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## Failure to Confirm XMRV/MLVs in the Blood of Patients with Chronic Fatigue Syndrome: A Multi-laboratory Study

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### Abstract

Murine leukemia viruses (MLV), including xenotropic-MLV-related virus (XMRV), have been controversially linked to chronic fatigue syndrome (CFS). To explore this issue in greater depth, we compiled coded replicate samples of blood from 15 subjects previously reported to be XMRV/MLV-positive (14 with CFS) and from 15 healthy donors previously determined to be negative for the viruses. These samples were distributed in a blinded fashion to nine laboratories which performed assays designed to detect XMRV/MLV nucleic acid, virus replication, and antibody. Only two laboratories reported evidence of XMRV/MLVs; however, replicate sample results showed disagreement and reactivity was similar among CFS subjects and negative controls. These

<sup>16</sup>A description of the SRWG is available as supporting material in Science Online.

results indicate that current assays do not reproducibly detect XMRV/MLV in blood samples and that blood donor screening is not warranted.

Novel murine leukemia virus (MLV)-like sequences were identified in, and implicated as a potential infectious cause of, human prostate cancer in 2006 (1). These sequences appeared to be closely related to xenotropic MLV (X-MLV) and were termed X-MLV-related virus or XMRV. In 2009, similar viral sequences were identified in a cohort of patients with chronic fatigue syndrome (CFS) (2). In that study XMRV could be directly cultured from both peripheral blood mononuclear cells (PBMC) and plasma from the majority of patients with CFS, and XMRV sequences were detected by PCR and RT-PCR (2, 3). Furthermore, evidence of an immune response to MLVs was observed in patient plasma (2, 3). In an independent study, other patients with CFS were reported to harbor MLV-related virus sequences, but not XMRV, in PBMC and plasma (4). These sequences were derived from viruses resembling polytropic MLVs (P-MLV), rather than X-MLV. Importantly, both studies identified XMRV/P-MLV in the majority (67 to 86%) of patients with CFS but also in substantial numbers of healthy controls including blood donors (4-7%) (2, 4).

Subsequent studies cast doubt on the association between XMRV/P-MLVs and CFS, and indeed on the detection of XMRV/P-MLVs in human populations (reviewed in (5)). Many, although not all (6, 7), of these negative studies focused on nucleic acid detection and/or serology and did not include cell culture assays for virus (8-11). Several additional findings raised uncertainty about the high rates of XMRV/P-MLV in patients with CFS that had been described in the two seminal papers: (i) clinical samples and PCR reagents were found to be contaminated by XMRV and mouse DNA containing endogenous MLVs (12); (ii) XMRV and P-MLV lack the sequence diversity that would be expected to arise following transmission, infection, and repeated cycles of replication of a retrovirus in humans (13, 14), and (iii) evidence was presented which strongly suggested that XMRV originated in the early 1990s by recombination of endogenous MLVs following serial passage of a human prostate xenograft in laboratory mice (15). It was postulated that this laboratory passage resulted in the generation of several prostate cancer cell lines harboring integrated XMRV sequences that produced high levels of infectious virions. These XMRV-infected cell lines were subsequently widely disseminated and likely produced inadvertent XMRV contamination of laboratories and reagents (15).

We report here the results of a comprehensive study where multiple laboratories analyzed the same blood samples for XMRV/P-MLV. The blood samples which were drawn from persons who were previously reported to be XMRV- (2) or P-MLV-positive (4) and from blood donors who previously tested negative for XMRV, were aliquoted into replicate tubes and assembled into coded panels together with replicates of experimentally prepared positive control samples. The testing was performed fully blinded to remove bias. These samples were tested by nine laboratories using highly sensitive and previously validated nucleic acid, serological and culture assays (tables S1 to S5) for XMRV and other MLVs (16). The two laboratories that had previously found an association for the MLVs with CFS participated in this study (2, 4). All nine laboratories used XMRV/P-MLV nucleic acid amplification testing (NAT), serological and/or culture assays of their own choosing which were incorporated into parallel or serial testing algorithms to generate final results. The majority of laboratories included assays to detect murine DNA contamination either on all samples or on all NAT positive samples.

Fourteen patients with CFS, together with one person reporting contact with a CFS patient [described in supporting online material (17)], all of whom were previously reported to be XMRV/P-MLV-positive by at least one method (table S6) were enrolled into the study at two clinical sites using IRB-approved protocols and consents (referred to as the XMRV/P-

MLV cohorts henceforth). Per study protocol, none of the 15 subjects were on antiretrovirals, but several later disclosed that they were taking other antivirals (e.g., valacyclovir) and two were on immunosuppressive medications (the latter are indicated in table S6). In the case of the P-MLV-like viruses described by Lo and colleagues (4), only PCR detection had been performed in the original study; four of five patients enrolled into the current study were reported to be P-MLV reactive on the archived samples from the original cohort study and on a second sample collected 15 years later (2010) whereas one patient was PCR-positive only on the original archived sample (4). The Whittemore Peterson Institute (WPI) patient cohort was more intensively characterized as positive by PCR, serology and/or culture, although none of the study subjects tested positive in all assays at all time points (table S6).

To minimize introduction of potential contaminants, we took extensive precautionary measures during the collection of specimens and the laboratory processing of blood and preparation of sample aliquots (17). Blood specimens were collected by independent phlebotomists, shipped to the central laboratory (17), and processed into coded PBMC, plasma and whole blood (WB) aliquots. Similarly, fifteen control specimens from blood donors (n=12) or laboratory controls (n=3) that had been established as negative for XMRV and MLVs by PCR, serology and culture by multiple laboratories, were collected, processed and aliquoted in parallel (17). Finally, a separate facility in the central laboratory prepared and characterized stocks of the XMRV-infected human cell line 22Rv1 (15, 18) and supernatant, which were used to spike samples to create a set of low-level positive controls (17).

A total of eleven NAT, five serology and three culture assays were performed on the samples (17). The WPI laboratory did not report culture assay results because their target cells had become contaminated with mycoplasma. Other than this, all sites reported results on all distributed and coded sample aliquots to the central laboratory. The results were then decoded and compiled into analysis datasets specific to the panels.

Few positive NAT results were reported, other than on the coded spiked positive control replicate aliquots (table 1) (table S7). Six of seven laboratories that performed NAT on three sample types (plasma, PBMC and WB) reported no positive result for coded clinical samples (XMRV/P-MLV cohorts or negative controls), whereas these laboratories detected XMRV in 100% of the spiked controls (table 1). These laboratories included those that employed the most sensitive XMRV/P-MLV assays available, based on our previous blinded analytical sensitivity performance study (16). Of particular note, the FDA/Lo laboratory failed to detect MLV-like sequences using the same nested PCR assay as previously published, in either the known negative controls or in the XMRV/P-MLV cohort samples. The samples scored as negative by this laboratory included the replicate samples from five patients with CFS reported as P-MLV positive in their previous study, four of whom had also tested positive on a second specimen collected over a decade after the archived CFS cohort panel (4).

The only positive NAT results on some of the replicates from clinical samples were reported by WPI. The WPI assays appeared less sensitive than those used by the other laboratories, based on the fact that only 3 of 5 plasma and 4 of 5 PBMC-spiked positive control replicates were scored as positive by WPI (table 1) (table S7). However, two plasma clinical aliquots were reported as positive in the WPI nested RT-PCR *gag* assay. These samples were from two different negative controls, and only one out of the three replicates was positive in each case. Sequencing of the excised bands revealed 1-3 base changes compared to XMRV derived from 22Rv1 (supporting online text). A clinical PBMC sample, derived from one of the nine WPI CFS patients, was also positive in WPI's nested *gag* PCR assay. However,

only one of two PBMC replicates for this individual was positive, and all replicates of plasma and WB from this patient were reported as negative by WPI. All positive samples tested negative for mouse DNA contamination as assessed by mouse mitochondrial DNA PCR (4). Reactivity rates did not significantly differ between samples from negative controls and the XMRV/P-MLV cohorts ( $p > 0.05$ ) (supporting online text, table S10).

In the initial study, Lombardi et al. reported that the most effective and consistent method of determining whether an individual was XMRV-positive was by isolation of replication-competent virus through co-culture of target prostate cell lines with either patient PBMCs or plasma (2, 3). Although culture results were not reported by WPI in the present study, the NCI/Ruscetti laboratory also successfully performed virus culture using both plasma and PBMC in the Lombardi et al. study (2, 3). Additionally, virus culture was performed by the FDA/Hewlett laboratory, which used two methods, one of which (LNCaP cell culture) was established in their laboratory for this study based on WPI procedures and on-site training by the lead investigators from the WPI and NCI/Ruscetti laboratories, and hence viral culture in this laboratory would be expected to have equivalent sensitivity to the culture method used by Lombardi et al. (17). Both laboratories successfully detected all five replicates of the spiked positive controls ( $\sim 10^6$  RNA copies/ml). However, while neither of the FDA/Hewlett assays detected confirmed positive cultures in the 30 coded clinical aliquots, the NCI/Ruscetti laboratory reported nine aliquots as positive (table 1, 2). Six of the positive results were from negative control samples (40% positive rate); these six subjects/samples had previously been pedigreed by the same laboratory as culture-negative (17). In contrast, only three (20%) of the 15 XMRV/P-MLV-cohort subjects (including ten subjects who had previously been found to be culture-positive by the WPI and NCI/Ruscetti laboratories) tested positive in the coded panel (table S1). There was no significant difference between the rate of reported positive culture results among negative controls and the XMRV/P-MLV cohort subjects ( $p$ -value = 0.43, table S8).

Finally, serology was performed by four laboratories (17). Although plasma with human antibodies to XMRV/P-MLVs was not available to produce spiked controls for serology, all four laboratories performed their own internal controls (17). Three assays --a Western blot test using purified XMRV (CDC) (19) and two chemiluminescent immunoassays using recombinant XMRV gp70 and p15E (Abbott Diagnostics) (20)-- failed to detect positive results for any of the coded replicates prepared from the 30 clinical samples. A flow cytometry-based serologic assay run by two laboratories (NCI/Ruscetti and WPI), utilizing mouse cells expressing the spleen focus-forming virus (SFFV) envelope as employed in the original Lombardi et al. study, reported a number of positive results on samples from both the XMRV/P-MLV cohorts and the negative-plasma controls. The NCI/Ruscetti laboratory reported 13 positive samples, including eight (53%) from 15 known negatives and five (33%) from 15 XMRV/P-MLV cohort subjects (table 1) (table 2). None of the positive results from the XMRV/P-MLV cohorts or controls were reported for more than one of the uniquely coded replicates, despite the fact that every sample was represented in the panel in duplicate or triplicate (table 2). There was no significant difference between the proportions of negative controls and XMRV/P-MLV cohort subjects identified as serology-positive ( $p$ -values  $> 0.20$  regardless of how positivity was defined [supporting online text, Table S9]).

Among all serologic replicates tested, the WPI detected 22 positives, including 10 reactive results among the negative controls, and six each in the subjects previously reported as positive by WPI and by FDA/Lo (table 1) (table S7). Three of the six known negative controls with a positive serology result had at least two of three replicates positive (table 2). All five patients previously identified as P-MLV positive by FDA/Lo had a replicate called serology positive, but only one had both replicates reported as positive. Similarly for the 10 subjects previously identified as XMRV positive by WPI, four subjects had one of two



replicates reported as serology positive, while both replicates from one patient were reported positive (table 2). There was no significant difference in the rates of positive WPI serology results between negative controls and XMRV/P-MLV cohort subjects ( $p$ -value = 0.27). There was no statistical agreement between the samples reported as serology positive by the NCI/Ruscetti and WPI laboratories, despite the fact that they used similar assays (supporting online text, tables S9, S10). Kappa values were calculated for each criterion and for all subjects combined using standard procedures (17, 21). The Kappa values for level of agreement of results between these two laboratories ranged from  $-0.20$  for WPI XMRV/P-MLV-positive subjects (no agreement) to  $0.21$  for all negative controls combined (fair agreement). However, the most telling Kappa value between the WPI and NCI/Ruscetti serology results is the one computed for all subjects combined, which is  $0.01$  indicating no agreement.

In summary, our study demonstrates that no XMRV/P-MLV assay in any of the nine participating laboratories could reproducibly detect XMRV/P-MLV in fifteen subjects (fourteen with CFS) who had previously been reported as XMRV/P-MLV-infected usually at multiple time points and often by multiple assays (2, 4). The two laboratories (WPI and NCI/Ruscetti labs) that reported positive results in this study reported similar rates of reactivity among XMRV/P-MLV subjects and known negative control donor samples. The results from both laboratories were inconsistent when their assays were performed in parallel on replicate sample aliquots derived from individual subject specimens. There was also no agreement of reactivity when comparing results between these two laboratories for the 30 blinded XMRV/P-MLV cohorts and control samples. In contrast, assays developed by FDA (Lo and Hewlett), CDC, NCI/DRP, Abbott Diagnostics, Abbott Molecular and Gen-Probe, all of which have been designed to detect XMRV and relevant MLVs with high sensitivity and specificity, failed to detect evidence of viral infection in any of the previously positive subjects, including CFS patients, or negative control specimens represented in the study.

Altogether, 15 XMRV/P-MLV cohort subjects were represented in this study, the maximum number of subjects who could be recruited by the cohort investigators (2, 4). Since most patients were selected based on having previously tested positive for XMRV/P-MLV 1-3 years ago, it is possible that levels of viremia and/or antibody could have waned by the time samples were drawn in our study; however, this is contradictory to Lo et al.'s finding that 4 of 5 patients retested positive 15 years later (4). The inconsistent reactive results from the two laboratories that previously reported detection of XMRV (NCI/Ruscetti and WPI) and the negative results from all other laboratories, including the laboratory that previously reported detection of P-MLV (FDA/Lo), strongly suggest that the positive reactivity in this study represents false positive results due to assay non-specificity or cross-reactivity (e.g. to other endogenous or exogenous retroviruses). However, we cannot definitely exclude the possibility that the levels of XMRV/P-MLV markers in blood may be at or below the limit of detection of all assays and/or fluctuate over time as recently described in experimentally infected macaque studies (22).

Based on these findings, we conclude that currently available XMRV/P-MLV assays, including the assays employed by the three participating laboratories that previously reported positive results on samples from CFS patients and controls (2, 4), cannot reproducibly detect direct virus markers (RNA, DNA, or culture) or specific antibodies in blood samples from subjects previously characterized as XMRV/P-MLV positive (all but one with a diagnosis of CFS) or healthy blood donors. Finally, our findings are reassuring with respect to blood safety and indicate that routine blood donor screening for XMRV/P-MLV is not warranted at this time.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

All XMRV/P-MLV assay results from all laboratories

Test	Laboratory	Sample type			
		Negative Controls*	WPI XMRV/P-MLV Subjects*	Lo et al. XMRV/P-MLV Subjects*	Spiked Controls*
NAT/Plasma	Abbott-M <sup>†</sup>	0/15	0/10	0/5	5/5
	CDC	0/15	0/10	0/5	5/5
	FDA/Lo	0/15	0/10	0/5	5/5
	FDA/Hewlett	0/15	0/10	0/5	5/5
	Gen-Probe	0/15	0/10	0/5	5/5
	NCI/DRP	0/15	0/10	0/5	5/5
	WPI	2/15 <sup>‡</sup>	0/10	0/5	3/5
	Abbott-M	0/3	0/10	0/5	5/5
	CDC	0/3	0/10	0/5	5/5
	FDA/Lo	0/3	0/10	0/5	5/5
	FDA/Hewlett	0/3	0/10	0/5	5/5
	Gen-Probe	0/3	0/10	0/5	5/5
	NCI/DRP	0/3	0/10	0/5	5/5
	WPI	0/3	1/10 <sup>‡</sup>	0/5	4/5
	NAT/WB	Abbott-M	0/15	0/10	0/5
CDC		0/15	0/10	0/5	5/5
FDA/Lo		0/15	0/10	0/5	5/5
FDA/Hewlett		0/15	0/10	0/5	5/5
Gen-Probe		0/15	0/10	0/5	5/5
NCI/DRP		0/15	0/10	0/5	5/5
Culture	WPI	0/15	0/10	0/5	5/5
	FDA/Hewlett	0/15	0/10	0/5	5/5
	NCI/Ruscetti	6/15	3/10 <sup>‡</sup>	0/5	5/5
Serology	Abbott-D	0/15	0/10	0/5	N/A
	CDC	0/15	0/10	0/5	N/A

Test	Laboratory	Sample type		
		Negative Controls*	WPI XMRV/P-MLV Subjects*	Lo et al. XMRV/P-MLV Subjects* Spiked Controls*
	NCI/Ruscetti	8/15	3/10	2/5 <sup>‡</sup> N/A
	WPI	6/15	5/10	5/5 <sup>‡</sup> N/A

\* = Number positive/number tested. A single reactive replicate out of 1, 2 or 3 tested for a given individual was considered positive

<sup>†</sup> = Abbott-M is Abbott Molecular, Abbott-D is Abbott Diagnostics, WB is whole blood, N/A is Not Applicable

<sup>‡</sup> = No significant association was seen when the reactivity rates of control negatives and XMRV/P-MLV cohort subjects were compared (p values are discussed [supporting online text])

**Table 2**

Results of replicates for assays with positive results

Sample Type	Subject	Assay					
		WPI NAT/Plasma	WPI NAT/PBMC	WPI Serology	NCI/Ruscetti Serology	NCI/Ruscetti Culture	
Negative Controls	1	0/1	0/2	0/2	0/2	0/1	
	2	0/1	0/1	0/2	0/2	1/1	
	3	0/1	0/1	2/2	1/2	0/1	
	4	0/3	NT*	1/3	1/3	0/1	
	5	0/3	NT	0/3	0/3	0/1	
	6	1/3	NT	0/3	1/3	0/1	
	7	0/3	NT	0/3	0/3	0/1	
	8	0/3	NT	2/3	0/3	0/1	
	9	0/3	NT	3/3	1/3	1/1	
	10	0/3	NT	0/3	1/3	1/1	
	11	0/3	NT	1/3	1/3	0/1	
	12	0/3	NT	1/3	0/3	1/1	
	13	0/3	NT	0/3	1/3	1/1	
	14	1/3	NT	0/3	1/3	1/1	
	15	0/3	NT	0/3	0/3	0/1	
†							
<b>WPI XMRV/P-MLV Subjects</b>							
	1	0/1	0/2	0/2	1/2	0/1	
	2	0/1	0/1	1/2	0/2	0/1	
	3	0/1	0/1	1/2	0/2	0/1	
	4	0/2	0/2	2/2	0/2	1/1	
	5	0/1	1/2	0/2	0/2	1/1	
	6	0/1	0/2	0/2	0/2	0/1	
	7	0/2	0/2	0/2	0/2	0/1	
	8	0/1	0/2	1/2	1/2	0/1	

Sample Type	Subject	Assay						
		WPI NAT/Plasma	WPI NAT/PBMC	WPI Serology	NCI/Ruscetti Serology	NCI/Ruscetti Culture		
	9	0/1	0/3	1/2	0/2	0/1	0/1	
	10	0/1	0/2	0/2	1/2	1/1	1/1	
$\ddagger$								
<b>Lo et al. XMRV/P-MLV Subjects</b>								
	1	0/3	0/2	1/2	0/2	0/1	0/1	
	2	0/3	0/2	1/2	0/2	0/1	0/1	
	3	0/3	0/2	1/2	1/2	0/1	0/1	
	4	0/3	0/2	1/2	1/2	0/1	0/1	
	5	0/3	0/1	2/2	0/2	0/1	0/1	
$\S, \P$								
<b>Spiked Controls</b>								
	1	3/5	4/5	NT	NT	5/5	5/5	

\* NT is Not Tested

$\ddagger$  The kappa for the serology for the negative controls between NCI/Ruscetti and WPI is 0.21

$\S$  The kappa for the serology for the WPI XMRV/P-MLV subjects between NCI/Ruscetti and WPI is -0.20

$\P$  The kappa for the serology for the Lo et al. XMRV/P-MLV subjects between NCI/Ruscetti and WPI is 0.00

$\P$  The kappa for the serology for all cohort subjects between NCI/Ruscetti and WPI is -0.08