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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
61/321,147	04/06/2010		110	40000377-0001Var		

CONFIRMATION NO. 7100

FILING RECEIPT

26263
SONNENSCHEIN NATH & ROSENTHAL LLP
P.O. BOX 061080
WACKER DRIVE STATION, WILLIS TOWER
CHICAGO, IL 60606-1080

Date Mailed: 04/23/2010

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Applicant(s)

Judy A. Mikovits, Reno, NV;
Francis W. Ruscetti, New Market, MD;

Power of Attorney:
Saul Zackson--52391

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The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 61/321,147**
Projected Publication Date: None, application is not eligible for pre-grant publication
Non-Publication Request: No
Early Publication Request: No
** **SMALL ENTITY** **
Title

Diagnostic Identification of Variants of Xenotropic Murine Leukemia Virus-Related Virus

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PTO/SB/16 (12-08)
Approved for use through 06/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PROVISIONAL APPLICATION FOR PATENT COVER SHEET – Page 1 of 2

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. _____

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Judy A	Mikovits	Reno, NV
Francis W.	Ruscetti	New Market, MD

Additional inventors are being named on the _____ *separately numbered sheets attached hereto.*

TITLE OF THE INVENTION (500 characters max):

Diagnostic Identification of Variants of Xenotropic Murine Leukemia Virus-Related Virus

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☐ Application Data Sheet. See 37 CFR 1.76

☐ CD(s), Number of CDs _____

☒ Drawing(s) *Number of Sheets* 19

☐ Other (specify) _____

☒ Specification (e.g. description of the invention) *Number of Pages* 20

Fees Due: Filing Fee of $220 ($110 for small entity). If the specification and drawings exceed 100 sheets of paper, an application size fee is also due, which is $270 ($135 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

METHOD OF PAYMENT OF THE FILING FEE AND APPLICATION SIZE FEE FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☒ Yes, the name of the U.S. Government agency and the Government contract number are: NCI/NIH CA104943, USPHS HHSN26120080001E, DoD Prostate Cancer Program W81XWH-07-1338

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SIGNATURE /Saul L. Zackson/ Date 4/6/2010

TYPED or PRINTED NAME Saul L. Zackson REGISTRATION NO. 52,391 *(if appropriate)*

TELEPHONE (314) 259-5817 Docket Number: 40000377-0001Var

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Diagnostic Identification of Variants of Xenotropic Murine Leukemia Virus-Related Virus

Judy Mikovits

Francis Ruscetti

Summary

The present inventors disclose the following aspects herein:

1. A method of diagnosing an XMRV-related disease other than prostate cancer, comprising:

a) providing a sample from a subject comprising, or suspected of comprising, at least one XMRV subgroup;

b) determining at least one sequence of at least one XMRV nucleic acid, if any, comprised by the sample,

wherein the at least one sequence can be diagnostic for an XMRV-related disease other than prostate cancer if the at least one sequence i) differs in sequence identity by at least 2% with a homologous sequence of reference sequence VP62 and ii) has at least 90% sequence identity with the homologous sequence.

2. A method in accordance with Aspect 1, wherein the at least one sequence can comprise at least 50 contiguous nucleotides of an XMRV nucleic acid sequence.

3. A method in accordance with Aspect 1, wherein the at least one sequence can comprise at least 60 contiguous nucleotides of an XMRV nucleic acid sequence.

4. A method in accordance with Aspect 1, wherein the at least one sequence can comprise at least 70 contiguous nucleotides of an XMRV nucleic acid sequence.

5. A method in accordance with Aspect 1, wherein the at least one sequence can comprise at least 80 contiguous nucleotides of an XMRV nucleic acid sequence.

6. A method in accordance with Aspect 1, wherein the at least one sequence can comprise at least 90 contiguous nucleotides of an XMRV nucleic acid sequence.

7. A method in accordance with Aspect 1, wherein the at least one sequence can comprise at least 100 contiguous nucleotides of an XMRV nucleic acid sequence.

8. A method in accordance with Aspect 1, wherein the at least one sequence can comprise at least 1 mutation compared to VP62.

9. A method in accordance with Aspect 1, wherein the at least one sequence can comprise at least 1 SNP compared to VP62.

10. A method in accordance with Aspect 1, wherein the at least one sequence can comprise at least 2 SNPs compared to VP62.

11. A method in accordance with any one of Aspects 8, 9. and 10, wherein a mutation or SNP can be a mutation or SNP set forth in table 3.

12. A method in accordance with Aspect 1, wherein the sample comprises a body fluid.

13. A method in accordance with Aspect 12, wherein the body fluid can be selected from the group consisting of blood, plasma, serum, cerebrospinal fluid, saliva, urine, and a combination thereof.

14. A method in accordance with Aspect 12, wherein the body fluid can be selected from the group consisting of blood, plasma, serum, cerebrospinal fluid and a combination thereof.

15. A method in accordance with Aspect 12, wherein the body fluid can be selected from the group consisting of blood, plasma, serum and a combination thereof.

16. A method in accordance with Aspect 12, wherein the body fluid can be peripheral blood.

17. A method in accordance with Aspect 12, wherein the sample can be selected from the group consisting of a blood sample, a serum sample, a plasma sample, and a cerebrospinal fluid sample.

18. A method in accordance with Aspect 12, wherein the sample comprises peripheral blood.

19. A method in accordance with Aspect 1, wherein the determining at least one sequence can comprise a polymerase chain reaction amplification.

20. A method in accordance with Aspect 19, wherein the polymerase chain reaction amplification can comprise a nested RT-PCR.

21. A method in accordance with Aspect 1, wherein the determining at least one sequence can comprise pyrosequencing.

22. A method in accordance with Aspect 1, wherein the determining at least one sequence can comprise a hybridization assay.

23. A method in accordance with Aspect 1, wherein the at least one XMRV nucleic acid can comprise at least 10 contiguous nucleotides encoding an amino acid sequence of an XMRV gag protein.

24. A method in accordance with Aspect 23, wherein the at least 10 contiguous nucleotides encoding an amino acid sequence of an XMRV gag protein can comprise at least 10 contiguous nucleotides encoding an amino acid sequence of the core protein matrix (MA).

25. A method in accordance with Aspect 1, further comprising assigning the at least one XMRV to an XMRV subgroup, wherein the XMRV subgroup can be selected from the group consisting of XMRV subgroup A, XMRV subgroup B and XMRV subgroup C.

26. A method in accordance with Aspect 25, wherein if the sample comprises at least one of XMRV subgroup B and XMRV subgroup C, the subject can be diagnosed with an XMRV-related neuroimmune disease or an XMRV-related cancer other than prostate cancer.

27. A method in accordance with Aspect 26, wherein the XMRV-related neuroimmune disease can be selected from the group consisting of fibromyalgia, Chronic Fatigue Syndrome (CFS), Multiple Sclerosis (MS), Parkinson's Disease, Amyotrophic Lateral Sclerosis (ALS) and autism.

28. A method in accordance with Aspect 27, wherein the Multiple Sclerosis can be Atypical Multiple Sclerosis.

29. A method in accordance with Aspect 26, wherein the XMRV-related cancer can be an XMRV-related lymphoma.

30. A method in accordance with Aspect 29, wherein the XMRV-related lymphoma can be selected from the group consisting of an XMRV-related Mantle Cell Lymphoma (MCL) and an XMRV-related Chronic Lymphocytic Leukemia lymphoma (CLL).

In various aspects of the present teachings, a sequence of an XMRV detected in a sample from a subject can be compared to XMRV strain VP62, comprising a nucleic acid of the following sequence:

```
1 gcgccagtca tccgatagac tgagtcgccc gggtacccgt gttcccaata aagccttttg
 61 ctgtttgcat ccgaagcgtg gcctcgctgt tccttgggag ggtctcctca gagtgattga
121 ctacccagct cgggggtctt tcatttgggg gctcgtccgg gattcggaga cccccgccca
181 gggaccaccg acccaccgtc gggaggtaag ccggccggcg atcgttttgt ctttgtctct
241 gtctttgtgc gtgtgtgtgt gtgccggcat ctaatcctcg cgcctgcgtc tgaatctgta
301 ctagttagct aactagatct gtatctggcg gttccgcgga agaactgacg agttcgtatt
361 cccggccgca gccctgggag acgtcccagc ggcctcgggg gcccgttttg tggcccattc
421 tgtatcagtt aacctacccg agtcggactt tttggagtgg ctttgttggg ggacgagaga
481 cagagacact tcccgccccc gtctgaattt ttgctttcgg ttttacgccg aaaccgcgcc
541 gcgcgtctga tttgttttgt tgttcttctg ttcttcgtta gttttcttct gtctttaagt
601 gttctcgaga tcatgggaca gaccgtaact acccctctga gtctaacctt gcagcactgg
661 ggagatgtcc agcgcattgc atccaaccag tctgtggatg tcaagaagag gcgctgggtt
721 accttctgtt ccgccgaatg gccaactttc aatgtaggat ggcctcagga tggtactttt
781 aatttaggtg ttatctctca ggtcaagtct agagtgtttt gtcctggtcc ccacggacac
841 ccggatcagg tcccatatat cgtcacctgg gaggcacttg cctatgaccc ccctccgtgg
901 gtcaaaccgt ttgtctctcc taaacccccc cctttaccga cagctcccgt cctcccgccc
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1141 gaagaagagg cggccaccac ctccgaggtt tccccccctt ctcccatggt gtctcgactg
1201 cggggaagga gagaccctcc cgcagcggac tccaccacct cccaggcatt cccactccgc
1261 atgggggggag atggccagct tcagtactgg ccgttttcct cctctgattt atataattgg
1321 aaaaataata acccttcctt ttctgaagat ccaggtaaat tgacggcctt gattgagtcc
1381 gtcctcatca cccaccagcc cacctgggac gactgtcagc agttgttggg gaccctgctg
1441 accggagaag aaaagcagcg ggtgctccta gaggctagaa aggcagtccg gggcaatgat
1501 ggacgcccca ctcagttgcc taatgaagtc aatgctgctt ttccccttga gcgccccgat
1561 tgggattaca ccactacaga aggtaggaac cacctagtcc tctaccgcca gttgctctta
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5281 actttgacca aattaacgct tgcatctggc actagagact gggtactcct actccccttа
5341 gcccctctacc gagcccggaa tactccgggc ccccacggac tgactccgta tgaaattctg
5401 tatggggcac ccccgccccct tgtcaatttt catgatcctg aaatgtcaaa gttaactaat
5461 agtccctctc tccaagctca cttacaggcc ctccaagcag tacaacaaga ggtctggaag
5521 ccgctggccg ctgcttatca ggaccagcta gatcagccag tgataccaca cccttccgt
5581 gtcggtgacg ccgtgtgggt acgccggcac cagactaaga acttagaacc tcgctggaaa
5641 ggaccctaca ccgtcctgct gacaacccccc accgctctca aagtagacgg catctctgcg

5701 tggatacacg ccgctcacgt aaaggcggcg acaactcctc cggccggaac agcatggaaa
5761 gtccagcgtt ctcaaaaccc cttaaagata agattaaccc gtggggcccc ctgataatta
5821 tggggatctt ggtgagggca ggagcctcag tacaacgtga cagccctcac caggtcttta
5881 atgtcacttg gaaaattacc aacctaatga caggacaaac agctaatgct acctccctcc
5941 tggggacgat gacagacact ttccctaaac tatattttga cttgtgtgat ttagttggag
6001 acaactggga tgacccggaa cccgatattg gagatggttg ccgctctccc gggggaagaa
6061 aaaggacaag actatatgat ttctatgttt gccccggtca tactgtatta acagggtgtg
6121 gagggccgag agagggctac tgtggcaaat ggggatgtga gaccactgga caggcatact
6181 ggaagccatc atcatcatgg gacctaattt cccttaagcg aggaaacact cctaagggtc
6241 agggcccctg ttttgattcc tcagtgggct ccggtagcat ccagggtgcc acaccggggg
6301 gtcgatgcaa ccccctagtc ctagaattca ctgacgcggg taaaagggcc agctgggatg
6361 ccccccaaaac atggggacta agactgtatc gatccactgg ggccgacccg gtgaccctgt
6421 tctctctgac ccgccaggtc ctcaatgtag ggccccgcgt ccccattggg cctaatcccg
6481 tgatcactga acagctaccc cccticccaac ccgtgcagat catgctcccc aggactcctc
6541 gtcctcctcc ttcaggcgcg gcctctatgg tgcctggggc tccccccgcct tctcaacaac
6601 ctgggacggg agacaggctg ctaaacctgg tagaaggagc ctacctagcc ctcaacctca
6661 ccagtcccga caaaacccaa gagtgctggc tgtgtctagt atcgggaccc ccctactacg
6721 aaggggtggc cgtcctaggt acttactcca accataccic tgccccggct aactgctccg
6781 tgacctccca acacaagctg accctgtccg aagtgaccgg gcagggactc tgcataggag
6841 cagttcccaa aacccatcag gccctgtgta ataccaccca gaagacgagc gacgggtcct
6901 actatttggc ctctcccgcc gggaccattt gggcttgcag caccgggctc actccctgtc
6961 tatctactac tgtgcttaac ttaaccactg attactgtgt cctggttgaa ctctggccaa
7021 aggtaaccta ccactcccct aattatgttt atggccagtt tgaaaagaaa actaaatata
7081 aaagagagcc ggtgtcatta actctggccc tgctgttggg aggacttact atgggcggca
7141 tagctgcagg agttggaaca gggactacag ccctagtggc caccaaacaa ttcgagcagc
7201 tccaggcagc catacataca gaccttgggg ccttagaaaa atcagtcagt gccctagaaa
7261 agtctctgac ctcgttgtct gaggtggtcc tacagaaccg gaggggatta gatctactgt
7321 tcctaaaaga aggaggatta tgtgctgccc taaaagaaga atgctgtttt tacgcggacc
7381 acactggcgt agtaagagat agcatggcaa agctaagaga aaggttaaac cagagacaaa
7441 aattgttcga atcaggacaa gggtggtttg agggactgtt taacaggtcc ccatggttca
7501 cgaccctgat atccaccatt atgggccctc tgatagtact tttattaatc ctactcttcg
7561 gaccctgtat tctcaaccgc ttggtccagt ttgtaaaaga cagaatttcg gtagtgcagg
7621 ccctggttct gacccaacag tatcaccaac tcaaatcaat agatccagaa gaagtggaat
7681 cacgtgaata aaagatttta ttcagtttcc agaaagaggg gggaatgaaa gaccccacca
7741 taaggcttag cacgctagct acagtaacgc cattttgcaa ggcatggaaa agtaccagag
7801 ctgagttctc aaaagttaca aggaagttta attaaagaat aaggctgaat aacactggga
7861 cagggggcaa acaggatatc tgtagtcagg cacctgggcc ccggctcagg gccaagaaca
7921 gatggtcctc agataaagcg aaactaacaa cagtttctgg aaagtcccac ctcagtttca
7981 agttccccaa aagaccggga aatacccccaa gccttattta aactaaccaa tcagctcgct

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8041 tctcgcttct gtacccgcgc ttttgctcc ccagtcctag ccctataaaa aaggggtaag
8101 aactccacac tcggcgcgcc agtcatccga tagactgagt cgcccgggta cccgtgttcc
8161 caataaagcc ttttgctgtt tgcaa
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In various embodiments, a sequence of an XMRV comprised by a sample can be compared to the sequence of XMRV VP62. In various configurations, homologous sequences of the XMRV sample and XMRV VP62 can be identified using methods well known to skilled artisans. In some configurations, a comparison of homologous sequences can reveal if the sequences are identical or contain one or more differences. Using the sequence of XMRV VP62 as a standard for comparison, in various configurations, a difference between a sequence of a sample XMRV and that of XMRV VP62 can be an insertion of one or more nucleotides, a deletion of one or more nucleotides, an inversion, or a substitution of one or more nucleotides such as a single nucleotide polymorphism (a "SNP").

In various embodiments, based on the differences between a sample XMRV sequence and the sequence of XMRV VP62, an XMRV comprised by a sample can be assigned to an XMRV subgroup, such as XMRV subgroup A, XMRV subgroup B, or XMRV subgroup C. In various configurations, a subject having one or both of XMRV subgroup B and XMRV subgroup C can be considered to be at risk or can be diagnosed with a neuroimmune disease and/or a cancer other than prostate cancer. In some configurations, a neuroimmune disease can be selected from the group consisting of fibromyalgia, Chronic Fatigue Syndrome (CFS, or Myalgic Encephalomyelitis, or ME/CFS), Multiple Sclerosis (MS), Parkinson's Disease, Amyotrophic Lateral Sclerosis (ALS) and autism. In some aspects, the Multiple Sclerosis can be Atypical Multiple Sclerosis.

In some aspects, an XMRV-related cancer can be an XMRV-related lymphoma. In some configurations, an XMRV-related lymphoma can be selected from the group consisting of an XMRV-related Mantle Cell Lymphoma (MCL) and an XMRV-related Chronic Lymphocytic Leukemia lymphoma (CLL).

In some embodiments, the identification of XMRV subgroups infecting a subject can influence choice of treatment. For example, a person infected with subgroups B and C, or subgroups A, B, and C, can require more aggressive treatment compared to a subject infected with only subgroup B or subgroup C. In some configurations, a subject infected with only XMRV subgroup may not be a candidate for any treatment.

Detailed Description

The experiments utilize standard laboratory techniques and equipment, such as those set forth in Sambrook, J., et al., Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001; Spector, D. L. et al., Cells: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1998; and Ausubel, F. M., et al., ed., Current Protocols in Molecular Biology, or as described in a reference cited herein.

DNA and RNA isolation. Whole blood can be drawn from subjects by venipuncture using standardized phlebotomy procedures into 8-mL greencapped Vacutainers containing the anti-coagulant sodium heparin (Becton Dickinson). Plasma can be collected by centrifugation, aspirated and stored at -80 °C for later use. The plasma can be replaced with PBS and the blood resuspended and further diluted with an equal volume of PBS. PBMCs can be isolated by layering the diluted blood onto Ficoll-Paque PLUS (GE Healthcare), centrifuging for 22 min at 800 g, aspirating the PBMC layer and washing it once in PBS. The PBMCs (approximately 2 x 10^7 cells) can be centrifuged at 500 g for 7 min and either stored as frozen unactivated cells in 90% FBS and 10% DMSO at -80 °C for further culture and analysis or resuspended in TRIzol (Invitrogen) and stored at -80 °C for DNA and RNA extraction and analysis. DNA can be isolated from TRIzol according the to manufacturer's protocol and also can be isolated from frozen PBMC pellets using the QIAamp DNA Mini purification kit (QIAGEN) according to the manufacturer's protocol and the final DNA can be resuspended in RNase/DNase free water and quantified using the Quant-iTTM Pico Green dsDNA Kit (Invitrogen). RNA can be isolated from TRIzol according to the manufacturer's protocol and quantified using the Quant-iT Ribo Green RNA kit (Invitrogen). cDNA can be made from RNA using the iScript Select cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol.

[0037] PCR. Nested PCR can be performed with separate reagents in a separate laboratory room designated to be free of high copy amplicon or plasmid DNA. Negative controls in the absence of added DNA can be included in every experiment. Identification of XMRV gag and env genes can be performed by PCR in separate reactions. Reactions can be performed as follows: 100 to 250 ng DNA, 2 μL of 25 mM MgCl2, 25 μL of HotStart-IT FideliTaq Master Mix (USB Corporation), 0.75 μL of each of 20 μM forward and reverse oligonucleotide primers in reaction volumes of 50 μL. For identification of gag, 419F (5'-

ATCAGTTAACCTACCCGAGTCGGAC-3') (SEQ ID NO: 5) and 1154R (5'-GCCGCCTCTTCTTCATTGTTCTC-3') (SEQ ID NO: 6) can be used as forward and reverse primers. For env, 5922F (5'- GCTAATGCTACCTCCCTCCTGG-3') (SEQ ID NO: 7) and 6273R (5'-GGAGCCCACTGAGGAATCAAAACAGG-3') (SEQ ID NO: 8) can be used. For both gag and env PCR, 94 °C for 4 min initial denaturation can be performed for every reaction followed by 94 °C for 30 seconds, 57 °C for 30 seconds and 72 °C for 1 minute. The cycle can be repeated 45 times followed by final extension at 72 °C for 2 minutes. Six microliters of each reaction product can be loaded onto 2% agarose gels in TBE buffer with 1 kb+ DNA ladder (Invitrogen) as markers. PCR products can be purified using Wizard SV Gel and PCR Clean-Up kit (Promega) and sequenced. PCR amplification for sequencing full-length XMRV genomes can be performed on DNA amplified by nested or semi-nested PCR from overlapping regions from PBMC DNA. For 5' end amplification of R-U5 region, 4F (5'-CCAGTCATCCGATAGACTGAGTCGC-3') (SEQ ID NO: 9) and 1154R can be used for first round and 4F and 770R (5'-TACCATCCTGAGGCCATCCTACATTG-3') (SEQ ID NO: 10) can be used for second round. For regions including gag-pro and partial pol, 350F(5'-GAGTTCGTATTCCCGGCCGCAGC-3') (SEQ ID NO: 11) and 5135R (5'-CCTGCGGCATTCCAAATCTCG-3') (SEQ ID NO: 12) can be used for first round followed by second round with 419F and 4789R (5'-GGGTGAGTCTGTGTAGGGAGTCTAA-3'). (SEQ ID NO: 13) For regions including partial pol and env region, 4166F (5'-CAAGAAGGACAACGGAGAGCTGGAG-3') (SEQ ID NO: 14) and 7622R (5'-GGCCTGCACTACCGAAAT TCTGTC-3') (SEQ ID NO: 15) can be used for first round followed by 4672F (5'GAGCCACCTACAATCAGACAAAAGGAT-3') (SEQ ID NO: 16) and 7590R (5'- CTGGACCAAGCGGTTGAGAATACAG-3') (SEQ ID NO: 17) for second round. For the 3' end including the U3-R region, 7472F (5'-TCAGGACAAGGGTGGTTTGAG-3') (SEQ ID NO: 18) and 8182R (5'-CAAACAGCAAAAGGCTTTATTGG-3') (SEQ ID NO: 19) can be used for first round followed by 7472F and 8147R (5'-CCGGGCGACTCAGTCTATC-3') (SEQ ID NO: 20) for second round. The reaction mixtures and conditions can be as described above except for the following: For larger fragments, extension can be done at 68 °C for 10 min instead of 72 °C. All second round PCR products can be column purified as mentioned above and overlapping sequences can be determined with internal primers. Nested RT-PCR for gag sequences can be done as described (5) with modifications. GAG-O-R primer can be used for 1st

strand synthesis; cycle conditions can be 52 °C annealing, for 35 cycles. For second round PCR, annealing can be at 54 °C for 35 cycles.

[0038] Phylogenetic Analysis Sequences can be aligned using ClustalX Clustal alignments can be imported into MEGA4 to generate neighbor-joining trees using the Kimura 2-parameter plus Γ distribution (K80+Γ) distance model. Free parameters can be reduced to the K80 model, and α values can be estimated from the data set using a maximum likelihood approach in PAUP*4.0 (Sinauer Associates, Inc. Publishers, Sunderland, MA, USA). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Accession numbers from GenBank (http://www.ncbi.nlm.nih.gov/Genbank): FLV (NC_001940), MoMLV (NC_001501), XMRV VP35 (DQ241301), XMRV VP42 (DQ241302) XMRV VP62 (EF185282). Genomic Nonecotropic MLV Provirus Sequences can be downloaded from PLOS Genetics 3(10): e183 .

We previously 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 two studies from England 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 by passing 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 (Lombardi, V.C., et al., Science 326: 285, 2009) we established XMRV infection in the blood products of our patient population by four 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 Fig 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 nested PCR, which inevitably raises questions of contamination, is not essential to detect XMRV in highly viremic ME/CFS patients. Of the other 90 samples described in the paper, very few exhibited XMRV-specific PCR products following single round DNA PCR. Only 11% of the 101 DNA samples from PBMCs exhibited products by nested PCR. In contrast, when cDNA was prepared from PBMCs, sixty of the 90 other 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 1 A of the Science paper, patient 1118 was negative by single round PCR on DNA from unstimulated PBMCs, but positive in other assays (Science Fig 2A, 2D, 4A, 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 indicator cell lines, as XMRV gag and env PCR products were detected in the cell lines 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 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 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 11% of our 101 patients' PBMCs exhibit products upon DNA PCR, 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 reports of negative PCR tests for XMRV in two UK patient cohorts has raised questions whether our findings could be due to contamination of our PCR experiments by mouse genomic DNA, which contain gag and env sequences highly similar to XMRV. We point out that 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 samples were obtained from patients residing in the U.S., Canada, and Europe coming to be treated at the well-known Sierra Internal Medicine practice, located in Incline Village, Nevada. Once collected, 45 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 45 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 were negative for mouse contamination.

In our experience, the most sensitive blood-based assays for detection of XMRV in decreasing order are: 1) performing nested PCR for gag sequences from LNCaP cells that have been co-cultured with subject's plasma or activated PBMCs, 2) detection of viral proteins expressed by

activated PBMCs with appropriate antisera, 3) the presence of antibodies to XMRV Env in subject's plasma, 4) nested RT-PCR of plasma nucleic acid or PCR from cDNA from unactivated PBMCs and nested 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 (4). As in the case with HTLV, the lack of diversity implies that XMRV recently descended from a common ancestor (5). The high degree of similarity to xenotropic murine leukemia virus suggests that a cross-species transmission event was likely involved in evolution of XMRV (6). 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.

References cited herein are incorporated by reference, each in its entirety.

Table 1 XMRV detection using cDNA from 20 unstimulated PBMCs

GAG Gene				ENV Gene			
Lane	1st	2nd		Lane	1st	2nd	
1				1			
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 14 patients PCR negative

GAG Gene				ENV Gene				
Lane	1st	2nd		Lane	1st	2nd		Type
6	-	-	1169	26	+	+	1169	(RNA)
7	+	+	1221	27	+	+	1221	(RNA)
8	-	+	1150	28	+	+	1150	(RNA)
9	-	+	1199	29	-	+	1199	(RNA)
10	+	+	1220	30	+	+	1220	(RNA)
11	-	-	LNCaP	31	-	-	LNCaP	(RNA)
12	-	-	1186	32	-	-	1186	(RNA)
13	-	+	1150	33	-	-	1150	(DNA)
14	-	+	1132	34	-	-	1132	(DNA)
15	-	+	1111	35	-	-	1111	(DNA)
16	-	+	1186	36	-	-	1186	(DNA)
17	-	+	1189	37	+	+	1189	(DNA)
18	-	+	1172	38	+	+	1172	(DNA)
19	-	+	1173	39	-	+	1173	(DNA)
20	-	+	1103	40	+	+	1103	(DNA)

Sequence variations of the gag gene of XMRV form three distinct subgroups

To investigate the diversity of XMRV, peripheral blood mononuclear cells (PBMCs) from XMRV-infected individuals were isolated, and the sequence of the region of gag that encodes the core protein matrix (MA) was determined using nested RT-PCR. (Figure 1A). This protein is the most diverse of the gag proteins of gammaretroviruses: sequence analysis of several different murine, feline, and primate gammaretroviruses have revealed low sequence and residue identity (Supplemental Figure 1). In contrast, the MA sequences of XMRV available on GenBank show significant conservation, differing by 0-2 of 387 (<1%) nucleotides.

To further investigate the genetic diversity of additional XMRV isolates, RNA was isolated directly from the PBMCs of XMRV-infected individuals and regions encoding the MA protein were amplified by nested RT-PCR. Comparison of the sequences amplified from 17 individuals revealed a significant amount of variation in this region (Figure 1B). All sequences analyzed directly from XMRV-infected subjects differed by at least one nucleotide from the XMRV reference strain VP62 (Fig. 1B, line 1). Overall, 15/327 residues had nucleotide substitutions relative to VP62, and all but two of these changes were observed in two or more of the isolates examined. In addition, 7 of the 17 samples had a 21 bp deletion from nt 127- 147.

Analysis of the MA sequences revealed the variants could be classified into three subgroups (Figure 1B). These subgroup delineations are supported by unrooted neighbor-joining analysis of the MA nucleotide sequence fragment (Figure 1C). Seven of the sequences, which fell into subgroup A, are closely related to the previously published sequences of XMRV in this region (Fig. 1B, lines 2-8, compare with line 1). At most, this group differed by 3 nucleotides from the reference strain VP62; one resulted in a synonymous change (i.e., the same residue was encoded). and two were non-synonymous (Table 1). The non-synonymous substitution, nt 178: G→A, is present in all of the sequences in subgroup A (G178A), and has also been previously reported to be present in other XMRV sequences (Table 1).

Eight of the seventeen (8/17) sequences analyzed fell into a second group (subgroup B), all of which had a 21 bp deletion, resulting in an in-frame deletion of seven amino acid residues. All sequences in subgroup B also had seven specific nucleotide substitutions relative to the sequence of the XMRV reference strain (nt 75: C→T, nt 85: G→A, nt 91: A→G, nt 92: A→G, nt 304: C→G, nt 315: C→T, and nt 316: C→T) (Fig. 1B lines 9-17), of which were four were synonymous and three were non-synonymous changes (Table 1).

Subgroup C contained two sequences and was characterized by three unique nucleotide

substitutions (nt 106: G→A, nt 175: G→A, and nt 192: C→T) (Fig. 1B, lines 17 and 18 and Table 1), of which two were synonymous and one was a non-synonymous change. This group also had three nucleotide substitutions relative to VP62 that were in common with members of groups A and B (nt 92: A→G, nt 178: G→A, nt 325: A→G).

To gain insight into whether the variation observed in the XMRV sequences could be tolerated by the MA protein and persist in nature, we examined the MA protein sequences of gammaretroviruses from other mus muculus and other species. Alignment of MA proteins of other members gammaretrovirus genus revealed that 5 of the 6 amino acid changes in the XMRV variants are present in other infectious gammaretroviruses (Table 2 and Fig. S1).

Analysis of XMRV MA sequences in lymphocytes following ex vivo culture

XMRV RNA could not always be detected in the PBMCs of subjects from which infectious virus had been isolated from plasma. This suggests that the virus is expressed at very low frequency in PBMCs isolated directly from infected individuals. We have observed that culturing these PBMCs under conditions that induce activation of T cells (Lombardi) increases the frequency of XMRV detected by RT-PCR in the cells maintained in culture. This increase appears to be dependent on the spread of the virus, since the addition of a reverse transcriptase inhibitor to the cultures prior to activation prevents the increase XMRV expression, as measured by cell surface expression of Env (Fig. S2). To biologically increase the level of XMRV and increase the probability that XMRV sequences could be detected by PCR, PBMCs were cultured under conditions that activated T cells for 7-10 days, the RNA isolated, and nested RT-PCR analysis performed as described above.

All the MA sequences amplified following ex vivo culture could be classified into two out of the three subgroups observed in the analysis of RNA from unactivated PBMCs. Sequences for 4/11 individuals were similar to the previously published sequences (subgroup A) (Figure 2A, lines 2-5). Sequences amplified from another 6 individuals fell into subgroup C. (Figure 2A, lines 6-12). Unrooted neighbor-joining analysis of nucleotide sequences direct from subject PBMCs and after ex vivo culture reflected the variability noted in the sequence analysis and confirmed that post-culture, only variants A and C can be detected (Figure 2B).

Evidence for multiple variants in XMRV-infected individuals.

None of the sequences amplified following ex vivo culture were similar to the sequences

of subgroup B. One explanation for this would be that the PBMCs contained multiple strains of XMRV and, because of differences in replication capacity and/or tropism, the major variant present following spread in the cultures differed from the major variant present in unstimulated PBMCs of infected individuals. Reexamination of direct sequencing data obtained from unactivated PBMCs suggested that several of the sequence chromatograms *which did not give a clear sequence* might reflect the presence of more than one virus. Analysis of samples from three individuals indicated that they were infected with both subgroup B and C viruses (Fig. 2D). For all three of these individuals, the sequences detected following ex vivo activation and culture of T cells all subgroup C consistent with the hypothesis that the subgroup B variants have a decreased replicative capacity (Figure 2A, lines 6 and 10, and supplemental 3).

Identification of endogenous polytropic MLVs highly homologous to MA of subgroups B and C

Previous analyses of the major coding regions of XMRV with ecotropic and non-ecotropic MLV sequences indicated that, while the pol and env sequences of XMRV cluster with xenotropic viruses, the gag region of XMRV is more similar to the to polytropic and modified polytropics viruses than xenotropic viruses. In the current study, comparison of XMRV MA subgroup A sequences with sequences in Genbank indicates that, similar to the previously published XMRV sequences, subgroup A is most closely related to endogenous MLVs (i.e., AY349138.1 and S80082.1) and differ in 5 of the 327 nucleotides.

In contrast, analysis of XMRV MA sequences from subgroup B with sequences in Genbank revealed a 100% identity with a recently characterized murine endogenous polytropic retrovirus, clone 51 (Evans JV 2009) (Figure 3A). Although, clone 51 contains several deletions and is not infectious, it is expressed in certain strains of mice. Clone 51 genomes in mice infected with an infectious ecotropic MLV (Fr-MLV) can be packaged into the Fr-MLV virions and transferred to other cells.

Subgroup C sequences are also most highly related to the MA of a polytropic murine leukemia virus sequence. This virus, the Rmcf provirus, is an expressed endogenous MLV with large deletions in gag and pol (Christine). One variant in subgroup C (1281) was identical to the on a nucleotide level; the others in this group differed by 1-6 nucleotides (Fig. 3B). However, the protein sequence of the different members of subgroup C differed at most by 1 residue from the Rmcf provirus.

Thus, XMRV subgroup B and C appear to be more closely related to known endogenous MLV sequences than the XMRV subgroup A viruses.

References:

1. Fukuda K, Straus S, Hickie I, et al. 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, et al. Myalgic encephalomyelitis/chronic fatigue syndrome: Clinical working case definition. diagnostic and treatment protocols. J Chronic Fatigue Syndrome 2003; 11:1-12.

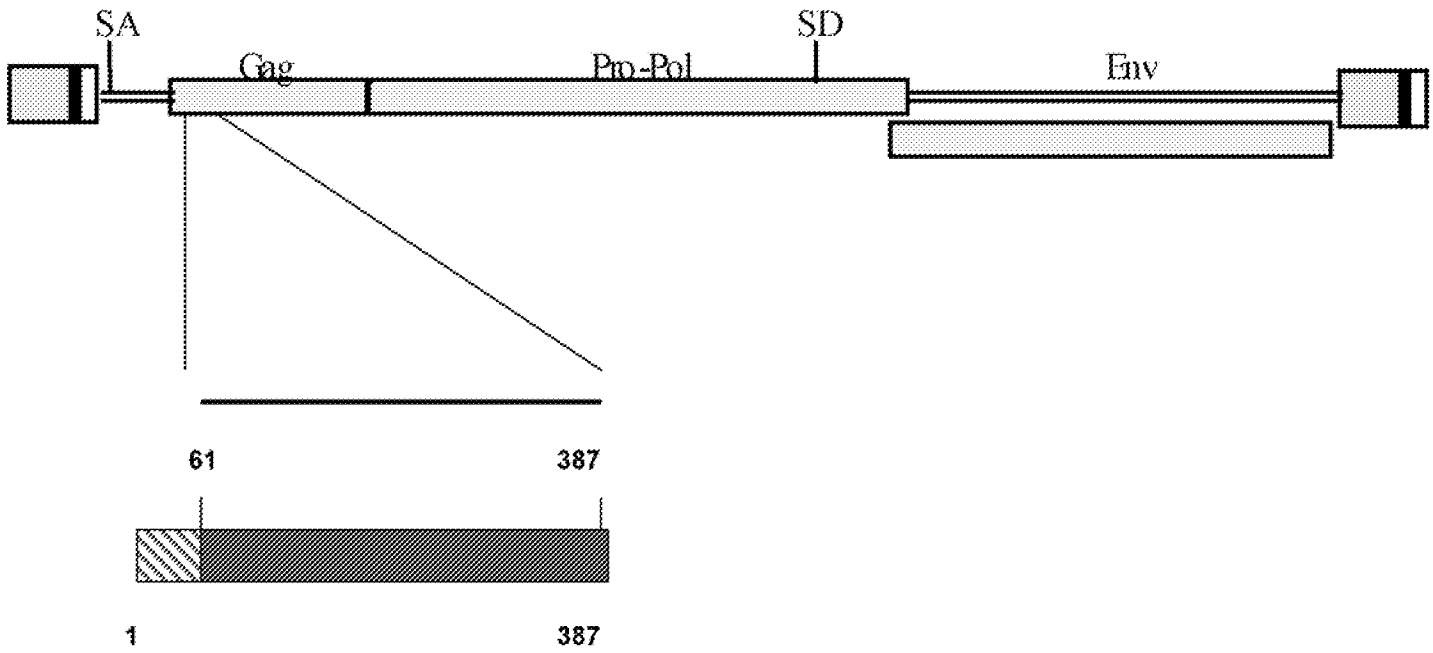
3. Jason L, Torres-Harding S, Jurgens A, Helgerson J. Comparing the Fukuda et al. criteria and the Canadian definition for chronic fatigue syndrome. J Chronic Fatigue S 2004; 12:37-52

4. Ratner L, Philpott T.and DB Towbridge. Nucleotide sequence analysis of isolates of Human T - lymphotropic virus type 1 of diverse georgraphical regions. AIDS Rs Hum Retroviruses. 1991 Nov: 7(11): 923-41

5. Verdonck K, Gonzalez E, Van Dooren S, Vandamme AM, Vanham G and Gotuzzo E (2007) Human Tlymphotropic virus 1: recent knowledge about an ancient infection. Lancet Infect Dis 7(4):266-281.

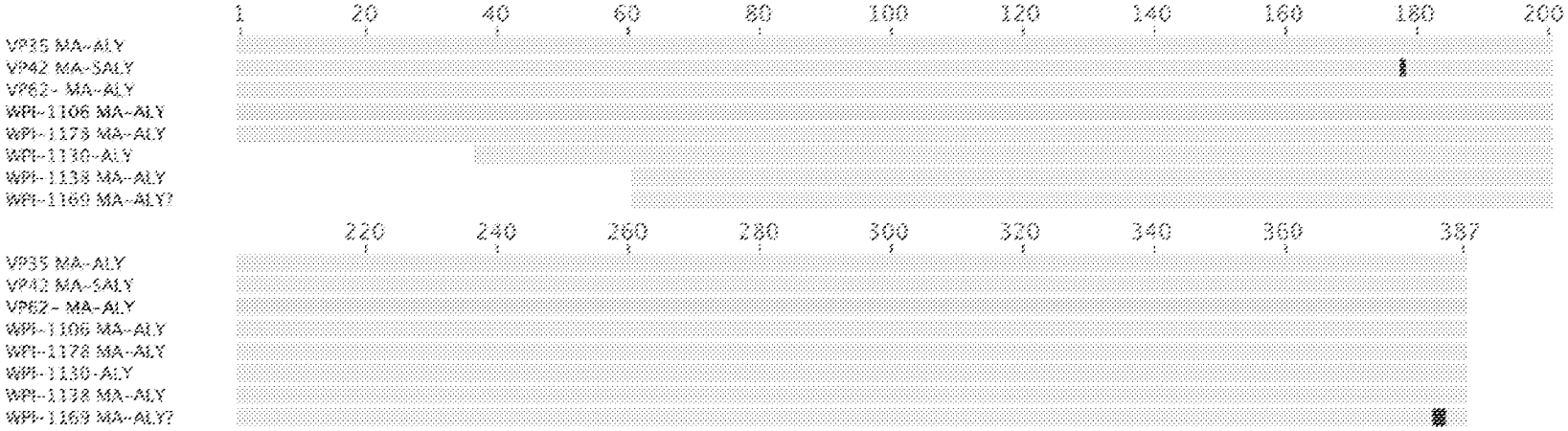
6. Yan Y, Liu Q and Kozak CA (2009) Six host range variants of the xenotropic/polytropic gammaretroviruses define determinants for entry in the XPR1 cell surface receptor. Retrovirology 6:87.

Figure 1A. Schematic diagram of the XMRV genome and the gag region examined in this study



Supplement A

Figure 1B. Variation in MA in published XMRV sequences



Note that EK2, which was isolated from an infected cell line, has a deletion.

Fig. 1C. Alignment of gag sequences from individuals with XMRV

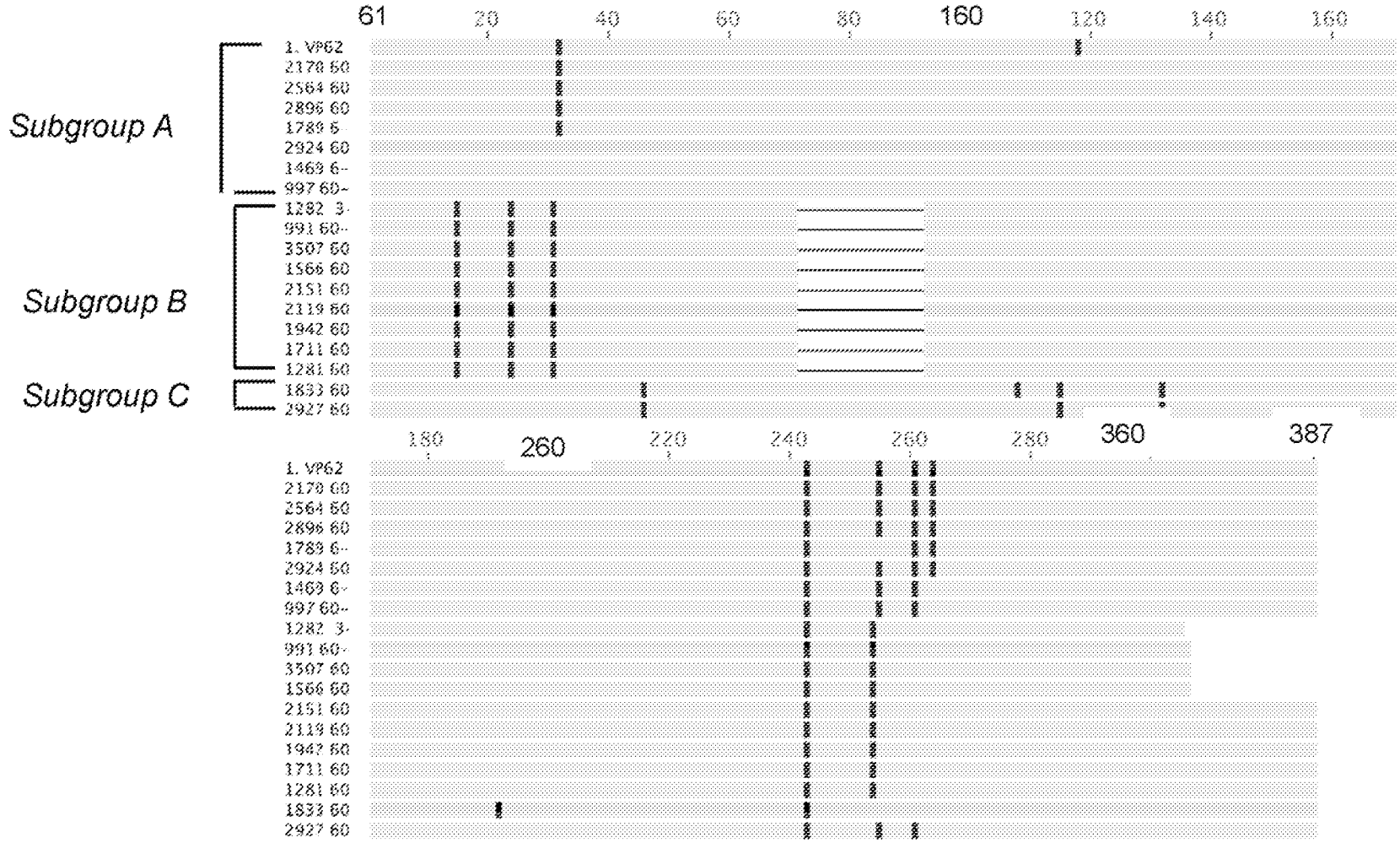


Table 1. Changes seen (reference is VP62)

Location	Substitution(s)	Groups with change	aa change
C75	T	B	None
G85	A	B	None
A91	G	B	Lys (31)-Gly
A92	G	A,B,C	Lys (31)-Arg
G106	A	C	Val (36)- Ile
A132-152	21 bp deletion*	B	7 aa deletion*
T168	C	C	None
G175	A	C	Gly (59)-Ser
G178#	A	A,B,C	Val (60)-Ile
C192	T	C	None
C242	T	C	None
C303	T	C	None
C303	G	B	None
C314	T	B	Pro (105)-Leu
C315	T	B,C	None
T321	C	B,C	None
A324	G	A,B,C	None

* because of repeat (starts and ends TGGCCT), this could be moved up 5 bp from where Clustal put it.. AA WPTFNVGWP…

place where VP42, EK1 and EK2 have same substitutions relative to the other published

Table 2. Amino acid substitutions of XMRV MA found in other gammaretroviruses

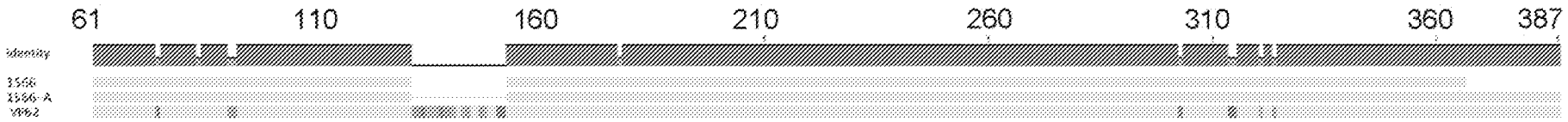
aa change	aa identical to substitution
Lys (31)-Arg/Gly	FeLV, Fr-MLV, KoRV (Arg); none (Gly)
Val (36)- Ile	FeLV
Gly (59)-Ser	GaLV, KoRV,
Val (60)-Ile	AKV-MLV, Ampho-MLV,Cas-BrE, Fr-MLV, Mo-MLV,X-MLV
Pro (105)-Leu	AKV-MLV, X-MLV

Accession numbers: AKV MLV (MLOCG), Amphotropic MLV (AF411814), Cas-BrE (X57540), FeLV (AF052723) Friend MLV (Fr-MLV) (NC 001362), GaLV (NC 001885), KoRV (QT9TTC2), Moloney-MLV (NC 001501), and xenotropic MLV (X-MLV)(EU035300).

*

Supplemental Figure 2:

A.



B.

C.

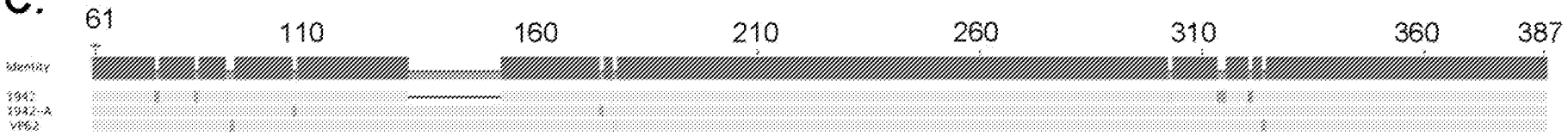


Figure 1A.

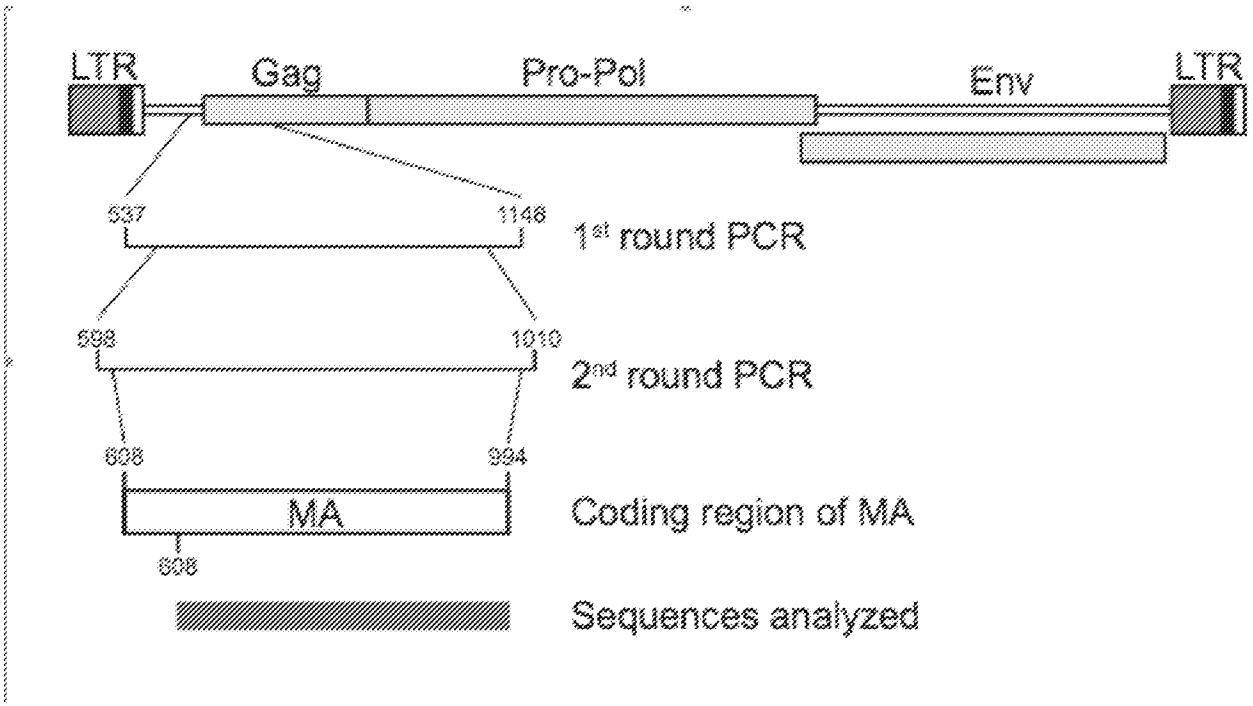

LTR
Gag
Pro-Pol
Env
LTR
537
1148
1st round PCR
588
1010
2nd round PCR
608
994
MA
Coding region of MA
608
Sequences analyzed

Fig. 1B.

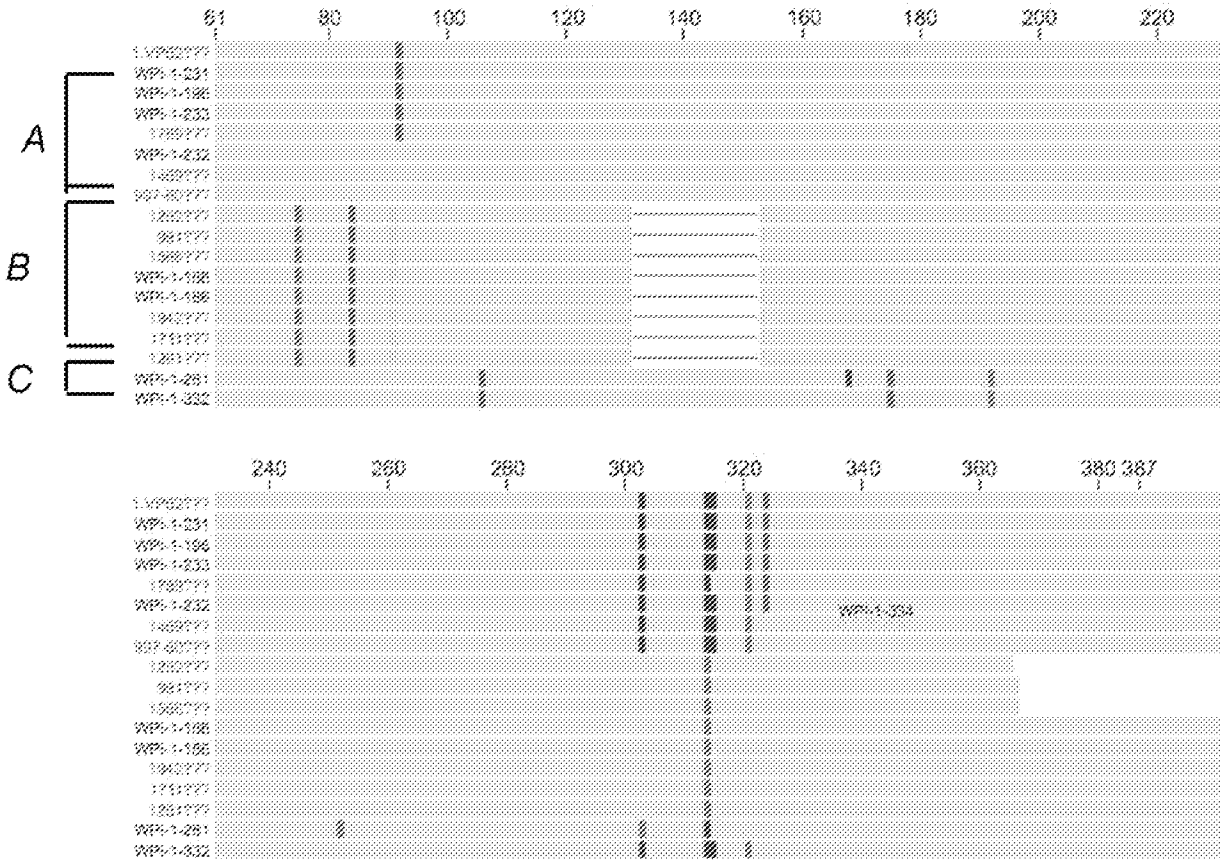


Table 1.

Location	Substitution(s)	Groups with Change	aa Change
C75	T	B	None
G85	A	B	None
A91	G	B	Lys (31)-Gly
A92	G	A,B,C	Lys (31)-Arg
G106	A	C	Val (36)- Ile
A132-152	21 bp deletion*	B	7 aa deletion*
T168	C	C	None
G175	A	C	Gly (59)-Ser
G178#	A	A,B,C	Val (60)-Ile
C192	T	C	None
C242	T	C	None
C303	T	C	None
C303	G	B	None
C314	T	B	Pro (105)-Leu
C315	T	B,C	None
T321	C	B,C	None
A324	G	A,B,C	None

 # This substitution is present in other XMRV sequences available in GenBank: VP42, EK1 and EK2 [ksj will add accession #]

Table 2.

aa Change	**aa Identical to Substitution**
Lys (31)-Arg/Gly	FeLV, Fr-MLV, KoRV (Arg); none (Gly)
Val (36)- Ile	FeLV
Gly (59)-Ser	GaLV, KoRV,
Val (60)-Ile	AKV-MLV, Ampho-MLV,Cas-BrE, Fr-MLV, Mo-MLV,X-MLV
Pro (105)-Leu	AKV-MLV, X-MLV

Accession numbers: AKV MLV (MLOCG), Amphotropic MLV (AF411814), Cas-BrE (X57540), FeLV (AF052723) Friend MLV (Fr-MLV) (NC 001362), GaLV (NC 001885), KoRV (QT9TTC2), Moloney-MLV (NC 001501), and xenotropic MLV (X-MLV)(EU035300).

*

Figure 2A.

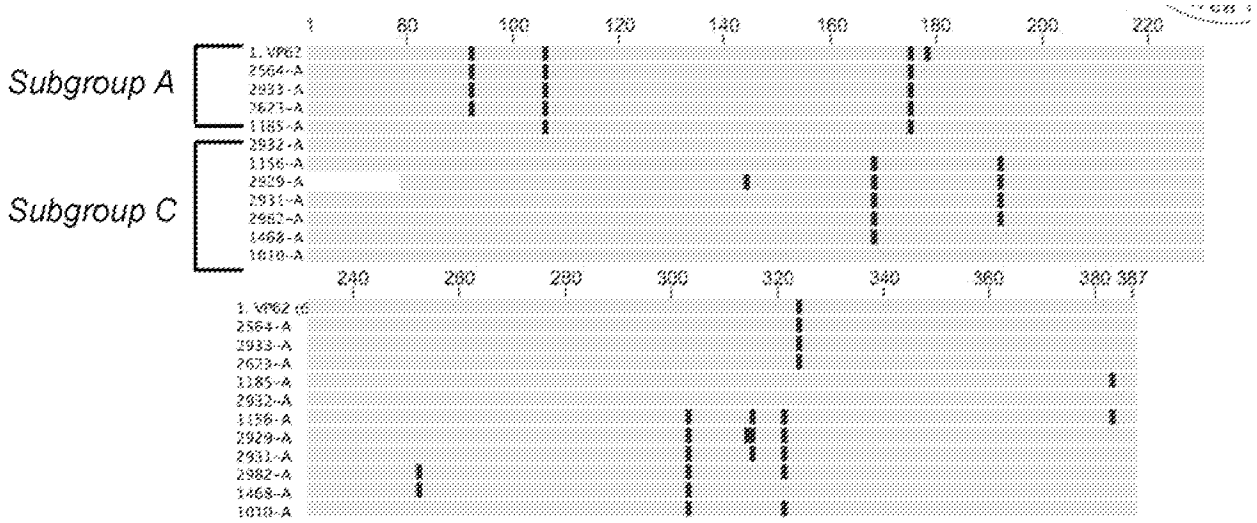
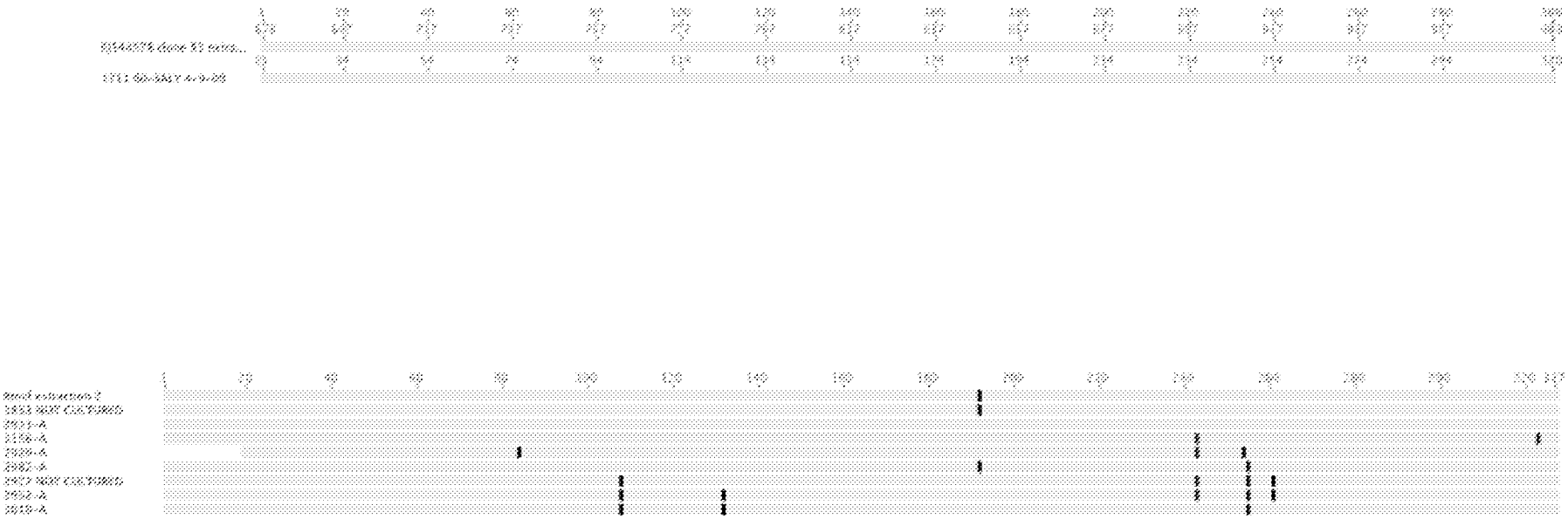

Subgroup A
Subgroup C
1
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
387

Fig. 2C.

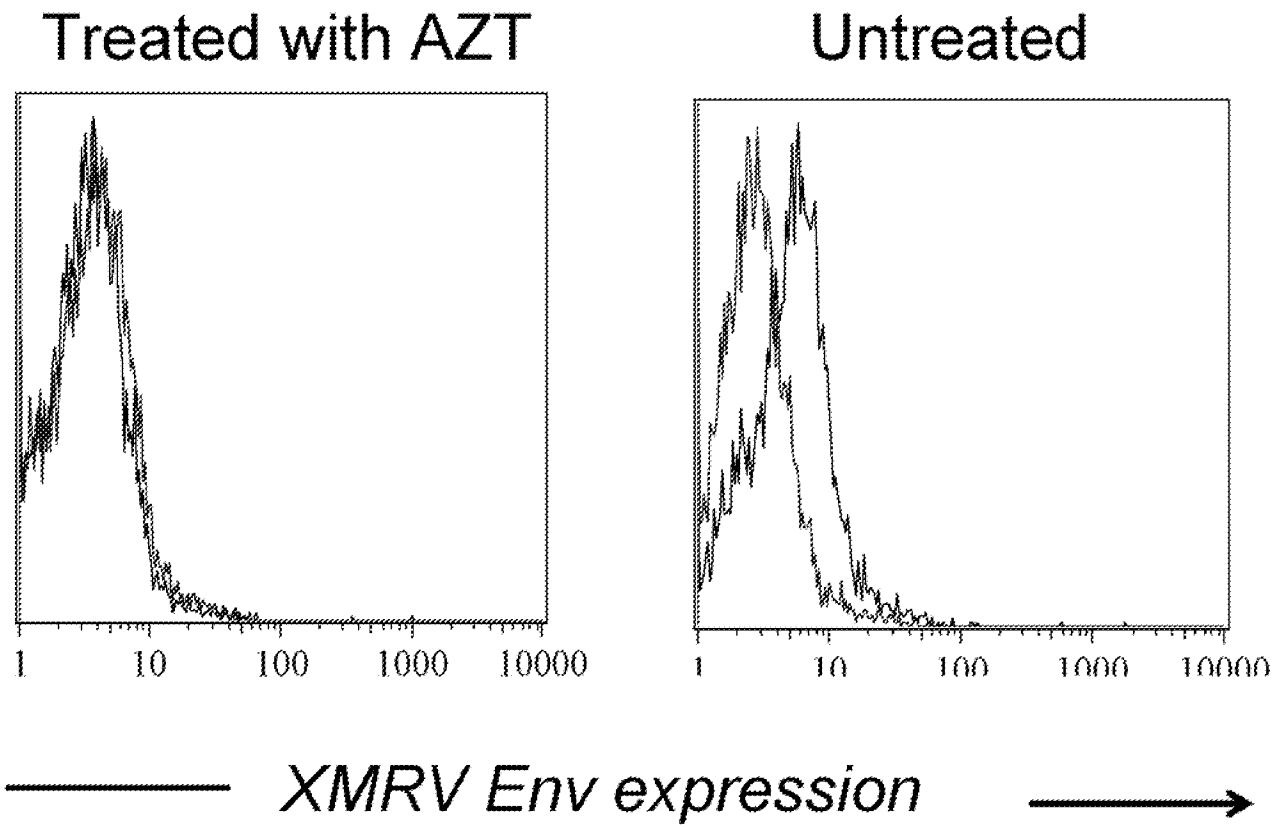
		B	C	
991		~50%	~50%	
1468		~80%	~20%	
1156		~20%	~80%	

Figure 3A and B

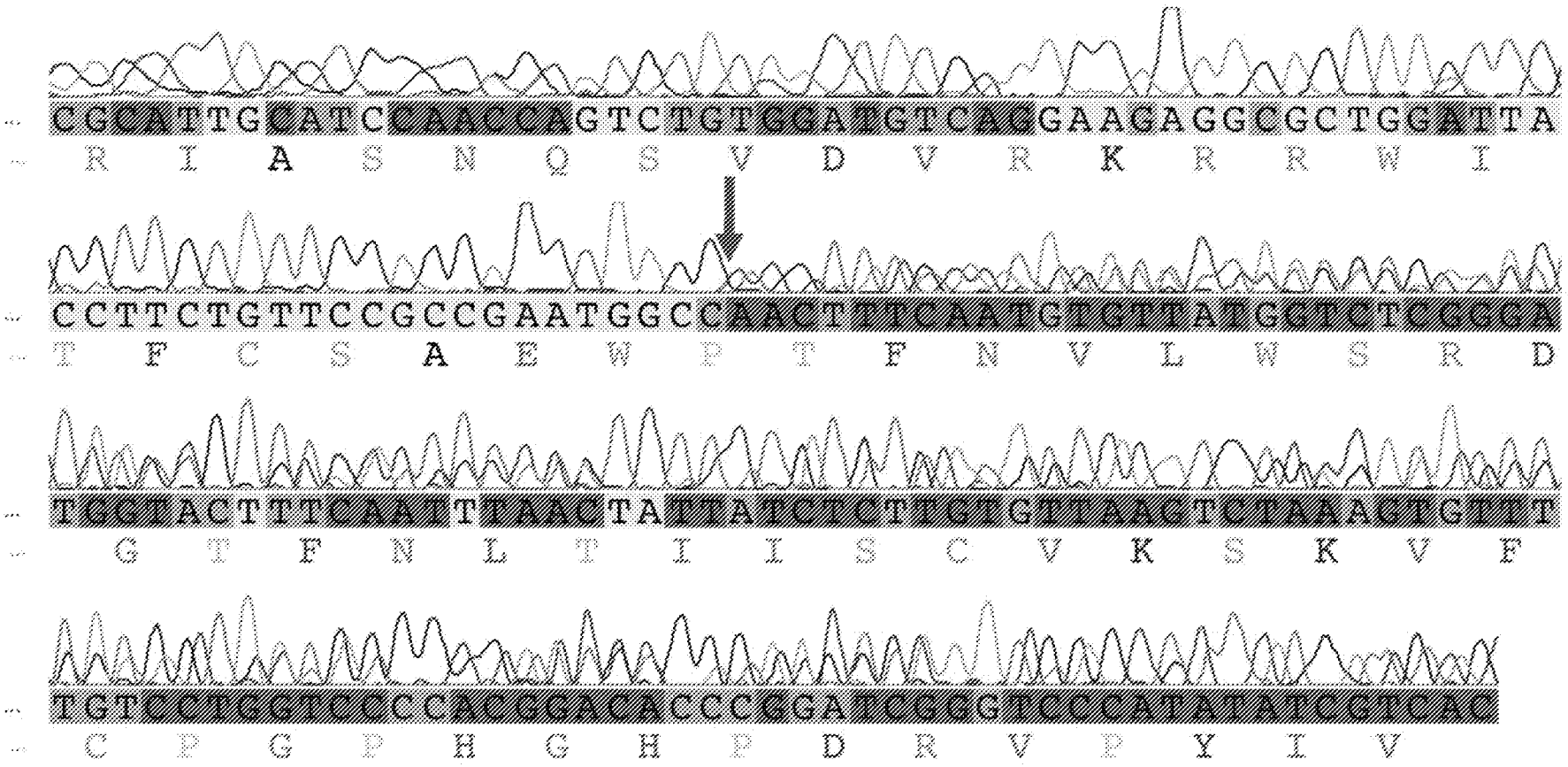


Supplement 1

Supplement 2

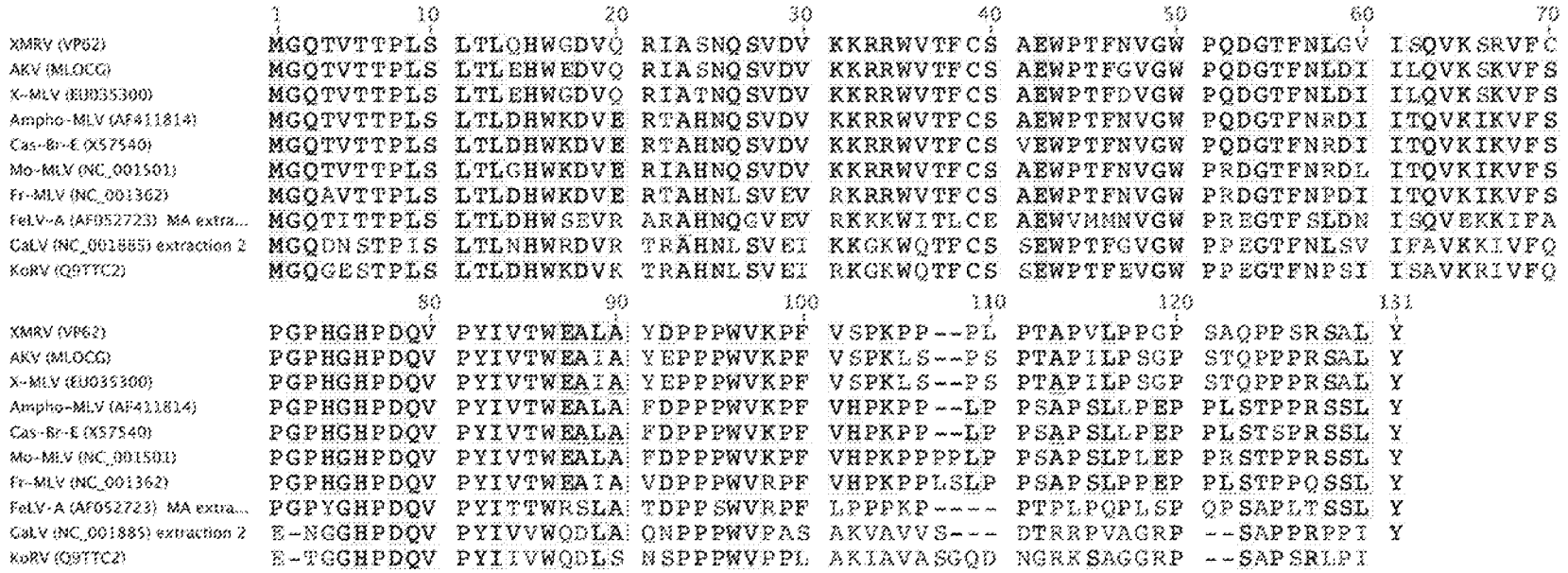


Supplemental Figure 3:

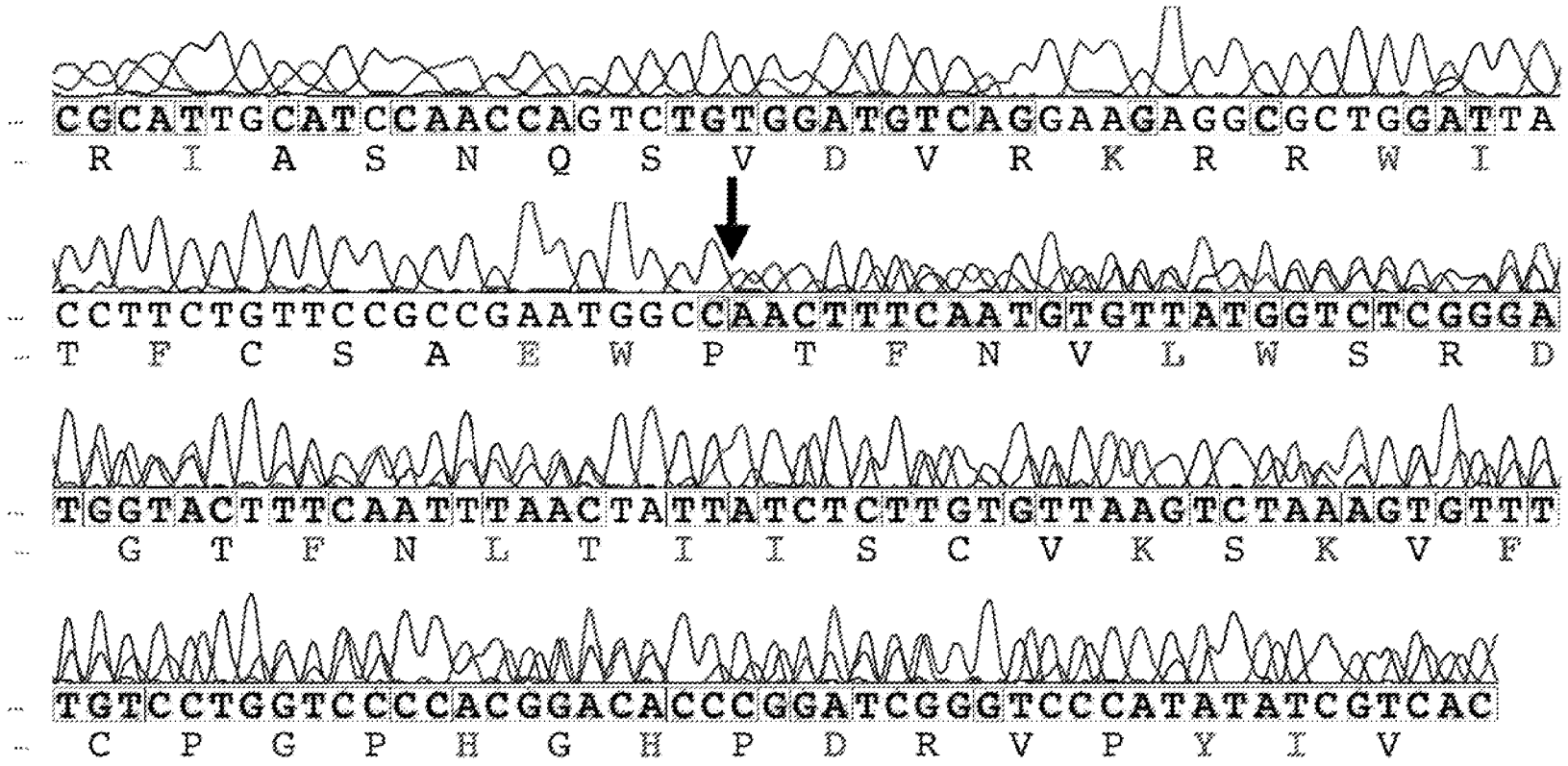


Red arrow denotes where the deletion begins

Supplement A



Supplemental Figure 3:



arrow denotes where the deletion begins

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	Diagnostic Identification of Variants of Xenotropic Murine Leukemia Virus-Related Virus
First Named Inventor/Applicant Name:	Judy A Mikovits
Filer:	Saul L. Zackson
Attorney Docket Number:	40000377-0001Var

Filed as Small Entity

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Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
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Provisional Application filing fee	2005	1	110	110
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**110**

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EFS ID:	7356333
Application Number:	61321147
International Application Number:	
Confirmation Number:	7100
Title of Invention:	Diagnostic Identification of Variants of Xenotropic Murine Leukemia Virus-Related Virus
First Named Inventor/Applicant Name:	Judy A Mikovits
Customer Number:	26263
Filer:	Saul L. Zackson
Filer Authorized By:	
Attorney Docket Number:	40000377-0001Var
Receipt Date:	06-APR-2010
Filing Date:	
Time Stamp:	06:10:39
Application Type:	Provisional

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