No Evidence of Murine Leukemia Virus-Related Viruses in Live Attenuated Human Vaccines

William M. Switzer1*, HaoQiang Zheng1, Graham Simmons2, Yanchen Zhou1, Shaohua Tang1, Anupama Shankar1, Beatrix Kapusinszky2, Eric L. Delwart2, Walid Heneine1

1 Laboratory Branch, Division of HIV/AIDS Prevention, National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention, Centers for Disease Control and Prevention, Atlanta, Georgia, United States of America, 2 Blood Systems Research Institute and Department of Laboratory Medicine, University of California (UCSF), San Francisco, San Francisco, California, United States of America

Abstract

Background: The association of xenotropic murine leukemia virus (MLV)-related virus (XMRV) in prostate cancer and chronic fatigue syndrome reported in previous studies remains controversial as these results have been questioned by recent data. Nonetheless, concerns have been raised regarding contamination of human vaccines as a possible source of introduction of XMRV and MLV into human populations. To address this possibility, we tested eight live attenuated human vaccines using generic PCR for XMRV and MLV sequences. Viral metagenomics using deep sequencing was also done to identify the possibility of other adventitious agents.

Results: All eight live attenuated vaccines, including Japanese encephalitis virus (JEV) (SA-14-14-2), varicella (Varivax), measles, mumps, and rubella (MMR-II), measles (Attenvax), rubella (Meruvax-II), rotavirus (Rotateq and Rotarix), and yellow fever virus were negative for XMRV and highly related MLV sequences. However, residual hamster DNA, but not RNA, containing novel endogenous gammaretrovirus sequences was detected in the JEV vaccine using PCR. Metagenomics analysis did not detect any adventitious viral sequences of public health concern. Intraclasternal A particle sequences closest to those present in Syrian hamsters and not mice were also detected in the JEV SA-14-14-2 vaccine. Combined, these results are consistent with the production of the JEV vaccine in Syrian hamster cells.

Conclusions: We found no evidence of XMRV and MLV in eight live attenuated human vaccines further supporting the safety of these vaccines. Our findings suggest that vaccines are an unlikely source of XMRV and MLV exposure in humans and are consistent with the mounting evidence on the absence of these viruses in humans.

Introduction

A gammaretrovirus, called the xenotropic murine leukemia virus (MLV)-related virus (XMRV), has been reported in persons with prostate cancer (PC), chronic fatigue syndrome (CFS), and blood donors with PCR prevalences up to 67% [1,2]. A related MLV was also reported in about 86% of CFS persons in a separate study [3]. The finding of XMRV and MLV in humans is controversial as subsequent studies have failed to confirm the initial reports [1,4,5,6,7,8,9,10]. Recent data showing that XMRV was generated in the laboratory during the passage of a human prostate cancer xenograft in nude mice during the generation of the XMRV-infected prostate cancer cell line 22Rv1 raises further doubts about the association of XMRV with human disease [11]. The origin of MLV in humans has also been questioned by the finding of reagents, human cell lines and specimens that are contaminated with MLV sequences [12,13,14,15,16,17,18]. However, additional studies aimed at defining the prevalence of XMRV and related viruses in humans and their association with diseases using an array of diagnostic tests are currently in progress [19]. MLVs are endogenous gammaretroviruses that constitute about 8–10% of the mouse genome and can cause leukemia, lymphoma, and neurological disorders in mice [20]. XMRV shares about 96% nucleotide identity with MLVs classified as xenotropic and which replicate only in non-mouse cells [1]. Thus, while mice are the likely species origin of MLV-related viruses, exposures that may have led to possible cross-species infections may be diverse, ranging from natural exposure to mice to possible exposures of biologicals, such as vaccines [21]. Mice and other rodents have been, or are currently used, in the production of vaccines. For example, the first live polio vaccine was grown in mice and tested on humans in 1950, smallpox, yellow fever, and rabies viruses were cultured in the brains of mice for vaccine production and several live, attenuated vaccines are produced on mammalian cell lines from mice, pigs, chickens, and cats [22]. Thus, MLV may have been introduced into vaccines during attenuation of the master seed stock during successive passage in rodents or during production in the absence of these viruses in humans.
vaccine production from contaminated reagents or growth in mouse or other rodent cell lines.

The Japanese encephalitis virus (JEV) vaccine (SA14-14-2, Rongsheng, China) is an example of a live vaccine that was attenuated via passage in rodents, including mouse brain, primary hamster kidney (PHK) cells, mouse spleen and skin, Syrian hamster (Mesocricetus auratus) spleens, and suckling mice skin [23]. Production of the SA-14-14-2 vaccine is done in the hamster PHK cell line. According to the manufacturer, the master seed virus of the SA-14-14-2 JEV vaccine and the PHK cell lines used for vaccine production were shown to be free of adventitious agents and pathogens. The SA-14-14-2 JEV vaccine has been used for over 20 years and administered to over 300 million children in China, South Korea, Nepal, and India.

Although the master seeds and cell substrates used for vaccine production are prescreened for adventitious agents, newer technologies, including sequence-independent amplification, followed by ultra-deep DNA sequencing, and microarrays have shown that some cell substrates and live-attenuated vaccines still contain adventitious viruses, including endogenous retroviruses like avian leukosis virus (ALV), and porcine circovirus [24]. Previous studies have also documented the presence of endogenous avian retroviruses in currently used avian cell-derived vaccines [25,26]. Endogenous retroviruses exist in all mammals as proviral DNA integrated in the germ line of the host and are passed from parent to offspring. Thus, endogenous retroviruses cannot be eliminated from cell lines or live animals by prescreening. While most endogenous retroviruses are replication defective, some exist as intact genomes that are capable of expressing infectious virus.

We screened eight live attenuated human vaccines for XMRV and related MLV and adventitious agents using PCR and metagenomics. The eight vaccines included JEV (SA-14-14-2), varicella (Varivax), measles, mumps, and rubella (MMR-II), measles (Attenuvax), rubella (Meruvax-II), rotavirus (Rotateq and Rotarix), and yellow fever virus. All eight vaccines were negative for XMRV and closely related MLV sequences using these two approaches. We found novel hamster genomic and retrovirus sequences in the JEV vaccine mostly likely originating from vaccine production in Syrian hamster cells. Our findings do not support the hypothesis that vaccines are a possible source of XMRV or MLV introduction into humans and are consistent with accumulating evidence on the absence of these viruses in humans.

Results

Absence of MLV and XMRV sequences in live attenuated vaccines by PCR testing

Total nucleic acids and particle-associated RNA from eight live attenuated vaccines were tested for XMRV and MLV sequences using a generic polymerase (pol) and a specific (gag) nested PCR test capable of detecting at least 10 DNA and 100 RNA sequences per reaction [2,3,27]. In addition, we used a new generic, quantitative real-time PCR (qPCR) test capable of detecting protease (pro) sequences of all MLV and XMRV with a reported sensitivity of 10 copies per reaction (fragment 1, Fig. 1) [28]. All eight vaccines were negative for XMRV and MLV DNA and RNA sequences using this combination of PCR tests, except the JEV vaccine which was estimated to contain about 960 copies of MLV-like DNA sequences/ml by using the qpro test (Tables 1 and 2). The MLV-like DNA sequences detected in the JEV vaccine by qPCR were confirmed by gel electrophoresis and sequence analysis. However, the amount of MLV-like sequences in the qpro amplification product appeared greater in the gel image than that quantified by qPCR when compared to the qPCR assay standards (data not shown). These results indicated detection of a variant with some...
level of sequence divergence from the generic MLV probes used in the qPCR assay. Indeed, analysis of this 91-bp pro sequence identified a distinct gammaretrovirus that is equidistant from murine endogenous retroviruses (mERVs), XMRV, and MLV sharing only 72–75% nucleotide and amino acid identity (data not shown).

### Table 1. Identification of Hamster DNA and Retroviruses in Eight Live, Attenuated Human Virus Vaccines.

| Vaccine | Virus | Manufacturer | Lot Nos. | Virus Preparation | Animal Passage | Metagenomics analysis (non-vaccine viruses) | XMRV/MLV PCR | Murine DNA PCR |
|---------|-------|--------------|----------|-------------------|----------------|-------------------------------------------|--------------|---------------|
| SA-14-2-2 | Japanese encephalitis | Rongsheng | 201002A014-3 200906A087-1 | live, attenuated | Primary hamster kidney cells, mice, hamsters, primary chick embryo | Autographa c. nucleopolyhedrous virus, HERV-H | Pos¹ | Syrian hamster DNA |
| Varivax | varicella | Merck | 1526X | live, attenuated | embryonic guinea pig | None | Neg | NT³ |
| MMR-II | measles, mumps, rubella | Merck | 1732X | live, attenuated | chick embryo cell culture | None | Neg | NT |
| Attenuvax | measles | Merck | 1440X | live, attenuated | chick embryo cell culture | avian leukemia virus | Neg | NT |
| Meruvax-II | rubella | Merck | 1198X | live, attenuated | none | None | Neg | NT |
| RotaTeq | rotavirus | GlaxoSmithKline | A41FA799A | live, attenuated | MA104 (rhesus macaque kidney), Vero cells (AGM kidney) | None | Neg | NT |
| Yellow Fever | yellow fever virus | Sanofi Pasteur | UF430AA-5188 | live, attenuated | embryonated chicken eggs | None | Neg | NT |

¹Metagenomic results for vaccines other than JEV were reported previously [24].  
²Distinct gammaretrovirus sequences were obtained that were equidistant from MLV and XMRV.  
³Not tested.  

**Table 2. Detection of Japanese encephalitis virus (JEV) RNA, hamster endogenous retrovirus (ERV) DNA, and hamster genomic DNA in the live, attenuated JEV vaccine (SA-14-14-2).**

| PCR Assay | JEV SA-14-14-2 | Nuclease¹−(copies/ml) | Nuclease²(copies/ml) |
|-----------|----------------|------------------------|-----------------------|
| JEV RNA   |                 | 4.0×10⁵                 | 4.0×10³                |
| Murine mtDNA (MCOX2) | negative | negative |
| IAP-pol DNA² | Syrian hamster + murine specific | 6.32×10⁷ | 1.94×10⁴ |
| IAP-pol DNA | (Chinese hamster specific) | negative | negative |
| MLV RNA (qpro) | 9.6×10² | negative |
| Hamster ERV RNA (qpro-based)³ | 2.4×10⁸ | negative |
| MLV RNA (ext-qpro)⁴ | positive | negative |
| Hamster ERV RNA (ext-qpro-based)⁵ | 2.1×10⁷ | negative |
| MLV RNA (qgag) | negative | negative |
| MLV RNA (nested gag) | negative | ND⁶ |
| MLV RNA (nested pol) | negative | ND |

¹Viral filtrates treated with (+) or without (−) DNase and RNase.  
²IAP, intracisternal A particle; pol, polymerase.  
³Primers were based on amplicons generated with MLV quantitative protease (qpro) assay.  
⁴Ext, extended qpro assay and is equivalent to Fragment 2 in Fig. 1.  
⁵Primers were based on amplicons generated with extended qpro assay.  
⁶ND, not done.  

**doi:**10.1371/journal.pone.0029223.t002
Detection and characterization of novel hamster retrovirus and genomic sequences in JEV vaccines

To confirm the presence of the distinct gammaretrovirus sequence in the JEV vaccine we designed a new qPCR test specific for the detection and quantification of this sequence. Using this test we estimated that the JEV vaccine contained 2.4 x 10⁶ copies/ml of this sequence (Table 2). To obtain longer sequences for further viral characterization, we designed additional generic primers flanking the qpro primers that are based on a nucleotide alignment of MLV and XMRV complete genomes (fragment 2, Fig. 1). We used these new pro primers to test nucleic acids from the JEV vaccine. In addition, we tested DNA from Chinese (Chinese hamster ovary, CHO) and Syrian (baby hamster kidney, BHK) hamster cell lines since the JEV vaccine is produced in hamster cells [23,29]. Longer pro sequences 200–235-bp in length were detected in the JEV vaccine while 235-bp sequences were amplified from the CHO and BHK cell lines, after removal of the primer sequences. BLAST analysis showed that these longer pro sequences in the vaccine and BHK were equidistant (~80% nucleotide identity) from MLVs while those from the CHO cell line were 94% identical to a transcriptionally active endogenous retrovirus (Genbank U09104) found in Chinese hamsters (Cricetulus griseus). Interestingly, these 200–235-bp pro sequences were distinct from the 91-bp pro sequences sharing only 68–75% nucleotide identity. Phylogenetic analysis of 82-bp containing the overlapping regions of the 91-bp and 200–235-bp pro sequences confirmed these genetic relationships and showed that these sequences are distinct from MLV and XMRV and were closer in identity to gammaretroviruses in the Chinese (CHO) and Syrian hamster cell lines (BHK and HAK) (Fig. 2a).

We next designed additional forward PCR primers based on the consensus qpro and the 200–235-bp pro sequence and new generic reverse primers based on MLV and XMRV prototype pro-pol sequences and applied these to the JEV vaccine and hamster cell line nucleic acids to obtain longer and more phylogenetically informative sequences (Fig. 1). A total of three different primer combinations were used (Fig. 1) which generated three different groups of DNA sequences 950-bp in length (fragments 3–5) from the HAK cell line DNA and the JEV vaccine and all were distinct from the 92-bp pro sequences. These pro-pol sequences were cloned and analyzed and determined to be equidistant from other murine retroviruses sharing only 70% nucleotide identity and were 77% identical to each other. Phylogenetic analysis of an alignment containing 910-bp of the 950-bp fragments alone or with the overlapping pro regions (82-bp) confirmed that these new pro-pol sequences are distinct from other gammaretroviruses in rodents and other mammals (Fig. 2). When all five PCR fragments were aligned and analyzed, six different gammaretrovirus phylogenetic lineages were inferred (Fig. 2a). The first lineage included the original qpro (Fragment 1) sequences that were distinct from MLV, XMRV and other gammaretroviruses in rodents and other mammals (Fig. 2). When all five PCR fragments were aligned and analyzed, six different gammaretrovirus phylogenetic lineages were inferred (Fig. 2a). The first lineage included the original qpro (Fragment 1) sequences that were distinct from MLV, XMRV and other gammaretroviruses, including those found in the JEV vaccine and hamster cell lines. The second lineage contained fragment two sequences from the JEV vaccine and Syrian hamster cell line, BHK, while the third lineage only contained gammaretrovirus sequences from the Chinese hamster. Lineage four contained distinct JEV (fragment 3) and Syrian hamster sequences from the cell line HAK, while the fifth lineage consisted of JEV fragment 4 and 5 sequences and distinct Syrian hamster cell line (HAK) gammaretrovirus sequences. The sixth lineage was composed of a divergent JEV sequence using the fragment three PCR primers and a third set of Syrian hamster sequences obtained from the HAK cell line. The sixth lineage was also the most divergent, sharing a common ancestor with MLV/XMRV, RaLV, FeLV, and the other hamster and JEV gammaretrovirus sequences (Fig. 2a).

To further characterize the hamster and JEV gammaretroviruses, we phylogenetically analyzed the larger 910-bp alignment consisting of only the fragment 3 to 5 sequences and the appropriate reference sequences (Fig. 2b). Like the phylogenetic analysis of the shorter 82-bp fragments, two distinct fragment 3 lineages and a single lineage containing the fragment 4 and 5 JEV and hamster sequences were inferred. The two groups of 910-bp pro-pol sequences from the JEV vaccine and HAK DNA containing fragments 3 to 5 were also distinct from, but clustered with the endogenous retrovirus (Genbank U09104) found in Chinese hamsters (Cricetulus griseus) (Fig. 2b). The additional 910-bp pro-pol fragment 3 sequences clustered more closely with rat leukemia viruses (RaLV) but with moderate bootstrap support (Fig. 2b). All three 910-bp pro-pol sequence lineages also contained sequences from the HAK Syrian hamster cell line with strong bootstrap support suggesting they are all of hamster origin (Fig. 2b). 950-bp pro-pol sequences were not detected in the BHK and CHO cell line DNAs using these primer combinations. The finding of highly divergent gammaretrovirus sequences using these pro-pol primer combinations is consistent with the detection of endogenous retroviruses present in hamster DNA.

We evaluated these new sequences for open reading frames (ORFs) indicative of intact viral genomes with replication potential. The two different, 91-bp qpro sequences (fragment 1) and all 950-bp pro-pol fragment 4 and 5 sequences in the JEV SA-14-14-2 vaccine contained ORFs. In contrast, a single distinct fragment 3 sequence found in the JEV vaccine and three HAK pro-pol fragment 3 sequences (950-bp) that clustered weakly with rat leukemia virus RaLV were all defective. A mixture of cloned sequences with ORFs and defective ORFs were present in the second class of fragment 3 pro-pol sequences (950-bp) from the SA-14-14-2 vaccine and HAK cell line (71% and 50%, respectively). Almost 69% of the fragment 2 pro sequences in the SA-14-14-2 vaccine and all BHK and CHO sequences were defective. Thus, the majority of the cloned gammaretrovirus sequences detected in the attenuated JEV vaccine and hamster cell lines were defective in the subgenomic regions examined.

To investigate whether the retroviral sequences in the JEV vaccines originated from endogenous proviral elements present in DNA or were particle-associated RNA, we PCR-tested viral filtrates that were first treated with and without nucleases. In the presence of nucleases only JEV viral RNA was detected in the filtrates indicating that the gammaretrovirus sequences in this vaccine were not particle associated. Testing particle-associated RNA using primers specific for the five new classes of gammaretrovirus sequences identified in the vaccine yielded negative results (Table 2) confirming that these sequences are not particle-protected and likely represent genomic DNA from the hamster cell substrate in which the vaccine was grown (Table 1). Furthermore, essentially equivalent amounts of pro signals were obtained when vaccine nucleic acids were tested by RT-PCR with and without reverse transcriptase documenting retroviral DNA in these extracts (data not shown). However, the presence of particle-associated JEV RNA in the two vaccines was confirmed by the detection of 4 x 10⁷ copies/ml JEV in both the untreated and the nuclease-treated SA-14-14-2 vaccine filtrates. The attenuated JEV vaccine was also negative for murine mtDNA and IAP sequences but was positive for Syrian hamster, but not Chinese hamster, IAP sequences (~4 x 10⁷ copies/ml) using new real-time PCR tests that detect murine and hamster IAPs (Table 2). These new IAP tests are extremely sensitive for detecting mouse DNA contamination in a specimen and can detect 10 attograms of mouse DNA in a
background of 1 ug human DNA (data not shown). Our finding of residual Syrian hamster IAP DNA sequences even after filtration and nuclease treatment of vaccine material demonstrates the sensitivity of this new IAP assay for detecting low levels of IAP DNA. These results most likely reflect incomplete digestion of DNA in the material (Table 2). Combined with the presence of endogenous hamster retrovirus DNA sequences, the IAP results further demonstrate that the JEV SA-14-14-2 vaccine contains residual hamster and not mouse DNA.

Absence of adventitious agents in attenuated JEV vaccines by metagenomics analysis

We have previously shown by metagenomics analysis that the attenuated Attenuvax measles contained viral particle-associated RNA for ALV and endogenous avian retrovirus (EAV) [24]. Likewise, yellow fever and MMR-II vaccines, also made in chick embryo cells, were previously found to contain particles with ALV and EAV RNA [25,26]. The rotavirus vaccine Rotarix was shown to contain porcine circovirus 1 (PCV1) [24] while PCR studies showed RotaTeq to contain low levels of PCV1 and PCV2 (http://www.ncbi.nlm.nih.gov/pubmed/21569811). Using viral metagenomics and a BLASTx E score cutoff of 0.001 to any viral sequences we found no evidence of adventitious viruses in the live, attenuated JEV (SA-14-14-2) (Table 1). A total of 1993 pyrosequence reads of over 100 nucleotides in length were generated. These sequences included 48 contigs containing 281 identical sequences obtained for that fragment. Asterisks indicate sequences without open reading frames. The MLV/XMRV and PERV branches were collapsed to fit the trees to single pages. Stability of the tree topology was tested using 1000 bootstrap replicates in both neighbor joining (NJ) and maximum likelihood (ML) methods. Bootstrap values >60 are shown (NJ/ML). New sequences (fragments 1–4) from the JEV vaccine and hamster cell lines are in light blue and red text, respectively. Fragment 5 sequences amplified from the JEV SA-14-14-2 vaccine are in dark blue text.

doi:10.1371/journal.pone.0029223.g002
common insect baculovirus sprayed on vegetable fields to control insect pests and therefore most likely represents an environmental contaminant. A 365-bp sequence with 99% nucleotide identity to a human genomic region (GenBank accession no. AJ289710) annotated as the human endogenous retrovirus type H was also present in the JEV vaccine and most likely represents human DNA contamination (Table 1). The inability of the metagenomics analysis to detect the novel gammaretrovirus and hamster IAP sequences reflects that these DNAs are not particle-associated.

Discussion

Despite the production of most strains of attenuated viruses on primary animal-derived cells, live attenuated vaccines are safe, inexpensive, and highly effective at reducing mortality and morbidity from numerous diseases, and thus are used worldwide. We tested eight live attenuated vaccines used globally to prevent viral diseases. We found no evidence of either XMRV or MLV in all eight vaccines using sensitive PCR testing for both viral RNA and DNA sequences. We also show by ultra-deep sequencing of particle-protected nucleic acids the absence of XMRV and MLV in these vaccines. Thus, our data do not support the hypothesis that these vaccines are a possible source of human exposures to XMRV or MLV. These results are consistent with the production of all these vaccines in non-mouse cells which substantially reduces the risks of adventitious endogenous murine retroviruses.

There are other mouse-derived biological products that may be at a higher risk of carrying MLV, such as monoclonal antibodies (mAbs) that are used in humans for the treatment of cancer, inflammatory, and autoimmune diseases [30]. Indeed, mouse hybridomas that have been shown to be contaminated with endogenous xenotropic MLVs [30], but the production of mouse mAbs for clinical use requires purification steps that inactivate and remove viral particles. Our study is also limited by the testing of only eight vaccines and does not include other live attenuated vaccines that have a history of passage in rodents, such as early versions of the oral polio vaccine. We also did not test master virus seed stocks (MVSS) and master cell banks (MCB) used in the manufacture of the live attenuated vaccines from our study since these materials were not readily available. Nonetheless, our testing of vaccines used on humans was a more direct public health risk assessment for adventitious agents that vaccine recipients may have been exposed to compared to MVSS and MCB. Furthermore, the recent finding that XMRV originated as a laboratory artifact during passaging of a prostate tumor xenograft in inbred mice [11], the ability of human serum to inactivate MLV, and the accumulating evidence from a large number of epidemiologic studies showing the absence of XMRV or MLV in humans, all cast doubts on the links of XMRV or MLV to prostate cancer and CFS and their endemicity in human populations [4,5,10,14,15,16,17,28,31,32,33,34]. Likewise, the proposed laboratory origin of XMRV in the 22Rv1 cell line around 1996 [4,5,10,14,15,16,17,28,31,32,33,34,35]. The ability to use sensitive PCR tests to detect these sequences is therefore not unusual.

Conclusions

We found no evidence of XMRV or MLV in eight globally used live attenuated vaccines and thus our results do not support the hypothesis that these vaccines contributed to the iatrogenic introduction of XMRV or MLV into the human population and are in agreement with the accumulating evidence on the absence of these viruses in human populations. We identified residual hamster DNA containing multiple endogenous gammaretrovirus sequences, but not retroviral RNA, in the JEV vaccine and show that these sequences are of Syrian hamster origin consistent with the production history of the vaccine.

Methods

Vaccines

Single dose, live attenuated SA 14-14-2 (Rongsheng; lot nos. 200906A087-1 and 201002A014-3) JEV vaccines were stored at 4°C and were re-suspended in the supplied 1.0 ml PBS. Additional lyophilized live attenuated vaccines, as previously described [24], were resuspended in 200 μl of manufacturer-appropriate sterile diluent (Merck; lot no. 4089) or sodium chloride solution (Sanofi Pasteur; lot no. UF198AB). Rotarix (rotavirus, GlaxoSmithKline [GSK]; lot no. A41X799A) and Rotateq (rotavirus; Merck; lot no. 1724X) were resuspended in a 1-dose volume of accompanying oral diluent. A total of 200 μl of Meruvax (rubella; Merck; lot no. 1198X), Attenuvax (measles; Merck, lot no. 1440X), YF-VAX (yellow fever; Sanofi Pasteur; lot, no. UF430AA-5188), MMR-II (measles, mumps, rubella; Merck; lot no. 1732X), Rotateq, or Varivax (varicella virus; Merck; lot no. 1526X) was filtered through a 0.45 μM filter (Millipore).

Viral particle purification and nucleic acid extraction

Vaccines were passed through a 400 nm filter and filtrate containing viral particles was treated with a mixture of DNase and RNase to remove exogenous nucleic acids as described previously [24]. Particle-associated nucleic acids were then prepared using a QIAamp viral RNA extraction kit (Qiagen). Nucleic acids were also extracted directly from the JEV vaccine using the QIAamp viral RNA Mini extraction kit (Qiagen).

Virus-specific PCR for MLV, XMRV, and JEV

Both MLV-generic and XMRV-specific PCR tests were used to screen the vaccine nucleic acids. Nested and real-time PCR tests were used to generically detect MLV and XMRV gag and pro sequences, respectively. The gag PCR test used the primers 419F and 1154R in the primary amplification and GAG-I-F/GAG-I-R for the nested PCR reaction [2,3,27]. The pol PCR test used the primers XPOL-OF and XPOL-OR in primary and XPOL-IF and XPOL-IR in the nested PCR reactions [27]. The quantitative real-time PCR test in the protease (pro) region, called qpro, used the Taqman primers Pro-UNV-F1 and Pro-UNV-R1 and probes Pro-UNV-PIC and Pro-UNV-PR1 [28] (Fig. 1). The XMRV and MLV generic gag quantitative real-time PCR test was performed with the primers GAG-UNV-F1, 5′AGGTAGGAACACACGA-TA-GTYC3′ and GAG-UNV-R1, 5′GGCTCAGG GTCATAAGGAG3′ and probes GAG-UNV-PIC, 5′FAMACCGGGTGTC-
Metagenomics analysis of live, attenuated vaccines

Viral cDNA synthesis and random PCR amplification were performed as previously described [24]. Briefly, 100 pmol of primer consisting of an arbitrarily designed 20-base oligonucleotide followed by a randomized octamer (8N) sequence at the 3' end was used in a reverse transcription (RT) reaction (Superscript III; Invitrogen). Two distinct primers containing different 20-base fixed sequences were used in two separate RT reactions targeting the virus-enriched nucleic acids from the live-attenuated JEV vaccine. A single round of DNA synthesis was then performed using Klenow fragment polymerase (New England Biolabs), and then PCR amplification of double-stranded DNA using a primer consisting of only the 20 fixed bases was performed. Independent duplicate PCRs were performed for each random primer, generating a total of 4 separate reactions. Random PCR DNA products were pooled and separated on an agarose gel, and fragments from 500 bp to 1,000 bp were excised and extracted. DNA was sequenced using GS FLX Titanium reagents.

Sequence read classification

The 454 sequence reads were trimmed of their random PCR primer sequences and assembled into longer contigs using the program Sequencher (GeneCodes), with an overlap set as 95% similarity over 50-bp to merge fragments. The contigs and singlets greater than 100 bp were compared to the NCBI nonredundant nucleotide and protein databases using BLASTn and BLASTx, respectively. Viral sequences were classified based on their best alignment E value and those with values <10e-3 were deemed unclassifiable.

Cell line DNA

Syrian baby hamster kidney (BHK), Syrian hamster kidney (HAK), and Chinese hamster ovary (CHO) cryopreserved cell lines were obtained from the CDC Biologics Branch and DNA was extracted using the Qiagen Flexigene DNA kit.

Identification of novel hamster gammaretrovirus sequences in the JEV vaccines

To further characterize the gammaretrovirus sequences identified in the JEV SA-14-14-2 vaccine additional PCR primers were designed based on an alignment of endogenous murine and hamster retrovirus sequences available at GenBank and the new sequences obtained with the qpro primers and subsequent amplicons. The primers PRO-UNV-OF 5'TAGGGAGGTC AGGGTCAGGACG3' and PRO-UNV-OR 5'GGAAAA-GAGTTGRGTCACCT TACGGGT3' were used to amplify a second fragment (283-bp) surrounding the qpro sequences from the SA-vaccine and BHK and CHO cell line DNAs (Fig. 1). To increase the sensitivity of detecting divergent murine or hamster gammaretroviruses we used the following nested PCR primer combinations to amplify three additional fragments about 550-bp in size from the JEV SA-14-14-2 vaccine and HAK cell line DNA (Fig. 1). The primary PCR amplification for fragment 3 used the primers PRO-UNV-F1 and XPOLIR 5'AGGTGCGCCGCCACG CAGTAAGGTCAT3', while the outer primers for fragments 4 and 5 were PRO-UNV-OF and XPOLIR. The internal primers for fragments 3 and 4 were PRO-SA-F1 5'GAGCAACCGAT- CACCTTCCCTA3' and POL-IRM 5'TCTGGGTGCTGATC CGGA3', while the internal primers for fragment 5 were PRO-UNV-IF2 5'GGGGCCGCRGTCACTTCCTG3' and POLIRM (Fig. 1). The Expand High Fidelity PCR System (Roche) was used for the primary amplification and AmpliTaq (ABI) for the nested PCR. 25 ul of extracted nucleic acids or 100 ng cell line DNA were used as templates in the primary PCR amplification. Forty cycles of each round of PCR was done using 95°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min.

PCR detection of mouse and hamster DNA contamination

Mouse mitochondrial DNA (mtDNA) and intracisternal A particle (IAP) sequences were detected using the primers MCOX2F2 and MCOX2R1 and probes MCOX2PR1 and MCOX2PR2 and IAP-F and IAP-R using the conditions previously reported [16,28]. Given that the JEV SA-14-14-2 vaccine was also passed in hamsters we also designed two new real-time PCR tests that genetically detect mouse and Syrian hamster IAP or specifically detect Chinese hamster (CHO) IAP pol sequences. The generic IAP primers and probes are IAP-MH-POLF2: 5' GCCCTCAYATGTG ATCCAACTTG 3' and IAP-MH-POLR2: 5' TTGRGASGTATAWGCCGTG CCATT 3' and IAP-MH-POLP2: 5'FAM TTAGGGGACTGAGTGGT GCTG TTCGGGCAGA 3'BHQ1, respectively. The CHO-specific IAP primers and probe are IAP-MH-POLF3 5'GCCGGCGATGTTG TCAAACCTGG3', IAP-MH-POLR3 5' TGAGAGGTATAAACGCGCCCA T3', and IAP-MH-POLP3 FAM5'TAGAGGCTTGGGTGCTGAGTGGTG AAAC CCTGAT3'BHQ1. The assay was performed with a hot start at 95°C for 9 min followed by 55 cycles of PCR at 95°C for 30 sec and 62°C for 30 sec. This assay was 1000X more sensitive in detecting IAP sequences in mouse DNA than that using the IAP-F and IAP-R primers though both and the mtDNA PCR tests could detect 1 copy each in a background of 1 ug of human DNA (data not shown).

Sequence analysis

PCR products were purified with QiaQuick PCR or gel purification kits (Qiagen, Valencia, CA) and were directly sequenced on both strands by using ABI Prism BigDye terminator kits and an ABI 3130 Genetic Analyzer or a 3100 capillary sequencer (Foster City, CA) or following cloning in the TOPO vector (Invitrogen). Initial sequence identity was determined using BLAST analysis at the National Center for Biotechnology Information web server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using either the megablast or blastn search options. Sequences were aligned with those retrieved from the BLAST analysis with the highest nucleotide identity, and other MLV prototypes available at GenBank, using Clustal W in the MEGA v5.03 program (http://megasoftware.net/). Following manual editing and removal of indels, substitution models and phylogenetic relationships were inferred using the neighbor joining (NJ) method implemented in MEGA v5.03. Phylogenies were also inferred using the program PhyML plugin in the Geneious software package v5.3.6 (www.geneious.com) that implements the fast maximum likelihood (ML) method. Support for the branching order was evaluated using 1,000 nonparametric bootstrap replicates.

Nucleotide sequence accession numbers

The new hamster gammaretrovirus pro-pro sequences generated in the current study are available at GenBank with accession numbers JN652837–JN652873. An alignment of the 91-bp pro
sequences is available from the authors upon request. GenBank does not accept sequences less than 200-bp in length.

Acknowledgments
Use of trade names is for identification only and does not imply endorsement by the U.S. Department of Health and Human Services, the Public Health Service, or the Centers for Disease Control and Prevention (CDC). The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the CDC.

Author Contributions
Conceived and designed the experiments: WMS ELD WH GS HZ. Performed the experiments: HZ AS ST GS YZ BK ELD. Analyzed the data: WMS HZ ELD GS WH. Contributed reagents/materials/analysis tools: WMS HZ ELD. Wrote the paper: WMS ELD GS HZ WH.

References

1. Silverman RH, Nguyen C, Weight CJ, Klein EA (2010) The human retrovirus XMRV in prostate cancer and chronic fatigue syndrome. Nat Rev Urol 7: 392–402.
2. Lombardi VC, Ruscitti FW, Das Gupta J, Ploet MA, Hagen KS, et al. (2009) Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome. Science 326: 305–309.
3. Lo SC, Pripuzova N, Li B, Komaroff AL, Hung GC, et al. (2010) Association of the XMRV-related virus with prostate cancer. Nat Med 16: 1426–1429.
4. Shim CH, Bateman L, Schlaberg R, Bunker AM, Leonard CJ, et al. (2010) Comparative analysis of the XMRV-related virus compared with HIV-1 and murine leukemia virus. PLoS One 5: e15874–15879.
5. Satterfield BC, Garcia RA, Jia H, Tang S, Zheng H, et al. (2011) Serologic and PCR testing of persons with chronic fatigue syndrome in the United States shows no association with xenotropic or polytropic murine leukemia virus-related viruses. Retrovirology 8: 12.
6. Lintas C, Gaidi F, Manzi B, Mancini A, Curatolo P, et al. (2011) Lack of Infection with XMRV or Other MLV-Related Viruses in Blood, Post-Mortem Brains and Parenteral Gametes of Autistic Individuals. PLoS One 6: e16609.
7. Furuta RA, Miyazawa T, Sugiyama T, Kuratsune H, Ikeda Y, et al. (2011) No association of xenotropic murine leukemia virus-related virus with prostate cancer or chronic fatigue syndrome in Japan. Retrovirology 8: 20.
8. Erlich O, Robinson MJ, Kaye S, Wills G, Iuzzi S, et al. (2011) Investigation into the presence of and serologic response to XMRV in CFS patients. PLoS One 6: e17592.
9. Hong P, Li J, Li Y (2010) Failure to detect xenotropic murine leukemia virus-related virus in Chinese patients with chronic fatigue syndrome. Virol J 7: 224.
10. Knox K, Carrigon D, Simmons G, Teppe F, Zhou Y, et al. (2011) No Evidence of Murine-Like Gammaretroviruses in CFS Patients Previously Identified as XMRV-Infected. Science.
11. Paprotka T, Debvks-Frankenberry KA, Cingož O, Martinez A, Kung HJ, et al. (2011) Recombinant Origin of the Retrovirus XMRV. Science.
12. Tuke PW, Tettmar KI, Tamuri A, Stoye JP, Tedder RS (2011) PCR master mix harbour murine DNA sequences. Caevat emport! PLoS One 6: e19953.
13. Stanos KS, Aloia AL, Hicks JL, Esopi DM, Steranka JP, et al. (2011) Identification of replication competent murine gammaretroviruses in commonly used prostate cancer cell lines. PLoS One 6: e20074.
14. Smith RA (2010) Contamination of clinical specimens with MLV-encoding nucleic acids: implications for XMRV and other candidate human retroviruses. Retrovirology 7: 108.
15. Sato E, Furuta RA, Miyazawa T (2010) An endogenous murine leukemia virus genome contaminant in a commercial RT-PCR Kit is amplified using standard primers for XMRV. Retrovirology 7: 110.
16. Robinson MJ, Erlich OW, Kaye S, Weber J, Cingoz O, et al. (2010) Mouse DNA contamination in human tissue tested for XMRV. Retrovirology 7: 108.
17. Oakes B, Tai AK, Cingoz O, Henefield MH, Levine S, et al. (2010) Contamination of human DNA samples with mouse DNA can lead to false detection of XMRV-like sequences. Retrovirology 7: 109.
18. Erlich O, Robinson MJ, Duskan S, Weber J, Kaye S, et al. (2011) DNA extraction columns contaminated with murine sequences. PLoS One 6: e23484.
19. Simmons G, Glynn SA, Holmberg JA, Coffin JM, Hedwett JK, et al. (2011) The Blood Xenotropic Murine Leukemia Virus-Related Virus Scientific Research Working Group: mission, progress, and plans. Transfusion 51: 643–653.
20. Morse HC (2007) Retroelements in the Mouse. In: Fox JG, Barthold S, Davison M, Newcomer CE, Quimby FW, Smith A, eds. The Mouse in Biomedical Research. 2nd ed. St. Louis, MO: Elsevier, Inc. pp 269–279.
21. van der Kuy AC, Cornelis M, Berkhout B (2011) Of mice and men: on the origin of XMRV. Frontiers in Microbiology 1: 1–7.
22. Miyazawa T (2010) Endogenous retroviruses as potential hazards for vaccines. Biologicals 38: 371–376.
23. Yu Y (2010) Phenotypic and genotypic characteristics of Japanese encephalitis attenuated live virus vaccine SA14-12-4 and their stabilities. Vaccine 28: 3637–3641.
24. Victoria JG, Wang CL, Jones MS, Jiaing C, McLaughlin K, et al. (2010) Viral Nucleic Acids in Live-Attenuated Vaccines: Detection of Minion Variants and an Adventitious Virus. Journal of Virology 84: 6033–6040.
25. Hussain AM, Johnson JA, Freire MD, Heneine W (2005) Identification and characterization of avian retroviruses in chicken embryo-derived yellow fever vaccines. Investigation of transmission to vaccine recipients. Journal of Virology 77: 1105–1111.
26. Tsang SX, Switzer WM, Shamguvam V, Johnson JA, Goldsmith C, et al. (1999) Evidence of avian leukaosis virus subgroup E and endogenous avian viruses in measles and mumps vaccines derived from chicken cells. Investigation of transmission to vaccine recipients. Journal of Virology 73: 5043–5051.
27. Switzer WM, Jia H, Holan O, Zheng H, Tang S, et al. (2010) Absence of evidence of xenotropic murine leukemia virus-related virus infection in persons with chronic fatigue syndrome and healthy controls in the United States. Retrovirology.
28. Switzer WM, Jia H, Zheng H, Tang S, Heneine W (2011) No Association of Xenotropic Murine Leukemia Virus-Related Viruses with Prostate Cancer. PLoS One 6: e19065.
29. Beasley DW, Lethewhale P, Solomon T (2008) Current use and development of vaccines for Japanese encephalitis. Expert Opin Biol Ther 8: 95–106.
30. Shepherd AJ, Wilson NJ, Smith KT (2003) Characterisation of endogenous retrovirus in rodent cell lines used for production of biologicals. Biologicals 31: 251–260.
31. Garson JA, Kellam P, Towers GJ (2011) Analysis of XMRV integration sites from human prostate cancer tissues suggests PCR contamination rather than genuine human infection. Retrovirology 8: 13.
32. Sakuma T, Hue S, Squillace KA, Tonme JM, Blackburn PR, et al. (2011) No evidence of XMRV in prostate cancer cohorts in the Midwestern United States. Retrovirology 8: 23.
33. Qin X, Swanson P, Tang N, Leckie G, Devare S, et al. (2011) Prevalence of XMRV in blood donors, HTLV and HIV cohorts. Retrovirology 8: A222.
34. Maric R, Pedersen F, Kjeldbjerg A, Moeller-Larsen A, Bahrami S, et al. (2011) Prevalence of polytropic gammaretroviruses in a Danish multiple sclerosis cohort. Retrovirology 8: A213.
35. Lobigs M, Pavé M, Hall RA, Lobigs P, Cooper P, et al. (2010) An inactivated Vero cell-grown Japanese encephalitis vaccine formulated with Advax, a novel adjuvant, induces protective neutralizing antibody against homologous and heterologous flaviviruses. J Gen Virol 91: 1407–1417.
36. Shirato K, Miyoshi H, Kariwa H, Takashima I (2005) Detection of West Nile virus and Japanese encephalitis virus using real-time PCR with a probe common to both viruses. Journal of Virological Methods 126: 119–125.