Author’s response to reviews

Title: BOVINE LEUKEMIA VIRUS DISCOVERED IN HUMAN BLOOD

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Although the reviewers are apparently satisfied with the revised paper, as an editor, however, I have identified a number of issues that need to be addressed. Fig. 1 is completely missing from the revised paper. As it is referred to in the text, I assume it has been deleted in error.

Response: The figure was uploaded on Editorial Manager, but we assume that somehow it did not go through. It has been uploaded again on this resubmission and I am sending you a copy personally in case it does not go through again.
The references are messy. For instance, line 360, ref. 20 is not Blast; ref. 19 (Blast) is not the proper reference in lines 342 and 446. Please check the other refs carefully.

Response: It again appears that Editorial Manager has messed things up (has happened to me before both as an author and a reviewer). The reference numbers you mention correspond to the numbers used for the first submission, not the revised version. Since the references have to be submitted as a separate attachment, it is likely that Editorial Manager attached the references from the first submission instead of the revised submission. Because of the mistakes made for the figure and the references, I am sending you the manuscript directly in addition to sending it through Editorial Manager.

Other comments:

Line 235: why not show the results of patients with sequence identity to the cultured virus? Now it appears that the finding needs hiding.

Response: We have revised Figure 1 to include the LTR sequences of all of the 23 study subjects who were positive for the LTR region. We have also added as reference sequences, one representative sequence from each of the 10 BLV genotypes described in Polat et al. The identity and geographical source of the reference sequences is given in the Figure 1 legend. All bases are written as letters to verify that nothing is hidden. However, in order to meet the journal’s limitation of figure size (11.5cm wide and 18cm long), the size of the figure has also been adjusted, which makes the size of the letters very small and difficult to read.

Lines 301-302: no retrovirus produces its own RNA; viral genomic DNA is transcribed by host RNA polymerase II. Please reword.

Response: The text of the second paragraph of the discussion has been reworded to use the phrase "host cell assisted production of RNA copies of its genome”

Lines 310-312 are a repeat of lines 309-310 ("the results of this study...")
Response: Once again, this must be an Editorial Manager mishap; the revised submission did not have a repeat of these lines;

Line 335: when the whole viral genome is deleted, the resulting LTR reminds one of the solitary LTRs of endogenous retroviruses. As suggested in lines 305-306, the majority of BLV viral DNA are linear or circular DNA genomes residing in the cytoplasm. But solitary LTRs are remnants of genomes integrated into nuclear DNA whereby the coding parts are deleted by homologous recombination between the LTRs. The reasoning to target the LTR because it would not be deleted from the genome makes little sense when apparently targeting cytoplasmic viral genomes. Please revise.
Response: We have revised the text to indicate that “genome” means the BLV genome not the human genome. We have also revised the text to clarify how BLV is different from some other families of retroviruses in that it does not usually integrate into the host cell genomic DNA and it is common to have unintegrated copies in the cytoplasm (Discussion section, paragraph 2, line 5).

Although HIV integrates into host cell DNA relatively soon after infection, BLV is in a different retrovirus family and does not behave the same way. Lentiviruses such as HIV have a mechanism to pass through the nuclear membrane once they have entered the cell. However, Deltaretroviruses (HTLV and BLV) do not have this mechanism and have to wait until cell division, when the temporary dissolution of the nuclear membrane occurs and allows the viruses to enter the nucleus. Evidence from studies on BLV in cattle has indicated that BLV usually does not integrate into the host genome DNA until very late stages of the development of leukemias and lymphomas when leukocyte cell division is more frequent. It exists primarily as linear and circular forms in the host cell cytoplasm.

Line 355: “Another strength of this study is that the primer sequences were chosen because of their high homology with BLV”. This statement should be modified. When testing the first LTR primer given in Table 1 myself, Blast analysis displayed 100% homology, next to BLV at the bottom of the list, with just about everything in the nucleotide database. Also, the primers used here are very short, 16-17 nt, which increases their ability to amplify non-target sequences.

Response: This is a very important issue and we are grateful that you mentioned it. The BLAST site can be very complicated and give misleading results if it is not used correctly. We have added more detail (Discussion section, paragraph 5) to indicate more clearly exactly how we utilized the BLAST site. The purpose of testing primer specificity on the BLAST site was to compare primer specificity for only two species: bovine leukemia virus and homo sapiens, and to insure that the primers we used were highly specific for bovine leukemia virus and were not specific for the human genome and would not be likely to amplify any stretch of human genomic DNA. Since the nucleotide data base includes sequences for 49,985,097 different species of organisms, we followed the advice of the BLAST instruction site on “Home” to narrow the search in the nucleotide database to a particular species (filling in box entitled “organism”) to get accurate information. Therefore, under the section “Choose search set,” we entered “bovine leukemia virus” in the box labeled “organism.” The default setting on the BLAST site is to search for sequences with “somewhat similar sequences.” When using BLAST we always set the parameters to search only for “highly similar sequences,” which greatly reduces the number of nonspecific matches. BLAST reverts to the default setting of “somewhat similar searches” after each search, so it has to be reset to “highly similar searches” before each separate search. When the search results came up we looked only at genomes, not “transcripts” or “protein” matches, since our study was based strictly on DNA genome similarities. In addition, as stated on BLAST, the program automatically controls for short sequences, which helps reduce probability of amplifying non-target sequences homologous with the primer being evaluated. Using these BLAST settings, we did not find 100% homology of the primer sequence with anything except BLV.
The prevalences given here are much higher than those shown in the tables. Please re-examine.

Response: You are quite correct and very observant. Thank you so much for catching this error! After bringing this to our attention, we realized that the Excel spreadsheet sent to our biostatisticians was the earlier version that was not updated. The newer, correct spreadsheet contained the antibody results with the ROC corrections which the previous spreadsheet did not have. ROC corrections correct for potentially false positive results and tend to lower the number of positive ELISA tests. The newer spreadsheet also included the final 7 subjects tested for antibodies, which the earlier spreadsheet did not include. We reexamined the total number of subjects for whom we had information for all 3 factors included in the statistical analysis: age, antibody test data, and PCR test data, and it is 95. A new statistical analysis has been completed based on the updated final spreadsheet and we have corrected all numerical ratios given throughout the manuscript to be based on the full population of 95. This correction does not change any conclusion or basic ideas related to the overall manuscript. However, hopefully it should eliminate the numerical discrepancies in the manuscript.

And my last remark on the original manuscript, “When claiming such an important finding as stated above, a much more thorough sequencing and phylogenetic analysis would be expected. All fragments should be sequenced and trees should be constructed which incorporate reference sequences from all ten known BLV genotypes, not only a single refseq to compare a fragment of the LTR by eye””, is still valid and has not been addressed. The reader should get an idea of global sequence variation within BLV to estimate the importance of the current findings.

Response: We have added to Figure 1 at least one reference sequence representing eight of the 10 BLV genotypes described in Polat et al [1] for which a sequence for the LTR region has been deposited in GenBank. Now the reader can directly compare the sequenced segments from our LTR positive subjects with these published sequences. This should give the reader an idea of the degree of global LTR sequence variation of cattle and how the sequences of the US human subjects compare to the GenBank BLV LTR sequences from cattle in different global regions. As Figure 1 illustrates, sequence variations among both reference sequences and sequences from the study subjects are extremely few. For all human specimens positive for the BLV LTR region and sequenced for this region, only 4 showed variations from the consensus sequence of reference sequences. Three of the 4 variations did not match the sequence of any sequence posted on GenBank.

Regarding sequencing all fragments of the BLV genome and constructing phylogenetic trees, how can that be done if all genome fragments are not present i.e. were already deleted in vivo when we acquired the specimen? Deletion is common in cattle and apparently also happens in humans. Only 5% of all BLV positive specimens in this study contained even 3 genome regions (LTR, tax, and gag) and most BLV positive specimens had only 1-2 genome regions present.
Not a single one of our 23 specimens sequenced for LTR region had the entire BLV genome present. Furthermore, there were so few LTR variations from the reference sequences that it was not possible to obtain a meaningful phylogenetic tree. While it certainly would be useful and important to have more sequences and do phylogenetic analyses, humans are not as easy as cattle to get ideal specimens from, especially given the smaller volume of blood obtainable. Our goal in doing this research project was to determine if the blood cells of any humans were infected with BLV. It was not our goal to determine the phylogenetic relationships of BLV isolates from different parts of the globe. This is an important future project, but as stated in the final paragraph of the discussion, it is not possible without the contributions of researchers in other parts of the world who are willing to collect human blood specimens and analyze them for the presence of BLV. This was emphasized in the final paragraph of the manuscript and, hopefully, the publication of this manuscript will stimulate other researchers to participate in a global endeavor.

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Reviewer reports:

James Lawson (Reviewer 1):

The Editor and the second reviewer raised highly relevant questions. These questions are important because of the serious implications of the outcomes of this current study.

The authors appear to have carefully and successfully answered these questions.

The outcomes of this current study, when considered in the context of previous reports of bovine leukemia infections and cancer in humans, indicate a need to control bovine leukemia viral infections in cattle on a global basis.

In my opinion the manuscript should be published without further changes.

Maria Carolina Ceriani (Reviewer 2): To the authors: I understand that the authors have made all the changes, and add the additional information that was required by the reviewers and the editor. The manuscript is really well written and there were only some recommended changes and/or doubts to be clarified or introduced in order to be accepted for publication. I strongly congratulate the authors for their efforts.