

Bulletin of the IACFS/ME

A Quarterly Publication of the International Association for CFS/ME

Letter To the Editor

Judy A. Mikovits, PhD, Director of Research, Whittemore Peterson Institute, Reno, Nevada

Since the publication of the *Science* article in October 2009 concerning Xenotropic murine leukemia-related virus (XMRV) in patients with chronic fatigue syndrome (CFS) (1), there have been three PCR-only papers published that have been unable to detect this agent (2-4). As these papers have generated questions concerning the presence of XMRV in persons with CFS, we wish to document the precise methods used when XMRV was amplified from the patient samples. We would urge researchers not to rely solely on DNA PCR on unactivated PBMCs, and offer our assistance if needed.

Why are reports of negative PCR studies occurring?

The first possibility is that like HTLV-1, and unlike HIV-1, the worldwide distribution of XMRV is low, particularly in Europe, and that chronic disease such as CFS may have varied environmental triggers in different parts of the world. In our subsequent work since the publication of the *Science* paper, we have found XMRV in persons from around the world and we think this explanation is unlikely.

A second possibility is that there is more sequence diversity in XMRV than previously reported. That is, because of strain differences, PCR detection will occur only if the primer sequences employed are correct for that particular strain. However, PCR sequences generally are from conserved regions of the viral genome, so this explanation may also be unlikely.

The replication rate for XMRV could be very low and/or the levels could fluctuate in a given individual over time. This explanation may apply for individuals, but would be unlikely to apply to large numbers as the three negative studies have used. However, if any of the last three are true, single round PCR of genomic DNA isolated from PBMCs, using primers based on published sequences from using highly specific PCR based on the sequence of a single molecular clone, (VP62) might not result in the amplification of XMRV, even from an infected individual.

A fourth explanation is that peripheral blood mononuclear cells might not be the main reservoir for the virus. It should be noted that early in HIV infection, PCR of peripheral blood mononuclear cells will not detect the virus, as the reservoir is in macrophages. It is only later that CD4 cells become infected and PCR of peripheral blood becomes positive. From *in vitro* studies we are aware of tropism of XMRV, however no full investigation of *in vivo* tropism has been carried out to date. There are other possible explanations for the disparity of results between the Lombardi et al study and the PCR-only studies which are currently being explored.

However, despite the current assumption that PCR answers all questions, there are serious flaws in this assumption, and PCR-only papers are rarely published without supporting data. For this reason, the *Science* paper employed several other biologic technologies in asserting that XMRV is an infectious retrovirus, and a human pathogen linked to persons with CFS. It should be noted that the question of XMRV "causing" CFS was never discussed in the *Science* paper.

Because of these issues, we will describe below the methods we and other investigators currently use to reproducibly detect XMRV. Because we wish to accurately share technical information, the following will be quite technical, and possibly difficult for clinicians. Again we would like to offer any assistance we might be able to give in discussing these methods.

AMPLIFICATION METHODS FOR XMRV DETECTION:

1) REVERSE TRANSCRIPTION (RT)-[PCR]

To avoid problems with laboratory DNA contamination, we perform nested PCR with separate reagents in a separate laboratory room designated free of high copy amplicon or plasmid DNA. Negative controls (absence of added cDNA or DNA) were included in every experiment. Two methods are used. TRIzol method is used to extract RNA. PBMC (2-5 million) or plasma (250 µL) are added to 1 mL TRIzol reagent (or 750 µL TRIzol LS reagent) and RNA prepared according to manufacturer's instructions. The final RNA pellet was suspended in 14 to 30 µL of RNase-free water, and quantified using the Quant-iT RiboGreen RNA (Invitrogen). Reverse transcription (RT) with SuperScript VILO cDNA synthesis kit (Invitrogen), cDNA was made by a mixture of oligo d(T) and random primer with 2.5 µg total RNA. Subsequent optimization of the nested PCR revealed that an increase in starting template leads to a reduction in false negatives.

Identification of XMRV *gag* and *env* genes was performed by PCR in separate reactions. Reactions were performed as follows: 750 to 1000 ng DNA/cDNA, 2 µL of 25 mM MgCl₂, 25 µL of HotStart-IT Fidelity Master Mix (USB Corporation), 0.75 µL of each of 20 µM forward and reverse primers in reaction volumes of 50 µL. For identification of *gag*, 419F (5'-ATCAGTTAACCTACCCGAGTCGGAC-3') and 1154R (5'-GCCGCCTCTTCTTCATTGTTCTC-3') were forward and reverse primers. For *env*, 5922F (5'-GCTAATGCTACCTCCCTCCTGG-3') and 6273R (5'-GGAGCCCACTGAGGAATC AAAACAGG-3') were used. For both *gag* and *env* PCR, 94 °C for 4 min initial denaturation was performed for every reaction followed by 94 °C

for 30 seconds, 57°C for 30 seconds and 72 °C for 1 minute. 35 cycles was performed followed by final extension at 72°C for 2 min. For second round PCR, annealing was performed at 54°C for 35 cycles. Six µL of each reaction product was loaded onto 2% agarose gels in TBE buffer with 1 kb+ DNA ladder as markers. PCR products were purified using Wizard SV Gel and PCR Clean-Up kit (Promega) and sequenced.

Alternatively, in the first round of PCR, cDNA is synthesized followed immediately by the first round of PCR amplification using USB Taq polymerase and master mix according to the manufacturer's instructions, using 0.5 ug RNA as template and the previously described GAG-O-F (F542) and GAG-O-R (R1153) as the sense and antisense primers, respectively (1). The second round of PCR was performed using 5 ul of the reaction mix, the primers GAG-I-F (F603) and GAG-I-R (R1015) using 0.5 ul USB Taq polymerase and Invitrogen PCR buffer, supplemented with an additional 0.5mM MgCl₂. The PCR products were then separated on a 1% agarose gel, and the PCR products of the expected size (413 bp) were recovered with a QIAEX II gel extraction kit (Qiagen) and sequenced. Using a similar approach for the amplification of *env* sequences using previously described primers only resulted in the amplification of a small percentage (<than 2%) of the *gag* positive samples.

2-VIRAL AMPLIFICATION BY TRANSMISSION TO LNCaP (Biological amplification)

Two methods are used to detect virus following transmission to LNCaP cells. The first uses nested PCR: Plasma from 20 mL of anti-coagulant blood is flash frozen and PBMC isolated by ficoll-hypaque density centrifugation. The PBMC are activated for three days RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate and antibiotics supplemented with PHA (1 µg/mL) and IL-2 (20 units/mL). In 15 mL centrifuge tubes, 5 x 10⁵ detached LNCaP, 1 x 10⁵ activated PBMC free of IL-2 and 50-250 µL of autologous plasma in 250 µL of RPMI complete media are centrifuged for 10 min at 1500 rpm. The entire contents of the cell pellet are cultured in a T-25 flask in complete RPMI 1640 media for 4-5 days until the LNCaP cells are confluent. DNA is then extracted from the cells and nested PCR for *gag* is performed as previously described. A companion negative normal donor is always run under the same conditions.

3-VIRAL ISOLATION

Cell-free plasma (100-150 µL diluted to 300 µL with serum-free tissue culture media) is added to a six-well culture plate with the LNCaP cell line (30-50% confluent). Negative normal plasma is included in one well in each plate as a control. The plates are centrifuged 5 min at 1500 RPM, rotated 180° to prevent drying out part of the culture and centrifuged again for 5 min. It is important to minimize toxicity in the plate and conditions will vary with type of centrifuge used. Each well is given 3-4 mL of complete RPMI. For cell-cell transmission, 1 x 10⁶ PBMC given PHA but no IL-2 are added to a six-well culture plate with the LNCaP cell line (30-50% confluent) are centrifuged and cultured as above. For PCR determination the cell/plasma samples are culture for 5

days then harvested. For viral isolation, after overnight incubation, the plasma/PBMC is removed and fresh media is added. The cultures are grown until confluent, expanded by transferring to T-75 flask. Productive virus isolation is determined at passage 2 by immuno-blotting or PCR detection and sequencing.

In sum, we have had success in identifying XMRV with the above methods. We remain confident that XMRV is linked to CFS and further studies are proceeding. We are aware that the research concerning XMRV's role in persons with CFS and other neuro-immune illnesses is in its infancy, and eagerly look forward to emerging knowledge in this field.

1. Lombardi V, Ruscetti F, Gupta J, Pfost M, Hagen K, Peterson D, et al. Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome. *Science*. 2009;326(5952):595-89.
2. Erlwein O, Kaye S, McClure M, Weber J, Wills G, Collier D, et al. Failure to detect the novel XMRV in chronic fatigue syndrome. *PLoS ONE*. 2010;5: e8519. doi:10.1371/journal.pone.0008519.
3. Groom H, Boucherit V, Makinson K, Randal E, Baptista S, Hagen S, et al. Absence of xenotropic murine leukaemia virus-related virus in UK patients with chronic fatigue syndrome. *Retrovirology*. 2010;7(10 [Epub ahead of print]).
4. Kuppeveld F, de Jong A, Lanke K, Verhaegh G, Melchers W, Swanink C, et al. Prevalence of xenotropic murine leukaemia virus-related virus in patients with chronic fatigue syndrome in the Netherlands: retrospective analysis of samples from an established cohort. *BMJ* 2010;340(doi:10.1136/bmj.c1018).