XMRV Prevalence in Patients with Chronic Fatigue Syndrome or Chronic Immunomodulatory Conditions

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Abstract

We investigated the prevalence of xenotropic murine leukemia virus-related virus (XMRV) in 293 participants seen at academic hospitals in Boston, Massachusetts. Participants were recruited from five groups of patients: chronic fatigue syndrome (CFS, n = 32), HIV infection (n = 43), rheumatoid arthritis (RA, n = 97), hematopoietic stem-cell or solid organ transplant (n = 26), or a general cohort of patients presenting for medical care (n = 95). XMRV DNA was not detected in any participant samples. We found no association between XMRV and patients with CFS or chronic immunomodulatory conditions.

Keywords

XMRV; chronic fatigue syndrome; HIV infection; rheumatoid arthritis; hematopoietic stem-cell transplantation; solid organ transplantation

Introduction

Murine and human xenotropic viruses were first described over 30 years ago [1]. The xenotropic murine leukemia virus-related virus (XMRV) was discovered in prostate tissue from prostate cancer patients homozygous for a reduced-activity variant of RNase L [2–4]. RNase L gene abnormalities have been described in patients with CFS [5], although these findings have not been verified in a more recent investigation [6].
A recent study in the United States found evidence of integrated XMRV DNA in 67% of subjects with CFS, compared to 3.7% of healthy controls [7]. Three subsequent CFS studies in European cohorts have not demonstrated the presence of XMRV DNA in CFS subjects or healthy controls [8–10]. The explanation for these contrasting results is unknown.

We designed a cross-sectional cohort study to determine XMRV prevalence in a variety of North American clinic populations, including healthy subjects, participants with CFS, and participants with states of chronic immune activation or suppression. We incorporated three different PCR primer sets from published XMRV studies into our testing strategy to determine if differences existed in their ability to amplify XMRV DNA.

Methods

Participant enrollment and study design

Samples were obtained from adult patients presenting to outpatient clinics, or from pre-existing repositories and cohorts at Brigham and Women’s Hospital, Massachusetts General Hospital, and Dana-Farber Cancer Institute. Participants prospectively enrolled had a prior diagnosis of HIV infection, hematopoietic stem-cell or solid organ transplant, or CFS. This study was powered to detect XMRV prevalence with an upper confidence limit of 1.2% for the combined cohorts.

All CFS participants enrolled met the Centers for Disease Control and Prevention (CDC) revised CFS case definition (http://www.cdc.gov/cfs/cfsdiagnosis.htm). CFS participants had 10 mL of whole blood collected for peripheral blood mononuclear cell (PBMC) isolation and XMRV PCR testing, and completed a questionnaire. Demographic and clinical information were obtained from chart review. Cryopreserved PBMC and clinical information were obtained from HIV-infected patients from the International HIV Controllers Study [11–12]. Cryopreserved PBMC DNA and demographic data for rheumatoid arthritis participants, and an age- and gender-matched cohort of participants who had presented for either inpatient or outpatient clinical care, were obtained from the Crimson Biospecimen Core [13]. This study was approved by the relevant Institutional Review Boards.

XMRV PCR amplification

Human DNA was extracted from at least 5×10^6 PBMC (Qiagen). PCR reaction conditions and primer sets are described in Table 1. First round PCR amplifications were performed with 1 unit of Platinum Taq DNA Polymerase High Fidelity (Invitrogen), 80–200ng of PBMC DNA, and 200nM of the outer primers of Lombardi, et al. (outer primer set) [7]. Nested PCRs were performed with first round product and 200nM of the inner primers of either Erlwein et al. (inner primer set #1) [8] or Urisman et al. (inner primer set #2) [4]. PBMC DNA from participant S6 was retested using an additional cryopreserved DNA aliquot, the original Platinum Taq HF Polymerase reagents, and USB Taq Polymerase (Affymetrix).

Each PCR amplification run included a negative control and positive controls of 10, 100, and 1000 copies of full-length XMRV (isolate VP62), generously provided by Robert H. Silverman, Cleveland Clinic. To test the efficiency of DNA extraction and PCR amplification, increasing amounts of XMRV plasmid, starting at 1 copy, were added to 200ng of PBMC DNA and to PBMCs prior to DNA extraction. Nested PCR amplification for each experiment was performed in triplicate for each primer set. To verify DNA integrity, human β-globin (hBG) was amplified from each participant sample with a single-round PCR reaction that used the outer hBG primer set of Erlwein, et al. [8]. All but one sample, from a general clinic participant, demonstrated β-globin amplification. This sample
was excluded from our analysis. Laboratory personnel who performed PCR amplification were blinded to the identity of each sample.

**XMRV cloning and sequence analysis**

DNA bands near the length of interest were purified with the QIAquick Gel Extraction kit (Qiagen), cloned into a pCR4-TOPO vector (Invitrogen) and bi-directionally sequenced (ABI 3730 automated DNA sequencer, Applied Biosystems).

**Data analysis**

Chi-squared ($\chi^2$) testing with continuity correction was used to compare inter-group variation between non-parametric variables. Fisher’s exact tests were used if expected counts were less than five. One-way analysis of variance testing was performed to define inter-group comparisons of continuous variables; a Bonferonni correction was used in pairwise tests of individual hypotheses (SPSS version 10). To estimate the probability of detecting XMRV in CFS participants based on sample size, the Blyth-Still-Casella 95% exact confidence intervals (CIs) for disease prevalence were calculated.

**Results**

**Participant cohorts**

PBMC samples from 293 participants were tested for the presence of XMRV DNA (Table 2). Thirty-two subjects with a previous CFS diagnosis and 26 patients that had undergone either hematopoietic stem-cell or solid-organ transplant from participating outpatient hospital clinics were prospectively enrolled. Samples from 43 HIV-infected subjects were included either from an existing sample cohort or enrolled prospectively from the Brigham and Women’s Hospital infectious disease clinic. Twenty-two of the HIV-infected patients (51%) were virologically suppressed on ART; the remainder were chronically infected persons not on treatment. We tested 97 samples from patients with rheumatoid arthritis and 96 samples from age- and gender-matched controls obtained from the Crimson Biospecimen Core.

Participants with rheumatoid arthritis and those from the age- and gender-matched general clinic/hospital cohort had the highest proportion of women, greater than 80%, within each group. The majority of CFS participants were women (66%), whereas the HIV-infected and hematopoietic stem-cell transplantation or organ transplantation cohorts contained fewer women (19% and 46%, respectively; significant inter-group difference compared to CFS cohort, p < 0.001). Mean CD4+ T lymphocyte counts for HIV-infected participants on or off ART were similar (566 and 554 cells/μl respectively, P = 0.89); 95.5% of participants receiving ART has plasma HIV-1 RNA levels <50 copies/mL. A majority of transplant patients had undergone solid organ transplantation, including eleven kidney, seven liver, two lung, two heart, and one liver/kidney transplants. One liver transplant recipient had underlying treated HIV infection. The remaining three transplant participants had undergone allogeneic hematopoietic stem-cell transplantation.

A majority of CFS participants had daily symptoms (75.9%), stopped work as a direct result of CFS symptoms (69%), or experienced fever, lymphadenopathy or swollen glands at the start of their illness (75%); 20.7% of participants had household contacts with similar symptoms or a diagnosis of CFS and 7% noted a tick-bite just prior to onset of their symptoms. The mean reported duration of symptoms for the CFS participants was 11.6 years.
PCR analysis

We could reliably detect 10 copies of XMRV per 200ng of PBMC DNA when control XMRV plasmid was added to PBMC aliquots either before or after DNA extraction. We were able to detect 1 copy of XMRV DNA in 2 of 3 assays when the VP62 plasmid was added to extracted PBMC DNA aliquots, a result consistent with the Poisson distribution. Using the PCR strategy described, we did not detect XMRV gag DNA in any of the 293 participant samples. In seven nested PCR reactions, DNA bands of a size similar to the expected XMRV gag PCR product were observed (data not shown). Cloning and sequence analysis of the PCR amplicon from participant S6, a subject with rheumatoid arthritis, demonstrated >99% sequence identity to a mouse endogenous retrovirus that was not XMRV [14]. Multiple repeat PCR amplifications with participant S6 PBMC DNA that used the original reagents, an additional aliquot of DNA, or another commercially available Taq polymerase failed to generate a PCR amplicon; this finding suggests that the original amplicon most likely resulted from contamination. Amplicons from the other six nested PCR reactions had sequences matching a portion of the human genome and shared no homology with XMRV.

Discussion

We assessed the prevalence of XMRV in a cohort of 293 American patients with CFS or chronic conditions associated with immune activation and/or immune deficiency and did not detect XMRV in any participant sample. A previous report showed that XMRV DNA could be detected in patient samples after a single round of 45-cycle PCR [7]. Our XMRV amplification strategy used similar amounts of input PBMC DNA and identical primer sets as were used in previous reports, and we increased the sensitivity of our methods by adding a nested PCR amplification that used two additional previously published XMRV primer sets [4,7–8]. These negative findings demonstrate that XMRV was not associated with any specific group that we investigated; the choice of PCR primers did not affect XMRV prevalence estimates. XMRV DNA could be present at levels below our detection threshold. However, we used PCR methodology that was comparable to previously published methods that detected XMRV DNA in CFS and healthy control subjects [7,15].

The upper limit of the 95% confidence interval around our CFS participant XMRV prevalence estimate (0%) was 9.5%. This result is similar to reports from Europe and suggests a far lower rate of XMRV infection, if any, in patients with CFS compared to the initial report [7]. Regional differences in XMRV prevalence among CFS patients could reflect geographical clustering of XMRV infection, and weakens the epidemiological link between XMRV infection and CFS.

To further characterize our CFS cohort and provide a basis of comparison to other CFS groups described in the literature, we administered a 43-item CFS questionnaire. Although our questionnaire may be confounded by recall bias, a majority of our CFS participants reported ongoing symptoms at the time of study entry. These symptoms had been present for an average of 12 years and were debilitating enough to cause the majority of participants to stop working.

Endogenous or latent viruses can become activated in patients with altered immune function. We explored the effect that immune activation or suppression could have on XMRV prevalence by including participants with rheumatoid arthritis, HIV infection (both treatment naïve and virologically suppressed), and hematopoietic stem-cell and solid organ transplant. We did not identify an association between XMRV prevalence and immune status. Healthy individuals were not included per se in this study and preclude us from drawing conclusions about the prevalence of XMRV in the general population. Our rheumatoid arthritis age- and
gender-matched hospital cohort may contain healthy patients that presented for routine clinical care, but we would reasonably expect greater morbidity in this control group, relative to the population-at-large in Boston. We did detect a mouse endogenous retroviral sequence in PBMC DNA from one participant, but could not replicate this finding. Mouse endogenous retroviral sequences are not present in the human genome; reagent testing did not identify the source of this contaminating sequence.

In summary, we found no evidence of XMRV infection in a cohort of patients cared for at Boston-area hospitals and no association of XMRV with either chronic fatigue syndrome or chronic conditions with altered immune function. Further research should be performed to define the demographic and geographic distribution of XMRV and to clarify its relationship with chronic fatigue syndrome.

Acknowledgments

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References


<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Location(^a)</th>
<th>PCR Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>XMRV Forward outer [7]</td>
<td>ATCAGTTAACCTACCGGAGTCGGAC</td>
<td>424–448</td>
<td>94°C × 2m</td>
</tr>
<tr>
<td>XMRV Reverse outer [7]</td>
<td>GCGGCTCTTCATTTGCTTCTC</td>
<td>1154–1132</td>
<td>40 cycles: 94°C × 30s, 57°C × 30s, 68°C × 60s, 68°C × 5m</td>
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<tr>
<td>XMRV Forward inner #1 [8]</td>
<td>GACCTTTTGAGTGGCTTTGT</td>
<td>446–466</td>
<td>94°C × 2m</td>
</tr>
<tr>
<td>XMRV Reverse inner #1 [8]</td>
<td>ACAGAAGAAACAAAACAAACAAATC</td>
<td>571–549</td>
<td>30 cycles: 94°C × 30s, 55°C × 30s, 68°C × 30s, 68°C × 5m</td>
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<tr>
<td>XMRV Forward inner #2 [4]</td>
<td>TCTCGAGATCATGGGACAGA</td>
<td>603–622</td>
<td>94°C × 2m</td>
</tr>
<tr>
<td>XMRV Reverse inner #2 [4]</td>
<td>AGAGGGTAAGGGCAGGGTAA</td>
<td>1015–996</td>
<td>30 cycles: 94°C × 30s, 60°C × 30s, 68°C × 30s, 68°C × 5m</td>
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<tr>
<td>hBG Forward</td>
<td>TGTTGGTCTACCTTTGGACC</td>
<td>148–162</td>
<td>94°C × 2m</td>
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<tr>
<td>hBG Reverse</td>
<td>GAGGTTGTCACCGTGAGCCA</td>
<td>296–277</td>
<td>40 cycles: 94°C × 30s, 55°C × 30s, 68°C × 30s, 68°C × 2m</td>
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</tbody>
</table>

\(^a\)Locations in XMRV isolate VP62 (GenBank accession DQ399707) and human beta-globin (GenBank accession NM000158.4) genes. XMRV gag-pro-pol gene starts at nucleotide location 613.
### Table 2

Demographics of Screened Participant Cohorts

<table>
<thead>
<tr>
<th>Cohort</th>
<th>CFS</th>
<th>HIV</th>
<th>SOT/HCT</th>
<th>Rheumatoid Arthritis (RA)</th>
<th>RA-Matched Hospital Control</th>
<th>All Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>32</td>
<td>43</td>
<td>26</td>
<td>97</td>
<td>95</td>
<td>293</td>
</tr>
<tr>
<td>Median Age (Years)</td>
<td>46</td>
<td>46</td>
<td>56</td>
<td>66</td>
<td>62</td>
<td>58</td>
</tr>
<tr>
<td>Female</td>
<td>21</td>
<td>8</td>
<td>12</td>
<td>83</td>
<td>84</td>
<td>208</td>
</tr>
<tr>
<td>Gender</td>
<td>(65.6%)</td>
<td>(18.6%)</td>
<td>(46.2%)</td>
<td>(85.6%)</td>
<td>(88.4%)</td>
<td>(71.0%)</td>
</tr>
</tbody>
</table>

**Note:**
- CFS = chronic fatigue syndrome; SOT = solid organ transplant; HCT = hematopoietic stem-cell transplant
- DNA obtained from discarded in- or outpatient phlebotomy samples, age and gender matched with the rheumatoid arthritis cohort
- 22 subjects currently receiving and 21 subjects not receiving antiretroviral therapy
- 3 HCT, 11 kidney, 2 lung, 2 heart, 7 liver, and 1 liver and kidney transplants. 1 liver transplant patient also had concurrent HIV infection
- N (percent female gender within specific cohort)