

## Xenotropic Murine Leukemia Virus–Related Virus Prevalence in Patients with Chronic Fatigue Syndrome or Chronic Immunomodulatory Conditions

Timothy J. Henrich,<sup>1,3,4,a</sup> Jonathan Z. Li,<sup>1,3,4,a</sup> Donna Felsenstein,<sup>3,4</sup> Camille N. Kotton,<sup>3,4</sup> Robert M. Plenge,<sup>2,4</sup> Florencia Pereyra,<sup>1,4,5</sup> Francisco M. Marty,<sup>1,4,6</sup> Nina H. Lin,<sup>3,4</sup> Paul Grazioso,<sup>3</sup> Danielle M. Crochiere,<sup>3</sup> Daniel Eggers,<sup>3</sup> Daniel R. Kuritzkes,<sup>1,3,4</sup> and Athe M. N. Tsibris<sup>1,3,4</sup>

Divisions of <sup>1</sup>Infectious Diseases and <sup>2</sup>Allergy, Immunology, and Rheumatology, Brigham and Women's Hospital, <sup>3</sup>Division of Infectious Diseases, Massachusetts General Hospital, <sup>4</sup>Harvard Medical School, <sup>5</sup>Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard University, Cambridge, and <sup>6</sup>Dana-Farber Cancer Institute, Boston, Massachusetts

(See the article by Danielson et al, on pages 1470–1477, the brief report by Barnes et al, on pages 1482–1485, and the editorial commentary by Kearney and Maldarelli, on pages 1463–1466.)

We investigated the prevalence of xenotropic murine leukemia virus-related virus (XMRV) among 293 participants seen at academic hospitals in Boston, Massachusetts. Participants were recruited from the following 5 groups of patients: chronic fatigue syndrome ( $n = 32$ ), human immunodeficiency virus infection ( $n = 43$ ), rheumatoid arthritis ( $n = 97$ ), 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 chronic fatigue syndrome or chronic immunomodulatory conditions.

Murine and human xenotropic viruses were first described >30 years ago [1]. The xenotropic murine leukemia virus–related virus (XMRV) was discovered in prostate tissue from patients with prostate cancer who were homozygous for a reduced-ac-

tivity variant of RNase L [2–4]. RNase L gene abnormalities have been described in patients with chronic fatigue syndrome (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 with 3.7% of healthy controls [7]. Three subsequent CFS studies in European cohorts have not demonstrated the presence of XMRV DNA in subjects with CFS 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 3 different polymerase chain reaction (PCR) primer sets from other XMRV studies into our testing strategy to determine whether differences existed in their ability to amplify XMRV DNA.

**Methods.** Samples were obtained from adult patients presenting to outpatient clinics, or from preexisting 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 human immunodeficiency virus (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 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 PBMCs 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 sex-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.

For XMRV PCR amplification, human DNA was extracted from at least  $5 \times 10^6$  PBMCs (Qiagen). PCR reaction conditions and primer sets are described in Table 1. First-round PCR amplifications were performed with 1 unit of Platinum

Received 16 May 2010; accepted 2 July 2010; electronically published 11 October 2010.  
Potential conflicts of interest: none reported

Financial support: National Institutes of Health grants T32 AI07387 (to J.L.), KL2 Medical Research Investigator Training (MeRIT) program of Harvard Catalyst, The Harvard Clinical and Translational Science Center UL1 RR025758 (to T.J.H.), K24 RR016482 (to D.R.K.), and K08 AI081547 (to A.M.N.T.).

<sup>a</sup> T.J.H. and J.Z.L. contributed equally to this work.

Reprints or correspondence: Athe M. N. Tsibris, Div of Infectious Diseases, Massachusetts General Hospital, 65 Landsdowne St, Rm 422, Cambridge, MA 02139 ([atsibris@partners.org](mailto:atsibris@partners.org)).

The Journal of Infectious Diseases 2010;202(10):1478–1481

© 2010 by the Infectious Diseases Society of America. All rights reserved.

0022-1899/2010/20210-0004\$15.00

DOI: 10.1093/infdis/jiq168

**Table 1. Xenotropic Murine Leukemia Virus–Related Virus (XMRV) and Human  $\beta$ -Globin (hBG) Oligonucleotide Primers and Polymerase Chain Reaction (PCR) Conditions**

Reaction, primer	Sequence	Location <sup>a</sup>	PCR conditions
XMRV outer			
Forward outer [7]	5'-ATCAGTTAACCTACCCGAGTCGGAC-3'	424–448	94°C × 2 min; 40 cycles: 94°C × 30 s, 57°C × 30 s, 68°C × 60 s; 68°C × 5 min
Reverse outer [7]	5'-GCCGCCTCTTCTTCATTGTTCTC-3'	1154–1132	
XMRV inner no. 1			
Forward inner no. 1 [8]	5'-GACTTTTTGGAGTGGCTTTGT-3'	446–466	94°C × 2 min; 30 cycles: 94°C × 30 s, 55°C × 30 s, 68°C × 30 s; 68°C × 5 min
Reverse inner no. 1 [8]	5'-ACAGAAGAACAACAAAACAATC-3'	571–549	
XMRV inner no. 2			
Forward inner no. 2 [4]	5'-TCTCGAGATCATGGGACAGA-3'	603–622	94°C × 2 min; 30 cycles: 94°C × 30 s, 60°C × 30 s, 68°C × 30 s; 68°C × 5 min
Reverse inner no. 2 [4]	5'-AGAGGGTAAGGGCAGGGTAA-3'	1015–996	
hBG			
Forward	5'-TGGTGGTCTACCCTTGGACC-3'	148–162	94°C × 2 min; 40 cycles: 94°C × 30 s, 55°C × 30 s, 68°C × 30 s; 68°C × 2 min
Reverse	5'-GAGGTTGTCCAGGTGAGCCA-3'	296–277	

<sup>a</sup> Locations in XMRV isolate VP62 (GenBank accession no. DQ399707) and hBG (GenBank accession no. NM000158.4) genes. XMRV gag-pro-pol gene starts at nucleotide location 613.

*Taq* DNA Polymerase High Fidelity (Invitrogen), 80–200 ng of PBMC DNA, and 200 nmol/L of the outer primers described by Lombardi et al (outer primer set) [7]. Nested PCRs were performed with first-round PCR product and 200 nmol/L of the inner primers described by 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 200 ng 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  $\beta$ -globin was amplified from each participant sample with a single-round PCR reaction that used the outer human  $\beta$ -globin primer set described by Erlwein et al [8]. All but 1 sample, from a general clinic participant, demonstrated  $\beta$ -globin amplification. This sample was excluded from our analysis. Laboratory personnel who performed PCR amplification were blinded to the identity of each sample.

For XMRV cloning and sequence analysis, DNA bands near the length of interest were purified with the QIAquick Gel Extraction kit (Qiagen). These amplicons were cloned into a pCR4-TOPO vector (Invitrogen) and were bidirection-

ally sequenced (ABI 3730 automated DNA sequencer; Applied Biosystems).

$\chi^2$  testing with continuity correction was used to compare intergroup variation between nonparametric variables. Fisher's exact tests were used if expected counts were <5. One-way analysis of variance testing was performed to define intergroup comparisons of continuous variables; a Bonferroni correction was used in pair-wise tests of individual hypotheses (SPSS, version 10; SPSS). To estimate the probability of detecting XMRV in CFS participants on the basis of sample size, the Blyth-Still-Casella 95% exact confidence intervals for disease prevalence were calculated.

**Results.** PBMC samples from 293 participants were tested for the presence of XMRV DNA (Table 2). Thirty-two subjects who had previously received a CFS diagnosis and 26 patients who 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 and receiving antiretroviral therapy; the remainder were chronically infected persons who were not receiving treatment. We tested 97 samples from patients with rheumatoid arthritis and 96 samples from age- and sex-matched controls obtained from the Crimson Biospecimen Core.

Participants with rheumatoid arthritis and those from the age- and sex-matched general clinic/hospital cohort had the

**Table 2. Demographic Characteristics of Screened Participant Cohorts**

Characteristic	Cohort					
	CFS	HIV	SOT/HCT	RA	RA-matched hospital control <sup>a</sup>	All subjects
No. of participants	32	43 <sup>b</sup>	26 <sup>c</sup>	97	95	293
Age, median years	46	46	56	66	62	58
Female sex, no. (%) of participants	21 (65.6)	8 (18.6)	12 (46.2)	83 (85.6)	84 (88.4)	208 (71.0)

**NOTE.** CFS, chronic fatigue syndrome; HCT, hematopoietic stem-cell transplant; HIV, human immunodeficiency virus; SOT, solid organ transplant.

<sup>a</sup> DNA obtained from discarded inpatient or outpatient phlebotomy samples, age- and sex-matched with the RA cohort.

<sup>b</sup> Twenty-two subjects currently receiving and 21 subjects not receiving antiretroviral therapy.

<sup>c</sup> Three HCT, 11 kidney, 2 lung, 2 heart, 7 liver, and 1 liver and kidney transplants. One liver transplant patient also had concurrent HIV infection.

highest percentage of women (>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 intergroup difference, compared with CFS cohort;  $P < .001$ ). Mean CD4<sup>+</sup> T lymphocyte counts for HIV-infected participants receiving or not receiving antiretroviral therapy were similar (566 and 554 cells/ $\mu$ L, respectively;  $P = .89$ ); 95.5% of participants receiving antiretroviral therapy had plasma HIV RNA levels <50 copies/mL. A majority of transplant patients had undergone solid organ transplantation, including 11 kidney, 7 liver, 2 lung, 2 heart, and 1 liver/kidney transplants. One liver transplant recipient had underlying treated HIV infection. The remaining 3 transplant participants had undergone allogeneic hematopoietic stem-cell transplantation.

A majority of participants with CFS 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.

With PCR analysis, we could reliably detect 10 copies of XMRV per 200 ng 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. With use of the PCR strategy described, we did not detect XMRV *gag* DNA in any of the 293 participant samples. In 7 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 ampli-

fications 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 6 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. Another 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 other reports, and we increased the sensitivity of our methods by adding a nested PCR amplification that used 2 additional 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 other 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 with 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 a mean 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 naive 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 sex-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 1 participant, but we 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 CFS 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

We thank the study participants for their participation. We thank Robert Silverman for his kind gift of the XMRV VP62 plasmid. We also thank the clinic staff of the Dana Farber Cancer Institute Brigham and Women's Hospital and Massachusetts General Hospital Infectious Diseases clinics and the Massachusetts General Hospital transplant clinic for their efforts in support of this study. We are grateful to Hongying Wang for her statistical analysis advice.

**Note added in proof.** A recent study detected a high prevalence of murine leukemia virus–related virus DNA, but not XMRV DNA, in patients with CFS [16]. The outer PCR primers and a nested PCR primer set used in that report are identical to primers used in our study.

### References

1. Levy JA. Xenotropic type C viruses. *Curr Top Microbiol Immunol* **1978**;79:111–213.
2. Fischer N, Hellwinkel O, Schulz C, et al. Prevalence of human gammaretrovirus XMRV in sporadic prostate cancer. *J Clin Virol* **2008**;43:277–283.
3. Schlager R, Choe DJ, Brown KR, Thaker HM, Singh IR. XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors. *Proc Natl Acad Sci U S A* **2009**;106:16351–16356.
4. Urisman A, Molinaro RJ, Fischer N, et al. Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNA-SEL variant. *PLoS Pathog* **2006**;2:e25.
5. Suhadolnik RJ, Reichenbach NL, Hitzges P, et al. Upregulation of the 2–5A synthetase/RNase L antiviral pathway associated with chronic fatigue syndrome. *Clin Infect Dis* **1994**;18(Suppl 1):S96–S104.
6. Gow JW, Simpson K, Behan PO, Chaudhuri A, McKay IC, Behan WM. Antiviral pathway activation in patients with chronic fatigue syndrome and acute infection. *Clin Infect Dis* **2001**;33:2080–2081.
7. Lombardi VC, Ruscetti FW, Das Gupta J, et al. Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome. *Science* **2009**;326:585–589.
8. Erlwein O, Kaye S, McClure MO, et al. Failure to detect the novel retrovirus XMRV in chronic fatigue syndrome. *PLoS One* **2010**;5:e8519.
9. Groom HC, Boucherit VC, Makinson K, et al. Absence of xenotropic murine leukaemia virus-related virus in UK patients with chronic fatigue syndrome. *Retrovirology* **2010**;7:10.
10. van Kuppeveld FJ, de Jong AS, Lanke KH, et al. Prevalence of xenotropic murine leukaemia virus-related virus in patients with chronic fatigue syndrome in the Netherlands: retrospective analysis of samples from an established cohort. *BMJ* **2010**;340:c1018.
11. Walker BD. Elite control of HIV Infection: implications for vaccines and treatment. *Top HIV Med* **2007**;15:134–136.
12. Pereyra F, Addo MM, Kaufmann DE, et al. Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy. *J Infect Dis* **2008**;197:563–571.
13. Murphy S, Churchill S, Bry L, et al. Instrumenting the health care enterprise for discovery research in the genomic era. *Genome Res* **2009**;19:1675–1681.
14. Policastro PF, Fredholm M, Wilson MC. Truncated gag products encoded by Gv-1-responsive endogenous retrovirus loci. *J Virol* **1989**;63:4136–4147.
15. Fischer N, Schulz C, Stieler K, et al. Xenotropic murine leukemia virus-related gammaretrovirus in respiratory tract. *Emerg Infect Dis* **2010**;16:1000–1002.
16. Lo SC, Pripuzova N, Li B, et al. Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors. *Proc Natl Acad Sci U S A* **2010**;107:15874–15879.