

XMRV Infection in Patients With Prostate Cancer: Novel Serologic Assay and Correlation With PCR and FISH

Rebecca S. Arnold, Natalia V. Makarova, Adeboye O. Osunkoya, Suganthi Suppiah, Takara A. Scott, Nicole A. Johnson, Sushma M. Bhosle, Dennis Liotta, Eric Hunter, Fray F. Marshall, Hinh Ly, Ross J. Molinaro, Jerry L. Blackwell, and John A. Petros

OBJECTIVES

To develop a serum-based assay to detect neutralizing antibodies to the xenotropic murine leukemia virus-related virus (XMRV) retrovirus and to use this assay with polymerase chain reaction and fluorescence in situ hybridization to identify patients with prostate cancer previously exposed to XMRV infection and those who carry XMRV viral sequences in their prostate.

METHODS

Patients who had undergone radical prostatectomy were enrolled, and biologic specimens were obtained at surgery. The patients were genotyped for the R462Q RNASEL variant using a TaqMan genotyping assay on DNA from the peripheral blood. A serum assay that detects XMRV neutralizing antibodies was developed and used to determine which patients had serologic evidence of previous infection with XMRV virus. Some of these patients were also tested for the presence of XMRV nucleotide sequences in their prostate using polymerase chain reaction and fluorescence in situ hybridization analysis.

RESULTS

At a serum dilution of 1:150, our assay detected 11 (27.5%) of 40 patients with XMRV neutralizing antibodies, including 8 (40%) of 20 with the RNASEL genotype QQ and 3 (15%) of 20 with either the RQ or RR genotype. These results were in complete concordance with 2 other assays (polymerase chain reaction and fluorescence in situ hybridization), which were designed to detect XMRV infection.

CONCLUSIONS

XMRV infects some patients with prostate cancer. Neutralizing antibodies against XMRV correlated with 2 independent methods of detecting the virus in the prostate. The antibody response suggests that with clinical serologic assay development, it might be possible to screen patients for XMRV infection. The cases presented in the present report provided biologic samples that can be used for the development of a clinically relevant assay. UROLOGY 75: 755–761, 2010. Published by Elsevier Inc.

Currently, conflicting published data are available regarding whether the recently discovered retrovirus xenotropic murine leukemia virus-related virus (XMRV) is actually found in human prostate cancer, and, if so, whether it is a relatively common or uncommon occurrence. A substantial reason for this controversy is that no standardized diagnostic tests are available to determine either the presence of the virus or the host immune response (eg, antibody production). In ad-

dition, the virus is likely present at low copy numbers, requiring very sensitive methods of detection, such as nested polymerase chain reaction (PCR) or reverse transcriptase-PCR, which are susceptible to both false-positive and false-negative results. An additional difficulty in the laboratory analysis of this virus is the DNA sequence homology it shares with the endogenous retroviruses existing in the mouse genome, which can also produce false-positive PCR products in academic laboratories in which mouse work is performed. Finally, to date, no highly specific monoclonal antibodies are available that can recognize XMRV proteins, an advance that could potentially allow for accurate immunohistochemical analysis of clinical specimens.

A very brief review of the published data on XMRV in clinical prostate cancer illustrates the current lack of consensus. The original report by Urisman et al.¹ in 2006 included 86 patients with prostate cancer and found XMRV in 9 (10%). In 2008, Fischer et al.² found a single

This work was supported in part by the Research Scholar Award to Hinh Ly by the American Cancer Society (grant RSG-06-162-01-GMC) and by the Emory Biomarker Service Center.

From the Departments of Urology, Pathology and Laboratory Medicine, Chemistry, and Medicine, Division of Infectious Diseases, Emory University School of Medicine, Atlanta, Georgia; Atlanta Veterans Affairs Medical Center, Decatur, Georgia; and Yerkes National Primate Research Center, Emory Vaccine Center, Emory University, Atlanta, Georgia

Reprint requests: John A. Petros, M.D., Department of Urology, Emory University School of Medicine, 1365 Clifton Road, Building B, Atlanta, GA 30322. E-mail: jpetros@emory.edu

XMRV-positive case in 105 tissue samples of 87 German patients with nonfamilial prostate cancer (1.1%), in addition to a single positive case from 70 men with benign prostatic hyperplasia (1.4%). More recently, Schlaberg et al.³ reported that 27% of 334 patients with prostate cancer had XMRV either by immunohistochemical or PCR analysis of primary tissues. One month later, an analysis of 589 German patients reported a complete lack of any XMRV positive cases (0 of 589).⁴ Although it is possible that regional rates of XMRV infection might account for the difference in the reported incidence of infection in patients with prostate cancer (0% versus 27%), it is also possible that the technical challenges outlined might account for some of the apparently contradictory data in published reports.

To confirm the presence or absence of XMRV in clinical prostate cancer, we developed a novel method of detecting serologic evidence of previous infection with the virus adapted from our work with human immunodeficiency virus (HIV) and confirmed the results using 2 independent methods of detecting XMRV nucleic acids (PCR and fluorescence in situ hybridization [FISH]) in a subset of these patients. We believe that until large studies have proved the accuracy of any single clinical diagnostic test, multiple independent assays will be required to accurately confirm XMRV infection. We also believe that cases ascertained in this manner could provide a reference set of biologic samples for additional assay development for screening purposes.

MATERIAL AND METHODS

All collection and use of human samples was performed using approved Emory University institutional review board protocols.

Sample Preparation

Peripheral Blood DNA. Blood was collected in the operating room in heparin vacutainers and stored at 4°C. Preparation of the DNA was performed using the Qiagen Flexigene Kit according to the recommended protocol. The DNA aliquots were stored at -20°C.

Serum. After the blood had been collected in the operating room in vacutainers and stored at 4°C, the vacutainers were centrifuged at 4000g for 20 minutes at 4°C to separate the serum from the remaining contents. The serum was then separated into multiple tubes and stored at -80°C.

Tissue. The prostatic tissue was divided into subsections, and a portion of the sections was placed in OCT, frozen in liquid nitrogen, and stored in liquid nitrogen. The remaining sections were fixed in formalin fixed and embedded in paraffin.

Genotyping

The patients were genotyped for the R462Q (1385G → A)RNASEL variant using a premade TaqMan genotyping assay (Applied Biosystems, Foster City, CA) on DNA isolated from the peripheral blood. A total of 5 ng of genomic DNA were

assayed according to the manufacturer's protocol in the Emory Biomarker Service Center.

Tissue DNA Preparation

The OCT-embedded prostatic tissue was serially sliced into 15- μ m sections, combining 10 sections per Eppendorf tube, for (on average) 12 tubes, and stored at -80°C. Every third tube was thawed, and the samples were washed by adding 1 mL phosphate-buffered saline, vortexing, briefly centrifuging at 13 000g, and removal of the supernatant. DNA was prepared using Qiagen's QIAmp DNA Mini Kit according to manufacturer's protocol. In 1 patient (patient 22), DNA was prepared from formalin-fixed tissue. A slice of tissue was deparaffinized in xylene, washed twice for 5 minutes each, dried, and scraped into a tube. The DNA was extracted using PicoPure DNA extraction kit (Molecular Devices, Sunnyvale, CA), according to the manufacturer's protocol.

Nested PCR

Tissue DNA was analyzed using the AmpliTaq gold Kit (Applied Biosystems) for the presence of XMRV using nested PCR analysis. The first-round PCR consisted of 0.25-2.0 μ g DNA, 1 \times buffer, 2.5 mM MgCl₂, 0.25 μ M each of dNTP (Roche, Indianapolis, IN), 200 nM 6200R:CCCATGATGATGATGGCTTCCAGTATGC, 200 nM 5922F:GCTAATGCTACCTCCCTCCTGG,⁵ 2.5 U of Taq under the following conditions: 94°C for 5 minutes followed by 40 cycles of 94°C for 30 seconds, 54.4°C for 30 seconds, 72°C for 45 seconds, and ending with 72°C for 2 minutes. The second round of PCR consisted of 5 μ L of the first round PCR product, 1 \times buffer, 2.5 mM MgCl₂, 0.25 μ M each of dNTP, 200 nM 5942F:GGGACGATGACAGACACTT-TCC, 200 nM 6159R: CACATCCCCATTTGCCACAG-TAG, 2.5 U of Taq under the following conditions: 94°C for 5 minutes followed by 40 cycles of 94°C for 30 seconds, 51°C for 30 seconds, 72°C for 45 seconds, and ending with 72°C for 2 minutes. The PCR products were separated on a 3% TAE agarose gel. The expected product for first- and second-round PCR was 278 bp and 217 bp, respectively.

Sequencing

Bands from the DNA agarose gels that were of the correct size were excised from the agarose and the DNA purified using a Qiagen QIAquick gel Extraction Kit according to the recommended protocol. Single-strand PCR was performed on the purified DNA using BigDye Terminator version 3.1, Sequencing Kit (Applied Biosystems, Foster City, CA). In brief, purified DNA, 0.16- μ M primer (either 5942F or 6159R) and 1 \times BigDye were reacted under the following conditions: 96°C for 1 minute followed by 25 cycles of 96°C for 10 seconds, 55°C for 5 seconds, 60°C for 4 minutes. After purification using CentriSep 96 (Princeton Separations, Adelphia, NJ), the samples were sequenced on an ABI Prism 3100 Genetic Analyzer, and basic nucleotide basic alignment search tool (BLAST) performed.

FISH Assay

FISH was performed as previously described,¹ with the following modifications. The template to generate the probe of the FISH assay was generated by excising full-length XMRV cDNA from the pXMRV plasmid¹ using NotI and HindIII restriction enzymes (New England Biolabs, Ipswich, MA). Digested XMRV DNA template was purified (Qiagen gel purification

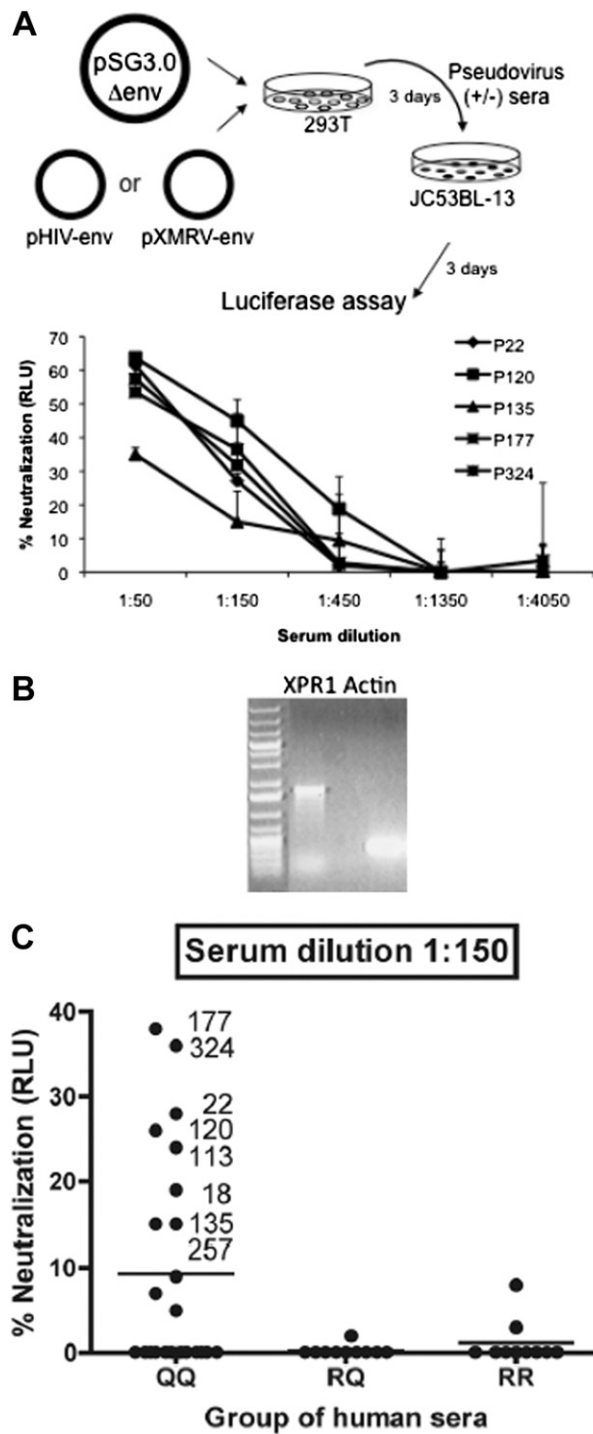


Figure 1. Detection of XMRV neutralizing antibodies in patient serum and receptor expression of reporter cells. **(A, Top)** Diagram of single-round reporter gene assay. 293T cells were co-transfected with pSG3Δenv and XRMV env- or HIV env-expression plasmids. Three days later, media was transferred to JC53BL-13 in presence or absence of serially diluted antisera. **(A, Bottom)** Two days later, cells were lysed and luciferase activity in measured. Relative neutralization (percentage of control) calculated by dividing number of luciferase units at each serum dilution by values in wells containing no test serum and subtracting that value from values in wells containing no test serum. Samples tested in triplicate; error bars represent standard deviation. **(B)**

kit) and used for a subsequent nick translation procedure to generate SpectrumGreen dUTP-labeled probes. Nick translation was performed according to the manufacturer's protocol (Abbot Molecular, Des Plaines, IL). Baked slides containing 4-μm sections of prostatic tissues were deparaffinized with xylene, rehydrated, and treated with target retrieval solution (Dako Denmark, Glostrup, Denmark) for 40 minutes at 95°C. The slides were cooled for 20 minutes and then treated with 1:5000 dilution of proteinase K (Dako) in 50 mM Tris-HCl (pH 7.6) for 20 minutes. The slides were rinsed in water to remove proteinase K and placed overnight in a ThermoBrite hybridizer (Abbot Molecular) at 37°C with XMRV-specific probe. After soaking the slides in 2× standard saline citrate (pH 5.3), coverslips were removed and samples were washed in high stringency 0.01× standard saline citrate, 0.4% NP-40 solution at 75°C, for 5 minutes. The FISH samples were counterstained with Vectashield mounting media (Vector Laboratories, Burlingame, CA) containing 46c-diamidino-2-phenylindole-2 HCl. As a positive control, the tissue sections were also probed with TelVysion 3q SpectrumOrange-labeled probe specific for a single human chromosome arm. The FISH images were visualized using Axioplan 2 imaging microscope (Zeiss, Thornwood, NY) using 100× objective. XMRV nucleic acid images were acquired using green (488 nm) and 46c-diamidino-2-phenylindole-2 HCl filters. Spectrum orange was visualized using Texas Red filter (data not shown). The Z-stack images were acquired using MetaSystems Isis software. The slides were subsequently counterstained with hematoxylin-eosin stain, and the cell morphology of the FISH-positive cells was analyzed at 40× and 100× magnification using a Nikon Eclipse E600 microscope.

Reverse Transcriptase-PCR

RNA isolated from JC53BL-13 cells (Fig. 1) was used as a template to synthesize Xpr1 cDNA using Superscript II (Invitrogen, Carlsbad, CA), which were then amplified by PCR using the GoTaqDNA polymerase (Promega, Madison, WI) at 94°C for 4 minutes, 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds for 30 cycles, and, finally, 72°C for 10 minutes in a reaction that contained the XPR1out-F (5'-CACTGGTGTACTACGCTG-3') and XPR1out-R (5'-GCAACAAAGTTGTAGAGGT-3') primers specific to the XPR-1 mRNA. In addition, a set of universal primers was used to PCR amplify the β-actin gene as a control.

Assay of XMRV Neutralizing Antibodies in Patient Serum

To evaluate the immune responses, we applied a single-round reporter gene assay based on a similar approach used to quantify neutralizing antibodies in HIV-1 infected patient sera.⁶ A schematic presentation of the single-round infection neutralization assay is shown in Figure 1A.

The pSG3.0ΔEnv plasmid carries all genes required to produce a replication deficient HIV virus, with the exception of the envelope (Env) gene. This plasmid is combined with either

Detection of XPR1 receptor expression in JC53BL-13 cells. **(C)** Serum from 20 QQ patients, 10 RQ patients, and 10 RR patients analyzed for neutralizing activity. Horizontal lines represent average percentage of neutralization of samples tested.

Table 1. Characterization of XMRV pseudovirus used for neutralization assay

Pseudovirus	p24 Content (pg/mL)	Infectivity (IU/mL)	50% Neutralization With Monoclonal Antibody ($\mu\text{g/mL}$)	
			83A25	B12
XMRV Env-HIV	15 ± 3	$4 \times 10^6 \pm 660$	11.3	ND
HIV Env-HIV	14.3 ± 3	$1.5 \times 10^5 \pm 33$	ND	0.14

XMRV, xenotropic murine leukemia virus-related virus; Env, envelope; HIV, human immunodeficiency virus; ND, no detectable neutralization at greatest concentration of antibody tested (ie, 10 $\mu\text{g/mL}$).

Concentration extrapolated using linear regression-least squares fit method.

the HIV envelope gene (pHIV-env plasmid) or the XMRV envelope gene (pXMRV-env plasmid) in 293-T cells (by cotransfection) in which a complete pseudovirus carrying the corresponding envelope proteins is produced and released into the culture medium. The resulting XMRV-HIV pseudovirus (XMRV-HIV) and HIV-HIV pseudovirus (NL4.3-HIV) were each found to efficiently infect the reporter cell line JC53BL-13 by detection of β -galactosidase expression 48 hours after infection of JC53BL-13 cells (Table 1).

The JC53BL-13 cell line (National Institute of Health Acquired Immunodeficiency Syndrome Research and reference reagent program catalog no. 8129, TZM-bl) is a HeLa cell clone that was engineered for successful HIV infection. JC53BL-13 cells express CD4 and co-receptors CCR5 and CXCR4 and the XMRV receptor XPR1 (Fig. 1B) and contain integrated reporter genes for firefly luciferase and *Escherichia coli* β -galactosidase under control of an HIV-1 long-terminal repeat sequence.⁷⁻⁹ Reporter gene expression is stimulated on activation with Tat protein of HIV (expressed by both HIV and XMRV pseudoviruses in these experiments). The read out (of either luciferase or β -galactosidase) has been shown to directly correlate with the amount of infections virus present in the media. The Jc53-BL-13 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (Cellgro, Mediatech, Manassas, VA).

Heat-inactivated human sera were assayed for neutralizing antibody activity against HIV-1 virions pseudotyped with XMRV envelope protein using a single-round pseudotype reporter assay, as previously described.¹⁰ In brief, JC53BL-13 cells were plated and cultured overnight. A total of 2000 infectious units of pseudotyped virus were combined with 3-, 5-, and 10-fold dilutions of heat-inactivated test serum and incubated for 1 hour at 37°C. Heat-inactivated human serum from a healthy donor was added as necessary to maintain a constant overall serum concentration. The virus-antibody mixture was then added to JC53BL-13 cells, and, after 2 days, the cells were lysed, and the luciferase activity of each well was measured using a luciferase assay reagent (Promega) and Synergy HT luminometer (Bio-Tek, Winooski, VT). The background luminescence was determined using the uninfected wells and subtracted from all experimental wells. Cell viability and toxicity were monitored by basal levels of luciferase expression and visual inspection. Relative neutralization (percentage of control) was calculated by dividing the number of luciferase units at each serum dilution by the values in the wells containing no test serum and subtracting that value from the values in wells containing no test serum (Fig. 1A). At a dilution of 1:150, the positive values were defined as all values greater than the average percentage of neutralization for all samples in any individual RNASEL category (represented by horizontal lines in Fig. 1C).

Table 2. Complete concordance of all results for 7 patients who had all 3 tests performed

Sample ID	DNA	FISH Status	Serum Analysis (Percentage of Neutralization) (RLU)
22	Positive	Positive	28
69	Negative	Negative	0
120	Positive	Positive	45
135	Positive	Positive	15
143	Negative	Negative	0
177	Positive	Positive	38
324	Positive*	Positive	36

RLU, relative luminescence units.

* Results for patient 324 were positive using nested polymerase chain reaction and longer template than for other specimens.

Reporter gene expression was directly proportional to the amount of pseudovirus used to infect the JC53BL-13 cells. For the control of XMRV-specific neutralization, we used 83A25 monoclonal antibody (mAb) that moderately neutralizes murine leukemia virus (MuLV) and interacts with an antigenic epitope on MuLV envelope glycoprotein that is also present on the XMRV envelope.¹¹ XMRV Env-HIV virus was neutralized by 83A25 mAb but was not neutralized by the HIV-specific mAb, B12. In contrast, HIV Env-HIV pseudovirus was neutralized by B12 mAb but was not neutralized by 83A2 mAb (Table 1). To investigate the neutralizing activity of the patients' sera, XMRV Env-HIV pseudoviruses were preincubated with different dilutions of the selected patients' sera, and inhibition of infectivity was used as an expression of neutralizing activity. Titration of the neutralizing activity in the serum of patients detected positive for the presence of XMRV in prostatic tissues is shown in Figure. 1A (lower graph).

RESULTS

The serum from 40 patients with prostate cancer was tested for the presence of neutralizing antibodies against XMRV. At a serum dilution of 1:150, 11 (27.5%) of 40 patients showed positive results, including 8 (40%) of 20 with the RNASEL genotype QQ and 3 (15%) of 20 with either the RQ or RR genotype (Fig. 1C).

Sufficient biologic material was available for 7 of the patients to conduct all 3 assays (DNA PCR, FISH, and serum neutralizing antibodies) with unequivocal results. In each of these index cases, the results of the multiple assays for each patient were completely concordant, with 5 patients positive for infection using all 3 assays and 2 negative using all 3 assays (Table 2). These patients

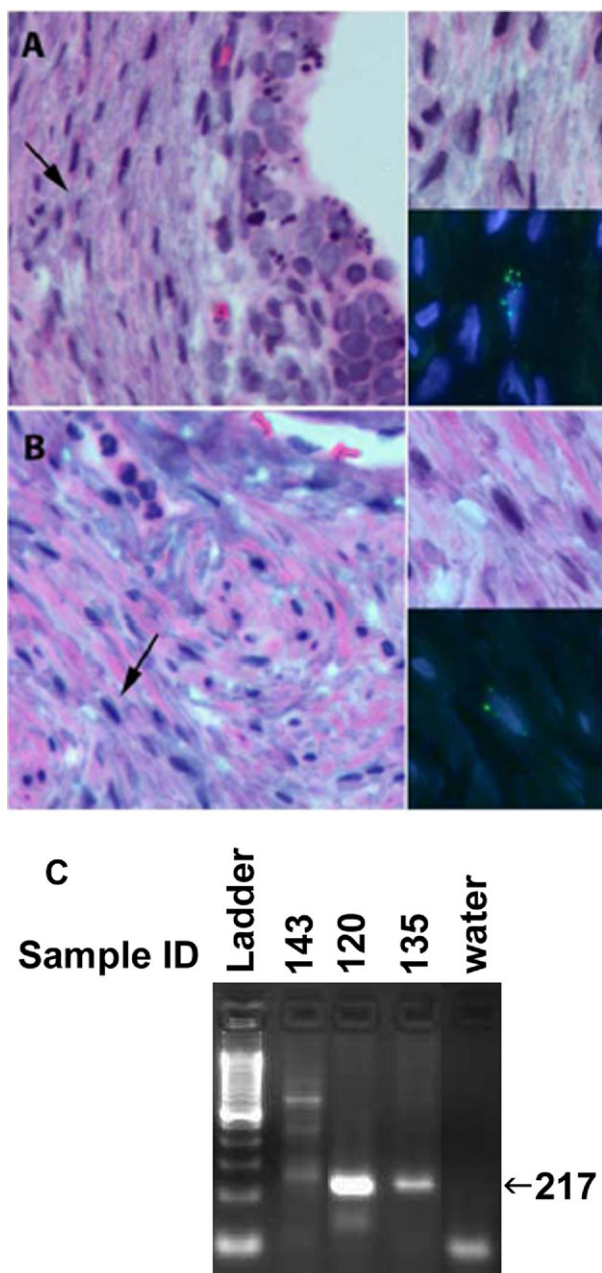


Figure 2. PCR and FISH analysis in prostatectomy tissue. **(A,B)** Two fields of hematoxylin-eosin–stained prostatic tissue and corresponding XMRV FISH positives shown for patient 177. Large panels show FISH positive cells (arrow). Smaller panels show magnified field of hematoxylin-eosin stain and fluorescent image of XMRV FISH-positive cells. Similar findings were observed for all FISH-positive patients but absent in FISH-negative patients. **(C)** Representative gel of bands resulting from second round of nested PCR using patient prostatic tissue DNA as template and XMRV-Env gene specific primers. Expected size of band was 217 bp. Each positive PCR product was sequenced to verify XMRV unique sequences. All patient specimens were also negative for mouse mitochondrial DNA, ruling out contamination (data not shown). Sample ID numbers for labeling lanes correspond to sample ID numbers in tables.

Our results suggest that it is feasible to develop a clinically useful serologic test for previous infection with XMRV. This is in concordance with other known retroviral infections, specifically HIV. Although the assay used in the present report involved the inhibition of infection of target cells by viral-like particles with the XMRV envelope protein expressed on their surface, our results also suggest that more standard serologic tests for antibodies against specific viral antigens can be developed in the future.

Finally, our report adds to the growing body of evidence that XMRV is indeed a novel gamma-retrovirus capable of infecting humans and that at least some patients with prostate cancer have been infected with XMRV. We have reported serologic evidence of infection and that the serology correlated with tissue-based assays. The concordance of 3 independent methods of detecting infection added confidence to the assertion that this recently discovered virus is real and possibly related to human disease. Robust clinical assays are needed to detect XMRV infection, and much work remains to be done in determining whether XMRV is indeed an oncogenic virus or simply an associated epiphenomenon.

CONCLUSIONS

The novel gamma-retrovirus XMRV has been confirmed to have infected humans. We have reported the detection of neutralizing antibodies in patient serum, and it correlated with 2 independent methods of detecting viral nucleotide sequences in prostatic tissue. The presence of a host antibody response suggests that future clinical serologic assay development could be possible. The cases presented in the present report could provide a set of biologic samples for the development of a clinically relevant assay.

Acknowledgment. To Dennis Liotta (Department of Chemistry, Emory University) for his generous financial support of this research, his encouragement and mentoring and his ongoing critical review of the research.

References

1. Urisman A, Molinaro RJ, Fischer N, et al. Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant. *PLoS Pathog.* 2006;2:e25–e25.
2. Fischer N, Hellwinkel O, Schulz C, et al. Prevalence of human gammaretrovirus XMRV in sporadic prostate cancer. *J Clin Virol.* 2008;43:277–283.
3. Schlager R, Choe DJ, Brown KR, et al. XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors. *Proc Natl Acad Sci USA.* 2009;106:16351–16356.
4. Hohn O, Krause H, Barbarotto P, et al. Lack of evidence for xenotropic murine leukemia virus-related virus(XMRV) in German prostate cancer patients. *Retrovirology.* 2009;6:92–92.
5. Hong S, Klein EA, Das Gupta J, et al. Fibrils of prostatic acid phosphatase fragments boost infections with XMRV (xenotropic murine leukemia virus-related virus), a human retrovirus associated with prostate cancer. *J Virol.* 2009;83:6995–7003.

6. Montefiori DC, Metch B, McElrath MJ, et al. Demographic factors that influence the neutralizing antibody response in recipients of recombinant HIV-1 gp120 vaccines. *J Infect Dis*. 2004;190:1962-1969.
7. Platt EJ, Wehrly K, Kuhmann SE, et al. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J Virol*. 1998;72:2855-2864.
8. Meng G, Wei X, Wu X, et al. Primary intestinal epithelial cells selectively transfer R5 HIV-1 to CCR5+ cells. *Nat Med*. 2002;8:150-156.
9. Wei X, Decker JM, Liu H, et al. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob Agents Chemother*. 2002;46:1896-1905.
10. Li B, Decker JM, Johnson RW, et al. Evidence for potent autologous neutralizing antibody titers and compact envelopes in early infection with subtype C human immunodeficiency virus type 1. *J Virol*. 2006;80:5211-5218.
11. Burkhart MD, D'Agostino P, Kayman SC, et al. Involvement of the C-terminal disulfide-bonded loop of murine leukemia virus SU protein in a postbinding step critical for viral entry. *J Virol*. 2005;79:7868-7876.