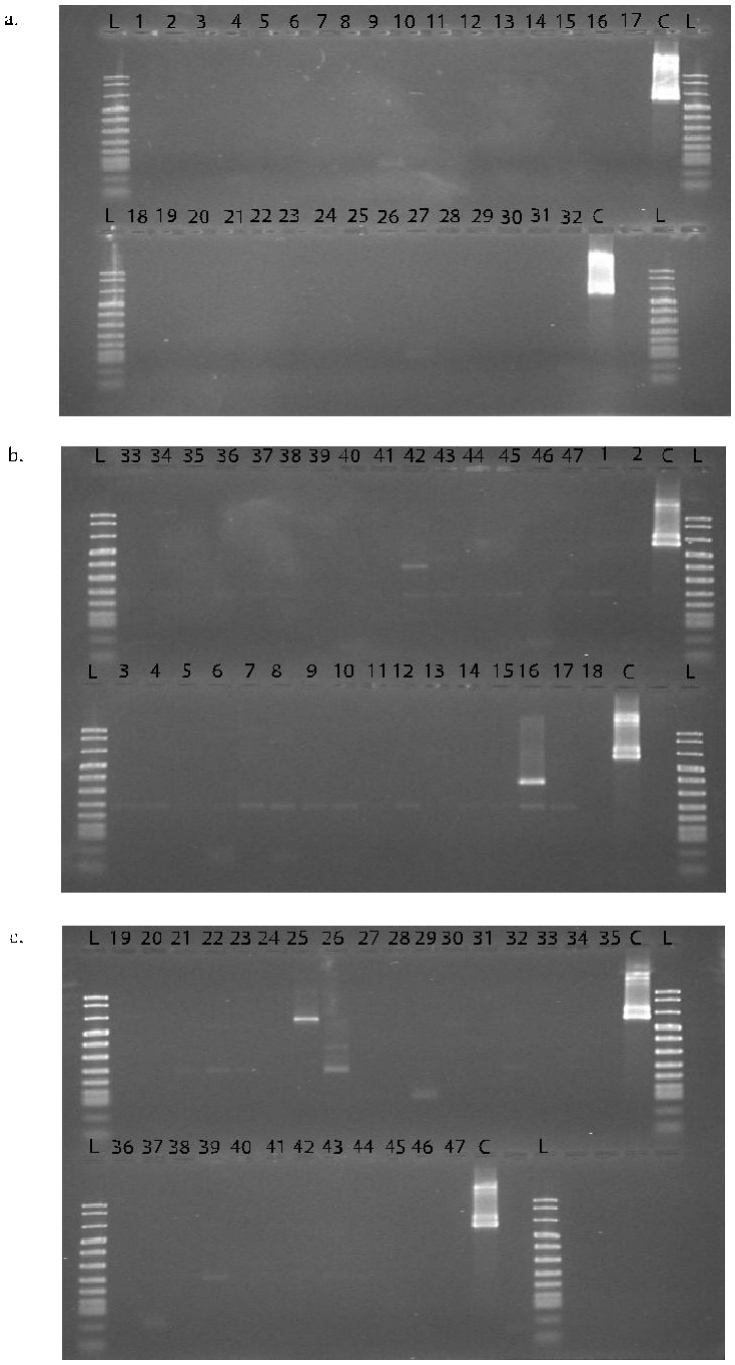

Figure 1. Gel images showing the results of the second PCR step in the nested PCR for XMRV for all patients and controls. Each gel row also contains a size marker and the PCR product from the positive control. The first 47 samples are from the twins affected with CSF and the remaining PCRs are from the healthy twins. No PCR products matching the XMRV control could be detected.

virus was found in the metagenomic search for viruses that was carried out in these samples (Sullivan et al., 2011).

The results of this study of XMRV are in concordance with other negative studies of European CFS cases and controls. As mentioned previously, multiple recent studies have indicated that positive XMRV findings were caused by laboratory contamination and that XMRV is not involved in human disease (Hue et al., 2010; Smith, 2010; Sato et al., 2010; Oakes et al., 2010; Robinson et

al., 2010; Shin et al., 2011; Knox et al., 2011). Our results do not conflict with this possibility. Our results thus support the conclusion that XMRV is not involved in the etiology of CFS. Other explanations, such as that the pattern of XMRV infection is different depending on the geographic location, or that the virus variants present in Europe are significantly different from those present in the two American sample cohorts where it was detected, appear unlikely in the light of recent findings.

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