

A Different Kind of XMRV? Dr. Mikovits and Dr. Racaniello on XMRV by Cort Johnson

by **Cort** Published on July 11th, 2010 05:39 PM

Prelude: XMRV's status seems more up in the air than ever. Yes, a number of negative studies have been published but every study has had its problems. With the Alter paper unpublished and questions about the CDC study arising just as quickly as it was published, the only thing that seems to be clear, amazingly enough, after almost nine months, is that there is still no consensus on XMRV.

The Real Watchdog in Town? Mindy Kitei reported the DHHS officials were apparently asleep at the switch as the CDC and Alter papers raced towards publication. It was the CDC that played watchdog, and alerted senior public officials at the DHHS about the impending clash of results, but it was the DHHS officials who then asked both research groups to do more testing.

Our attention has been focused on the fact that the negative paper has been published (by the CDC no less) while the positive paper has been temporarily withdrawn - a disquieting series of events coming on top of the breakthrough CFS patients had been waiting for. The CDC's temporary withdrawal of their paper last month resulted in hullabaloo that erupted when we expected the CDC paper to be released and it wasn't. Perhaps because the DHHS asked the CDC to do less testing, they finished their testing first and the paper was published (unchanged).

Now we await the publication of the Alter paper which the CFIDS Association reported should be published in several weeks. Only time will tell whether Dr. Alter's conclusions will change (Mindy Kitei reports they will not).



The Central Issue Remains the Same - How to Find XMRV - The big new issue concerning the CDC's inability to find XMRV in the samples the WPI samples is simply another version of that. I asked Dr. Mikovits and Dr. Racaniello about XMRV and both were good enough to give their viewpoints on where we stand now. This is a long and rather technical article; in it they talk about

- how to test for XMRV
- whether a different type of XMRV is present in CFS patients and how it got there
- the importance of subsets
- the history between the CDC and the WPI
- how the CDC study may have gone wrong.

Dr. Racaniello, a Professor of Microbiology at Columbia University Medical Center, has been studying a wide variety of viruses including enteroviruses and polioviruses in his laboratory for over 20 years. You can find his [This Week in Virology](#) blog here and a [Wikipedia page about him here](#). Over time he's provided an objective voice on the XMRV/CFS connection. He will join Dr. Bateman and Dr. Vernon in the [CFIDS Association's Webinar on July 15th on XMRV](#).

Dr. Judy Mikovits is the [Research Director of the Whittemore-Peterson- Neuroimmune Institute](#). She spent more than 20 years at the National Cancer Institute where she directed the Lab on Antiviral Drug Mechanisms, studying a wide array of viruses including HHV-6 and HIV. She also served as the senior scientist at Biosource International and was the Chief Scientific Officer and VP of Drug Discovery at Epigenix Biosciences. She co-authored the paper that found XMRV in CFS.

To PCR Or Not To PCR? - PCR has been the main focus for validation studies thus far, something that has sometimes irked Dr. Mikovits after the extensive efforts the WPI went through to find XMRV in other ways. In his recently published overview of XMRV in CFS, Dr. Silverman reported four ways the PCR finding of XMRV was validated (note how many times activation/culturing appears): XMRV was found in activated PBMC's, T and B cells., activated PBMC's and plasma from CFS patients but not controls transferred XMRV to LnCap cells when they were cultured, and that antibodies to an envelope protein of XMRV were detected in CFS patients but not controls. Given the multiple, overlapping layers of evidence of XMRV Dr Silverman concluded that "these data strongly support the presence of XMRV in these patients with CFS"

Given the many other ways to detect XMRV I asked Dr. Racaniello why the research community was focusing on PCR so much? He stated that PCR was the logical method to use at this point because

"PCR is the most sensitive way to detect nucleic acids, it's cheap, and very easy to do."

Given the difficulty of finding XMRV using PCR thus far was the research community putting the cart before the horse, so to speak, by focusing mostly on PCR? He didn't think so.

"Not at all" he said, "PCR is the gold standard for diagnosing viral infections. When you think you have flu, your doc gives you a rapid test in the office. Those are antigen-based and are lousy. If the answer is negative, you go to PCR."

Was it necessary to use PCR to validate XMRV ? He felt no but there was a catch

"No. The best way would be to find infectious virus. PCR doesn't detect infectious virus; nor do serological methods. But not everyone knows how to do virus culture. Nearly anyone can do PCR. To validate the role of XMRV in CFS requires examining many patient samples (thousands) and to do this by virus culture would impede progress. PCR will do the job."

To Culture or Not to Culture? The most salient factor regarding XMRV methodology appears to be the role that culturing cells should play in the search for XMRV. Dr. Mikovits remains adamant that the virus is so hard to find that you must culture first and she stated

"this is why our first step and the entire paper was focused on the biological amplification and isolation of XMRV in the LNCaP cell line"

It seemed remarkable that Dr. Mikovits could put so much emphasis on culturing and yet have it not appear in the first four studies. I asked Dr. Racaniello why other studies had not used it to date?

He pointed out that the section of the Science study on PCR did not mention culturing.

"The PBMC (approximately 2×10^6 cells) were centrifuged at 500x g for 7 min and either stored as unactivated cells in 90% FBS and 10% DMSO at -80 °C for further culture and analysis or resuspended in TRIzol (Invitrogen, Carlsbad, CA) and stored at -80 °C for DNA and RNA extraction and analysis."

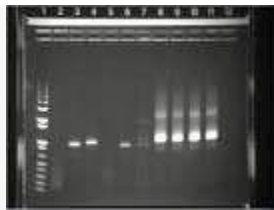
Notice that the PBMC were stored for DNA and RNA extraction - which was then used for PCR. The activated PBMC were used for detecting virus and viral proteins; (ie culturing in the LNCap cell line was used for the antibody and other tests). He stated

"It's not clear to me why the need for activating/culturing was not indicated in the paper. That's probably one reason why no one else is doing it - they are trying to reproduce the results using methodology written in the paper."

I asked Dr. Mikovits about Dr. Racaniello's comment and she stated that culturing was used in every aspect of the Science paper except in Figure 1 - which was the PCR results. If I understand this correctly, least at the time of the Science paper, the WPI was using culturing extensively but not in the cells used in the PCR tests.

An Important Issue- One problem is that XMRV is not replicating in the type of immune cells that have been examined thus far. These were the first cells looked at because they are central sites of replication for the other human infectious retroviruses - but not, it turns out, for XMRV.

Dr. Mikovits "A key issue here is everyone (including us) assumed that the T and B cells are major cellular reservoirs as they are in HIV ...this is clearly not the case with XMRV....I think that most of the virologists who are not retrovirologists simply think of HIV where the copy number in white blood cells is high..This is NOT the case with either HTLV or now apparently XMRV as retrovirologists always culture the cells to amplify the virus... Culture is not needed to do PCR effectively. Culture is needed to detect the human retroviruses, XMRV and HTLV1"



(Frank Ruscetti, one of the co-authors of the Science paper has quite a history with retroviruses; he - was a co-author of the paper that first described HTLV-1, the first human infectious retrovirus found. That paper was published in another almost equally prestigious journal -Nature.)

Still Not Sensitive Enough? The low levels of XMRV present mean the techniques used to find it need to be very sensitive. As researchers have refined their tests they have become more and more sensitive, I asked Dr. Mikovits if she felt they've become

sensitive enough to find the virus without culturing the cells it occurs in yet? She felt not stating

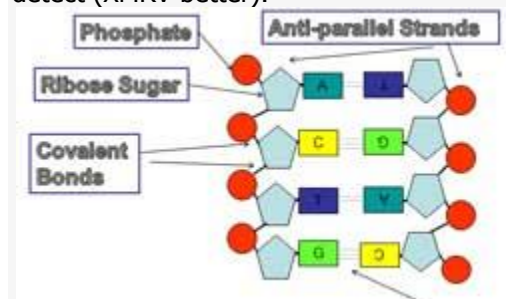
"the virus is in too low a copy number in the genomic DNA (that is the provirus..integrated into the cells DNA) ..there may be less than 10 copies of provirus in 10 million cells..so unless you amplify the 1 microgram of genomic DNA from uncultured cells you might miss it"

The low levels of the XMRV in the cells tested thus far mean the techniques used to search for it need to have high sensitivity. Regarding the sensitivity of the assays Dr. Racaniello stated that

"I don't know that Dr. Mikovits or anyone else yet knows the copy number of XMRV in human DNA. (Not producing the sensitivity figures for the assays was a critique of the Science paper.) But I do think that at least some of the published studies were sufficiently sensitive to detect XMRV if it were there. Note that Dr. Mikovits says that you might need to amplify 1 microgram of DNA from unactivated, cultured human cells, which is what was done in the CDC study. "

. I think all the groups are very capable of detecting XMRV by PCR, as long as they are looking for proviral DNA (by definition viral DNA integrated into the human genome)."

A Different Kind of XMRV? Dr. Mikovits, however, brought a startling new idea into play, that she felt might explain the differing results. She indicated that two papers, one in March by **Bishop and Groom** and one by **Paprotka in June**, played a big role in helping her understand how to look for XMRV. "There were a lot of clues in the biology of the paper by Bishop and Groom that helped us figure out the PCR strategy that would detect (XMRV better)."



Researchers are looking for XMRV in CFS in the immune cells (T and B and others) in the blood. The papers indicated that the genetic sequences of the virus in T and B cells can get altered (or edited) over time by what are called APOBEC3 editing enzymes. Cells use these enzymes to attack retroviruses that have become integrated into our genome. Simply by exchanging the guanine bases in the retroviral sequences to alanin APOBEC3 stops the virus from replicating. Basically, except for the few virions that manage to escape this defense mechanism, T and B cells in the blood are a dead end for XMRV.

The XMRV in prostate cancer cells may be different, however, because the APOBEC3 enzyme is not found in those cells, thus allowing them to escape this editing process. Since XMRV was first discovered in prostate cancer cells it's not surprising that prostate cancer XMRV forms the basis for many of the PCR probes but it does mean studies using this form of the XMRV could miss the different looking virus in the T and B cells.

From the Paprotka paper:

"XMRV proviral genomes were extensively hypermutatedin T-cell lines" where APOBEC3 inhibits XMRV replication greatly (200-fold) but is not present in prostate cancer cells.

Only the WPI has verified their results using patient derived XMRV samples. So far as we know, only the WPI is culturing XMRV from patients and then analyzing it. All the other studies to date have used artificially manufactured samples. Researchers have always wondered if geographically derived genetic variations in XMRV were getting in the way of finding it. This finding suggests that variations in XMRV makeup in the body, no less, could be getting in the way.

In Dr. Mikovits words.

"The R22V1 cell line does not have the APOBEC3 editing enzymes ...The patient isolated viruses have undergone significant APOBEC3 editing where Gs are changed to As ...the sequence changes but the changes are synonymous (that is the amino acids in the resultant protein don't change..thus the virus is still infectious as we demonstrated)...thus PCR would and does miss these" (The R22V1 cell line was used in the Kuppeveld study)

This kind of altered XMRV, however, may be just the kind of XMRV likely to be found in people with CFS. From Dr. Mikovits

"Since patients have been infected with their immune system trying to chop up these bad guys for decades there can be many of these sequence changes...

Interestingly, the alterations in the virus do not affect the antibody results

"The really cool thing is that the virus escapes and the amino acid stays the same, so the antibody assay detects it fine and it is perfectly infectious.

(This appears to suggest that amino acids in the provirus - the one embedded in the DNA get altered - but the virus still produces normal virions. Thus the antibody tests are still accurate - it's only the PCR tests that experience the editing glitch.



The Catch? Those Genetically Identical

Clones? - The WPI, however, produced two XMRV clones from two patients that were almost genetically identical to the standard XMRV strain from prostate tissues, which suggests that editing wasn't a problem in those patients. (One would imagine that the samples sent to the CDC in Oct 2009 were from those patients as well, which suggests that editing might not have been a problem there either). WPI researchers were also able to find XMRV in their samples before they learned about the editing problem ..

The Cohort Question - The CFS patients in the Science study were rigorously defined and quite ill. (First studies typically focus on patients researchers feel are most likely to prove them right - usually this means focusing on the really ill patients.)

APOBEC3 is a powerful cellular antiviral enzyme that's strong enough to knock some variants of HIV down. Could illness in CFS, in part, be a function of poor APOBEC3 functioning? Might very ill patients have normal, less disturbed, more functional XMRV while less ill patients have edited versions of XMRV that are a) less functionally harmful and b) more difficult to pick using normal PCR? Does the editing problem show up more in less ill patients?

There are still many questions regarding the role editing plays in the difficulty finding XMRV in PCR such as how extensive it is and in which types of patients it appears but it's clear that it is occurring and that it is capable of changing the makeup of XMRV in immune cells; ie it's a new factor that must be accounted for.

Dr. Racaniello, interestingly, believes that the cohorts probably play the biggest role in the differing results.

"I suspect that the single most important variable in the negative studies is how you define the patient population. They are probably looking at very different subsets."

Dr. Mikovits would agree and noted that a similar situation occurs with regard to XMRV in prostate cancer as well. She stated that prostate cancer, like ME/CFS, is a very heterogeneous disease with XMRV showing up in much larger percentages in some groups of patients.

"Prostate cancer in younger men is a totally different disease with inflammation and the tumor microenvironment playing a big role. We looked at a cohort of men from the NIH Clinical Center (these are the

sickest young men and most aggressive tumors) and we found evidence of infection in >50%...a big difference!!

Of course we don't expect to see XMRV in 80% of everyone that is diagnosed with CFS and Dr Racaniello is perfectly correct. That said, there are going to be millions of people with diseases which would satisfy the CCC for CFS.

CDC Study

The WPI had their overlapping levels of evidence but so did the CDC group; neither they or the two independent labs they worked with were able to find XMRV using antibodies or PCR. The CDC used some of the same PCR tests that the WPI did but their reagents were different and that apparently changes things dramatically.

PCR and antibody tests, it turns out, have to be validated for specificity (to ensure they only pick up XMRV) and sensitivity (to ensure that they can pick it up at a certain level) using positive XMRV samples. The CDC did test their results against an XMRV sample but it was a laboratory sample not a patient sample. Validating tests against a patient sample, not a laboratory derived standard, however, is the gold standard. Dr. Mikovits noted that antibodies.....

"are biological reagents that must also be clinically validated (for specificity and sensitivity) as we did with the reagents in our paper. If a publication uses different antibodies they should show that they can detect XMRV in a clinical sample"

She reported the WPI sent positive XMRV samples to the CDC two weeks prior to the publication of the Science paper. The CDC apparently quickly reported that they were negative for XMRV. The WPI researchers were surprised to learn, however, that the CDC, eight months later, still had never validated their test results against a human sample.

"We sent the CDC 20 confirmed positive plasma samples on September 29, 09 (two weeks before the paper was published). They declared them all negative and that was the last we heard of them until last week. when it was revealed...to the Blood Working Group that...they had NEVER demonstrated that the methods in the paper could detect a clinical positive sample. In fact their methods had FAILED to detect any clinical positive".

Dr. Mikovits - "There is a huge difference in the CLINICAL sensitivity of the PCRs and the analytical sensitivity.. If one-10 edited copies were there THEY would miss them in all the negative papers. But we do NOT..we culture the virus."

"The difference is the analytical sensitivity vs the clinical sensitivity.. Cloned isolate vs a natural isolate.. which in this case makes all the difference in the world. This is why I am glad I was trained before PCR and always to let the biology tell me the answer - Frank Ruscetti preached that for > 20 years.. I am a slow learner:)"(editor underlined)

Regarding the importance of having a positive patient sample to test against Dr. Racaniello was in agreement.

"In my view the CDC paper should not have been published without a proper positive control, eg patient samples known to contain XMRV. If I had reviewed the CDC paper that's what I would have asked for."

A New And Different Virus - A main issue is that XMRV is a new virus that whose secrets researchers are clearly just beginning to fathom. Dr. Mikovits view appears to be that the process the research community has taken over the past eight months has been backwards. Because XMRV is a new virus the most efficient path would have been to focus on validating the WPI's procedures first and then trying new ones. Instead all the research community has achieved thus far, by taking this scattershot approach, is to demonstrate what does not work in XMRV.

Indeed in an earlier chat Dr. Mikovits noted that when the WPI tried their techniques they couldn't find the virus either: "when we looked at our isolates with their primers we would miss them"

"We know very little about XMRV...this is all the more reason to use our methods and our clinical positive samples ...to validate new methods. That is why we have been working very hard with the Blood Working Group (at the DHHS) to develop sensitive and clinically validated methods that everyone can follow".

The WPI probably has a reputation as something of an outsider. They're the small Research Institute that made the big splash and they have, at times, engaged in a kind of verbal brawl with dissenting research groups, but Dr. Mikovits statement indicates they are also working inside the fence as well. Dr. Mikovits is a member of the DHHS Blood Working Group and the fact that the WPI is a partner in the biggest and most comprehensive research effort on XMRV is a hopeful one for the WPI and the CFS community. (The DHHS group is looking at the editing question.)

She evinced some frustration at the way at the reception given to XMRV by the biggest source of retroviral funding, the NIAID, given the promise she feels XMRV presents.

"It is still amazing to me that when I entered the field everyone said the most important thing was to find biomarkers in order to better subgroup. XMRV would be a huge benefit to the medical profession and our economy in prostate cancer if it could predict indolent vs latent prostate cancer as billions of dollars are currently spent on unnecessary treatments and millions more die because no one know which man will die so the current best practice is "watch and wait"

Similarly in CFS and other XMRV associated diseases the millions lost in human productivity could be gained if one could simply identify this infection early in the disease course treat as therapies are developed (both immune modulating and anti-retroviral) and of course ..best of all prevention strategies...vaccines...

My point overall is that there is no doubt that this is the third human exogenous retrovirus (family) identified...like HTLV and HIV it is firmly associated with a neurological disease and cancer...so why is our NIAID not funding XMRV research...not talking about CFS research talking about XMRV research...because from my point of view, if you are infected with a human retrovirus, it will not likely be benign..."

Here we are, eight months after the publication of the Science paper, with four published negative studies behind us but still no replication studies, I asked Dr. Mikovits if she knew of any true replications studies that were underway and the news was good.

"I know of at least two true replication studies that are being done in very well characterized CFS cohorts."