資料4

XMRVに関する文献報告（続報）（平成23年12月13日）

血液事業部会運営委員会委員　岡田　義昭

文献番号	文献名	報告国	要約
1	Tang S, Zhao J, Haleyur, et al., PLoS One.2011, 6(11):e27391 Absence of Detectable XMRV and Other MLV-Related Viruses in healthy Blood Donors in the United States	米国	以前の報告で献血者の6.8%からマウス白血病ウイルスに関連したウイルス遺伝子が検出されたNIH Blood Bankから提供を受けた71検体の末梢単核球と110検体の血漿から 核酸を抽出し、XMRVのgag領域を増幅するPCR法を行った。また、血漿の33検体については、レトロウイルスが感染するとGFPが発現する遺伝子改変細胞を用いて血漿中の感染性ウイルスの有無を解析した。計算上は少なくとも3～4検体が陽性となると考えられたが、PCR法及び培養法による検討では全て陰性であった。以上から米国の供血者においてXMRV、及びマウス白血病ウイルスに関連したウイルスは存在しないことが示された。
2	Dodd RY, Hackett Jr J,Linnen JM , et al., Transfusion 2011, Nov 21 Xenotropic murine leukemia virus-related virus does not pose a risk to blood recipient safety	米国	全米の6つの血液センターからの13,399検体の血液からp15Eとgp70の2つの抗原を用いてXMRVのenvelopeに対する抗体とgag(p30)に対する抗体の有無を検討した。p15Eに対して29検体、gp70は93検体、p30では2検体が抗体陽性であった。しかし、2つの envelopeに対して共に抗体陽性となった検体はなかった。これらの抗体陽性となった検体は核酸増幅検査（TMA)では全て陰性であった。また、米国赤十字とエール大学で保管していた供血者3741検体ではp15Eに対して5検体、gp70に対して20検体が抗体陽性であったが、2つの抗原に共に抗体陽性となった検体はなかった。さらに頻回に輸血を受けた患者109人由来の830検体を検査したところ、p15Eに対して1検体、gp70に対しては20検体（2人の受血者由来）が抗体陽性であった。これら3名への供血者に抗体陽性者はいなかった。以上から、供血者と受血者計17,249におけるXMRVに対する抗体の存在は確認できなかった。また、109人の受血者と供血者計1763検体の 核酸増幅検査（TMA)では、XMRVの遺伝子は検出できなかった。これらは、XMRV及び類似ウイルスが大きなドナー集団に存在しないこと、及び輸血による感染の証拠がないことを示している。
3	Qiu X ,Swanson P ,Tang N , et al., Transfusion. 2011, Oct 24 Seroprevalence of xenotropic murine leukemia virus-ralated virus in normal and retorovirus-infected blood donors	米国	1000人の米国献血者、100人の HIV-1に感染したカメルーン人、1988年に採血された486人のHTLV-1に感染した日本人及び156人のHTLV-1非感染の日本人、311人の性感染症の検査を受けた患者、以上の血漿を用いてXMRVのエンベロープ（p15Eとgp70）に反応する抗体の有無を化学発光免疫測定法を用いてスクリーニングし、陽性の場合はウエスタンブロット法を行った。米国の献血者3名と性感染症の検査を受けた患者2名が gp70だけに陽性であった。一方、HTLV-1感染者は20名がp15 Eに、4名がgp70に対して陽性となった。何れもPCRではXMRVの遺伝子は検出されなかった。HTLV-1感染者が陽性となった理由としてp15EにXMRVとHTLV-1との間に良く似たアミノ酸配列が存在することが推定され、数例では、この類似した配列のペプチドを合成して抗体測定系に添加したところ抑制が確認された。
4	Stieler K,Schindler S ,Schlomm T, et al., PLoS One. 2011.6(10):e25592 No detection of XMRV in Blood Samples and Tissuue Sections from Prostate Cancer Patients in Northern Europe	ドイツ	北ヨーロッパの前立腺癌患者におけるXMRV感染を検出するために前立腺癌患者92例とコントロール7例から末梢血を採取した。単核球を活性化後に核酸を抽出し PCRを実施した。また、その内の67例は前立腺癌細胞株(LNCap)と混合培養し8週間後にPCRとウエスタンブロット法にてXMRVの感染の有無を検索した。さらに培養後の上清を逆転写酵素の発現があると蛍光を発する細胞に添加し、感染の有無を調べた。これらの全てでXMRVの感染は検出できなかった。また、前立腺肥大、gradeの異なる前立腺癌及び乳がんや結腸がん等の組織アレイを2種のXMRVに対する特異的抗体用いてXMRVのウイルス抗原の有無を検索したがウイルス抗原が発現している確証はなかった。
5	Katzourakis A,Hue Stephane,Kellam P, et al., J.Virol 2011.vol.85. 10909-10913 Phylogenetic Analysis of Murine Leukemia Virus Sequences from Longitudinally Sampled Chronic Fatigue Syndrome Patients Suggests PCR Contamination Rather than Viral Evolusion	英国	PNAS誌(vol.107:15874-15879,2010)に Alterらは、慢性疲労症候群患者からXMRVとは異なるマウスレトロウイルスが検出された、と報告した。ウイルスが検出された患者8例は初回のサンプリングから15年後に再度サンプリングすることができ、内7例の患者から gag遺伝子が検出されていた。6例の配列がデーターベースに登録されていたのでこれを用いてウイルスの変異を解析した。初回の塩基配列と15年後の塩基配列を比較したところ、感染者の体内でのウイルスの分子進化とは異なり、初回のウイルスとは関連しない内因性のマウスレトロウイルスの配列であった。これらはウイルスの進化よりも検体のコンタミを示唆している。

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Absence of Detectable XMRV and Other MLV-Related Viruses in Healthy Blood Donors in the United States

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Abstract

Background: Preliminary studies in chronic fatigue syndrome (CFS) patients and XMRV infected animals demonstrated plasma viremia and infection of blood cells with XMRV, indicating the potential risk for transfusion transmission. XMRV and MLV-related virus gene sequences have also been detected in 4–6% of healthy individuals including blood donors in the U.S. These results imply that millions of persons in the U.S. may be carrying the nucleic acid sequences of XMRV and/or MLV-related viruses, which is a serious public health and blood safety concern.

Methodology/Principal Findings: To gain evidence of XMRV or MLV-related virus infection in the U.S. blood donors, 110 plasma samples and 71 PBMC samples from blood donors at the NIH blood bank were screened for XMRV and MLV-related virus infection. We employed highly sensitive assays, including nested PCR and real-time PCR, as well as co-culture of plasma with highly sensitive indicator DERSE cells. Using these assays, none of the samples were positive for XMRV or MLV-related virus.

Conclusions/Significance: Our results are consistent with those from several other studies, and demonstrate the absence of XMRV or MLV-related viruses in the U.S. blood donors that we studied.

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Introduction

Xenotropic murine leukemia virus-related virus (XMRV) was originally identified in prostate cancer tissues in 2006 [1], and proposed to be associated with PC [1,2,3,4,5] and chronic fatigue syndrome (CFS) [6,7]. However, a causal relationship has not been validated and several controversial findings have been reported [8,9,10,11,12]. Furthermore, XMRV as a human pathogen has been questioned since mouse DNA contamination has been found in human samples tested [13,14,15,16], and XMRV may be the result of a recombination of two MLV ancestors [17]. As a newly identified retrovirus, XMRV can infect human tissues and cells including lymphoid organs [18] and peripheral blood mononuclear cells (PBMCs) [6], indicating potential transfusion transmission of XMRV. XMRV has also been detected in 3.7% of healthy individuals [6] and 5.9% of non-prostate cancer patients [2] in the U.S.. In addition, Lo et al reported that 6.8% of U.S. healthy blood donors carried MLV-related sequences, which are molecularly different from but very similar to XMRV [19]. These results, if confirmed, imply that millions of persons in the U.S. may harbor XMRV and/or MLV-related viruses and thus pose a serious threat to public health, including blood safety and organ transplantation. To ensure blood safety, suggestions and preventive measures have been proposed, such as developing screening tools and deferring CFS patients for blood donation [20]. However, these recommendations and measures have been questioned in the absence of the conclusive consensus of the prevalence of XMRV infection in blood donors and causality for human diseases. In order to address blood safety concerns, the Blood XMRV Scientific Research Working Group (SRWG) composed of members from academia, government and blood organizations was formed by the National Heart, Lung, and Blood Institute (NHLBI) [21]. The major goals of this group were to 1) validate the testing methods for XMRV since one of the possible reasons for the conflicting findings was attributed to differences in testing methods, and 2) to investigate possible infection of blood donors with XMRV or MLV-related viruses.

During the past two years, our laboratory actively participated in assay validation and assessment of the threats posed by XMRV on blood safety. We previously reported that our RT-PCR assay could detect 10 copies and 1 copy of plasmid DNA in the 1st and 2nd round PCR, respectively [22] by using primers described by Silverman et al [1] and Mikovits et al [6]. Our quantitative PCR assay could detect 1–10 copies of XMRV plasmid DNA, which is comparable to the results reported by Schlaberg et al [2]. Our PCR assays were able to achieve similar levels of sensitivity and specificity based on the spiked XMRV panels created by the Blood XMRV SRWG [21]. For virus culture, we set up an infectivity assay using the Detectors of Exogenous Retroviral Sequence Elements (DERSE) indicator cells where plasma samples are co-

cultured with modified LNCaP cells which are susceptible to XMRV infection and virus replication monitored using a fluorescence signal [23]. Mikovits et al who reported the association of XMRV with CFS claimed that culture of virus from plasma was the most sensitive blood-based assay for detection of XMRV [7]. By using these highly sensitive assays, we screened U.S. blood donors for XMRV or MLV-related viruses in order to provide further evidence of the status of these possible new viruses in the blood donors from the NIH Blood Bank, the same blood bank from which donors had previously reported to harbor polytropic MLV-related virus sequences in 6.8% of the individuals tested [19].

Materials and Methods

Ethics Statement

The Food and Drug Administration Research Ethics Committee has waived the need for consent due to the fact the blood donor material used was fully anonymised.

Collection and PCR testing

A total of 71 PMBC samples and 110 plasma samples from blood donors were enrolled in our study. Both plasma and PBMCs were recovered from the entire buffy coat that was received from the NIH Blood Bank. Briefly, the entire buffy coat was centrifuged at 1500 rpm for 15 minutes and plasma was carefully removed. Cells were resuspended in 15 ml of Ficoll solution and centrifuged for 30 minutes at 400g. The PBMCs, seen as a ring or band at the top of the Ficoll solution, were removed, placed in a fresh 50 ml tube and filled with PBS saline for further use.

Viral RNA was extracted from 140 µl of plasma using QIAamp MiniElute Virus Spin kit (Qiagen, Valencia, CA), and genomic DNA of 1×10^6 PBMCs was extracted using the QIAamp DNA Blood mini kit. Reverse transcription was performed with SuperScript III for First-strand Synthesis System (Invitrogen) using 8 ul of viral RNA or total nucleic acid from PBMC and XMRV gag reverse primer 1154R [6]. For amplification of XMRV gag gene, first-round PCR was performed in a 20 ul volume containing 5 ul of cDNA or 200~500 ng of genomic DNA, 10 ul of 2xPCR buffer (Extensor Hi-Fidelity ReddyMix PCR Master Mix, ABgen House, Surrey, UK) and 2.5 pmol each primer (GAG-O-F and GAG-O-R) [1]. Reaction conditions were one cycle at 94°C, 5′, 45 cycles at 94°C, 1′, 58°C , 1′, 72°C, 1′ and one cycle at 72°C, 7′. Two microliters of 1st round PCR products were added to 2nd round PCR with the same reaction conditions as those in the 1st PCR except that the different primers (GAG-I-F and GAG-I-R) and the annealing temperature of 60°C were used [1]. Each PCR run included both XMRV positive control (a full-length XMRV plasmid DNA, isolate VP62, gifted by Dr R. Silverman) and negative control (water). PCR amplification products were visualized on a 2% agarose gel stained with ethidium bromide. Each sample was tested in triplicate, the band equivalent to the correct size of positive control was excised from 2% agarose gel using the QIAquick gel extraction kit (Qiagen Inc., Valencia, CA) for sequence analysis. Alternatively, a specific PCR product was purified using ExoSAP-IT reagent (usb, Santa Clara, CA). Purified PCR products were sequenced directly using the ABI Prism BigDye Terminator Cycle Sequencing kit in the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence and phylogenetic analyses were performed using the MEGA5 software package and the Invitrogen Vector NTI software, version 11.3.0 (Invitrogen, Carlsbad, CA). A positive test result was defined as one where at least one band of the correct size was detected in triplicate PCR reactions, and confirmed by sequencing as XMRV. A negative result was defined as one where no bands of the correct size were detected in triplicate PCR reactions or at least one band of correct size was observed but the sequence analysis did not confirm as XMRV. To ensure integrity of extracted DNAs, human GAPDH gene was amplified with the same PCR primers (hGAPDH-66F and hGAPDH-291R) and conditions published previously [1]. To avoid possible mouse DNA contamination, PCR assays for amplifying mouse intracisternal A particle (IAP), mouse mitochondrial DNA were performed as previously described [13,15,19]. The experiments were performed by two laboratory personnel to ensure that results were scored based on reproducibility of data obtained by two independent operators.

Cell culture assay for detection of infectious virus

A co-culture assay was adopted to monitor XMRV infection by using Detectors of Exogenous Retroviral Sequence Elements (DERSE) cells that are LNCaP-iGFP cell clones displaying sensitivity to XMRV infection that leads to expression of a GFP reporter [23]. In this 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. In this indicator cell line, GFP is only expressed after mobilization by an infecting gammaretrovirus during a second round of infection. Briefly, 0.4×10^5 DERSE cells/well were added in 24-well plate. After 24–48 hours, the cells were mixed with 200 ul of plasma samples or normal plasma spiked with XMRV. The plate was centrifuged at 1500 rpm (Eppendorf Centrifuge # 5810 R) for 5 minutes, and then incubated at 37°C overnight. Plasma was very carefully replaced with fresh RPMI complete media, and transferred to a 6-well plate to expand as required (usually after 4–5 days post infection). When cells became confluent, they were transferred to a T-25 flask and maintained for 21 days post infection. GFP expression in cells at different days post-infection was determined using fluorescence microscopy.

Results

By using serial 1:10 dilutions of XMRV plasmid DNA with known copy numbers based on absorbance A260 of the purified plasmid VP62, 10 copies and one copy of plasmid DNA were detected in the first- and second-round PCR, a lower detection limit of one copy of proviral DNA using our current nested PCR conditions was achieved. The sensitivity of the PCR assays was also evaluated using XMRV DNA extracted from a series of 1:10 dilutions of 22Rv1 cells (CRL-2505, ATCC, Gaithersburg, MD) that harbor multiple copies of integrated XMRV provirus and constitutively produce infectious virus [24]. The current nested PCR assay could detect XMRV DNA from single 22Rv1 cells (data not shown). Using this assay, none of the 110 plasma samples were positive for XMRV or MLV-related virus with either XMRV gag primer sets although the positive control was successfully amplified in each PCR run (Fig. 1A). Total nucleic acid from 71 PBMC samples was also tested but found to be negative for XMRV or MLV-related virus using both nested DNA PCR and RT-PCR assays (Fig. 1B, Table 1). Both assays were used since it was reported that RT-PCR could be more sensitive than DNA PCR for detection of XMRV in activated PBMCs [7]. Any bands with similar size of XMRV positive control were excised from the gel, purified and sequenced. No XMRV sequences were found on sequence analysis. A specific hGAPDH gene was amplified from all 71 PBMC samples (Fig. 1C), indicating the integrity of the extracted DNA.

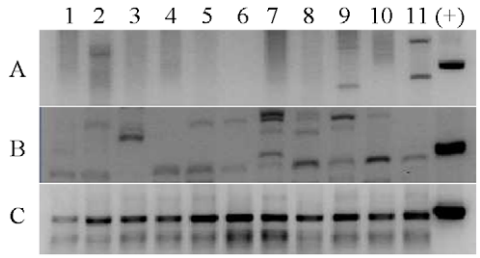

Figure 1. PCR screening for XMRV or MLV-related virus. (A) PCR products of 11 plasma samples (lane 1–11) collected in NIH Blood Bank with XMRV gag gene primer pair. Lane 12 was positive control of XMRV. (B) PCR products of 11 PBMC samples (lane 1–11) collected in the NIH Blood Bank with XMRV gag gene primer pair. Lane 12 was positive control of XMRV. (C) hGAPDH gene. Lane 1–11 was results for 11 PBMC samples while lane 12 was positive control for hGAPDH.
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Using DERSE cells, the GFP signal could be detected within three days of XMRV infection, with the number of GFP-positive cells increasing over subsequent days. The DERSE GFP culture method is highly sensitive as it can detect around 2000 copies of XMRV. In our study, DERSE cells could be successfully infected by culture supernatant of the 22Rv1 cell line which carries XMRV [24] (Fig. 2A) and displayed fluorescence 4 days after infection (Fig. 2B). GFP expression was observed 18 days post infection in cells that were infected with 2000 copies of XMRV. However, none of the 33 plasma samples tested displayed visible fluorescence signal even after 21 days post infection (Fig. 2D). The culture supernatants were also negative for XMRV using both quantitative PCR and RT-PCR (data not shown).

Discussion

The above results strongly support the conclusion that XMRV and other MLV-related viruses are absent in healthy blood donors in the population we studied. The rigorous testing employed and use of highly sensitive PCR and cell culture methods to evaluate the presence of both nucleic acid and infectious virus provide strong evidence to support this conclusion. The failure to detect XMRV in U.S. blood donor samples is unlikely due to the sensitivity of PCR assays because they have been shown to be at least as sensitive as those previously reported [22], and comparable to those used by other labs enrolled in the assay evaluation study sponsored by the Blood XMRV SRWG [21]. XMRV positive and negative controls were correctly identified in both PCR and co-culture experiments in our study indicating the accuracy of test performance and validity of assay runs. In addition, the sample size we tested was sufficiently large enough to potentially identify at least 3–4 XMRV or MLV-related virus positive samples since between 4–6% of healthy controls including blood donors were reported to be positive for XMRV or MLV-like viruses in previous studies conducted in the U.S. [2,6,19]. Therefore, based on testing using highly sensitive detection assays we did not find evidence of XMRV or MLV-related virus infection in the U.S. blood donor samples we tested.

Table 1. Detection of XMRV in the plasma and PBMC samples from the NIH Blood Bank[1].

Sample	PCR results		DERSE results	
	No. tested	No. positive	No. tested	No. positive
Plasma	110	0	33	0
PBMCs	71	0	0	0

[1]Viral RNA isolated from plasma was analyzed for XMRV and HIV-1 using RT-nested PCR while genomic DNA extracted from PBMCs was analyzed for XMRV and HIV-1 using nested PCR and (q)PCR.
GAPDH was amplified in parallel as an internal control.
doi:10.1371/journal.pone.0027391.t001

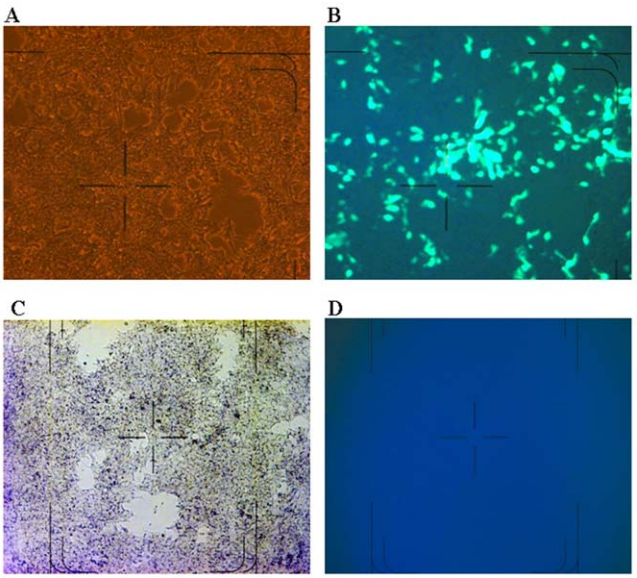

Figure 2. GFP signal detection in DERSE cell culture. (A) Light microscopy image for positive control. DERSE cells were infected with culture supernatant from 22Rv1 cell. (B) Fluorescence microscopy image for XMRV positive control. Panel C (light microscopy image) and D (fluorescence microscopy image) for blood donor plasma in which no XMRV was detected.
doi:10.1371/journal.pone.0027391.g002

Our results are consistent with other recent findings that have been reported in the U.S. Gao et al tested 425 plasma samples from U.S. blood donors using a transcription mediated amplification (TMA) assay and did not detect XMRV in these samples [25]. Their assay was reported to be one of the most sensitive assays in the assay evaluation study sponsored by the Blood XMRV SRWG [21]. Qiu et al reported that only 0.1% of the U.S. blood donors were positive for anti-XMRV antibodies by using their prototype direct chemiluminescent immunoassays (CMIAs) on the automated ARCHITECT® instrument for detecting anti-XMRV assay, which is the first immunoassay that has been evaluated by the well characterized XMRV infected animal bleeds [26]. Switzer et al were unable to detect XMRV infection in 51 healthy controls and 43 U.S. blood donors using PCR and serology assays [27]. Kunstman et al tested 996 samples from the Chicago Multicenter AIDS Cohort Study (562 HIV-1 positive and 434 at high risk for HIV-1 infection, but HIV-1 negative individuals), none of them were XMRV positive [28]. Henrich et al were unable to detect XMRV infection in PBMC samples from 43 HIV positive individuals, 97 rheumatoid arthritis patients, 26 transplant recipients and 95 general patients [29].

XMRV was also not or rarely detected in general populations worldwide. Only about 1% of control groups were found to be positive for XMRV in Germany [10], the U.K [12] and Japan [30], but no XMRV was detected in Chinese blood donors [31]. Negative results were reported for XMRV testing of blood donors or individuals infected HIV-1 in Africa [22]. These results indicate

that XMRV or other MLV-like viruses may be very rare, or absent in the general population overall. In contrast, our results support the recent findings that the current positive detection of XMRV or MLV-related virus in human samples may be due to mouse DNA contamination rather than a true human infection. Robinson et al reported that XMRV positive prostate cancer tissues and 21.5% of XMRV negative cases were positive for mouse IAP sequence [15]. Oakes et al found that by using a less specific PCR assay, both XMRV and/or MLV were detected in CFS patients. However, all positive samples were also positive for mouse IAP while no contamination was observed in any of the negative control samples [13]. Sato et al reported that endogenous MLV was amplified in a commercial RT-PCR kit using standard primers for XMRV [16]. The contamination originated from the hybridoma cell line from which the monoclonal antibody used in the polymerase reaction mixture to facilitate hot-start PCR was prepared. Hue et al also demonstrated that XMRV specific primers can amplify murine endogenous viral sequences [14]. These results indicate that mouse DNA contamination is widespread and can confound XMRV detection in human samples.

Furthermore, Hue et al compared the published XMRV sequences with those from 22Rv1 cell, which is infected with XMRV and found that the genetic distance among 22Rv1-derived sequences exceeds that of patient-associated sequences, indicating that patient-associated XMRV sequences are consistent with laboratory contamination rather than a true human infection [14]. The 22Rv1 cell line was derived from a human prostate cancer xenograft (CWR22) that was serially passaged in nude mice in 1990s. Interestingly, it was recently shown by Paprotka et al that XMRV resulted from recombination between two endogenous MLVs during passage of the CWR22 PC xenograft [17], suggesting that the laboratory-derived virus may have contaminated samples for more than a decade and thereby contributed to the inconsistent positive detection reported by various laboratories that had used them for these studies and over extended periods of time. The relevant published studies on XMRV and MLRV findings in CFS, PCA and blood donors are listed in the Table S1.

In summary, we screened 110 plasma samples and 71 PBMC samples collected from U.S. blood donors using well characterized and highly sensitive PCR and culture assays. The testing employed independent test operators and rigorous testing conditions aimed at avoiding contamination. Under these conditions, none of the samples were found to be positive for XMRV or MLV-related virus sequences or infectious virus. Our results failed to demonstrate the presence of XMRV or MLV-related viruses in the samples we tested, and provide strong evidence for the absence of XMRV or MLV-related virus in the U.S. blood donor population we studied.

Supporting Information

Table S1 Relevant Published Studies on XMRV and MLRV Findings in CFS, PCA and Blood Donors.
(DOC)

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Author Contributions

Conceived and designed the experiments: ST IH. Performed the experiments: ST JZ MH KD DG RV OW PZ. Analyzed the data: ST MH KD IH. Wrote the paper: ST JZ MH KD DG RV OW PZ IH.

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Xenotropic murine leukemia virus–related virus does not pose a risk to blood recipient safety

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BACKGROUND: When xenotropic murine leukemia virus–related virus (XMRV) was first reported in association with chronic fatigue syndrome, it was suggested that it might offer a risk to blood safety. Thus, the prevalence of the virus among blood donors and, if present, its transmissibility by transfusion need to be defined.

STUDY DESIGN AND METHODS: Two populations of routine blood donor samples (1435 and 13,399) were obtained for prevalence evaluations; samples from a linked donor-recipient repository were also evaluated. Samples were tested for the presence of antibodies to XMRV-related recombinant antigens and/or for XMRV RNA, using validated, high-throughput systems.

RESULTS: The presence of antibodies to XMRV could not be confirmed among a total of 17,249 blood donors or recipients (0%; 95% confidence interval [CI], 0%-0.017%); 1763 tested samples were nonreactive for XMRV RNA (0%; 95% CI, 0%-0.17%). Evidence of infection was absent from 109 recipients and 830 evaluable blood samples tested after transfusion of a total of 3741 blood components.

CONCLUSIONS: XMRV and related murine leukemia virus (MLV) markers are not present among a large population of blood donors and evidence of transfusion transmission could not be detected. Thus, these viruses do not currently pose a threat to blood recipient safety and further actions relating to XMRV and MLV are not justified.

Xenotropic murine leukemia virus–related virus (XMRV) was first reported in selected patients with prostate cancer in 2006.[1] Subsequently, it was also reported in 67% of patients with chronic fatigue syndrome (CFS) and among 3.7% of healthy controls.[2] At the same time, it was suggested that this gammaretrovirus might offer a risk to blood safety.[3] A second study then reported the presence of mouse-derived retroviral *gag* sequences representing polytropic murine leukemia viruses (MLVs) among 87% of CFS patients sampled in the 1990s and 6.7% of contemporary blood donor controls.[4] Whether these two studies are mutually supportive is unlikely. In addition, at least 11 other published studies have failed to confirm a relationship between XMRV and/or MLVs and CFS.[5] Recently, there has been an editorial expression of concern[6] regarding the original study[2] demonstrating XMRV in CFS patients and some of the data have been retracted.[7] A similar relationship between XMRV and prostate cancer has not been confirmed, with more than half of the published studies showing no association.[5] Thus, there is

ABBREVIATIONS: ARC = American Red Cross; CFS = chronic fatigue syndrome; CMIA(s) = microparticle-based chemiluminescence immunoassay(s); MLV(s) = murine leukemia virus(-es); S/CO = sample-to-cutoff ratio; SRWG = Scientific Research Working Group; TMA = transcription-mediated amplification; XMRV = xenotropic murine leukemia virus–related virus.

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TRANSFUSION **;**:**-**.

significant controversy about the relationship of XMRV and/or MLVs to human disease. Furthermore, there is increasing evidence that XMRV is a laboratory artifact resulting from recombination of two endogenous murine retroviral proviruses during passage of prostate tumor cells in mice.[8] Several additional studies have suggested that the findings of the two positive studies reporting a linkage between XMRV and/or MLV and CFS are attributable to contamination via one or more routes (e.g., reagents, samples, techniques) rather than to human infection.[5,6] A recent multicenter study involving nine laboratories testing replicate samples from 15 patients or individuals with a history of XMRV positivity and 15 control subjects has shown that currently used tests, including those from two groups that were part of the study by Lombardi and colleagues,[2] do not reliably detect markers of XMRV. While all but two assays correctly identified blinded negative and positive control samples, no assay could detect XMRV in patients previously characterized as XMRV-infected.[9] However, it has become apparent that XMRV is a bona fide virus with the capability of readily infecting human cells, at least in vitro,[8] although recent data suggest that the virus itself may not be effective in infecting humans in vivo.[10] It has been shown that XMRV can elicit a weak, transient virus-specific immune response in experimentally infected rhesus macaques.[11] Nevertheless, unexpectedly high reported prevalence rates for XMRV and/or MLV markers in healthy human controls warrant further evaluation.

In the context of blood safety, there is concern about the impact of retroviruses, irrespective of their relationship to disease. More specifically, the potential for mutation and acquisition of new pathogenic properties (particularly in the context of a species jump) must always be considered. With respect to XMRV, the NIH has sponsored two groups to examine the risk of this virus to blood safety and its relationship to CFS; each of the two study groups involves multiple investigators with considerable expertise and prior publications in this arena. The first, sponsored by the National Heart, Lung, and Blood Institute, included the examination of patients who were previously found positive in the two referenced studies, described above,[2,4] and the second, an NIH-sponsored study group that focused on patients with well-defined CFS; the second study has not yet been completed. Reports of the activities of the NHLBI group, referred to as the Scientific Research Working Group (SRWG) have been published.[9,12] In addition, the AABB has also provided information and advice to their membership regarding management of presenting blood donors with a history of CFS.[13]

The objective of this study was to determine the prevalence of XMRV infection in US blood donors and to examine the risk of transfusion transmission. High-throughput microparticle-based chemiluminescence immunoassays (CMIAs)[14,15] and transcription-mediated amplification (TMA)[16] were used to evaluate the prevalence of XMRV and/or MLV antibodies and XMRV RNA among routine blood donors and in a population of highly transfused patients and their donors. For the transfusion transmission study, we used a previously characterized donor-recipient repository maintained by the American Red Cross (ARC) and Yale University.[17,18]

MATERIALS AND METHODS

Samples

Four groups of blood donor samples were obtained for XMRV RNA or XMRV and/or MLV antibody testing. First, plasma containing ethylenediaminetetraacetate (EDTA) as an anticoagulant from 1435 unlinked surplus blood donation samples collected in the Charlotte, North Carolina area by the ARC in early 2010 were submitted to Gen-Probe, Inc. (San Diego, CA) for an initial evaluation of the specificity of their assay. Submitted samples tested nonreactive in all routine blood donor screening tests and were frozen within 72 hours of collection. In addition, as part of the specificity evaluation, 97 human T-lymphotropic virus (HTLV)-1 and/or -2 antibody confirmed-positive, ACD-plasma samples identified through routine blood donation screening at the ARC from calendar years 2008 through 2010 were submitted for testing. HTLV confirmed-positive samples including those containing antibodies to HTLV-1 (45), HTLV-2 (30), or both HTLV-1 and -2 (22) were frozen within 24 hours of collection. The HTLV confirmed-positive samples were obtained directly from the retained frozen plasma component of the donated blood unit.

The prevalence study samples included a total of 13,399 unlinked paired serum and EDTA-plasma samples obtained from surplus blood donation samples found nonreactive in all routine blood donor screening tests; samples were frozen within 72 hours of collection. The samples were collected from six ARC blood center collection areas (Atlanta, GA; Baltimore, MD; Boston, MA; Detroit, MI; Los Angeles, CA; and Portland, OR) during June to September 2010. Each location contributed between 2000 and 2600 samples.

Finally, 3741 serum samples were obtained that represented the available retention samples from all blood units transfused to a population of frequently transfused recipient-patients, as previously described.[17,18] For the recipients of this latter group of blood components, there were a total of 830 evaluable blood samples (CPD or EDTA-plasma) representing pre- and sequential post-transfusion samples from 109 of the patients in the same study.[17,18] Recipients in this study only received blood from the 3741 donors, except on rare occasions where other components were transfused.

TABLE 1. XMRV and/or MLV antibody reactivity to individual recombinant antigens by metropolitan area for six US regions*

Region*	Number tested	Number p15E reactive	Percent†	Number gp70 reactive	Percent†	Number p30 reactive
Atlanta, GA	2,385	6	0.25	0		1
		0		19	0.80	1
Boston, MA	2,631	4	0.15	0		0
		0		21	0.80	0
Los Angeles, CA	2,142	3	0.14	0		0
		0		19	0.89	0
Detroit, MI	2,020	8	0.40	0		0
		0		9	0.45	0
Portland, OR	2,008	5	0.25	0		0
		0		12	0.60	0
Baltimore, MD	2,213	3	0.14	0		0
		0		13	0.59	0
Total	13,399	29	0.22	93	0.69	

* Chi-square for p15E (5 d.f.) = 5.06, p = 0.42. Chi-square for gp70 (5 d.f.) = 4.38, p = 0.50.
† The differences in regional prevalence rates are not statistically significant.

Antibody testing

Samples were separately tested for antibodies to XMRV and/or MLV p15E (transmembrane) and gp70 (surface envelope) antigens using prototype CMIAs, processed on an automated analyzer (ARCHITECT, Abbott Diagnostics, Abbott Park, IL).[9,10,14,15] These assays have been shown to be both specific (99.5%-99.9%) and highly sensitive (100%), on the basis of studies performed in rhesus macaques and blood donors.[14] Any sample that gave a signal equal to or greater than the cutoff value (sample-to-cutoff ratio [S/CO] $\geq$ 1.00) was repeated in duplicate and those samples with repeatedly reactive results for either marker were also tested for antibodies to the XMRV and/or MLV p30 antigen (capsid), using the same test method. Repeat reactivity (S/CO $\geq$ 1.0) to all three antigens is required to confirm a positive antibody finding.

RNA testing

Samples were tested for XMRV RNA sequences using a research TMA assay[9,16] and processed on an automated analyzer (TIGRIS System, Novartis Diagnostics, Emeryville, CA); initial reactive samples would have been retested in duplicate had they occurred. This assay has been shown to be more sensitive than other assays when used to test panels of dilutions of the VP62 isolate of XMRV.[12] Analytic analysis using the VP62 isolate demonstrates a sensitivity of 2.5 copies/mL at 95% confidence (95% confidence interval [CI], 1.8-4.8).[12,16]

Statistical analysis

Frequencies of positive findings were calculated including 95% CIs by the mid P exact method (OpenEpi, http://www.openepi.com/OE2.3/Menu/OpenEpiMenu.htm); chi-square analysis was used to estimate regional differences where p values of less than 0.05 were considered significantly different.

TABLE 2. p15E antibody–reactive blood donor sample results from six US regions*

Number	p15E	gp70	p30	RNA
1	1.04	0.08	0.25	0.00
2	2.27	0.09	0.18	0.00
3	9.01	0.10	0.17	0.00
4	1.81	0.10	**7.40†**	0.00
5	1.57	0.12	0.21	0.00
6	1.94	0.12	0.18	0.00
7	1.18	0.09	0.23	0.00
8	1.19	0.14	0.15	0.00
9	13.06	0.10	0.18	0.00
10	5.96	0.09	0.78	0.00
11	5.50	0.10	0.16	0.00
12	2.00	0.13	0.19	0.00
13	1.84	0.11	0.15	0.00
14	6.22	0.11	0.16	0.00
15	3.12	0.08	0.15	0.00
16	1.61	0.27	0.18	0.00
17	3.50	0.09	0.14	0.00
18	1.21	0.07	0.23	0.00
19	1.09	0.09	0.20	0.00
20	3.79	0.10	0.18	0.03
21	1.26	0.13	0.14	0.00
22	7.33	0.09	0.17	0.00
23	1.15	0.10	0.17	0.00
24	2.68	0.05	0.21	0.00
25	2.25	0.09	0.15	0.00
26	3.20	0.06	0.20	0.00
27	1.70	0.05	0.14	0.00
28	1.63	0.06	0.24	0.00
29	1.17	0.06	0.33	0.00

* Reactive results are expressed as means of duplicate retests; n = 29 of 13,399 tested.
† Bolded value is greater than or equal to 1.0 and represents reactive results.

Human subjects review

All studies were approved by the ARC Institutional Review Board; the donor-repository study was also approved by the Yale University Institutional Review Board.

RESULTS

RNA testing

A total of 1435 blood donor samples, nonreactive on all routine screening for blood-borne infections, were tested for XMRV RNA using TMA; none was found to be reactive, with a mean S/CO of 0.18. An S/CO of 1.0 or greater is regarded as reactive. Additionally, none of 97 donor samples confirmed positive for HTLV antibodies was found to be reactive for XMRV RNA (S/CO mean of 0.04). Thus, specificity of the XMRV TMA assay for the 1532 evaluated blood donor samples was 100% (95% CI, 99.8%-100%; n = 1532).

Antibody testing

CMIA was used to identify individual antibodies to recombinant XMRV and/or MLV p15E (transmembrane), gp70 (surface envelope), and p30 (capsid) antigens. Among 13,399 blood donor samples collected from each of six US regions and tested for antibodies to XMRV and/or MLV by CMIA, 29 (0.22%) had isolated reactive results for antibody to p15E, whereas 93 (0.69%) had isolated reactive results for antibody to gp70; no sample had reactivity to both antigens. Isolated antibody reactivity ranged by US region from 0.14% to 0.40% for p15E and 0.45% to 0.89% for gp70 (Table 1): there was no significant difference in prevalence between regions. The respective ranges of S/CO values (mean of duplicate retest determinations) were 1.04 to 13.06 and 1.02 to 32.13. Two samples, one of which was reactive for antibody to p15E and the other for gp70, were found reactive for antibody to p30, with S/CO values of 7.40 and 1.66, respectively (Tables 2 and 3). The distribution of S/CO values for p15E and gp70 antibody reactivity is illustrated in Fig. 1 using a natural log scale to better illustrate samples with weak reactivity. No donor sample had all three XMRV antibody markers. The 122 (29 p15E plus 93 gp70) isolated antibody-reactive samples were also tested for XMRV RNA and were found nonreactive, with S/CO values ranging from 0.00 to 0.14 (Tables 2 and 3). Therefore, there was no evidence of XMRV and/or MLV infection in this population. The prevalence of XMRV and/or MLV antibodies in contemporary US blood donors from six US regions was 0% (95% CI, 0%-0.023%) with no indication of ongoing infection as demonstrated by the lack of viral RNA.

Repository samples

Donors

Among the 3741 donor samples from an established donor-recipient repository,[17,18] five (0.13%) had antibody

TABLE 3. gp70 antibody–reactive blood donor sample results from six US regions*

Number	p15E	gp70	p30	RNA	No.	p15E	gp70	p30	RNA	No.	p15E	gp70	p30	RNA
1	0.30	**1.74**	0.17	0.00	32	0.11	**1.34**	0.16	0.00	63	0.14	**6.10**	0.16	0.00
2	0.17	**3.64**	0.20	0.00	33	0.16	**3.03**	0.26	0.00	64	0.14	**1.29**	0.16	0.00
3	0.15	**2.06**	0.45	0.00	34	0.12	**3.16**	0.47	0.00	65	0.13	**6.11**	0.15	0.00
4	0.13	**4.40**	0.19	0.00	35	0.15	**1.93**	0.16	0.00	66	0.15	**2.51**	0.48	0.00
5	0.13	**3.08**	0.17	0.00	36	0.11	**2.29**	0.15	0.00	67	0.12	**12.53**	0.15	0.00
6	0.13	**1.13**	0.15	0.00	37	0.12	**3.24**	0.16	0.00	68	0.13	**1.31**	0.20	0.00
7	0.15	**5.94**	**1.66**	0.00	38	0.12	**1.14**	0.15	0.00	69	0.13	**5.35**	0.19	0.00
8	0.23	**11.87**	0.14	0.00	39	0.12	**1.86**	0.16	0.00	70	0.13	**23.65**	0.16	0.00
9	0.14	**4.86**	0.19	0.00	40	0.12	**1.02**	0.15	0.00	71	0.14	**6.86**	0.14	0.00
10	0.15	**1.59**	0.16	0.00	41	0.13	**1.45**	0.17	0.00	72	0.12	**1.02**	0.20	0.00
11	0.11	**3.95**	0.20	0.03	42	0.14	**30.30**	0.17	0.00	73	0.10	**5.29**	0.16	0.00
12	0.13	**1.80**	0.18	0.00	43	0.13	**1.57**	0.17	0.00	74	0.13	**1.72**	0.21	0.00
13	0.10	**7.70**	0.17	0.00	44	0.15	**1.53**	0.26	0.00	75	0.14	**1.16**	0.18	0.14
14	0.13	**2.84**	0.17	0.00	45	0.14	**1.57**	0.15	0.00	76	0.28	**3.82**	0.15	0.00
15	0.12	**1.37**	0.20	0.00	46	0.11	**8.83**	0.18	0.00	77	0.11	**3.96**	0.17	0.00
16	0.13	**3.02**	0.19	0.00	47	0.09	**10.66**	0.17	0.00	78	0.13	**8.23**	0.27	0.00
17	0.11	**3.70**	0.18	0.00	48	0.10	**3.16**	0.21	0.00	79	0.14	**1.72**	0.20	0.00
18	0.10	**1.20**	0.26	0.00	49	0.17	**4.37**	0.91	0.12	80	0.16	**1.38**	0.18	0.00
19	0.13	**1.78**	0.25	0.00	50	0.11	**1.02**	0.15	0.00	81	0.18	**1.33**	0.22	0.00
20	0.10	**1.08**	0.17	0.00	51	0.11	**3.05**	0.16	0.00	82	0.16	**1.08**	0.18	0.00
21	0.11	**5.36**	0.18	0.00	52	0.13	**1.16**	0.15	0.00	83	0.13	**1.12**	0.22	0.00
22	0.13	**4.05**	0.20	0.00	53	0.12	**32.13**	0.16	0.00	84	0.14	**1.75**	0.98	0.00
23	0.09	**4.72**	0.15	0.00	54	0.12	**3.65**	0.32	0.00	85	0.13	**4.45**	0.18	0.00
24	0.11	**1.76**	0.28	0.00	55	0.16	**1.04**	0.16	0.00	86	0.13	**1.71**	0.29	0.00
25	0.11	**12.78**	0.21	0.05	56	0.15	**1.24**	0.16	0.00	87	0.17	**1.32**	0.16	0.00
26	0.12	**8.29**	0.17	0.00	57	0.13	**24.05**	0.29	0.01	88	0.15	**11.14**	0.25	0.00
27	0.09	**7.92**	0.18	0.00	58	0.11	**3.70**	0.15	0.00	89	0.13	**2.28**	0.24	0.00
28	0.12	**3.00**	0.27	0.00	59	0.11	**4.86**	0.15	0.00	90	0.10	**2.31**	0.32	0.00
29	0.12	**18.99**	0.16	0.00	60	0.12	**1.45**	0.14	0.00	91	0.17	**1.30**	0.18	0.00
30	0.12	**2.59**	0.18	0.00	61	0.12	**1.54**	0.15	0.00	92	0.13	**2.63**	0.25	0.00
31	0.16	**1.54**	0.19	0.00	62	0.14	**8.57**	0.17	0.00	93	0.13	**21.78**	0.18	0.00

* Reactive results are expressed as means of duplicate retests; n = 93 of 13,399 tested.
Bolded values are greater than or equal to 1.0 and represent reactive results.

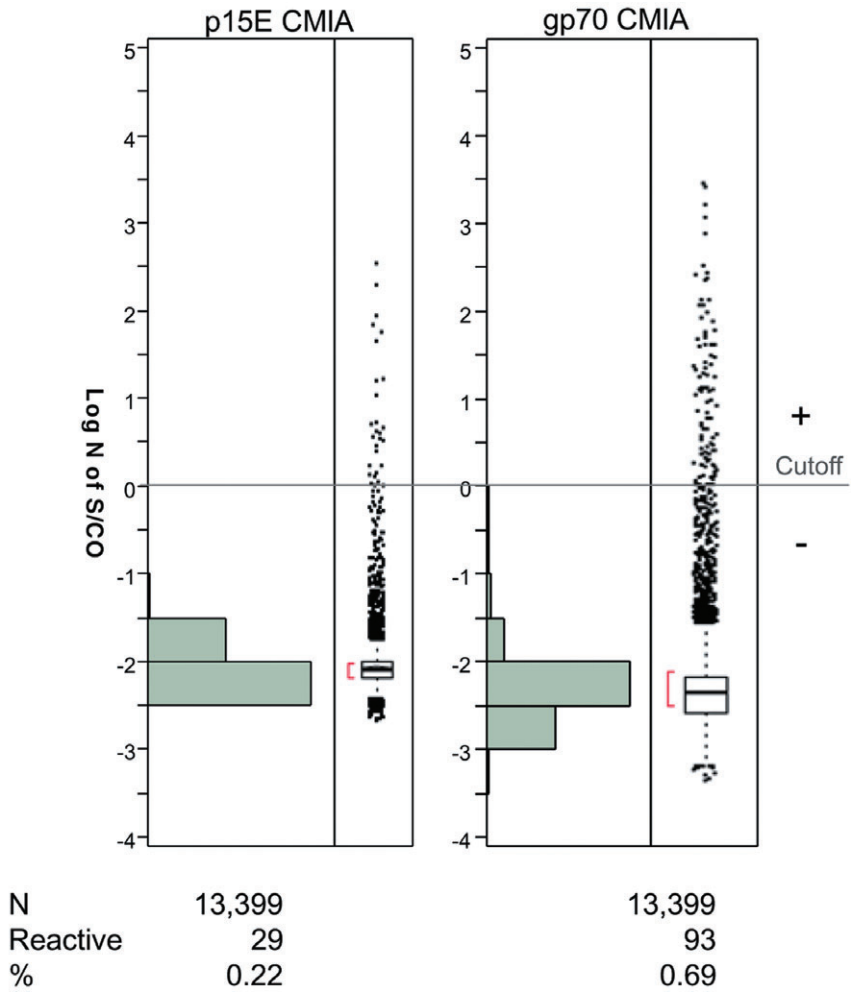

Fig. 1. Signal distributions of p15E CMIA and gp70 CMIA on 13,399 US blood donors from six US regions. The *X* axis shows the sample frequency expressed as number of samples/total population. The *Y* axis represents the CMIA signal expressed in units of natural log-transformed signal ratio of sample to the cutoff (Log N S/CO); values greater than 0 are considered positive. The number (N) of donors tested, number reactive samples, and percentage (%) of reactive samples are summarized beneath each figure.

reactivity to p15E and 20 (0.53%) had antibody reactivity to gp70; no sample had reactivity to both antigens. The distribution of natural log S/CO values is shown in Fig. 2 and a listing of samples with isolated antibody reactivity is shown in Table 4. Among the 25 reactive donor samples, one gp70 antibody-reactive sample was also reactive to p30 (S/CO 4.76 and 4.73, respectively). Thus, no donor sample was confirmed as XMRV and/or MLV antibody positive. The S/CO values for the 20 gp70-reactive samples ranged from 1.04 to 49.11 with the corresponding nonreactive results ranging from 0.11 to 0.17 for p15E and from 0.17 to 0.30 for p30. The S/CO values for the five p15E-reactive samples ranged from 1.09 to 12.47 with corresponding nonreactive results of 0.06 for gp70 and a range of 0.18 to 0.22 for p30. RNA testing was not performed on the donor samples as their storage conditions precluded such testing. These samples represented more than 96% of blood components transfused to the recipient population studied.

Recipients

Among the 830 repository samples representing 109 individual highly transfused recipients, one sample had p15E antibody reactivity and 20 samples, representing two recipients, had reactivity to gp70. No recipient sample had reactivity to both antigens or to p30 (Table 5). Recipient 1 was a 44-year-old male with beta thalassemia: his first sample, taken on September 29, 2004, had a weak isolated antibody signal to gp70 (S/CO, 1.53). Subsequently, he received a total of 73 red blood cell (RBC) and six fresh-frozen plasma units through the period ending May 23, 2006. Among the 21 additional samples tested during the period of transfusion, 18 had weak isolated reactivity to gp70 (S/CO ranged from 1.06-1.64). The remaining three samples were gp70 nonreactive with reactivity just under the assay cutoff (S/CO range, 0.93-0.99); antibodies to p15E and p30 were nonreactive for all samples (S/CO ranges of 0.13-0.16 and 0.19-0.26, respectively). This same recipient had tested immunoglobulin (Ig)G weakly reactive to three of four markers for which the repository samples had previously been tested (parvovirus B19, cytomegalovirus, and *Chlamydia pneumoniae*). Recipient 2 was a 63-year-old male with coronary artery disease; he had weak isolated p15E reactivity (S/CO, 1.64) in a pretransfusion sample with nonreactive results for both gp70 and p30 (S/CO, 0.08 and 0.32, respectively); no follow-up samples were available for further evaluation. Recipient 2 was also IgG weakly reactive for parvovirus B19 and *C. pneumoniae*. Recipient 3 was an 18-year-old female with sickle cell disease. Her initial sample, on April 28, 2005, was nonreactive for p15E and gp70 (S/CO, 0.18 and 0.10, respectively). She received a total of 41 RBC units in the period up to April 19, 2007. Among seven follow-up samples tested, one had weak isolated reactivity to gp70 (S/CO, 1.12) with nonreactive results for p15E and p30 (S/CO, 0.17 and 0.23, respectively); all six subsequent samples were gp70 nonreactive (S/CO range, 0.06-0.08). Recipient 3 was also IgG weakly reactive for parvovirus

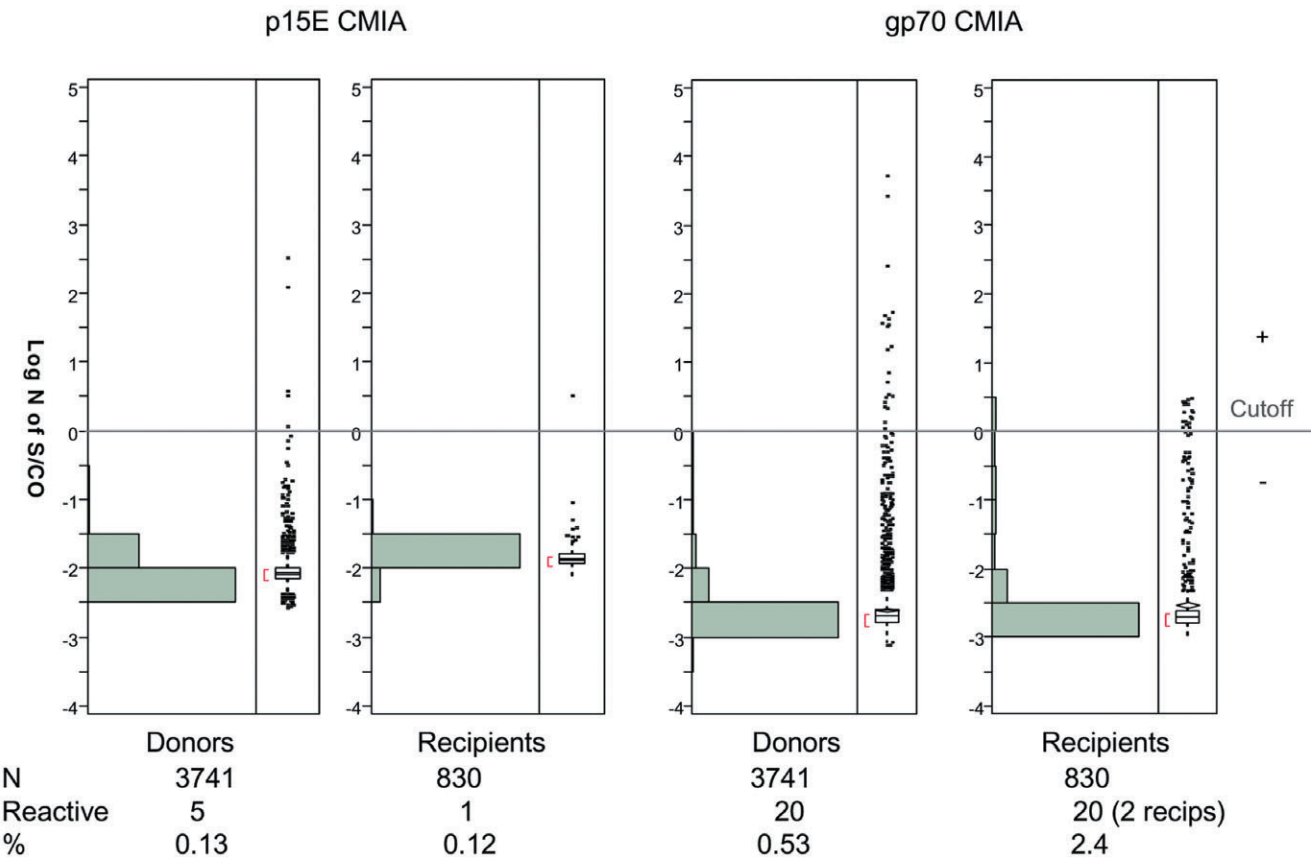

Fig. 2. Signal distributions of p15E CMIA and gp70 CMIA on 3741 US blood donations and 830 recipient samples from a well-characterized donor-recipient repository. The *X* axis shows the sample frequency expressed as number of samples/total population. The *Y* axis represents the CMIA signal expressed in units of natural log-transformed signal ratio of sample to the cutoff (Log N S/CO); values greater than 0 are considered positive. The number (N) of donors and recipients (recips) tested, number of reactive samples, and percentage (%) of reactive samples are summarized beneath each figure.

B19, cytomegalovirus (CMV), *C. pneumoniae*, and *Babesia microti* (*B. microti* unpublished observation). None of the three recipients with isolated antibody reactivity had received blood products from the 25 donors described as having detectable but unconfirmed XMRV and/or MLV antibody reactivity. All 830 recipient samples were nonreactive for XMRV RNA (S/CO, 0.00-0.59). A listing of all observed antibody reactivity and associated RNA-nonreactive results for tested samples from the three described recipients is shown in Table 5.

In summary, 17,249 antibody-tested blood donors and recipients (13,399 + 3741 + 109) and 1763 RNA-tested blood donors and recipients (1435 + 97 + 122 + 109) showed no evidence of XMRV infection, for an antibody prevalence of 0% (95% CI, 0%-0.017%) and RNA prevalence of 0% (95% CI, 0%-0.17%) as shown in Table 6.

DISCUSSION

In this study, we used well-validated, automated tests to evaluate the prevalence of XMRV and/or MLV antibody and XMRV RNA in a large number of blood donors. Using interpretive criteria already established for the antibody tests, we have not found any evidence of confirmed XMRV and/or MLV antibodies among 17,140 (13,399 + 3741) blood donors, implying an upper 95% confidence bound of less than 0.02% prevalence. Furthermore, we have been unable to demonstrate any detectable XMRV RNA in 1435 routine blood donation samples, in any sample with isolated antibody reactivity (122), in donation samples that are positive for HTLV antibodies (97), or from 830 samples collected from 109 highly transfused recipients.

These negative findings among blood donors make the question of transmissibility of XMRV by transfusion somewhat academic. Nevertheless, we have examined an existing donor-recipient repository for evidence of such transmission. We did find a low frequency of antibodies to individual XMRV and/or MLV recombinant antigens among both donors and recipients; however, antibody positivity could not be confirmed by the presence of all three antibody markers and no recipient tested RNA positive. Furthermore, donations with reactive, unconfirmed

TABLE 4. Sample results from antibody-reactive blood donors from the donor-recipient repository*

Donor sample	p15E	gp70	p30
1	0.16	**1.47**	0.30
2	0.17	**5.14**	0.17
3	0.13	**4.74**	4.73
4	0.14	**1.42**	0.19
5	0.13	**11.19**	0.17
6	0.12	**1.76**	0.17
7	0.12	**3.51**	0.22
8	0.13	**5.38**	0.20
9	0.12	**1.83**	0.18
10	0.13	**1.51**	0.18
11	0.12	**1.44**	0.22
12	0.13	**2.20**	0.19
13	0.15	**31.96**	0.19
14	0.12	**6.36**	0.20
15	0.13	**49.11**	0.22
16	0.11	**1.04**	0.23
17	0.11	**4.94**	0.22
18	0.12	**2.18**	0.23
19	0.11	**4.48**	0.18
20	0.12	**3.76**	0.24
1	**2.04**	0.06	0.22
2	**1.09**	0.06	0.18
3	**12.47**	0.06	0.21
4	**1.68**	0.06	0.18
5	**9.30**	0.06	0.20

* Reactive results expressed as means of duplicate retests; n = 25 of 3741 tested.
Bolded values are greater than or equal to 1.0 and represent reactive results.

TABLE 5. Sample results from antibody-reactive recipients from the donor-recipient repository*

Subject	Date of collection	p15E	gp70	p30	RNA
Recipient 1					
1	September 29, 2004	0.15	**1.53**	0.26	0.01
2	October 27, 2004	0.16	**1.54**	0.22	0.05
3	November 17, 2004	0.14	**1.64**	0.20	0.08
4	December 6, 2004	0.15	**1.36**	0.22	0.06
5	January 10, 2005	0.15	**1.34**	0.22	0.05
6	January 26, 2005	0.16	**1.06**	0.21	0.04
7	February 25, 2005	0.14	**1.29**	0.22	0.04
8	March 18, 2005	0.14	**1.49**	0.23	0.05
9	April 25, 2005	0.14	**1.45**	0.20	0.20
10	June 6, 2005	0.14	**1.24**	0.26	0.06
11	June 27, 2005	0.14	**1.28**	0.24	0.41
12	July 18, 2005	0.13	**1.20**	0.24	0.09
13	August 8, 2005	0.14	0.97		0.00
14	August 29, 2005	0.15	**1.10**	0.22	0.13
15	October 10, 2005	0.13	0.99		0.01
16	November 7, 2005	0.13	0.93		0.59
17	November 28, 2005	0.14	**1.19**	0.22	0.00
18	January 11, 2006	0.14	**1.18**	0.24	0.00
19	February 6, 2006	0.13	**1.44**	0.19	0.02
20	March 6, 2006	0.15	**1.16**	0.22	0.04
21	March 27, 2006	0.15	**1.14**	0.20	0.00
22	April 17, 2006	0.15	**1.12**	0.22	0.05
Recipient 2					
1	October 22, 2004	**1.64**	0.08	0.32	0.08
Recipient 3					
1	April 28, 2005	0.18	0.10		0.00
2	November 30, 2005	0.17	**1.12**	0.23	0.10
3	March 2, 2006	0.16	0.06		0.03
4	October 4, 2006	0.21	0.07		0.00
5	November 22, 2006	0.21	0.07		0.05
6	February 20, 2007	0.19	0.08		0.04
7	March 21, 2007	0.27	0.08		0.05
8	April 19, 2007	0.35	0.08		0.03

* Reactive results expressed as means of duplicate tests; n = 21 of 830 tested.
Bolded values are greater than or equal to 1.0 and represent reactive results.

antibody markers were not associated with the three recipients that demonstrated such markers; no isolated antibody-reactive donation was associated with a reactive recipient. Therefore, we conclude that, whatever the origin of the isolated and unconfirmed antibody reactivity that we observed, these were not attributable to transfusion-transmitted XMRV and/or MLV. The presence of isolated IgG antibodies to either or all agents previously investigated in these studied recipients (i.e., parvovirus B19, CMV, *C. pneumoniae*, and *B. microti*) indicates either specific immune responses to these agents (in some case attributable to passive transfer) or just as likely, nonspecific reactivity attributable to the fact that these recipients were highly transfused.

Other studies have reported the absence of reliable detection of markers of XMRV and/or MLV among small numbers of blood donors (236 human immunodeficiency virus–infected blood donors in Africa by polymerase chain reaction [PCR] and 391 routine blood donors in China by PCR and culture),[19,20] but our study is the first to examine a large and geographically diverse population of healthy, routine US blood donors for evidence of active XMRV and/or MLV infection. Although the SRWG group concluded that their study indicated "that routine blood donor screening for XMRV/P-MLV is not warranted at this time," this conclusion was based on the inability of the evaluated tests to reliably detect the presence of XMRV markers among samples drawn from only 15 subjects (14 CFS patients and one relative of a patient), previously found positive for XMRV and/or MLV. There were also 15 healthy controls who were blood donors or laboratory staff.[9] It should be noted that the tests used in our studies were also evaluated in the SRWG studies and are well-validated and are sensitive when used on relevant control preparations.[9-12,14,15] We have shown that, in contrast to the studies of Lombardi and colleagues[2] and Lo and colleagues[3] the measured prevalence of markers of infection among healthy donors is zero and that the upper 95% CI for XMRV-related antibody and RNA is, respectively, 0.017 and 0.17%. Further, direct observation of viral markers among blood donors and the recipients of their blood showed no evidence of any transmission of XMRV-related viruses. We recognize that studies in rhesus macaques showed that markers of XMRV in the blood of inoculated animals were transient and weak,[11] suggesting that these

TABLE 6. Blood donor and recipient studies for XMRV-related antibodies and RNA

Population	Test: number reactive/total tested or reactive subset tested						Number confirmed positive
	Anti-p15E	Anti-gp70	Anti-p30*	Anti-p15E and p30	Anti-gp70 and p30	RNA	
Random blood donors, n = 1,435 (TMA validation)	ND	ND	ND	ND	ND	0	0
HTLV-positive blood donors, n = 97 (TMA validation)	ND	ND	ND	ND	ND	0	0
Random blood donors, n = 13,399	29	93	2/122	1/29	1/93	0/122	0
Repository blood donors, n = 3,741	5	20	1/25	0/5	1/20	ND	0
Repository blood recipients (samples), n = 830†	1	20	0/21	0/1	0/20	0/830	0
Repository blood recipients (patients), n = 109	1	2	0/3	0/1	0/2	0/109	0
Total (%) individuals antibody tested, n = 17,249 (13,399 + 3,741 + 109)	35 (0.20)	115 (0.67)	3/150 (2.00)	1/35 (2.86)	2/115 (1.72)		0
Total individuals RNA tested, n = 1,763 (1,435 + 97 + 122 + 109)	ND	ND	ND	ND	ND	0	0

* Anti-p30 testing was performed on anti-p15E– or anti-gp70–reactive samples.
† Results not part of total.
ND = not done.

markers would be more likely to occur after recent infection. Thus, a limitation of our study is that detection of XMRV markers might be expected to be infrequent. Nevertheless, we feel that it is important to contrast our findings with the high prevalence rates among controls that were previously reported by Lombardi and coworkers[2] and Lo and coworkers.[4]

The lessons learned from the 2-year exercise since the threat of XMRV to blood safety was initially raised[3] warrant mention. The United States and the world monitored the scientific literature closely, mobilized technical, regulatory, and policy groups to identify gaps and investigate immediate actions to protect donor and recipient safety including the ongoing exclusion of donors with a medical diagnosis of CFS. Through extensive efforts, the scientific process was invoked and successful in that the threat of XMRV was given a priority status and fully investigated and through the generation of data using targeted study populations can now be concluded to pose no current threat. The models for investigating emerging infectious diseases that challenge transfusion safety will by necessity differ depending on the specific agent, its epidemiology, and a host of other factors. The findings of our study, coupled with the prior studies that did not confirm any association of XMRV and/or MLV with human disease, indicate that these viruses do not currently pose a threat to blood recipients or to public health.[9,10,12,21] Thus, we conclude that no further action relating to XMRV and/or MLV and blood safety is necessary.

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CONFLICT OF INTEREST

RYD, KD, YW, SZ, DEK, and SLS have no conflicts. JH, XQ, PS, and GS are employees and shareholders of Abbott Laboratories. JML, KG, and JMC are employees of Gen-Probe, Inc.

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Seroprevalence of xenotropic murine leukemia virus–related virus in normal and retrovirus-infected blood donors

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BACKGROUND: Xenotropic murine leukemia virus–related virus (XMRV) has been reported in patients with prostate cancer and chronic fatigue syndrome. Although results have been conflicting, the potential of XMRV as an infectious human retrovirus has raised concerns about transfusion safety. To address this issue, normal and retrovirus-infected blood donors were screened for evidence of XMRV infection.

STUDY DESIGN AND METHODS: Plasma from 1000 US, 100 human immunodeficiency virus Type 1–infected Cameroonian, and 642 human T-lymphotropic virus Type I (HTLV-I)-infected or uninfected Japanese blood donors as well as 311 sexually transmitted disease diagnostic specimens were screened for antibodies to XMRV gp70 and p15E using chemiluminescent immunoassays (CMIAs). CMIA-reactive samples were evaluated by p30 CMIA, Western blot, and real-time reverse transcriptase polymerase chain reaction.

RESULTS: XMRV seroreactivity was low (0%-0.6%) with the exception of the HTLV-I–infected donors (4.9%). Antibody was detected against only a single XMRV protein (p15E or gp70); none of the seroreactive samples had detectable XMRV *pol* or *env* sequences. The elevated seroreactivity in HTLV-I–infected donors was due to an increased p15E seroreactive rate (4.1%). Inspection of XMRV and HTLV sequences revealed a high level of conservation within the immunodominant region (IDR) of the transmembrane protein. In some cases, HTLV IDR peptide competitively reduced the XMRV p15E signal.

CONCLUSIONS: Based on the low prevalence of seroreactivity, detection of antibody to only a single XMRV protein and the absence of XMRV sequences, this study finds no compelling evidence of XMRV in normal or retrovirus-infected blood donors. The increased p15E seroreactivity observed in HTLV infection is likely due to cross-reactive antibodies.

Xenotropic murine leukemia virus–related virus (XMRV), a novel gammaretrovirus, was originally identified in 2006 in human prostate tumor tissues using Virochip DNA microarray technology.[1] Subsequent studies using quantitative polymerase chain reaction (PCR) and immunohistochemistry confirmed the presence of XMRV: viral nucleic acid or proteins were detected in 6% to 23% of prostate tissue obtained from 233 prostate cancer patients and in 2% to 4% of 101 benign controls.[2] In 2009, XMRV sequences were reported in the blood of 67% (68/101) of patients with chronic fatigue syndrome (CFS) and in 3.6% (8/218) of healthy controls.[3] Although these findings generated tremendous interest in studying the association of XMRV with human diseases, results have been controversial. XMRV has not been consistently detected in prostate cancer samples[4-10] and many studies failed to detect evidence of XMRV in CFS patients from the United States, Europe, China, and Japan.[11-18] Of note, one group reported finding sequences of polytropic murine leukemia virus (MLV), a virus related to but unique from XMRV, in 87% (32/37) of CFS samples and in 7% (3/44) of blood donors.[19]

ABBREVIATIONS: CFS = chronic fatigue syndrome; CMIA(s) = chemiluminescent immunoassay(s); CO = cutoff; HPR = hydroxypyruvate reductase; IC(s) = internal control(s); IDR = immunodominant region; MLV = murine leukemia virus; NC = negative control; PC(s) = positive control(s); RLU = relative light units; STD(s) = sexually transmitted disease(s); TM = transmembrane; WB = Western blot; XMRV = xenotropic murine leukemia virus–related virus.

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TRANSFUSION **;**:**-**.

Although the etiologic role of XMRV in human disease remains unclear, the virus is highly infectious. In vitro, XMRV has been shown to infect a wide range of cell types and host species, including human hematopoietic cell lines, prostrate stromal fibroblasts, and peripheral blood mononuclear cell (PBMNC) cultures.[16,20,21] Moreover, XMRV reportedly can be transmitted from activated lymphocytes and cell-free plasma of PCR-positive individuals and replicates efficiently in prostate carcinoma cell lines.[3,22] In vivo, animal models of XMRV infection in rhesus macaque and wild-derived mouse (*Mus pahari*) showed that XMRV infects lymphoid cells and is widely disseminated to other tissues.[23,24] These results suggest that XMRV has the capacity to infect humans and may be a transfusion-transmissible infectious agent posing a potential risk to public health and blood transfusion safety.

In this study, plasma from normal blood donors and individuals with retrovirus infections (human immunodeficiency virus [HIV] or human T-lymphotropic virus [HTLV]) as well as diagnostic samples from individuals tested for sexually transmitted diseases (STDs) were screened for the presence of XMRV or other MLV antibodies using two prototype chemiluminescent immunoassays (CMIAs). The CMIAs utilize recombinant XMRV antigens for capture and detection of antibodies to p15E (transmembrane [TM]) or gp70 (glycosylated envelope) on the automated ARCHITECT instrument system.[25] CMIA-reactive samples were further evaluated by a third CMIA for antibodies to p30 (capsid protein), Western blot (WB), and real-time reverse transcriptase PCR (RT-PCR) for XMRV *pol* and *env* sequences (N. Tang, A. Frank, G. Leckie, J. Hackett Jr, G. Simmons, M. Busch, K. Abravaya, manuscript in preparation).

MATERIALS AND METHODS

Study population

Plasma samples from 1000 random blood donors were obtained from the Gulf Coast Regional Blood Center (Houston, TX). All samples were nonreactive for bloodborne infectious diseases on donor screening tests including hepatitis B surface antigen, hepatitis C virus antibody (anti-HCV), antibody to hepatitis B core antigen, anti-HIV-1/HIV-2, HIV-1 nucleic acid testing (NAT), HCV NAT, anti-HTLV-I/II, syphilis, West Nile virus, and Chagas.

Plasma specimens from 311 individuals undergoing testing for STDs were provided by Dr M. Pandori of the San Francisco Department of Public Health (San Francisco, CA). Plasma specimens from 100 HIV-1–seropositive Cameroonian blood donors collected during 2007 in accordance with local country regulations were provided by Drs L. Kaptué (Université des Montagnes, Bangangté, Cameroon) and L. Gürtler (Max von Pettenkofer Institut-Virologie Ludwig-Maximilian University, Munich, Germany). Plasma specimens from 486 HTLV-I–infected and 156 HTLV-uninfected Japanese blood donors collected in 1988 were provided by Dr S. Hino (Nagasaki University, Nagasaki, Japan).

XMRV CMIAs

Specimens were screened with two prototype ARCHITECT CMIAs (Abbott Diagnostics, Abbott Park, IL) that utilize a direct assay format in which *Escherichia coli*–expressed XMRV p15E or mammalian-expressed XMRV gp70 are used as both capture and detection antigens.[25] Assay positive controls (PCs) were derived from XMRV-infected rhesus macaque plasma at 1:1000 (PC1) or 1:4000 (PC2). A pool of normal human plasma was used as negative control (NC). Cutoff (CO) values of the ARCHITECT CMIAs were calculated based on the following formulas: CO = 0.45 × (PC2 mean relative light units [RLU]) for p15E CMIA and CO = 0.078 × (PC1 mean RLU) for gp70 CMIA. Assay results were reported as the ratio of the sample RLU to the CO RLU (S/CO) for each specimen. Specimens with S/CO values of less than 1.00 were considered nonreactive; specimens with S/CO values of 1.00 or more were considered initially reactive. Neat plasma samples (100 μL) were first screened by both p15E and gp70 CMIAs. Initially reactive specimens were retested in duplicate by either ARCHITECT p15E or gp70 CMIAs. Repeatedly reactive specimens were further analyzed by the ARCHITECT p30 CMIA, by investigational WB assays, and by real-time RT-PCR for XMRV *pol* and *env* sequences.

The ARCHITECT p30 CMIA also utilizes the direct assay format with *E. coli*-expressed XMRV p30 (capsid protein) to capture and detect anti-p30.[25] The same sample volume (100 μL) and controls were used for the p30 CMIA. The p30 CMIA CO was calculated based on the formula CO = 0.27 × (PC1 mean RLU).

Competitive inhibition of p15E-reactive HTLV samples was performed by incubation of the samples with HTLV gp21 peptides (100 μg/mL) at room temperature for 30 minutes to block specific antibodies. The preabsorbed samples along with unabsorbed samples were subsequently tested in duplicate by p15E CMIA as described above. The HTLV gp21 peptides were synthesized by Genscript USA, Inc. (Piscataway, NJ).

WB analysis

WB analysis using purified XMRV viral lysate or recombinant gp70 protein was performed as described.[25] Briefly, viral lysate (65 μg/gel) or recombinant gp70 protein (25 μg/gel) was separated by electrophoresis on a 4%-12% NuPAGE Bis-Tris two-dimension gel (Invitrogen, Carlsbad, CA) in the presence of sodium dodecyl sulfate. The protein bands on the gel were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen). After blocking, the PVDF membrane was cut into 2-mm strips. Strips were incubated overnight at 2 to

8°C with human samples diluted 1:100 or XMRV-infected macaque plasma diluted 1:200. After removal of unbound antibodies, strips were incubated with alkaline phosphatase–conjugated goat anti-human immunoglobulin G (Southern Biotech, Birmingham, AL) for 30 minutes at room temperature. The strips were washed and chromogenic substrate solution was added to visualize the reactive bands.

Real-time XMRV reverse transcriptase-PCR assays

Two high-throughput real-time reverse transcriptase polymerase chain reaction (RT-PCR) prototype assays were performed on the automated *m*2000 system (Abbott Molecular, Inc., Des Plaines, IL) as described (N. Tang, A. Frank, G. Leckie, J. Hackett Jr, G. Simmons, M. Busch, K. Abravaya, manuscript in preparation). Briefly, 25 µL of RNA extracted from 0.4 mL of sample was amplified with two primer sets designed to target *pol* or *env* regions of the XMRV genome. Tris ethylenediaminetetraacetic acid buffer containing 1.5 µg/mL poly(dA : dT) was used as assay NC. XMRV VP62 DNA plasmid diluted in the NC was used as assay PC. 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 55°C for 30 minutes and denaturation at 95°C for 1 minute followed by 55 cycles of 93°C for 15 seconds and 60°C for 60 seconds. Fluorescence signals from the amplified XMRV and pumpkin-HPR sequences were simultaneously measured during the 60°C incubation step at each of the 55 cycles. Amplification cycle threshold was set at 0.1 for XMRV and 0.05 for pumpkin-HPR IC. Using serial dilutions of the XMRV VP62 PC, assays for *pol* or *env* target regions could reliably detect five or more copies of DNA per reaction. Performance of sensitivity and specificity was also assessed by testing two blinded panels containing XMRV proviral DNA from cell line 22Rv1 or viral RNA prepared by the XMRV Scientific Research Working Group.[26] The limit of detection for both assays was at least 0.5 XMRV-containing 22Rv1 cells/mL for the whole blood panel and at least 80 XMRV copies/mL for the plasma panel (N. Tang, A. Frank, G. Leckie, J. Hackett Jr, G. Simmons, M. Busch, K. Abravaya, manuscript in preparation).

Amino acid sequence homology analysis

Amino acid sequences of TM proteins from XMRV (isolate VP62 Accession Number Q27ID8), HTLV-I (strain Japan MT-2 subtype A Accession Number P23064), and HTLV-II (Accession Number P03383) were obtained from the web site of UniProt (http://www.Uniprot.org). Multiple sequence alignments were performed using ClustalW program available on the UniProt Web site.

Statistical analysis

Two-sided Fisher's exact test was used to determine significance of seroreactivity for each population. A p value of less than 0.05 was considered to be significant.

RESULTS

XMRV seroprevalence in normal blood donors

Normal blood donations prescreened as negative for known blood-borne infectious agents were selected to assess XMRV prevalence in a low-risk population using the ARCHITECT p15E and gp70 CMIAs. Of 1000 plasma samples tested, only one sample was repeatedly reactive in the p15E CMIA (Fig. 1A). In the gp70 CMIA, seven samples were repeatedly reactive (Fig. 1A). However, no samples were reactive against both p15E and gp70. Subsequent testing with p30 CMIA showed no detectable anti-p30 (Fig. 1A). Further analysis by viral lysate WB showed that no samples were reactive to native p15E protein including the p15E CMIA-positive Sample 12 (Fig. 1B). However, three of the seven gp70 CMIA-positive samples were reactive by recombinant gp70 WB (Figs. 1A and 1B). Thus, based on combined CMIA and WB reactivity, XMRV gp70 seroreactivity was 0.3% in the normal US blood donor population (Table 1).

In an attempt to confirm the observed seroreactivity, the eight CMIA-reactive samples were subject to analysis by two prototype real-time RT-PCR XMRV assays (N. Tang, A. Frank, G. Leckie, J. Hackett Jr, G. Simmons, M. Busch, K. Abravaya, manuscript in preparation). As shown in Fig. 2, although XMRV *pol* and *env* sequences were readily amplified from control XMRV VP62 plasmid DNA (Fig. 2A), after 55 cycles of amplification none of the eight seroreactive blood donors had detectable XMRV (Fig. 2B). The pumpkin-HPR ICs were amplified as expected (Fig. 2C), confirming the integrity of RNA extraction and absence of sample inhibition. Thus, the RT-PCR testing did not confirm XMRV infection in these samples.

XMRV seroprevalence in potential risk populations

To assess XMRV seroprevalence in the presumed risk populations, plasma samples from blood donors with retroviral infections (HIV or HTLV) or individuals undergoing diagnostic testing for STD infection were screened by both p15E and gp70 CMIAs. None of the 100 HIV-1–infected blood donors from Cameroon were reactive in either assay (Fig. 3A). Among the 311 STD diagnostic samples, two samples had detectable p15E antibody (S/CO = 1.7 and 2.7 or Log N S/CO = 0.53 and 0.99, Fig. 3B) but no visible p15E band on the viral lysate WB (data not shown). Two additional samples had detectable gp70 antibody by CMIA (S/CO = 2.4 and 4.4 or Log N S/CO = 0.89 and 1.5, Fig. 3B) and by recombinant gp70 WB, resulting in 0.6%

A

Blood Donor ID	CMIA (S/CO) S/CO≥1: Positive			WB
	p15E	gp70	p30	p15E/gp70
12	**3.3**	0.07	0.19	-
58	0.16	**4.3**	0.22	gp70+
200	0.16	**5.7**	0.21	gp70+
334	0.13	**4.7**	0.20	-
462	0.14	**21**	0.23	-
721	0.14	**8.1**	0.21	gp70+
831	0.13	**1.8**	0.23	-
873	0.14	**1.2**	0.28	-

B

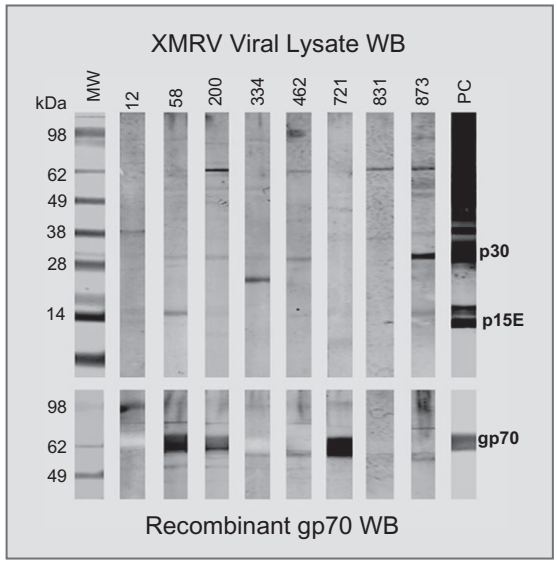

Fig. 1. Serologic characterization of XMRV CMIA-reactive blood donors. (A) Summary of XMRV antibody reactivity by CMIA and WB. Note that the CMIA-positive results (S/CO ≥ 1) represent the mean of three test results, whereas the negative results (S/CO < 1) are the initial testing values. (B) WB analysis of CMIA-reactive samples with XMRV viral lysate proteins and mammalian expressed recombinant gp70 antigen. XMRV-infected macaque plasma was used as the PC. Molecular weight (MW) markers are shown in kilodaltons (kDa).

TABLE 1. XMRV seroreactivity in selected populations*

Population	No	CMIA p15E antibody positive	CMIA gp70 antibody positive	WB p15E antibody positive	WB gp70 antibody positive	Total seroreactive†
US blood donors	1000	1 (0.1)	7 (0.7)	0 (0)	3 (0.3)	3 (0.3)
US STD diagnostic	311	2 (0.6)	2 (0.6)	0 (0)	2 (0.6)	2 (0.6)
HIV-1–infected Cameroonian blood donors	100	0 (0)	0 (0)	NT	NT	0 (0)
Japanese blood donors						
HTLV-1–infected	486	20 (4.1)	8 (1.6)	20 (4.1)	4 (0.8)	24 (4.9)
HTLV-1–uninfected	156	1 (0.6)	0 (0)	1 (0.6)	NT	1 (0.6)

* Data are reported as number (%).
† The summation of the WB confirmed p15E and gp70 reactives.
NT = not tested.

gp70 seroreactivity in the STD diagnostic population (Table 1). None of the four p15E or gp70 CMIA-reactive samples were reactive in the p30 CMIA. Sample volume limitations precluded testing these samples by RT-PCR.

Of the Japanese blood donor samples, none of 156 HTLV-uninfected subjects had detectable gp70 antibody (Fig. 3C). Eight of 486 HTLV-I–infected donors were repeatedly reactive by gp70 CMIA with S/CO ranging from 1.2-4.7 (Log N S/CO = 0.18-1.5, Fig. 3D); four of these samples were reactive in the recombinant gp70 WB (data not shown) resulting in 0.8% (4/486) gp70 seroreactivity (Table 1). One of 156 HTLV-uninfected blood donors (0.6%) had detectable p15E antibody by CMIA (S/CO = 2.0 or 0.69 Log N S/CO, Fig. 3C) and by XMRV lysate WB (Fig. 4, Lane 1). In contrast, 20 of 486 HTLV-I–infected blood donors were repeatedly reactive by p15E CMIA with S/CO values ranging from 1.1 to 36 (Log N S/CO = 0.1-3.6, Fig. 3D). In fact, eight had S/CO values of more than 10, considerably elevated levels relative to the S/CO values observed for the p15E-reactive samples from US blood donors and HTLV-uninfected and STD diagnostic populations. Viral lysate WB confirmed the p15E-specific reactivity; all 20 of the p15E CMIA-positive HTLV-I–infected samples had clearly visible bands against the native p15E protein (Fig. 4, Lanes 2-21), whereas none of the gp70 CMIA-reactive samples had evidence of specific reactivity against the p15E protein (Fig. 4, Lanes 22-29). Thus, the WB-confirmed p15E seroreactivity among HTLV-I–infected Japanese blood donors was 4.1%, significant ($p = 0.02$) relative to the 0.6% seroreactivity among the

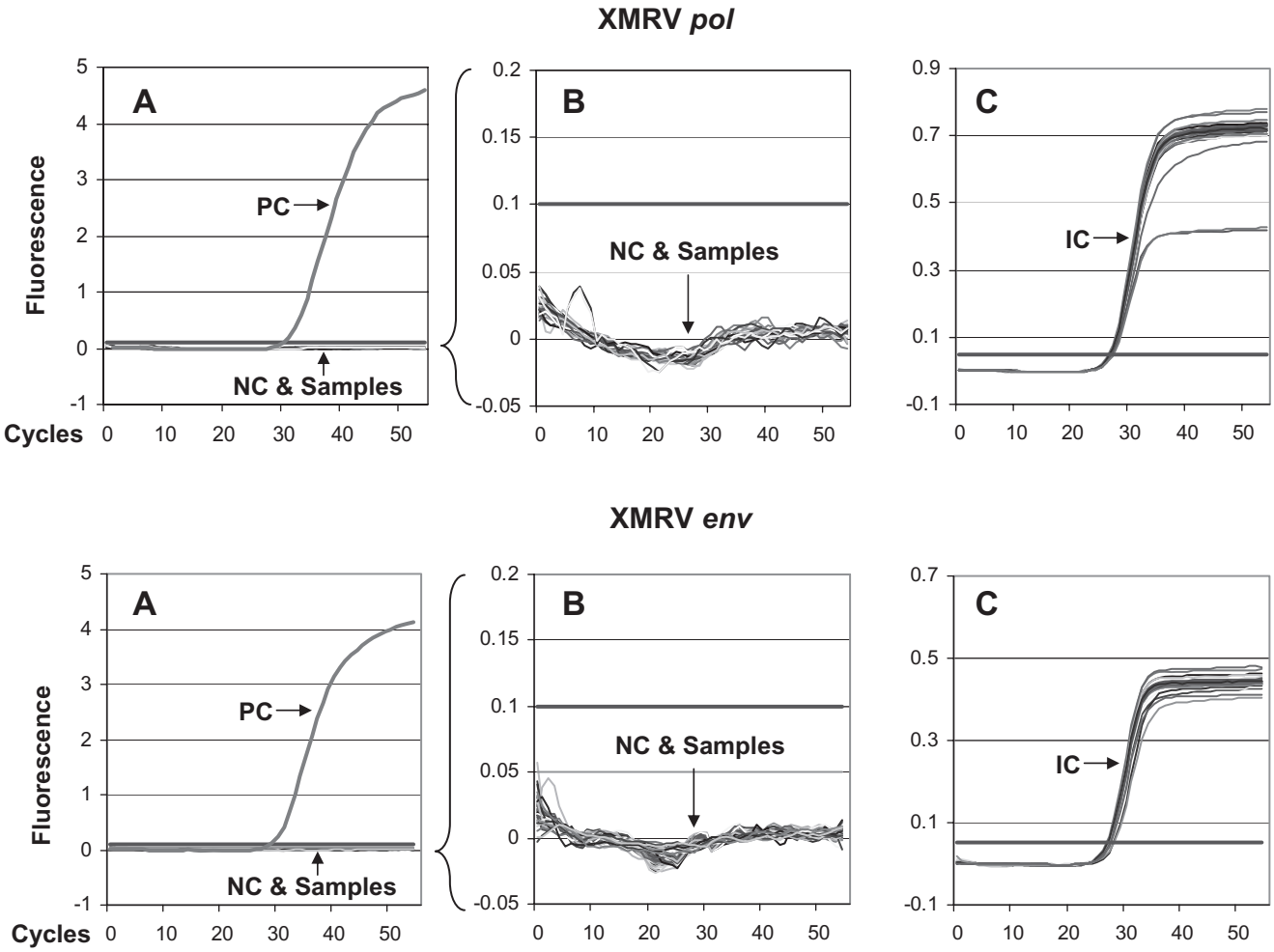

Fig. 2. Real-time RT-PCR evaluation of XMRV CMIA-reactive samples. PCR curves represent amplification of (A) XMRV VP62 plasmid DNA PC, NC of Tris ethylenediaminetetraacetic acid buffer with1.5 μg/mL poly(dA : dT), and 37 CMIA-reactive samples (eight US normal blood donors, 28 HTLV-I–infected, and one uninfected Japanese blood donors) using XMRV *pol* (top) and *env* primers (bottom). (B) Amplification curves of NC and 37 CMIA-reactive samples are shown on an expanded fluorescence signal scale. (C) Amplification curves of the pumpkin-HPR gene IC for each sample and control. The bold horizontal line represents cycle threshold: 0.1 for XMRV and 0.05 for pumpkin-HPR, respectively.

HTLV-uninfected blood donors. There was no significant difference ($p = 0.32$) in the gp70 reactivity between the HTLV-I–infected and -uninfected blood donors. Further analysis with p30 CMIA and RT-PCR showed that neither anti-gp30 nor XMRV sequences (*pol* or *env*) were detected in any of the p15E or gp70 reactive Japanese blood donors (Fig. 2B). The lack of availability of PBMNCs or whole blood precluded further confirmation by PCR.

Characterization of p15E reactivity from HTLV-I–infected blood donors

Based on the p15E seroreactivity in the HTLV-I–infected Japanese blood donors, it was of interest to examine sequence homology between XMRV, HTLV, and HIV across the envelope, the TM, and the capsid proteins. Of interest, the highest homology (40%) was observed between XMRV and HTLV in the TM region. Analysis of sequence alignments revealed a particularly high level of conservation within the immunodominant region (IDR) of HTLV TM gp21 protein (Fig. 5A), suggesting that the increased p15E reactivity may be due to cross-reactive antibody elicited by HTLV infection. To test this hypothesis, two synthetic peptides derived from the IDR region of HTLV gp21 (Fig. 5A) were used to competitively inhibit p15E CMIA reactivity. HTLV Peptide 1, containing all 21 amino acids comprising the gp21 IDR, reduced signals for 5 of 21 p15E CMIA-reactive samples by at least 30%. Notably, four of the five inhibited samples had high signals (>20,000 RLU) in the p15E CMIA (Fig. 5B). In contrast, HTLV Peptide 2, the truncated IDR peptide, exhibited minimal inhibition (Fig. 5B).

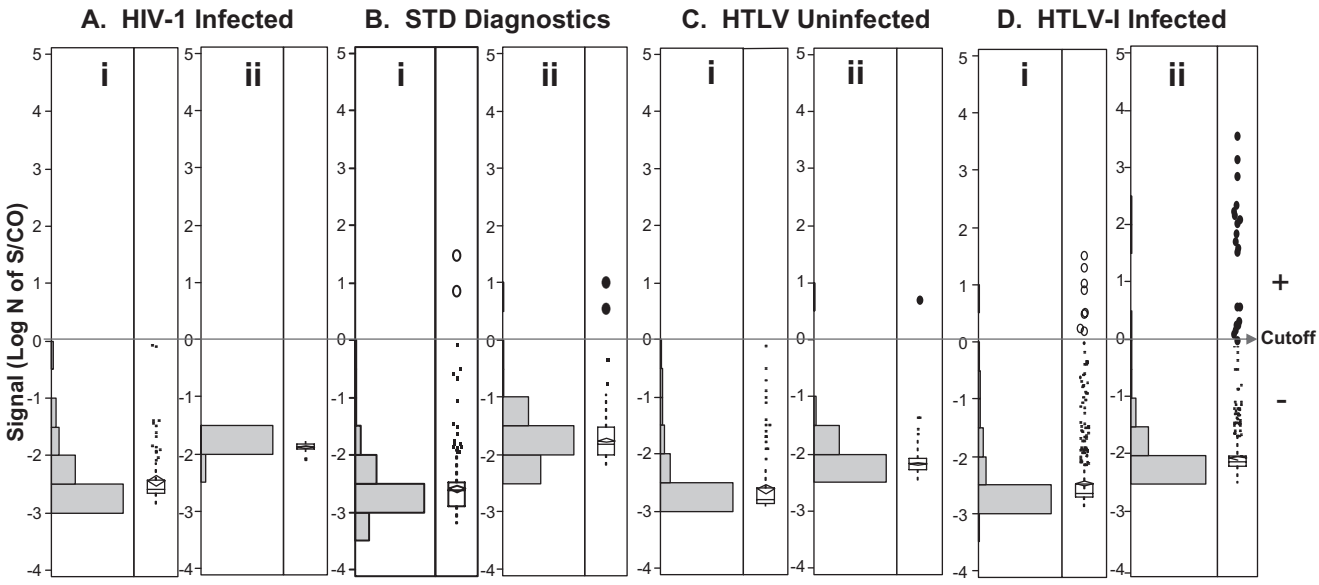

Fig. 3. Detection of XMRV antibodies in retrovirus-infected blood donors by ARCHITECT CMIAs. Signal distributions of gp70 CMIA (i) and p15E CMIA (ii) on (A) 100 HIV-1–infected Cameroonian blood donors, (B) 311 STD diagnostic specimens, (C) 156 HTLV-uninfected Japanese blood donors, and (D) 486 HTLV-I–infected Japanese blood donors. The *X* axis shows the sample frequency expressed as number of samples/total population. The *Y* axis represents the CMIA signal expressed in units of natural log-transformed signal ratio of sample to the CO (Log N S/CO); values greater than 0 are considered positive. CMIA-positive samples are highlighted as closed circles for p15E and open circles for gp70.

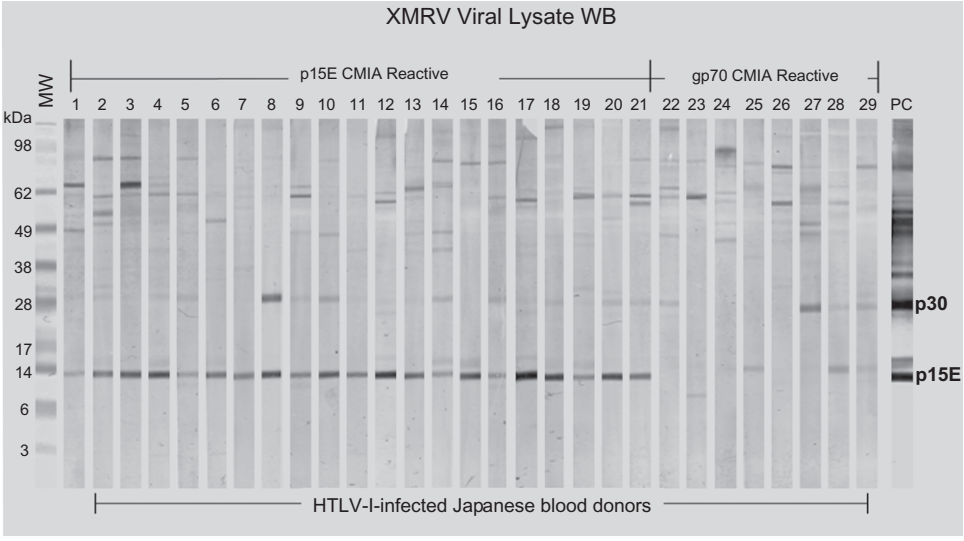

Fig. 4. WB analysis of CMIA-reactive Japanese blood donors. XMRV viral lysate WB was used to analyze 21 p15E and eight gp70 CMIA-reactive Japanese blood donors. With one exception (Lane 1), all of the CMIA-reactive samples are from HTLV-I–infected donors (Lanes 2-29). XMRV-infected macaque plasma was used as the PC. Molecular weight (MW) markers are shown in kilodaltons (kDa).

DISCUSSION

In this study, the prevalence of individuals with antibodies to XMRV or other MLVs was assessed in a low-risk population of blood donors and the presumed risk populations of individuals with retroviral or potential STD infections. Serologic screening using two prototype immunoassays showed that XMRV gp70 seroreactivity among normal blood donors free of blood-borne infectious diseases was 0.3% (3/1000), substantially lower than reports of a 4% to 7% XMRV infection rate among healthy individuals.[2,3,19] Furthermore, with the exception of HTLV-infected indi-

A

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--AVPVAVWLVSALAMG---AGVAGGITGSMSLASGKSLLHEVDKDISQLTQAIVKNHKN 55 ENV_HTLV1
--AVPIAVWLVSALAAG---TGIAGGVTGSLSLASSKSLLLEVDKDISHLTQAIVKNHQN 55 ENV_HTLV2
EPVSLTLALLLGGLTMGGIAAGVGTGTTALVATKQFEQLQAAIHTDLGALEKSVSALEKS 60 ENV_XMRV

      YAAQNRRGLDLLFWEQGGLCK  (HTLV peptide 1)
                  FWEQGGLCKALQEQC  (HTLV peptide 2)
      ************************
LLKIAQYAAQNRRGLDLLFWEQGGLCKALQEQCCFLN----ITNSHVSILQERPPLENRV 111 ENV_HTLV1
ILRVAQYAAQNRRGLDLLFWEQGGLCKAIQEQCCFLN----ISNTHVSVLQERPPLEKRV 111 ENV_HTLV2
LTSLSEVVLQNRRGLDLLFLKEGGLCAALKEECCFYADHTGVVRDSMAKLRERLNQRQKL 120 ENV_XMRV

LTGWGLNWDLGLS---QWAREALQTGITLVALLLLVILAGPCILRQLRHLPSR------- 161 ENV_HTLV1
ITGWGLNWDLGLS---QWAREALQTGITILALLLLVILFGPCILRQIQALPQR------- 161 ENV_HTLV2
FE-SGQGWFEGLFNRSPWFTTLISTIMGPLIVLLLILLFGPCILNRLVQFVKDRISVVQA 179 ENV_XMRV

--VRYPH--YSLINPESSL--- 176 ENV_HTLV1
--LQNRHNQYSLINPETML--- 178 ENV_HTLV2
LVLTQQYHQLKSIDPEEVESRE 201 ENV_XMRV
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B

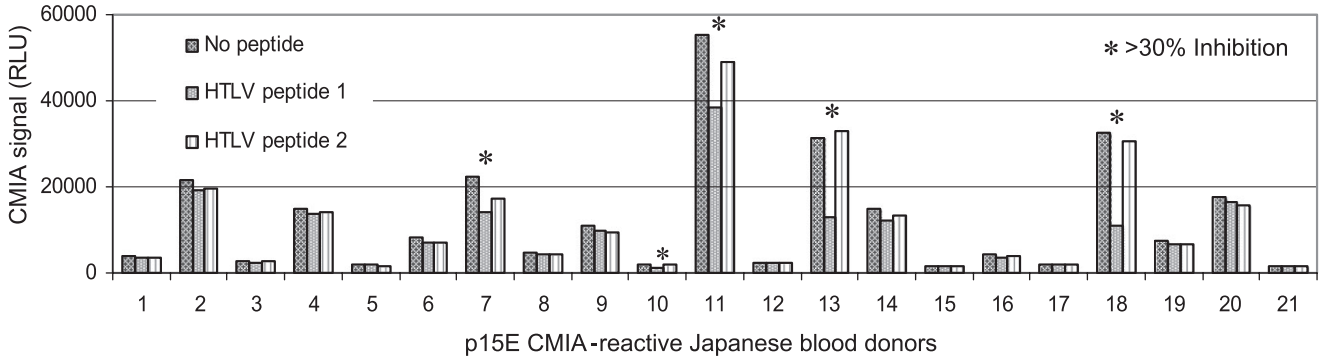

Fig. 5. Sequence homology of TM protein between XMRV and HTLV. (A) Sequence alignment of the amino acid sequences of XMRV p15E (Accession Number Q27ID8), HTLV-I (P23064), and HTLV-II (P03383) gp21. Identical amino acids are highlighted in gray. Box indicates the highly conserved region between XMRV and HTLV. *Amino acids involved in the IDR of HTLV gp21. Two synthetic peptides (HTLV Peptide1 and HTLV Peptide 2) used for inhibition in p15E CMIA are shown above the corresponding sequence. The alignment was generated using ClustalW program available on the UniProt Web site (http://www.Uniprot.org). (B) Inhibition of p15E CMIA reactivity by HTLV IDR peptides on 20 HTLV-I–infected (Lanes 2-21) and one uninfected (Lane 1) Japanese blood donors. Percent inhibition was determined based on signal ratio of preabsorbed samples with HTLV peptides to the unabsorbed samples.

viduals, there was no evidence of an increased XMRV seroreactivity in the potentially at-risk populations such as individuals with HIV or other STDs. Although the route of transmission has not been elucidated, evidence suggests that XMRV may be transmitted similarly to other human retroviruses, HIV and HTLV.[3,16,27] Therefore, if the virus is circulating in the general population, XMRV seroprevalence might be expected to be higher among individuals with HIV or HTLV infections than the normal blood donor population. However, in this study, no XMRV antibodies were detected in the 100 HIV-1–seropositive blood donors from Cameroon. Zero or low levels of seroreactivity have been reported in HIV-1–infected Spanish subjects (0%, 0/149) and US multicenter AIDS cohort patients (0.9%, 3/332)[28] (M. Arredondo, J. Hackett Jr, F. de Bethencourt, A. Treviñ, D. Escudero, A. Collado, X. Qiu, P. Swanson, V. Soriano, C. Carmen de Mendoza, manuscript in preparation). These serologic results are consistent with several other studies that have failed to detect XMRV DNA or RNA in HIV-1–infected patients from Europe, United States, and Africa.[29-33] This study also showed that XMRV seroreactivity among the STD diagnostic samples (0.6%, 2/311) was not significantly different ($p = 0.09$) from the seroreactivity among normal blood donors (0.3%, 3/1000). The apparent increased seroreactive rate among the HTLV-I–infected Japanese blood donors (24/486, 4.9%) is most likely due to cross-reactive antibodies. If the 20 p15E cross-reactive HTLV-infected donors are excluded, there is no significant difference ($p = 0.22$) in the seroreactive rate between the HTLV-I–infected (4/466, 0.8%) and -uninfected (1/156, 0.6%) Japanese blood donors. In summary, this study showed a low

XMRV seroreactivity (0-0.8%) in the normal and retrovirus-infected blood donor populations.

It should be emphasized that the XMRV seroreactivity reported here represents the summation of isolated p15E- and gp70-seroreactive rates since no samples had reactivity against both antigens. None of the p15E or gp70 CMIA-reactive samples were positive by p30 CMIA even though several had weak reactivity at the expected position of p30 on the viral lysate WB (Figs. 1B and 4). Of note, subsequent WB analysis of CMIA-negative normal blood donors showed the presence of a p30 band indicative of nonspecificity or cross-reactivity (data not shown). Others have also observed the nonspecific reactivity against XMRV p30 protein by WB.[17] Although bona fide XMRV antibody–positive human specimens are currently unavailable, recent animal studies showed that XMRV infection elicited a potent humoral immune response in rhesus macaques. Similar to HIV or HTLV infection in humans, the experimentally infected macaques developed XMRV-specific antibodies against all three structural proteins: the envelope protein gp70, TM p15E, and capsid protein p30.[25] The prototype CMIAs used in this study were optimized for sensitivity and validated using seroconversion samples from the animal model; all three CMIAs were capable of detecting p15E-, gp70-, and p30-specific antibodies in the XMRV-infected macaques with high signal (S/CO ranges: 10-82 for p15E, 15-292 for gp70, and 2.5-49 for p30). Notably, both p15E and gp70 CMIAs were able to detect p15E- or gp70-specific antibodies as early as Day 9 postinfection.[25] Thus, the restricted antibody response observed in human samples is unlikely due to limitations of the CMIAs. Of note, these CMIAs can also detect antibodies to other MLVs.[25] Considered in combination with the negative PCR data, the observed weak and restricted seroreactivity against p15E or gp70 proteins is most likely nonspecific or due to cross-reactive antibodies and not elicited by XMRV infection. Consequently, our data provide no compelling serologic evidence of XMRV or other MLVs infection in the normal blood donor population as well as in the selected risk populations. These results are consistent with several recent studies that have raised serious concerns as to whether XMRV is a naturally occurring infection in humans.[18,34-36] However, one limitation of this study is that the molecular confirmation of XMRV infection was restricted to the plasma compartment (cell-free virus) since whole blood or blood cells were not available for analysis.

In summary, with exception of the HTLV-I–infected group, low XMRV seroreactivity (0%-0.6%) was observed in both normal blood donors and potential risk populations. In contrast to antibody responses in the XMRV-infected primates as well as in HIV- or HTLV-I–infected humans, the detected antibody responses were weak and restricted to a single XMRV protein (p15E or gp70). Furthermore, none of the seroreactive samples had detectable XMRV *pol* or *env* sequences. Collectively, these results suggested that the observed antibody responses were probably not elicited by XMRV infection. The apparent increased frequency of seroreactivity for XMRV in the HTLV-I–infected population most likely is due to cross-reactive antibodies elicited by HTLV-I infection. This study finds no compelling serologic evidence of XMRV infection in the general population or in several selected high-risk populations and provides a valuable reminder of the limitation of interpreting serologic reactivity to isolated retroviral proteins.

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CONFLICT OF INTEREST

XQ, PS, NT, GWL, SGD, GS, and JH are employed by and shareholders of Abbott Laboratories.

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文献4

No Detection of XMRV in Blood Samples and Tissue Sections from Prostate Cancer Patients in Northern Europe

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Abstract

Background: We recently published the rare detection of xenotropic murine leukemia virus-related virus (XMRV) (1/105) in prostate cancer (PCA) tissue of patients in Northern Europe by PCR. The controversial discussion about the virus being detected in PCA tissue, blood samples from patients suffering from chronic fatigue syndrome (CFS), as well as from a significant number of healthy controls prompted us to deepen our studies about detection of XMRV infection applying different detection methods (PCR, cocultivation and immunohistochemistry [IHC]).

Methodology/Principal Findings: Peripheral blood mononuclear cells (PBMCs) from 92 PCA and 7 healthy controls were isolated, PHA activated and cocultivated with LNCaP cells for up to 8 weeks. Supernatant of these cells was applied to a reporter cell line, DERSE-iGFP. Furthermore, the PBMCs and cocultivated LNCaP cells were tested for the presence of XMRV by PCR as well as Western Blot analysis. While all PCR amplifications and Western Blot analyses were negative for signs of XMRV infection, DERSE-iGFP cells displayed isolated GFP positive cells in three cases. In all three cases XMRV presence could not be confirmed by PCR technology. In addition, we performed XMRV specific IHC on PCA tissue sections. Whole tissue sections (n = 20), as well as tissue microarrays (TMA) including 50 benign prostate hyperplasia (BPH), 50 low grade and 50 high grade PCA sections and TMAs including breast cancer, colon cancer and normal tissues were stained with two XMRV specific antisera. XMRV protein expression was not detected in any cancer sections included. One BPH tissue displayed XMRV specific protein expression in random isolated basal cells.

Conclusion: We were unable to conclusively detect XMRV in the blood from PCA patients or from healthy controls and there is no conclusive evidence of XMRV protein expression in PCA, breast cancer and colon cancer tissue sections tested by IHC staining.

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Introduction

Currently, the detection of Xenotropic Murine Leukaemia Virus related Retrovirus (XMRV) in human bio specimens is controversially discussed ranging from XMRV being associated with two major human diseases, chronic fatigue syndrome (CFS) [1,2] and prostate cancer (PCA) [3,4] to being a men generated laboratory contaminant due to xenograft passaging through mice [5–18].

In 2006, XMRV has been identified in prostate tissue from patients with familiar prostate cancer (PCA) carrying a homozygous mutation within the RNaseL gene (R462Q) [19]. The association between XMRV and PCA was severely strengthened by studies demonstrating XMRV protein expression as well as the presence of XMRV sequences in up to 26% of all PCA cases [3,4,20]. XMRV protein expression was predominantly seen in malignant epithelium suggesting a more direct role in tumorigenesis. However, there are multiple studies only rarely or completely failing to detect XMRV in prostate cancer samples using PCR or IHC methods [3,4,9,21–26]. We recently detected XMRV at low frequency (1%) in sporadic PCA samples from Northern Europe using PCR amplification methods and RNA isolated from fresh frozen tissue specimens [27]. Expression of XMRV protein as well as the presence of XMRV sequences in up to 26% of all analysed PCA samples was demonstrated in 2009 by applying immunohistochemistry (IHC) of whole mount PCA sections with an anti-XMRV specific antiserum [4,20]. However, a recent report using Rauscher MLV gag antisera which also recognizes XMRV gag protein, did not confirm these findings [24]. The study by Schlaberg et al. prompted us to revisit the prevalence of XMRV in PCA samples by IHC since focal infections seen by IHC might be missed in PCR analysis. In addition, we evaluate the presence of XMRV protein expression in sections of other malignancies as well as normal tissue by IHC. By using the recently published anti-XMRV antiserum [4] as well as an XMRV gag specific antiserum we were unable to detect XMRV gag specific staining of cells in PCA or other cancerous tissue. However, one benign prostate

hyperplasia (BPH) section clearly displayed positive stained cells using anti-XMRV gag k121 serum.

In 2009 XMRV was identified in up to 68% of PBMC (peripheral blood mononuclear cells) samples from patients with chronic fatigue syndrome and 3–4% of the control cohort showed signs of XMRV infection [2]. PCR data were strengthened by cell dependent as well as cell free transmission of the virus from blood samples of CFS patients to indicator cells. However, several subsequent studies by other labs failed to confirm the PCR data and no virus transmission experiments have been reproduced to date [6,9,10,11,13,15,17,18,28,29,30,31]. Recently, blood samples from CFS patients previously reported to contain XMRV sequences were retested, however were identified as XMRV negative by PCR amplification strategies and serology methods [12,32].

Earlier this year, while this study was in progress, several publications addressed the risk of contaminations by traces of mouse DNA (paraffin sections, cell lines or other sources) [7,13,15] and the risk of false positive PCR products by some commercial amplification kits [17,33]. In addition, Hue and colleagues argue that due to the lack of sequence variability of XMRV gene fragments in patient isolates compared to sequence variability identified in a XMRV positive cell line 22Rv1, XMRV might be a laboratory contaminant rather than a true exogenous human virus [11]. A strong indication that XMRV is a virus circulating in the human population is the identification of viral integration sites in the host genome [34]. However, more recent findings demonstrate that two integration sites published earlier are identical to XMRV integration sites in an in vitro infected cell line DU145 [35]. Furthermore, Paprotka and colleagues provide evidence that XMRV derived from two mouse endogenous pre-viruses which underwent retroviral recombination in cell culture thereby suggesting that all XMRV sequences reported to date did most likely originate from this cell culture event [14]. In the presented study we addressed the detection of XMRV and related MLV sequences in peripheral blood cells of prostate cancer patients and healthy controls motivated by the detection of XMRV in blood cells of 3–4% of healthy controls [2] and our hypothesis that XMRV replication could be activated due to immunosuppression accompanying PCA and subsequently detectable in the blood of patients. A total of 100 blood samples were included in our study. PBMCs were isolated, stimulated and subsequently used for genomic DNA isolation or cocultivation experiments following published protocols [1,2]. Furthermore, protein extracts from activated PBMCs were generated and analysed for XMRV protein expression. We show that PBMCs in general can be in vitro infected with XMRV, resulting in 1–2% infected cells which can be easily monitored by PCR or protein expression analyses thereby confirming recently published results [10]. Although viral genomes are highly edited due to Apobec restriction, supernatant from XMRV infected PBMCs efficiently infects a reporter cell line, DERSE-iGFP. This cell line (generated by Vineet N. KewalRamani, National Cancer Institute, Frederick, USA) expresses a GFP reporter which is activated by reverse transcriptase expression. Although the sensitivity of all techniques used in our study is fairly high, no XMRV sequences or XMRV specific protein expression was detected in activated PBMCs. Interestingly, we detected in supernatant from 3/67 activated PBMCs and 2/67 cocultivation experiments of PBMCs with LNCaP cells, RT activity resulting in GFP positive DERSE-iGFP cells, however, we were unable to unambiguously proof that these PBMCs have been infected with XMRV, other sources of RT activity can not be excluded.

Materials and Methods

Ethics statement

The study was approved by the Ethics Committee of the Federal State Hamburg (no. OB-052-04).

Study population and specimen collection. Study population and specimen collection

Blood samples of 92 prostate cancer patients (age 44–77) were collected one day prior radical prostatectomy. Clinical data are summarized in Table 1. Additionally, blood samples from 7 men (age 30–44) without any evidence of PCA were included in the study. All patients gave written informed consent for the scientific use of blood samples; EDTA-blood from patients and healthy controls were processed by density gradient centrifugation using Ficoll (Biocoll, Biochrom L6715). Primary blood mononuclear cells (PBMCs) were separated and cultivated as described below.

Cell lines

The human prostate cancer cell line LNCaP (ATCC #CRL-1740), LNCaP DERSE-iGFP (kindly provided by Vineet N. KewalRamani, National Cancer Institute, Frederick, USA) and the XMRV positive human prostate cancer cell line 22Rv1 (ATCC #CRL-2505) were grown in RPMI 1640 (Gibco) supplemented with 10% FCS, 5% Penicillin/Streptomycin and L-glutamine. Chronically infected LNCaP cells (XMRV) were generated by transfection of proviral XMRV VP62 DNA as published previously [36] and maintained for several weeks. PBMC were isolated from 10 ml EDTA blood and cultured in RPMI 1640 (Gibco) similar to established prostate cancer cell lines but additionally supplemented with PHA (5 µg/ml, Thermo Fisher Scientific) and rhIL-2 (180 IU/ml, R&D Systems).

Table 1. Summary of clinical data.

Patients PBMCs	n (%) 92
Age at surgery	
mean (years)	63
median (years)	63
range (years)	44–77
Gleason	
≤3+3	7 (7.6)
3+4	69 (75)
4+3	14 (15.2)
≥4+4	2 (2.2)
T stage	
pT2a	7 (7.6)
pT2c	57 (62)
pT3a	20 (21.7)
pT3b	8 (8.7)
N staus	
N0	69 (75)
N1	4 (4.3)
Nx	19 (20.7)

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Cocultivation experiments

1ml cell suspension containing 1×10^6–3×10^6 PBMCs activated for 7 days was added to 2×10^5 LNCaP cells maintained in 2ml RPMI containing 8 µg/ml polybrene in 6-well plates. Plates were centrifuged for 30 min at 37°C and 800 × g. PBMCs were removed 24h later. LNCaP cells were cultured for 6–8 weeks. Cells were split when reaching 100% confluence. Supernatants were taken after 6 and 8 weeks and applied to DERSE-iGFP cells (see below).

For positive controls human PBMC were infected with XMRV-containing supernatant from LNCaP XMRV cells. Indicated amount of virus containing supernatant from XMRV producing cells (at least 80% confluence) was sterile filtered and added to 3×10^6 PBMCs pre-activated for two days. Plates were centrifuged for 30 min at 37°C and 800 × g. XMRV containing supernatant was removed the next day by pelleting cells at 200 × g, washing them with 10 ml PBS (Gibco) and disseminating after an additional centrifugation step in a new 6-well plate in 2 ml RPMI containing PHA and rhIL-2. PBMCs were cultivated for 7 days before analyzing supernatant, co-cultivation, nucleic acid and protein extraction.

Infection using replication competent XMRV

XMRV VP62 proviral DNA was transfected into LNCaP cells to produce virus containing supernatant as described earlier [34,36].

PCR

Genomic DNA was extracted from PBMCs using Qiagen QIAamp mini kit and stored at 4°C. Nucleic acid concentrations were determined using a Nanodrop (Peqlab). Different nested PCRs targeting gag and env sequences were performed as recently published [1,3,19], using 650 ng template DNA per reaction. Gag outside primer: 419F 5′- ATCAGTTAACCTACCCGAGTCGGAC-3′, 1154R 5′-GCCGCCTCTTCTTCATTGTTCTC-3′; inside primer: NP116F 5′-CATGGGACAGACCGTAACTACC-3′and NP117R 5′-GCAGATCGGGACGGAGGTTG-3′. To determine the sensitivity of the Gag PCR originally published by Urisman et al. the following primers were applied: GAG OF 5′-CGCGTCTGATTT-GTTTTGTT-3′, GAG OR 5′- CCGCCTCTTCTTCATTGTTC-3′, GAG IF 5′- TCTCGAGATCATGGGACAGA-3′ and GAG IR 5′- AGAGGGTAAGGGCAGGGTAA [19]. The env PCR was performed as recently published [3] using the following primer pairs F 5′-ACCAGACTAAGAACTTAGAACCTCG-3′, R5′-AGCTGTT-CAGTGATCACGGGATTAG-3′, IF 5′-GAACAGCATGGAAA-GTCCAGCGTTC-3′ and IR 5′-CAGTGGATCGATACAGTCT-TAGTCC-3′. The integrity of the DNA samples and the presence of putative inhibiters were controlled by amplifying GAPDH, F 5′-GAAGGTGAAGGTCGGAGTC-3′ and R 5′- GAAGATGG TGATGGGATTTC-3′.

Western Blot

Cell lysates were generated using RIPA buffer containing 1% Triton-X 100 and protease inhibitor mix (Roche). Specific protein bands were detected by polyclonal Env antibody Rauscher 77S85 (gift of C. Stocking, Heinrich-Pette Institute, Hamburg, Germany), XMRV specific rabbit polyclonal Gag antiserum k121 and p30-Gag recognizing hybridoma supernatant from CRL-1912 cells (ATCC). Equal protein amounts per lane were ensured with anti-human actin antibody mAB 1501 (Chemicon) incubation. For the detection of XMRV particles in cell culture supernatants, sterile filtered culture medium of infected cells was ultracentrifuged 1 h, 110.000×g at 4°C (Beckman SW60Ti). The pellet of 11ml supernatant was resuspended in 10 µl PBS and analyzed by immunoblotting.

Cell line paraffin sections and TMAs

1×10^7 cells (LNCaP, LNCaP chronically infected with XMRV, 293T, 293T chronically infected with XMRV and mouse SC1 cells) were fixed for 20 h in 10% phosphate buffered formalin, embedded in agar and processed to paraffin wax [37].

A preexisting TMA containing prostate tissue (50 low grade PCA, 50 high grade PCA and 50 benign prostate hyperplasia (BPH)) was used for IHC.

Immunhistochemistry

Slides with paraffin sections of prostate cancer patients were initially deparaffinized using xylene. For antigen retrieval sections were heated 4×2 min in a citrate buffer using a microwave (650W) and then cooled down to room temperature for 30 min. Blocking was performed for 30 min at RT with 10% swine serum in antibody dilution buffer (Dako). Afterwards endogenous biotin was blocked using Avidin/Biotin Kit (Dako). Primary antibody (diluted in antibody dilution buffer with 2% swine serum, anti-XMRV 1:7500; XMRV anti-gag k121 1:5000) was incubated for 2 h at room temperature in a humid chamber. Controls were either coated with the corresponding pre serum (same dilution) or only with antibody dilution buffer with 2% swine serum. The incubation with the secondary antibody – biotin/streptavidin labeled – was performed for 30 min at RT. For a later detection of bound antibodies labeled sections were coated with alkaline phosphatase solution (Dako, AK 5000) according to manufactures instructions. IHC staining solution containing levamisole to inhibit endogenous alkaline phosphatase was added to the slides for 15–20 min, while counterstaining was performed with Mayers hamin solutions. The anti-XMRV serum was kindly provided by Ila Singh (University of Utah, USA).

Results

XMRV protein expression in PCA tissue by IHC methods

In 2009, the finding of 23% of PCA sections positive for XMRV protein expression has been reported [4]. XMRV protein expression which in the majority of cases localized to the tumor epithelium strongly correlated with higher Gleason grades. Interestingly, the protein expression data did not correlate with PCR results. One putative explanation being few focal infected XMRV cells in the prostate which are hardly detectable by PCR using DNA from whole mount tissue sections as template. However, these findings were not confirmed by another study [24]. To contribute to the explanation of the discrepancies we screened whole PCA sections as well as TMAs using the recently published anti-XMRV serum [4] and a rabbit polyclonal anti-XMRV gag serum (gag k121).

Both sera have been tested in Western Blot analyzes with gag k121 serum specifically recognizing xenotropic gag protein while displaying no cross reactivity with any cellular proteins. In contrast the anti-XMRV serum [4] also recognized cellular proteins in non infected human and mouse cell lines (supplementary Figure S1). We generated paraffin sections representing human cell lines 293T, LNCaP, both cell lines infected with XMRV and a mouse cell line SC1. Both antisera recognize XMRV protein expressing cells in paraffin sections showing granular staining of the cytoplasm (Figure 1). No staining of uninfected cells and no staining of SC1 mouse cells was detected. A total of 100 PCA (low grade and high grade PCA) and 50 BPH represented on a TMA as well as 10 large sections of prostate cancer (with high Gleason Score) were analyzed with gag k121 serum (Table 2). In addition a TMA containing breast, colon and prostate cancer as well as several normal tissues was tested for XMRV protein expression. Each IHC staining was controlled by including positive controls (paraffin sections of cell lines) and negative controls (without

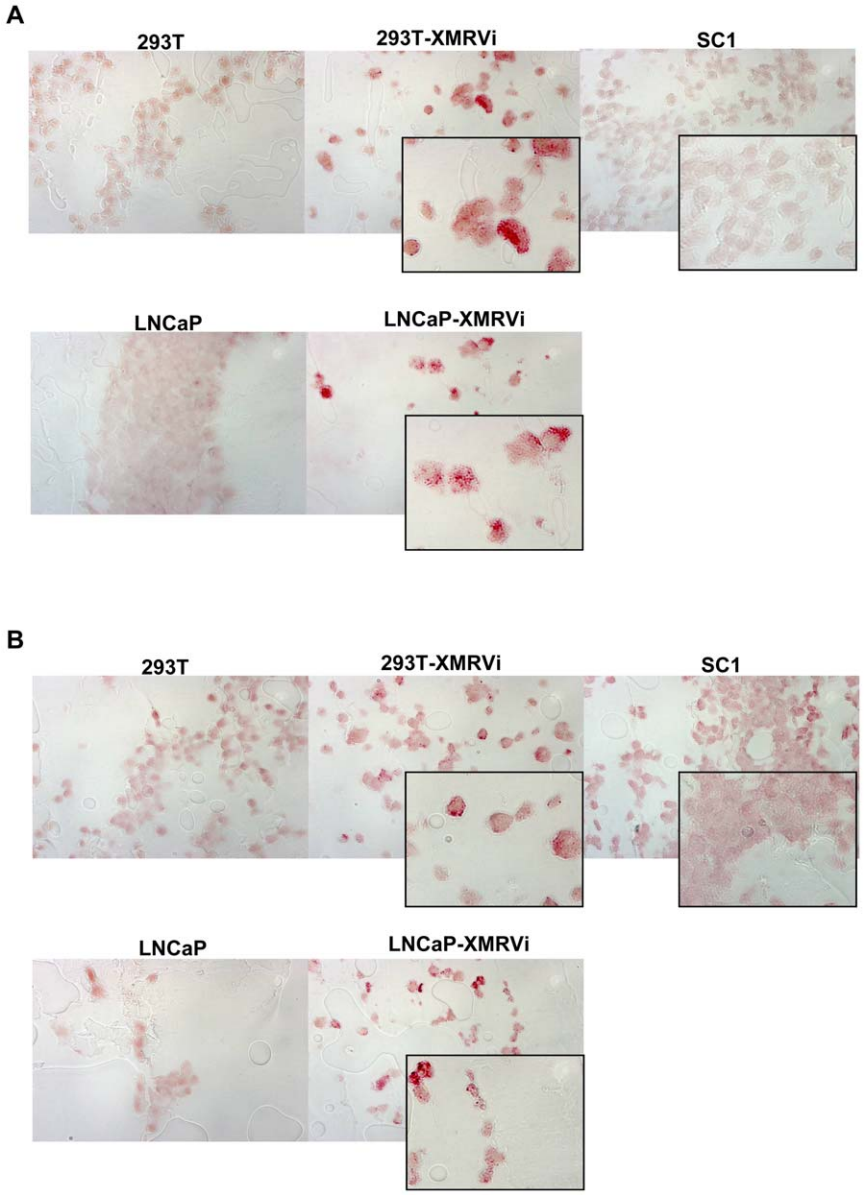

Figure 1. XMRV specific immunohistochemistry staining on cell line paraffin sections. Paraffin sections of cell line array containing XMRV infected cell lines as well as non infected cell lines were stained for XMRV protein expression using anti-XMRV serum (A) or anti-gag k121 polyclonal rabbit serum (B). Larger magnifications are displayed for XMRV infected cells as well as for a feral mouse cell line, SC1.
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Table 2. Summary of XMRV IHC on PCA sections and other common malignancies.

	α-XMRV (Schlaberg et al., PNAS 2009)	α-gag 121
PCA TMA	n.t.	0/50 high grade PCA
		0/50 low grade PCA
		1/50 BPH
TMA*	n.t.	0/114
PCA tissue sections	0/10 (high grade)	0/10 (high grade)

*: **Neoplasia**: Breast cancer, colon cancer; prostate cancer; **Normal tissue**: Adrenal gland, colon, endometrium, epididymis, heart, kidney, lung, pancreas, placenta, parotid gland, prostate, skin, spleen, stomach, striated muscle, thymus, tonsil, testis.
doi:10.1371/journal.pone.0025592.t002

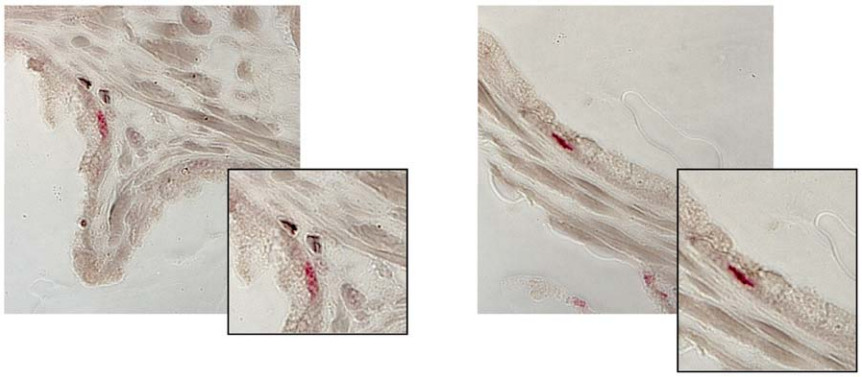

Figure 2. Immunohistochemistry staining using anti-gag k121 polyclonal rabbit serum on TMAs representing prostate cancer sections as well as benign prostate hyperplasia (BPH). In 1/50 BPH random positive stained cells were observed, which might be basal cells based on their localization in the prostate.
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addition of first antibody) as well as higher dilutions of the first antibody. No staining of cancer sections was observed as well as the majority of control tissues was negative for gag k121 staining. Only one section of BPH displayed very few random basal cells staining positive with anti-gag k121 serum (Figure 2). None of the TMA was tested with the anti-XMRV serum since high background due to the TMA generation procedure has been observed.

Activated PBMCs can be infected with XMRV, however XMRV replication is restricted in PBMCs. Following the hypothesis published by Lombardi et al, that XMRV can be detected in PBMCs from up to 67% of CFS patients as well as in up to 4% of healthy controls [2] we intended to activate PBMCs from PCA patients and control patients and screen for XMRV infection applying different methods. We first established our XMRV detection methods on PBMCs which have been in vitro infected with viral supernatant containing VP62 XMRV. Proviral DNA was used to produce XMRV infectious supernatants in LNCaP cells which strongly support XMRV replication due to strong activation of the LTR as well as the lack of retroviral restriction factors Apobec 3G expression [36,38–41]. PHA activated PBMCs were in vitro infected with the indicated amounts of viral supernatant (Figure 3) which were cultured in the presence of IL2 for another 7 d. Virus containing supernatant was then subjected to ultracentrifugation and viral pellets (Figure 3A) as well as cell lysate (Figure 3B) from the infected PBMCs were analyzed by Western Blotting ensuring the expression of XMRV specific proteins. Based on Western Blot experiments using chronically infected LNCaP cells diluted with the indicated cell number of uninfected 293T cells (Figure S2) we can estimate that approximately 1–2% of PBMCs are infected with XMRV. Only if we infect PBMCs with high viral titers we efficiently detected XMRV in the viral pellet after ultracentrifugation and Western Blot analysis (Figure 3A). Genomic DNA isolated from these in vitro XMRV infected PBMCs was positive for XMRV sequences by PCR using 650 ng genomic DNA and two different primer sets targeting gag and env (Figure 4A and Figure S3). Sensitivity of all PCR reactions is indicated in supplementary Figure S4 with all PCR detecting 1–10 infected cells in a background of 10^6 uninfected cells.

Cocultivation of XMRV infected PBMCs with LNCaP cells significantly increases sensitivity of XMRV detection. DERSE-iGFP cells were exposed to filtered culture supernatant from XMRV infected PBMCs. 500 µl of supernatant was added to 5×10^4 DERSE-iGFP cells which were scored for GFP expression 7 d p.i. by microscopy and FACS analysis (Figure 3C). In general, viral supernatant from PBMCs is infectious, however only very few GFP positive cells were detected. Interestingly, if we cocultivate the XMRV infected PBMCs with LNCaP cells for 5 d, harvest the supernatant and reinfect DERSE-iGFP cells with filtered supernatant, sensitivity of XMRV detection using DERSE-iGFP cells was 100fold increased Figure 3D and Figure 4B.

PBMCs of PCA patients are negative for XMRV detection by PCR analysis

Using this approach we isolated PBMC from 92 PCA patients and 7 healthy volunteers by Ficoll gradient; isolated PBMCs were PHA activated and cultured in the presence of IL-2 for 7 d. PBMCs were subjected to different assays as outlines in Figure 5A: genomic DNA isolation followed by XMRV specific nested PCR applying two published XMRV PCR strategies [1,3,19]; cocultivation of activated PBMCs with LNCaP cells for 8 weeks with subsequent infection of DERSE-iGFP cells using supernatant 6 weeks and 8 weeks after cocultivation. Localization of the different primer sets used is shown in Figure S3 and sensitivity of the different XMRV PCRs is reflected in Figure S4. The integrity of the genomic DNA together with the absence of putative PCR inhibitors was ensured by GAPDH amplification (Figure S4). The culturing of PBMCs, DNA preparations and the PCR amplification were performed in laboratories of the Heinrich-Pette Institute where no other XMRV studies were performed. In addition, all nested PCRs to detect XMRV sequences using two different primer pairs targeting gag, both recently published, as well as an env PCR were run by two operators using 650 ng genomic DNA as template. All DNA samples were found to be consistently negative (Table 3). PCR reactions were routinely controlled for mouse contamination using primers directed against retrotransposons, intracisternal A particle (IAP), as recently published [15]. None of the PCR reactions was positive for mouse DNA sequences (data not shown).

67 PBMC samples were cocultured with LNCaP cells for up to 8 weeks and SN of the LNCaP cells was applied to the reporter cell line DERSE-iGFP. This cell line carries a MLV vector, which leads to expression of a GFP reporter if reverse transcriptase is expressed. 72 h p.i. DERSE-iGFP cells were monitored for GFP expression by microscopy. Of 67 samples supernatant from PBMCs cocultured with LNCaP cells, two resulted in 2–3 GFP positive cells in 5×10^4 cells (Figure 5B). We did not observe an increase of GFP positive cells over time indicating that there was no spread of viral infection. Interestingly the supernatant of the

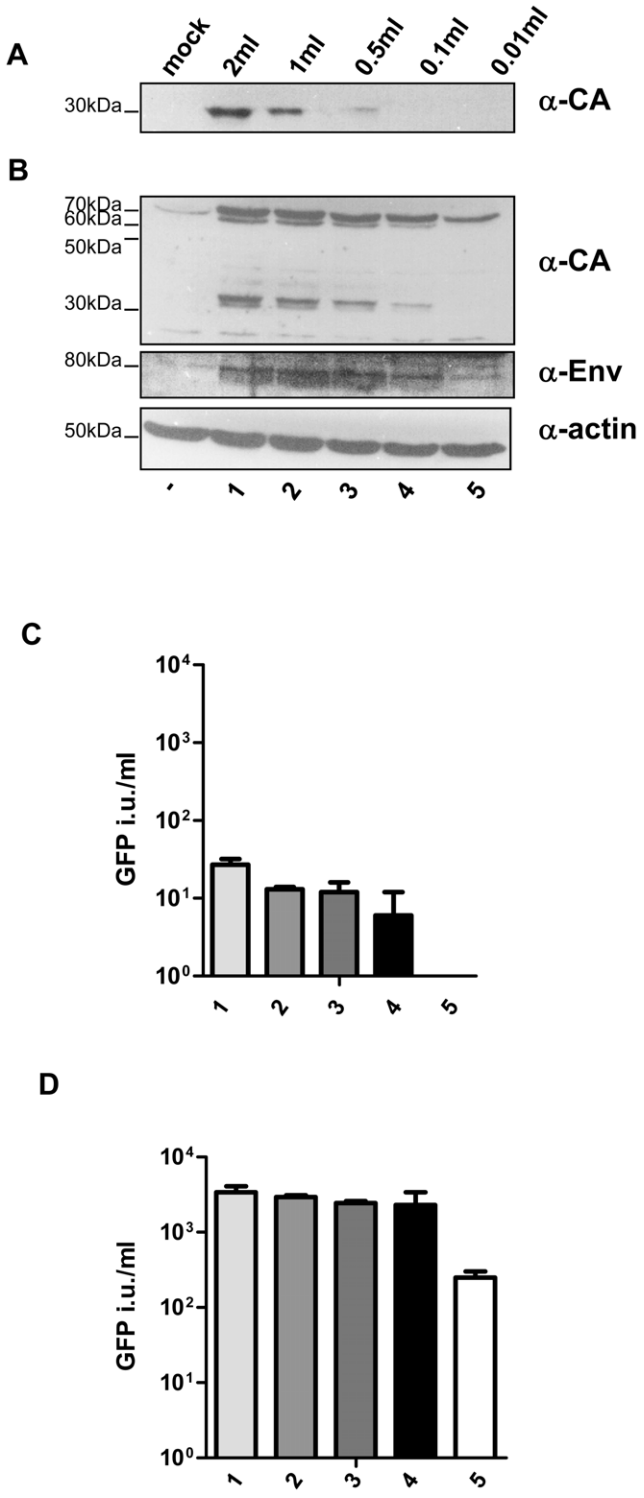

Figure 3. XMRV efficiently infects and replicates in peripheral blood mononuclear cells (PBMCs). PBMCs from two different donors were isolated, pooled, PHA stimulated and subsequently infected with the indicated amounts of XMRV containing supernatant (lane 1–5). Western Blot analysis of cell lysate from infected PBMCs was performed 7 d past infection (B). Supernatant of the infected PBMCs was enriched for virus particles by ultracentrifugation and stained for CA expression (A). (C) 500 µl of XMRV containing supernatant originated from PBMCs shown in A and B was used to infect DERSE-iGFP cells which were analysed for GFP expression 7 d past infection by FACS. Titers are indicated as GFP infectious units/ml. (D) Infection of DERSE-iGFP cells is 100fold increased by cocultivation of infected PBMCs (shown in (A)) with LNCaP cells for 7 d, SN of LNCaP cells was then applied to DERSE-iGFP cells, which were analysed by FACS 5 d p.i.. doi:10.1371/journal.pone.0025592.g003

activated PBMCs from these two patients without cocultivation also resulted in 1–2 GFP positive DERSE-iGFP cells per well. In one case two independent PBMC isolations from the same patient were performed (#99 and #100) which both resulted in 1–2 GFP positive DERSE-iGFP cells. However, both isolations were performed at the same day by the same operator. PCR from LNCaP cells cocultured with PBMCs of these two patients did not result in detection of XMRV specific sequences as well as we were unable to culture and expand GFP positive DERSE-iGFP cells for subsequent analyses.

Discussion

In this study we have examined the detection of XMRV in prostate cancer patients by studying different diagnostic bio specimens for the presence of XMRV or related MLV sequences. In particular, we analyzed PCA tissue specimens as well as tissue sections from other malignancies and normal tissues for XMRV protein expression by IHC. Furthermore, PBMCs from 92 PCA and 7 healthy controls were screened for the presence of XMRV sequences and recovery of infectious virus. PBMCs were PHA activated, cocultured for up to 8 weeks and XMRV presence was examined by either nested PCR targeting two different XMRV regions, Western Blot analyzes using different anti-XMRV antibodies or infection of DERSE-iGFP cells applying supernatant from activated PBMCs or supernatant from LNCaP cells cocultured with PBMCs for up to 8 weeks.

We were unable to conclusively show that XMRV sequences can be detected in activated PBMCs of PCA patients although in two patients GFP positive DERSE-iGFP cells were detected. In both cases subsequent PCR analyses of activated PBMCs as well as cocultured LNCaP cells were negative for XMRV sequences as well as we did not find XMRV protein expression in PCA sections of one of these patients.

We previously published that XMRV sequences are only rarely detected in Germany using cDNA generated from PCA tissue RNA amplified by PCR [27]. Similar results for a study in the US have been recently published by Switzer et al., [26]. However, there are multiple studies not identifying any XMRV sequences in PCA tissue as well as there are studies with higher prevalence of XMRV in PCA [3,9,21–24,31,42]. Considering the possibility of focal XMRV infection in the prostate which might be missed by PCR amplification due to only a minority of cells infected we established IHC staining using the published anti-XMRV serum and an XMRV specific anti-gag serum. We failed to detect XMRV protein expression in PCA tissue, breast cancer or colon cancer tissue as well as most control tissue (including 10 sections each: adrenal gland, colon, endometrium, epididymis, heart, kidney, lung, pancreas, placenta, parotid gland, spleen, stomach, striated muscle, thymus, tonsil, and testis) did not show any positive staining for gag k121 serum. Interestingly, using the anti-gag k121 serum we detected 1/50 BPH sections positive for XMRV protein expression. Protein expression was identified in a few isolated basal cells in the prostate epithelium. Basal cells are absent in PCA, supporting the fact that XMRV most likely is not directly involved in PCA development. The small number of whole mount tissue sections examined could account for the discrepancy between our findings and earlier findings by Schlaberg et al. [4]. We only stained ten whole mount tissue

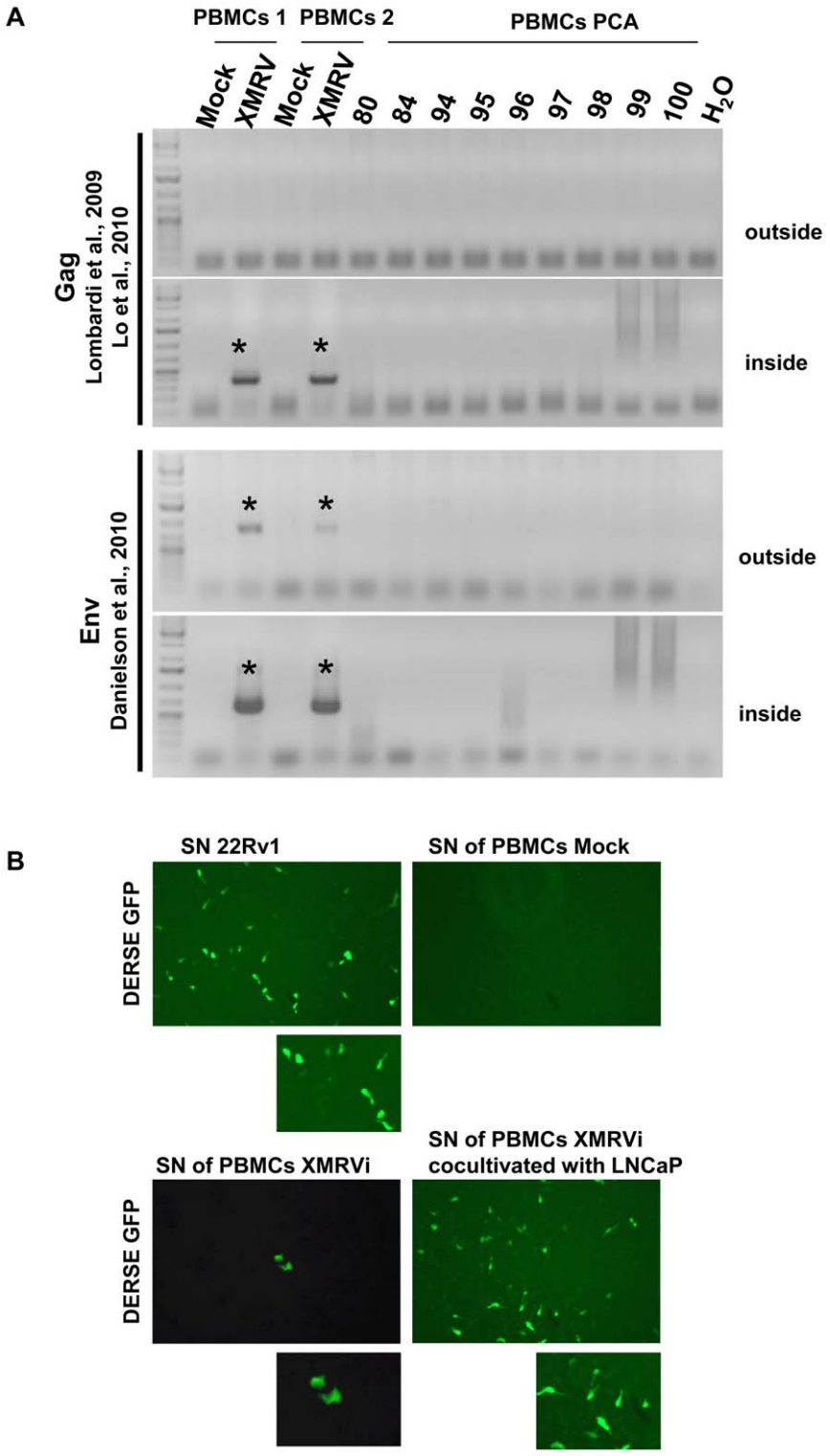

Figure 4. Detection of XMRV infection in PBMCs in vitro infected with XMRV by PCR (A), 650 ng genomic DNA isolated from PBMCs 7 d past infection were used as template. (B) DERSE-iGFP cells were infected with 500 µl supernatant from 22Rv1 cells, mock infected cells or LNCaP cells cocultured with XMRV infected PBMCs for 14 d. 72 h past infection DERSE-iGFP cells were monitored for GFP positive cells by microscopy.
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sections with both antisera, the anti-XMRV serum [4] was not used on TMA sections due to high background staining. Aloia et al. and Sakuma et al. both discuss a cross reactivity of anti-XMRV serum with human protein antigens resulting in IHC positive staining in PCA sections [16,24]. We detect some cross reactivity with the published anti-XMRV serum on Western Blots analyzing cell lysates from infected and non infected cells, however there was no background observed on paraffin sections of cell lines or on

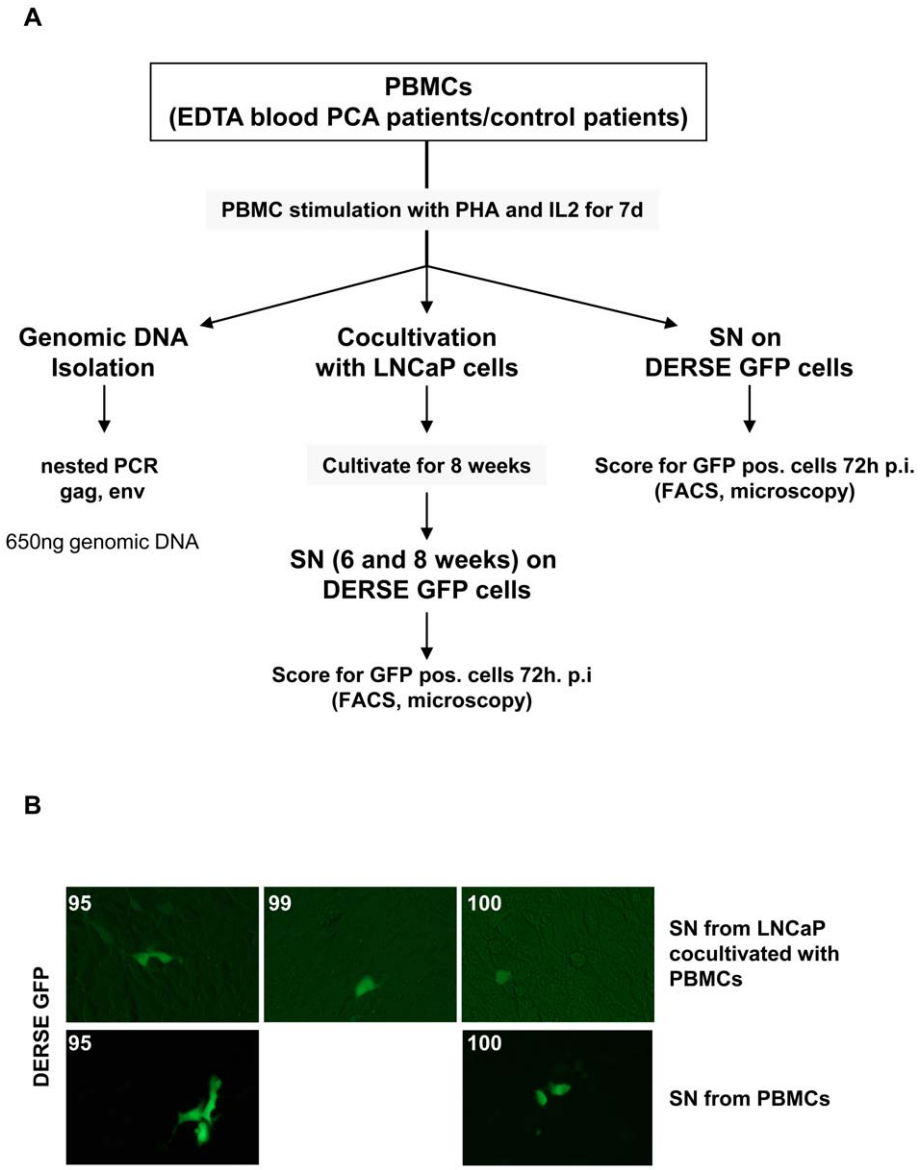

Figure 5. Detection of XMRV in PBMCs isolated from PCA patients and healthy controls. (A) Methods used to screen for XMRV in PBMCs of PCA patients and healthy controls. (B) DERSE-iGFP cells 72 h p.i. with SN from LNCaP cells cocultured for 8 weeks with patient derived PBMCs (upper panels). The lower panels display DERSE-iGFP cells 72 h p.i. with SN from patient derived PBMCs which were activated with PHA for 7d.
doi:10.1371/journal.pone.0025592.g005

Table 3. Summary of XMRV detection in activated PBMCs from PCA patients using nested PCR amplification from genomic DNA and coculture experiments on DERSE-iGFP cells.

	Nested PCR			Cell Culture	
	GAG (Urisman et al. PLoS Pathog. 2006)	GAG (Lo et al. PNAS 2010)	ENV (Danielson et al. JID 2010)	PBMCs cocultured with LNCaP[1]	SN from PBMCs on DERSE-iGFP cells[2]
PCA Patients	0/93	0/93	0/93	2/67	3*/10
Healthy Controls	0/7	0/7	0/7	n.t.	n.t.

[1]Activated PBMCs were cocultured with LNCaP cells for 8 weeks. Supernatant of these LNCaP cells was applied to DERSE-iGFP cells.
[2]Supernatant from activated PBMCs was applied to DERSE-iGFP cells without cocultivation with LNCaP cells.
*#99 and #100 derived from the same patient.
doi:10.1371/journal.pone.0025592.t003

whole sections of PCA tissue using serum at the indicated dilutions. Negative IHC staining does not exclude the possibility of few cells carrying XMRV proviral sequences which we might miss by PCR amplification. We did not apply DNA FISH technology to detect XMRV proviral integration in human tissue. Evaluation of FISH positive signal in 0.1% or less of the cells especially if only one viral copy per cell is to be expected, is highly error prone.

Recently, Lombardi et al. reported detection and transmission of infectious XMRV from PBMCs or plasma of patients with CFS by coculturing with LNCaP cells [2]. Interestingly, 3-4% of PBMCs isolated from control patients were identified to be positive for XMRV infectious virus resulting in the general concern about the safety of blood products. Several subsequent studies motivated by these results were unable to confirm these original findings. Reasons for the discordance are unclear and are currently investigated. While the majority of studies focussed on PCR techniques as well as detection of XMRV specific antibodies only one study included cocultivation of activated PBMCs from CFS patients with LNCaP cells [10] and a more recent study tested the transmission of XMRV from plasma (derived from CFS patients) to LNCaP cells [43]. Both studies did not detect XMRV in any of the samples tested. Focusing on the possibility that XMRV is a bystander virus reactivated in prostate cancer patients together with the finding that XMRV can be detected in PBMCs of patients [2] we searched for signs of XMRV infection in blood cells of PCA patients applying PCR technology and cocultivation of activated PBMCs with indicator cells. To our knowledge the current study is the first analyzing the presence of XMRV in blood samples from PCA patients in general and from a larger number of PBMCs (n = 92) tested by labor intensive coculturing of activated PBMCs with LNCaP cells for up to 8 weeks. A previous report by Hohn et al. also used cocultivation of activated PBMCs with subsequent genomic DNA isolation and XMRV specific amplification. Here we cocultivated activated PBMCs with LNCaP cells for up to 8 weeks (which increases sensitivity up to 100fold) and tested supernatant of these LNCaP cells for XMRV release by infection of DERSE-iGFP cells and subsequent FACS analysis or microscopy study.

In two patients we identified isolated GFP positive DERSE-iGFP cells when applying supernatant of activated PBMCs after 7 d as well as from the supernatant of LNCaP cells cocultivated for 8weeks with PBMCs. In all cases only very few positive cells were detected which could not be subcultivated to achieve significant cell numbers for subsequent experiments.

Taken together our data generated by analyzing different bio specimen, in particular tissue sections and PBMCs, for signs of XMRV infection do not support the association of XMRV with prostate cancer. Since we did not apply FISH technology to detect proviral integration we cannot exclude that few cell might show XMRV integration. However, the question of XMRV existence is different from the question of disease association. Our data are in concordance with recently published results demonstrating that XMRV can infect PBMCs in vitro [10,44]. We find that 1–2% of PBMCs are infected when high amounts of viral titers are used for in vitro infection. These PBMCs release XMRV, however less viral particles are released compared to LNCaP cells and the virus is highly edited. Nevertheless, XMRV released from PBMCs is able to efficiently infect cells. Although we observed by two different experiments that DERSE-iGFP cells after incubation with supernatant from activated PBMCs express GFP in a few cells, we were unable to conclusively show that XMRV can be reactivated from PBMCs and infect an indicator cells line: no PCR detection of XMRV was achieved as well as the ultimate proof, cloning of integration sites from patients, is impossible from this material. At no time did we observe spontaneous GFP expression of DERSE-iGFP cells or GFP expression due to exogenous contamination of our cell culture, still contamination can not be experimentally ruled out.

In summary, we applied multiple methods to detect XMRV in bio specimen of prostate cancer patients; the results of our study do not support an association of XMRV and prostate cancer.

Supporting Information

Figure S1 Western Blot analysis of XMRV negative (293T; LNCaP), XMRV positive human cell lines (22Rv1), chronically infected human cell lines (293T-XMRV; LNCaP-XMRV) as well as mouse cell lines (inbred NIH3T3 and feral mouse cells SC1) using rabbit polyclonal α-gag k121 serum (A) or rabbit polyclonal α-XMRV serum [4] (B) for detection.
(TIF)

Figure S2 Western Blot analysis of diluting amounts of chronically XMRV infected LNCaP cells mixed with non infected 293T cells. 25 µg total protein lysate was loaded per lane. Blots were immunoblotted using goat-anti env serum and rabbit-anti gag k121 serum. To ensure equal protein amounts loaded per lane the blot was reprobed with anti-actin monoclonal antibody.
(TIF)

Figure S3 XMRV VP62 Gag sequence 407-1160 (GI:89889045). Primers are indicated as arrows, GAG-O/I dark grey, 419F/1154R and NP116/NP117 light grey. Sequence variability between XMRV and MLV related sequences located in the indicated primer sequences are labeled with a star (*).
(TIF)

Figure S4 Genomic DNA was isolated from 1×10^6 cells (indicated number of chronically XMRV infected LNCaP cells mixed with non infected 293T cells in 10 fold dilutions of infected cells in non infected cells). Nested PCR was performed using the oligos GAG-O and GAG-I [19], 419F/1154R and NP116/NP117 [1] as well as env primers 5604F/6491R and 5742F/6394R [3]. The highest dilution still showing XMRV specific amplification products in labelled with an *.
(TIF)

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Author Contributions

Conceived and designed the experiments: KS MS NF. Performed the experiments: KS SS SM OH NF. Analyzed the data: NF. Contributed reagents/materials/analysis tools: TS MS OH NB NF RS SM. Wrote the paper: NF.

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文献5

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Phylogenetic Analysis of Murine Leukemia Virus Sequences from Longitudinally Sampled Chronic Fatigue Syndrome Patients Suggests PCR Contamination Rather than Viral Evolution▽‡

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Xenotropic murine leukemia virus (MLV)-related virus (XMRV) has been amplified from human prostate cancer and chronic fatigue syndrome (CFS) patient samples. Other studies failed to replicate these findings and suggested PCR contamination with a prostate cancer cell line, 22Rv1, as a likely source. MLV-like sequences have also been detected in CFS patients in longitudinal samples 15 years apart. Here, we tested whether sequence data from these samples are consistent with viral evolution. Our phylogenetic analyses strongly reject a model of within-patient evolution and demonstrate that the sequences from the first and second time points represent distinct endogenous murine retroviruses, suggesting contamination.

Detection of murine leukemia virus (MLV) DNA in patient samples has suggested that the human population may be infected with murine gammaretroviruses (4). A particular xenotropic MLV named xenotropic murine leukemia virus-related virus (XMRV) has been cloned from human prostate cancer tumors as well as blood samples from individuals suffering from chronic fatigue syndrome (CFS) (1, 18, 29, 39). XMRV detection in a small percentage of samples from healthy controls suggested widespread infection (7, 18, 29). Controversy has since surrounded XMRV detection, partly because many laboratories have been unable to detect XMRV in patient samples (2, 5, 6, 8–11, 14, 16, 19–21, 28, 30, 35, 37, 40) and partly because an almost identical virus has been found infecting a common prostate cancer cell line called 22Rv1 (12, 15, 23). These data strongly suggest that XMRV in patient material is the result of DNA contamination from laboratory cell lines or mouse DNA. Importantly, a recent study demonstrated that XMRV arose by recombination during the experiments in which the 22Rv1 cell line was developed by xenografting prostate tumors in mice (22). This observation confirms a date for XMRV genesis in the cell line at between 1990 and 1996 and rules out any human XMRV infection before this time. These observations have raised concerns that previous XMRV detection in humans is likely to be artifactual (3).

An important study in support of MLV infection in humans is that by Lo, Alter, and colleagues (17). These authors suggested that they could confirm human infection of MLV by PCR amplifying a variety of MLV sequences from the blood of CFS patients as well as healthy controls. A PCR test for mouse mitochondrial DNA was used to control for contamination with mouse DNA and found to be negative, but recently, more sensitive intracisternal type A particle (IAP)-based PCR tests for murine contamination reveal that in some cases mouse contamination is not detected by amplification of mitochondrial DNA (26). Surprisingly, Lo et al.'s study did not find XMRV but found a set of MLV sequences almost identical to known endogenous nonecotropic gammaretroviruses of mice. These MLV sequences were characterized as type 1 (18 patients), type 2 (2 patients), and type 3 (1 patient), based on their *gag* gene sequences. Importantly, the authors suggested that evolution of patient viruses could be demonstrated by the accumulation of significant sequence variation over time. Longitudinal samples were taken from eight individuals apparently infected with type 1 viruses 15 years after the first sampling. Seven of these had detectable MLV *gag* at the second time point (28). The sequences derived from six of these longitudinal samples have been deposited in GenBank under accession numbers HQ601957 to HQ601962. Here, we used phylogenetic analyses to consider whether MLV sequences described in this study are consistent with viral infective evolution, a conservative test of whether they are likely to represent genuine human MLV infections.

The shape of a phylogenetic tree reflects the evolutionary processes under which it has grown. The expectation for longitudinally sampled retroviral sequences from the same patient, or from a population of infected patients, is that they cluster with the initial sequences and to the exclusion of all other sequences in the data set. Phylogenetic analysis of the human-derived MLV sequences as well as a variety of known MLV sequences (see the supplemental material for details)

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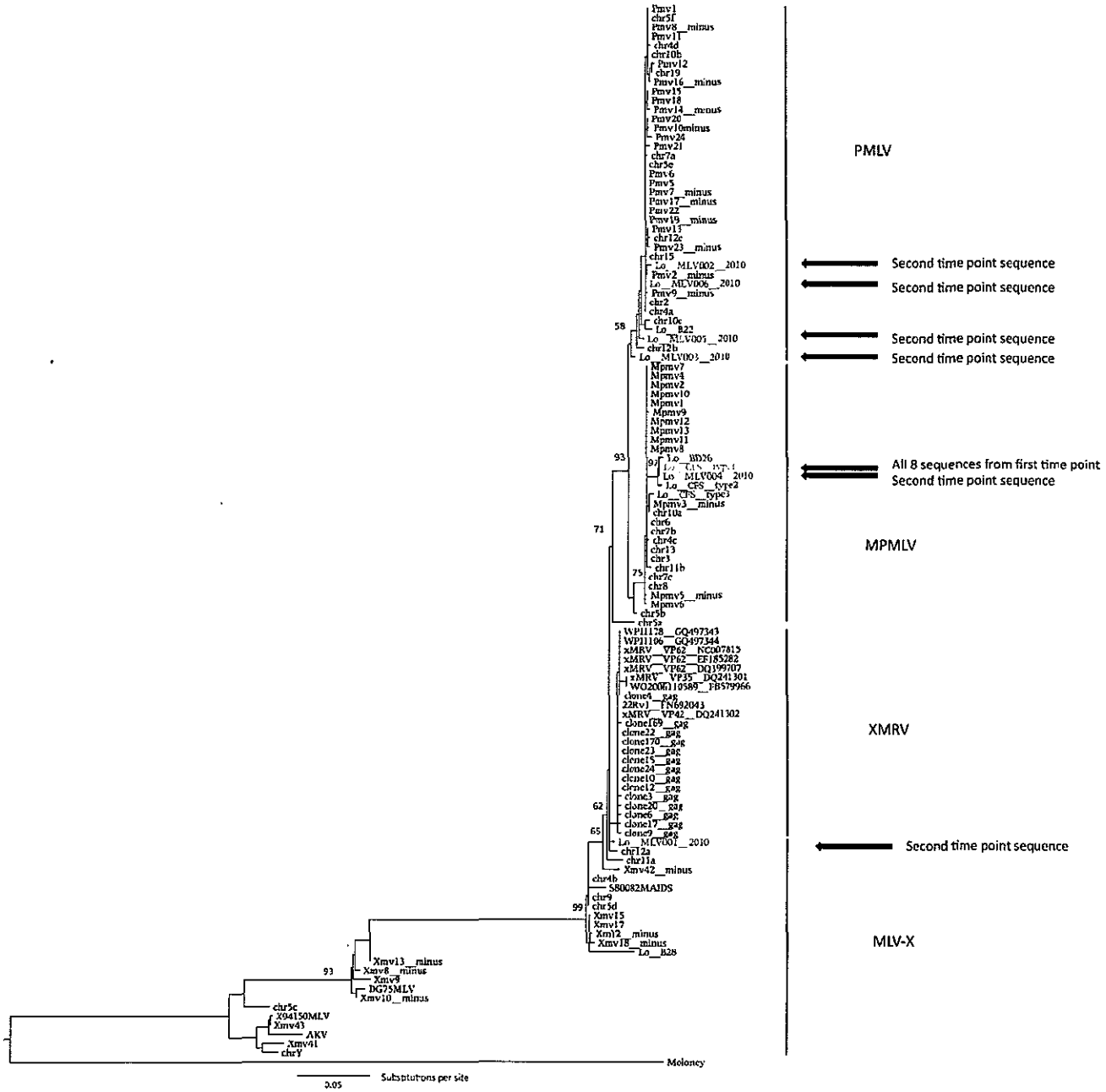

FIG. 1. Maximum likelihood phylogeny of XMRV, xenotropic MLV (MLV-X), polytropic MLV (PMLV; Pmv), and modified polytropic MLV (MPMLV; Mpmv) *gag* gene sequences (710 nt). The initial chronic fatigue syndrome patient-derived XMRV sequences from Lo et al. (17) are indicated in blue. The eight sequences taken from the first time point are represented by a single branch, colored green. XMRV sequences sampled 15 years later from the same patients (GenBank accession numbers HQ601957 to HQ601962) are colored in red, while other sequences that do not have corresponding second-time-point sequences from Lo et al. are colored blue. The tree is rooted with Moloney MLV (GenBank accession number AF033811). Bootstrap scores of >50% are indicated on the corresponding branches. The scale bar represents the number of nucleotide substitutions per site.

indicates that the three patient-derived sequence types, CFS types 1 to 3, fall within the modified polytropic MLV clade. The overall shape of the phylogenetic tree, including the three main groupings (polytropic, modified polytopic, and xenotropic) and the relationships between them, is consistent with previous studies based on full-length proviruses (Fig. 1) (12, 13). Sequences described in Lo et al.'s original report are colored blue and green. While the type 3 sequence is clearly separated from types 1 and 2, the latter sequences form a strongly supported monophyletic cluster (97% bootstrap support) together with the sequence BD26, derived from a healthy donor, and the sequence MLV004_2010, a longitudinal CFS

TABLE 1. Comparison of maximum likelihood phylogenetic tree with hypotheses consistent with within-patient viral evolution[a]

Constraint	Log likelihood	Decrease in likelihood	Standard deviation	*P* value		Expected likelihood wt
				SH	AU	
Best tree	−3,658.76	0.00	NA	NA	NA	0.99
All 5 MLV 2010	−3,721.22	62.46	14.92	<0.001	<0.0001	<0.0001
MLV001 2010	−3,689.93	31.17	9.62	0.001	<0.0001	<0.0001
MLV002 2010	−3,696.66	37.90	10.80	0.002	<0.0001	<0.0001
MLV003 2010	−3,679.73	20.96	9.31	0.022	0.002	0.0086
MLV005 2010	−3,687.62	28.86	10.15	0.004	0.0003	0.0004
MLV006 2010	−3,701.63	42.87	12.90	0.002	<0.0001	<0.0001

[a] The most stringent constraint involved all 5 MLV sequences from the second time point, while each of the 5-s time point sequences was also constrained individually to cluster with the CFS type 1 sequence from the first time point. The trees were compared using pairwise Shimodaira-Hasegawa (SH) tests, the approximately unbiased (AU) test, and expected likelihood weights. All of the constrained trees were significantly worse than the maximum likelihood tree. NA, not applicable.

patient sample (Fig. 1). All the resampled patients originally yielded identical polytropic MLV CFS type 1 sequences (S. C. Lo, personal communication); in other words, all eight sequences derived from patients at the first time point are represented by a single branch in the phylogeny. Under a model of within-patient viral evolution, we would expect all of the 2010 daughter sequences to branch from the parental CFS type 1 sequence with longer but approximately equidistant branches. This is true for all other longitudinally sequenced viruses (reviewed in reference 24).

We assessed the fit of the data to this model by inspection of the phylogenetic tree and by maximum likelihood-based model testing. The phylogenetic placement of the longitudinal sequences does not fit this expected model. When the sequences from the second time point were examined, we found that 5 of 6 are phylogenetically distinct from the parental CFS type 1 sequence and from each other. The more recent longitudinally sampled sequences are shown in red in Fig. 1. While the originating CFS type 1 sequences belong to the modified polytropic clade, longitudinally sampled sequences from the same CFS type 1-infected patients are derived from three strongly supported and distinct regions of the tree, namely, the polytropic, modified polytropic, and xenotropic clades. Surprisingly, 3 of the new sequences (MLV002_2010, MLV005_2010, and MLV006_2010) do not even form sister taxa within the polytropic clade. In fact, the two most distantly related sequences from these longitudinal patient samples are about as different from each other as the biggest distance possible within the polytropic clade. Another 2010 (MLV001_2010) sequence is placed within the xenotropic clade, at the base of the XMRV cluster. It also contains a large deletion in *gag*, which would be expected to inactivate the virus. Although this sequence is basal, aside from the deletion, it is very similar to other XMRV sequences, differing at only a single nucleotide position across its 330-nucleotide (nt) length from the prostate cancer patient sequence VP62 (39). It is therefore substantially more similar to XMRV than to either the polytropic or modified polytropic MLV sequences previously reported by Lo et al. (17). There is no evidence for hypermutation mediated by APOBEC proteins in the patient-derived sequences as might be expected during an infection, given the susceptibility of MLV to mutation by these proteins (23). Thus, these new patient-derived MLV sequences show tremendous variation from the parental CFS type 1 sequence and as such are extremely unlikely to have evolved from the CFS type 1 sequence.

The probability that the data are consistent with a model of within-patient evolution can be explicitly tested by comparing the maximum likelihood phylogenetic tree directly derived from the data with a tree in which sequences from the second time point are constrained to cluster with sequences from the first time point. The difference in likelihood of these two topologies was determined by using the Shimodaira-Hasegawa test (33), the approximately unbiased test (31), and expected likelihood weights (34) as implemented in CONSEL (32). We can reject the hypothesis of clustering of sequences from the second time point with those from the first (Table 1). In order to explore the robustness of this test to the possibility that any one individual sequence is consistent with within-patient evolution, and that the *P* value may be unduly influenced by any of the other sequences, we also explored the relative fit of each sequence from the second time point to a model consistent with within-patient evolution (i.e., constrained to cluster with sequences from the first time point). We compared the likelihood of five additional trees, where each patient sample was constrained in turn to cluster with the CFS type 1 sequence while the other patients were allowed to assume the most likely position within the tree. For each patient individually, the tree that is consistent with within-patient evolution was rejected (Table 1). The use of these likelihood-based tests is robust to the short sequences used in the alignment, which account for the low bootstrap support scores for many nodes in the phylogenetic tree.

To calculate the chance of a modified polytropic virus evolving into a polytropic virus, we reconstructed the common ancestors of the polytropic and modified polytropic clades by taking a consensus of each sequence set. We then estimated the probability of a virus making the specific changes required to evolve from one clade to the other by running 10,000 simulations in Seq-Gen and counting the number of times the mutations arose (25). The hypothesis that the characteristic mutations could have arisen by chance, given that a number of mutations equivalent to the distance between these groups had occurred, can be rejected with a *P* value of <0.0001. Indeed, the fact that 3 of the newly sampled viruses appear to have independently made these specific changes underlines the fact that these sequences represent different viruses rather than CFS type 1 descendants. The last of the longitudinal sequences is 100% identical to the CFS type 1 sequence identified in 18 patients at the initial time point, 15 years earlier. This observation indicates that in this case, there has been no viral evolution throughout 15 years of infection. Some slowly

TABLE 2. Comparison of patient-derived MLV sequences and known MLV sequences within the mouse genome[a]

Sequence	GenBank accession no.	Sampling yr(s)	Closest relative in the C57BL/6J mouse genome (July 2007 assembly)							
			Chr.	Strand	Span (nt)	Start (nt)	End (nt)	% identity	Changes[b]	Gaps (length in nt)
BD22	HM630560	Mid-1990s	10	−	697	8269377	8270073	99.30	5/697	0
			10	−	696	50145707	50146402	99.30	5/696	0
			X	+	696	15052167	15052862	99.30	5/696	0
BD26	HM630561	Mid-1990s	6	−	339	73242571	73242909	98.30	6/339	0
			2	−	339	15949590	15949928	98.30	6/339	0
			10	−	339	4627251	4627589	98.30	6/339	0
BD28	HM630557	Mid-1990s	9	+	331	62288048	62288378	98.10	6/318	1 (21)
			5	+	331	23722164	23722494	98.10	6/318	1 (21)
			4	+	331	133716363	133716693	98.10	6/318	1 (21)
CFS type 1	HM630562	Mid-1990s	8	+	697	125689652	125690348	99.30	5/697	0
			11	+	697	102946013	102946709	99.30	5/697	0
			6	−	696	73242413	73243108	98.90	5/696	0
CFS type 2	HM630558	Mid-1990s	8	+	698	125689652	125690349	99.00	7/698	1 (1)
			11	+	698	102946013	102946710	99.00	7/698	1 (1)
			6	−	697	73242412	73243108	98.60	7/697	1 (1)
CFS type 3	HM630559	Mid-1990s	2	−	697	15949431	15950127	99.90	1/697	0
			13	+	697	21905315	21906011	99.80	2/697	0
			6	−	696	73242413	73243108	99.30	1/696	1 (1)
MLV001	HQ601957	2010	12	+	340	19250254	19250593	99.70	1/276	2 (63; 1)
			9	+	339	62288048	62288386	99.00	2/276	1 (63)
			5	+	339	23722164	23722502	99.00	3/276	1 (63)
MLV002	HQ601958	2010	4	−	339	107826090	107826428	99.80	1/339	0
			2	−	339	57074273	57074611	99.80	1/339	0
			15	−	339	76395902	76396240	99.80	1/339	0
MLV003	HQ601959	2010	6	−	339	73242571	73242909	99.20	3/339	0
			4	−	339	107826090	107826428	99.20	3/339	0
			2	−	339	57074273	57074611	99.20	3/339	0
MLV004	HQ601960	2010	6	−	339	73242571	73242909	98.60	5/339	0
			2	−	339	15949590	15949928	98.60	5/339	0
			10	−	339	4627251	4627589	98.60	5/339	0
MLV005	HQ601961	2010	4	−	339	107826090	107826428	99.50	2/339	0
			2	−	339	57074273	57074611	99.50	2/339	0
			15	−	339	76395902	76396240	99.50	2/339	0
MLV006	HQ601962	2010	4	−	339	107826090	107826428	100.00	0/339	0
			2	−	339	57074273	57074611	100.00	0/339	0
			15	−	339	76395902	76396240	100.00	0/339	0

[a] Due to the short lengths of the patient-derived sequences, several mouse sequences are equally similar. Thus, three sequences are shown. The span column refers to the total length of the best match in the murine chromosome (Chr.).
[b] Number of nucleotides different/total number of nucleotides.

evolving retroviruses (e.g., simian foamy virus) can remain virtually identical over many years (36); however, this patient sample contrasts markedly with all the other samples from the second time point. In summary, in some patients, viral diversity is as vast as the diversity that the whole set of known nonecotropic MLVs allows, and yet from another patient, an identical sequence was amplified 15 years later. The repeat samples could not have been derived from the initial samples via a process of viral evolution. In fact, they represent different endogenous murine viruses. It is theoretically possible that the 6 patients were originally coinfected with diverse viruses, but this possibility is rather undermined by the fact that the original samples each gave rise to the same identical type 1 sequence from 18 independent patients. An alternative possibility is that all but one of the patients that retested positive for MLV were superinfected with a distinct MLV prior to the samples being taken at the second time point and that in each of these patients, the viral infection from the first time point was cleared.

Examination of the viral sequences amplified reveals that they are almost identical to known sequences in mouse genomic DNA. The numbers of differences between the pa-

tient amplified samples and their nearest relatives in the published mouse C57BL/6J genome are shown in Table 2. For example, MLV002 is 1 nucleotide out of 339 nucleotides different from polytropic MLV on the C57BL/6J mouse chromosomes 4, 2, and 15, most likely due to a single nucleotide polymorphism within the mouse population. The only realistic explanation that could account for these observations is that all of the MLV-positive patient samples, or the PCRs performed using patient DNA as template, were contaminated with mouse DNA. This would act as a source for amplification of the diverse viruses found and could perhaps have occurred as a result of repeated handling of patient-derived samples (see reference 41 for a discussion). There is no credible hypothesis that could explain these observations in the absence of PCR contamination. It appears to be extremely difficult to do mouse-free PCR, and we note that other studies in which contamination has been demonstrated have also amplified a diverse range of MLVs (27, 38). We propose that the detection of murine virus in human samples be more rigorously controlled using IAP PCR (26) to rule out murine DNA contamination and robust phylogenetic analysis to rule out random amplification of endogenous proviruses (12), which can exist at a high copy number in the genomes of mice or in cell lines that become infected with mouse viruses during routine experimentation.

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