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Supporting Online Material for

Failure to Confirm XMRV/MLVs in the Blood of Patients with Chronic Fatigue Syndrome: A Multi-Laboratory Study

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Table S7 as a PDF

SUPPORTING ONLINE MATERIAL

MATERIALS AND METHODS

Collection and processing of XMRV/P-MLV-positive cohort blood specimens

Fourteen patients with CFS, together with one person reporting contact with a CFS patient, all of whom were previously reported to be XMRV/P-MLV-positive by at least one method (table S6), were enrolled into the study. Ten subjects were females and five were males. Duration of illness among patients diagnosed with CFS varied from 6 to 28 years with 12 patients ill for 20 or more years. As described in Table S1, all five Harvard CFS patients from the Lo et al. study, and their criteria for diagnosis, were previously described (*4*). Similarly, six of the 10 subjects from the WPI cohort were previously described (*2*), and the remaining four fulfill the diagnostic CFS criteria reported in Lombardi et al. None of the patients had undergone experimental anti-retroviral treatment at the time of blood collection, and only two of the fifteen were receiving immunosuppressive/anti-inflammatory therapies. Several patients were reported to receive antiviral (such as anti-herpes treatment), antibiotic, and antifungal agents. One person, who was in contact with CFS patients did not fulfill all criteria necessary to establish a diagnosis of CFS using the 1994 CDC Fukuda Criteria definition (*23*). All specimens were collected in accordance with local IRB approval.

A single lot of EDTA BD Vacutainer Blood collection tubes (BD Biosciences) was purchased and distributed to two laboratories (CDC and NCI/DRP) for testing to ensure contamination with XMRV or mouse DNA was not present. Following validation, these tubes were used for all blood collections from the WPI and laboratory controls. Collection kits, including the XMRV-free certified tubes, were distributed to independent phlebotomists (Phlebotomy Services International, Central Point, OR). Phlebotomies were performed in patient homes in accordance with IRB approval, with anonymizing of the samples performed by Phlebotomy Services International. Samples were then shipped by overnight courier to BSRI. Immediately upon arrival, all samples were processed into plasma, peripheral blood mononuclear cells (PBMC) and whole blood (WB). After centrifugation, initially 1mL and 0.25 mL plasma aliquots were made, using most of the plasma. This was followed by mixing each blood tube five times and then making two 0.5 mL WB aliquots per tube. Finally, the tubes were reconstituted to the original volume using Dulbecco's phosphate buffer saline (DPBS) without calcium and magnesium salts. PBMCs were isolated from these tubes using the traditional ficoll paque gradient technique. The PBMCs were aliquoted at $5.0x10^6$ cells/mL. All aliquots were stored at -80°C.

Negative controls

Blood specimens were collected multiple times from three consenting laboratory controls under local IRB approval and WB, plasma and PBMCs were tested by all of the participating laboratories for XMRV/MLVs, using nucleic acid amplification testing (NAT), serology and virus culture techniques. In addition, twelve whole blood donations from local blood donors were unlinked and then separated into a buffy coat and corresponding plasma unit (Blood Centers of the Pacific, San Francisco). Aliquots of these twelve WB and plasma samples were distributed to five of the laboratories (CDC,

FDA/Lo, NCI/DRP, NCI/Ruscetti, WPI) for similar testing. The working group unanimously concluded that all fifteen of these individuals were negative for XMRV/MLV. These control specimens were processed in the same manner as described for the XMRV/P-MLV positive controls except that 1 mL and 0.25 mL aliquots were made from the plasma units and 1mL WB aliquots were made from the whole blood-derived buffy coats.

Spiked controls

22Rv1 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. In order to create positive controls for NAT assays for PBMC and WB, ten 22Rv1 cells per aliquot of PBMC and WB from one of the three laboratory controls were added.

For spiking of plasma, a large batch of supernatant from 22Rv1 cells was prepared from a T-75 flask plated at 60% confluency and grown for four days. Supernatant was collected and 0.45 μm filtered. An aliquot was tested by the CDC laboratory using six different real-time RT-PCR assays (*19, 24-26*). An average of the six assays gave a result of 1.5×10^9 RNA copies/mL. This was diluted to give a final dilution of 100 RNA copies per aliquot for the NAT plasma and 10^6 RNA copies for the culture controls. The cultivation of 22Rv1 cells, spiking of plasma, WB and PBMC and aliquoting of samples was all performed in a separate facility than where the handling of clinical materials was done.

Creation of Panels

All the above aliquoted samples were coded (total of 1554 aliquots, each with a uniquely numbered label) and compiled into 14 distinct panels. Depending on the available quantities, between one and three replicate aliquots of each XMRV/P-MLV-positive and negative-control sample, and five replicates of the spiked positive controls, were included in the panels. Seven panels of plasma, PBMCs and WB for NAT, four plasma panels for serology and three plasma panels for viral culture were produced. These coded panels without personal identifiers were then distributed to the nine participating laboratories.

Nucleic acid testing assays

Summaries of the NAT assays performed on the different sample types are given in tables S1, S2 and S3. The analytic sensitivity of many of the assays in plasma and WB were directly compared in a previous publication (*16*). Particular extraction columns (*27*), and enzymes (*6, 7, 28*) have been demonstrated to be contaminated with XMRV-like and other mouse endogenous MLV sequences. Only one of the laboratories used any product reported to be tainted with such materials – The laboratory of FDA/Lo continued to use Platinium taq (Invitrogen) as previously described (*4*) for PCR on PBMC and whole blood. However, all laboratory reagents (other than primers) including this enzyme were UV-irradiated. In addition, a number of laboratories quality controlled their reagents to confirm they were negative for XMRV/MLVs and mouse DNA prior to use in this study.

Abbott Molecular Inc. performed two real-time reverse transcriptase polymerase chain reaction (RT-PCR) assays on the automated *m*2000 system. Briefly, RNA was extracted from 0.8 mL of plasma, and total nucleic acid (TNA) was extracted from 0.4 mL of WB, or 0.2 ml of PBMC (diluted 1:1 in TE buffer). The extracted RNA or TNA

(1/3 of the eluate per reaction) was amplified with two primer sets designed to target the polymerase (*pol*) or envelope (*env*) regions of the XMRV genome. Armored RNA of pumpkin hydroxypyruvate reductase (HPR) gene was used as an internal control (IC) to assess RNA extraction recovery, PCR inhibition and amplification efficiency. RT-PCR was performed using the following conditions: reverse transcription at 55ºC for 30 min; denaturation at 95ºC for 1 min followed by 55 cycles of 93ºC for 15 seconds and 60ºC for 60 seconds. Fluorescence signals from the amplified XMRV and pumpkin-HPR sequences were simultaneously measured during the 60ºC incubation step at each of the 55 cycles. Amplification cycle threshold (Ct) was set at 0.1 for XMRV and 0.05 for pumpkin-HPR IC. Using serial dilutions of the XMRV VP62 plasmid controls, both assays could reliably detect ≥5 copies of DNA per reaction. Using the previously described WB and plasma panels (*16*), these assays were equal to the most sensitive assays tested. New reagents, master mixes and stock solutions were all tested for performance and potential mouse DNA contamination by testing of large numbers of positive and negative controls.

The CDC performed several different NAT assays as previously described (*16, 19, 26, 29*). Briefly, plasma was ultracentrifuged at 45,000 rpm and RNA was extracted from the pellet using a QIAamp Viral RNA minikit (Qiagen), while RNA and DNA was extracted from PBMCs with a QIAamp Viral RNA minikit (Qiagen). DNA was extracted from WB using QIAamp DNA blood mini kit (Qiagen). A quantitative RT-PCR assay for generic MLV/XMRV protease (*pro*) detection(*26*) was performed on RNA from plasma and PBMC in addition to a quantitative RT-PCR assay for generic MLV/XMRV *gag* (*19*) performed on RNA from plasma alone. A quantitative PCR version of the *pro* assay was also performed using 0.5 ug of WB DNA. cDNA synthesis and amplification of plasma RNA was done using RobusT I RT-PCR (Finnzymes) and the primers XPOLOF and XPOLOR followed by a nested PCR with primers XPOLIF and XPOLIR for the generic detection of MLV/XMRV *pol* sequences(*26*), while a PCR version of the nested *pol* assay was performed on 0.5 μg of WB and PBMC DNA. Positive bands of the correct size (216-bp) in the nested *pol* PCR were excised, purified and sequenced, and found to match XMRV 22Rv1 (GenBank accession # FN692043). Finally, PCR for β-actin sequences was performed on PBMC and WB DNA to verify the presence of amplifiable DNA in these specimens, as previously described (*29*). Testing for mouse intracisternal A particle (IAP) sequences in XMRV/MLV-positive samples was done using a quantitative PCR assay.

The FDA laboratory of Lo and colleagues performed nested RT-PCR and PCR assays as previously described with some modifications (*4*). Briefly, 500 μl plasma was lysed by mixing with 2 ml buffer AVL (Qiagen) containing 5 μg carrier RNA, and nucleic acids were purified using a QIAamp Viral RNA kit (Qiagen). cDNA synthesis and the first round PCR was carried out with rTth DNA polymerase (Applied Biosystems). Genomic DNA was extracted from WB and PBMCs using DNeasy blood kits (Qiagen). Nested PCR for MLV gag was performed in replicates of six using the 419F/1154R outer primer pair and GAG-I-F/GAG-I-R inner primer set using conditions as previously described (*4*). Any amplicon band of approximately the correct size (~746-bp for the outer primer pair and ~410-bp for the internal primer pairs) was excised, purified and sequenced. PCR for detection of mouse mitochondrial DNA (mtDNA) was performed on all the samples using a semi-nested PCR targeting the mouse-specific D-

loop sequence as described previously (*4*). The mtDNA assay detected 3 fg of both mouse spleen DNA and EL4 mouse cell DNA spiked into 50 ng of human DNA run in parallel as the positive controls. Detection of murine IAP sequences were also performed on all samples using the IAP PCR assay (*30*) with some revisions (45 amplification cycles and 72ºC annealing temperature) provided by Dr. Coffin in December 2010 to all the members of the working group. The IAP PCR assay detected 100 fg of both mouse spleen DNA and EL4 mouse cell DNA spiked into 50 ng of human DNA run in parallel as the positive controls.

The FDA laboratory of Hewlett and colleagues performed nested RT-PCR and PCR assays as previously described (*11*). Briefly, RNA was extracted from plasma and PBMCs using a QIAamp Viral RNA mini kit (Qiagen), and immediately reverse transcribed into cDNA using SuperScript III for First-strand Synthesis Kit (Invitrogen). Nucleic acids were extracted from WB using a QIAamp DNA Blood mini kit (Qiagen), with half of the sample reverse transcribed into cDNA with Superscript III, and the rest directly used in PCR. Nested PCR for MLV *gag* was performed in replicates of four by two operators using the 419F/1154R outer primer pair and GAG-I-F/GAG-I-R inner primer set and conditions as described (*11*), except that the number of cycles for the nested step was increased to 45. This modification improved the limit-of-detection of the RT-PCR assay in plasma from 4.5 RNA copies to 1.5 copies per mL. For positive samples, bands of the correct size was excised and sequenced to verify the presence of XMRV. Positive samples were also tested for mouse DNA contamination by PCR for mouse mtDNA sequences and the mouse IAP PCR assay (*30*).

The Gen-Probe XMRV TMA Assay is a qualitative NAT that uses the same technology as the FDA licensed PROCLEIX® blood screening assays. The assay includes three main steps, which take place in a single tube: 1) sample preparation by magnetic-based target specific capture, 2) amplification by transcription-mediated amplification (TMA), and 3) detection of the amplification products (amplicon) with chemiluminescent probes. The XRMV TMA assay simultaneously targets two separate regions in the XMRV genome: the long terminal repeat (LTR)-region and the *pol* gene. For the studies described here, nucleic acids were prepared from 500 µL of sample, with the exception of WB and PBMC samples which used a 50 µL sample volume. An internal control RNA, based on a modified XMRV sequence, was added to each reaction (by way of the Target Capture Reagent) to determine individual reaction validity. Assay results were reported in relative light units (RLU) values, which were used to derive signal-to-cutoff (S/CO) values. A sample was considered reactive if the analyte S/CO was ≥ 1.0 and non-reactive if the analyte S/CO was < 1.0. The XMRV assay is compatible with the fully-automated TIGRIS System but because of the limited sample volumes used in this study, all testing was done manually on the PROCLEIX semi-automated system.

The Drug Resistance Program (DRP) laboratory at NCI utilized a single-copy assay (SCA) similar to that developed for human immunodeficiency virus (HIV) (*31*). Briefly, plasma was ultracentrifuged at 170,000 x g and RNA was extracted using a 5.8 M guanidinium isothiocyanate extraction protocol (*31*). An internal avian retrovirus (RCAS) control was added to each plasma sample prior to ultracentrifugation to quantify nucleic acid recovery from plasma samples as described previously. Genomic DNA was extracted from WB and PBMC samples using the Promega genomic DNA Extraction Kit

(Cat # A1120) according to the manufacturer's suggested protocol. Although the primers target a gag leader region conserved between XMRV and generic MLVs (forward 5-TGTATCAGTTAACCTACCCGAGT-3', reverse 5-AGACGGGGGCGGGAAGTGTCTC-3'), the Taqman probe (5'fam-TGGAGTGGC TTTGTTGGGGGACGA- tamra3') used for detection spans a signature 24 nucleotide deletion in the XMRV, allowing for the differentiation between XMRV and other MLVs, based on the resulting fluorescence plateau. Reactions were performed in triplicate using a Lightcycler 480 with 45 cycles of 95ºC for 15 seconds, 60ºC for 1 minute after an initial 10 minute, 95ºC polymerase activation step. RCAS amplification was preformed as previously described (*31*). Reagents used in the NCI/DRP assay were tested for mouse DNA by running full 96-well plates of no template controls. The assay is able to detect mouse DNA in the environment at a rate of about 2 positives per 100 reactions, or about 4 positives per 100 when negative plasma is used as a template. Therefore, the criterion was established that replicate tests from the same sample must be positive in order to report a positive result.

WPI performed nested RT-PCR and PCR assays essentially as previously described (*2*). RNA was extracted from the plasma using a QIAamp Viral RNA kit (Qiagen). The PBMC aliquot was split into two fractions with half being used for RNA extraction using a RNeasy kit (Qiagen). All RNA was converted to cDNA using a VILO kit (Invitrogen). Genomic DNA was extracted from the rest of the PBMCs and WB using QIAamp blood kit (Qiagen). Nested PCR was performed using the Gag-O-F & Gag-O-R outer and Gag-I-F & Gag-I-R inner primers and conditions as described (*2*), except 2.5 mM $MgCl_2$, primers are each at 200 nM were employed, Phusion taq (Thermo) was used. All products were gel excised and purified for sequencing. As a control for mouse DNA contamination PCR for mouse mtDNA sequences was performed on all positive samples (*4*).

Virus culture assays

Virus culture was performed by two laboratories as described in table S4.

The NCI laboratory of Ruscetti and colleagues performed virus culture as described previously (*2*). Briefly, 15,000 cells of the human prostate cell line, LNCaP, were cultured in a T12.5 flask for 72-96 hours. Reagent grade $CaCl_2$ (5 mM) was added to the plasma to neutralize EDTA. Medium was removed from the flasks, 200 μl of fresh LNCaP medium was added together with 20-100 μl of plasma and 8 μg/ml of polybrene. After 2-3 hours 0.5 mL of media was added and the cells were incubated overnight. The next day the medium was removed and 3 ml of fresh complete medium was added. Cells were passaged into T25 flasks after 3-4 days. After a further 14 days, cells were lysed and analyzed as described by western blot using anti-gp70 and p30 antibodies (*2*).
Given the recent studies showing that 22Rv1 virus could spread in tissue culture (*32, 33*), additional experiments were performed. XMRV negative non co-cultured LNCaP cells were subcultured in the same hood following the subculturing of the cells co-cultured with samples spiked with 22Rv1 cells. They became positive after 19 days confirming the possibility of false positives due to spread of 22Rv1 virus. This is not an issue in the original culture experiments as no XMRV infected cell line was cultured in the same laboratories as patient cells. The virus that spread to negative LNCaP cultures was sequenced in the gag region as above and found to be identical to the spiked virus.

The FDA laboratory of Hewlett and colleagues performed two virus culture assays. In the first assay, culture was performed essentially as described above for NCI using LNCaP cells, except assays were performed in duplicate using the spinoculation method rather than polybrene and infection was assayed using a real-time PCR assay (*11*) on days 14 and 21 post-infection rather than western blot. In addition, approximately 40,000 cells were used for infection which is within the range of 15,000-50,000 cells used in the NCI/Ruscetti method. In the second assay, a derivative of LNCaP cells termed DERSE.LiGP cells (a gift from Dr Vineet KewalRamani, NCI) were used. DERSE cells were selected to express pBabe.iGFP-puro, a MLV proviral vector encoding an intron-interrupted GFP reporter gene. Thus, in this indicator cell line, GFP is only expressed after mobilization by an infecting gammaretrovirus during a second round of infection. Cells were plated 72 hours before infection at 3 x 10^4 cells/ml in 6-well plates. Medium was removed and 300 µl fresh medium and 100 µl of plasma were added per well in duplicate and the plates were centrifuged at 1,200 rpm for 1 hour. 0.5ml fresh medium was then added. The inoculum was removed the next day and the cells were cultured in 2ml fresh medium and monitored for GFP expression on days 7, 16 and 20 post-infection.

The use of DERSE.LiGP offers an opportunity to determine the sensitivity of viral transmission assays. Serial 10-fold dilutions of viral stocks from 22Rv1 culture were performed. With the caveat that the number defective particles in the stock is not known, it was determined that the assay could detect approximately 10^3 viral particles at both the NCI and FDA/Hewlett laboratories. An assay with similar sensitivity using dog osteosarcoma cells has been published (*34*).

Serological assays

Serology was performed by four laboratories as described in table S5.

Abbott Diagnostics performed two previously described (*6, 20*) chemiluminescent immunoassays (CMIA), whereby a direct assay format was used in which *E. coli*-expressed XMRV p15E or mammalian-expressed XMRV gp70 acts as both capture and detection antigens. Assays were run on the ARCHITECT® system using neat or 1:2 diluted plasma (diluted in pooled normal human plasma). XMRV-infected macaque plasma at 1:1000 (PC1) or 1:4000 (PC2) dilutions and a pool of normal human plasma acted as positive and negative controls respectively. Cutoff (CO) values of the ARCHITECT® CMIAs were calculated based on the following formulas: CO=0.45 X (PC2 Mean RLU) for p15E and CO=0.078 X (PC1 Mean RLU) for gp70. Results were reported as the ratio of the sample RLU to the cutoff RLU (S/CO) for each specimen. Specimens with S/CO values <1.00 were considered non-reactive; specimens with S/CO values ≥1.00 were considered reactive. The S/CO values of the negative and positive controls were 0.14, 12.83 and 3.43 for gp70 and 0.24, 7.93 and 2.22 for p15E.

CDC performed Western blot testing on plasma samples as previously described (*19*). Briefly, purified XMRV from infected DU145 prostate cells (C7) was denatured with SDS-PAGE sample buffer at 95°C for 10 minutes, separated by gel electrophoresis and transferred to Nytran membranes. Viral proteins were probed using test plasma and control antisera and detected with horseradish peroxidase conjugated protein G and

chemiluminescence. Specimens were considered reactive if they showed reactivity to viral Env and/or Gag proteins of the expected size as seen in the positive control antisera.

Both the WPI and the NCI laboratory of Ruscetti and colleagues performed a flow cytometry-based serologic assay which was modified from what was previously reported (*2*). 1 x 10^5 murine BaF3ER cells or BaF3ER cells expressing the spleen focus-forming virus (SFFV) envelope (S8) were incubated with 1:10, 1:100 and 1:1000 dilutions of the test specimens or control antisera for 20 minutes at room temperature. This assay was chosen because SFFV and XMRV are highly similar in the N-terminus (Genbank Accession # FJ556972) and a cell-based assay would recognize conformational antibodies. A monoclonal antibody (7C10) recognizes the N-terminus of XMRV but not the C-terminus. XMRV envelope blocks the binding of patient plasma to cell surface XMRV. Cells were washed, then incubated with anti-human IgG phycoerythrin (PE) (1:200) in FBS/PBS media incubated for 20 minutes at 4ºC, before washing in cold PBS and analysis. The cells were then examined using a BD FACSCAN. Negatives were determined by running Baf3ER cells (parental cells, not expressing SFFV envelope) that were exposed to the patient sera at 1:10 and stained with the same anti-human IgG as the experimental samples. Instrument settings were adjusted so that the center of the resulting negative histogram was in the center of the first log. Using the Baf-3ER cells, the live cells were determined from forward versus side scatter with the resulting plot set as negative and copied and pasted to the other samples in that group. This comparison was used to determine if the plasma was positive based on the level of fluorescence intensity of viable cells. As with any cell-based assay, the shape and the size (and therefore the gate) of the Baf3ER cells changes. All the other samples were run in this manner. The human sera does not always titrate; sometimes the 1:100 or 1:1000 dilutions have higher anti-human IgG binding on the BAF3-ER-SFFV envelope cells than the 1:10 distribution. The whole population can be recognized by some human plasma samples whereas only a subset of the population is recognized by other plasmas. Plasma was determined to be positive if two of three dilutions gave a positive signal (3-fold above background) on SFFV envelope, but not non-envelope, expressing cells. Positive signal could be a result of reactivity to XMRV or another gammaretrovirus or cellular cross-reactivity. Analysis was done using Flowjo software (Tree Star, Ashland, OR).

Statistics

We compared reactivity rates between negative controls and XMRV/P-MLV patients in several ways. First, we assigned "reactive" status to a given subject if any replicate sample was positive. Reactivity rates were then calculated as the ratio of reactive subjects to all subjects; rates were compared between controls and XMRV/P-MLV patients using Fisher's exact test (*35*). We performed a second analysis where reactive status was assigned only if the subject had all positive replicates. Reactivity rates were again calculated as the ratio of reactive to all subjects; in this analysis, all reactivity rates were 0.0%, so that p-values could not be calculated. We performed a third analysis based on individual replicates, where rates were calculated as the ratio of reactive replicates to all replicates. For this analysis, p-values based on two-sample z-score tests, clustered at the subject level. Specifically, replicate responses (reactive or non-reactive) were aggregated

to the subject level and standard deviations for each sample were calculated by applying standard formulas.

Kappa values were calculated for the first two methods described above (reactive subject defined as any positive replicate vs. reactive subject defined as all positive replicates) using standard procedures (*21*). In cases where one laboratory indicated that all subjects were negative, Kappa was calculated as 0.00 when the second laboratory indicated that one or more subjects were positive. Kappa statistics were not calculated if both laboratories assigned negative (or positive) status to all subjects. Kappa statistics were not calculated for the third comparison described above of all replicates.

SUPPORTING TEXT

Sequence Results

Sequencing of the excised bands was performed by all of the laboratories performing nested PCR assays. In all cases sequencing of results from spiked controls revealed sequence that matched sequences isolated from 22Rv1 cells (*13, 18*). Sequencing of the two clinical plasma samples that tested positive in the WPI assays revealed one sequence that varied from the published VP62 XMRV sequence (GenBank accession #DQ399707) by two bases and from the 22Rv1 sequence (GenBank accession #FN692043) by three bases, while the other was identical to VP62 and varied from the 22Rv1 sequence by one base. In comparison, upon decoding, it was found that bands from all the spiked controls were identical to each other and the 22Rv1 sequence, varying from the VP62 sequence by one base. The sequences identified from the positive WPI PBMC CFS patient sample varied from VP62 by two bases and from 22Rv1 by three bases.

Statistical analysis

We compared the WPI PCR reactivity rates between the 15 negative controls and the 15 XMRV/P-MLV subjects (14 of whom carried a diagnosis of CFS) by several different analyses. First, we assigned a "reactive" status to a given subject if any replicate sample was positive. In this analysis, there were no statistically significant differences between negative controls and the XMRV/P-MLV cohorts: reactivity rates were 13.3% vs. 0.0%, respectively, based on plasma ($p = 0.48$), 0.0% vs. 6.7% for PBMC ($p = 1.00$), and 0.0% vs. 0.0% for WB (all p-values were based on exact tests.) We performed a second analysis where a subject must have all positive replicates to be considered reactive; in this analysis, all reactivity rates were 0.0% (no p-values calculated). Finally, we performed a third analysis based on individual replicates, where there were no significant differences between negative controls and the XMRV/P-MLV cohorts: reactivity rates were 5.1% vs. 0.0%, respectively, based on plasma ($p = 0.14$), 0.0% vs. 3.6% for PBMC ($p = 0.38$), and 0.0% vs. 0.0% for WB (p-values based on two-sample z-score tests, clustered at the subject level.) Similar results were obtained when reactivity rates were compared between the 15 negative controls and the 14 patients with CFS (for the any-reactive subject definition, reactivity rates were 13.3% vs. 0.0% for plasma ($p = 0.48$), 0.0% vs. 7.1% for PBMC ($p = 1.00$), and 0.0% vs. 0.0% for WB; using the replicate-level definition, reactivity rates were 5.1% vs. 0.0% for plasma ($p = 0.15$), 0.0% vs. 3.9% for PBMC ($p = 0.38$), and 0.0% vs. 0.0% for WB).

Laboratory	Extraction method	Plasma Vol (µl)	Assay #	Target[a]	Assay type	Real-Time/ Nested
Abbott Molecular	*m*2000*sp* RNA	800	1	*env*	RT-PCR	Real-Time
			2	*pol*	RT-PCR	Real-Time
CDC	Ultra[b]/QIAamp Viral RNA	1000	1	*pol*	RT-PCR	Nested
			2	*pro*	RT-PCR	Real-Time
			3	*gag*	RT-PCR	Real-Time
FDA/Lo	QIAamp Viral RNA	500	1	*gag*	RT-PCR	Nested
FDA/Hewlett	QIAamp Viral RNA	140	1	*gag*	RT-PCR	Nested
Gen-Probe	Magnetic-based target capture	500	1	LTR/*pol*	TC-TMA	NA
NCI/DRP	Ultra/ GITC[c]	500	1	5' UTR of *gag*	RT-PCR SCA	Real-Time
WPI	QIAamp Viral RNA	140	1	*gag*	RT-PCR	Nested

Table S1. Methods for detection of XMRV/MLV nucleic acids in plasma panel samples. a. *env* is envelope, *pol* is polymerase, *pro* is protease and *gag* is group-specific antigen, LTR is long terminal repeat, UTR is untranslated region b. Ultra is ultracentrifuged. c. GITC is guanidinium isothiocyanate

Laboratory	Extraction method	Volume (µl)	Assay #	Target[a]	Assay type	Real-Time/ Nested
Abbott Molecular	*m*2000*sp* TNA	200	1	*env*	RT-PCR	Real-Time
			2	*pol*	RT-PCR	Real-Time
CDC	QiaAmp viral RNA	1000	1	*pol*	PCR	Nested
			2	*pro*	RT-PCR	Real-Time
FDA/Lo	Qiagen DNeasy blood	500	1	*gag*	PCR	Nested
FDA/ Hewlett	QIAamp Viral RNA	200	1	*gag*	RT-PCR	Nested
Gen-Probe	Magnetic-based target capture	50	1	LTR/*pol*	TC-TMA	NA
NCI/DRP	Promega Wizard Genomic DNA	1000	1	5' UTR of *gag*	qPCR SCA	Real-Time
WPI	QIAamp DNA mini blood **and** RNeasy	500 each	1	*gag*	PCR and RT-PCR	Nested

Table S2. Methods for detection of XMRV/P-MLV nucleic acids in PBMC panel samples. a. *env* is envelope, *pol* is polymerase, *pro* is protease and *gag* is group-specific antigen, LTR is long terminal repeat, UTR is untranslated region

Laboratory	Extraction method	Blood Vol (µl)	Assay #	Target[a]	Assay type	Real-Time/ Nested
Abbott Molecular	*m*2000*sp* TNA	400	1	*env*	RT-PCR	Real-Time
			2	*pol*	RT-PCR	Real-Time
CDC	QIAamp DNA blood mini	200	1	*pol*	PCR	Nested
			2	*pro*	qPCR	Real-Time
FDA/Lo	Qiagen DNeasy blood	500	1	*gag*	PCR	Nested
FDA/ Hewlett	QIAamp DNA blood mini	200	1	*gag*	PCR and RT-PCR	Nested
Gen-Probe	Magnetic-based target capture	50	1	LTR/*pol*	TC-TMA	NA
WPI	QIAamp DNA blood mini	200	1	*gag*	PCR	Nested
NCI/DRP	Promega Wizard Genomic DNA	500	1	5' UTR of *gag*	qPCR SCA	Real-Time

Table S3. Methods for detection of XMRV/MLV nucleic acids in whole blood panel samples. a. *env* is envelope, *pol* is polymerase, *pro* is protease and *gag* is group-specific antigen, LTR is long terminal repeat, UTR is untranslated region

Lab	Assay #	Cell type	Length of culture	Detection
FDA/ Hewlett	1	DERSE[a] cells	20 days	GFP[b]
	2	LNCaP cells	21 days	Real-Time PCR
NCI/ Ruscetti	1	LNCaP cells	19 days	Western blot

Table S4. Methods for culturing XMRV/P-MLV in plasma panel samples. a. DERSE cells are a LNCaP-based indicator cell line for MLV infection.
b. GFP is green fluorescent protein. Infection was assayed by fluorescence microscopy

Laboratory	Method	Assay #	Antigen	Reference
Abbott Diagnostics	CMIA[a]	1	gp70	Qiu et al. (*20*)
		2	p15E	
CDC	Western blot	1	Purified XMRV	Satterfield et al. (*19*)
NCI/ Ruscetti	Flow Cytometry	1	SFFV[b] Env	Lombardi et al. (*2*)
WPI	Flow Cytometry	1	SFFV Env	Lombardi et al. (*2*)

Table S5. Methods for detection of antibody reactivity to XMRV/MLV in plasma panel samples. a. CMIA is chemiluminescent immunoassay. b. SFFV Env is spleen focus forming virus envelope

Identifier	Age	Gender	Duration of illness (yrs)	anti-Retrovirals	Immuno-suppressives	PCR PBMC 1990s	PCR PBMC 2009/10	PCR Cultured PBMC	PCR Plasma	Serology	Culture
Lo et al. XMRV/P-MLV Subjects											
1*	55	F	24	No	No	**Positive**	Negative	NT	NT	NT	NT
2*	69	F	26	No	No	**Positive**	**Positive**	NT	NT	NT	NT
3*	59	F	23	No	No	**Positive**	**Positive**	NT	NT	NT	NT
4*	41	F	6	No	No	**Positive**	**Positive**	NT	NT	NT	NT
5*	41	F	21	No	No	**Positive**	**Positive**	NT	NT	NT	NT
WPI XMRV/P-MLV Subjects											
1~	55	F	18	No	No	NT	NT; NT	NT; NT***	NT	NT	**Positive**
2~	59	M	20	No	No	NT	0/2;**1/2****	NT; NT	Negative	**Positive**	**Positive**
3	58	M	23	No	No	NT	NT; NT	NT; NT	**Positive**	**Positive**	**Positive**
4~	59	F	21	No	No	NT	**1/2; 1/2**	**1/2**; NT	Negative	**Positive**	**Positive**
5~	52	F	20	No	No	NT	NT; NT	**1/2; 1/2**	Negative	**Positive**	**Positive**
6~	58	F	0	No	No	NT	0/2; NT	NT; **1/2**	**Positive**	**Positive**	**Positive**
7	64	F	28	No	No	NT	**1/2**; NT	NT; NT	NT	NT	**Positive**
8~	29	M	7	No	No	NT	NT; NT	**1/4**; NT	Negative	NT	**Positive**
9	47	M	21	No	Yes	NT	NT; NT	NT; **1/2**	NT	NT	**Positive**
10	63	M	27	No	Yes	NT	NT; NT	NT; 0/2	Negative	**Positive**	**Positive**

* = in Lo et al.

~ = in Lombardi et al

** = indicates number of positives/number of times patient was sampled. First set of results is PCR results on cDNA; second set is PCR results on genomic DNA

*** = PBMC were stimulated and grown in culture prior to being assayed by either RT-PCR (first set of results) or PCR (second set of results).

Table S6. Demographic characteristics, disease duration, and historical laboratory results in XMRV/P-MLV cohorts

		NAT-PLASM Abbott-M*	NAT-PLASM CDC*	NAT-PLASM Gen-Probe	NAT-PLASM FDA/Lo	NAT-PLASM FDA/Hewlett	NAT-PLASM NCI/DRP	NAT-PLASM WPI	NAT-PBMC Abbott-M*	NAT-PBMC CDC*	NAT-PBMC Gen-Probe	NAT-PBMC FDA/Lo	NAT-PBMC FDA/Hewlett	NAT-PBMC NCI/DRP	NAT-PBMC WPI	NAT-WB Abbott-M*	NAT-WB CDC*	NAT-WB Gen-Probe	NAT-WB FDA/Lo	NAT-WB FDA/Hewlett	NAT-WB NCI/DRP	NAT-WB WPI	Serology Abbott-D*	Serology CDC	Serology NCI/Ruscetti**	Serology WPI	Culture FDA/Hewlett-1	Culture FDA/Hewlett-2***	Culture NCI/Ruscetti
Negative Laboratory Controls																													
	1	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
		Negative	Negative	Negative	Negative	ND	ND	ND	Negative	Negative	Negative	ND	ND	ND	Negative	Negative	Negative	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
	2	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive
		Negative	Negative	Negative	ND	ND	ND	ND	Negative	Negative	Negative	ND	ND	ND	ND	Negative	Negative	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
	3	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Negative	Negative	Negative
		Negative	Negative	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	ND	ND	ND	Negative	Negative	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Positive	ND	ND	ND
Negative Blood Donor controls																													
	4	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Negative	Indeterminate	Negative
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
	5	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
	6	Negative	Negative	Negative	Negative	Negative	Negative	Positive	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Negative
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Indeterminate	Negative	ND	ND	ND
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
	7	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
	8	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Indeterminate	Positive	Negative	Indeterminate	Negative
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	ND	ND	ND
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
	9	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Negative	Negative	Positive
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Indeterminate	Positive	ND	ND	ND
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	ND	ND	ND
	10	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Indeterminate	Positive
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
	11	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Negative	Negative	Negative
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
	12	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Positive
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
	13	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Positive
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Indeterminate	Negative	ND	ND	ND
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Indeterminate	Negative	ND	ND	ND
	14	Negative	Negative	Negative	Negative	Negative	Negative	Positive	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Positive
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Indeterminate	Negative	ND	ND	ND
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
	15	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Indeterminate	Negative	Negative	Negative	Negative
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
Lo et al XMRV/P-MLV Subjects (all with CFS)																													
	1	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Negative
		ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	ND	ND	ND	Negative	ND	Negative	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	ND	ND	ND
	2	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Indeterminate	Positive	Negative	Negative	Negative
		ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	ND	ND	Negative	ND	Negative	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	ND	ND	ND
	3	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Negative	Negative	Negative
		ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	ND	ND	ND	Negative	ND	Negative	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	ND	ND	ND
	4	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Negative	Negative	Negative
		ND	ND	ND	ND	ND	ND	ND	Negative	Negative	Negative	ND	ND	ND	Negative	ND	Negative	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	ND	ND	ND
	5	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
		Negative	Negative	Negative	ND	Negative	ND	ND	Negative	Negative	Negative	Negative	ND	ND	Negative	Negative	Negative	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	ND	ND	ND
		Negative	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	6	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Indeterminate	Negative
		Negative	Negative	Negative	ND	Negative	ND	ND	Negative	Negative	Negative	ND	ND	ND	Negative	Negative	Negative	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	ND	ND	ND
	7	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
		Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	Negative	Negative	Negative	ND	ND	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND
		Negative	ND	Negative	Negative	ND	Negative	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	8	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Negative	Negative	Negative
		Negative	Negative	Negative	Negative	ND	Negative	ND	Negative	Negative	Negative	Negative	ND	ND	Negative	Negative	Negative	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	ND	ND	ND
		ND	ND	ND	ND	ND	ND	ND	Negative	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	9	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Negative
		Negative	Negative	Negative	ND	Negative	ND	ND	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	ND	ND	ND
		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	Negative	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	10	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Positive
		Negative	Negative	Negative	Negative	ND	Negative	ND	Negative	Negative	Negative	ND	ND	ND	Negative	Negative	Negative	ND	ND	ND	ND	ND	Negative	Negative	Negative	Negative	ND	ND	ND
Spiked Controls																													
	1	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	ND	ND	ND	ND	Positive	Positive
	2	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	ND	ND	ND	ND	Positive	Positive
	3	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	ND	ND	ND	ND	Positive	Positive
	4	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	ND	ND	ND	ND	Positive	Positive
	5	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	ND	ND	ND	ND	Positive	Positive

Table S7. Complete XMRV/P-MLV assay results from all laboratories on all tested replicates in the blinded panel

* = Abbott and CDC ran multiple assays for each sample. Although algorithms were in place for calling positives, in practice all samples in multiple assays apart from the spiked controls were negative

**=NCI/Ruscetti serology was performed at three dilutions. Only if two out of three were above background were samples called positive. If one out of three was above background the sample was deemed indeterminate.

***=FDA/Hewlett Culture was performed on LNCaP cells with real-time PCR read-out [1] or on DERGE cells with GFP read out [2]. Assays were performed in duplicate and both replicates were required to be positive for sample to be called positive. Otherwise it was deemed indeterminate. Note - No sample was consistently positive for all time-points, even for one replicate.

	Sample size		Reactivity rates			Kappa statistics		
	Subjects	Replicates	FDA/Hewlett-1	FDA/Hewlett-2	NCI/Ruscetti	NCI/Ruscetti vs. FDA/Hewlett-1	NCI/Ruscetti vs. FDA/Hewlett-2	FDA/Hewlett-1 vs. FDA/Hewlett-2
Negative Laboratory controls	3	1	0.0%	0.0%	33.3%	0.00	0.00	□
Negative blood donor controls	12	1	0.0%	0.0%	41.7%	0.00	0.00	□
Combined negative controls	15	1	0.0%	0.0%	40.0%	0.00	0.00	□
Lo et al XMRV/P-MLV subjects	5	1	0.0%	0.0%	0.0%	□	□	□
WPI XMRV/P-MLV subjects	10	1	0.0%	0.0%	30.0%	0.00	0.00	□
Combined XMRV/P-MLV subjects	15	1	0.0%	0.0%	20.0%	0.00	0.00	□
All subjects	30	1	0.0%	0.0%	30.0%	0.00	0.00	□
Spiked positive controls	5	1	100.0%	100.0%	100.0%	□	□	□
p-value for negative controls vs. XMRV/P-MLV subjects			□	□	0.4270			

Note: Kappa statistics provide a measure of agreement:
< 0 indicates no agreement
0 to 0.20 indicates slight agreement
0.21 to 0.40 indicates fair agreement
0.41 to 0.60 indicates moderate agreement
0.61 to 0.80 indicates substantial agreement
0.81 to 1.0 indicates almost perfect agreement

Table S8. Statistical analysis of viral culture results

	Sample size		Reactivity rates[3]				Kappa[1-3] for NCI/Ruscetti vs. WPI
	Subjects	Replicates	Abbott-Diag	CDC	NCI/Ruscetti	WPI	
By subject (any positive = reactive)							
Negative Laboratory controls	3	2	0.0%	0.0%	33.3%	33.3%	1.00
Negative blood donor controls	12	3	0.0%	0.0%	58.3%	41.7%	0.03
Combined negative controls	15	2-3	0.0%	0.0%	53.3%	40.0%	0.21
Lo et al XMRV/P-MLV subjects	5	2	0.0%	0.0%	40.0%	100.0%	0.00
WPI XMRV/P-MLV subjects	10	2	0.0%	0.0%	30.0%	50.0%	-0.20
Combined XMRV/P-MLV subjects	15	2	0.0%	0.0%	33.3%	66.7%	-0.08
All subjects	30	2-3	0.0%	0.0%	43.3%	53.3%	0.01
p-value for negative controls vs. XMRV/P-MLV subjects			□	□	0.4621	0.2723	
By subject (all positive=reactive)							
Negative Laboratory controls	3	2	0.0%	0.0%	0.0%	33.3%	0.00
Negative blood donor controls	12	3	0.0%	0.0%	0.0%	8.3%	0.00
Combined negative controls	15	2-3	0.0%	0.0%	0.0%	13.3%	0.00
Lo et al XMRV/P-MLV subjects	5	2	0.0%	0.0%	0.0%	20.0%	0.00
WPI XMRV/P-MLV subjects	10	2	0.0%	0.0%	0.0%	10.0%	0.00
Combined XMRV/P-MLV subjects	15	2	0.0%	0.0%	0.0%	13.3%	0.00
All subjects	30	2-3	0.0%	0.0%	0.0%	13.3%	0.00
p-value for negative controls vs. XMRV/P-MLV subjects			□	□	□	1.0000	
By replicate							
Negative Laboratory controls	3	2	0.0%	0.0%	16.7%	33.3%	
Negative blood donor controls	12	3	0.0%	0.0%	19.4%	22.2%	
Combined negative controls	15	2-3	0.0%	0.0%	19.0%	23.8%	
Lo et al XMRV/P-MLV subjects	5	2	0.0%	0.0%	20.0%	60.0%	
WPI XMRV/P-MLV subjects	10	2	0.0%	0.0%	15.0%	30.0%	
Combined XMRV/P-MLV subjects	15	2	0.0%	0.0%	16.7%	40.0%	
All subjects	30	2-3	0.0%	0.0%	18.1%	30.6%	
p-value for negative controls vs. XMRV/P-MLV subjects			□	□	0.7596	0.2013	

Note 1: Kappa statistics provide a measure of agreement:
< 0 indicates no agreement
0 to 0.20 indicates slight agreement
0.21 to 0.40 indicates fair agreement
0.41 to 0.60 indicates moderate agreement
0.61 to 0.80 indicates substantial agreement
0.81 to 1.0 indicates almost perfect agreement

Note 2: When one lab has all negative results and the second lab has one or more positive results, the Kappa statistic will be 0.00, regardless of the number of positive results in the second lab.

Note 3: Indeterminate results are interpreted as negative in this analysis.

Table S9. Statistical analysis of serology results

	Serology (NCI/Ruscetti) vs. NAT/plasma (WPI)	Serology (WPI) vs. NAT/plasma (WPI)	Culture (NCI/Ruscetti) vs. NAT/plasma (WPI)	Serology (NCI/Ruscetti) vs. PBMC (WPI)	Serology (WPI) vs. PBMC (WPI)	Culture (NCI/Ruscetti) vs. PBMC (WPI)	Serology (NCI/Ruscetti) vs. culture (NCI/Ruscetti)	Serology (WPI) vs. culture (NCI/Ruscetti)
By subject (any positive = reactive)								
All subjects combined	0.17	-0.13	-0.11	-0.11	-0.11	-0.10	0.06	-0.03

Note 1: Kappa statistics provide a measure of agreement:
< 0 indicates no agreement
0 to 0.20 indicates slight agreement
0.21 to 0.40 indicates fair agreement
0.41 to 0.60 indicates moderate agreement
0.61 to 0.80 indicates substantial agreement
0.81 to 1.0 indicates almost perfect agreement

Note 2: Indeterminate results are interpreted as negative in this analysis.

Table S10. Inter-assay statistical analysis for laboratories with positive results

Blood XMRV Scientific Research Working Group (SRWG) members

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Branch Chief, Transfusion Medicine and Cellular Therapeutics Branch
Division of Blood Diseases and Resources, National Heart, Lung, and Blood Institute
Jerry A. Holmberg, PhD
Co-Chair
Senior Advisor for Blood Policy
Executive Secretary of the Advisory Committee on Blood Safety and Availability
U.S. Department of Health and Human Services, Office of the Secretary
Office of the Assistant Secretary for Health

Celso Bianco, MD
Executive Vice President
America's Blood Centers
Michael P. Busch, MD, PhD
Director, Blood Systems Research Institute
Vice President, Research/Scientific Affairs, Blood Systems
Professor of Laboratory Medicine, UCSF
Roger Y. Dodd, PhD
Vice President, Research and Development
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Office of the Director
National Institutes of Health
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