No evidence of xenotropic murine leukemia virus-related virus infection in Brazilian multiply transfused patients with sickle cell disease and beta-thalassemia major

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SUMMARY

Although xenotropic murine leukemia virus-related virus (XMRV) has been regarded as a laboratory contaminant, it remains one of the most controversial viruses. The objective of the study was to determine if XMRV is present in 44 patients with beta-thalassemia major, 48 with sickle cell disease, and 89 volunteer blood donors. After RNA/DNA extraction from plasma/buffy coat the samples were screened for XMRV sequences by conserved nested *GAG* primers. None of the RNA samples showed a positive result. Surprisingly, four DNA samples obtained from blood donors were positive for XMRV provirus. The subsequent phylogenetic analysis revealed that these sequences are identical to the positive control (murine leukemia retrovirus) and are probably consistent with laboratory contamination. XMRV infection (provirus and viral RNA) was absent in multiply transfused patients and volunteer blood donors. The positive result obtained from some blood donors probably reflects laboratory contamination. We believe that XMRV does not pose risk to blood transfusion.

KEY WORDS: Xenotropic murine leukemia virus-related virus, XMRV, Sickle cell disease, Beta-thalassemia major, Blood donors.

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Xenotropic murine leukemia virus-related virus (XMRV) remains a paradigm and during its short existence as an emerging viral agent has caused serious controversy. The main concern was whether XMRV really contributes to the development of clinical conditions like prostate cancer and chronic fatigue syndrome. The subsequent denial that XMRV is a human pathogen was so outstanding that all approaches for virus research were influenced. As a result special caution must be taken when characterizing any emerging virus. It is important therefore, to rule out possible DNA contamination from external sources (generally animal origin). Nowadays, DNA contamination has been more frequent since there is huge variety of amplification techniques with increased sensitivity. Moreover, XMRV discovery was also a reflection of how special caution must be taken,when associating an emerging virus to a specific disease and thus providing hope of treatment for millions of patients.

In 2006, genome sequences of unknown gam-

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maretrovirus were detected by the research group of Robert Silverman (Cleveland Clinic, Ohio). The detection was made by Virochip microarray/PCR in USA patients with a rare type of prostate cancer (Urisman *et al*., 2006). The genetic similarity between these isolates and murine leukemia viruses (xenotropic/endotropic) led to the final designation of this emerging virus as xenotropic murine leukemia virus-related virus (Urisman *et al.*, 2006). Shortly thereafter, the infectious nature of the provirus, viral receptors, and XMRV integration sites in the human genome were characterized (Dong *et al*., 2007). Clinical studies performed by that time demonstrated that patients with prostate cancer have a high prevalence of both anti-XMRV IgG (Arnold *et al*., 2010) and provirus (Fischer *et al*., 2008; Schlaberg *et al*., 2009). In 2009, a study published in *Science* established a relationship between XMRV and chronic fatigue syndrome, with detection of XMRV proviral DNA in 67% of the blood samples obtained from patients with this entity (Lombardi *et al*., 2009).

However, accumulating studies were not able to confirm previously obtained results (Urisman *et al*., 2006; Lombardi *et al*., 2009) or to establish a relationship between XMRV and disease (Hohn *et al*., 2009; Erlwein *et al*., 2010; van Kuppeveld *et al*., 2010). This controversy led to questioning about XMRV existence as a viral pathogen (Dolgin, 2010). Between 2010 and 2011, data describing XMRV as an artifact due to laboratory contamination with endogenous murine sequences were alerting (Robinson *et al*., 2010; Oakes *et al*., 2010; Sato *et al*., 2010; Kaiser, 2011; Yang *et al*., 2011). Concerns that such a contamination can be explained by the continuous use of mouse cell lines for the production of laboratory reagents or extraction kits have been raised (Bacich *et al*., 2011; Wolff and Gerritzen, 2011; Zheng *et al*., 2011; Erlwein *et al*., 2011). Due to the significant concern expressed for the validity of the results reported by Lombardi *et al*. (2009) in *Science*, that article was retracted in 2011 (Alberts, 2011). As a consequence, XMRV has been declared a contamination artifact with non-existent transfusion risk (Dodd *et al*., 2012).

Although studies on XMRV have been performed generally in the USA (Sakuma *et al*., 2011; Ali *et al*., 2011; Gingaras *et al*., 2012), Europe (Gray *et al*., 2011; Korn *et al*., 2012; Touinssi *et al*., 2012; Maggi *et al*., 2012; Oltra *et al*., 2013), Australia (Rezaei *et al*., 2013), some Asiatic (Hong *et al*., 2010; Furuta *et al*., 2011; Mi *et al*., 2012; Matsumoto *et al*., 2012) and African countries (Tang *et al*., 2011; He *et al*., 2012), there is still no information on this virus in South America. As a result, we performed a study evaluating the presence of murine retroviral sequences/XMRV in Brazilian multiply transfused patients (sickle cell disease, beta-thalassemia major) and volunteer blood donors. In order not to introduce any murine retrovirus contaminant in our laboratory, the positive control sequence was obtained from murine leukemia retrovirus (MLV) detected in laboratory mice. Peripheral blood mononuclear cells (provirus) and plasma (viral RNA) were analyzed simultaneously for the presence of XMRV/MLV sequences by the use of conserved *GAG* region primers.

From May to August 2013, 181 blood samples were collected from 92 multiply transfused patients (44 patients with beta-thalassemia major and 48 patients with sickle cell disease), and 89 volunteer blood donors. The patients with beta-thalassemia major (median age, 26.6 years; range, 4-62 years; 56% male), and the patients with sickle cell disease (median age, 22.2 years; range, 2-52 years; 51% males) were continuously receiving transfusion therapy with packed red cell concentrates obtained from Brazilian donors. The patients with beta-thalassemia major showed 25% (n=11/44) anti-HCV IgG seroprevalence, and no anti-HIV-1/2 IgG seropositivity. The patients with sickle cell disease showed 2% (1/48) anti-HCV IgG prevalence and no anti-HIV 1/2 IgG seroreactivity. The volunteer blood donors (mean age, 35.7 years; range, 18-65 years; 55% males) did not report signs of acute infection prior to blood donation. They were seronegative for anti-HIV-1/2, anti-HCV, anti-HBc, anti-HTLV-1/2, anti-*Treponema pallidum* and anti-*Trypanosoma cruzi* IgG. Both patients and donors attended the Regional Blood Center of Ribeirão Preto, Ribeirão Preto city, São Paulo State, Brazil and all gave their written informed consent. The study was approved by the Institutional Ethics Committee of the General Hospital at the School of Medicine of Ribeirão Preto, University of São Paulo (HC-FMRP-USP), process N. HCRP-5701/2013.

Four milliliters of total blood was collected in EDTA sterile tubes (Vacuette, Greineir Bio-One, São Paulo, Brazil) by brachial vein puncture. Plasma was separated by low speed centrifugation (1426 × *g* for 10 min) and was stored at -80°C until use. Mononuclear cells were separated by erythrocyte lysis using specific buffer (0.144 M NH_4Cl, 0.01M NH_4HCO_3). Viral RNA was extracted from 140 µl of plasma using QIAamp Viral RNA Mini Kit (QIAGEN, São Paulo, Brazil) according to the manufacturer´s instructions. Cellular DNA was extracted using Gentra Puregene Purification Kit (QIAGEN, São Paulo, Brazil). In brief, after cell lysis, DNA was precipitated by isopropyl alcohol and co-purified by 70% ethanol. After draining, the pellet was resuspended in 100 µL DNAse/RNAse free water.

To obtain cDNA, the extracted RNA was submitted to reverse transcription. The High Capacity cDNA Reverse Transcription Kit (Life Technologies, São Paulo, Brazil) was used. In brief, 10X RT Buffer, 100 mM dNTPs, 10X RT random primers and MultiScribe™ Reverse Transcriptase (50 IU/µl) were applied in a 25 µl reaction volume. The reaction conditions included initial activation step at 25ºC for 10 min, and reverse transcription step at 37ºC for 120 min. After reverse transcription, the samples were submitted directly to PCR amplification.

MLVs (including XMRV) were detected by in-house nested-PCR with conserved primers for a partial *GAG* gene sequence. The first primer pair gagF (5´TGGGAGGCACTTGCCTATGA3´) and gagR (5´GGTGATGAGGACGGACTCAATCA3´) amplified a 524 bp gene portion. The reaction conditions included initial denaturation at 95ºC/5 min, followed by 40 cycles of 95ºC/30s, 55ºC/30s, and 72ºC/1:30s and a final elongation step at 72ºC/10 min. The reaction mix included 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 250 nM forward and reverse primer, 1.25 U Taq DNA polymerase (Life Technologies, São Paulo, Brazil), and ~ 50 ng DNA in 50 µl final volume. A second reaction was performed immediately using a second primer pair XMRV-FN (5´CTAAACCCCCTCCTTTACCG3´) and XMRV-RN (5´CCGCCTCTTCTTCATTGTTC3´). The reaction was applied in 50 µl final volume and contained 5 µl amplification product of the first reaction, 1X PCR buffer, 3 mM $MgCl_2$, 300 µM dNTPs, 250 nM of each primer, and 2 U Taq polymerase (Life Technologies, São Paulo, Brazil). The cycling conditions were the same as the first reaction. The estimated sensitivity of the reaction was 10 copies/reaction. In case of a positive result, and to exclude reaction contamination, another nested-PCR targeting the *ENV* gene of XMRV was applied. The primer sequences and the reaction conditions have already been described by Danielson *et al.* (2010). In order to consider our laboratory XMRV-free, as a positive control we used a xenotropic murine retroviral *GAG* fragment obtained after amplification of spleen DNA from discarded laboratory C57BL/6J mice (black 6). The sequence was characterized genotypically and phylogenetically as belonging to the murine leukemic viruses group. In all of the reactions, the positive control was used at a concentration of 10^3 copies/reaction. The amplification products were revealed in 2% agarose gel (Life Technologies, São Paulo, Brazil) under UV light (ImageQuant, General Electric, Fairfield, CT, USA). For phylogenetic analysis, a 233 bp partial conserved *GAG* sequence, obtained from nested PCR was submitted to sequencing. The sequencing was performed using Big Dye® Terminator Cycle Sequencing Kit v. 3.1 (Life Technologies, São Paulo, Brazil) and 500 nM of each XMRV-FN/XMRV-RV primers. The cycling conditions included initial hold of 95ºC for 1 min, followed by 25 cycles consisting of denaturation step at 96°C for 10 s, annealing at 50°C for 5 s and elongation at 60ºC for 4 min. For all phylogenetic procedures the MEGA v.6 software was used.

All plasma samples obtained from patients and donors were negative for XMRV RNA. Similarly, all peripheral blood samples obtained from patients were negative for XMRV provirus. Surprisingly, four peripheral blood samples samples obtained from blood donors demonstrated positive results for murine provirus. Consequently, they were sequenced and analyzed phylogenetically to confirm the origin of the sequence. The phylogenetic analysis demonstrated full identity of the obtained sequences with the positive control (100% genetic identity). The BLAST comparison revealed 99% identity with mouse chromosomes 7 (nt 93,923-94,046) and chromosome 19 (nt 19,583-19,706), and 98% with a wide range of MLVs (Figure 1).

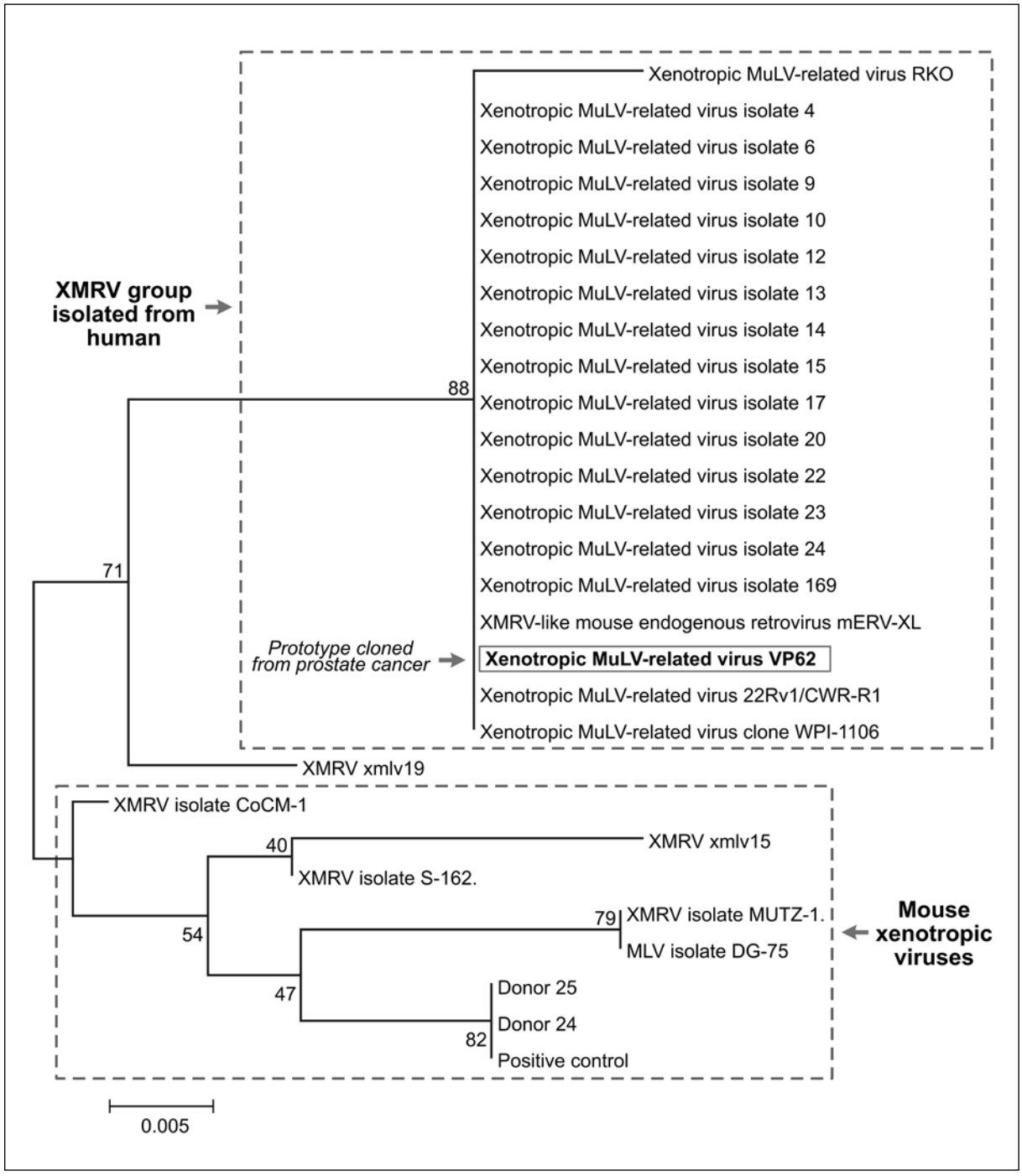

FIGURE 1 - *Phylogenetic analysis of XMRV isolates obtained from volunteer blood donors. Neighbor-joining phylogenetic tree based on 233 bp partial nucleotide XMRV/MLVs GAG sequence derived from 19 XMRV, six MLVs and three new isolates and inferred by the MEGA v.6. software package. The bootstrap probabilities are expressed in percents on all branches. The tree was characterized with separation of the isolates into two main clusters: the human-derived XMRV and murine leukemia retroviruses. The isolates obtained from the positive samples (Donor 24 and Donor 25) were genetically identical to the positive control (derived from mouse leukemia retrovirus), and belonged to the group of the murine leukemia viruses. These results are consistent with laboratory contamination, probably during manipulation with reagents. Bar: 0.005 nucleotide substitutions per site per year.*

Once our positive control was derived from a cloned partial MLV *GAG* gene, we believe that the obtained results represent laboratory contamination which occurred during manipulation with PCR reagents. This is additionally confirmed by the nested PCR performed for another viral gene (*ENV*), which showed negative results for all XMRV *GAG* positive samples. Therefore, no XMRV-like sequences could be confirmed in Brazilian multiply transfused patients and volunteer blood donors. We confirm previous studies demonstrating a high risk of cross contamination with XMRV/MLV sequences but we believe that the risk of transfusion-transmitted XMRV infection is low or nonexistent.

In this article, we discuss the detection of four suspected XMRV provirus sequences in peripheral blood samples from healthy blood donors. These samples have already been determined as XMRV RNA negative. The phylogenetic analysis performed confirmed that they are genetically identical with the positive control which presents a partially cloned MLV *GAG* sequence. Therefore, the detected sequences seem to have resulted from possible laboratory contamination. The genetic BLAST analysis performed additionally revealed 99% similarity between both positive samples and control with mouse chromosomes 7 and 19 (containing mouse endogenous retroviral elements) reconfirming the murine origin of the detected isolates.

XMRV has doubtful existence but it is genetically very close to MLVs. Nevertheless, the constructed phylogenetic tree, using partial *GAG* sequences, demonstrated that both positive control and donor isolates were located in the group of MLVs (Figure 1). This analysis and the additional negative results obtained for the amplification of the XMRV *ENV* gene confirm that contamination with MLV sequences probably occurred during manipulation with laboratory reagents. The repeated freezing and thawing as well as the close proximity of patient and donor samples must also have contributed to the contamination. Lo *et al*. (2010) detected sequences much closer to MLV than XMRV in blood samples from patients with chronic fatigue syndrome, which was consistent with laboratory contamination (Hong and Li, 2012). If the sequences obtained from donor samples in our study were independently derived, they would show increased genetic diversity compared to the positive control (Knox *et al*., 2011). Therefore, any similarity to XMRV will be detected in the phylogenetic analysis, where all reference XMRV sequences formed a highly identical cluster with the prototype strain VP62 (obtained from prostate cancer tissue) in comparison to the reference MLV sequences.

The other possibility as discussed by other authors is that the positive samples were previously contaminated with mouse DNA. Such hypothesis is unacceptable, as all patient samples were obtained from laboratories that never worked with mouse retroviral sequences. The obtained samples were opened only prior to starting laboratory work and the laboratory of performance had never come into contact with murine sequences. Hué *et al*. (2010) demonstrated a high probability of obtaining false-positive MLV results using primers detecting conserved XMRV/MLV regions in human samples contaminated occasionally by murine DNA (Hué *et al*., 2010). All studies therefore, establishing relationship between XMRV and disease should consider possible PCR contamination. Any study reporting identification of XMRV DNA in patients or healthy individuals should include rigorous PCR tests and phylogenetic studies to exclude the possibility of contamination. In our case, the contamination was suggested regarding the results of the phylogenetic analysis and the absence of detectable XMRV RNA in any of the samples.

Contamination of laboratory reagents with murine retroviral sequences could also contribute to the detection of positive XMRV samples using conserved primers (Hué *et al*., 2010). Special concern has been raised about the contamination of commercial hot-start *Taq* polymerase-containing monoclonal mouse antibodies with traces of MLV sequences (Sato *et al*., 2010; Zheng *et al*., 2011). We do not believe that this is our case due to the low number of positive results (four DNA samples in a total of 362 performed tests). Therefore, we attribute this contamination to handling out laboratory reagents or continuous manipulation of samples (freezing/thawing).

It has not been established whether XMRV can exert pathogenetic effects on humans, nor if it is the ethiologic agent involved in human dis-

eases like cancer or chronic fatigue syndrome (Hong and Li, 2012). Patients with hematological disorders like beta-thalassemia major and sickle cell disease who receive multiple blood transfusions present suitable model to evaluate the risk of transfusion-transmitted emerging viruses (in this respect XMRV). As our results do not confirm real XMRV sequences in all patients with sickle cell disease and beta-thalassemia major, we believe that XMRV/MLV does not pose a risk for the transfusion medicine and blood recipients. One question which cannot be ignored is that the number of patients and donors tested was relatively small and this study may not reflect the real situation with XMRV in Brazil. However, we tested all multiply transfused patients with sickle cell disease and beta-thalassemia major attending our Blood Center (92 individuals) and, therefore, this number is regionally significant. It is pertinent, nevertheless, that other blood centers including those located in large metropolitan zones perform studies regarding the transmission risk of this doubtful virus in Brazil.

In conclusion, we have found no evidence for XMRV/MLV sequences in blood samples obtained from multiply transfused patients. The positive results obtained from the peripheral blood of some healthy blood donors reflect possible laboratory contamination with sequences of the positive control. In this respect, we demonstrate the ease of contamination with murine sequences during manipulation with laboratory reagents. Such an event can lead to wrong interpretation of the results and false positives. Therefore, we believe that the detection of XMRV DNA in other groups of patients, especially those with prostate cancer and chronic fatigue syndrome, reflects laboratory contamination. We also believe that XMRV does not pose a risk to blood transfusion.

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