Absence of XMRV Retrovirus and Other Murine Leukemia Virus-Related Viruses in Patients with Chronic Fatigue Syndrome

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Chronic fatigue syndrome (CFS) is a multisystem disorder characterized by prolonged and severe fatigue that is not relieved by rest. Attempts to treat CFS have been largely ineffective primarily because the etiology of the disorder is unknown. Recently, CFS has been associated with xenotropic murine leukemia virus-related virus (XMRV) as well as other murine leukemia virus (MLV)-related viruses, though not all studies have found these associations. We collected blood samples from 100 CFS patients and 200 self-reported healthy volunteers from the same geographical area. We analyzed these in a blind manner using molecular, serological, and viral replication assays. We also analyzed samples from patients in the original study that reported XMRV in CFS patients. We did not find XMRV or related MLVs either as viral sequences or infectious viruses, nor did we find antibodies to these viruses in any of the patient samples, including those from the original study. We show that at least some of the discrepancy with previous studies is due to the presence of trace amounts of mouse DNA in the Taq polymerase enzymes used in these previous studies. Our findings do not support an association between CFS and MLV-related viruses, including XMRV, and the off-label use of antiretrovirals for the treatment of CFS does not seem justified at present.

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dations for an accurate study (23), we incorporated all of these factors in the design of the investigation reported here and have performed what we believe is the most comprehensive study to date on the proposed association of XMRV and other related viruses with CFS.

We enrolled 105 CFS patients, including 100 who fulfilled both the 1994 case definition of the CDC (4) as well as the criteria defined by the Canadian consensus document on myalgic encephalomyelitis (ME)/CFS (1). Patients and 200 healthy volunteers were all from the greater Salt Lake City area. Blood samples from both patients and healthy volunteers were prospectively collected and processed in parallel. In conjunction with a third-party phlebotomy service, we also collected samples in a blind manner (blinded samples) from 14 patients in the cohort used in the original CFS-XMRV study performed at the Whittomere Peterson Institute (WPI) (12).

For virus detection, we utilized four different TaqMan quantitative PCR (qPCR) assays, PCR assays that had resulted in the detection of XMRV or MLV-like sequences in previous studies (11, 12), and an enzyme-linked immunosorbent assay (ELISA) to look for XMRV sequences and antibodies in all of our samples. A subset of samples was analyzed by Western blotting. Using some of the samples, we also attempted to grow virus in a cell culture, a technique outlined in the original study (12) which, though labor-intensive, has been proposed to be the most sensitive method for viral detection (14). All samples were processed and tested in a blind manner.

**MATERIALS AND METHODS**

**Patient and participant selection.** We initially identified 150 patients from a clinic that specializes in the diagnosis and management of CFS and fibromyalgia. All patients had been diagnosed with CFS using the CDC-Fukuda criteria (4) in a clinical setting by a board-certified clinician (L.B.). The vast majority of these patients had been serially assessed and managed by L.B. for many years to verify CFS onset, duration, and life impact. Controls consisted of 100 healthy males and 100 healthy females by self-report, and all were employed in Salt Lake City. Participants were recruited via e-mail and enrolled after informed consent under University of Utah IRB protocol number 7740.

CFS onset, duration, and life impact. CFS onset was reported to be associated with virus-like symptoms in 72% of patients. Seventeen patients had participated in the phase III clinical trial of Ampligen (AMP516), which required virus-like symptoms in 72% of patients. The 157-bp product in repeat (LTR), 157-bp product in gag, and 65-bp product in env. The 157-bp product in gag was added to the tube to clear red blood cells. The tube was inverted 5 times, incubated at room temperature for 10 min, and centrifuged at 3000 rpm for 5 min. The supernatant was discarded, and the pellet resuspended in 10 ml of wash buffer (phosphate-buffered saline [PBS], 2 mM EDTA). After the supernatant was spun for 5 min at 3000 rpm, the pellet was divided into three aliquots: one in 1 ml of fetal calf serum containing 10% (vol/vol) dimethyl sulfoxide (DMSO), another in 1 ml of RLT buffer containing guanidine isothiocyanate and 1% beta-mercaptoethanol (Qiagen), and the third without any buffer. All aliquots were stored in cryovials at −80°C.

Nucleic acid extraction from buffy coat and whole blood. Nucleic acid extraction was performed using the DNeasy blood and tissue kit (Qiagen) by following the manufacturer’s directions. Each extraction control was included for each batch of controls and each assay run.

Quantitative and nested PCRs. All qPCRs were done using the TaqMan probe system on a 7900HT real-time PCR system with a standard 96-well block module (Applied Biosystems). Each 20-µl reaction mixture contained 1× TaqMan universal PCR master mix, 900 nM forward and reverse primers, 250 nM TaqMan probe, and 400 to 1,000 ng of DNA or 5 µl of water. Thermocycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 45 or 50 cycles of 95°C for 15 s and 60°C for 1 min. Four XMRV amplicons were used, 63 bp in the long terminal repeat (LTR), 157-bp product in gag, 65-bp product in pol, and 86-bp product in env. The 63-bp LTR product corresponded to 47F (5′-AATAAGGGCTTTTGC TGTGGTAGCA-3′), 109R (5′-GAGGAAGCACCTCCAAAGGAAA-3′), and 74MGB (5′-6FAM-AACGCGTG CCTGGC-3′). The 157-bp gag product corre-

33 years), and the average BMI was 27.6. None of the participants had ever been diagnosed with prostate cancer, 12% of which included blood relatives (4% did not know about family history of prostate cancer). One female reported a diagnosis of fibromyalgia, and 17% reported a family history of CFS/fibromyalgia. The average age of males was 34.6 years (median, 33 years), and the average BMI was 27.6. None of the participants had ever been diagnosed with prostate cancer. Fourteen percent had a family history of prostate cancer, 12% of which included blood relatives (4% did not know about family history of prostate cancer). None of the males reported a diagnosis of fibromyalgia; however, 13% reported a family history of CFS/fibromyalgia, 10% of which were blood relatives.

**Blinded sampling protocol.** Immediately after arrival at the clinic, subjects were given full details about the study verbally and in writing, with all of their questions answered, and they provided informed consent in writing according to a protocol approved by the University of Utah IRB. For 15 min on average, they sat quietly and completed self-report questionnaires and then had blood drawn. The clinical research division at ARUP Laboratories, Salt Lake City, UT, collected and stored samples from all individuals within a period of 2 weeks. Blood was collected into 8.5-ml Vacutainer tubes (Becton Dickinson): 2 EDTA tubes and 1 serum separator tube. After the blood was allowed to clot at room temperature for 30 min, the serum separator tube was spun for 10 min at 3,000 rpm. Serum aliquots of 1 ml were frozen in cryovials at −80°C. From the EDTA tubes, 1 ml of whole blood was removed and stored at −80°C in cryovials (Nunc). The remaining volume was spun for 10 min at 3,000 rpm, and plasma aliquots of 1 ml were frozen in cryovials at −80°C. The bloody coats were removed and combined into a 15-ml Falcon tube for each individual, and 7 ml of ACK lysis buffer (28) was added to the tube to clear red blood cells. The tube was inverted 5 times, incubated at room temperature for 10 min, and centrifuged at 3,000 rpm for 5 min. The supernatant was spun for 5 min at 3,000 rpm, the pellet was divided into three aliquots: one in 1 ml of fetal calf serum containing 10% (vol/vol) dimethyl sulfoxide (DMSO), another in 1 ml of RLT buffer containing guanidine isothio-
cyanate and 1% beta-mercaptoethanol (Qiagen), and the third without any buffer. All aliquots were stored in cryovials at −80°C.
TGGCTGA-3'). The Mlu assay used to look for XMRV plasmin contamination utilized Mlu-F (5’-GGTGGGCCCCCTTGGC-3’), MluR (5’-AGTTACGCTGCTGATCCATTTT-3’) and Mlu-MGB (5’-6FAM-CGTTGAGCCAGCCTAT- MGB-3’).

For the nested PCR, we made two modifications to the original protocol (11). We used 1.0 U of Platinum Taq instead of 0.5 U and added dUTP to the master mix to prevent subsequent PCR contamination with amplicons. XMRV SU recombinant protein. The forward primer with the Xhol site (5’-ATTATCCTCCAGACGACACGTACAGCCTC-3’) and the reverse primer with the HindIII site (5’-ATTATCAAGCTTCTTTTACTGAGCC TAA3-3’) were used to PCR amplify the SU sequence from pXMRV1 (22). The forward primer with the NheI site (5’-ATTATCGTAGACTAATGAGCGCGTTCAG-3’) and the reverse primer with the Xhol site (5’-ATTATACGCTGGGACGCGACGCTA-3’) occurred in chilled transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 20 V for 40 min in a semidry apparatus (Bio-Rad). The membranes were blocked in 5% milk (1 ml of PBS, 0.01% Tween 20) for 1 h at room temperature (RT). Human sera were then added at a 1:150 dilution in Sea Block with 0.05% Tween 20. Following incubation for 2 h at RT, plates were washed five times with high-salt wash buffer with 0.05% Tween 20 and a 1:15,000 dilution of horseradish peroxidase conjugated AffiniPure F(ab)2 fragment goat anti-human IgG antibody was added (Jackson ImmunoResearch). The plates were then incubated for 1 h at RT and washed again five times with high-salt wash buffer with 0.05% Tween 20. TRIS-TUBE substrate was added and allowed to incubate for 30 min at RT. Development was stopped with 1 N sulfuric acid, and the absorbance was measured at 450 nm and 650 nm. Results were expressed as the difference in optical density at 450 nm (OD450) and OD650 to correct for irregularities in the plate.

Western blot assays with human sera. Five micrograms of purified XMRV SU protein or 5 µg of uninfected 293T cell lysate was diluted in 2× sample buffer (2% SDS, 50 mM Tris, pH 6.8, 10% glycerol) and heated for 3 min at 95°C. Proteins were loaded into 4- to-20% gradient Precise protein gels (Pierce) in 1X Tris-HEPES-SDS running buffer (Pierce). The gel was run at 150 V for 50 min. The transfer to polyvinylidene fluoride (PVDF) Immobilon-FL membranes (Millipore) occurred in chilled transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 20 V for 40 min in a semidry apparatus (Bio-Rad). The membranes were blocked in 5% milk (1X PBS, 0.01% Tween 20) for 1 h. The membranes were probed with human serum and diluted 1:50 in 5% milk overnight at 4°C. The membranes were washed 4 times with PBS (1X PBS, 0.01% Tween 20) and probed with goat anti-human antibody IR-700 (Rockland) at 1:10,000 in 5% milk for 2 h at RT. The membranes were washed 4 times with PBST before imaging was done on an Odyssey scanner (Licer). Viral replication assay using spin inoculation. The protocol for the viral replication assay using spin inoculation was adapted from the one used in the original study that found XMRV in CFS patients (12), with extensive help from Frank Ruscetti (Leukocyte Biology Section, NCI). LNCaP cells transfected into 293T cells with Lipofectamine 2000 (Invitrogen). The supernatant of transfected into bacteria. DNA was prepared and verified by sequencing and transformed into E. coli occurred in chilled transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 20 V for 40 min in a semidry apparatus (Bio-Rad). The transfer to polyvinylidene fluoride (PVDF) Immobilon-FL membranes (Millipore) were blocked in 5% milk (1 ml of PBS, 0.01% Tween 20) for 1 h at room temperature (RT).

Western blotting using spin inoculation samples. We employed full time. Among the CFS patients, a total of 43% were unable to work or attend college even part time, including 13% who were on disability.

The clinical research department at ARUP Laboratories, Salt Lake City, UT, collected blood samples from all 300 individuals within a period of 3 weeks (see Materials and Methods) (Fig. 1). Anticoagulated whole blood was separated into white blood cells (for DNA isolation and qPCR assays) and plasma (for inoculation of cultured cells to assay for viral replication). Whole blood was also allowed to clot, and the serum was used for ELISA and Western blot assays designed to detect anti-XMRV antibodies.

qPCR assays for XMRV are sensitive to at least five viral copies. In order to be confident of detecting XMRV in clinical samples, we developed our PCR assays to the robust and reliable standards of clinically used assays. We developed four distinct qPCR (TaqMan) assays that target different regions of
fewer than 5 copies of XMRV plasmid DNA in a background of 400 ng of human placental DNA, and the assay was linear over a large range, viz: 5,000 to 5 copies of viral DNA. This sensitivity was matched by the assays targeting the env and pol regions. The gag assay was also able to reliably detect at least 5 viral copies with an average of a 3-cycle delay in crossing the threshold (threshold cycle, $C_T$) (see Fig. 2A). We also demonstrated that the assay had good precision and reproducibility as demonstrated by the $R^2$ values of the $C_T$s being close to 1. To determine intrarun precision, 4 different amounts of XMRV plasmid DNA, ranging from 5 copies to 5,000 copies, were amplified in 3 different reactions in the same run. To determine interrun precision, the 4 different levels of XMRV DNA were amplified in 3 different runs on 3 different days. The assays had good intrarun precision, with a mean coefficient of variation (CV) of 0.99%, and also good interrun precision, with a mean CV of 1.36%. We also verified that the tests were specific for XMRV and did not detect other common human pathogens, including other human retroviruses (Fig. 2C).

**Blood from CFS patients and healthy volunteers is negative for XMRV by qPCR.** Using our four qPCR assays, we looked for XMRV and related viral sequences in DNA made from white blood cells of 100 CFS patients and 200 healthy volunteers. We did not find any positive samples even when reactions were carried out for 45 cycles. Positive control reactions were reliably positive for 50 and 5 copies of XMRV plasmid DNA. To verify that the DNA extracted from samples was of adequate and comparable quality, each sample was also tested by a qPCR targeting a single-copy gene encoding vesicle associated membrane protein 2 (VAMP2) (22), and was found to be positive at a $C_T$ of 21 to 23 cycles. Water controls that were subjected to the same extraction method as samples were consistently negative. Each plate of 96 PCRs also contained 12 wells with water instead of template DNA; these were always negative, as expected.

**Absence of XMRV anti-SU antibodies in sera of CFS patients and healthy volunteers.** Infection of rhesus macaques with XMRV has shown that the most prominent antibody response is to the XMRV Env protein, gp70 (SU) (16). We tested sera from CFS patients and healthy volunteers for reactivity to recombinant XMRV SU protein in an ELISA that we developed. Rabbit anti-XMRV antisera were used as controls. We found no difference in the reactivities to XMRV SU protein between patients and healthy volunteers ($P = 0.541$, Kruskal-Wallis test) (Fig. 3). Samples with reactivities greater than 2 standard deviations from the mean were tested by Western blotting against recombinant His-tagged XMRV SU protein. For controls on the Western blots, we used a His-tagged protein that is unrelated to XMRV, as well as uninfected cell lysates. While we saw a good response with the XMRV antisera, no reactivity was seen with any of the human sera.

**Absence of infectious XMRV in plasma of CFS patients and healthy volunteers.** Inoculating cultured cells with patient plasma and monitoring for evidence of XMRV replication have been proposed to constitute the most sensitive method for XMRV detection in plasma samples from CFS patients (14). Because of the labor-intensive nature of this method, we decided to perform this procedure on a subset of our samples ($n = 65$) chosen by a random-number generator. We inoculated LNCaP cells with 100 μl of plasma from 31 patients and 34 healthy volunteers and passaged the cells weekly for 6

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**FIG. 2.** Defining qPCR assay characteristics. (A) Reproducibility of all XMRV qPCR assays (pol, env, LTR, gag) performed in triplicate with pXMRV1 template amounts of 500, 50, and 5 copies. $R^2$ values show high reproducibility for each assay. (B) Sensitivity of XMRV LTR qPCR assay with a 6-carboxyfluorescein (FAM) reporter showing that the assay was linear with as low as 5 copies of pXMRV1 template added in a background of 400 ng of human placental DNA. $R_n$ is the ratio of fluorescence between the FAM reporter and the standard reference ROX dye. (C) Testing for cross-reactivity (or specificity) of XMRV qPCR assays against other common human pathogenic viruses. A positive clinical sample or plasmid DNA from a variety of common pathogenic viruses was amplified using the LTR and pol (shIN) qPCR assays. No significant cross-reactivity was seen with any of the following: BK virus, cytomegalovirus (CMV), Epstein-Barr virus (EBV), enterovirus (EV), human herpesvirus 6 variant A (HHV6-A), human herpesvirus 6 variant B (HHV6-B), human immunodeficiency virus (HIV), human metapneumovirus (HMPV), influenza A virus (FLUA), and influenza B virus (FLUB). Each sample was extracted with an exogenous internal control (IC) plasmid IC2, containing the Caenorhabditis elegans pax1/9 (FLUA), and influenza B virus (FLUB). Each sample was extracted with an exogenous internal control (IC) plasmid IC2, containing the Caenorhabditis elegans pax1/9 gene fused to green fluorescent protein (GFP), added to each aliquot of whole blood prior to sample extraction. This internal control plasmid was coamplified with each sample to identify potential inhibitors of PCR and to monitor extraction efficiency. Extraction was efficient, as shown by the IC $C_T$ range of 33.5 to 35.9. The XMRV proviral sequence. One, targeting the pol gene region, has been used extensively by us (22) and others (15, 21, 25) and, of all the published PCR-based tests for XMRV, has been shown to be the most specific (8). We improved on the sensitivity of this assay so that it could reliably detect at least 5 viral copies of XMRV DNA (see Fig. 2A). To allow for possible variations in viral sequence in our subjects, we developed three additional qPCR tests that targeted the LTR, gag, and env regions of XMRV proviral DNA. We characterized each of these assays in detail to determine their limits of detection, specificity, and reproducibility. Assay characteristics for the LTR qPCR are shown in Fig. 2B. We could reliably detect fewer than 5 copies of XMRV plasmid DNA in a background of 400 ng of human placental DNA, and the assay was linear over a large range, viz: 5,000 to 5 copies of viral DNA. This sensitivity was matched by the assays targeting the env and pol regions. The gag assay was also able to reliably detect at least 5 viral copies with an average of a 3-cycle delay in crossing the threshold (threshold cycle, $C_T$) (see Fig. 2A). We also demonstrated that the assay had good precision and reproducibility as demonstrated by the $R^2$ values of the $C_T$s being close to 1. To determine intrarun precision, 4 different amounts of XMRV plasmid DNA, ranging from 5 copies to 5,000 copies, were amplified in 3 different reactions in the same run. To determine interrun precision, the 4 different levels of XMRV DNA were amplified in 3 different runs on 3 different days. The assays had good intrarun precision, with a mean coefficient of variation (CV) of 0.99%, and also good interrun precision, with a mean CV of 1.36%. We also verified that the tests were specific for XMRV and did not detect other common human pathogens, including other human retroviruses (Fig. 2C).

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intracisternal A-type particles (IAP), which are present in mouse DNA. This assay targeted the sequences coding for TaqMan assay to detect small amounts of contaminating source of XMRV-like sequences (15, 19). Based on an assay mouse DNA, since that has been shown to be a potential contamination with the XMRV infectious clone. we determined that none of the positive reactions were due to plasmid pXMRV1 at various copy numbers. Using this assay, the MluI site, is consistently lower than the MluI-containing from the reporter for plasmid pAO-H4, which does not have to the MluI site. Figure 5A shows that the peak fluorescence is isolated of XMRV by a qPCR assay where the probe would bind Thus, the laboratory plasmid can be distinguished from a wild不曾的 nested-PCR assay. Since we were unable to find any evidence of XMRV using our sensitive qPCR assays, serological methods, or viral growth assays, we decided to test our samples using the PCR primers first used in the original study that found XMRV in CFS patients (12). Another study utilized a modified, nested version of this assay to discover the presence of polytropic murine leukemia virus-like sequences in CFS patients (11). Using this assay (11), we found approximately 5% of our samples to be positive for products of the expected size, regardless of whether they were patients or healthy volunteers. It was possible that our samples were contaminated with XMRV plasmid DNA, even though work with the plasmid had been done in a separate laboratory. We decided to test for this possibility with qPCR primers flanking a restriction site for endonuclease MluI that had been introduced during the construction of our infectious clone (22). Thus, the laboratory plasmid can be distinguished from a wild isolate of XMRV by a qPCR assay where the probe would bind to the MluI site. Figure 5A shows that the peak fluorescence from the reporter for plasmid pAO-H4, which does not have the MluI site, is consistently lower than the MluI-containing plasmid pXMRV1 at various copy numbers. Using this assay, we determined that none of the positive reactions were due to contamination with the XMRV infectious clone.

We next checked if our samples were contaminated with mouse DNA, since that has been shown to be a potential source of XMRV-like sequences (15, 19). Based on an assay first introduced by Oakes et al. (15), we developed a qPCR TaqMan assay to detect small amounts of contaminating mouse DNA. This assay targeted the sequences coding for intracisternal A-type particles (IAP), which are present in approximately 2,000 copies per diploid genome of many mouse strains (13). As seen in Fig. 5B, our qPCR assay was linear down to as little as 62.5 fg of C57BL/6 mouse DNA and could reproducibly detect as little as 625 ag of mouse DNA per reaction, making it a remarkably sensitive method for detection of contaminating mouse DNA. Using this assay, we determined that our samples did not contain any mouse DNA, and the nested-PCR results could not be positive due to mouse DNA in the samples.

When repeating the nested PCR assays, we noticed that the initially positive samples were not consistently positive in subsequent nested assays. However, the proportion of positive reactions remained constant at approximately 5%. Even though extraction and amplification controls (1 per 7 samples) were consistently negative, we suspected that contamination of a PCR reagent might cause the lack of reproducibility and the consistent positivity rate of 5%. We tested 36 replicates of genomic DNA from uninfected LNCaP cells with the nested PCR and found that 2 produced a positive result (Fig. 5C, a subset of the data). Sequencing these products revealed MLV-related sequences with 95 to 100% similarity to sequences published previously (11; data not shown). In contrast to the nested-PCR results, this DNA tested consistently negative with all our XMRV qPCR assays and the IAP qPCR assay for detection of trace amounts of mouse DNA. We next tested each component of the nested PCR using the IAP qPCR assay in replicates of 8. We discovered that both recombinant Taq polymerase (Invitrogen) and the Platinum Taq polymerase (Invitrogen) tested positive for IAP sequences. Furthermore, adding increasing amounts of both Taq polymerases resulted in progressively lower Cₚₜ values (Fig. 5D). Along similar lines, when increasing amounts of both Taq polymerases were used as a template for the gag qPCR, positivity also increased. Positive reactions were obtained with four different batches of Taq polymerase. Applied Biosystems' AmpliTaq Gold Taq polymerase contained in the master mix of all of our qPCR TaqMan assays did not contain any IAP sequences (Fig. 5D),
indicating that it was free of mouse DNA. When additional AmpliTaq Gold Taq polymerase was added as a template for the IAP qPCR assay, as was done with the other polymerase preparations, all reactions remained negative. Contamination of Taq polymerase preparations with mouse RNA has been reported in an independent study (20). Taken together, our analysis shows that certain Taq preparations contain very small amounts of mouse DNA that can cause false-positive reactions when used in highly sensitive assays for XMRV.

A subset of blinded samples from the original XMRV-CFS study was negative for XMRV. To test whether we could detect XMRV in samples that had previously tested positive or negative for XMRV, we obtained a subset of samples from the original cohort that was used to make the association of XMRV with CFS (12). Using a third-party phlebotomy service that collected blood samples in home visits, we obtained blinded whole-blood and serum samples from 14 individuals. These individuals had repeatedly tested positive in the last 2 years when tested by the laboratories at the WPI, though this information was not available to us until the completion of our study. The clinical research department at ARUP Laboratories received these specimens and processed the blood using the same protocols as those used for our healthy volunteers and CFS patient samples. Thus, the samples were never opened in a research lab where XMRV might be present, until they reached us. We tested these samples using all of the assays we developed: four qPCR assays, ELISA, and Western blotting. None of the samples contained any evidence of XMRV. Serologically, there was no difference in the reactivities to XMRV SU between healthy volunteers and the WPI cohort ($P$ value = 0.667, Kruskal-Wallis test), indicating that there was no detectable antibody response that was specific to XMRV in the WPI cohort. Furthermore, we also analyzed the WPI samples using tests from the two studies that found XMRV or XMRV-like viruses in CFS patients, viz. a PCR assay for gag sequences, both in single-round (12) and nested formats (11), and a test for viral growth in cultured cells (12). Neither of these tests revealed any evidence of XMRV.

**DISCUSSION**

We examined blood samples from 100 CFS patients and 200 regionally matched healthy volunteers. The patients met both CDC-Fukuda and Canadian criteria for CFS/ME, and over 70% reported the association of a flu-like illness with the onset of their disorder. All blood samples had been freshly collected, “blinded”, processed, and analyzed identically. Special care was taken to avoid contamination using proper controls during DNA extraction, spin inoculation, and PCR analysis. Despite using a number of carefully characterized tests that were capable of detecting small amounts of XMRV and related MLVs, we did not detect XMRV in any of our samples. These tests consisted of sensitive qPCR assays, ELISA, and Western blotting that we developed. We also performed PCR assays for gag sequences used in the studies that found XMRV or XMRV-like viruses in CFS patients, viz. a PCR assay for gag sequences, both in single-round (12) and nested formats (11), and a test for viral growth in cultured cells (12). Neither of these tests revealed any evidence of XMRV.
was our inability to detect any XMRV in samples from patients that had tested positive for XMRV in the original study. We report here a repeat testing of samples obtained from CFS patients that were recruited, diagnosed, and defined as positive exemplars of XMRV infection by the investigators who performed the original WPI-based study. This testing was performed in an independent laboratory (ours), using many of the same techniques as in the original study. To our knowledge, this is the first study to report negative findings after a full repetition of all assay methods in patients who have previously tested positive for XMRV.

Our experience has taught us that the detection of XMRV in blood is fraught with difficulties. In our own laboratory, starting with aliquots of samples from the same patients that we report here, we initially found some samples to be positive for XMRV. DNA from these aliquots had been extracted on a BioRobot (Qiagen) in a 96-well format. Twelve wells spread throughout the plate served as negative extraction controls, and a few of these also tested positive. It turns out that a few months prior to the extraction of our blood samples, the same BioRobot had been used to extract DNA from tissue culture cells that had been infected with XMRV. Despite the several-month interval between the two extractions and the use of sterile, disposable reagents in the BioRobot, we obtained false positives in our negative extraction controls and some patient samples. Once we abandoned the BioRobot, and used new aliquots of samples to extract DNA manually, we did not find any patient or healthy volunteer samples to be positive. We continued this process of extreme care not to contaminate samples in all of our techniques, especially the viral replication assay. Because the viral replication assay consists of passing cells inoculated with patient samples and controls inoculated with infectious XMRV, every week for 6 to 8 weeks, this assay is especially vulnerable to contamination. We prevented this by handling only one set of cultures in the biosafety cabinet at a time and meticulously decontaminated the cabinet between cultures with 70% ethanol and UV irradiation. This made the viral replication assay very time-consuming and labor-intensive, and we could perform it only on a subset of our samples. But it is easy to see how the sample extraction and tissue culture processes might be the most vulnerable to contamination. We prevented this by handling only one set of cultures in the biosafety cabinet at a time and meticulously decontaminated the cabinet between cultures with 70% ethanol and UV irradiation. This made the viral replication assay very time-consuming and labor-intensive, and we could perform it only on a subset of our samples. But it is easy to see how the sample extraction and tissue culture processes might be the most vulnerable to contamination, providing a possible explanation for why the 14 samples from individuals tested repeatedly by the WPI over a period of 2 years were positive in their hands and negative in ours. Our early false-positive findings did have one benefit: they confirmed beyond a doubt that our assay methods were highly sensitive to even tiny quantities of XMRV, and thus we would have every expectation of detecting the virus if it had been present in any of the samples that we tested.

The presence of mouse DNA in PCR reagents emphasizes the critical importance of proper controls and carefully chosen, sensitive assays to detect trace amounts of mouse DNA. Sato et al. (20), using a sensitive RT-PCR kit, found that Platinum Taq polymerase (Invitrogen) contained RNA from polytropic endogenous MLV. This is not too surprising because the mouse monoclonal antibody used to prevent enzyme activity prior to heat activation might be the source of mouse DNA in the enzyme. What was surprising, however, was our finding mouse sequences in Invitrogen’s recombinant Taq polymerase that is expressed in *Escherichia coli*; we are not sure what the source of mouse DNA is, in this case. We did confirm, however, that Applied Biosystems’ AmpliTaq Gold polymerase that was used in all of our qPCRs, both here and in previous studies (5, 22, 25), did not contain any detectable mouse DNA. Lo et al. used the finding of negative results with the mouse mitochondrial seminested-PCR assays to support the assertion that their samples were free of mouse DNA. Like others (15, 19), we propose the detection of IAP sequences instead of mouse mitochondrial DNA as a better way to look for contamination. We demonstrate that our qPCR assay for IAP sequences is exquisitely sensitive and can detect attogram quantities of mouse DNA.

The question remains how mouse DNA in the *Taq* polymerase could lead to a disproportionate number of positives in patients versus controls in the two studies linking XMRV to CFS. It is possible, as has been suggested before (27), that patient samples were handled more than control samples and thus had a higher likelihood of contamination. In our study, both patient and control samples were handled in the same manner with the same frequency, in a blind manner. We also suggest that any planned studies proposed to screen for XMRV carefully check their reagents, equipment, and all possible, and seemingly not possible, sources of contamination with exogenous XMRV and mouse DNA. Obviously, all such studies should be conducted in a blind manner to prevent unintended experimental bias on the part of investigators and staff.

Unlike molecular and cell culture amplification assays, serological assays have the advantage of being difficult to contaminate. However, serological assays are still susceptible to false positives because of nonspecific binding of antibodies to related antigens. Serologically, our patient samples appear indistinguishable from control samples, as do the samples from the WPI cohort. It is possible that assays that have found anti-XMRV reactivity in CFS patients are due to cross-reactivity to related antigens.

Given the lack of evidence for XMRV or XMRV-like viruses in our cohort of CFS patients, as well as the lack of these viruses in a set of patients that previously tested positive, we feel that that XMRV is not associated with CFS. We are forced to conclude that prescribing antiretroviral agents to CFS patients is insufficiently justified and potentially dangerous. It is also vital to state that there is still a wealth of earlier data (2, 10) to encourage further research into the involvement of other infectious agents in CFS, and these efforts must continue.

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