No Association of Xenotropic Murine Leukemia Virus-Related Viruses with Prostate Cancer


Laboratory Branch, Division of HIV/AIDS Prevention, National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention, Centers for Disease Control and Prevention, Atlanta, Georgia, United States of America

Abstract

**Background:** The association of the xenotropic murine leukemia virus-related virus (XMRV) with prostate cancer continues to receive heightened attention as studies report discrepant XMRV prevalences ranging from zero up to 23%. It is unclear if differences in the diagnostic testing, disease severity, geography, or other factors account for the discordant results. We report here the prevalence of XMRV in a population with well-defined prostate cancers and RNase L polymorphism. We used broadly reactive PCR and Western blot (WB) assays to detect infection with XMRV and related murine leukemia viruses (MLV).

**Methodology/Principal Findings:** We studied specimens from 162 US patients diagnosed with prostate cancer with an intermediate to advanced stage (Gleason Scores of 5–10; moderate (46%) poorly differentiated tumors (54%)). Prostate tissue DNA was tested by PCR assays that detect XMRV and MLV variants. To exclude contamination with mouse DNA, we also designed and used a mouse-specific DNA PCR test. Detailed phylogenetic analysis was used to infer evolutionary relationships. RNase L typing showed that 9.3% were homozygous (QQ) for the R462Q RNase L mutation, while 45.6% and 45.1% were homozygous or heterozygous, respectively. Serologic testing was performed by a WB test. Three of 162 (1.9%) prostate tissue DNA were PCR-positive for XMRV and had undetectable mouse DNA. None was homozygous for the QQ mutation. Plasma from all three persons was negative for viral RNA by RT-PCR. All 162 patients were WB negative. Phylogenetic analysis inferred a distinct XMRV.

**Conclusions and Their Significance:** We found a very low prevalence of XMRV in prostate cancer patients. Infection was confirmed by phylogenetic analysis and absence of contaminating mouse DNA. The finding of undetectable antibodies and viremia in all three patients may reflect latent infection. Our results do not support an association of XMRV or MLV variants with prostate cancer.


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* E-mail: bis3@cdc.gov

Introduction

Prostate cancer is one of the most frequent, slow growing, noncutaneous malignancies of men in the developing world [1]. For example, it is estimated that over 192,000 men in the US, mostly of African-American descent, will be diagnosed with prostate cancer this year [2,3]. Although the natural history of prostate cancer is currently unknown, multiple etiologies have been hypothesized, including genetic defects [4,5,6]. In 2006, using microarray and RT-PCR analysis xenotropic murine leukemia virus (MLV)-related virus (XMRV) was first identified in about 40% of familial prostate cancer patients containing the R462Q mutation in the RNase L gene, a component of the antiviral innate immunity [7]. MLVs are endogenous gammaretroviruses that have integrated into the mouse genome and can cause cancer, neurologic disease, and immunodeficiency disorders in mice and are classified into three groups based on their host tropism [8]. Xenotropic MLV (XMLV) replicate only in non-mouse cells. In contrast, ecotropic MLV (EMLV) replicate only in mice, while polytropic MLV (PMLV) have a broader tropism and can replicate in mouse and non-mouse hosts [8]. XMRV shares about 93% and 89% nucleotide identity with XMLV and PMLV across the genome [7]. Identification of a possible viral cause of prostate cancer is highly significant because it could facilitate treatment and prevention of this debilitating disease.

Additional evidence for XMRV infection of persons with prostate cancer has been reported in a US study showing a higher prevalence of XMRV DNA detection by PCR (6%) and viral proteins by immunohistochemistry (IHC) (23%) in prostate tissues from 334 prostate cancer patients compared to 101 benign controls (1.9% by PCR and 3.9% by IHC) [9]. This study also reported finding XMRV more frequently in patients with a higher prostate tumor grade suggesting a causal link between virus and disease. A similar trend was reported in another study that reported a 22% XMRV prevalence rate in prostate cancer patients from Texas but found virus in both tumor and non-tumor tissues [9,10]. XMRV neutralizing antibodies were identified
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Results

Rare XMRV sequences in tumor tissues from prostate cancer patients

β-actin sequences were detected by PCR in the DNA extracts from all prostate tissues confirming their integrity for PCR testing. DNA from only two (5956 and 6203) of 162 patients (1.23%) tested positive for XMRV sequences in the initial screening (Fig. 1, Table 1). Of the 77 DNA specimens that were additionally tested in triplicate by the pol PCR assay, only one patient (5935) (1.3%) was found positive. This specimen was positive in only one of three replicates. Thus, the overall PCR prevalence was 3/162 or 1.85%. Patients 5956 was positive for gag and pol sequences in 3/5 and 4/6 repeat tests, respectively, and env sequences were also detected in 1/3 repeat tests (Table 2). Specimen 5956 was also positive in all three replicate pol PCR tests. Only XMRV pol and env sequences were detected in patient 6203 prostate tissue DNA in 1/4 and 1/1 repeat tests, respectively. Additional testing of DNA extracted from another prostate tissue section of 6203 was repeatedly negative, likely reflecting an uneven distribution of low copy XMRV in this tissue. DNA from all three patients repeatedly tested negative for murine mtDNA demonstrating the absence of contaminating mouse DNAs. To test for viremia we analyzed RNA extracts from plasma specimens from all three patients. All samples had undetectable sequences by the qRT-PCR and nRT-PCR tests.

Identification of XMRV diversity in prostate cancer patients

Sequence analysis showed that patients 5935, 5956 and 6203 are infected with variant XMRV strains. The 168-bp pol sequences from all three patients showed 90.5–100% nucleotide identity to each other, 94–100% to XMRV, 91.7–98.8% to XMLV, 94–100% to PMLV, and 91–100% to ecotropic MLV (EMLV) in this short region. 164-bp env sequences from persons 5956 and 6203 were identical to each other and shared the highest nucleotide identity (94.9–100%) to XMRV and other xenotropic MLV strains, respectively, [25]. The env sequences from both persons were highly divergent from EMLVs sharing only about 46% nucleotide identity. 413-bp gag sequences were only amplified from the prostate tissue DNA of person 5956 and had about 98% nucleotide identity to XMRV but was identical to a xenotropic mERV found on mouse chromosome 8 (GenBank accession # AC127575).

Figure 1. Identification of XMRV sequences in prostate cancer patients. Representative nested pol PCR results using prostate tumor DNA. Lanes 1–24, prostate cancer patients, including patients 5956 (lane 8) and 6203 (lane 19); lane 25, negative human PBMC DNA control; lanes 26 and 27, water only controls for primary and nested PCR tests, respectively; lanes 28 and 29, assay sensitivity controls consisting of 10 and 10³ copies of XMRV VP62 plasmid DNA diluted in a background of 1 μg of human PBMC DNA, respectively.

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Phylogenetic analysis of the env sequences inferred three distinct clusters with strong bootstrap support based on viral tropism as expected, since this region includes a portion of the viral surface membrane involved in cellular tropism (Fig. 2a). The env sequences from both patients clustered with XMLV sequences but were distinct from previously reported XMRV and prototypical PMLV (Fig. 2a). In contrast, the inferred phylogenetic relationships of pol sequences from all MLV groups showed that this region does not cluster by host range (Fig. 2b). The three pol sequences from the prostate cancer patients formed a well-supported lineage containing a neuropathogenic Moloney MLV recombinant (strain ts-1-92b, GenBank accession # AF462057) (Fig. 2b). XMRV pol sequences from previously reported prostate cancer patients (coded with VP), except two (VP29 and VP184, kindly provided by Joe DeRisi), clustered with those from persons with CFS (coded with WPI) with significant bootstrap support. VP29 and VP184 pol sequences were identical to each other but 1.2% divergent from other XMRV and formed a weakly supported cluster containing a mixture of EMLV and PMLV (Fig. 2b). These results demonstrate a broader diversity of XMRV in this region than previously seen, but are consistent with recombination with Moloney MLV as reported recently [7,25]. VP29, VP79, VP86, VP88, VP90, and VP184 are prostate cancer patients reported in the Urismann et al. paper but for which the XMRV pol sequences are not yet available at GenBank [7]. Newly reported gag and pol sequences found in human tumor cell line DNA were located outside the regions used in our study and thus were not included in the analyses [25].

Table 1. XMRV/MLV infection is rare in prostate cancer1,2.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Sample Total</th>
<th>RR (45.7)</th>
<th>RQ (45.1)</th>
<th>QQ (9.3)</th>
<th>GAG (0.6)</th>
<th>POL (1.9)</th>
<th>WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate Tissue DNA</td>
<td>162</td>
<td>74</td>
<td>73</td>
<td>15</td>
<td>1</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Plasma</td>
<td>162</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0/162 (0)</td>
</tr>
</tbody>
</table>

1Percentages in parentheses.
2Dashes indicate test not performed on these sample types.
3nPCR, nested PCR.
doi:10.1371/journal.pone.0019065.t001

Phylogenetic analysis of the env sequences inferred three distinct clusters with strong bootstrap support based on viral tropism as expected, since this region includes a portion of the viral surface membrane involved in cellular tropism (Fig. 2a). The env sequences from both patients clustered with XMLV sequences but were distinct from previously reported XMRV and prototypical PMLV (Fig. 2a). In contrast, the inferred phylogenetic relationships of pol sequences from all MLV groups showed that this region does not cluster by host range (Fig. 2b). The three pol sequences from the prostate cancer patients formed a well-supported lineage containing a neuropathogenic Moloney MLV recombinant (strain ts-1-92b, GenBank accession # AF462057) (Fig. 2b). XMRV pol sequences from previously reported prostate cancer patients (coded with VP), except two (VP29 and VP184, kindly provided by Joe DeRisi), clustered with those from persons with CFS (coded with WPI) with significant bootstrap support. VP29 and VP184 pol sequences were identical to each other but 1.2% divergent from other XMRV and formed a weakly supported cluster containing a mixture of EMLV and PMLV (Fig. 2b). These results demonstrate a broader diversity of XMRV in this region than previously seen, but are consistent with recombination with Moloney MLV as reported recently [7,25]. VP29, VP79, VP86, VP88, VP90, and VP184 are prostate cancer patients reported in the Urismann et al. paper but for which the XMRV pol sequences are not yet available at GenBank [7]. Newly reported gag and pol sequences found in human tumor cell line DNA were located outside the regions used in our study and thus were not included in the analyses [25].

Table 2. Detection of XMRV in three prostate cancer patients1.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Specimen Type2</th>
<th>RNase L Q462R genotype</th>
<th>XMRV/MLV nPCR3</th>
<th>qRT-PCR4</th>
<th>nRT-PCR5</th>
<th>Murine PCR6</th>
<th>MCOX2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5935</td>
<td>PTT DNA</td>
<td>RQ</td>
<td>gag (0/3)</td>
<td>pol (1/4)</td>
<td>env (0/3)</td>
<td>pro (1)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Plasm 4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5956</td>
<td>PTT DNA</td>
<td>RR</td>
<td>gag (3/5)</td>
<td>pol (7/9)</td>
<td>env (1/3)</td>
<td>pro (2/0)</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>Plasm 4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6203</td>
<td>PTT DNA</td>
<td>RQ</td>
<td>gag (0/4)</td>
<td>pol (1/4)</td>
<td>env (1/1)</td>
<td>pro (2/0)</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>Plasm 4</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1Dashes indicate test not performed.
2PTT, prostate tumor tissue.
3nPCR, nested PCR; gag, group specific antigen; pol, polymerase; env, envelope.
4qRT-PCR, quantitative RNA PCR; pro, protease gene.
5nRT-PCR, nested RNA PCR.
6Murine PCR, test for detection of specimen contamination with mouse cells or DNA using mitochondrial primers (MCOX2).

For specimens 5935 and 5956 testing includes results of triplicates and the initial screening. Quantity of DNA for 6203 was insufficient for triplicate testing.
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A

B

C

4.8 - 6.5 % divergence

≤ 2.9% divergence

163-4p env

167-4p pol

233-4p gag
The XMRV sequences presented in the current paper are available at GenBank with the accession numbers HM003608–HM003612, and HQ116790. Sequences from other studies with XMRV-positive prostate cancer patients were not available at GenBank for inclusion in our analyses [9,11,12,16].

Absence of antibodies in plasma from prostate cancer patients

All 162 prostate cancer patient plasma samples were found to be negative for antibodies to XMRV/MLV in the WB test, including plasma from persons 5935, 5956, and 6203 (Fig. 3). In most plasma specimens some level of nonspecific reactivity was observed to the uninfected antigen but without evidence of specific reactivity to Gag and/or Env proteins in the infected antigen blot (Fig. 3).

RNase L R462Q allelic distribution in the study population and clinical characteristics of the three XMRV-infected persons

15 of the 162 persons (9.3%) were determined to be homozygous (QQ) for the R462Q RNase L mutation, 74 (45.6%) had the homozygous RR allele, and 73 (45.1%) were heterozygous (RQ) for the mutation (Table 1).

Patient 5935 is the heterozygous RQ RNase L genotype. He has a poorly differentiated adenocarcinoma with a Gleason’s score of 6, T2C pathologic stage, PSA level of 4.6 ng/mL. Patient 5956 is the wild-type homozygous RR RNase L genotype. He had a poorly differentiated adenocarcinoma with a Gleason’s score of 7, T3A pathologic stage, a 12.4 ng/mL PSA level. Patient 6203 is the heterozygous RQ RNase L genotype, and had a moderately differentiated adenocarcinoma with a Gleason’s score of 6, T2C pathologic stage, and a 7.1 ng/mL PSA level. Thus, we were unable to confirm an association of XMRV infection with the homozygous QQ RNase L allele. Given the low XMRV prevalence, we did not have the statistical power to determine if there is an association of XMRV with tumor grade.

The predominantly negative PCR results were observed despite the use of 1 ug of DNA which is 4–10× higher than the input DNA used in previous studies that reported XMRV detection [7,9,14,16]. Likewise, the observed low prevalence of XMRV may not be explained by a decreased PCR assay sensitivity since we screened specimens with the same assay used originally by Urisman et al. [7]. In the three specimens with detectable sequences, we noted that the copy number was very low as nested PCR and replicate testing was often needed for detection. Importantly, in all three specimens we were unable to detect mouse mtDNA despite the use of a highly sensitive assay, which suggests that the source of XMRV is unlikely the result of contamination with mouse DNA. These results are important since mouse DNA contamination was reported to be the source of XMRV in a recent study of prostate cancer tissues [26]. The authors of this study used a PCR assay for the mouse intracisternal A particle (IAP) which they reported to be more sensitive than a mouse-specific D-loop mtDNA-based assay [26]. Although DNA specimens were not available for testing in the IAP test, we have found that the IAP assay is equally sensitive to our COX2 real-time mtDNA assay (data not shown), thus further excluding mouse DNA contamination as a source for our positive PCR results. In addition, the DNA specimens were prepared at FCCC where only human biological specimens, and not cell lines, are handled, reducing further the possibility of murine contamination.

Sequence analysis of the PCR-positive specimens was highly informative because it confirmed that all three specimens were XMRV-related. Also, the finding of a viral strain in three prostate cancer patients that is distinct from the XMRV seen in previous studies is significant and demonstrates a broader viral diversity [7,9,14,16]. This would be an expected result consistent with virus evolution during spread and persistence. The absence of antibodies and plasma viremia in these three patients is noteworthy because it may reflect sequestered or latent infections. Loss of antibody during a latent infection, while atypical of most retroviral infections, has been described previously for natural infection of macaques with simian type D retroviruses (SRV) [27]. SRV in macaques is associated with outbreaks of severe immune deficiency in primate colonies and latent SRV infection in these antibody negative animals is confirmed with greater sensitivity using PCR analysis [27]. Our results are also consistent with those seen recently in macaques experimentally infected with XMRV in which tissues at necropsy are PCR-positive but viremia and detection of provirus in PBMCs disappear quickly, followed by loss of antibody detection [28,29]. Although one study reported the detection of XMRV neutralizing antibody in 11 patients with prostate cancer, these data were not confirmed by more sensitive methods such as WB, or by PCR testing in all cases, and thus may represent nonspecific reactivity [11,17]. Longitudinal studies may better define host responses to XMRV infection.

**Discussion**

We used PCR and serologic testing to study the prevalence of XMRV in 162 well-characterized North American patients with prostate cancer. We surveyed cancers with intermediate to advanced stages, and utilized multiple PCR assays, including tests with conserved primers to allow the detection of diverse XMRV and MLV-related sequences. Nearly half the specimens were also tested in triplicate to maximize the detection sensitivity of low-copy sequences. We used a broadly reactive WB assay to test all patients for antibodies. Our findings document a very low prevalence (1.9%) of XMRV sequences in prostate tissue DNA and absence of antibody positivity in all specimens. Combined these data do not support an association of XMRV or related MLV with prostate cancer.
Our study population contained 15 persons with the mutant QQ RNase L allele. However, none were XMRV-positive, which contrasts with the original findings by Urismann et al. [7]. The XMRV-infected persons in our study had either the homozygous wild-type (RR) or heterozygous (RQ) RNase L R462Q alleles. Our results are consistent with other US and European studies which also identified higher frequencies of the homozygous QQ mutant allele in XMRV-negative persons [9,10,16]. Combined, these results suggest that XMRV infection is not associated with this allelic form of RNase L.

Although our data do not support an association of prostate cancer with XMRV, it is important to understand whether XMRV has any causal role in prostate cancer when it is infrequently detected. In general, gammaretroviruses like XMRV induce malignant transformation by insertional mutagenesis, so that malignant cells in a tumor are all clonally infected. This mechanism of carcinogenesis has been found in animals as well as in children exposed to MLV-derived vectors in gene-therapy trials [30,31]. Therefore, the low-frequency of XMRV-infected cells found in all three patients are inconsistent with a direct role of XMRV in the prostate carcinogenesis in these patients. Previously, a human prostate cancer cell line, 22Rv1, has been shown to express high levels of XMRV, a finding that supports a role of XMRV in prostate cancer tumorigenesis [32]. However, it was reported recently that the XMRV expressed by 22Rv1 may not be of human origin, but most likely arose via recombination of two overlapping XMRV-like genomes during passage of the prostate tumor in inbred mice (T. Paprotka et al. 18th Conference on Retroviruses and Opportunistic Infections). Furthermore, others have shown that XMRV integration sites cloned from prostate tissues of two of nine patients may have been the result of contamination with DNA from experimentally infected DU145 cells [33], while other XMRV integration sites are not near tumor suppressor genes or proto-oncogenes [34]. Combined, these results raise questions about the role of XRMV in prostate cancer. Nonetheless, more work is needed to better understand the prevalence of XMRV and MLVs in humans and their role in human disease.

Materials and Methods

Study population

Anonymous, archived plasma and prostate tissue were available from 162 U.S. prostate cancer patients collected by the Fox Chase Cancer Center (FCCC) Biosample Repository Core Facility between 1997 and 2004. Whole blood specimens were collected at the time of pre-surgical testing or on the day of surgery prior to anesthesia from consenting cancer patients as part of a study approved by the FCCC Human Subjects Committee. Written informed consent was obtained from all participants. The FCCC Human Subjects Ethics Committee approved collection, storage, and future testing of the specimens collected from all consenting study participants. A non-research determination was approved for testing of the anonymized samples at CDC for XMRV and related viruses. The age at diagnosis of the 162 participants ranged from 36 to 71 years old with an average and median of 57 years. 86% were Caucasian, 12% African American, and 1% were Asian, not recorded, or other. The average and median serum prostate-specific antigen (PSA) levels were 7.22 ng/mL and 5.5 ng/mL, respectively. The tumor grade for 75 of 162 (46%) of the participants was moderately differentiated, while 54% had poorly differentiated tumors. Using the Gleason system, 42% of the study population had grade 5–6 cancer, 40% had grade 7, and 18% had high grade adenocarcinoma (Gleason’s score 8–10). Pathologic staging classified the prostate tumors as T1C (0.6%), T2A (9.3%), T2B (3.1%), T2C (61.7%), T3A (17.9%), T3B (5.6%), and T4 (1.8%).

Specimen preparation

Plasma was aliquoted and stored at −80°C within 4 hours of blood collection. Plasma samples were aliquoted again at the CDC for XMRV and related viruses. The age at diagnosis of the 162 participants ranged from 36 to 71 years old with an average and median of 57 years. 86% were Caucasian, 12% African American, and 1% were Asian, not recorded, or other. The average and median serum prostate-specific antigen (PSA) levels were 7.22 ng/mL and 5.5 ng/mL, respectively. The tumor grade for 75 of 162 (46%) of the participants was moderately differentiated, while 54% had poorly differentiated tumors. Using the Gleason system, 42% of the study population had grade 5–6 cancer, 40% had grade 7, and 18% had high grade adenocarcinoma (Gleason’s score 8–10). Pathologic staging classified the prostate tumors as T1C (0.6%), T2A (9.3%), T2B (3.1%), T2C (61.7%), T3A (17.9%), T3B (5.6%), and T4 (1.8%).
procedures. Only human tissues are processed at FCCC. For selected samples the QIAamp DNA minikit (Qiagen, Carlsbad, CA) was used at the CDC. For plasma RT-PCR testing a volume of 0.5–1.0 ml was ultracentrifuged at 45,000 rpm to concentrate virus. 360 ul to 860 ul plasma supernatant was carefully removed and the pellet was resuspended in 140 ul of centrifuged plasma remaining in the tube and then RNA was extracted using the QIAamp Viral RNA minikit (Qiagen, Valencia, CA). Nucleic acid concentrations were determined by spectrophotometry using the Nanodrop instrument (Thermo Scientific, Wilmington, DE). Integrity of the DNA and RNA specimens was determined using β-actin or GAPDH PCR, respectively, as described [35,36]. All specimen preparation, tissue culture, and PCR testing was done in physically isolated rooms to prevent contamination.

Molecular detection of XMRV

To detect XMRV and other MLV variants we used separate PCR assays with primers specific for the detection of XMRV, or conserved primers for the generic detection of MLV and XMRV, as previously described [21]. DNA specimens were screened using two nested PCR assays. The first assay uses the PCR primers GAGOF, GAGOR, GAGIF, and GAGIR employed by Urismann et al. and by Lombardi et al. to detect 413-bp XMRV gag sequences in prostate cancer patients and in persons with CFS, respectively [7,18]. The second PCR assay generically detects MLV and XMRV polymerase (pol) sequences about 216-bp in length using the primers XPOLOF, XPOLOR, XPOLIF, and XPOLOR [21]. Both the gag and pol PCR tests can detect 10 copies of XMRV plasmid DNA diluted in a background of 1 ug of human DNA [21]. Specimens testing positive for either gag or pol sequences were re-tested with both assays and were also tested with a third nested PCR test for XMRV envelope (env) sequences that is also generic for XMRV/MLV. The external XENVOF (5′ GGG GAT CAT GGT GAG GGC AGG AGC 3′) and XENVOR (5′ CAG AGA GAA GAC CCT CGG GTC 3′) and internal primers XENVIF (5′ AGG GCT ACT GTG GCA AAT GGG GAT 3′) and XENVIR (5′ GCC TTT ACC GGC GTG AGT GAA TTC 3′) amplify a 215-bp env sequence. In addition, a subset of specimens (n = 77) for which sufficient DNA was available were tested in triplicate using the nested pol PCR assay to improve detection of low copy XMRV/MLV.

PCR was performed using 1 ug of prostate tissue DNA in a 100 ul reaction volume using standard conditions of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec for 40 cycles on an ABI 9700 thermalcycler (Foster City, CA). PCR products were visualized by electrophoresis in an ethidium bromide-stained 1.8% agarose gel. To increase the sensitivity and specificity of the assays, amplified gag, pol, and env sequences were confirmed by Southern blot analysis using the biotinylated oligoprobes XGAGP2 (5′ ATG AGG CAC TGC ACA GAG ACC 3′) and XPOLOR (5′ ATG AGG CAC TGC ACA GAG ACC 3′) and XENVIF (5′ TGG GAG GCC AGG AGC 3′) and XENVIR (5′ GAA CAG GGT CAC CGG GTC 3′) sequences that is also generic for XMRV/MLV. In addition, a subset of specimens (n = 77) for which sufficient DNA was available were tested in triplicate using the nested pol PCR assay to improve detection of low copy XMRV/MLV.

Quantitative PCR for murine mitochondrial DNA (mtDNA) cytochrome oxidase subunit 2 (COX2) sequences

Contamination of specimens with mouse DNA will produce a false positive PCR result since mouse DNA contains endogenous MLV sequences that amplify by the diagnostic XMRV/MLV PCR assays. Thus, to distinguish true infection from specimen contamination with murine DNA, all specimens testing positive for XMRV/MLV sequences were also tested for murine mtDNA COX2 sequences using a newly developed PCR assay. The primers MCOX2F2 (5′ TGG TGC CAC CCG GTA TCA TCG TTA 3′) and MCOX2R1 (5′ AGT TGG TTA GGT GTG TGG GGA T 3′) and probes MCOX2F2 (5′ FAM-CGT AGC TCT AGT ATC ATT GGT GCC CTA TGG T-BHQ 5′) and MCOX2R1

Plasma samples from persons with positive XMRV PCR results in the prostate tissue DNA were tested further for viral RNA using two RT-PCR tests. The first assay, referred to as qRT-PCR was also capable of detecting 10 copies of XMRV gag sequences per reaction in replicate dilutions (40/40, 100%) and a linear range from 103 to 109 copies. Plasma from 32 US human blood donors and 32 HIV-1-infected persons tested negative by this assay.

For the nested gag RT-PCR tests, cDNA was synthesized with the XMRV-specific reverse primer GAG OR by using iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA) in a 20 ul reaction following the manufacturer’s instructions. Briefly, 10 ul RNA representing extract from 126 ul plasma was mixed with 10 μM GAGOR, 2 ul GSP enhancer solution and Nuclease-free water in a 15 ul reaction volume. Following incubation at 65°C for 5 minutes and cooling to 4°C, 4 ul of 5 × iScript Select reaction mix and 1 ul reverse transcriptase were added and incubated at 42°C for 1 hr. The reaction was stopped by heating to 85°C for 5 minutes. For first round PCR, 10 ul of the RT product was mixed with 0.2 μM of the external primers GAG OF and GAG OR, 2.5 μM of each dNTP, 2.5 mM MgCl2 using the Expand High Fidelity PCR System (Roche, Indianapolis, IN) and 5 μl 10× PCR buffer in a 50 ul reaction volume for 35 cycles at 94°C for 30 sec, 52°C for 30 sec, and 72°C for 45 sec. For nested PCR, 0.2 μM of the internal primers GAG IF and GAG IR in first round PCR reaction and run for 40 cycles with annealing temperature increased to 54°C. Both primary and nested PCR products were electrophoresed on 1.8% agarose gels and specific amplicons were detected by Southern blot as previously described. The nested gag RT-PCR assay had a sensitivity between 25 and 100 copies per reaction in replicate testing of XMRV RNA 92.5% (37/40) and 97.5% (39/40), respectively, but could also detect 5 copies in 50% (20/40) of the replicates. Plasma from 40 HIV-1-infected persons were all negative by this assay. 32 of these samples were negative by the pro qRT-PCR test.
References


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Author Contributions

Conceived and designed the experiments: WMS WH. Performed the experiments: HJ HZ ST. Analyzed the data: WMS HJ ST WH. Contributed reagents/materials/analysis tools: WMS. Wrote the paper: WMS HJ HZ ST WH. Sequence analysis: WMS.


