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Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome

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In October 2009, we reported the first direct isolation of infectious xenotropic murine leukemia virus-related virus (XMRV). In that study, we used a combination of biological amplification and molecular enhancement techniques to detect XMRV in more than 75% of 101 patients with chronic fatigue syndrome (CFS). Since our report, controversy arose after the publication of several studies that failed to detect XMRV infection in their CFS patient populations. In this addenda, we further detail the multiple detection methods we used in order to observe XMRV infection in our CFS cohort. Our results indicate that PCR from DNA of unstimulated peripheral blood mononuclear cells is the least sensitive method for detection of XMRV in subjects' blood. We advocate the use of more than one type of assay in order to determine the frequency of XMRV infection in patient cohorts in future studies of the relevance of XMRV to human disease.

Patient selection poses a challenge to any study of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). In our October 2009 paper, samples banked from 2006 to 2008 were selected for our study from severely disabled patients who fulfilled the 1994 CDC Fukuda Criteria for chronic fatigue syndrome[1] as well as the 2003 Canadian Consensus Criteria (CCC) for ME/CFS.[2] The CCC requires post-exertional malaise, which many clinicians feel is the *sine qua non* of ME/CFS. Furthermore the CCC further requires that patients exhibit post exertional fatigue, unrefreshing sleep, pain and neurological/cognitive manifestations, rather than these being optional symptoms.[3] Many clinicians interested in CFS are switching to the Canadian criteria because they feel it is more descriptive of the clinical entity being defined. The Fukuda criteria have the advantage of a longer period of usage and existence of many publications that have added modifications. Suffice it to say that the clinician author of the *Science* paper elected to use both criteria, thus bypassing the argument of which criteria were better. Moreover, the emphasis in the *Science* paper was directed toward the virology, not the clinical description of ME/CFS.

In our October 2009 publication, we established XMRV infection in the blood products of our patient population by five different methods. Of these methods, single-round PCR on DNA from peripheral blood mononuclear cells (PBMCs), the least sensitive method, required us to use samples from a subset of chronically ill patients we had observed to have persistent viremia. In Figure 1A of our *Science* paper, we showed that DNA of 7 of 11 patients exhibited the expected *gag* and *env* PCR amplification products from single-round PCR with XMRV primers. We included this figure to demonstrate that

Table 1. XMRV detection using cDNA from 22 unstimulated PBMCs

gag gene				*env* gene			
Sample	**1st**	**2nd**		**Sample**	**1st**	**2nd**	
1	-	-	Normal	1	-	-	Normal
2	-	+	1104	2	-	-	1104
3	+	+	1110	3	-	-	1110
4	-	-	1113	4	-	-	1113
5	-	-	1114	5	-	-	1114
6	-	+	1115	6	-	+	1115
7	-	-	1117	7	-	-	1117
8	-	+	1125	8	-	-	1125
9	-	+	1130	9	-	-	1130
10	+	+	1135	10	-	-	1135
11	-	-	1142	11	-	-	1142
12	+	+	1150	12	-	-	1150
13	-	-	1155	13	-	-	1155
14	-	+	1161	14	-	-	1161
15	-	+	1165	15	-	-	1165
16	-	+	1166	16	-	-	1166
17	+	+	1168	17	-	-	1168
18	-	+	1169	18	-	-	1169
19	-	+	1177	19	-	-	1177
20	-	+	1178	20	-	-	1178
21	-	-	1182	21	-	-	1182
22	-	-	1199	22	-	-	1199

Table 2. Co-culture with LNCaP of PBMCs from 12 patients PCR negative for *env*

gag gene				*env* gene				
Sample	**1st**	**2nd**		**Sample**	**1st**	**2nd**		**Type**
1	-	-	Normal	1	-	-	Normal	cDNA
2	-	+	1169	2	+	+	1169	cDNA
3	+	+	1221	3	+	+	1221	cDNA
4	-	+	1150	4	+	+	1150	cDNA
5	-	+	1199	5	-	+	1199	cDNA
6	+	+	1220	6	+	+	1220	cDNA
7	-	-	LNCaP	7	-	-	LNCaP	cDNA
8	-	+	1186	8	-	+	1186	cDNA
9	-	+	1132	9	-	+	1132	DNA
10	-	+	1111	10	-	-	1111	DNA
11	-	+	1189	11	+	+	1189	DNA
12	-	+	1172	12	+	+	1172	DNA
13	-	+	1173	13	-	+	1173	DNA
14	-	+	1103	14	+	+	1103	DNA

nested PCR, which inevitably raises questions of contamination, is not essential to detect XMRV in highly viremic ME/CFS patients. The remaining 90 samples described in the paper exhibited very few XMRV-*gag* specific PCR products and no *env* specific PCR products following single round DNA PCR of DNA of unstimulated PBMCs. In contrast, when cDNA was prepared from PBMCs, 67% of the samples exhibited *gag* products upon nested PCR, though PCR with nested *env* primers did not result in detectable products from these samples (**Table 1**).

Samples that are negative for XMRV by one of our PCR assays are sometimes positive by other assays. For example, in Figure 1A of the *Science* paper, patient 1118 was negative by single round PCR on DNA from unstimulated PBMCs, but positive in other assays (*Science* Figs. 2A and D, 4A and S5). Of the 34 patients whose PBMCs were negative for XMRV by DNA or cDNA PCR, 17 were positive for infectious virus when co-cultured with the LNCaP indicator cell line, as XMRV *gag* and *env* PCR products were detected in the cell line following their infection with XMRV from the patient PBMCs (**Table 2**). Both *gag* and *env* products obtained from either single-round or nested PCR were sequenced and shown to be 99% identical to XMRV VP62.

Subsequent to our October 2009 publication, two papers from the United Kingdom[4,5] and a paper from the Netherlands[6] have appeared in which the authors report the lack of detection of XMRV PCR products from DNA of unstimulated PBMCs, using patient populations selected by only the Fukuda criteria or the Oxford criteria rather than both Fukuda and CCC criteria. We regret that these authors did not request positive control samples of our patients who exhibit XMRV PCR products even when assayed by the least sensitive detection method, namely PCR of DNA from unstimulated PBMCs. Given that only 7% of our 101 patients' PBMCs exhibit products upon DNA PCR (**Table 3 and 4**), and that a number of patients were included in the UK studies who do not fulfill the CCC criteria, very few, if any, of the samples would be expected to be positive by DNA PCR. We also note that both studies followed different methods than ours for blood collection, DNA quantities and isolation and PCR, possible sources of the disparate results. The XMRV detection results of the 101 patients are listed in **Table 4**.

The negative reports of PCR tests for XMRV has raised questions whether our findings could be due to contamination of our PCR experiments by mouse genomic

Table 3. Summary of multiple viral assays from a group of 57 patients

Unstimulated PBMC		Stimulated PBMC		Co-Cultured LNCaP		Serology	Unstimulated PBMC
Nested gag		Nested gag		Nested gag		Env antibody	Single round gag
cDNA	DNA	cDNA	-	cDNA	-	Plasma	DNA
31/57	44/205*	41/57	-	51/57	-	47/57	4/57
54%	21%	72%	-	89%	-	82%	7%

*multiple DNA samples taken from some of the 57 patients on different dates.

Table 4. XMRV detection results of 101 patients

Patient ID	cDNA nested PCR	DNA nested PCR	LNCaP co-culture with PMCs	Antibody in plasma	LNCaP culture with plasma
1103	+	+	+	+	+
1104	+	+	+	+	+
1105	+	-	+	+	+
1106	+	+	+	+	+
1107	+	-	-	NT*	NT
1108	+	-	-	-	-
1109	+	-	NT	NT	NT
1110	+	-	+	+	+
1111	+	+	+	-	+
1112	+	-	NT	NT	NT
1113	+	-	+	NT	NT
1114	+	-	NT	NT	+
1115	+	-	+	+	+
1116	-	-	NT	NT	+
1117	-	-	NT	NT	NT
1118	+	-	+	+	+
1119	+	-	NT	NT	NT
1120	-	-	NT	NT	NT
1121	+	-	NT	NT	NT
1124	+	-	-	-	-
1125	+	-	+	+	+
1126	+	-	NT	NT	NT
1127	+	-	NT	NT	NT
1128	+	-	NT	NT	NT
1129	+	-	NT	-	NT
1130	+	-	NT	NT	NT
1131	+	-	NT	NT	NT
1132	+	+	+	NT	NT
1133	+	+	NT	NT	NT
1134	-	-	NT	NT	NT
1135	+	+	NT	NT	NT
1136	+	+	-	+	+
1137	+	+	-	+	+
1265	+	-	+	+	+
1138	+	-	NT	NT	NT
1335	+	-	NT	+	+
1139	-	-	-	-	-

*NT, not tested. Note not all assays were run on all samples and/or patients.

Table 4. XMRV detection results of 101 patients

1140	+	-	NT	-	+
1141	+	-	+	+	+
1142	-	-	NT	NT	+
1206	+	-	NT	-	+
1144	+	-	NT	NT	NT
1145	-	-	NT	NT	NT
1148	-	-	NT	NT	NT
1149	+	-	NT	NT	NT
1150	+	+	+	+	+
1151	+	-	NT	NT	NT
1230	+	-	+	NT	NT
1237	+	-	+	NT	NT
1154	-	-	NT	NT	NT
1155	-	-	NT	NT	NT
1156	-	-	NT	NT	+
1157	+	+	NT	NT	NT
1158	+	-	-	+	+
1159	+	-	NT	NT	NT
1231	+	-	+	NT	NT
1161	+	-	-	+	+
1220	+	-	+	NT	NT
1221	+	-	+	NT	+
1164	-	-	NT	NT	NT
1165	+	-	+	+	+
1166	+	-	-	+	+
1167	-	-	NT	NT	NT
1168	+	-	NT	NT	NT
1169	+	-	+	+	+
1170	-	-	NT	NT	NT
1235	-	-	+	NT	NT
1281	+	-	+	+	+
1172	+	+	+	+	+
1282	+	-	-	-	+
1173	+	+	+	+	+
1174	+	-	NT	NT	NT
1175	-	-	NT	NT	NT
1176	-	-	NT	NT	NT
1177	+	-	+	+	+
1178	+	-	+	+	+
1179	+	-	NT	-	+
1180	-	-	NT	+	+
1181	+	-	NT	NT	NT
1182	-	-	NT	+	+
1183	+	-	-	-	+
1236	+	-	+	NT	NT
1224	+	-	NT	NT	+
1186	+	+	+	+	+

*NT, not tested. Note not all assays were run on all samples and/or patients.

Table 4. XMRV detection results of 101 patients

1187	-	-	NT	+	+
1188	+	-	+	+	+
1189	+	+	+	+	+
1190	+	-	+	+	+
1191	+	-	+	+	+
1192	+	+	NT	+	+
1193	+	+	NT	+	+
1194	+	-	NT	-	+
1238	+	-	+	+	+

*NT, not tested. Note not all assays were run on all samples and/or patients.

DNA, which contain *gag* and *env* sequences highly similar to XMRV. Positive PCR results for XMRV were obtained independently in multiple laboratories led by co-authors of the *Science* paper. In the summer of 2006, prior to work on XMRV at the Reno Whittemore-Peterson Institute (WPI), 30 mL of heparinized peripheral blood were obtained from patients residing in the US, Canada and Europe coming to be treated at the well-known Sierra Internal Medicine practice, located in Incline Village, NV. Once collected, 48 of these blood samples were shipped directly to NCI where cDNA was prepared for planned microarray experiments. After the WPI observed an XMRV PCR product from a patient sample in 2009, the NCI began testing these stored samples by PCR. cDNA from 42 of the 48 samples sent to the NCI lab in February 2007 tested positive for XMRV *gag* by nested PCR. Neither the WPI nor NCI labs where PCR was performed had ever worked with mouse tissues or had been exposed to XMRV from other sources. The *env* sequences amplified from LNCaP cells infected by patient PBMCs exhibit less similarity to mouse genomic DNA than to XMRV VP62, further indicating the presence of XMRV infection rather than mouse genomic DNA contamination. After we developed a sensitive cell culture assay for detection of XMRV, we assayed our cell lines and patient material with a highly sensitive assay (developed and kindly provided by Bill Switzer, CDC) to detect the presence of mouse tissue contamination by the identification of murine mitochrondial cytochrome oxidase by real time PCR. All of the cell lines and 101 patient materials tested negative for mouse contamination.

In our experience from performing the multiple methods on the same 57 blood samples, the most sensitive blood-based assays for detection of XMRV in decreasing order (**Table 3**) are: (1) performing nested PCR for *gag* sequences from LNCaP cells that have been co-cultured with subject's plasma or activated PBMCs, (2) the presence of antibodies to XMRV Env in subject's plasma, (3) presence of gag products by nested PCR on stimulated PBMCs or detection of viral proteins expressed by activated PBMCs with appropriate antisera, (4) nested RT-PCR of plasma nucleic acid or PCR from cDNA from unactivated PBMCs and (5) PCR of DNA from unactivated PBMC prepared from subject's blood.

Despite association with both prostate cancer and CFS, many questions remain regarding the prevalence of XMRV in the human population, the incidence of XMRV in disease, and the extent of genetic variation between XMRV isolates. The genetic variation between XMRV isolates currently identified is only 0.03%, despite the fact that the viral sequences were obtained from isolates from two vastly different diseases in patients from geographically distinct areas. This variation is smaller than the variation observed between HTLV-1 isolates.[7] As in the case with HTLV, the lack of diversity implies that XMRV recently descended from a common ancestor.[8] The high degree of similarity to xenotropic murine leukemia virus suggests that a cross-species transmission event was likely involved in the evolution of XMRV.[9] Further examination of XMRV from human subjects may reveal more extensive sequence variation, which also may confound its detection unless PCR primers are designed with this possibility in mind.

We have not claimed in our October 2009 publication or in other venues that XMRV is the cause of CFS, only that its detection in the majority of our ME/CFS patient cohort allows us to form a testable hypothesis as to an infectious basis for this devastating disease. Future work should establish what role XMRV may play in development of prostate cancer, ME/CFS and other diseases.

References

1. Fukuda K, Straus S, Hickie I, Sharpe MC, Dobbins JG, Komaroff A. The chronic fatigue syndrome: A comprehensive approach to its definition and study. Ann Intern Med 1994; 121:953-9.
2. Carruthers B, Jain A, DeMeirlier K, Peterson DL, Klimas NG, Lerner AM, et al. Myalgic encephalomyelitis/chronic fatigue syndrome: Clinical working case definition. diagnostic and treatment protocols. J Chronic Fatigue Syndrome 2003; 11:1-12.
3. Jason L, Torres-Harding S, Jurgens A, Helgerson J. Comparing the Fukuda et al. criteria and the Canadian definition for chronic fatigue syndrome. J Chronic Fatigue S 2004; 12:37-52.
4. Erlwein O, Kaye S, McClure MO, Weber J, Wills G, Collier D, et al. Failure to detect the novel retrovirus XMRV in chronic fatigue syndrome. PLoS One 5:8519.
5. Groom HC, Boucherit VC, Makinson K, Randal E, Baptista S, Hagan S, et al. Absence of xenotropic murine leukaemia virus-related virus in UK patients with chronic fatigue syndrome. Retrovirology 7.
6. van Kuppeveld FJ, de Jong AS, Lanke KH, Verhaegh GW, Melchers WJ, Swanink CM ,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 340:1018.
7. Ratner L, Philpott T, Towbridge DB. Nucleotide sequence analysis of isolates of Human T-lymphotropic virus type 1 of diverse georgraphical regions. AIDS Rs Hum Retroviruses 1991; 7:923-41.
8. Verdonck K, Gonzalez E, Van Dooren S, Vandamme AM, Vanham G, Gotuzzo E. Human T-lymphotropic virus 1: Recent knowledge about an ancient infection. Lancet Infect Dis 2007; 7:266-81.
9. Yan Y, Liu Q, Kozak CA. Six host range variants of the xenotropic/polytropic gammaretroviruses define determinants for entry in the XPR1 cell surface receptor. Retrovirology 2009; 6:87.