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Development of sensitive single-round *pol* or *env* RT-PCR assays to screen for XMRV in multiple sample types

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Abstract

The potential association between xenotropic murine leukemia virus-related virus (XMRV) and prostate cancer and chronic fatigue syndrome (CFS) has been much debated. To help resolve the potential role of XMRV in human disease, it is critical to develop sensitive and accurate reverse transcriptase (RT)-PCR assays to screen for the virus.

Single-round RT-PCR assays were developed on the automated *m*2000™ system for detection of the *pol* or *env* regions of XMRV in whole blood, plasma, urine cell pellets and urogenital swab samples. Assay performance was assessed by testing two blinded panels, one comprised of whole blood and the other of plasma spiked with serial dilutions of XMRV-infected tissue culture cells and supernatant, respectively, prepared by the Blood XMRV Scientific Research Working Group (SRWG). For both whole blood and plasma panel testing, the assays showed excellent specificity and sensitivity as compared to the other tests included in the SRWG phase I study. Analytical specificity of the assays was also evaluated. Neither *pol* nor *env* PCR assays detected a panel of potential cross-reactive microorganisms, although some cross-reaction was observed with mouse genomic DNA. Screening of 196 normal human blood donor plasma, 214 HIV-1 seropositive plasma, 20 formalin-fixed paraffin-embedded (FFPE) prostate cancer specimens, 4 FFPE benign prostate specimens, 400 urine pellets from prostate cancer patients, 166 urine pellets from non-prostate cancer patients, and 135 cervical swab specimens, detected no samples as unequivocally XMRV positive.

Keywords

real-time RT-PCR assay; XMRV *pol*; XMRV *env*; Blood XMRV Scientific Research Working Group blinded panel

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Conflict of interest AF is an employee of Abbott laboratories. NT, GL, JH and KA are employees and shareholders of Abbott Laboratories.

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1. Introduction

The gammaretrovirus, XMRV was first identified in human prostate cancer tissue and was strongly associated with homozygosity for the R462Q reduced activity variant of the antiviral enzyme, RNAseL (Urisman et al., 2006). XMRV was later reported to be present in patients with CFS (Lombardi et al., 2009).

Subsequent studies have produced conflicting data about the association of XMRV and prostate cancer with some groups providing supporting evidence (Arnold et al., 2010; Danielson et al., 2010; Schlaberg et al., 2009; Verhaegh et al., 2010), and others seeing little or no evidence of XMRV infection in prostate cancer (D'Arcy et al., 2008; Hohn et al., 2009; Sakuma et al., 2011). The link between XMRV and CFS is even more tenuous as numerous studies have failed to detect evidence of XMRV infection in CFS patients (Erlwein et al., 2010; Groom et al., 2010; Knox et al., 2011; Satterfield et al., 2011; Schutzer et al., 2011; Shin et al., 2011; Switzer et al., 2010; van Kuppeveld et al., 2010;). Notably, one group reported detection of polytropic and modified polytropic MLV sequences (MLVs) in specimens collected from CFS patients (Lo et al., 2010). Various reasons have been proposed to explain the conflicting data. Methodological explanations include lack of standardized PCR assays for XMRV detection, amplicon contamination of PCR assays, and cross-reactivity of XMRV PCR assays with closely related endogenous murine leukemia virus sequences from trace quantities of mouse genomic DNA found in reagents and samples (Hué et al, 2010; Knox et al, 2011; Oakes et al, 2010; Robinson et al, 2010; Sato et al, 2010). Other explanations include potential differences in the geographical distribution of XMRV, sequence variation between XMRV genomes, and differences in patient selection criteria (Knox et al., 2011; Silverman et al., 2010; Singh I.R., 2010).

To address some of the methodological issues we developed prototype RT-PCR assays with the following characteristics: *pol* or *env* regions of XMRV were chosen to allow for confirmation of positive results using a different XMRV target region; sample-to-sample and amplicon contamination was minimized by use of the automated Abbott *m*2000*sp*™ and *m*2000*rt*™ system for real-time PCR analysis; cross-reactivity of these assays against mouse DNA and other potential confounders was assessed; and assay performance results were standardized against panels developed by the Blood XMRV Scientific Research Working Group (SRWG) (Simmons et al., 2011). Additionally, we used these newly developed prototype RT-PCR assays to screen a variety of clinical samples for the presence of XMRV.

2. Materials and Methods

2.1. Primers and probes

Two XMRV primer/probe sets were used. The first primer/probe set was designed to amplify a sequence of 128 nucleotides in the *pol* integrase region of the XMRV genome.

FP 5′ GCCCGATCAGTCCGTGTTT

RP 5′ TAGTTCTGTCCCGGTTTAACAT

Probe FAM- TCCCTACACAGACTCACC-BHQ

The second primer/probe set was designed to amplify a sequence of 61 nucleotides in the *env* region of the XMRV genome.

FP 5′ ATCAGGCCCTGTGTAATACC

RP 5′ GGAGAGGCCAAATAGTAGGACC

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Probe FAM-ACCCAGAAGACGAGCGAC-BHQ

To increase probe Tm, each C and T in both probes was modified to 5-propynyl dC and 5-propynyl dU. The probes were labeled with the fluorophore FAM at the 5′ end and with Black Hole Quencher (BHQ) at the 3′ end.

The Internal Control (IC) primer/probe set was designed to target a sequence of 136 nucleotides derived from the hydroxypyruvate reductase (HPR) gene of the pumpkin plant. The IC probe was labeled with the fluorophore CY5 at the 5′ end and BHQ at the 3′ end (Tang et al., 2007). When beta-globin was used in some tests as IC, a primer/probe set for detecting a region of 136 bases in the human beta-globin gene was used (Huang et al., 2009).

2.2. Controls

One positive control and one negative control were included in each run. The negative control was made with TE buffer and 1.5 ug/mL of poly dA:dT (pH 7.9-8.1). The positive control was made by diluting full length XMRV (VP62) plasmid DNA in TE buffer with 1.5 ug/mL of poly dA:dT (pH 7.9-8.1). IC Armored RNA (Tang et al., 2007) was diluted to the appropriate concentration in XMRV-negative plasma. IC was added at the start of sample preparation, serving as a control for sample preparation recovery, sample inhibition, and amplification efficiency. The IC threshold cycle (Ct) value was used to assess the validity of results of each sample result. For prostate FFPE sample isolation and testing, positive control was paraffin-embedded cell mixture of 22Rv1 and DU145 prostate cancer cells. For intracisternal A-type particles (IAP) PCR testing, the positive control was mouse DNA diluted in TE buffer. When cervical swab samples were tested, no Armored RNA IC was added to the sample preparation and amplification. A primer/probe for detecting the human beta-globin gene was used to control for specimen adequacy (Huang et al., 2009).

2.3. Sample preparation

The *m*2000*sp* ™ instrument was used for automatic sample preparation and master mix addition. Four protocols were developed: 0.4 mL plasma RNA protocol, 0.4 mL whole blood total nucleic acid (TNA) protocol, 0.4 mL DNA protocol, and 0.2 mL cell pellet (urine cell pellets or PBMC cell pellets) TNA protocol. Specimens and controls were loaded onto the m2000*sp* ™ instrument where nucleic acid was isolated and purified using magnetic microparticle technology. After the bound nucleic acids were eluted, a master mix with the primers and probes were loaded onto the *m*2000*sp* ™. The *m*2000*sp* ™ dispensed 25 μl aliquots of the master mix and 25 μl aliquots of the extracted eluates to a 96-well optical reaction plate. The plate was sealed and transferred to the *m*2000*rt* ™ for real-time RT-PCR. The eluate volume was sufficient to allow testing with a second set of primers/probe, if desired, and was accomplished by loading another master mix with the second set of primers/probes onto the *m*2000*sp* ™ after the first PCR plate was completed.

For formalin-fixed paraffin-embedded (FFPE) prostate cancer tissue curls or slide samples, total nucleic acid was purified using the QIA amp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, catalog # 56404). Total RNA was purified using the RNeasy FFPE kit (Qiagen catalog #: 74404).

2.4. Amplification and detection

The *m*2000*rt* ™ instrument was used for amplification and real-time fluorescence detection. Reverse transcription and PCR amplification was achieved using r*Tth* DNA Polymerase in the presence of manganese chloride. An aptamer-oligonucleotide was included in the reaction to prevent non-specific extension prior to the temperature being raised above 45°C.

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The following thermal cycling conditions were used: 1 cycle at 55°C for 30 minutes; 1 cycle at 95°C for 1 minute; and 55 cycles at 93°C for 15 seconds, 60°C for 60 seconds. Fluorescence measurements were recorded during the 60°C step of the 55 cycles. This amplification and detection system allowed for simultaneous detection of both XMRV and IC amplified products at each read cycle. If tests with both sets of primer/probes were required, one *m*2000*sp*™ run and two *m*2000*rt*™ runs were performed. One replicate of each clinical sample was tested using the *pol* RT-PCR and the *env* RT-PCR assay.

2.5. Panels and clinical specimens

Two blinded panels generated by the Blood XMRV Scientific Research Working Group (SRWG) from their phase I studies were used to assess assay performance (Simmons et al., 2011). The first panel consisted of whole blood panel members containing XMRV-infected 22Rv1 cells with concentrations varying from 9.9 × 10^3 cells/mL to 0.5 cells/mL. The second panel consisted of plasma panel members containing XMRV-infected 22Rv1 cell supernatant with concentrations varying from 2.5 × 10^5 virus copies/mL to 0.128 virus copies/mL. Assay results generated on blinded panels at Abbott Laboratories followed by decoding by representatives of the SWRG including theoretical levels of XMRV infected cells and virions and results of other XMRV detection assays on identical panels (Simmons et al., 2011).

Analytical specificity panel members were collected as follows: HIV-1 (subtype B), HCV high titer stocks, and plasmids containing the whole genome of HBV, HPV16, and HPV18 were obtained from Abbott Molecular. Viral lysates of HIV-2 and HTLV-1, and DNA from Epstein-Barr Virus (EBV), Herpes simplex virus 1, Herpes simplex virus 2, CMV, Human herpesvirus 6B, Human herpesvirus 8, Vaccinia virus, BK human polyomavirus, and flavivirus were obtained from Advanced Biotechnology Inc (Columbia, MD). Human placental DNA was obtained from Sigma-Aldrich (St. Louis, MO). Moloney/Amph MLV, strain pAMS plasmid in *E. coli*, and *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Mycobacterium gordonae*, and *Mycobacterium smegmatis* were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Laboratory inbred/hybrid (from mouse strains 129S7 and C57BL/6J) mouse tail genomic DNAs were obtained from Dr. Xiaozhong Wang of Northwestern University, Department of Molecular Biosciences.

A total of 20 prostate cancer FFPE samples were obtained from Dr. Imad Almanaseer of Advocate Lutheran General Hospital, Department of Pathology. For each sample, three 10 micron curls were collected for total DNA isolation and for total RNA isolation. For repeat extraction, 4 FFPE slides for each sample were used for TNA isolation. Four prostate non-cancer hyperplasia FFPE samples were obtained from Abbott Molecular FISH group. All specimens were collected per regulation in the US at the time of collection.

A total of 196 potassium EDTA normal plasma donor specimens were obtained from ProMedDx, LLC (Norton, MA). Additionally, 214 HIV seropositive EDTA plasma specimens (100 from Cameroon, 62 from Uganda and 52 from Thailand) were obtained from the Abbott Diagnostics HIV Global Surveillance Program. All specimens were collected per local regulations in the country of origin at the time of collection.

Four hundred prostate urine cell pellet specimens and 166 non-prostate cancer urine cell pellets specimens were collected by the Clinical Research Center of Cape Cod (CRCCC; Hyannis, MA).

One hundred and thirty five cervical swab specimens (89 with abnormal cytology of Atypical Squamous Cells of Undetermined Significance (ASCUS) or Low grade Squamous

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Intraepithelial Lesion (LSIL) or High Grade Squamous Intraepithelial Lesion (HSIL) and 46 with negative cytology) were obtained from ConVerge Diagnostic Services, LLC (Peabody, MA).

2.6. RNase L R462Q genotype PCR

The R462Q genotype primer/probe set was adapted from a reference paper (Shook et al., 2007). AgPath-ID One-Step RT-PCR Kit (Ambion: Austin, TX), catalog AM1005 was used for PCR. PCR was carried out using 1 cycle of 95°C for 10 minutes, 50 cycles of 95°C 15 seconds and 60°C for 1 minute. For each run the following controls were included: water negative control, Jurkat tumor cell line genomic DNA QQ control, MCF 7 tumor cell line genomic DNA RQ control, and HeLa tumor cell line genomic DNA RR control (all purchased from BioChain in Hayward, CA).

2.7. Mouse intracisternal A-type particles (IAP) PCR

The mouse IAP PCR assay primer/probe set was adapted from sequences that provided by Dr. Robert Silverman (Cleveland Clinic, Cleveland, OH). PCR conditions used were identical to the XMRV *pol* and *env* RT-PCR assays described above.

3. Results

3.1. SWRG blinded panels testing

The SWRG whole blood and plasma panels were tested in the real-time RT-PCR assays targeting XMRV *pol* and *env* on the *m*2000 ™ automated platform. The blinded panel test results were decoded by SWRG representatives. For the whole blood panel testing, the *pol* and *env* assays produced the same results. All six XMRV negative samples were assay negative, while all three replicates of each of the XMRV-positive samples were detected as XMRV positive (0.5 XMRV-containing 22Rv1 cells/mL and up). See Figure 1 for the real-time RT-PCR amplification plots for *pol* RT-PCR / *env* RT-PCR and for the result comparison with the other assays reported from the SRWG phase I study (Simmons et al., 2011).

For the plasma panel testing, only the *pol* assay was used. All six XMRV negative samples were assay negative, while the assay detected 0/3 of the 0.128 and 0.64 copies/mL XMRV panel members, 1/3 of the 3.2 copies/mL XMRV panel member, 2/3 of the 16 copies/mL panel member, and 3/3 of the panel members containing > 80 copies/mL. Using optical density quantitated transcript panels (spiked into lysis buffer then mixed with plasma), the estimated viral RNA detection limit for both *pol* and *env* RT-PCR assay with 0.4mL plasma sample input was approximately 29-60 copies/mL (data not shown). Figure 2 presents the real-time RT-PCR amplification plots and provides a comparison of results with the other assays reported from the SRWG phase I study (Simmons et al., 2011).

3.2. Analytical specificity evaluation

The analytical specificity of both assays was assessed by testing a panel of 24 potential cross-reactive microorganisms at concentrations ranging from 1×10^5 copies/mL to 1×10^6 copies/mL (see method section). No positive assay results were observed (data not shown).

GenBank database searches and sequence alignments showed that the *pol* primer/probe set should specifically detect XMRV (low homology with MuLV) whereas the *env* primer/probe has more homology to MuLV and therefore has the potential to amplify other xeno- and polytropic strians (data not shown). However, neither the *pol* RT-PCR nor the *env* RT-PCR assay detected the more divergent Moloney/Amph MuLV.

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Both *pol* RT-PCR and *env* RT-PCR assays were used to test mouse genomic DNA at 1×10^4 copies/mL and 1×10^6copies/mL, as well as XMRV DNA at 20 copies/mL, 100 copies/mL, and 1×10^4 copies/mL. Both assays detected mouse genomic DNA, although at significantly different levels. The *pol* assay detected mouse genomic DNA about two orders of magnitude (6.0 Ct) later than the equivalent XMRV target concentration and with suppressed signals. The *env* assay detected mouse genomic DNA and XMRV with similar sensitivity. Results of this comparison are presented in Figure 3. These data are consistent with the BLAST results that showed that the *pol* primer/probe set shares less homology with mouse DNA than the *env* primer/probe set. The impact of human genomic DNA on assay sensitivity was also evaluated. With 4-5 ug/mL of human genomic DNA in the input samples, and with a 0.2 mL of total nucleic acid preparation protocol, 10 copies/input (0.2 mL) of VP62 plasmid DNA was always detected (data not shown). A quantity of 4.5 ug/mL of human genomic DNA is equivalent to approximately 750,000 cells/mL or 50,000 cells/PCR reaction.

3.3. Clinical sample testing

All testing of clinical samples was performed using both the *pol* and *env* assays. No positive assay results were observed when 410 human plasma samples (196 normal, 214 HIV-1 seropositive), 135 cervical swab specimens (including 89 with abnormal cytology), and 166 non-prostate cancer urine pellets were tested (Table 1) while all the run controls and internal controls were valid.

Two of 400 (0.5%) prostate cancer urine pellets were detected positive with late Ct values. One sample was detected using the *pol* assay (Ct 40.82, or 10-25 copies/mL) but was not detected by the *env* assay. The other sample gave a positive result using the *env* assay (Ct 38.56, or 70-85 copies/mL) but was not detected by the *pol* assay. Limitations of sample volume precluded retest (Table 1).

Two of 20 total nucleic acids (TNA) purified from prostate cancer FFPE tissue curls were initially positive using the *env* assay with late Ct values (42.72 and 37.18 respectively), but were not detected by the *pol* assay. When RNA was purified from the same FFPE tissue curls and re-tested in duplicate, both the *env* and *pol* assays were negative for all the samples. To further investigate these samples, TNA was re-purified from FFPE slides of the two samples initially positive in the *env* assay. Results for both the *env* and *pol* assays were negative. These samples were also negative in the mouse IAP PCR assay (Table 2).

The 20 prostate cancer samples were genotyped for R462Q status. Four (20%) were homozygous for the QQ allele, seven (35%) were RQ heterozygous and nine (45%) samples were homozygous for the RR genotype (Table 2). The two initial *env* assay positive samples were not correlated with tumor grade or RNAseL QQ genotype (Table 2).

For the 4 non-prostate cancer hyperplasia FFPE samples, XMRV test results were negative for both the *pol* and *env* assays. Based on R462Q genotyping, all four samples were RR homozygous (data not shown).

4. Discussion

The debate about the role of XMRV in prostate cancer and CFS and potential concerns with available assays prompted us to develop sensitive real-time XMRV RT-PCR assays. There were four primary goals: (1) to develop accurate and reliable assays targeting at least two independent regions of the XMRV genome, (2) to incorporate a non-competitive internal control, (3) to standardize these assays utilizing the SWRG XMRV panels and, (4) to test a

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variety of clinical samples for XMRV, including HPV swab specimens, a sample type that has not been previously tested for the presence of XMRV.

The automated *m*2000 ™ system was chosen as the development system. Use of the *m*2000 ™ minimizes sample-to-sample contamination because sample manipulation and PCR plate set-up are fully automated. Despite potentially lower sensitivity, single-round RT-PCR assays were developed to reduce the higher likelihood of amplicon contamination associated with nested PCR assays. The IC is introduced into each sample at the beginning of the sample extraction process and is simultaneously amplified by RT-PCR and thus serves as a control for sample preparation, amplification efficiency, and inhibition. Moreover, automation and higher throughput allows these assays to be used for large scale epidemiological studies. Of note, results of the SWRG panel testing data showed that the single-round real-time *pol* and *env* RT-PCR assays were highly specific and more sensitive than most of the other comparator PCR assays. These included some nested PCR assays that theoretically could have had higher sensitivity.

Because of the high degree of sequence homology between XMRV and endogenous MLV-related proviral sequences present in mouse genomic DNA (~95%), it has been shown that XMRV PCR assays can cross-react with MLV proviral sequences found in mouse DNA. Moreover, trace quantities of mouse DNA have been detected in human samples and some commonly used PCR reagents (Knox et al., 2011; Robinson et al., 2011; Sakuma et al., 2011). Notably, both the *pol* and *env* PCR assays described herein detected endogenous MLV sequences present in mouse genomic DNA, although the *pol* assay has far greater sensitivity for XMRV than other MLV-related sequences, and thus showed higher specificity. To compensate for this cross-reaction, it was decided (when possible) to test all samples that were positive in the real-time RT-PCR assays with a PCR assay designed to detect IAP present in mouse DNA. None of the samples with positive results in the real-time XMRV assays were positive in the IAP assay. This suggests that mouse DNA contamination did not have a meaningful impact on this study. The *pol* and *env* assays were used to test a variety of clinical samples. No XMRV infection was seen in 196 normal blood donors or 214 HIV-1 seropositive samples. This result is consistent with several other recent studies (Barnes et al., 2010; Henrich et al., 2010; Tang et al., 2011) and suggests either that the prevalence of XMRV infection is very low (or zero) in these populations or that levels of XMRV in plasma are below the limit of detection of these assays. Studies using a rhesus macaque model system showed evidence of a transient XMRV plasma viremia, viral replication in lymphoid compartments (spleen, blood, lymph nodes, gastrointestinal mucosa) as well as reproductive tissues (prostate, seminal gland, and testis, vagina, cervix) (Villinger et al., 2010). In this study, negative *pol* and *env* RT-PCR assay results were obtained on urine pellet samples from 166 patients with normal prostate glands and for 135 cervical swab samples, which included 46 normal and 89 with abnormal cytology (ASCUS or LSIL or HSIL). Thus, no evidence of XMRV infection was observed in cell pellets from urine specimens obtained from normal males or in female cervical samples collected for HPV testing.

Given the potential association between XMRV infection and prostate cancer, it is of interest that out of 400 prostate cancer urine pellets screened, only two had preliminarily positive results (0.5 % positivity rate). Unfortunately, sufficient sample was not available for repeat testing. Notably, both positive results were characterized by very late Ct values and were either *pol* or *env* positive, but never both. Thus, neither could be confirmed as being truly positive. With these low level positive results for only a single target region, contamination cannot be fully ruled out. Of the FFPE prostate cancer samples, two of 20 TNA extractions were initially positive but only in the *env* assay and both had very late Ct values. No positives were obtained with total RNA extractions (repeated twice). A second

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TNA isolation was performed and retested in the XMRV *pol* and *env* assays as well as the mouse IAP assay. None of the samples were positive in the reflex testing. Taken together, these data could be interpreted in a number of ways. First, that XMRV is not present in this set of prostate cancer samples since the initial positive (*env* only) results could not be confirmed by repeat testing. Second, although IAP test results were negative for the two second extraction samples, we cannot completely exclude the possibility of trace amount mouse DNA contamination in the first round of sample extracts. A recent publication by Erlwein et al (Erlwein et al. 2011) showed that the QIAamp DNA FFPE Tissue Kit (Qiagen, catalog # 56404) occasionally contained trace quantities of mouse DNA. They detected mouse IAP sequence in 16% of columns with no tissue sample input. This could explain the two *env* positive samples in the first extraction which could not be repeated from the second extraction. Finally, XMRV may be present but at a concentration lower than the RT-PCR detection limit.

5. Conclusions

Prototype real-time RT-PCR assays targeting XMRV *pol* and *env* were developed on the automated *m*2000 ™ system to minimize sample-to-sample and amplicon contamination. Evaluation of assay performance on the SWRG XMRV panels demonstrated that the assays are highly sensitive and specific. Analysis of multiple types of human clinical samples revealed either no detectable XMRV infection (plasma from normal blood donors, HIV-1 seropositive patients, cervical swabs) or extremely low (or no) levels of XMRV in cell pellets from prostate cancer patient urine and prostate cancer tissue samples. These assays have utility for testing of a wide variety of sample types and thus have the potential to assist in elucidation of the role of XMRV in human disease and to increase our understanding of the biology of this novel gammaretrovirus.

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Highlights

- Single-round RT-PCR assays were developed on the automated *m*2000 ™ system for detection of the *pol* or *env* regions of XMRV.
- These assays demonstrated excellent specificity and sensitivity when evaluated with two blinded panels prepared by SRWG.
- Screened multiple clinical sample types, no samples were detected as unequivocally XMRV positive.
- Assist in elucidation of the role of XMRV in human disease.
- Increase our understanding of the biology of this novel gammaretrovirus.

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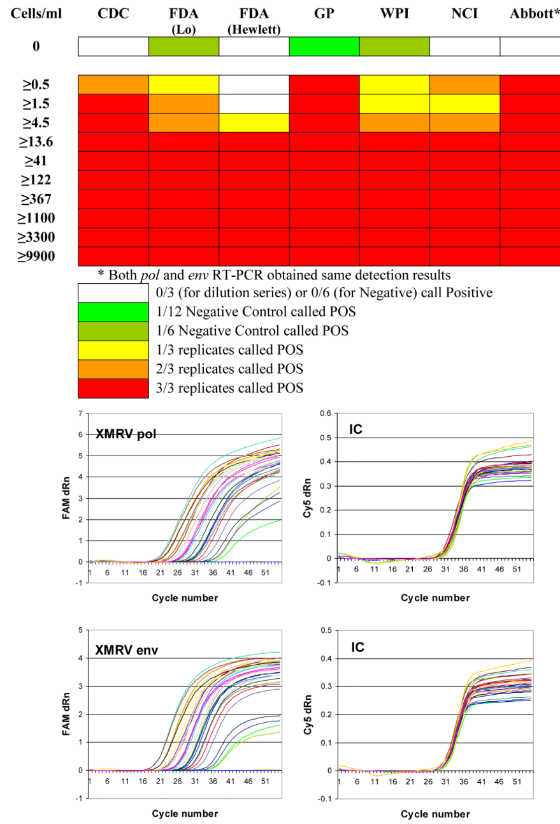

Fig.1.

Top graphic summarizes the performance of SRWG whole blood panel test results from the Abbott real-time prototype *pol* and *env* XMRV assays and the other assays (Simmons et al 2011) when used to test the SRWG whole blood panel. The Abbott *pol* and *env* assays provided no false positive results from all six negative panel members and detected all three replicates of each positive panel members. The other assays were as follows: CDC (Center for Disease Control) used *gag/pol* nested PCR, both FDA labs used *gag* nested PCR. GP (Gene Probe Inc) used duplex transcription-mediated amplification (TMA). WPI (Whittemore Peterson Institute) and NCI (National Cancer Institute) used 5′ UTR of *gag* qPCR (Simmons et al 2011). The lower four graphics show the real-time RT-PCR amplification plots of the *pol* primer/probe set and the *env* primer/probe set when used to test the SRWG whole blood panel. The left graphics are the XMRV (FAM) signals; the right graphs are the internal control (Cy5) signals amplified from the same run.

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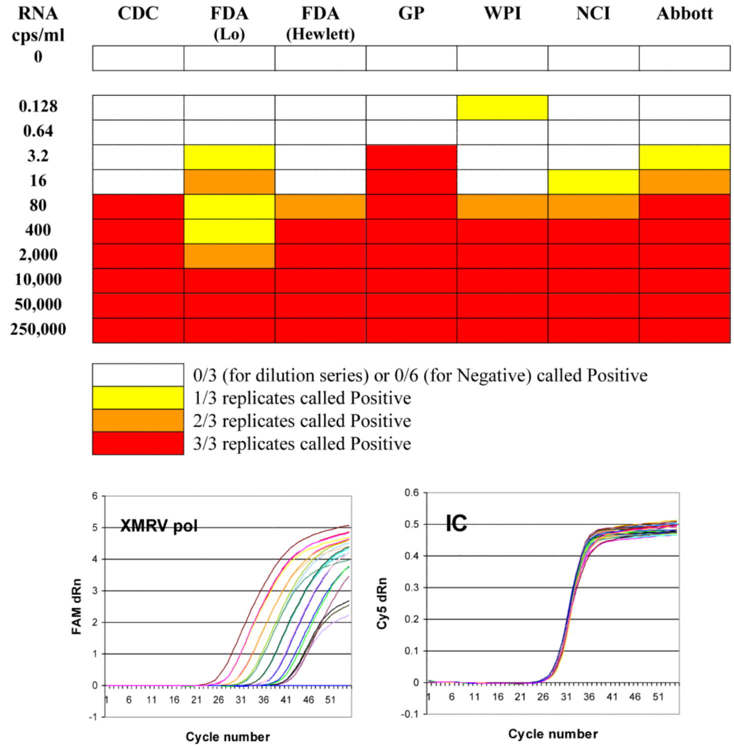

Fig.2.

Top graphic summarizes the performance of the Abbott real-time prototype *pol* XMRV assay and other assays (Simmons et al., 2011) when used to test the SRWG plasma panel. The Abbott real-time prototype *pol* XMRV assay did not detect all six negative panel members, failed to detect all three panel members containing 0.128 and 0.64 XMRV copies/mL, detected one of three replicates of the 3.2 copies/mL panel member and two of three of the 16 copies/mL panel member, and detected all panel members containing XMRV 80 copies/mL. The other assays were as follows: CDC (Center for Disease Control) used *gag/pol* nested PCR; both FDA labs used *gag* nested PCR; GP (Gene Probe Inc) used duplex transcription-mediated amplification (TMA); WPI (Whittemore Peterson Institute) and NCI (National Cancer Institute) used 5′ UTR of *gag* qPCR (Simmons et al. 2011). The lower two graphics show the real-time RT-PCR amplification plots of the SRWG plasma panel using the *pol* primer/probe set. The left graphic are the XMRV (FAM) signals; the right graphic are the internal control (Cy5) signals amplified from the same run.

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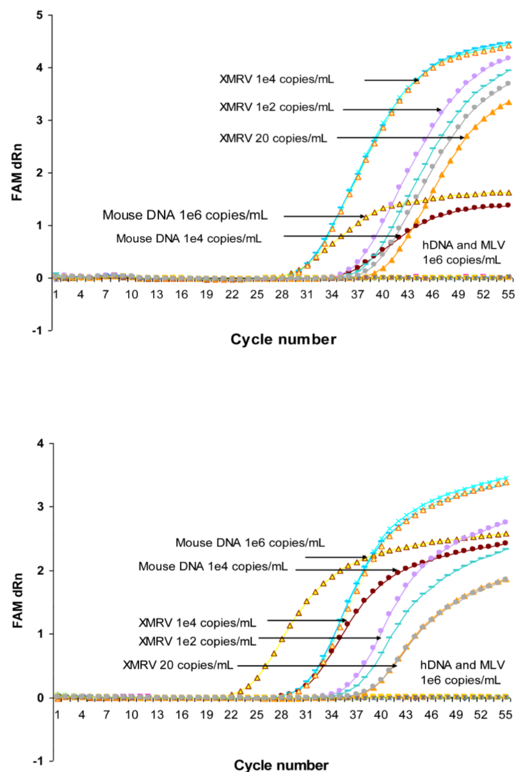

Fig.3.

The top graphic shows the *pol* primer/probe amplification of XMRV/human DNA/MuLV and mouse DNA. Neither human DNA nor Moloney/Amph MuLV was detected. However, amplified mouse DNA was detected, although with suppressed signals and at a two log (6.5Ct) delay as compared to a comparable level of XMRV target. The lower graphic shows the *env* primer/probe amplification of XMRV/human DNA/MuLV and mouse DNA. Neither human DNA nor Moloney/Amph MuLV was detected. Amplified mouse DNA was detected at a level similar to that for XMRV.

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Table 1

Multiple clinical sample type test using both the *pol* and *env* assays.

Sample/Cohort	Sample type	No tested	Preparation	pol pos	env pos	Total positive (%)
Normal blood donor	plasma	196	0.4mLTotal RNA	0	0	0
HIV-1 sero-positive						
--Cameroon	plasma	100	0.4mLTotal RNA	0	0	0
--Uganda	plasma	62	0.4mLTotal RNA	0	0	0
--Thailand	plasma 52	196	0.4mLTotal RNA	0	0	0
Prostate cancer	urine pellet	400	0.2mL TNA	1*	1*	0.5
Normal prostate	urine pellet	166	0.2mL TNA	0	0	0
Cervical swab						
--Abnormal cytology	swab	89	0.4mL DNA	0	0	0
-- Normal cytology	swab	46	0.4mL DNA	0	0	0

*One *pol* RT-PCR positive and one *env* RT-PCR positive results were from different samples.

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Table 2

Prostate cancer FFPE specimen characteristics, R462Q genotype determination, RT-PCR results and mouse IAP PCR results

								1st extraction[a]								2nd extraction[b]		
						% Tumor		TNA		TNA		RNA		RNA		TNA		
		Gleason	Age at	Years of	% Tumor	2nd HE	R462Q	Test 1	Test 1	Test 2	Test 2	Test 1	Test 1	Test 2	Test 2	Test 1	Test 1	Mouse
	SMP ID	**score**	**disgnosis**	**follow-up**	**1st HE**	**after3x10um**	**genotype**	**pol**	**env**	**pol**	**env**	**pol**	**env**	**pol**	**env**	**pol**	**env**	**IAP PCR**
1	97-2225	5	62	11	30	30	**QQ**	–	–	–	–	–	–	–	–			
2	97-9716	6	69	11	40	40	**RR**	–	–	–	–	–	–	–	–			
3	97-149292	6	66	11	25	25	**RQ**	+	–	–	–	–	–	–	–			
4	97-21235	7	64	11	30	30	**RR**	–	–	–	–	–	–	–	–			
5	98-587	7	61	10	50	45	**RR**	–	–	–	–	–	–	–	–			
6	99-16513	7	65	9	35	35	**RQ**	–	–	–	–	–	–	–	–			
7	00-823	6	65	8	25	25	**QQ**	–	–	–	–	–	–	–	–			
8	00-3228	6	60	8	40	40	**RQ**	–	–	–	–	–	–	–	–			
9	01-647	5	57	7	40	40	**QQ**	–	–	–	–	–	–	–	–			
10	99-7248	7	50	9	30	30	**RR**	–	–	–	–	–	–	–	–			
11	99-22765	7	65	9	50	40	**RR**	–	–	–	–	–	–	–	–			
12	99-21522	8-10	55	9	30	30	**RQ**	–	–	–	–	–	–	–	–			
13	99-5201	5	73	9	25	25	**RQ**	–	–	–	–	–	–	–	–			
14	99-4015	7	65	9	30	25	**RR**	–	–	–	–	–	–	–	–			
15	00-10288	7	69	8	40	35	**RQ**	–	–	–	–	–	–	–	–			
16	97-17123	7	64	11	45	40	**RR**	–	–	–	–	–	–	–	–			
17	00-11253	6	63	8	40	40	**RQ**	–	–	–	–	–	–	–	–			
18	97-7240	6	57	11	55	50	**RR**	–	+	–	+	–	–	–	–	–	–	–
19	98-17268	5	62	10	60	60	**QQ**	+	+	–	+	–	–	–	–	–	–	–
20	00-10321	6	56	8	40	40	**RR**	–	–	–	–	–	–	–	–			
	POS CTL[c]							+	+	+	+	+	+	+	+	+	+	+
	NEG CTL[d]							–	–	–	–	–	–	–	–	–	–	–

[a] 1st extraction was performed on FFPE curls and divided into total nucleic acid isolation and total RNA isolation. Average of 600ng purified TNA or RNA were used for RT-PCR.

[b] 2nd extraction was performed on FFPE slides for only two positive samples (4 slides for each sample since no curls leftover). Average of 500ng purified TNA was used for RT-PCR.

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[c]The positive control for XMRV was paraffin embedded cell mix from RV22 and Du145. The positive control for mouse IAP PCR was mouse DNA.

[d]The negative control was water and only for RT-PCR.