

## XMRVに関する文献報告(続報)(平成24年3月14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	Oakes B, Qiu X, Levine S, Hackett J Jr, Huber BT. <i>Adv Virol.</i> 2011;2011:854540. Epub 2011 Jul 27.  Failure to Detect XMRV-Specific Antibodies in the Plasma of CFS Patients Using Highly Sensitive Chemiluminescence Immunoassays.	米国	平成23年2月18日の運営委員会で紹介した論文の2報目である。1報目の論文では、XMRVの遺伝子が陽性となった検体の全てにマウスのDNAが混入していたことを報告したが、今回は前回用いた検体と同じ血漿(CFS:112、健康人:36)を用いてXMRVのエンベロープ(gp15Eとgp70)に対する抗体の有無を高感度化学発光法によって検討した。2検体を除き全て陰性であった。2検体はgp70にのみ弱陽性を示したが、ウエスタンブロット法では陰性であった。この2検体は、同一の供血者(健康人)が日を変えて提供したものであった。前回の報告でマウスDNAのコンタミによってXMRV遺伝子陽性となった検体も抗体は検出されなかった。
2	Robinson MJ, Tuke PW, Ertwein O, et al. <i>Adv Virol.</i> 2011;2011:782353. Epub 2011 Jun 9.  No Evidence of XMRV or MuLV Sequences in Prostate Cancer, Diffuse Large B-Cell Lymphoma, or the UK Blood Donor Population.	英国	英国の55例の新鮮な前立腺組織と日本(16例)とインド(20例)からのパラフィン包埋した前立腺切片、10例のパラフィン包埋したBリンパ腫からXMRV、及びマウス白血病ウイルスの遺伝子の検出を行い、インドからの検体からXMRVが2例とMuLVが4例検出されたが、全てマウスの遺伝子が混入していた。また、英国の献血者540名とミニプール400検体(19,200の供血者に相当)からもXMRV、及びマウス白血病ウイルスの遺伝子は検出されなかった。英国の供血者にXMRV、及びマウス白血病ウイルスの感染している証拠は見つけられなかった。
3	Kearney MF, Lee K, Bagni RK, et al. <i>Adv Virol.</i> 2011;2011:272193. Epub 2011 Nov 17.  Nucleic Acid, Antibody, and Virus Culture Methods to Detect Xenotropic MLV-Related Virus in Human Blood Samples.	米国	UC-Davisの前立腺癌患者由来の血液108検体からXMRV-RNAとエンベロープとgagに対する抗体の有無、NIHの医療センターの前立腺癌患者26症例からの血液ではXMRVのRNAとDNAの検出、その中の22症例ではエンベロープとgagに対する抗体の有無、さらに12例では培養法によるウイルス検出、また19症例では前立腺組織からのプロウイルスDNAの検体を行った。検討した全ての前立腺癌の患者の血液からXMRV感染の証拠は見つけられなかった。
4	Zhou Y, Steffen I, Montalvo L, et al. <i>Transfusion.</i> 2012 Feb;52(2):332-42.  Development and application of a high-throughput microneutralization assay: lack of xenotropic murine leukemia virus-related virus and/or murine leukemia virus detection in blood donors.	米国	XMRV由来のエンベロープをもつ pseudotypeウイルスを複製し、これを用いてReno/Tahoe地域の354人の献血者の血漿におけるXMRVとマウス白血病ウイルスに対する中和活性を測定した。6.5%の血漿に中程度の中和活性が認められたが、ウエスタンブロット法では何れの検体からもXMRVに対する抗体は検出できなかった。また、感染性ウイルスの検出や核酸増幅法によってもXMRVやマウス白血病ウイルスは検出できなかった。以上からXMRVのエンベロープを介しての感染を阻害する供血者は存在するが、XMRVやマウス白血病ウイルスに暴露されたことによって生じた抗体ではなく、非特異的な機構による阻害である。
5	Steffen I, Tyrrell D.L, Stein E, et al. <i>PLoS one.</i> 2011 volume 6.issue 11,e27870  No Evidence for XMRV Nucleic Acids, infectious Virus or Anti-XMRV Antibodies in Canadian Patients with chronic Fatigue Syndrome	カナダ	カルガリーとエドモントンに住む58人のCFSと診断された患者と57人の健康者から血液を採取し、PCRによるXMRV及び類似マウス白血病ウイルスの遺伝子の検出、ウイルス培養、XMRVに対する抗体検査を実施したが、全て陰性であった。以上から、CFSはこれらのウイルスと関係ないという結論になった。
6	Karafin MS, Stramer SL. <i>Transfusion.</i> 2012 Feb;52(2):222-5  The scientific method at work: xenotropic murine leukemia virus-related virus is neither a cause of chronic fatigue syndrome nor a threat to the blood supply.	米国	自然現象の理解はデータに基づいてなされるべきであり、現時点での自然現象の理解は科学的進歩によってより良いものに置き換えられる、というFrancis Baconの哲学を示しながら、XMRVのこれまでも経緯をまとめている。当初、前立腺癌やCFSの原因と関連していると推定され、健康人からもXMRVが検出されたことから血液の供給のリスクになるとされたが、その後の約30の追試では再現性が得られなかった。また、9つの研究施設が参加した同一検体を測定する研究では、これまで陽性例を報告した施設のみ陽性となった。さらに大規模な献血者での調査や保存されていた供血者と受血者の検体を用いた調査などからXMRVは、実験室由来のウイルスのコンタミであるという結果になった。それを受けてScienceのLombardiらの論文は編集者によって取り下げとなった。さらにPNASに掲載されたLoらの論文は著者らによって取り下げられた。なお、Ian LipkinらのCFS患者からのXMRV検出結果の結論は出されていない。

## Research Article

# Failure to Detect XMRV-Specific Antibodies in the Plasma of CFS Patients Using Highly Sensitive Chemiluminescence Immunoassays

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In 2009, Lombardi et al. reported their startling finding that the gammaretrovirus xenotropic murine leukemia virus-related retrovirus (XMRV) is present in 67% of blood samples of patients suffering from chronic fatigue syndrome (CFS), as opposed to only 3.7% of samples from healthy individuals. However, we and others could not confirm these results, using a nested PCR assay. An alternative to this highly sensitive, but contamination-prone, technique is to measure the serological response to XMRV. Thus, we tested the plasma samples from our cohorts of CFS patients and healthy controls for the presence of XMRV-specific antibodies. Using two novel chemiluminescence immunoassays (CMIA), we show that none of our samples have any XMRV-reactive antibodies. Taken together with our previous findings, we conclude that XMRV is not present in any human individual tested by us, regardless of CFS or healthy control.

## 1. Introduction

In 2006, Urisman et al. identified a new gammaretrovirus in prostate cancer samples harboring a mutation in a viral defense gene known as *RNASEL* [1]. This new virus, xenotropic murine leukemia virus-related retrovirus (XMRV), was found to be a close relative to known murine leukemia viruses (MLVs) and was the first documented case of human infection with a xenotropic retrovirus. Although XMRV was originally associated with the mutant variant of the *RNASEL* gene, further research could not confirm this association but did find it in about 10% of prostate cancers [2].

The discovery of a new virus that could infect humans lead Lombardi et al. [3] to test for the virus in patients suffering from chronic fatigue syndrome (CFS). CFS is a disease of unknown etiology that manifests as neurological, immunological, and endocrinological dysfunctions. A wide range of viruses have been investigated in the past as caus-

ative agents of CFS; however, findings were mixed, and no conclusive evidence of one virus causing CFS has been implicated [4]. Using a nested polymerase chain reaction (PCR), Lombardi et al. found that blood samples of 68 out of 101 (67%) CFS patients contained the XMRV *gag* sequence, as opposed to only 8 out of 212 (3.7%) samples from healthy individuals [3]. The finding of a virus linked to CFS reignited excitement in the field, leading many laboratories around the world to test for this new virus, but the excitement has been short lived. Although some support linking XMRV or MLVs and CFS has been published [3, 5, 6], it has been overshadowed by reports failing to detect the virus in CFS patients [7–20], including a study done by us.

In our original paper [17], we failed to find an association between CFS patients and XMRV, using PCR technology. However, we did detect some XMRV sequences as well as other MLV sequences in some of our samples. Due to the close relationship between XMRV and MLVs, which are

present throughout the mouse genome, we tested all of our samples for mouse DNA using a TaqMan qPCR assay for murine mitochondrial cytochrome oxidase, *cox2* [14], as well as a single PCR assay for the highly abundant intracisternal A-type particle (IAP) long terminal repeat sequence, developed by our group [17]. We found that every sample that contained an XMRV or MLV sequence was also positive for mouse DNA contamination. Although we did not claim that our findings provided a full explanation of the origin of XMRV, we put forward a cautionary tale about the risks of mouse DNA contamination in various common laboratory reagents.

One of the criticisms of our study [17] was that we only used PCR technology to test for the presence of XMRV, while the original paper also included serological analyses [3]. Specifically, some groups have developed novel serological tests utilizing western blots and ELISAs in the search for anti-XMRV antibodies, because the presence of antibodies could not be due to mouse DNA contamination [3, 8, 13, 14, 20, 21]. Recently, two prototype direct format chemiluminescent immunoassays (CMIA) were developed to detect XMRV-specific antibodies [22]. Both CMIA utilize a direct assay format in which recombinant p15E or gp70 protein serves as both capture and detection antigens. The assays demonstrated excellent sensitivity, detecting early seroconversion bleeds in XMRV-infected rhesus macaques [22]. Moreover, these assays were also shown to detect specific antibodies to MLVs [22]. In this study, we use these two sensitive CMIA to screen plasma samples from our blinded cohorts for the presence of XMRV-specific antibodies. No samples from our cohort of over 100 CFS patients were positive in either of these assays, while two samples from the healthy control cohort tested positive in one of the CMIA assays; however, reactivity of these same samples was not confirmed by western blot. Thus, these highly sensitive serological studies have confirmed our prior conclusion that the positive XMRV PCR results were a result of mouse DNA contamination, since no antibodies against XMRV were present.

## 2. Materials and Methods

**2.1. Sample Collection.** All samples were collected according to the institutional guidelines of Tufts University, after receiving informed consent. The 36 healthy individuals (15 females and 21 males) were recruited on a voluntary basis by the Huber laboratory and were between 18 and 65 years of age. The 112 CFS patients (90 females, 20 males, and 3 unknown), recruited by Dr. Susan Levine, were between 18 and 65 years of age and resided in the Northeastern United States. All patients were diagnosed for CFS according to the CDC criteria, and the majority was completely disabled. The cohort comprised a combination of those with an abrupt and others with a gradual onset of symptoms.

**2.2. Preparation of Human Blood and Plasma Samples.** Approximately 30 mL of blood were drawn into three heparinized tubes (Becton Dickinson) and shipped overnight (CFS patients) or processed immediately (healthy controls). The

blood collection tubes from each individual were consolidated into one 50 mL tube and diluted with PBS, containing CaCl<sub>2</sub> and MgCl<sub>2</sub> (sigma) at a 1 : 1 ratio. 15 mL of Ficoll (GE Healthcare) was added to two new 50 mL tubes, and 25 mL of the diluted blood was gently layered on top of the Ficoll, followed by a 30 min centrifugation in a Sorvall RT7 plus rotor at 2000 rpm at room temperature. The PBMCs were collected from the interface following the spin and were used for DNA isolation. Ten mL of plasma were also collected from each sample and stored at -80°C. One ml of plasma was sent to Abbott Labs on dry ice overnight for further testing.

**2.3. XMRV Chemiluminescent Immunoassays (CMIA).** A detailed procedure can be seen here [22]. Briefly, 100 µL of neat plasma were screened for antibodies to XMRV gp70 and p15E proteins using two prototype ARCHITECT chemiluminescent immunoassays (CMIA; Abbott Diagnostics, Abbott Park, Ill). The CMIA utilize a direct assay format in which *E. coli*-expressed XMRV p15E or mammalian-expressed XMRV gp70 were used as both capture and detection antigens. Assay positive controls were derived from XMRV-infected macaque plasmas at 1:1000 (PC1) or 1:4000 (PC2). A pool of normal human plasma was used as negative control (NC) and as sample diluents. Cutoff (CO) values of the ARCHITECT CMIA were calculated based on the following formulas:  $CO = 0.45 \times (\text{Calibrator 1 Mean Relative Light Units (RLU)})$  for p15E CMIA and  $CO = 0.078 \times (\text{Calibrator 2 Mean RLU})$  for gp70 CMIA. Assay results were reported as the ratio of the sample RLU to the cutoff RLU (S/CO) for each specimen. Specimens with S/CO values <1.00 were considered nonreactive; specimens with S/CO values ≥1.00 were considered initially reactive. The S/CO values of the NC, PC1, and PC2 were 0.16, 12.8, and 3.5 for the gp70 CMIA and 0.13, 7.4, and 2.2 for the p15E CMIA. Initially reactive specimens were retested in duplicate by either ARCHITECT p15E or gp70 CMIA. Repeatedly reactive specimens were analyzed at 1 : 100 dilution by investigational western blot assays using purified XMRV viral lysate as well as recombinant gp70 protein.

**2.4. Western Blot Analysis.** Western blot (WB) analysis using purified XMRV viral lysate as well as recombinant gp70 protein was performed as described [22]. Briefly, viral lysate (80 µg/gel) or recombinant gp70 protein (20 µg/gel) were separated by electrophoresis on a 4–12% NuPAGE Bis-Tris 2-dimension gel (Invitrogen, Carlsbad, Calif) in the presence of sodium dodecyl sulfate (SDS). 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 with human samples diluted 1:100 or XMRV-infected macaque plasma diluted 1:200 overnight at 2–8°C. After removal of unbound antibodies, strips were incubated with alkaline phosphatase conjugated goat antihuman IgG (Southern Biotech, Birmingham, Ala) for 30 minutes at room temperature. The strips were washed, and chromogenic substrate solution was added.

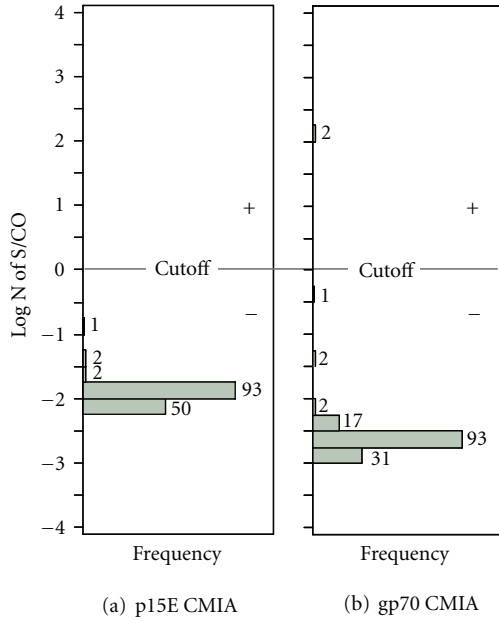


FIGURE 1: Distribution of p15E CMIA (a) and gp70 CMIA (b) log N of S/CO on 148 samples collected from 112 CFS patients and 36 healthy controls. Numbers of specimens within each log N of S/CO value are shown above the solid bars. Assay cutoffs were equivalent to mean 16 SD and 12 SD for p15E and gp70 CMIA, respectively, based on blood donor populations [22]. Log N of S/CO, natural log transformation of S/CO.

### 3. Results

148 blinded plasma samples from our original CFS and healthy control cohorts were analyzed for the presence of XMRV-specific antibodies, using the direct format ARCHITECT p15E and gp70 CMIA. None of the 148 plasma samples were reactive in the p15E CMIA (Figure 1(a)). Two of the 148 samples (ID = 137, 138) were positive in the gp70 CMIA (Figure 1(b)). Both specimens were weakly reactive in the gp70 CMIA with sample/cut-off (S/CO) values of 7.77 (log N of S/CO = 2.05) and 9.02 (log N of S/CO = 2.20), respectively. Although the samples were repeat reactive in the gp70 CMIA, they were not reactive by WB. As shown in Figure 2, both samples showed no visible WB bands using either XMRV viral lysate proteins (Figure 2(a)) or recombinant gp70 protein (Figure 2(b)). Unblinding of the samples revealed that the two gp70 reactive samples stemmed from two sequential blood collections of a single healthy control (Table 1).

### 4. Discussion

In our original study, we found no specific relationship between the presence of XMRV and CFS [17]. However, screening the genomic DNA from peripheral blood lymphocytes of both healthy control and CFS cohorts, we did detect PCR products that were identical to XMRV *gag* sequences, as well as other MLV *gag* sequences. Due to the high number of MLV sequences in the mouse genomic DNA, we found

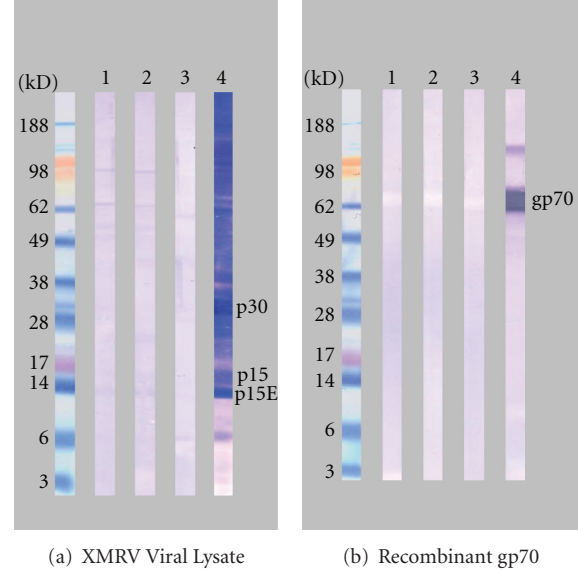


FIGURE 2: WB analysis of gp70 CMIA reactive samples with (a) native XMRV viral proteins and (b) recombinant gp70. WB strip key: 1 & 2: gp70 reactive samples 137 and 138; 3: normal blood donor plasma as negative control; 4: XMRV-infected macaque plasma as positive control. The faint white band in the 65–70 kD region in (B, strips no. 1–3) indicates a lack of specific anti-gp70 antibody.

it prudent to test for mouse DNA contamination in our samples. Using both a test developed by the Switzer lab at CDC for mouse mitochondrial DNA [14], as well as a test developed by the Coffin lab for the IAP [17], we found that every sample that was positive for XMRV or other MLVs PCR products was also positive for mouse DNA. Although these data provide an explanation for the detection of MLV sequences in our samples, they do not rule out the possibility that XMRV and mouse DNA contamination could be present in the same sample. To clarify this issue, we tested our plasma samples for the presence of XMRV-specific antibodies.

Recent animal studies showed that XMRV infection elicited a potent humoral immune response in rhesus macaques [22]. The infected macaques developed XMRV-specific antibodies within two weeks of infection and persisted more than 158 days. The predominant responses were to all three structural proteins of XMRV: the envelope protein gp70, the transmembrane protein p15E, and the capsid protein p30 [22]. Sensitivity of both p15E and gp70 CMIA was validated by the animal model; both CMIA were able to detect p15E or gp70 specific antibodies as early as day 9 after infection [22]. In contrast, we were unable to detect XMRV p15E or gp70 specific antibodies in the 112 CFS patients and the 36 healthy controls. Although 2 samples from the same healthy control had weak reactivity in gp70 CMIA, the reactivity was not confirmed by recombinant gp70 WB. Furthermore, both samples were nonreactive in p15E CMIA and had no detectable p15E and p30 antibodies by viral lysate WB. Considered in combination with the negative PCR data, the observed isolated and weak gp70 reactivity



TABLE 1: Results summary for XMRV positive PCR samples. All samples that tested positive for XMRV *gag* sequence in original study [17], as well as the two samples that reacted with the gp70 CMIA, are displayed. Bolded samples showed the VP42 *gag* sequence but did not react with the CMIA. The italic data shows the two samples that were reactive in the gp70 CMIA. CMIA values less than one are considered nonreactive. XMRV GAG: Nested *gag* PCR. Mcox: murine mitochondrial cytochrome oxidase qPCR. IAP: Intracisternal A-type particle PCR.

ID	Unblinded ID	XMRV GAG	PCR results			Initial test	Initial test	Repeat test
			Mcox	IAP	p15E S/CO	gp70 S/CO	gp70 S/CO	
72	TH72.1	+	+	+	0.38	0.06		
128	TH04.1	+	+	+	0.16	0.07		
129	TH01.7	+	+	+	0.15	0.06		
131	TH01.8	+	–	+	0.12	0.06		
132	TH01.3	+	+	+	0.15	0.06		
<b>134</b>	<b>TH06.1</b>	+	+	+	<b>0.15</b>	<b>0.07</b>		
135	TH01.1	+	+	+	0.14	0.09		
136	TH05.1	+	+	+	0.16	0.06		
<i>137</i>	<i>TH07.1</i>	+	+	+	<b>0.16</b>	<b>7.77</b>	<b>7.17, 7.21</b>	
<i>138</i>	<i>TH07.2</i>	–	–	–	<b>0.14</b>	<b>9.02</b>	<b>8.65, 8.77</b>	
143	TH10.1	+	+	+	0.14	0.07		
144	TH11.1	+	–	+	0.14	0.06		
147	TH02.1	+	+	+	0.14	0.07		
152	TH01.5	+	+	+	0.13	0.07		
153	TH21.1	+	+	+	0.15	0.07		
155	TH20.1	+	+	+	0.16	0.06		
156	TH02.2	+	+	+	0.17	0.07		
<b>158</b>	<b>TH08.1</b>	+	+	+	<b>0.13</b>	<b>0.07</b>		
<b>160</b>	<b>TH03.1</b>	+	+	+	<b>0.13</b>	<b>0.07</b>		
161	TH12.1	+	+	+	0.11	0.06		
163	TH19.1	+	+	+	0.16	0.72	0.75, 0.72	
164	TH16.1	+	+	+	0.15	0.07		

most likely represents nonspecific reactivity since specificity of the gp70 CMIA was reported as 99.5% [22]. In summary, the serologic data obtained in this study suggests a lack of XMRV infection in our CFS patients and healthy controls. It is theoretically possible that XMRV replicates at very low levels in humans and fails to induce a humoral immune response, or, alternatively, that it is sequestered or latent and specific antibody titers have declined to undetectable levels over time. Although these possibilities cannot be formally excluded, they seem unlikely given responses observed to other human retroviruses. The combination of negative molecular and serologic data do not support an association between CFS and XMRV or other MLVs. Furthermore, the recent demonstration that XMRV is a recombinant of two murine MLVs (23) raises doubts about the validity (24) of the original XMRV claims in CFS (3).

## 5. Conclusion

With the serological data added to our original finding, we can unequivocally conclude that XMRV is not present in our CFS patient or healthy control cohort samples. Although we have detected XMRV *gag* sequences in three of our samples,

they all tested positive for mouse DNA and tested negative for XMRV-specific antibodies. Laboratory mouse strains, as well as wild mice, all carry numerous endogenous MLVs, and extreme caution must be taken when testing for murine-related viruses.

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## Research Article

# No Evidence of XMRV or MuLV Sequences in Prostate Cancer, Diffuse Large B-Cell Lymphoma, or the UK Blood Donor Population

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Xenotropic murine leukaemia virus-related virus (XMRV) is a recently described retrovirus which has been claimed to infect humans and cause associated pathology. Initially identified in the US in patients with prostate cancer and subsequently in patients with chronic fatigue syndrome, doubt now exists that XMRV is a human pathogen. We studied the prevalence of genetic sequences of XMRV and related MuLV sequences in human prostate cancer, from B cell lymphoma patients and from UK blood donors. Nucleic acid was extracted from fresh prostate tissue biopsies, formalin-fixed paraffin-embedded (FFPE) prostate tissue and FFPE B-cell lymphoma. The presence of XMRV-specific LTR or MuLV generic *gag*-like sequences was investigated by nested PCR. To control for mouse DNA contamination, a PCR that detected intracisternal A-type particle (IAP) sequences was included. In addition, DNA and RNA were extracted from whole blood taken from UK blood donors and screened for XMRV sequences by real-time PCR. XMRV or MuLV-like sequences were not amplified from tissue samples. Occasionally MuLV *gag* and XMRV-LTR sequences were amplified from Indian prostate cancer samples, but were always detected in conjunction with contaminating murine genomic DNA. We found no evidence of XMRV or MuLV infection in the UK blood donors.

## 1. Introduction

In 2006, a new gammaretrovirus, xenotropic murine leukaemia virus-related virus (XMRV), was discovered by the Virochip analysis in prostate cancer tissue from patients homozygous for an RNase L mutation [1]. In these patients, the innate antiviral defence RNase L pathway is defective; hence,

these patients are likely to be susceptible to viral infection and a population more likely to find a novel virus with disease association in. When a second US study found that 6% of all prostate cancer patients, independent of RNase L mutations, were infected with the virus, thus broadening the population at risk [2], interest in XMRV intensified. However, subsequent studies from the USA [3, 4] and all

European studies [5–7] failed to confirm the presence of XMRV in prostate tissue. More recently it has been suggested that XMRV detection in prostate tissue in the US could be related to the specificity and conditions of the PCR used [8].

In 2009, Lombardi and colleagues reported the presence of XMRV proviral DNA in peripheral blood leucocytes from 3.7% of healthy controls and 67% of patients with chronic fatigue syndrome (CFS) [9]. The detection rate by PCR amplification of XMRV proviral DNA subsequently reduced the estimated CFS prevalence to 7%, with the explanation that RNA extraction and cDNA synthesis had been required to achieve the 67% prevalence originally reported [10]. Lo and colleagues (2010) using predominantly archival material from patients with CFS detected a high prevalence (86.5%) of pMuLVs. These are similar to, but constitute a different group from, the xenotropic endogenous MuLVs to which XMRV belongs [11]. However, questions were raised about how these data were generated [12], and a number of other studies have failed to demonstrate a link between XMRV or pMuLV infection and CFS [13–19].

The causes of B-cell lymphoma are not fully understood [20], but the clinical and epidemiological characteristics are suggestive of the involvement of an infectious agent [21]. Several viruses [22, 23] have been linked to the risk of B-cell lymphoma, most notably EBV [24–26], and retroviruses are implicated in animal leukaemias. Retroviral integration could cause somatic DNA changes leading to clonal expansion of B cells resulting in leukaemia as has been previously described for adult T-cell leukaemia (ATL) and HTLV-1 [27].

The geographical discrepancy of XMRV and pMuLV prevalence remains unexplained. To explore this further, we have tested a variety of tissues from diverse populations; prostate cancer (PC) formalin-fixed paraffin-embedded (FFPE) tissue from Japan and India, fresh prostate tissue samples received from the Urology Clinic at St Mary's Hospital, London, and peripheral blood from English blood donors.

A series of recent papers [28–31] have demonstrated the ease with which specimens can be contaminated with murine DNA sequences. To control for this, all tissue specimens were tested by PCR specific for intracisternal A particle (IAP), a retrotransposon present in multiple copies (~1000) within the mouse genome [32].

## 2. Methods and Materials

**2.1. Samples and Nucleic Acid Isolation.** Prostate biopsies were collected from 55 patients admitted to the Urology Department, St. Mary's Hospital, London, UK to undergo routine biopsy for prostate cancer screening. All patients gave written informed consent for their tissue to be banked for the purposes of research (ethics number 99/CCC/166, August 1999). The DNA was extracted using the QIAamp DNA mini kit (Qiagen, Crawley, UK) following the manufacturer's instructions.

B-cell lymphoma samples were provided by Professor Kikkiri Naresh, Centre for Pathology, Hammersmith Hospital, London, UK. The DNA from 10 Diffuse Large B-cell

Lymphoma (DLBCL) patients was extracted from FFPE tissues of lymph nodal or extranodal diffuse large B-cell lymphoma using the DNeasy Blood & Tissue Kit (Qiagen). Briefly, two 15  $\mu$ m sections were cut and transferred to 1.5 mL Eppendorf tubes. Blades were changed between samples to avoid cross-contamination. Sections were deparaffinised with xylene and ethanol, rehydrated, and incubated with proteinase K and lysis buffer in a shaking water bath at 55°C overnight and the extraction was completed according to the manufacturer's instructions.

Twenty FFPE prostate specimens including 10 prostate cancer (PC) and 10 benign prostatic hyperplasia (BPH) samples were supplied by Professor Ganesh Golpalakrishnan of Vedanayagam Hospital, RS Puram, Coimbatore, India and sixteen specimens from Dr. Takahiro Kimura of the Department of Urology, The Jikei University School of Medicine, Japan. From the Indian blocks, two 10  $\mu$ m sections were extracted with the QIAamp DNA FFPE tissue kit (Qiagen), according to the manufacturer's instructions. The Japanese samples were provided presliced on glass slides.

Random anonymous whole-blood samples were obtained from the Donation Testing Department at the National Health Service Blood and Transplant (NHSBT) Centre at Colindale, London, UK. Plasma minipools were similarly obtained from NHSBT. All blood and plasma samples were extracted on a Qiagen MDx Biorobot and eluted with 80  $\mu$ L of Qiagen buffer AVE.

**2.2. XMRV, MuLV, and Control Nested PCR.** Samples were tested for the presence of XMRV and MuLV proviral DNA using nested PCR, as described previously [14]. Briefly, we used a set of primers that encompasses the 24 bp deletion in the XMRV *gag* leader region, originally described to distinguish XMRV as a new human virus, along with a second set of primers reflecting a sequence conserved amongst most MuLVs. The positive control for the XMRV and MuLV PCRs was plasmid VP62 [1]. The PCR method has been shown to be sensitive enough to pick up one copy of XMRV VP62 plasmid in a background of 500 ng DNA [28]. As a control for sample addition and PCR inhibition, primers to the human beta-globin (hBG) gene were used. DNA extracted from LNCaP (human prostate cancer cells) was used as a positive control for human beta globin. To control for contamination of samples with murine DNA, primers specific to mouse IAP were used as described previously [28]. The positive control for IAP was DNA from the McCoy cell (murine fibroblast cells, ECAAC 90010305). In all PCRs, at least 6 "no template" controls were set up. All PCR products were visualised on Ethidium Bromide-stained 2% agarose gels.

**2.3. XMRV, MuLV, and Control Real-Time PCRs for Blood Donor Studies.** Real-time PCR was performed as detailed in Table 1. For the proviral DNA analysis, 10  $\mu$ L of the nucleic acid extract were analysed separately in three individual quantitative PCRs (Q-PCRs).

**2.3.1. XMRV Q-PCR and Internal Control.** Samples were tested by Q-PCR for XMRV, as described by McCormick et al. [33] and modified as detailed in Table 1. In a Q-PCR to



TABLE 1: Details of PCRs used to test blood sample.

Sample tested	PCR	Target	Primers and probes	Cycles ( <i>N</i> )	Reagents
540 DNAs from whole blood	XMRV Taq Man	gag	XMRV Probe, F, R	60	Qiagen QuantiTect Probe kit
540 DNAs from whole blood	SBCMV Taq Man	SBCMV plasmid	SBCWMVCPF, SBCWMVCPR, SBWMV237F	45	ABgene ABsolute QPCR ROX mastermix
540 DNAs from whole blood	PDH Taq Man	PDH human gene	PDH Probe, F, R	45	ABgene ABsolute QPCR ROX mastermix
600 NAs from whole blood 400 NAs from plasma minipools	XMRV/pMuLV RT Taq Man with BMV RT Taq Man	gag BMV	P2, F3, R4 BMV Probe, F, R	45	Qiagen QuantiTect Probe RT-PCR kit

The TaqMan assay conditions were 15 min at 95°C (15 secs 95°C, 1 min 60°C) × *N* cycles. 400 nM concentrations of primers, and 200 nM probes were used in all the TaqMan assays with the exception of the CDC MuLV Taq Man where the concentration of each probe was 100 nM and the PDH TaqMan where the primer concentrations were 50 nM.

control for the extraction efficiency and amplification inhibition coextracted soil-borne cereal mosaic virus (SBCMV) plasmid DNA was used, ( $5.4 \times 10^6$  copies were added to the 33 mL of Qiagen lysis buffer AL used for extracting 96 samples on the MDx Biorobot). This reaction was as described by Ratti et al. [34]. The primer sequences for this reaction were *SBCWMVCPF* (5'-CAC TCA GGA CGG TGA CGA GAT-3'), *SBCWMVCPR* (5'-GTG ATA CTG TGA GTC TGG TGA TGA TTT-3') and probe *SBWMV237Fa* (5' JOE-TTT TGT GAC CTT GGA GGT GAG GCA GTT ATG-BHQ1-3').

**2.3.2. Q-PCR for Quantification of Human DNA.** The input of human DNA in each extract was measured by a Q-PCR for the Pyruvate dehydrogenase (PDH) gene. Primers used *PDH Taq 1* (5'-TGA AAG TTA TAC AAA ATT GAG GTC ACT GTT-3'), *PDH Taq 2* (5' - TCC ACA GCC CTC GAC TAA CC -3') with probe *PDHP* (5'-VIC-CCC CCA GAT ACA CTT AAG GGA TCA ACT CTT AAT TGT-Tamra-3'). Positive control for this reaction was a dilution series of human male DNA (Applied Biosystems, Warrington, UK, Catalogue no. 4312660). The XMRV Q-PCR results were validated when the PDH threshold cycle (Ct) value was greater than the mean Ct minus 3 SD, and the SBCMV control was greater than the mean Ct minus 2 SD. Samples invalid on either control were excluded from the analysis.

**2.4. Detection of Gag Sequences by Nested PCR in Blood Donors.** Nuclease-free water (Severn Biotech, Kidderminster, UK) was used throughout for the cDNA and PCR mix preparations and as no-template controls. Nucleic acid extracts were tested by nested PCR using the *gag* primers as described by Lombardi et al. [9] and Lo et al. [11], but using Applied Biosystems Taq Gold LD PCR enzyme (Table 1) to overcome the problem of false positives that have arisen from the use of Invitrogen Taq Polymerase [30].

**2.5. QRT-PCR Amplification of XMRV/pMuLV in Blood Donors.** An XMRV/pMuLV *gag* QRT-PCR assay described

by Lo and colleagues [11] but modified to detect the pMuLVs was used to test nucleic acid from whole blood, plasma, and from plasma minipools. Further details of all QPCR and QRT-PCR reactions are listed in Table 1. The primers for this reaction were *F3* (5'-ACC GTT TGT CTC TCC TAA AC-3') and *R4* (5'-AGG GTA AAG GGC AGA TCG-3'), with probe *P2* (5'-Fam-CCG ACA GCT CCC GTC CTC CCG-Tamra-3'). Nuclease-free water (Severn Biotech) was used throughout for the RT-PCR mix preparations and as no template controls. RT-PCR was performed in a total volume of 50  $\mu$ L, containing 1x Qiagen QuantiTect RT-PCR buffer and primers, and probes as detailed in Table 1. Synthesis conditions were 50°C for 30 mins, followed by 95°C for 15 mins and 45 cycles of 95°C for 15 secs 60°C for 1 min. Twenty  $\mu$ L of nucleic acid was analysed in a QRT-PCR which multiplexed the XMRV/pMuLV TaqMan with the internal control TaqMan reaction (Brome mosaic virus (BMV)) [35]. The BMV RNA was added to the Qiagen AL lysis buffer and co-extracted with the sample. A sample was valid if the BMV Ct value was greater than the mean Ct minus 2 SD. Samples invalid on the BMV control were excluded from the analysis. The sensitivity of this QRT-PCR was determined as 150 RNA copies/mL (75 viral particles/mL) by calculation from the observed frequency of negatives using the Poisson distribution.

### 3. Results

**3.1. XMRV Detection in Tissue Samples by Nested PCR.** A representative stained gel following nested PCR is shown in Figure 1. For routine analysis, 0.11 pg of plasmid DNA (representing approximately 7000 copies/PCR) was used as positive control for XMRV and MuLV. All samples were positive for hBG sequences by PCR. The sensitivity of the IAP PCR has been shown previously to detect as little as 0.0011 pg DNA in a background of 500 ng DNA [28]. The results are summarised in Table 2(a). No evidence of XMRV or MuLV was found in any of the FFPE prostate tissue samples from Japan or the fresh prostate tissues from the

TABLE 2

(a) Amplification from fresh and FFPE tissues by nested PCR

	Fresh prostate tissue	Japan samples	Indian samples	LCBCL samples
Number of cancerous samples	16/55	16	10/20	10
Number of noncancerous samples (unknown status)	18/55 (21/55)	0	10/20	0
Mean age (range)	unknown	unknown	72 (62–85)	43 (27–83)
Beta globin +	55/55	16/16	20/20	10/10
XMRV +	0	0	2/20	0
MuLV +	0	0	4/20	0
IAP +	0	0	5/20	0
mtDNA +	nd	nd	2/10	nd

(b) Specific PCR results from Indian samples

Indian sample number	Cancer status	PCR result using specific primers				
		$\beta$ -globin	IAP	mtDNA	MLV <i>gag</i>	XMRV LTR
6489c/10	cancer	+	+	+	+	+
5383c/10	cancer	+	+	–	+	+
5406a3/10	cancer	+	+	–	–	–
2896c/10	BPH	+	+	+	+	–
5349c/10	cancer	+	+	–	+	–

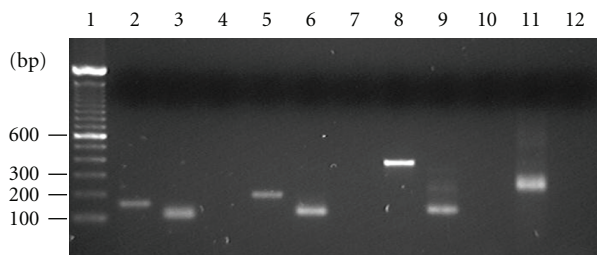


FIGURE 1: lane 1: MWM; lanes 2–4:  $\beta$ -globin PCR on LNCaP DNA template 1st round product, 2nd round product, and no-template control; lanes 5–7: XMRV LTR PCR on VP62 plasmid DNA template 1st round product, 2nd round product, and no-template control; lanes 8–10: MuLV *gag* PCR on VP62 plasmid DNA template 1st round product, 2nd round product, and no-template control; lanes 11–12: IAP PCR on McCoy cell DNA template and no-template control.

UK. Of the 20 Indian samples, four (20%) produced a PCR signal with the MuLV *gag* primers (three prostate cancer, one benign prostatic hyperplasia) and of these, 2/4 were positive with XMRV LTR primers (both prostate cancer). The IAP PCR was applied to the same samples to see if the positive signal was due to mouse DNA contamination. All MuLV/XMRV amplification was concordant with IAP amplification, except for one prostate cancer sample which was positive for IAP without MuLV/XMRV amplification. Confirmation of murine DNA contamination was achieved using PCR primers specific to mouse mitochondrial DNA (mtDNA). Although this PCR has been shown to be less sensitive than IAP PCR [28], 2/20 of the Indian samples

(one prostate cancer, one benign prostatic hyperplasia) were positive for mtDNA. In both of these samples, IAP and MuLV *gag* sequences were amplified. Additionally, one was positive for XMRV (detailed in Table 2(b)).

No evidence of MuLV or XMRV sequences was discovered in the DLBCL samples and none of the DCBCL samples gave an IAP specific product.

**3.2. XMRV Detection in Whole Blood by Real-Time PCR.** XMRV proviral DNA was not amplified from whole-blood extracts derived from 540 donors. The average DNA input for each amplification was 93,000 cells (approx 0.56  $\mu$ g). Detection of XMRV/MuLV RNA was undertaken on a further 600 donors and 400 plasma minipools, derived from 19,200 individual donations. All samples tested negative for XMRV and MuLV sequences.

## 4. Discussion

Using highly sensitive PCRs with primers that detect XMRV and primers that detect MuLV-like sequences, no proviral DNA was detected in any of the prostate cancer samples independently of murine DNA contamination. This served to confirm our previous studies in which FFPE prostate tissue was tested and XMRV/MuLV sequences failed to be amplified [28]. Here we have added further data to show that no XMRV or MuLV-like sequences can be detected in fresh UK prostate tissue or in prostate cancer samples collected from Japan. Samples from India showed evidence of MuLV and XMRV sequences when viral genomic sequences were amplified by nested PCR. However, this was concordant with murine genomic DNA contamination detected using primers

to IAP. IAPs are retrotransposons present at the level of around 1000 copies per mouse genome [30]. Thus, IAP PCR represents a highly sensitive detection method for murine DNA. Although the sample size was small ( $n = 10$ ), we found no evidence to suggest that XMRV might be involved in other cancers, such as diffuse large B-cell lymphoma.

It was reported last year that XMRV had been detected in greater than 60% of 50 samples from English blood donors [36]. In contrast, we found no evidence of XMRV or pMuLV in any of 540 whole-blood samples from unselected NHSBT donors nor were we able to detect MuLV-like sequences in either the DNA from whole blood or cDNA prepared from the plasma minipools from donors in England. There are three possible explanations for this. Firstly, there are no MuLV infections in blood donors in England. Secondly, there are MuLV infections, but that the assays used failed to detect them, either due to sensitivity or sequence variation. Thirdly, there are MuLV infections, but the prevalence is too low to be detected in the sample sizes tested.

Research into the presence of MuLVs in the human population is contentious, given discrepant findings [37–39]. Contamination from sequences contained in apparently XMRV-positive samples, amplified products, or plasmids has been suggested as a reason for the finding of MuLVs in human samples [30, 40]. A study of XMRV in patients with CFS or chronic immunomodulatory conditions, using Invitrogen Platinum Taq (IPT), reported a *gag* sequence with >99% homology to a mouse endogenous retrovirus [19]. This was designated as contamination, although the paper failed to speculate on the source of this sequence. Sato and colleagues (2010) recently reported finding predominantly RNA sequences, related to a pMuLV, in IPT containing reagents [30]. Another study concluded that the detection of MuLV-related sequences in human samples could be due to contamination with mouse DNA, most likely contained in various laboratory reagents [29]. We have demonstrated that murine sequences can be present in prostate sections, resulting in false positive detection of XMRV [28]. A phylogenetic overview concluded that the proviral sequences present in the genome of 22Rv1 cell line were ancestral to the published XMRV sequences [31]; finally, it has been shown that the mapping of integration sites of XMRV in prostate cancer tissues, thought to unequivocally confirm the existence of XMRV in clinical samples, was at least partially contaminant derived [41], further emphasising the ease with which contamination can occur.

The sources of contamination are still to be fully elucidated. However, given that most retroviral laboratories have worked with MuLV or MuLV-derived vector systems, or at least used murine reagents, it is essential that sufficient appropriate controls are included in all PCRs.

The absence of MuLVs from all the samples analysed in this study, where there was no concomitant detection of murine genomic sequences, adds weight to the growing body of data questioning the evidence for murine retrovirus infection of humans [42]. It is always challenging to prove a negative result, but it is likely that XMRV will be added to the long list of RNA tumour viruses [43].

## Conflict of Interests

The authors declare no conflict of interests.

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## Research Article

# Nucleic Acid, Antibody, and Virus Culture Methods to Detect Xenotropic MLV-Related Virus in Human Blood Samples

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The MLV-related retrovirus, XMRV, was recently identified and reported to be associated with both prostate cancer and chronic fatigue syndrome. At the National Cancer Institute-Frederick, MD (NCI-Frederick), we developed highly sensitive methods to detect XMRV nucleic acids, antibodies, and replication competent virus. Analysis of XMRV-spiked samples and/or specimens from two pigtail macaques experimentally inoculated with 22Rv1 cell-derived XMRV confirmed the ability of the assays used to detect XMRV RNA and DNA, and culture isolatable virus when present, along with XMRV reactive antibody responses. Using these assays, we did not detect evidence of XMRV in blood samples ( $N = 134$ ) or prostate specimens ( $N = 19$ ) from two independent cohorts of patients with prostate cancer. Previous studies detected XMRV in prostate tissues. In the present study, we primarily investigated the levels of XMRV in blood plasma samples collected from patients with prostate cancer. These results demonstrate that while XMRV-related assays developed at the NCI-Frederick can readily measure XMRV nucleic acids, antibodies, and replication competent virus, no evidence of XMRV was found in the blood of patients with prostate cancer.

## 1. Introduction

Xenotropic murine leukemia virus-related virus (XMRV) is a recently discovered gammaretrovirus reportedly associated with prostate cancer and chronic fatigue syndrome (CFS) [1, 2]. The discovery of XMRV arose from studies investigating a potential viral cause for diseases in patients with an *RNAseL* gene variant. This genotype, which is observed in a varying subset of patients in cohorts with prostate cancer [1, 3–8], has been associated with impairment of innate immune responses to viral infections [5]. Seeking an etiologically significant viral infection associated with impaired *RNAseL*-dependent responses, Urisman et al. first identified XMRV

in 2006 in a cohort of prostate cancer patients [2]. The association of XMRV with prostate cancer, but not its association with the *RNAseL* variant, was corroborated by Schlaberg et al. in 2009 [9]. The prostate cancer studies were followed by a report from Lombardi et al. presenting evidence for XMRV infection in 67% of individuals with severe CFS, compared to 3.7% of healthy individuals [1]. These high reported frequencies of XMRV infection and putative linkage to a debilitating illness prompted concerns about the possibility of a new, widespread retroviral epidemic and stimulated additional research towards determining the prevalence of XMRV infection in different populations worldwide.

Several studies supporting high prevalence of XMRV infection followed. For example, Arnold et al. detected anti-XMRV antibodies in 27% of individuals with prostate cancer [10], Schlager et al. found XMRV nucleic acid in 23% of prostate cancers and 4% of controls [11], and Danielson et al. detected XMRV in 22.8% of extracted prostate tissues from individuals who had radical prostatectomies [12]. However, controversy arose when other laboratories could not demonstrate comparable findings in similar cohorts not only in the US [13] but in Germany [14], The Netherlands [15], and England [16, 17]. Adding to the controversy, Lo et al. reported the presence of mouse retroviral sequences, but not XMRV, in 86.5% of CFS patients [18]. Claims were made that such findings supported the association of XMRV infection with CFS, complicating an already controversial field.

Several factors were speculatively proposed to contribute to the differential detection of XMRV/MLVs by different laboratories. It was suggested that inconsistencies in detection of XMRV/MLVs in patient samples could result from varied prevalence of infection in different populations, differing criteria for patient selection, and differing detection methodologies utilized [19]. It was also proposed that virus levels may be chronically low or episodic in patient plasma or tissues, making virus detection difficult [19]. Adding to the complexity, detection of XMRV by PCR is highly susceptible to false positive results due to the very close genetic relationship of XMRV with endogenous MLVs and the high prevalence of contaminating mouse genomic DNA in many specimens [20, 21]. Indeed, studies have suggested that XMRV detection is the result of laboratory contamination from infected cell lines [22–25] or contaminated reagents [26]. Further suggestions of laboratory contamination came after publication of a study by Paprotka et al. [25], showing that XMRV originated in a human cancer cell line generated by passaging prostate cancer cells through immunocompromised mice. This result indicates that XMRV could not have entered the human population until recently, yet was already being reported as prevalent in a sizeable fraction of prostatic cancers. Furthermore, it showed that most “XMRV-specific” detection assays could, in fact, detect one or the other of the two parental proviruses (PreXMRV-1 and 2) that gave rise to XMRV and are endogenous to some inbred and wild mice. In assessing this situation, it became clear that to rule out false positive results and reliably detect XMRV infection, one must apply several diagnostic methods used in conjunction with known positive and negative controls.

At the NCI-Frederick, we sought to help clarify the XMRV controversy by generating multiple assays, including rigorous methods to measure antibodies to XMRV through ELISA-based methods, to quantify XMRV proviral DNA and viral RNA through quantitative PCR and RT-PCR methods, and to measure infectious virus by viral isolation cultures using an indicator cell line system. We characterized these assays using available positive and negative control samples, including spiked samples and specimens from two pigtail macaques experimentally inoculated with XMRV. We then applied these methods to specimens from two cohorts of prostate cancer patients to determine the levels of XMRV in their blood. Overall, we observed a high level of concordance

between detection methods and were able to rule out false positive results by applying multiple assays on the same patient samples. Applying this approach, we did not find evidence of XMRV infection in any of the prostate cancer patient-derived specimens studied.

## 2. Methods

*2.1. Clinical Prostate Cancer Samples.* The XMRV detection assays developed at the NCI-Frederick were applied to samples collected from two cohorts of prostate cancer patients. In total, 134 patients were studied. Plasma samples from 108 patients were obtained at the UC Davis Cancer Center. Samples were collected between 2006 and 2010 from prostate cancer patients who were either newly diagnosed, on active treatment, or undergoing post-treatment monitoring. Plasma from all 108 patients was tested for XMRV RNA and antibodies to CA and TM. Institutional Review Board (IRB) approval was obtained from the UC Davis Cancer Center Biorepository, and all study subjects provided written informed consent.

Samples from an additional 26 recently diagnosed prostate cancer patients were obtained from the Urologic Oncology Branch, NIH Clinical Center, Bethesda, MD. All 26 blood samples were tested for the presence of XMRV RNA in plasma and DNA in whole blood. Tests for XMRV proviral DNA were also performed on prostate tissue from 19 of the 26 individuals in this cohort who had radical prostatectomies. Twenty-two of 26 blood samples were tested for antibodies to CA and TM. A subset of 12 samples was tested by virus rescue culture including those that had positive or indeterminate results by X-SCA or ELISA and matched negative controls. The study was approved by the IRB of NCI, NIH, Bethesda, MD, and all study subjects provided written informed consent.

*2.2. XMRV Nucleic Acid Detection with XMRV Single-Copy Assays (X-SCA).* Similar to the single-copy assay (SCA) for human immunodeficiency virus (HIV) [27], quantitative real-time PCR and RT-PCR assays for detection of XMRV, called XMRV single-copy assays (X-SCA), were developed to quantify XMRV nucleic acid in plasma, whole blood, and cell suspensions obtained from blood or tissue samples. The assays were designed using amplification primers targeting a *gag* leader region conserved between XMRV (as well as PreXMRV-2 [25]) and non-XMRV endogenous MLVs (forward 5-TGTATCAGTTAACCTACCCGAGT-3', reverse 5-AGACGGGGGCGGGAAGTGTCTC-3'). Consequently, efficient amplification is achieved from both target templates allowing detection of either XMRV or MLVs present in patient samples. The Taqman probe (5' fam-TGG AGT GGC TTT GTT GGG GGA CGA- tamra3') used for detection of amplified products was designed to span a signature 24 nucleotide deletion in the XMRV (PreXMRV-2) *gag* leader that differentiates these from all other MLV sequences (Figure 1(a)). In the event that a positive sample is identified by X-SCA, single-genome sequencing should be performed to confirm that the source of amplification was XMRV and

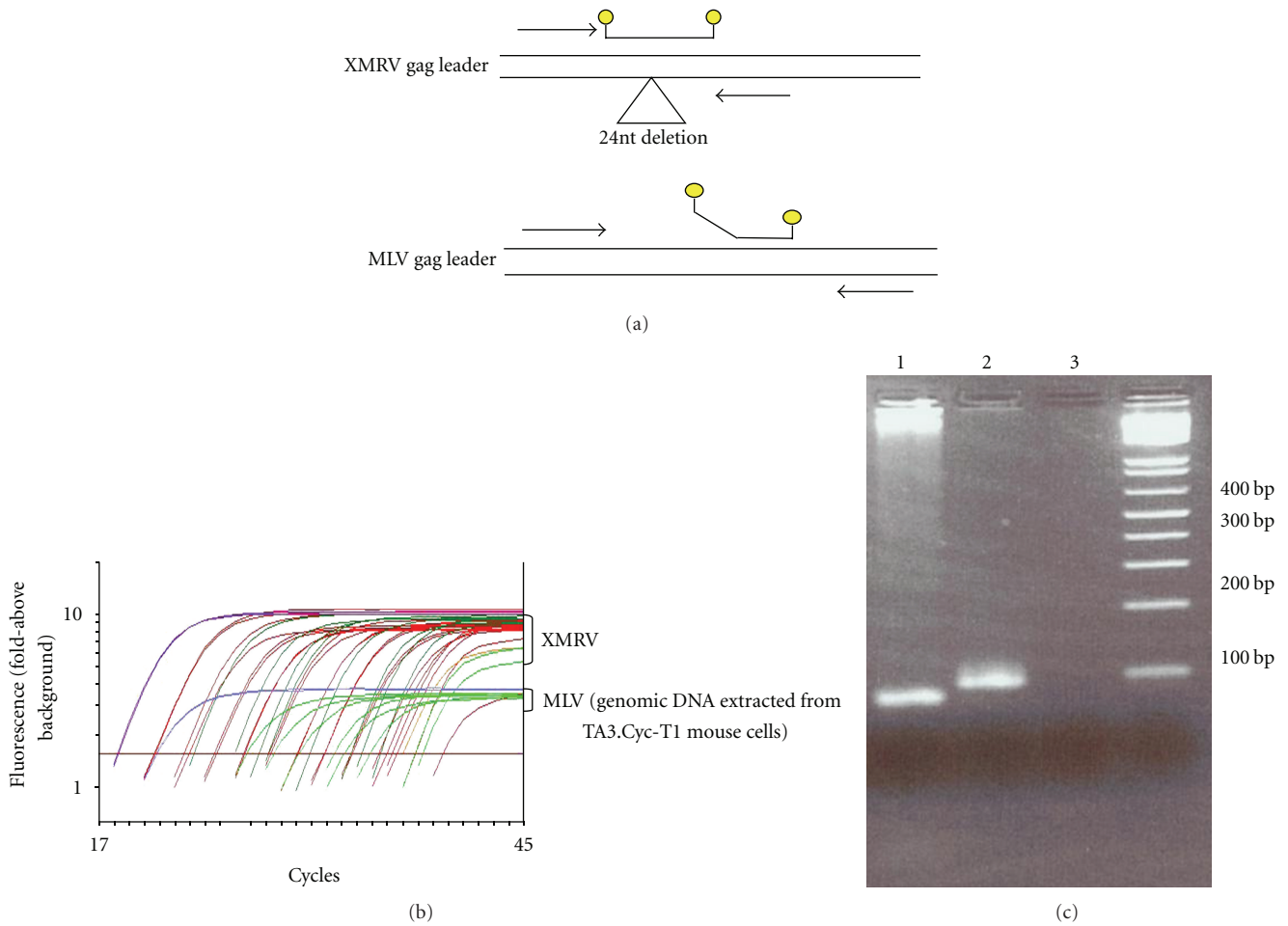


FIGURE 1: *XMRV* single-copy assay (X-SCA). X-SCA primers anneal to conserved regions in XMRV/MLV gag leader region while the probe spans a 24 nt deletion in XMRV compared to MLV (a) allowing for differential amplification profiles for XMRV and MLV (b). X-SCA amplification products run on a 2% agarose gel distinguish between the products being amplified since the XMRV product is 24 nt smaller than the MLV product. Lane 1 is the X-SCA product from the XMRV standard curve, Lane 2 is the MLV product from the genomic DNA extracted from TA3.Cyc-T1 mouse cells, and Lane 3 is the “no template” negative control (c).

not contaminating mouse DNA with a similar gag deletion, such as PreXMRV-2.

XMRV RNA was extracted from plasma samples following ultracentrifugation exactly as described for HIV SCA [27] and genomic DNA was extracted and whole blood samples using the Promega genomic DNA Extraction Kit (Cat no. A1120) according to the manufacturer’s suggested protocol. Reaction conditions for synthesizing cDNA and measuring RNA copy number were exactly as described previously for HIV SCA [27]. XMRV proviral copy number was determined using the Lightcycler 480 Probes Master (Cat no. 04707494001) according to protocol and by performing 45 cycles of 95°C for 15 seconds, 60°C for 1 minute after an initial 10 minute, 95°C polymerase activation step. Accurate detection of XMRV by X-SCA was verified by testing spiked human blood products [28] and by testing blood samples collected from XMRV inoculated macaques (Del Prete et al., in preparation). Pigtail macaques were experimentally inoculated with XMRV ( $\sim 4.8 \times 10^9$  RNA copy equivalents)

prepared from the supernatant of 22Rv1 cells (Lot SP1592, Biological Products Core, AIDS and Cancer Virus Program, SAIC-Frederick, Inc, NCI-Frederick). Plasma and PBMC samples were collected prior to inoculation and through 119 days after inoculation. These pre- and post-inoculation specimens were used as reference control samples in evaluating X-SCA methods for detection of XMRV. Details of the macaque infection study will be reported elsewhere (Del Prete et al. in preparation). Animals were housed and cared for in accordance with American Association for Accreditation of Laboratory Animal Care (AALAC) standards in an AAALAC accredited facility, and all animal procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the National Cancer Institute. Detection of MLV was qualified by extracting mouse genomic DNA from TA3.Cyc-T1 cells using the Promega genomic DNA Extraction Kit (Cat no. A1120) and performing X-SCA in duplicate on dilutions of 3000 to 0.03 cell equivalents.

All patient samples were tested by X-SCA in duplicate or triplicate with equal numbers of no template controls (NTC) to monitor the level of false positives due to either viral or mouse genomic DNA contamination. The level of detection for XMRV nucleic acid in clinical samples was determined by the volume of sample available for testing (100  $\mu$ L to 3 mL). Therefore, X-SCA sensitivity varied from 0.6 to 20.6 copies/mL of plasma and 0.9–10 copies/mL in whole blood. Because of the high frequency of false positives due to contaminating mouse DNA, we set strict criteria for declaring a sample positive for XMRV, requiring detection of viral sequence in all replicate PCR reactions from the samples being tested. These criteria result in a minimum detection of 1.8–41.2 copies XMRV RNA/mL in plasma and 2.7–30 copies XMRV DNA/mL in whole blood for a positive X-SCA test, depending on the volume of sample being tested. If discordant results are obtained from duplicate or triplicate wells, then the result is considered indeterminate and is repeated where sufficient sample is available.

**2.3. XMRV Serology.** XMRV antigens were prepared in the Protein Expression Laboratory, SAIC-Frederick, MD, as previously described [29]. Purified XMRV antigens were used to develop and optimize ELISA-based protocols (Bagni et al., in preparation). Briefly, purified CA and TM were spotted onto Meso Scale Discovery (MSD) (Gaithersburg, MD) standard 96-well plates at 8  $\mu$ g/mL and 2  $\mu$ g/mL, respectively. Samples were diluted 1 : 100 and incubated with individual XMRV antigens. Human antibodies were detected using biotin labeled anti-human IgG (Jackson ImmunoResearch, West Grove, Pa) and MSD-proprietary Sulfo-tagged streptavidin detection reagent and read on a SECTOR Imager 6000 (MSD) plate reader. The XMRV serology assays were qualified with samples obtained from XMRV-inoculated macaques (Del Prete et al., in preparation). Patient samples were considered reactive if the MSD electrochemiluminescent signal (ECL) was at least 50% relative to the ECL signal of the macaque positive control sera. Less reactive patient samples that were at least 2 standard deviations above the average negative human sample were considered indeterminate.

**2.4. XMRV Culture Detection.** The presence of replication-competent XMRV was determined in a virus rescue coculture assay using indicator cells designated DERSE (Detectors of Exogenous Retroviral Sequence Elements) and using expression of a GFP reporter as the readout. DERSE.LiGP cells are a subclone of LNCaP cells (gift from Dr. Francis Ruscetti, NCI) stably transfected with pBabe.iGFP-puro and screened for susceptibility to XMRV infection (Lee et al., in preparation). pBabe.iGFP-puro is an MLV proviral vector that encodes an intron-interrupted reporter GFP gene and is only expressed after mobilization by an infecting gammaretrovirus for a second round of infection of DERSE.LiGP cells. Similar MLV vectors that only express a reporter after being propagated in infection have been described previously using HEK293 cells [30]. The DERSE.LiGP assay will detect any MLV-related viruses that are capable of replicating in human prostate cancer cells. Virus replication can be detected by monitoring

GFP-positive cells either by fluorescence microscopy or FACS analysis.

DERSE.LiGP indicator cells were maintained in Roswell Park Memorial Institute (RPMI) media 1640 (Invitrogen) supplemented with 15% fetal bovine serum (FBS) (Hyclone), 1x Pen/Strep/Glutamine (100 U/mL Penicillin, 0.1 mg/mL Streptomycin, and 0.292 mg/mL Glutamine, Invitrogen) and 1  $\mu$ g/mL puromycin (Calbiochem). DERSE.LiGP cells were plated at  $1 \times 10^5$  cells/well in a 24-well tissue culture plate one day before infection. As a positive control, 22Rv1 cell supernatants were diluted in RPMI media and added to cells the next day in the presence of 5  $\mu$ g/mL of polybrene [31]. Culture medium was refreshed the following day by replacement or splitting cells at a 1 : 3 ratio depending on cell density. Although GFP can be detected in positive control samples within 3 days of infection, to maximize sensitivity for detection of low levels of virus, DERSE.LiGP cells exposed to clinical specimens were maintained in culture for at least two weeks and observed at intervals by fluorescence microscopy. After two weeks, cells were resuspended in a 2% paraformaldehyde (PFA) solution and GFP expression was measured by FACS (FACSCalibur, Becton Dickinson), indicative of a spreading infection. While DERSE.LiGP cells are relatively insensitive to heparin, plasma samples containing EDTA are toxic to the cultures. To mitigate toxicity, 200  $\mu$ L of EDTA containing plasma samples were distributed into Eppendorf tubes in the presence of 7.5 mM CaCl<sub>2</sub> to neutralize the EDTA and 30 U/mL heparin salt to minimize sample clotting. Tubes were incubated for 4 hrs at 4°C to separate the plasma from residual clotting. Accurate detection of XMRV by virus culture was verified using a dilution series of supernatants from 22Rv1 cells and XMRV-spiked human plasma samples containing approximately 10<sup>7</sup> to 10 copies of XMRV RNA. Using XMRV-spiked samples, we noted a loss of detection sensitivity of three- to fivefold in EDTA containing plasma samples treated in the above manner. A recent report of XMRV inactivation by human complement may explain in part the loss of infectivity after addition of plasma [24]. Prostate cancer samples with indeterminate results by X-SCA or ELISA were matched with negative samples and tested blinded in the virus culture assay.

We required that samples test positive for XMRV nucleic acid (RNA or DNA) and by at least one other detect method (immunoassay or culture assay) to be declared positive for XMRV infection.

All reagents developed at the NCI-Frederick and described here are being made available to the extramural research community through the NIH AIDS Research and Reference Reagent Program or AIDS and Cancer Virus Program, SAIC-Frederick, Inc., National Cancer Institute, Frederick.

### 3. Results

**3.1. Differentiating between XMRV and MLV with X-SCA Probe.** The X-SCA probe used for detection of amplified products spans a signature 24 nucleotide deletion in the XMRV [1] and in the PreXMRV-2 [32] *gag* leader that



differentiates these from all other MLV sequences (Figure 1(a)). Amplifications of XMRV from 22Rv1 DNA and MLV from mouse genomic DNA (extracted from TA3.CycT1 cells) show that the probe design results in a lower level of plateau fluorescence from non-XMRV MLV templates than from XMRV templates (Figure 1(b)), likely due to inefficient binding and/or degradation of the probe during MLV extension compared to XMRV extension. The result of the probe design is differential amplification profiles for XMRV and MLV, indicating which product is being detected in the assay and the proportions of each if both templates are detected. To confirm the result, the products were run on an agarose gel (Figure 1(c)). The XMRV X-SCA product is 86 nt long and the MLV product 110 nt, easily distinguishable on a 2% agarose gel.

**3.2. Qualifying XMRV Assay Detection Capabilities with Spiked Human Samples.** Assays for detection of XMRV nucleic acid and replication-competent virus were established using XMRV-spiked samples as positive control specimens. To determine the accuracy and sensitivity of X-SCA methods to detect XMRV in human blood products, we tested a full panel of plasma and whole blood samples that were spiked or not spiked with XMRV derived from 22Rv1 cells. The panel was blinded as to which samples were XMRV positive and which were XMRV negative and were provided to us by the XMRV Scientific Research Working Group for testing by X-SCA [28]. Results from the blinded panel of spiked samples were described previously by Simmons et al. [28] and demonstrated that we detected XMRV RNA and proviral DNA using X-SCA with 100% accuracy. The level of sensitivity for detecting XMRV RNA in the spiked plasma panel was limited by the volume of sample tested for XMRV (270  $\mu$ L) to 3.3 RNA copies/mL. The level of sensitivity for detecting XMRV proviral DNA was a single XMRV-infected 22Rv1 cell in whole blood samples. All unspiked samples were properly reported as negative for XMRV detection indicating a very low rate of false positivity.

The use of DERSE.L-iG-P cells to detect XMRV was verified using 22Rv1 culture supernatants and XMRV-spiked human plasma. Figure 2 shows the results from virus rescue experiments performed under the following conditions (i) 22Rv1 supernatant alone, (ii) 22Rv1 supernatant treated with  $\text{CaCl}_2$  and heparin, (iii) 22Rv1 supernatant spiked into human plasma treated with  $\text{CaCl}_2$  and heparin. DERSE.LiGP cells treated with EDTA-containing human plasma alone are not viable. Proportions of GFP-positive cells detected by FACS at day 4 and day 8 after infection are shown in Figures 2(a) and 2(b). DERSE.LiGP cells exposed to 0.01  $\mu$ L of 22Rv1 supernatant were GFP-positive by microscopy within 4 days of infection (Figure 2) demonstrating the sensitivity of this assay for detection of replication competent XMRV. The sensitivity of this detection decreased 3–5-fold in the presence of EDTA-containing plasma samples treated as described above. This decrease could in part be due to the presence of human complement as has been recently reported [24]. Additional days of culture increased the number of GFP-positive cells exposed to virus in the presence

TABLE 1: X-SCA Results on XMRV-inoculated macaques.

Monkey ID	Days after inoculation	Plasma XMRV RNA copies/mL	Copies of XMRV DNA per $10^6$ PBMCs
14232	0	<1.1	0
14232	5	534.11	197
14232	119	<1.1	23
8242	0	<1.1	0
8242	13	2153.56	2833
8242	119	<1.1	645

TABLE 2: Immunoassay of plasma from XMRV-inoculated macaques.

Monkey ID	Days after inoculation	Reactivity with	
		CA	TM
8242	0	19.5	248.5
8242	76	12713	544405
14232	0	14.5	145
14232	76	20108	285277

or absence of plasma. For this reason, cultures infected with human specimens were carried out for a minimum of two weeks.

**3.3. Verifying Assay Detection Capabilities with Blood Samples from XMRV-Inoculated Macaques.** To validate the specificity of X-SCA and ELISA, we used specimens from two pigtail macaques experimentally inoculated with XMRV. Detailed results from the macaque study will be reported elsewhere (Del Prete et al., in preparation). In short, samples tested by X-SCA revealed that peak viremia was achieved at 5 days after inoculation in one animal and at 13 days in the second (Table 1). By day 28, levels of XMRV RNA in plasma had declined to <1 copy/mL in both animals. PBMC-associated XMRV DNA was also measured by X-SCA. DNA levels peaked with similar kinetics as plasma viremia but persisted with levels of 23 and 645 copies/ $10^6$  PBMC in the two animals, respectively, at the end of the follow-up period, 119 days after inoculation. Antibody reactivity to XMRV capsid (CA) and transmembrane protein (TM) measured by ELISA was undetectable prior to inoculation but were robustly positive thereafter (Table 2) (Del Prete et al., in preparation). Replication competent XMRV cannot be cultured from macaque plasma or PBMC samples due to extensive hypermutation of the provirus post-inoculation, likely due to the effect of APOBEC proteins (Del Prete et al., in preparation). Consequently, XMRV-spiked human plasma was used to verify the DERSE.L-iG-P cells for detection of XMRV.

**3.4. Testing Prostate Cancer Samples for XMRV Nucleic Acid, Antibodies, and Isolatable Virus.** Samples obtained from the two cohorts of prostate cancer patients were assayed first for XMRV nucleic acid (X-SCA) and antibody reactivity against XMRV CA and TM protein (Tables 3 and 4). No

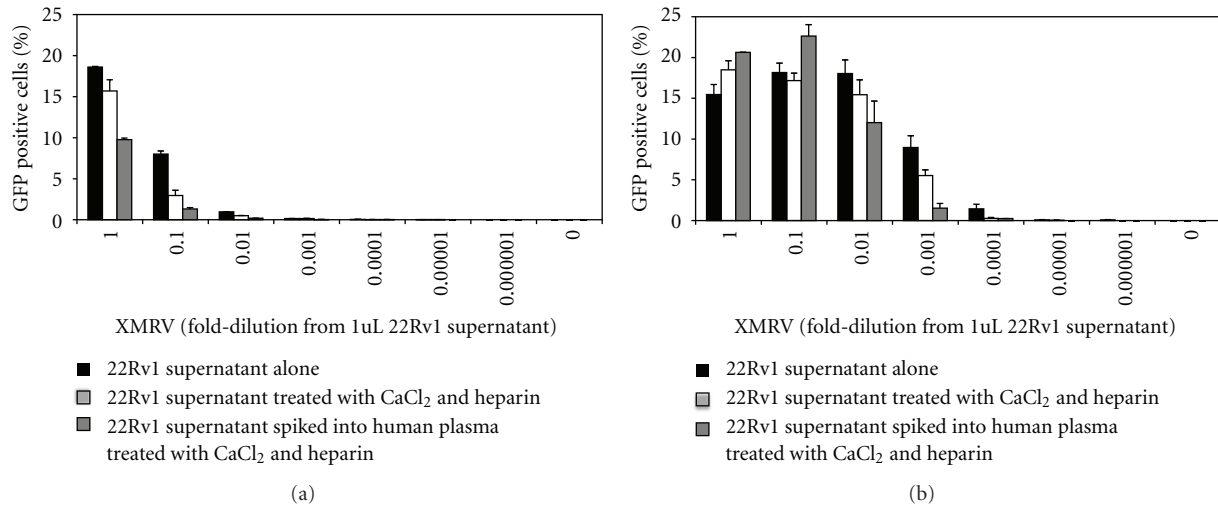


FIGURE 2: Verifying XMRV rescue by culturing on DERSE cells with 22Rv1 supernatants and with XMRV-spiked human plasma. XMRV culturing under the following conditions: (i) 22Rv1 supernatant alone (black bars), (ii) 22Rv1 supernatant treated with CaCl<sub>2</sub> + heparin (white bars), (iii) 22Rv1 supernatant spiked into human plasma treated with CaCl<sub>2</sub> + heparin (gray bars). GFP-positive cells were analyzed by FACS at day 4 (a) and day 8 (b).

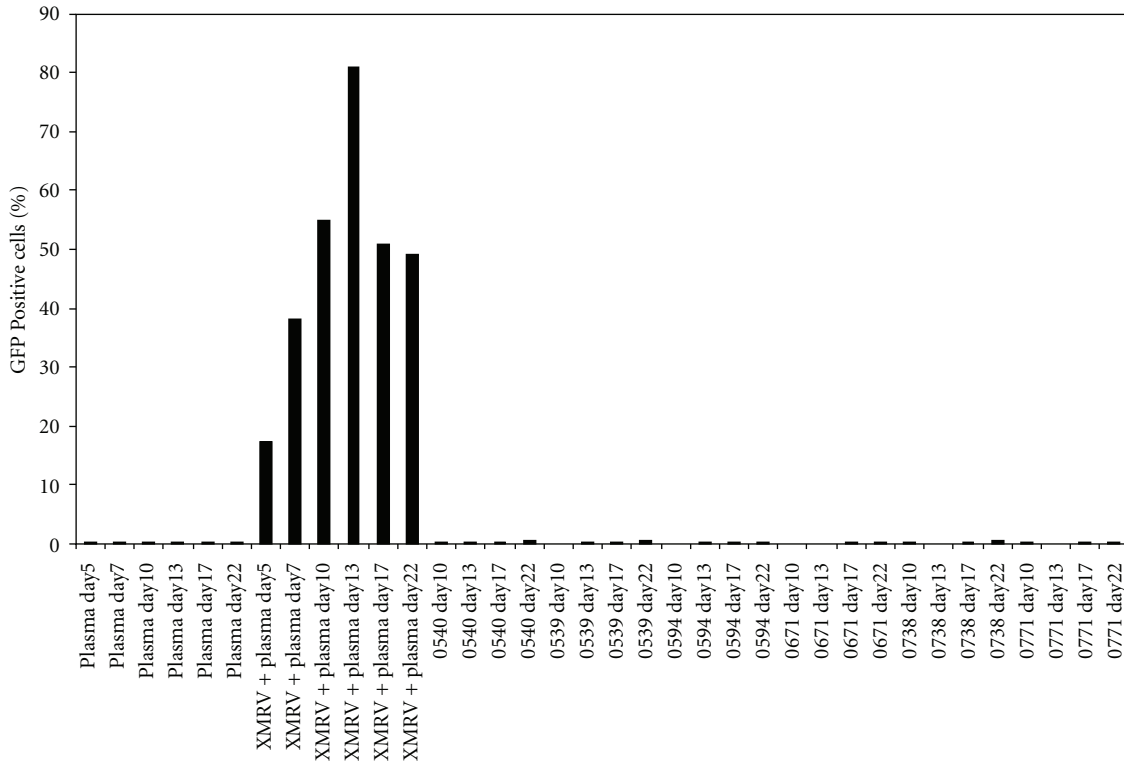
plasma or prostate tissue samples in the NIH prostate cancer cohort or the UC Davis prostate cancer cohort were positive for XMRV nucleic acids or antibodies (Tables 3 and 4). However, two plasma samples in the NIH cohort (0594, 0771) were indeterminate for XMRV RNA. One of these samples (0594) was negative by ELISA, and the other (0771) had an indeterminate ELISA result. One other patient sample in the NIH cohort (0781) was indeterminate for XMRV antibody reactivity but negative for XMRV nucleic acid (Table 3). All three of these samples, along with 9 matched negative samples, were blinded and tested for replicating virus using the DERSE.L-iG-P assay. Virus could not be cultured from any of these plasma samples while it was readily recovered from positive control samples (22Rv1-derived XMRV spiked into negative human plasma) (Figure 3). Consequently, by our prospectively defined criteria, none of the 26 patient samples in the NIH cohort were considered to be XMRV infected (positive for nucleic acid, antibody, and/or replication competent virus) (Table 3). All 108 plasma samples from prostate cancer patients obtained from UC Davis were assayed for XMRV RNA and antibodies (Table 4). All samples were negative for XMRV nucleic acid except one (0739), which was indeterminate. No sample was found to be antibody reactive by our ELISA criteria (at least 50% reactive relative to the macaque positive control sera). Twelve of the 108 samples were indeterminate for XMRV reactivity to either CA or TM (2 standard deviations above the average negative human sample) but were negative for nucleic acid (Table 4). No sample was indeterminate or positive for both XMRV nucleic acid and antibody, and therefore, all were determined to be negative for XMRV infection.

#### 4. Discussion

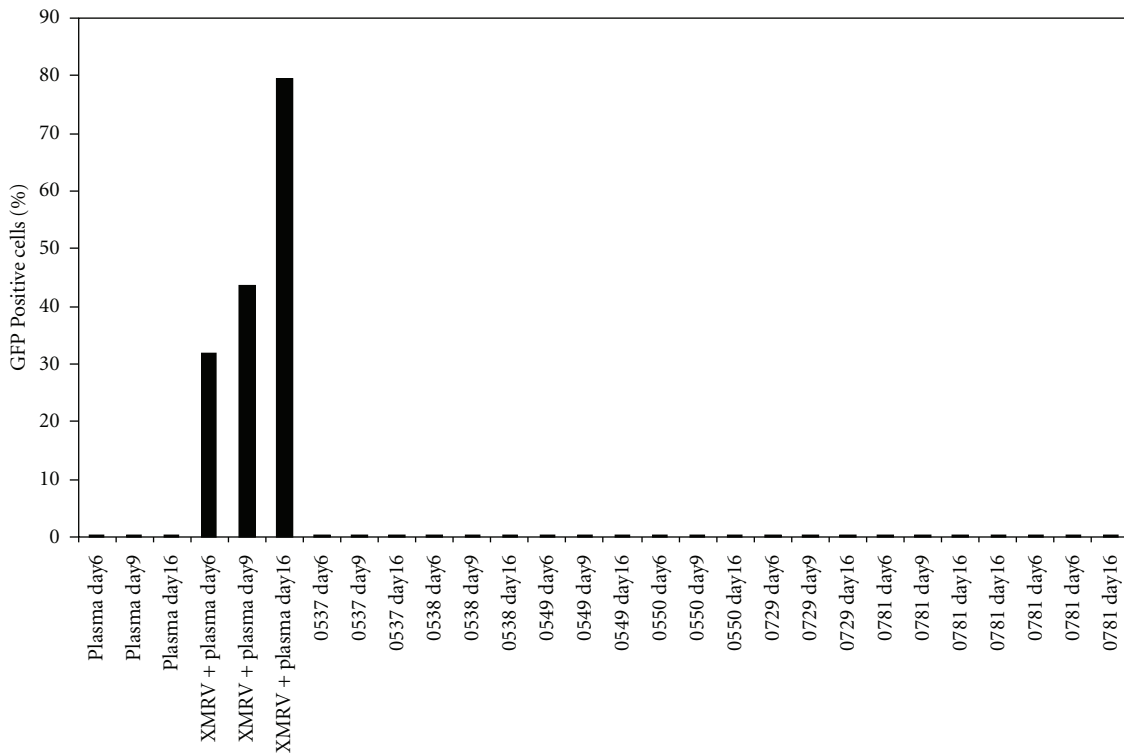
After publication of the XMRV study by Lombardi et al. in October 2009 suggesting a possible disease association with

CFS and a surprisingly high apparent seroprevalence for XMRV even among healthy control subjects, researchers at the NCI-Frederick set out to develop rigorous methods to evaluate the prevalence of XMRV infection. Using control samples, including spiked specimens where appropriate, we developed assays to measure plasma XMRV RNA viremia, cell-associated XMRV DNA levels, and antibodies to XMRV CA and TM. Because Lombardi et al. reported the presence of culture rescuable replication-competent virus from the blood of study subjects using coculture with a human cell line (LNCap), we created DERSE cells, derivatives of the same LNCap cells with a fluorescent reporter to detect XMRV replication. These cells broadly and sensitively detect the replication of different MLV-related gammaretroviruses that exhibit a tropism for human prostate cancer cells. In the absence of patient-derived definitive positive and negative control specimens, we applied our different assay methods to samples obtained from two pigtail macaques prior to and after experimental XMRV inoculation. XMRV plasma viremia was detectable in both inoculated macaques for 2-3 weeks after inoculation but then declined to undetectable levels (Del Prete et al., in preparation). However, XMRV DNA in PBMCs and serum antibodies remained at readily measurable levels for the duration of study follow-up in both animals (Del Prete et al., in preparation). Evaluation of samples from the inoculated macaques demonstrated the ability of our methods to reliably detect evidence of XMRV infection in blood samples and showed that XMRV provirus and antibodies persist even when viremia is not detectable.

In the development of diagnostic tools for XMRV infection, it became clear that a single method for XMRV detection would not be sufficient for definitive diagnosis due to a high frequency of false positives by PCR from contaminating nucleic acids (especially mouse genomic DNA) and high background reactivity seen by ELISA, even in samples



(a)



(b)

FIGURE 3: Testing plasma samples from prostate cancer patients for replication competent XMRV. Twelve samples were blinded as to their X-SCA and ELISA results and were tested for replicating virus using the DERSE.L-iG-P assay in two separate experiments. Six samples were tested in experiment 1 at passages 10, 13, 17, and 22 (a). All passages were negative for XMRV while virus was recovered from the positive control samples ( $10^7$  copies of XMRV from 22Rv1 cells spiked into human plasma). Six additional samples were tested in experiment 2 at passages 6, 9, and 16 (b). All passages were negative for XMRV while virus was recovered from the positive control samples.

TABLE 3: X-SCA, ELISA, and virus culture results on prostate cancer samples from NIH cohort.

Sample number	Sample ID	Date of blood draw	Plasma XMRV RNA		Nucleic acid testing by X-SCA		Serologic Testing by ELISA				Virus Culture	
			copies/mL	copies/mL in whole blood	XMRV DNA copies in prostate tissue	XMRV DNA copies in prostate cells tested	CA	TM	ELISA result	Virus replication	Overall result	
1	UB10-0533	8/5/2010	<0.6	<10	0	174000	999	985	NR	NT	NEGATIVE	
2	UB10-0537	8/6/2010	<0.8	<10	NA	NA	5713	5302	NR	NEGATIVE	NEGATIVE	
3	UB10-0538	8/6/2010	<0.7	<10	NA	NA	2323	2362	NR	NEGATIVE	NEGATIVE	
4	UB10-0539	8/6/2010	<0.8	<0.9	NA	NA	1505	1864	NR	NEGATIVE	NEGATIVE	
5	UB10-0540	8/6/2010	<0.8	<0.9	3.8	105600	1429	2811	NR	NEGATIVE	NEGATIVE	
6	UB10-0542	8/9/2010	<0.7	<10	0	307500	1796	1949	NR	NT	NEGATIVE	
7	UB10-0547	8/11/2010	<0.8	<10	0	81000	2248	4325	NR	NT	NEGATIVE	
8	UB10-0548	8/11/2010	<0.8	<10	NA	NA	2566	2826	NR	NT	NEGATIVE	
9	UB10-0549	8/11/2010	<0.8	<10	0	59025	5129	6059	NR	NEGATIVE	NEGATIVE	
10	UB10-0550	8/11/2010	<0.8	<10	0	54825	1412	1414	NR	NEGATIVE	NEGATIVE	
11	UB10-0578	8/19/2010	<0.8	<10	0	275250	1,412	1,460	NR	NT	NEGATIVE	
12	UB10-0594	8/21/2010	0.9	<0.9	0	144075	3,050	3,190	NR	NEGATIVE	NEGATIVE	
13	UB10-0643	9/14/2010	<0.7	<10	0	32400	5,359	5,430	NR	NT	NEGATIVE	
14	UB10-0665	9/16/2010	<0.7	<10	0	84300	2,736	3,817	NR	NT	NEGATIVE	
15	UB10-0671	9/21/2010	Invalid test	<0.9	NA	NA	1,490	1,375	NR	NEGATIVE	NEGATIVE	
16	UB10-0706	9/28/2010	Invalid test	<10.0	0	3502.5	2,514	2,500	NR	NT	NEGATIVE	
17	UB10-0729	9/30/2010	<0.7	<10.0	0	88950	1,453	1,620	NR	NEGATIVE	NEGATIVE	
18	UB10-0771	10/14/2010	10.4	<0.9	NA	NA	10,655	26,030	<i>Equiv</i>	NEGATIVE	Indeterminate	
19	UB10-0781	10/15/2010	<0.7	<10	NA	NA	8,151	8,451	<i>Equiv</i>	NEGATIVE	NEGATIVE	
20	UB10-0738	10/5/2010	Invalid test	<0.9	0	74325	2,280	2,312	NR	NEGATIVE	NEGATIVE	
21	UB10-0785	10/18/2010	<16.5	<10	0	27375	3,840	3,244	NR	NT	NEGATIVE	
22	UB10-0788	10/19/2010	<16.5	<10	0	20887	2,990	2,596	NR	NT	NEGATIVE	
23	UB10-0830	10/29/2010	<0.8	<10	0	63225	NT	NT	NT	NT	NEGATIVE	
24	UB10-0833	11/1/2010	<0.8	<10	0	41175	NT	NT	NT	NT	NEGATIVE	
25	UB10-0853	11/4/2010	<0.8	<10	0	8535	NT	NT	NT	NT	NEGATIVE	
26	UB10-0913	11/19/2010	<0.8	<10	0	7132	NT	NT	NT	NT	NEGATIVE	

NA: sample not available.

NT: sample not tested.



TABLE 4: X-SCA and ELISA results on prostate cancer samples from UC-Davis cohort.

Patient ID	Plasma RNA			Patient ID	Plasma RNA		
	Copies/mL	ELISA result	Overall result		Copies/mL	ELISA result	Overall result
P0005	<16.5	<i>Indeterminate</i>	NEGATIVE	P0566	<16.5	NR	NEGATIVE
P0013	<16.5	NR	NEGATIVE	P0572	<16.5	NR	NEGATIVE
P0015	<16.5	NR	NEGATIVE	P0592	<16.5	NR	NEGATIVE
P0024	<16.5	NR	NEGATIVE	P0593	<16.5	NR	NEGATIVE
P0026	<16.5	NR	NEGATIVE	P0605	<16.5	NR	NEGATIVE
P0027	<16.5	NR	NEGATIVE	P0611	<16.5	NR	NEGATIVE
P0031	<16.5	NR	NEGATIVE	P0612	<16.5	NR	NEGATIVE
P0034	<16.5	NR	NEGATIVE	P0617	<16.5	NR	NEGATIVE
P0036	<16.5	NR	NEGATIVE	P0632	<16.5	NR	NEGATIVE
P0044	<16.5	NR	NEGATIVE	P0637	<16.5	NR	NEGATIVE
P0045	<16.5	NR	NEGATIVE	P0641	<16.5	NR	NEGATIVE
P0118	<16.5	NR	NEGATIVE	P0650	<16.5	NR	NEGATIVE
P0133	<16.5	NR	NEGATIVE	P0657	<16.5	<i>Indeterminate</i>	NEGATIVE
P0144	<16.5	NR	NEGATIVE	P0659	<16.5	NR	NEGATIVE
P0154	<16.5	NR	NEGATIVE	P0672	<16.5	NR	NEGATIVE
P0156	<16.5	NR	NEGATIVE	P0673	<16.5	NR	NEGATIVE
P0162	<16.5	<i>Indeterminate</i>	NEGATIVE	P0675	<16.5	NR	NEGATIVE
P0167	<16.5	NR	NEGATIVE	P0679	<16.5	NR	NEGATIVE
P0170	<16.5	NR	NEGATIVE	P0685	<16.5	NR	NEGATIVE
P0172	<16.5	NR	NEGATIVE	P0710	<16.5	NR	NEGATIVE
P0177	<16.5	NR	NEGATIVE	P0721	<16.5	NR	NEGATIVE
P0185	<16.5	NR	NEGATIVE	P0723	<20.6	NR	NEGATIVE
P0195	<16.5	<i>Indeterminate</i>	NEGATIVE	P0726	<16.5	<i>Indeterminate</i>	NEGATIVE
P0209	<16.5	NR	NEGATIVE	P0733	<16.5	NR	NEGATIVE
P0219	<16.5	<i>Indeterminate</i>	NEGATIVE	P0739	55	NR	NEGATIVE
P0232	<16.5	NR	NEGATIVE	P0766	<20.6	NR	NEGATIVE
P0239	<16.5	NR	NEGATIVE	P0778	<16.5	NR	NEGATIVE
P0293	<16.5	NR	NEGATIVE	P0787	<16.5	NR	NEGATIVE
P0306	<16.5	NR	NEGATIVE	P0792	<16.5	NR	NEGATIVE
P0314	<16.5	NR	NEGATIVE	P0826	<16.5	NR	NEGATIVE
P0321	<16.5	NR	NEGATIVE	P0846	<16.5	NR	NEGATIVE
P0322	<16.5	NR	NEGATIVE	P0848	<16.5	NR	NEGATIVE
P0325	<16.5	NR	NEGATIVE	P0852	<20.6	NR	NEGATIVE
P0327	<16.5	NR	NEGATIVE	P0906	<16.5	<i>Indeterminate</i>	NEGATIVE
P0332	<16.5	<i>Indeterminate</i>	NEGATIVE	P0916	<16.5	NR	NEGATIVE
P0340	<16.5	<i>Indeterminate</i>	NEGATIVE	P0923	<16.5	NR	NEGATIVE
P0342	<16.5	NR	NEGATIVE	P0952	<16.5	NR	NEGATIVE
P0346	<16.5	<i>Indeterminate</i>	NEGATIVE	P0984	<16.5	NR	NEGATIVE
P0348	<16.5	NR	NEGATIVE	P0989	<16.5	NR	NEGATIVE
P0351	<16.5	NR	NEGATIVE	P0996	<16.5	NR	NEGATIVE
P0355	<16.5	NR	NEGATIVE	P0999	<16.5	NR	NEGATIVE
P0366	<20.6	NR	NEGATIVE	P1010	<16.5	NR	NEGATIVE
P0380	<16.5	NR	NEGATIVE	P1025	<16.5	NR	NEGATIVE
P0382	<16.5	NR	NEGATIVE	P1032	<16.5	NR	NEGATIVE
P0384	<16.5	NR	NEGATIVE	P1063	<16.5	NR	NEGATIVE

TABLE 4: Continued.

Patient ID	Plasma RNA			Patient ID	Plasma RNA		
	Copies/mL	ELISA result	Overall result		Copies/mL	ELISA result	Overall result
P0388	<16.5	NR	NEGATIVE	P1076	<16.5	NR	NEGATIVE
P0509	<16.5	<i>Indeterminate</i>	NEGATIVE	P1086	<16.5	NR	NEGATIVE
P0511	<16.5	NR	NEGATIVE	P1108	<16.5	<i>Indeterminate</i>	NEGATIVE
P0530	<16.5	NR	NEGATIVE	P1110	<16.5	NR	NEGATIVE
P0532	<16.5	NR	NEGATIVE	P1211	<16.5	NR	NEGATIVE
P0535	<16.5	NR	NEGATIVE	P1268	<16.5	NR	NEGATIVE
P0536	<16.5	NR	NEGATIVE	P1297	<16.5	NR	NEGATIVE
P0544	<16.5	NR	NEGATIVE	P1304	<16.5	NR	NEGATIVE
P0562	<16.5	NR	NEGATIVE	P1318	<16.5	NR	NEGATIVE

from healthy control subjects, presumably reflecting cross-reactivity. Therefore, we suggest a multiple assay approach to determine the XMRV status of patient samples. We established diagnostic criteria requiring that all replicates from X-SCA analysis must be positive and that serum antibodies and/or replicating virus must also be detectable in the same patient in order to report the patient XMRV positive. Samples resulting in discordant results from PCR replicates are reported as indeterminate. Despite earlier reports that evidence of XMRV infection was detected in as many as 20% of prostate tumors [2, 10–12], using the assays we developed, we did not find clear evidence for XMRV in the blood of two independent cohorts of patients with prostate cancer (total  $n = 134$ ) or in the prostate tissue of a small subset of these individuals ( $n = 19$ ). Based on previously reported frequencies of XMRV detection in prostate cancer patients, if XMRV is present in the blood of infected individuals, we expected that approximately 27 of the 134 patients in our study would be positive for XMRV. One patient from the NIH cohort (0771) had an indeterminate X-SCA result (2/3 reactions were positive for RNA). This sample was also positive for reactivity to CA and TM by ELISA. However, no XMRV DNA was found in the whole blood from this patient, and replication competent virus could not be recovered from the sample. Taken together, these data are considered an indeterminate result by our criteria. No other samples were positive by more than one diagnostic method.

The occasional positive X-SCA reaction is not above background for this assay. We regularly run 96-well plates of “no template controls” using both our X-SCA primers and primers targeting intracisternal A particles (IAP) [20, 21, 33] that are present in high copies in the mouse genome in order to monitor the levels of contaminating mouse DNA in the reagents and in the environment. We have found that about 5% of wells are positive with the X-SCA primers and about 20% with the IAP primers. Based on these backgrounds, we expect to detect low levels of mouse DNA contamination in samples tested, as seen in this study and in others [20, 21, 33]. Therefore, we required that all replicates of patient samples be positive to obtain a “positive” X-SCA result. We did not

test the samples directly with IAP primers since we have not successfully found reagents and an environment that are free from mouse genomic DNA (on average about 1/3000 of a mouse genome per PCR reaction).

Although we had an occasional indeterminate result for XMRV RNA in the plasma samples studied, we did not detect XMRV DNA in any sample tested, despite the ability of our assay to sensitively detect XMRV DNA in spiked control samples and in specimens from inoculated macaques [28] (Del Prete et al., in preparation). Results from the inoculated macaques showed that in experimental infection, XMRV proviral DNA is readily measurable in blood cells even when plasma viremia was not detectable (Del Prete et al., in preparation), further suggesting that these patients do not carry XMRV in their blood. Findings from previous studies reporting higher prevalence for XMRV in similar cohorts [2, 11, 12] typically involved testing of prostate tumors. None of these studies reported the detection of XMRV in blood samples or the isolation of infectious virus from clinical specimens, and only one measured the presence of reactive antibodies through a virus neutralization assay [10]. Detection of antibody responses to specific viral proteins by ELISA or by reactivity to XMRV immunoblots was not assessed. If we had used less rigorous criteria basing an overall diagnosis on a single, nonconfirmed test and not requiring all replicates to yield the same result, then our two cohorts would have given rise to an apparent, and in our view almost certainly incorrect, reported XMRV prevalence rate of approximately 12%. These considerations may explain conflicting prior reports for the prevalence of XMRV and are consistent with claims that XMRV detection is likely the result of laboratory contamination [22, 26, 33, 34]. Particularly given the potential for false positive results in PCR and serological assays for XMRV, our results suggest that applying multiple diagnostic methods including measuring levels of proviral DNA in blood cells provides a more reliable approach for investigating the prevalence of XMRV. These results also demonstrate that XMRV nucleic acid, and antibodies are undetectable in the blood of patients with prostate cancer.

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## Development and application of a high-throughput microneutralization assay: lack of xenotropic murine leukemia virus–related virus and/or murine leukemia virus detection in blood donors

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**BACKGROUND:** Xenotropic murine leukemia virus (MLV)-related virus (XMRV) and other related MLVs have been described with chronic fatigue syndrome and certain types of prostate cancer. In addition, prevalence rates as high as 7% have been reported in blood donors, raising the risk of transfusion-related transmission. Several laboratories have utilized microneutralization assays as a surrogate marker for detection of anti-MLV serologic responses—with up to 25% of prostate cancer patients reported to harbor neutralizing antibody responses.

**STUDY DESIGN AND METHODS:** We developed a high-throughput microneutralization assay for research studies on blood donors using retroviral vectors pseudotyped with XMRV-specific envelopes. Infection with these pseudotypes was neutralized by sera from both macaques and mice challenged with XMRV, but not preimmune serum. A total of 354 plasma samples from blood donors in the Reno/Tahoe area were screened for neutralization.

**RESULTS:** A total of 6.5% of donor samples gave moderate neutralization of XMRV, but not control pseudotypes. However, further testing by Western blot revealed no evidence of antibodies against MLVs in any of these samples. Furthermore, no evidence of infectious virus or viral nucleic acid was observed.

**CONCLUSION:** A microneutralization assay was developed for detection of XMRV and can be applied in a high-throughput format for large-scale studies. Although a proportion of blood donors demonstrated the ability to block XMRV envelope-mediated infection, we found no evidence that this inhibition was mediated by specific antibodies elicited by exposure to XMRV or MLV. It is likely that this moderate neutralization is mediated through another, nonspecific mechanism.

The short history of xenotropic murine leukemia virus (MLV)-related virus (XMRV) is one of controversy and discrepant results. Initial studies found XMRV nucleic acids and/or proteins in prostate cancers<sup>1,2</sup> and even a low percentage of prostate tissues from individuals with no history of prostate cancer.<sup>2</sup> In contrast, several other studies have failed to detect XMRV in prostate cancer tissue.<sup>3,4</sup> Much of this

**ABBREVIATIONS:** CFS = chronic fatigue syndrome; DEP = dual envelope pseudovirus; Lassa-GP = glycoprotein of Lassa virus; MLV = murine leukemia virus; MLV-P = polytropic MLV; qRT-PCR = quantitative reverse transcription–polymerase chain reaction; VSV-G = G protein of vesicular stomatitis virus; WB = Western blot; XMRV = xenotropic murine leukemia virus–related virus.

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controversy is likely explained by polymerase chain reaction (PCR) and other nucleic acid contamination.<sup>5,6</sup> Despite XMRV originally being isolated from human prostate cancer samples,<sup>1</sup> it is in all likelihood a laboratory artifact, created by the passage of human prostate tissue through mice.<sup>7</sup> This resulted in infection with, and subsequent recombination between, at least two endogenous MLVs.<sup>7</sup> Cell lines created from this tissue, and harboring XMRV, were likely distributed to many laboratories working on prostate cancer.

The controversy surrounding the association between XMRV and chronic fatigue syndrome (CFS) is, if anything, greater. It was reported by Lombardi and colleagues<sup>8</sup> that two-thirds of CFS patients from the United States harbored XMRV compared to 4% of controls. Importantly, this work was based on three separate lines of evidence: 1) direct and indirect nucleic acid detection in peripheral blood mononuclear cells (PBMNCs), stimulated PBMNCs, and plasma; 2) culture of replication-competent XMRV from plasma and PBMNC by coculture with human prostate cells; and 3) serologic evidence using a flow cytometry assay. In addition to the association with CFS, the presence of virus in plasma and blood cells, coupled with the relatively high prevalence observed in apparently healthy controls, suggested that XMRV may be both blood transfusion transmitted and a real threat to the safety of the US blood supply. However, many other groups failed to detect XMRV in PBMNC samples from CFS or healthy individuals.<sup>9-13</sup> At least two studies tried to fully replicate the initial study using PCR, culture, and serology, without any convincing evidence of XMRV in either CFS patients or healthy controls.<sup>14,15</sup> Furthermore, more recent testing of specimens from the study by Lombardi and coworkers revealed that some of the previously reported PCR-positive specimens were contaminated with XMRV-containing plasmid sequences leading to the partial retraction of these PCR results from the publication by Lombardi and colleagues.<sup>16</sup> Additionally, a recent multilaboratory blinded study using 15 previously reported XMRV- or MLV-positive subjects as well as validated negative controls demonstrated that virus culture assays used in the study by Lombardi and colleagues were prone to cross-contamination.<sup>17</sup> Thus, this leaves only the serologic results as possible evidence for the presence of XMRV or other MLVs in humans. In the same multilaboratory study<sup>15</sup> the assays used by Lombardi and colleagues detected a serologic response in some specimens; however, this reactivity was not consistent within replicates of the same plasma sample and no statistical association was observed in CFS patients compared to blood donors, while three other highly sensitive assays in the study failed to detect a serologic response in any specimen.<sup>17</sup>

Microneutralization assays have been used extensively as diagnostic and specificity tests for many viruses, including alphaviruses and influenza.<sup>18-20</sup> Indeed, neutral-

izing antibodies are typically formed as part of a highly specific response to conformational epitopes. Neutralization of XMRV in 11 of 40 (27.5%) serum samples was observed in prostate cancer patients<sup>21</sup> suggesting that a microneutralization assay for XMRV would be feasible and useful. In this study, we generated a microneutralization assay for studies of blood donors seeking serologic evidence of XMRV or MLV infection based on the dual envelope pseudovirus (DEP) assay system we recently developed,<sup>22</sup> which has been proven to be a rapid, sensitive, and specific high-throughput system for antiviral drug discovery targeting viral entry. This assay system is composed of two viruses. Entry of the target virus is driven by the XMRV envelope protein pseudotyped onto the core of a reporter retrovirus, while infection by a second, internal control pseudovirus is mediated by an unrelated envelope and is included to reduce the number of false positives. Using this assay, we screened 354 donors and identified a small number with a neutralization signature warranting further testing.

## MATERIALS AND METHODS

### Sample collection

Anonymized plasma and whole blood aliquots were prepared using residual samples left over from pilot tubes collected for routine blood donation testing. The samples selected were from 354 different donations from the United Blood Services Reno facility. One or two ethylenediaminetetraacetate (EDTA) plasma tube(s) were used for preparation of these aliquots depending on the unit collection type. From each EDTA tube two plasma aliquots were prepared, the remaining sample was gently inverted to resuspend, and then three or four whole blood aliquots were prepared. All aliquots were frozen the day of preparation. Donor samples were coded to retain linkage only to the donor's zip code of residence, age, sex, and race/ethnicity. Any linkage to personal donor information such as name, address, and telephone number was removed. All samples provided were anonymized before shipment to Blood Systems Research Institute for subsequent testing. The institutional review board of the University of California San Francisco approved the study protocol.

### Cells and reagents

Human embryonic kidney 293T cells clone 17 (293T/17) and human prostate LNCaP cells were obtained from the ATCC and grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and penicillin and streptomycin (10 U/mL). LNCaP iGFP cells (DERSE, detectors of exogenous retroviral sequence elements) were provided by V. KewalRamani (NCI, Frederick, MD).

LacZ encoding polytropic MLV (MLV-P; termed LacZ[MCF13]) viruses were generated by first infecting NIH3T3 cells with replication-competent MCF13. The resulting cell line was then infected with replication-defective lacZ(A-MLV) pseudotype virus to introduce the lacZ gene.<sup>23</sup> CHO cells overexpressing murine ecotropic MLV receptor mCAT-1 (CERD9) have previously been described.<sup>24,25</sup>

Sera from wild mice experimentally or mock-infected with XMRV for 12 weeks<sup>26</sup> were used as positive and negative controls. XMRV-infected rhesus macaque (RII10 and RYH10) sera were provided by J. Hackett (Abbott, Abbott Park, IL).<sup>27</sup>

### Plasmids

XMRV envelope (*env*) was PCR amplified from 22Rv1 cells with 100% nucleic acid sequence identity to the XMRV 22Rv1/CWR-R1 *env* sequence (GenBank Accession Number FN692043) and cloned into the pCAGGS vector with *KpnI* and *NheI* restriction sites. Plasmids encoding G protein of vesicular stomatitis virus (VSV-G), glycoprotein of Lassa virus (Lassa-GP), and the ecotropic MLV envelope have been described previously.<sup>28-32</sup>

Pseudotyped viruses with human immunodeficiency virus (HIV)-based retroviral backbone were generated from two plasmids, one encoding *env* and the other encoding the HIV backbone with a reporter gene. pNL4-3 Luc-R<sup>-</sup>E<sup>-</sup> (pNL-luc) encodes a replication-incompetent variant of the HIV-1 molecular clone NL4-3, in which the *nef* gene has been replaced by a firefly luciferase (*luc*) reporter, and the *env* and *vpr* genes were inactivated, as previously described.<sup>33</sup> Similarly, pNL4-3 Ren-R<sup>-</sup>E<sup>-</sup> (pNL-ren) was constructed by swapping the firefly luciferase gene for *Renilla* luciferase.<sup>22</sup> Pseudotyped viruses with MLV-based retroviral backbone were generated from three plasmids: XMRV *env*, MLV-based firefly luciferase reporter (MRP-luc),<sup>34</sup> and MLV gag/pol expression plasmid pHIT60.<sup>35</sup>

### Virion production

HIV-based pseudovirions were produced essentially as previously described<sup>30</sup> by transfecting 293T/17 cells with 10 µg of the corresponding HIV construct (pNL-luc or pNL-ren vector) and 30 µg of plasmid encoding the viral envelope per 10-cm dish using the calcium phosphate transfection method. Similarly, MLV-based pseudovirions were produced by transfecting 5 µg of each of the three plasmid constructs per 10-cm dish. The next day, expression was induced with sodium butyrate (10 mmol/L) for 6 hours before washing the cells once with phosphate-buffered saline and then replacing the medium. Forty hours after transfection, the supernatant was filtered through a 0.45-µm pore size filter and frozen at -80°C. If

required, virions were concentrated by ultracentrifuge concentration at 141,000 × g through a 20% sucrose cushion for 1.5 hours at 4°C. The pellets were resuspended in Hanks' buffered saline solution and aliquoted for storage at -80°C. Resulting reporter viruses were classified according to retroviral backbone, reporter system, and viral envelope, for example, MLV-luc(XMRV Env) or HIV-ren(Lassa-GP). LacZ encoding MLV-P was harvested from 3T3LacZMCF13 cells, filtered through a 0.45-µm pore size filter, and frozen at -80°C.

### Microneutralization assay

Neutralization assays were performed in 96-well white tissue culture plates (Nunc, Rochester, NY). Donor serum samples were prepared from plasma by adding thrombin (King Pharmaceuticals, Bristol, TN) in 0.5 mol/L MgCl<sub>2</sub>/CaCl<sub>2</sub> solution and then removing fibrin clots. The serum supernatant was transferred to a new tube and heat inactivated at 56°C for 30 minutes. A volume of 10 µL of serially diluted test sera or medium alone were transferred to assay wells, followed by 30 µL of either a single or a two-reporter virus mixture depending on the purpose of the assay and incubated for 1 hour at room temperature before addition of 40 µL of 293T/17 or LNCaP cells (500,000 cells/mL) to all wells. Plates were incubated for 2 days at 37°C and 5% CO<sub>2</sub> and firefly and *Renilla* luciferase reporter expression was determined sequentially as described in Zhou et al.<sup>22</sup> For the initial high-throughput microneutralization assays, sera samples with final dilutions of 80- and 240-fold were tested and each experiment repeated twice.

### Neutralization dose response

For generation of neutralization dose-response curves with selected donor sera, samples were serially diluted starting from 40- or 80-fold initial dilutions. Assays were performed in triplicate. Infection of pseudoviruses MLV-luc(XMRV Env) and MLV-luc(VSV G) in 293T/17 cells and infection of MLV-luc(MLV-E Env) and MLV-luc(VSV G) in CERD9 cells were detected using a luciferase assay system (Bright-Glo, Promega, Madison, WI). Infection of LacZ encoding MLV-P in 293T/17 cells was detected using a system for chemiluminescent reporter detection of β-galactosidase (Galacto-Light Plus, Applied Biosystems, Foster City, CA). Additionally, the percentage of cells infected with LacZ encoding MLV-P was measured with cell fixation and visualization of blue color development under a microscope using a β-gal staining kit (Invitrogen).

### Western blot

Western blot (WB) analysis was performed to detect XMRV or MLV antibodies in selected donor sera and

healthy controls as previously described.<sup>12,36</sup> Briefly, XMRV-infected DU145 prostate cells (C7) were grown in complete HuMEC serum-free medium supplemented with 1% HuMEC and 50 µg/mL bovine pituitary extract (Invitrogen). Tissue culture supernatants were clarified by centrifugation and by passage through a 0.45-µm filter. XMRV was purified from 150 mL of C7 supernatant using a retrovirus maxiprep kit (ViraTrap, Bioland Scientific LLC, Paramount, CA) following the manufacturer's protocol. A volume of 150 µL of purified XMRV was denatured with sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer at 95°C for 10 minutes and viral proteins were separated by gel electrophoresis in a NuPAGE 4%-12% Bis-Tris gel (Invitrogen) for WB testing as previously described but modified by using horseradish peroxidase–conjugated protein G instead of protein A/G.<sup>12,36</sup> Seroreactivity was defined by reactivity to viral envelope and/or gag proteins of the expected size as seen in the positive control antisera. This WB test accurately detected XMRV antibodies in three experimentally infected macaques equivalent to detection using recombinant proteins in recently described immunoassays.<sup>27</sup>

#### Quantitative reverse transcription–PCR

RNA was extracted from 100 µL of selected donor whole blood samples using Qiagen Viral RNA Mini kit. The isolated RNA was subjected to reverse transcription by MLV reverse transcriptase (RT; Roche, Indianapolis, IN). The resulting cDNA was amplified in a real-time PCR procedure and quantified in a commercially available system (LightCycler 480, Roche). Quantitative reverse transcription (qRT)-PCR was performed with FastStart *Taq* polymerase (Roche) in 45 amplification cycles of 95 and 60°C for 30 seconds each. Two primer pairs were used, integrase (F2 [5'-AACCTGATGGCAGATCAAGC-3'], R2 [5'-CCCAGTTCCCGTAGTCTTTTGGAG-3'], and XMRV probe [5'-FAM-AGTTCTAGAAACCTCTACTC-BHQ1-3'])<sup>13</sup> or *gag* (Q445F [5'-GGACTTTTTGGAGTGGCTTTGTT-3'], Q528R [5'-GCGTAAAACCGAAAGCAAAAT-3'], and XMRV probe F480PRO-BHQ [5'-FAM-ACAGAGACTTCCCGCCCCG-BHQ1-3']).<sup>37</sup> A cutoff of 40 CTs was used as evidence for the presence of XMRV or MLV sequences in a specimen. Positive controls represented recombinant plasmid spiked into whole blood samples in a dilution series from 10<sup>6</sup> to 10<sup>4</sup> copies/mL.

#### Nested RT-PCR amplification of XMRV sequences

Nested RT-PCR was performed as described.<sup>38</sup> Briefly, RNA was extracted from 0.5 mL of donor plasma using a virus kit (QIAamp Ultrasens, Qiagen) and subjected to reverse transcription employing a first-strand synthesis system for RT-PCR (Superscript III, Invitrogen). Culture supernatant of the XMRV-producing prostate cancer cell line

22Rv1 was used at a 10<sup>-5</sup> dilution as a positive control for RNA isolation. For amplification of XMRV *gag* sequences, 5 µL of the transcribed cDNA was used for the first round of 40-cycle amplification with primers 419F (5'-ATCAGTTAACCTACCCGAGTCGGAC-3') and 1154R (5'-GCCGCCTCTTCTTCATTGTTCTC-3')<sup>8</sup> and master mix (HotStart-IT FidelityTaq, USB Corp., Cleveland, OH). Nested PCR was performed for 45-cycle amplification with 5 µL of the first-round PCR product and two different primer pairs, Gag-I-F (5'-TCTCGAGATCATGGGACAGA-3') and Gag-I-R (5'-AGAGGGTAAGGGCAGGGTAA-3') or NP116 (5'-CATGGGACAGACCGTAACTACC-3') and NP117 (5'-GCAGATCGGGACGGAGGTTG-3').<sup>39</sup> To monitor assay sensitivity, plasmid DNA containing a cloned fragment of XMRV *gag*<sup>12</sup> was included in each PCR run at concentrations from 1 to 100 copies/µL. PCR and RT-PCR of GAPDH controls with primer pairs, forward (5'-CATGTTCCAA TATGATTCAC-3') and reverse (5'-CCTGGAAGATGGTG ATG-3'), were performed to ensure similar levels of DNA and RNA input in each round of amplification.

#### Propagation of infectious XMRV in indicator cells

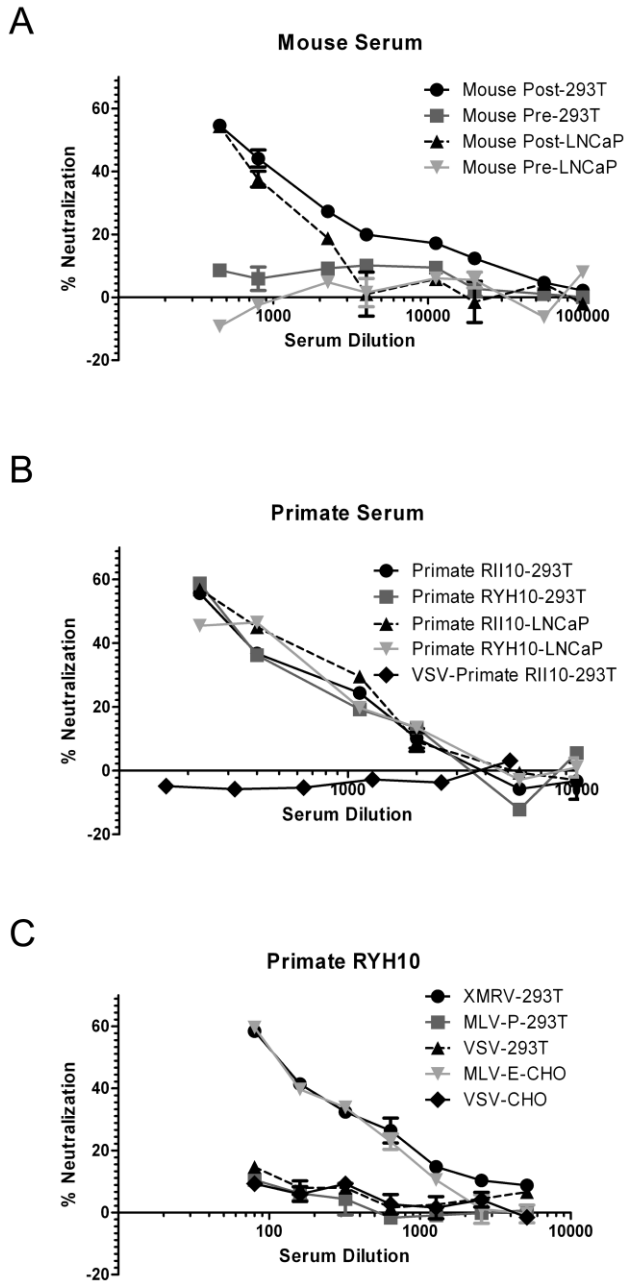
DERSE (detectors of exogenous retroviral sequence elements) indicator cells were developed at the National Cancer Institute by stable transfection of pBabe.iGFP-puro into LNCaP cells. The intron interrupted GFP gene from pBabe.iGFP-puro is only expressed after mobilization by an infecting gammaretrovirus for a second round of infection.<sup>40</sup> To test for the presence of infectious XMRV in selected donor plasma, DERSE.Li-G cells were inoculated with donor plasma or control plasma and spin infection, as described in Steffen and colleagues.<sup>38</sup> GFP expression was monitored every 3 to 4 days for a total period of 3 weeks. As a positive control, culture supernatant of the XMRV-producing prostate cancer cell line 22Rv1 (containing roughly 10<sup>9</sup> copies/mL) was used as an inoculum at 10<sup>-2</sup>, 10<sup>-4</sup>, and 10<sup>-6</sup> dilution.

## RESULTS

#### High-throughput microneutralization assay development

XMRV pseudoviruses (MLV-luc[XMRV Env]) were generated using a MLV-based retroviral backbone. These pseudoviruses infected both 293T and LNCaP cells. As expected from previous studies,<sup>41</sup> levels of infection mediated by the XMRV envelope were somewhat lower compared to control envelopes. For example, on 293T/17 cells, infection of unconcentrated MLV-luc(XMRV Env) was about equal to VSV-G pseudotyped virus stocks diluted 10-fold (67,714 and 63,742 relative light units, respectively). On both cell types MLV-luc(XMRV Env) was neutralized by sera from mice (Fig. 1A) and rhesus macaques





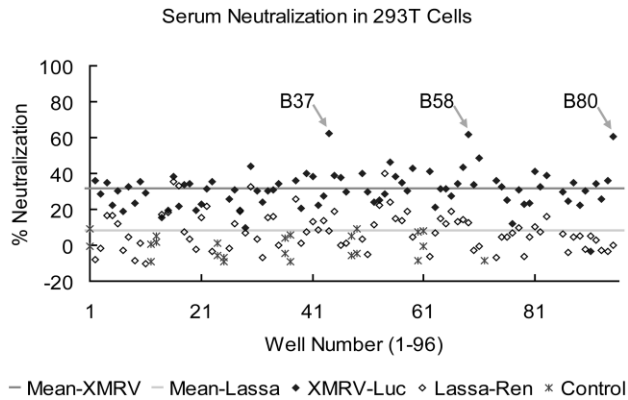
**Fig. 1. Detection of XMRV Env neutralizing antibodies in positive controls.** MLV-luc(XMRV Env) pseudovirus infection of 293T/17 and prostate LNCaP cells was neutralized by sera from both mice (A) and rhesus macaques (B) challenged with XMRV, whereas no clear neutralization was observed with preimmune sera or HIV-luc(VSV G) pseudoviruses. (C) MLV-luc(MLV-E Env) pseudoviruses were neutralized by sera from rhesus macaques challenged with XMRV in mCAT-1 expressing CHO cells (CERD9 cells), but no clear neutralization of LacZ encoding MLV-P or HIV-luc(VSV-G) pseudoviruses was observed in 293T/17 cells. Infection of pseudoviruses with firefly luciferase reporter was detected with a luciferase assay system (Bright-Glo, Promega), whereas infection of LacZ encoding MLV-P was measured using a system for detection of  $\beta$ -galactosidase (Galacto-Light Plus, Applied Biosystems). Absolute values for the no sera controls were as follows: MLV-luc(XMRV Env) gave 55,810 relative light units (RLU) on 293T cells and 20,213 RLU on LNCaP cells; HIV-luc(VSV-G) gave 65,961 RLU on 293T cells and 51,677 RLU on CHO cells; and MLV-P gave 32,356 RLU on 293T cells and MLV-luc(MLV-E Env) gave 41,771 RLU on CHO cells. Results are presented as percentage of neutralization and shown as mean  $\pm$  SD of triplicate measurements. A representative experiment of at least two experiments is shown.

(Fig. 1B) challenged with XMRV, whereas no clear neutralization was observed with preimmune sera. Similar results were obtained with HIV-luc(XMRV Env) (data not shown). Moreover, ecotropic MLV pseudoviruses (MLV-luc[MLV-E Env]) were also neutralized by sera from rhesus macaques challenged with XMRV (Fig. 1C). However, LacZ encoding MLV-P or HIV-luc(VSV G) pseudoviruses (Figs. 1B and 1C) were not neutralized.

To develop a reliable high-throughput assay system for the screening of large numbers of samples for XMRV infection, we generated a cell-based XMRV microneutralization assay system based on the internally controlled DEP assay we recently developed to screen for small-

molecule inhibitors,<sup>22</sup> which has been proven to be a rapid, safe, sensitive, and specific high-throughput system for antiviral drug discovery targeting viral entry. We adopted a similar approach here for the XMRV microneutralization assay. The assays were performed in 96-well plate format with the aid of liquid dispensing equipment for high-throughput applications. After preliminary experiments, a combination of MLV-luc(XMRV Env) and HIV-ren(Lassa-GP), which showed no clear interference between the two envelopes, was chosen for the sera screening. This combination proved to give very robust and reproducible results. A combination of MLV-luc(XMRV Env) and HIV-ren(Lassa-GP) from three 96-well plates indicated that the interplate coefficient of variation (CV)<sup>42</sup> was 8.2 and 5.2% for MLV-luc(XMRV Env) and HIV-ren(Lassa-GP), respectively. A set of 20 sera samples indicated that for the intraassays, the CV of every sample in triplicate was within 5% and for the interassays, the CV of every sample from three plates was within 12%, for both MLV-luc(XMRV Env) and HIV-ren(Lassa-GP) (data not shown).

Generally, sera showed relatively higher levels of neutralization of XMRV Env pseudoviruses (approx. 30%) than the Lassa-GP control (approx. 8%; Fig. 2). Similar results were obtained with sera at 240-fold dilutions, in individual virus alone, and in LNCaP cells (data not shown). Despite this higher level of background neutralization, neutralization with a number of sera was noticeably more pronounced. For example, in Fig. 2, three (B37, B58, and B80)



**Fig. 2. XMRV Env neutralizing antibody in blood donor sera using a cell-based XMRV microneutralization assay system. Shown is an example screen of 80 donor serum samples (80-fold dilutions) for XMRV neutralization with virus combinations of MLV-luc(XMRV Env) and HIV-ren(Lassa-GP) in a 96-well plate format. Three (B37, B58, and B80) of a total of more than 80 donor sera showed approximately 50% reduction in XMRV Env-, but not Lassa-GP-, mediated viral infection in 293T/17 cells.**

of more than 80 donor sera showed approximately 50% reduction in XMRV Env, but not Lassa-GP, mediated viral infection in 293T/17 cells.

### Screening of blood donors

We used this assay to screen a total of 354 blood donor sera collected within the United Blood Service region of Reno/Lake Tahoe. The Reno facility was chosen due to the collection territory including regions of Northern Nevada and California known to have clusters of CFS.<sup>43-45</sup> Patients from CFS clusters, including the Reno/Lake Tahoe area, formed the majority of subjects in the original demonstration of the presence of XMRV in blood.<sup>8</sup> Twenty-three sera gave more than 50% reduction in XMRV Env, but not Lassa-GP, mediated viral infection at either 80- or 240-fold dilutions. All 23 serum samples showed a dose-dependent neutralization of XMRV pseudoviruses (approx. 60% neutralization at 80-fold dilution), but unlike the mouse and macaque antisera, the blood donor sera demonstrated very limited neutralization for MLV-E pseudoviruses (<50% at 80-fold dilution; Fig. 3). No clear neutralization was detected for VSV pseudoviruses (Fig. 3) and LacZ encoding MLV-P (data not shown).

### Confirmatory testing

The 23 moderately neutralizing sera (>50%) as well as 14 additional poor neutralizers (approx. 30%-50%) and 12 donors with no clear neutralizing ability (<30%) were further assessed with a recently developed WB assay<sup>12,36</sup>

using purified, denatured XMRV antigen from XMRV-infected DU145 prostate cells (C7). All 50 of the tested blood donor sera were WB-negative (Fig. 4).

To further confirm whether there was any evidence of XMRV or other MLV infection in these individuals that would lead to a positive serologic response, we performed PCR assays and virus cultures that would detect both specifically XMRV and more broadly other MLVs. Whole blood samples of the selected donors were tested by qRT-PCR using primer sets located in either XMRV integrase<sup>40</sup> or *gag*. No positive signal was seen in any sample with either primer set (data not shown). Plasma samples of the 23 selected donors were also tested and found negative by nested RT-PCR using generic MLV primers previously shown to detect both XMRV and the broader family of xenotropic and polytropic MLVs<sup>39</sup> (Fig. 5).

To test for the presence of infectious MLVs in donor plasma, the indicator cell line DERSE was used. As a positive control, culture supernatant of the XMRV-producing prostate cancer cell line 22Rv1 (containing roughly RNA  $10^9$  copies/mL) was utilized as an inoculum. Whereas cells inoculated with 22Rv1 supernatants showed a concentration-dependent GFP expression on Day 7 and spread of the virus on Day 21 as previously described,<sup>38</sup> no GFP expression could be observed in any of the cells inoculated with donor plasma from the 23 seroreactive persons, even when spin infection was used to enhance the potential infection efficiency (data not shown).

## DISCUSSION

Determining whether serologic evidence of immune responses to gammaretroviruses in humans<sup>8,21</sup> is an indication of authentic infection or just nonspecific cross-reactivity is an important final step in the XMRV saga. In this study, we generated a robust, high-throughput microneutralization assay for the screening of large numbers of subjects for serologic evidence of XMRV and MLV infection based on the DEP assay system we recently developed.<sup>22</sup> This assay includes an internal control pseudovirus that is very useful for avoiding nonspecific inhibition and also controls for cytotoxicity. This method provides a reproducible high-throughput microneutralization research assay for large-scale testing for evidence of XMRV and MLV infection.

Currently, enzyme immunoassays (EIAs) and WB are the two most common serologic methods utilized for viral diagnosis.<sup>46,47</sup> WB is limited to the recognition of linear epitopes and is prone to high-background rates, while EIA can be restricted by the quality of the antigens, antibodies, and detection methods. Instead of directly detecting the existence of antiviral antibodies in the sera, the DEP-based microneutralization assay is based on the ability of a serum to neutralize pseudovirus infection. Compared with standard assays such as EIAs, the microneutraliza-

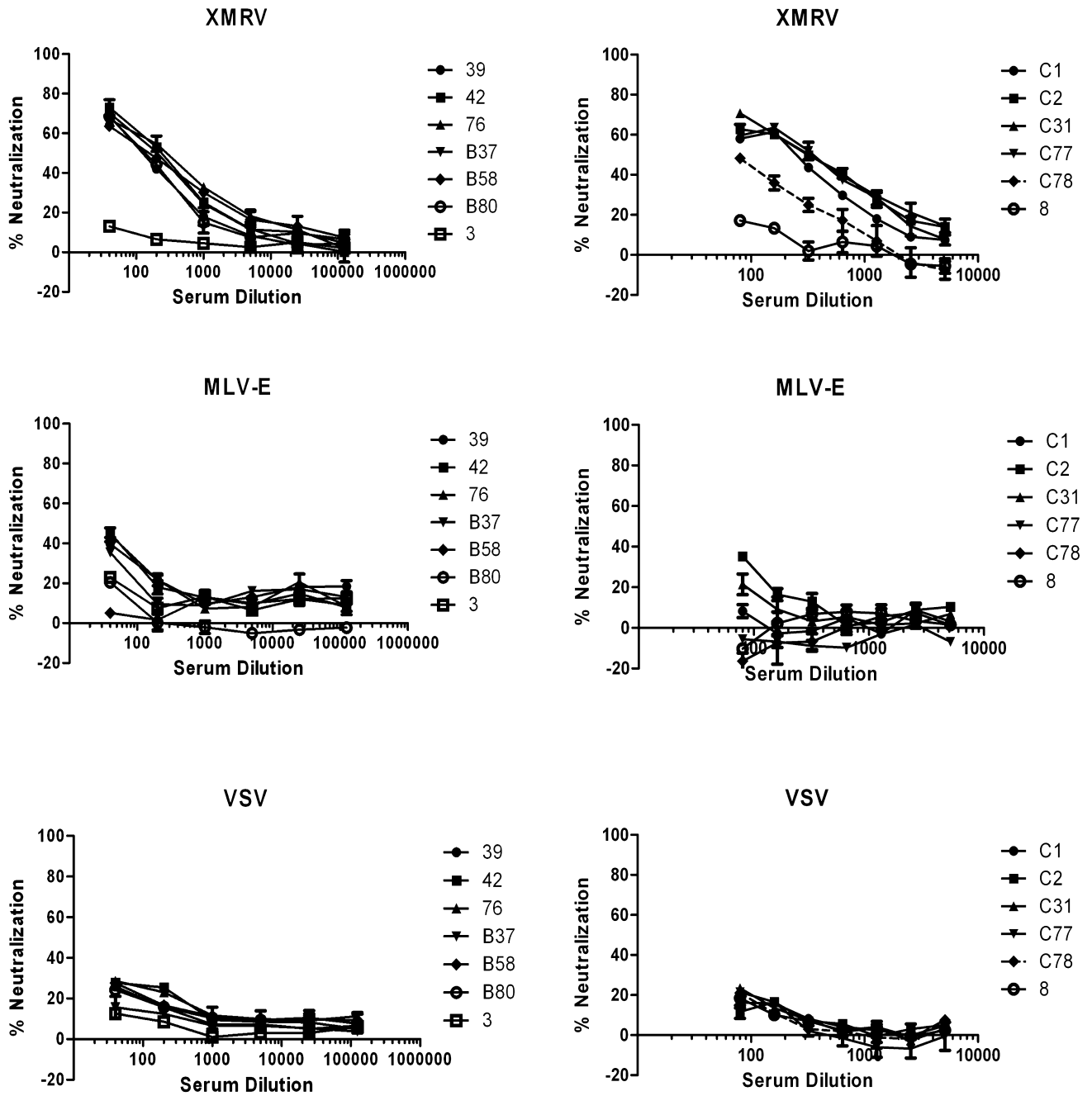
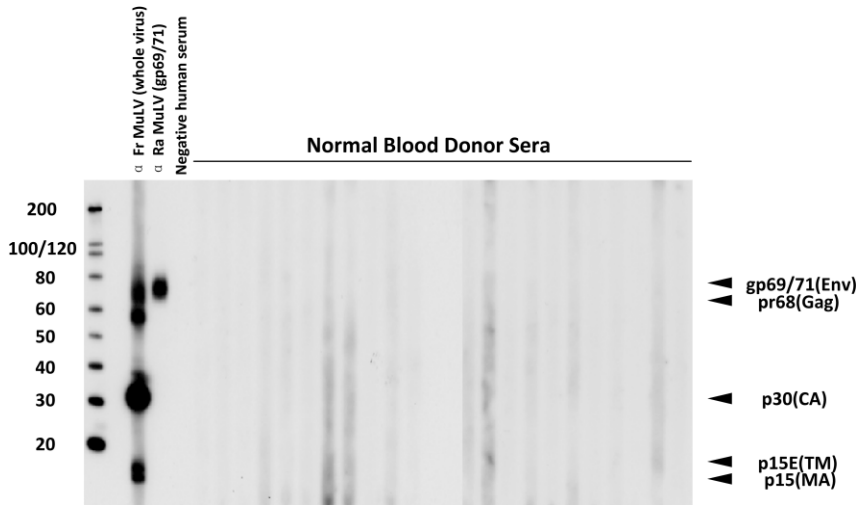


Fig. 3. Dose-response curves with selected blood donor sera. Neutralization of infection of HIV-luc(XMRV Env) and HIV-luc(VSV G) pseudoviruses with serially diluted donor sera samples were detected in 293T/17 cells and HIV-luc(MLV-E Env) in mCAT-1 expressed CHO cells (CERD9 cell). Results are presented as percentage of neutralization and shown as mean  $\pm$  SD of triplicate measurements. A representative of at least two experiments is shown.

tion assay has fewer steps and can be performed by automated liquid handling equipment, which may generate less SD. The disadvantage is a 2-day incubation period which impacts the clinical usefulness of the assay.

A recent study identified neutralizing activity against XMRV in approximately 14% of blood donor samples,<sup>10</sup>

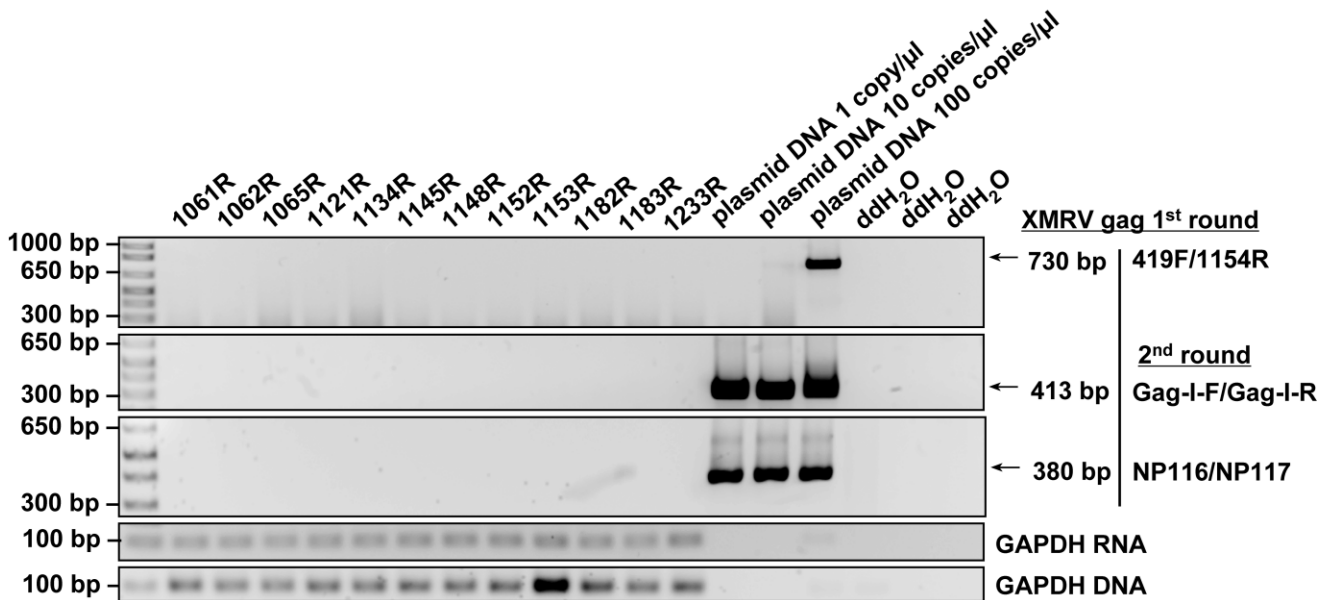
although in this instance many of these sera neutralized control viruses in addition to XMRV. In contrast, while we identified 23 of 354 blood donors (6.5%) able to moderately neutralize XMRV Env-mediated infection, control and other MLV envelopes were poorly or not at all neutralized. None of the samples tested showed any evidence of a serologic response to XMRV by WB testing. Furthermore,



**Fig. 4. Absence of XMRV/MLV antibodies in blood donor sera by WB analysis.** Purified, denatured XMRV antigen from XMRV-infected DU145 prostate cells (C7) was used for WB detection of XMRV or MLV antibodies in selected donor sera samples. Results of positive control antisera to purified XMRV antigen and 24 normal donor sera samples (B58, B80, E6, E8, E10, D17, D40, C5, C20, C30, C33, C35, C45, C47, C49, C50, C51, C67, 3, 4, 5, 6, 7, 8, from left to right) are shown; locations of reactivity to specific viral proteins are indicated. Env (gp69/71) = envelope; TM (p15E) = transmembrane; Gag (pr68); MA (p15) = matrix; CA (p30) = capsid. Molecular weight markers (kDa) are provided on the left of the WB.

all 23 seroreactive samples were negative for XMRV and MLV sequences using PCR or virus culture. These PCR and culture assays were designed to detect a broad range of gammaretroviruses, as well as XMRV specifically, thus excluding XMRV/MLV and other gammaretroviruses as a source of the nonspecific reactivity. The finding that neutralization by the 23 blood donors was specific to XMRV envelopes, but not other MLV envelopes, was surprising. Pairwise comparison of the amino acid sequence of the envelope region between XMRV and MLV-P or MLV-E shows the amino acid similarity is approximately 89 and 68%, respectively.

Given that the true XMRV neutralizing responses raised in animals were more broadly neutralizing (Fig. 1), this result strongly argues against specific neutralization, but rather suggests the moderate neutralization observed was mediated by other nonspecific means. This could be cross-reactive antibodies raised against endogenous retroviral elements, completely unrelated proteins, or other nonantibody serum factors. Human serum potentially inhibits



**Fig. 5. Absence of XMRV gag sequences in blood donor plasma by nested RT-PCR.** A representative result of 12 donor samples is shown with positive controls containing 1 to 100 copies/ $\mu$ L of a plasmid harboring a cloned fragment of XMRV gag<sup>12</sup> and negative water controls. First-round PCR amplification used primer pair 419F and 1154R and second-round PCR amplification used primer pairs Gag-I-F and Gag-I-R or NP116 and NP117. GAPDH RNA and DNA PCR results for the same samples are shown in the bottom two panels.



XMRV;<sup>14</sup> however, this is largely complement driven, and in our assay serum complement was inactivated by heating and did not influence our test results. The relatively high level of nonspecificity is greater than that seen with other microneutralization assays<sup>20,48</sup> and is partly due to the lack of known human positive cases that can be used to accurately set cutoffs for defining specific neutralization. Our results likely also explain other reported XMRV neutralization results in human samples.<sup>21</sup>

In addition to the initial association of XMRV to CFS made by Lombardi and colleagues,<sup>8</sup> a second publication by Lo and colleagues,<sup>39</sup> based only on PCR analysis, also yielded a strong association between CFS and MLV-like viruses.<sup>39</sup> These subsequent viruses demonstrated a far greater degree of sequence variation than XMRV, with the majority of sequences resembling P-MLV. Although Lo and coworkers<sup>39</sup> reported very stringent measures to minimize contamination, the most parsimonious explanation, given the extent of reported contamination of laboratory reagents, is that their PCR results are false positives resulting from reagent contamination. Indeed, Lo and colleagues used platinum *Taq* (Invitrogen) for PCR amplification, which several groups have convincingly demonstrated is contaminated with mouse DNA<sup>14,15,49</sup> due to the use of a mouse monoclonal antibody in the enzyme mix. Furthermore, recent detailed phylogenetic analysis of the longitudinal MLV-P sequences reported by Lo and coworkers showed that these sequences are inconsistent with retroviral evolution.<sup>50</sup> Nonetheless, the findings of Lo and colleagues raised the hypothesis that while XMRV itself is clearly a laboratory contaminant, the serologic responses detected in Lombardi and coworkers may be due to infection by other MLVs or gammaretroviruses. The serologic assay used by Lombardi and coworkers relies on antibody binding to the MLV spleen focus-forming virus (SFFV) Env expressed on the surface of cells. The logic of this assay is that conformationally dependent cross-reactive epitopes shared between this mouse gammaretrovirus and XMRV would bind XMRV antibodies, which would then be detected in a flow cytometry-based assay. However, it is likely that, as with our microneutralization assay, mammalian cell culture-based expression of an unrelated retrovirus Env would be highly prone to non-specific cross-reactivity that can confound the testing and which requires clarification by WB analysis using purified antigen. Indeed, when the Lombardi and colleagues' flow-based assay was used by two laboratories on plasma specimens in a blinded study, high levels of nonspecific reactivity were observed.<sup>15</sup>

In conclusion, we developed a robust, high-throughput microneutralization assay to conduct studies seeking evidence of infection with XMRV and MLV. Although a small proportion of blood donors demonstrated the ability to block XMRV-mediated infection, we found no evidence that this inhibition was mediated by

specific antibodies elicited by exposure to XMRV or related MLVs. It is likely that this moderate neutralization is mediated through another, nonspecific mechanism. Our findings also explain further the highly nonreproducible and nonspecific serologic responses detected with other assays.<sup>8,17</sup> In addition, this microneutralization assay system can be easily adapted to screen donor samples against other viruses with careful selection of matching partner virus envelopes, which will provide important information for neutralizing antibody responses and infectious disease profiles.

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#### CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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# No Evidence for XMRV Nucleic Acids, Infectious Virus or Anti-XMRV Antibodies in Canadian Patients with Chronic Fatigue Syndrome

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## Abstract

The gammaretroviruses xenotropic murine leukemia virus (MLV)-related virus (XMRV) and MLV have been reported to be more prevalent in plasma and peripheral blood mononuclear cells of chronic fatigue syndrome (CFS) patients than in healthy controls. Here, we report the complex analysis of whole blood and plasma samples from 58 CFS patients and 57 controls from Canada for the presence of XMRV/MLV nucleic acids, infectious virus, and XMRV/MLV-specific antibodies. Multiple techniques were employed, including nested and qRT-PCR, cell culture, and immunoblotting. We found no evidence of XMRV or MLV in humans and conclude that CFS is not associated with these gammaretroviruses.

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## Introduction

Chronic fatigue syndrome (CFS), also commonly referred to as myalgic encephalomyelitis (ME), is a complex disorder with an unknown etiology which is characterized by disabling physical and mental fatigue and pain that lasts for at least 6 months and lacks any obvious cause [1,2]. The sudden onset of symptoms and underlying activation of inflammatory pathways suggest an infectious agent as the triggering factor. Numerous viral and non-viral pathogens have been investigated in the context of CFS with as yet inconclusive results [1,2]. The xenotropic murine leukemia virus (MLV)-related virus (XMRV) was initially identified in human prostate cancer cells in 2006 [3]. It has since been thought to be the only member of the gammaretrovirus family known to infect humans and its possible role in the development of prostate cancer has been widely discussed [4]. In 2009, Lombardi *et al.* reported the detection of XMRV in both peripheral blood mononuclear cells (PBMC) and plasma of 67% of a CFS patient cohort compared to 3.7% in healthy controls [5]. This study has gained a high level of attention and was thought to mark a possible break-through in CFS research. Several studies have since addressed the possible connection between XMRV infection and CFS or prostate cancer, and the resulting evidence is controversially discussed in the field [4]. While one study reported the presence of other MLV-like sequences in CFS patients [6], others identified mouse DNA, human cell lines or commercial laboratory reagents to be a possible source of MLV contamination

[7]. Attempts to reproduce the initial findings in different CFS patient groups world-wide and in parts of the initial cohort have since failed [4,8,9]. Thus, more research is needed to resolve an association of MLV-like viruses in humans. In this study we performed an extensive analysis of whole blood and plasma samples from two well-characterized Canadian CFS patient cohorts and healthy controls utilizing multiple laboratory techniques, including nested and qRT-PCR, cell culture, and immunoblotting for the detection of XMRV/MLV nucleic acids, infectious virus, and XMRV/MLV-specific antibodies.

## Materials and Methods

### Ethics statement

All study protocols were reviewed and approved by the Human Research Ethics Boards of the University of Calgary and the University of Alberta and all study participants provided written informed consent. Laboratory testing of the samples was performed anonymously and blinded.

### Cohorts

All patients and controls examined in this study were part of cohorts from either Calgary or Edmonton, recruited in 2010 and 2011, respectively. All participants completed the De Paul Questionnaire [10] to gather demographic data and to elicit the Canadian Consensus Criteria (CCC) for ME/CFS as established by Carruthers *et al.* [1]. Moreover, all participants were screened



according to the Fukuda criteria [2]. Two participants did not meet the CCC and one participant did not meet Fukuda criteria, but all three were included on clinical grounds. The remainder of the CFS group met both the CCC and the Fukuda criteria. Healthy controls who showed more than one symptom of ME/CFS at moderate or greater severity were excluded. The CFS group (58 individuals) had a mean age of  $48.9 \pm 10.1$  years and 90% were female, compared to the healthy control group (57 individuals) with a mean age of  $47.6 \pm 10.6$  years and 89% female, reflecting the higher prevalence of the disease amongst women. A documented infectious onset could be reported by 59% of the CFS patients. Of the CFS patients, 93% have been sick for more than 2 years and 3% have been sick for 1–2 years, while 5% showed symptoms since childhood or adolescence.

### Nested RT-PCR

For detection of XMRV/MLV sequences by nested PCR, RNA was extracted from 0.5 ml plasma using the QIAamp Ultrasens Virus Kit (Qiagen). The isolated RNA was immediately subjected to reverse transcription employing the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen). Culture supernatant from the XMRV-producing prostate cancer cell line 22Rv1 was used at a  $10^{-5}$  dilution as a positive control for RNA isolation. For amplification of XMRV/MLV *gag* sequences, 5  $\mu$ l of the transcribed cDNA were used for the first round of amplification with primers 419F (5'-ATCAGTTAACCTACCCGAGTCG-GAC-3') and 1154R (5'-GCCGCCTCTTCTTCATTGTTCTC-3') [5] and HotStart-IT FidelityTaq Master Mix (USB) with the recommended component volumes. The amplification was initiated by incubation for 4 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 57°C and 1 min at 72°C, and a final incubation for 10 min at 72°C. Nested PCR was performed under the same conditions for 45 amplification cycles with 5  $\mu$ l of the first round PCR product and two different primer pairs, Gag-I-F (5'-TCTCGAGATCATGGGACAGA-3') and Gag-I-R (5'-AGAGGGTAAGGGCAGGGTAA-3') or NP116 (5'-CATGGGACAGACCGTAAGTACC-3') and NP117 (5'-GCAGATCGGGACGGAGGTTG-3'), both of which have been shown to detect both XMRV and MLV sequences [6]. To determine the assay sensitivity, serial dilutions of a cloned fragment of XMRV *gag* [9] ranging from 1 to 100 copies/ $\mu$ l were included in each PCR. The resulting PCR amplification products (730 bp for first round PCR and 413 bp or 380 bp for second round PCR, respectively) were analyzed by electrophoresis in 1.5% agarose gels. Any bands of approximately the correct size were excised and subjected to sequencing in order to determine homology to MLVs.

### qRT-PCR

For qRT-PCR analysis, RNA was extracted from 100  $\mu$ l of either whole blood or plasma using the Qiagen Viral RNA Mini Kit. The isolated RNA was subjected to reverse transcription by murine leukemia virus (MuLV) reverse transcriptase (Roche). The resulting cDNA was amplified in a real-time PCR reaction and quantified in a Roche LightCycler 480. Two different primer and probe sets were used for amplification of two distinct regions of the XMRV genome: primers XMRV-F2 5'-AACCTGATGGCAGATCAAGC-3' and XMRV-R2 5'-CCCAGTTCCCGTAGTCTTTTGGAG-3' and probe FAM-AGTTCTAGAAACCTCTCACTC-BHQ1 for amplification of the XMRV integrase gene [11], and WPI primers Q445F 5'-GGACTTTTGGAGTG-GCTTTGTT-3' and Q528R 5'-GCGTAAAACCGAAAGCAAAAAT-3' and probe FAM-ACAGAGACACTTCCCGCCCCG-BHQ1 for amplification of the XMRV-specific *gag* leader sequence [12] with FastStart Taq polymerase (Roche) in 45

amplification cycles of 95°C and 60°C for 30 sec each. Serial dilutions of a cloned fragment of XMRV *gag* [9] were used to produce standard curves (Fig. 1C). The sensitivity of the qRT-PCR assay was below  $10^3$  copies/ml plasma or whole blood.

### Virus culture

DERSE (Detectors of Exogenous Retroviral Sequence Elements) indicator cells were developed at the National Cancer Institute by stable transfection of pBabe.iGFP-puro into LNCaP cells. pBabe.iGFP-puro is an MLV vector encoding puromycin resistance and a CMV promoter driven GFP reporter gene which is interrupted by an intron placed in sense direction relative of the vector and transcribed antisense to the vector mRNA. The intron interrupted GFP gene is only expressed after mobilization by an infecting gammaretrovirus for a second round of infection. After screening clonal cell populations, the most sensitive clones were chosen and designated as DERSE.Li-G cells. To test for the presence of infectious MLVs in patient plasma, DERSE.Li-G cells were inoculated with CFS patient plasma or control plasma. Cells were seeded 72 hours before infection with  $3 \times 10^4$  cells/ml in 6-well plates. For spinoculation, the medium was removed and 300  $\mu$ l fresh medium and 50  $\mu$ l plasma were added per well. The plates were centrifuged at 1,200 rpm for 1 hour and 0.5 ml fresh medium was added. The inoculum was removed the next day and the cells were cultured in 2 ml fresh medium and monitored for GFP expression every 3 to 4 days for a total period of 3 weeks. As a positive control, culture supernatant from the 22Rv1 cell line (containing roughly  $10^9$  copies/ml as determined from the average of seven individual qPCR assays, data not shown) was used as an inoculum at  $10^{-4}$ , and  $10^{-6}$  dilution, respectively.

### Serology

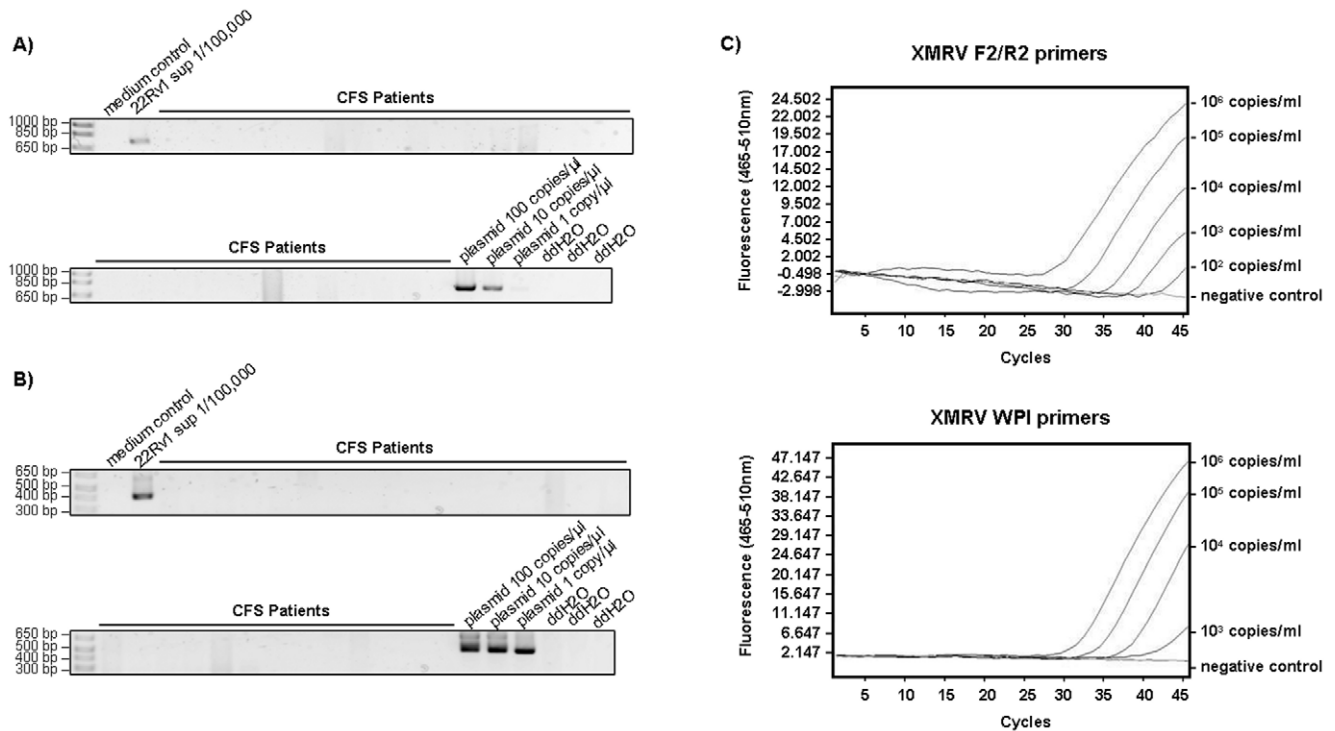
Western blot (WB) analysis was performed to detect anti-XMRV/MLV antibodies in CFS patient sera and healthy controls. Purified XMRV antigen from XMRV-infected DU145 prostate cells (C7) was denatured with SDS-PAGE sample buffer at 95°C for 10 min and analyzed by immunoblotting as previously described [9]. Seroreactivity was defined by reactivity to viral Env and/or Gag proteins of the expected size as seen in the positive control antisera (Fig. 2B).

### Results

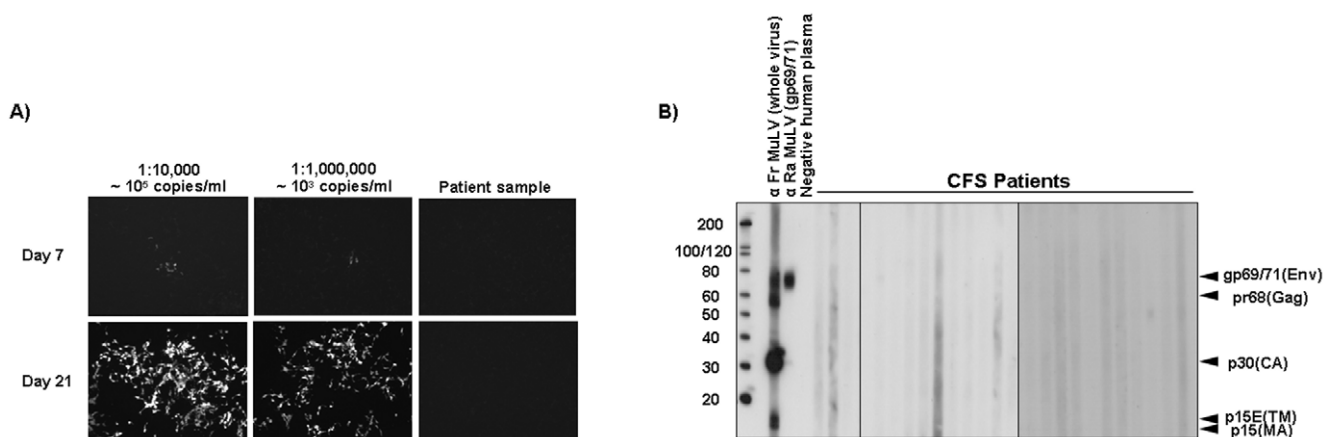
Whereas XMRV *gag* sequences were readily detectable in diluted 22Rv1 cell supernatants, XMRV and MLV were not detected in any of the patient plasma samples (Fig. 1A and B). The detection limit of the nested PCR assay was below 1 copy/ $\mu$ l isolated RNA or 5 copies/reaction as determined by the detection of known amounts of XMRV plasmid DNA (Fig. 1B). The sensitivity of the qRT-PCR assay was below  $10^3$  copies/ml plasma or whole blood. Regardless of whether whole blood or plasma was tested, all human samples were negative for detectable amounts of XMRV nucleic acid (data not shown).

DERSE.Li-G cells inoculated with 22Rv1 supernatants showed a concentration-dependent GFP expression on day 7 and spread of the virus on day 21. GFP expression was not observed in any of the DERSE.Li-G cells inoculated with patient plasma (typical example shown in Fig. 2A).

Seroreactivity was defined by Western blot reactivity to viral Env and/or Gag proteins of the expected size as seen in the positive control antisera (Fig. 2B). None of the 115 human plasma reacted with the purified XMRV antigen indicating an absence of antibodies to XMRV/MLV in the samples (typical example shown in Fig. 2B). Increased background noise as observed for one



**Figure 1. Failure of detection of XMRV nucleic acids in plasma and whole blood of CFS patients and healthy controls.** A) First round PCR products of a representative number of RNA samples isolated from patient plasma using primers 419F and 1154R. A  $10^{-5}$  dilution of 22Rv1 cell culture supernatant and three known concentrations of XMRV plasmid DNA were included as controls. B) Second round amplification products of nested PCR using primers Gag-I-F and Gag-I-R of samples shown in A). Identical results were obtained with primers NP116 and NP117 (see text, data not shown). The detection limit was below 1 copy/ $\mu$ l isolated RNA or 5 copies/reaction. C) Results of qRT-PCR for XMRV plasmid control in serial dilutions ranging from  $10^6$  to  $10^2$  copies/ml as well as negative controls for both primer pairs used, F2/R2 (upper panel) and WPI (lower panel). All patient plasma and whole blood samples were found to be negative after a total of 45 amplification cycles (data not shown). doi:10.1371/journal.pone.0027870.g001



**Figure 2. No evidence for infectious virus or XMRV-specific antibodies in plasma of CFS patients and healthy controls.** A) GFP expression of DERSE.Li-G cells 7 days (upper panels) or 21 days (lower panels) after spinoculation with two different dilutions of 22Rv1 cell culture supernatants ( $10^{-4}$  and  $10^{-6}$  dilution) or patient plasma. No GFP expression could be observed in any of the cells inoculated with human plasma. B) Immunoblotting of C7-purified XMRV antigen with patient plasma for detection of anti-XMRV/MLV antibodies. Representative WB results for CFS patients and healthy controls. Lane 1, anti-Friend MuLV whole virus, goat polyclonal antisera; lane 2, anti-Rauscher MuLV envelope, goat polyclonal antisera; lane 3, XMRV negative blood donor plasma. Locations of reactivity to specific viral proteins are indicated; Env (gp69/71), envelope; TM (p15E), transmembrane; MA (p15), matrix; Gag (pr68); CA (p30), capsid. doi:10.1371/journal.pone.0027870.g002

of the CFS patient samples (lane 5, Fig. 2B) is most likely due to the presence of cross-reactive epitopes.

## Discussion

In summary, we were unable to detect any evidence of XMRV or MLV infection in any of the 115 examined study participants, regardless of whether they were suffering from CFS or represented healthy controls. The 58 CFS patients enrolled in this study were carefully selected according to the Canadian Consensus Criteria for ME/CFS. Positively screened participants were only included if they showed symptoms in at least two categories of autonomous, neuroendocrine, and immune manifestations. The sensitivity of our assays reached copy numbers lower than 120 copies/ml of plasma for the detection of viral nucleic acids, and  $10^3$  copies/ml of plasma for the presence of infectious particles. While it is possible that XMRV and MLV are not predominantly blood-borne viruses and as such exist below the detection limit of most assays in plasma and whole blood, we believe that the assays used in this study are equally sensitive to those reported in previous positive studies. Moreover, our broad study design and the use of degenerate primers with specificity for highly conserved sequences in different MLV-like viruses and XMRV would have allowed us to identify nucleic acids, infectious particles, and antibodies for a number of related murine retroviruses. However, we could not detect any other murine retroviruses in any of our specimens, unlike the finding of MLV-like sequences reported by Lo *et al.* [6].

CFS patient cohorts have been tested for the presence of XMRV in the United States, Netherlands, Germany, China, and United Kingdom among others [4]. Being more aware of the possible risk of contaminants in commonly used laboratory reagents [13], none of these studies were able to reproduce the initial findings. Moreover, repeated testing of CFS patients previously reported to be infected with XMRV in the initial study performed by Lombardi *et al.* failed to detect any signs of XMRV infection in these patients [8]. On the contrary, it is now becoming increasingly clear that XMRV found in the prostate cancer cell line 22Rv1 originated from recombination of two MLVs present in the mouse strains used for passaging of the initial prostate cancer xenograft [14]. The fact that the viral sequences initially identified in prostate and CFS samples are virtually identical to those found in 22Rv1 cells [15] suggests that the assumed

association of XMRV with human diseases is due to sporadic laboratory contamination. Moreover, differential handling of patient samples compared to controls can introduce bias and was therefore carefully avoided in this study. Two independent studies could show that handling of human samples in laboratory environments with abundant endogenous MLV proviruses can lead to the false detection of XMRV/MLV-like sequences due to contamination as proven by PCR detection of the highly abundant intracisternal A-type particle (IAP) long terminal repeat in the same samples [16,17]. In the light of the accumulating evidence for the artefactual origin of XMRV and the high burden of MLV-like DNA contamination the initially reported connection of XMRV and prostate cancer is now being ruled out as well [18]. Thus, although XMRV was found to infect and replicate in a variety of human cells, natural XMRV/MLV infection of humans has not yet been reproduced and is believed to be a false-positive result from mouse DNA and/or MLV-contaminated PCR reagents [13]. This study examines a possible association of XMRV and chronic fatigue in a Canadian patient cohort and is consistent with a number of recently published reports declaring no evidence for the presence of MLV-like viruses in any human subjects. In conclusion, while this study and others fail to support an association between XMRV and CFS, they highlight the urgent need for further research into the root causes of CFS.

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Use of trade names is for identification only and does not imply endorsement by the U.S. Department of Health and Human Services, the Public Health Service, or the Centers for Disease Control and Prevention. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

## Author Contributions

Conceived and designed the experiments: DLT ES TL YZ WMS MH GS. Performed the experiments: IS LM KL ST HJ DH DMS ML AL JL. Analyzed the data: IS TL WMS MH GS. Wrote the paper: IS MH GS.

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## The scientific method at work: xenotropic murine leukemia virus–related virus is neither a cause of chronic fatigue syndrome nor a threat to the blood supply

Matthew S. Karafin and Susan L. Stramer

**S**ir Francis Bacon (1561–1626), the Lord Chancellor of England, was the first to provide a documented philosophical method for investigating a natural phenomenon. This method later became known as “the scientific method.” Unlike many before him, he suggested that our understanding of the world should be based on data, rather than faith or dogma. Moreover, his method required that our understanding of the world be provisional, with the hope that our current understanding of natural phenomena would eventually be replaced by better science. The events that have transpired over the past 2 years regarding the clinical relevance of xenotropic murine leukemia virus–related virus (XMRV) have shown that the method of scientific investigation first described 400 years ago is very much alive and well today.

The scientific method, as envisioned by Sir Francis Bacon, starts with observation. Urisman and colleagues<sup>1</sup> first observed that XMRV is associated with human disease in 2006, finding that 40% of men with prostate cancer and a low activity variant of RNase L, an enzyme involved in the interferon-induced antiviral response, were infected with this virus. Subsequently in 2009 and 2010, two groups of investigators also described finding XMRV or related sequences of polytropic murine leukemia viruses (MLVs) in association with chronic fatigue syndrome (CFS), a disease characterized by severe fatigue and other related symptoms lasting more than 6 months.<sup>2,3</sup>

XMRV is currently understood to be a retrovirus, but is unrelated to other well-described retroviruses such as

human immunodeficiency virus (HIV) and human T-cell lymphotropic virus (HTLV). XMRV specifically is a member of the family Retroviridae, subfamily Orthoretrovirinae, and genus *Gammaretrovirus* (see AABB XMRV Fact Sheet <http://www.aabb.org/resources/bct/eid/Pages/default.aspx>). XMRV is the first gammaretrovirus to be found in humans, and data indicate that it originated in mice after the recombination of two murine proviruses.<sup>4</sup> Virions are 80 to 100 nm in diameter, consisting of an envelope, nucleocapsid, and a nucleoid with a linear dimer of positive-sense, single-stranded RNA.<sup>5</sup>

From these initial observations, it was hypothesized that this virus could be causally related to both prostate cancer and CFS. Moreover, the finding of viral sequences in the blood of healthy controls in two studies<sup>2,3</sup> led to the concern that this virus could be transfusion transmitted, and thus the national blood supply could be at risk. Specifically, on June 18, 2010, the AABB issued a bulletin to its membership from its Interorganizational Task Force on XMRV that patients diagnosed with CFS be discouraged from donating blood. Consequently, the American Red Cross and a number of other blood donor centers started to offer educational information about CFS and have requested voluntary deferral of donors who ever have had a medical diagnosis of this debilitating condition.<sup>6</sup>

While the risk of transmission of XMRV by blood products was unknown at the time of release of this bulletin, the recommendation of the AABB Interorganizational Task Force was reasonable based on the initial hypothesis that CFS could have an infectious origin. First, XMRV is a gammaretrovirus, a genus that contains known animal pathogens (see AABB XMRV Fact Sheet). As other retroviruses, such as HIV and HTLV, are transfusion transmitted, it was plausible that an emerging retrovirus, such as XMRV, could also be transmitted by blood. Second, studies indicated that XMRV was physically present in blood. A rhesus macaque model of XMRV previously demonstrated that the virus can infect lymphoid cells, several tissues, and organs even though circulation of free virus was minimal.<sup>7</sup> Moreover, Lombardi and colleagues<sup>2</sup> found XMRV infection in the lymphocytes of the CFS patients

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studied. As lymphocytes are present in transfused cellular blood components, such as platelet products, red blood cells, and granulocytes, there is a substantial risk that this virus could be transmitted by transfusion.

The hypothesis that XMRV was a cause of human disease did not, however, withstand confirmation by other researchers who were investigating the same relationship. One review found that XMRV has been detected in 0% to 27.5% of prostate cancer patients in 12 studies.<sup>5</sup> Moreover, while the two original positive CFS studies found that XMRV/MLV sequences were present in up to 86.5% of CFS patients and up to 7% of healthy controls including blood donors,<sup>2,3</sup> as of 2011, at least 30 subsequent studies failed to reproduce these findings in CFS or other patient groups (see AABB XMRV Fact Sheet table). Additionally, these studies have failed to demonstrate a relationship between XMRV/MLV and CFS even with highly sensitive methods modeled after those described by Lombardi and coworkers,<sup>2</sup> including various molecular, serologic, and culture procedures, as well as enrolling many of the same CFS patients for study whose samples were previously identified as XMRV or MLV positive.<sup>8-11</sup>

Originally, several hypotheses were posed to explain the wide variations between study findings, including differences in cohort selection, testing methods, or sample source, preparation, and storage.<sup>6,10,12</sup> However, recent data indicate that laboratory, sample, and/or reagent contamination are the most likely causes for the results in those studies with positive findings.<sup>4,8,9</sup> The Scientific Research Working Group (SRWG) of the NHLBI, including several key authors of the original article associating XMRV/MLVs with CFS, sent samples in a blinded fashion to nine different laboratories, all with research tests for XMRV including detection of nucleic acids (11 laboratories), antibody (five laboratories), and virus following culture (three laboratories). They found that current testing methods do not reproducibly detect XMRV, even in blood samples from patients that were reported to have XMRV infection previously (including 14 with CFS) or in XMRV-negative, healthy controls.<sup>11</sup> Of note in this study, only the two laboratories associated with Lombardi and colleagues<sup>2</sup> obtained positive results for any samples (excluding the spiked positive controls); however, these laboratories found positive DNA and antibody results in healthy controls at the same rate as positive results in CFS patients with the additional caveat that positive patient results were inconsistent. Perhaps most importantly, Paprotka and colleagues<sup>4</sup> proposed that XMRV originated as the result of a laboratory recombination event involving two mouse proviruses that occurred during the serial passage of a human prostate cancer xenograft (CWR22) in nude mice in the 1990s. When aligned, these two proviruses were identical to the sequence of XMRV. Thus, the authors concluded that XMRV is not a real human pathogen and that positive findings were the result of contami-

nation by a laboratory-derived virus.<sup>4</sup> In a subsequent related study, it was shown that both proviruses occurred in laboratory mice but not in wild strains of mice, and no laboratory mouse strain could harbor XMRV replication due to the lack of the required receptor in laboratory mice, indicating that the xenografted human tumor cells were required for XMRV propagation.<sup>13</sup> In addition, the genetic distance among *env* and *pol* sequences from the persistently XMRV-infected prostate cell line, 22Rv1, derived from the CWR22 xenograft, exceeds that of patient-associated sequences, suggesting laboratory contamination versus human infectious transmission. Thus, XMRV derived from the 22Rv1 cell line is the genetic ancestor of all subsequent isolates from CFS or other patients.<sup>14</sup>

XMRV also does not appear to be a concern for blood recipient safety. Studies have recently demonstrated that XMRV would not be able to persist or replicate in human blood due to cell-mediated antiviral pathways.<sup>9</sup> A large recent study further demonstrated that no XMRV antibody could be detected from 17,249 blood donors or recipients, including 13,399 US blood donors from six different regions and 3741 donors linked to 109 recipients of which 830 samples were tested over a 2-year period. A positive antibody result required reactivity to three different XMRV proteins, and the tests used were the same as those used by the SRWG and represented those tests that were automated and could be used for blood donation screening if needed. Since RNA could also not be found in any recipient or any donor with isolated antibody reactivity, the study concludes that XMRV is not a current threat to blood safety.<sup>15</sup>

The mounting negative findings failing to associate XMRV/MLV with human disease, and now documentation of XMRV as a laboratory artifact, prompted the Editor of *Science* to call for a retraction of the 2009 publication by Lombardi and colleagues in an expression of concern.<sup>16</sup> However, the authors of the original study have not agreed to retract their original work entirely; a partial retraction initiated by one author, and signed by the other authors, has resulted in removal of the polymerase chain reaction data due to sample contamination with XMRV plasmid DNA.<sup>17</sup> Taken together, the scientific data to date indicate that XMRV/MLV is neither a human pathogen nor a risk to the national blood supply (see reviews detailing the chronology of events and investigations of potential XMRV disease associations since October 2009<sup>18-20</sup>). Most recently (December 23, 2011), the Editor-in-Chief of *Science* has issued an editorial retraction of Lombardi et al.<sup>2</sup> due to the inability of multiple laboratories to reproduce the study findings, including those of the original authors; questions of quality control related to a number of specific reported experiments; and an overall loss in confidence in the validity of the conclusions.<sup>21</sup> This was followed by a retraction of the Lo et al. manuscript<sup>3</sup> by the authors on December 27, 2011. One study examining the

potential link of XMRV with CFS is still pending. The study is sponsored by the NIAID and led by Ian Lipkin of Columbia University; this likely will be the last major study investigating the disease potential of these agents.

Thus, over the past 2 years, XMRV has transformed from an agent of potential human disease association, and a possible threat to the national blood supply, to a laboratory contaminant without a current threat to humans. This revolution of ideas regarding XMRV could only have been made possible by the scientific method. Sir Francis Bacon aptly described the scientific process with the following metaphor:

Those who have handled sciences have been either men of experiment or men of dogmas. The men of experiment are like the ant; they only collect and use: the reasoners resemble spiders, who make cobwebs out of their own substance. But the bee takes a middle course; it gathers its material from the flowers of the garden and the field, but transforms and digests it by a power of its own. Not unlike this is the true business of philosophy; for it neither relies solely or chiefly on the powers of the mind, nor does it take the matter which it gathers from natural history and mechanical experiments and lay it up in the memory whole, as it finds it; but it lays it up in the understanding altered and digested. Therefore from a closer and purer league between these two faculties, the experimental and the rational (such as has never yet been made) much may be hoped. (Book 1, Aphorism 95)<sup>22</sup>

Just as Sir Francis Bacon predicted, the astute combination of a rational evaluation of current knowledge with rigorous experimental observation allows the scientific community to move seamlessly from an unproven working hypothesis to a result based on the synthesized accumulation of data disproving the hypothesis. In conclusion, the scientific process remains to this day a powerful tool to understand our natural world, and XMRV clearly demonstrates the potency of a 400-year-old method. Sir Francis Bacon would be proud.

#### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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