Author’s response to reviews

Title: BOVINE LEUKEMIA VIRUS DISCOVERED IN HUMAN BLOOD

Authors:

Gertrude Buehring (buehring@berkeley.edu)
Anne DeLaney (anne.delaney@me.com)
HuaMin Shen (huamin@berkeley.edu)
David Chu (dchu1201@gmail.com)
Niema Razavian (niemaraz@umich.edu)
Daniel Schwartz (daniel.schwartz@ucsf.edu)
Zach Demkovich (demkovich@rti.org)
Michael Bates (michael_bates@berkeley.edu)

Version: 1 Date: 11 Dec 2018

Author’s response to reviews:

Editor Comments:

In addition to the comments made by the two reviewers, I have some editorial concerns as claiming such an important finding as widespread infection of the human population by a bovine oncovirus needs rigorous proof:

- Firstly, how did the authors control for contamination from their control cell line, as PCR reactions are exceptionally prone to contamination? Dedicated labs? Negative PCR controls?
This is one of the most important questions that can be asked. As you mentioned, DEDICATED LABS are an important aspect of preventing contamination among the specimens and the positive control. We have the following dedicated lab spaces that are used for separate functions:

1. Preparation of PCR reaction mix: This is done in a dedicated locked, DNA-free room with entrance and ventilation system separate from the main laboratory and with a dedicated laminar flow hood, small equipment (pipettors, etc.), plasticware and reagents. No specimens, cell lines, or other research materials containing DNA are allowed in this room.

2. Addition of template DNA to the PCR reaction mix: This is done in a different room in a small hood dedicated to specimen handling. Immediately after adding each specimen sample, the lid of the PCR tube is pressed tightly shut; then the hood, small equipment (pipettor), and gloved hands of the technician are decontaminated with DNA decontamination solution (RNase AWAY, Molecular BioProducts, San Diego, CA (USA). At the end of a session, the hood is exposed for 30 minutes to UV light at a dose that inactivates DNA.

3. Processing of the positive control cell line: This is done last after all specimens and the negative control have been added to the PCR tubes and the lids pressed tightly shut. The positive control DNA is added or otherwise manipulated ONLY in a fume hood (velocity=127fpm), which pulls air in from the general lab and vents it out to the outside atmospheric air, thus preventing any contaminating positive control aerosols from entering the general laboratory work area from the fume hood. The positive control DNA is NEVER manipulated in any way in the general laboratory area, including the small hood dedicated to specimen handing (item #2 above).

An abbreviated version of the above information about dedicated lab spaces has been added in the Methods section under the subheading “Precautions to prevent DNA contamination.”

NEGATIVE CONTROL: The negative control was sterile, filtered distilled water added to the reaction mix in the same volume as the samples added to the reaction mix. This information has been added to the text under the DNA extraction sub-heading.
As a means of quality control, we performed post PCR verification that BLV-positive human samples were not the result of contamination by the positive control cell line.

1. We tested all BLV positive DNA samples for the presence of a sheep housekeeping gene (mitochondrial cytochrome C oxidase) present in all cells of the positive control cell line but not present in the human genome [13]. None of the human specimens showed this sheep gene.

2. The DNA of all BLV-positive human samples was tested for a specific region in the envelope gene of BLV in which the FLK control line contains a signature mutation not present in any BLV sequences deposited in the NCBI repository of virus sequences. All human specimens were negative for this signature mutation. The results of both of these assays have been added to the text in the second paragraph of the results section (below Figure legend).

NEGATIVE CONTROL: The negative control was sterile, filtered distilled water added to the reaction mix in the same volume as the samples added to the reaction mix.

Many samples were only positive once, which could be due to low viral copies, but could also be due to contamination.

I am assuming you mean “positive for only one gene segment?” If this is what you mean, the best explanation is what is given in the second paragraph of the Discussion. The LTR and tax regions are the most highly conserved, but in cattle, the gag, pol, and env regions (coding for virion proteins) are frequently deleted, presumably to escape immune system detection.

- Why not do real-time PCRs? More convincing, faster and less prone to contamination.
It’s true that the lids of the tubes used in standard PCR may pop open more easily than the lids of smaller tubes previously used for RT-PCR. However, in the last 15 years that we have been doing standard PCR 2-5 times per week, and pressing the tube lids firmly shut, only one tube has ever popped open. The PCR machine was thoroughly cleaned afterwards and the hole each tube was in are cleaned after each run. All closed PCR tubes are thoroughly wiped with the nucleic acid decontaminant (RNAse away) mentioned above, before putting them into the machine. DNA contamination could conceivably also occur in current RT-PCR, techniques in which the reaction is done in a tray that is inserted into the machine to read. Although the tray is covered with a tight seal after the samples are loaded, the wells of the tray are extremely tiny, and loading the wells accurately requires good manual dexterity. Accidentally touching the pipette tip onto the edge of the incorrect well or misplacing a little liquid of the sample into the incorrect well could result in a false reading.

We agree that RT-PCR is faster and has remarkable ability to quantitate results. However, for purposes of the experiments reported in this manuscript, quantitation was not necessary since we are not analyzing or comparing viral loads. Also, for our project, speed was not the most important factor since our work is research and development, not clinical diagnosis. The fact that standard PCR may be less sensitive than RT-PCR means that the magnitude of BLV-positive samples in the human population may be even higher than we report here.

- Why was viral RNA not tested using an RT-PCR? In a real infection, viral RNA load is expected to be much higher than integrated DNA in haemolysed PBMC.

This is an excellent question and indicates that we need to include more background information about the life cycle of BLV and other Deltaretroviruses, which is very different from other families of retroviruses such as Lentiviruses, the family to which HIV belongs, and which might be more familiar to many scientists. Based on studies of BLV in cattle and experimental sheep, and HTLV (human T-cell leukemia virus) in humans, Deltaretroviruses rarely replicate and produce extracellular virions into the blood. Cells become infected by direct contact with other cells already infected, or by their exosomes, but not typically by extracellular virions. Upon infection of a cell, BLV RNA quickly retrotranscribes itself into a DNA form, so the predominant form of BLV within cells is DNA, not RNA. Unlike Lentiviruses, BLV viral DNA is not able to enter the nucleus unless the nuclear membrane dissolves during mitosis. Therefore in normal humans without leukemia (rapidly dividing cells), it would be rare to find BLV in the nucleus, and unlikely to find it in an integrated state. BLV usually does not integrate into the host cell genome, but commonly exists as linear and/or circular DNA forms in the cytoplasm and nucleosome. This information has been added to the manuscript (third paragraph of the Methods
and Materials section under subheading “DNA extraction). Viral load tests are typically used to measure virus particles suspended in blood, not intracellular viral nucleic acids. Therefore, for this study, extracting retrotranscribed DNA from the leukocytes within the blood was a more efficient means of viral detection than trying to measure RNA in virus particles which are rarely produced. This information has been incorporated into the manuscript in the first paragraph of the Discussion section.

- A very good indicator of infection are anti-BLV antibodies. Was serology performed on the samples, as blood plasma can be obtained from them with your isolation method?

This is a very important point and we are pleased that you mention this. Serology was performed on the samples and we have added information about the antibody assays in the Methods and the Results sections. In the Discussion section we discuss why, in the case of BLV infection in humans, antibodies may not be an accurate indicator of BLV infection, which is why we hesitated to include the data in the first submission. The three persons who did the extensive work on the ELISA antibody testing and the ROC curves have now been added as co-authors.

In humans, antibodies to BLV have been identified in previous studies[15]. However, these antibodies may not necessarily indicate infection, as humans consume large amounts of milk and other dairy products that are pasteurized and beef that is cooked (hopefully thoroughly). Multiple previous studies indicated that pasteurizations renders BLV non-infectious.

- When claiming such an important finding as stated above, much more thorough sequencing and phylogenetic analyses would be expected. All obtained 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.

This type of work has already been done using BLV retrotranscribed DNA from cattle. Phylogenetic trees of BLV full genome sequences and various segments have been constructed and published. A good review article of this field of research is by Polat et al., Epidemiology and genetic diversity of bovine leukemia virus, Virology Journal (2017) 14:209. To do the same for human isolates would require far more specimens than we had for this study under review and would require a massive global collaboration to collect specimens from all over the world. This
is beyond the scope of the investigation reported here. In order to undertake such a project and get both collaboration and funding, there would have to be some initial data to convince collaborators and funding source reviewers that human leukocytes were actually infected with BLV and that it would be possible and convenient to obtain BLV DNA from blood specimens. So we felt that publishing this report of the presence of BLV DNA in human leukocytes is an absolutely necessary first step upon which to build a more extensive global phylogenetic study.

BMC Infectious Diseases operates a policy of open peer review, which means that you will be able to see the names of the reviewers who provided the reports via the online peer review system. We encourage you to also view the reports there, via the action links on the left-hand side of the page, to see the names of the reviewers.

Reviewer reports:

James Lawson (Reviewer 1):

This is an excellent and important study. The concepts and methods are of high quality. The background outlined in the manuscript is excellent. The authors are obviously very well informed about the underlying science.

My only suggestion is for the authors to expand the discussion to include 2 issues. (i) that there is a correlation between red meat eating populations and high prevalence of breast cancer (which is compatible with consumption of meat infected by BLV.

Breast cancer is distinctly higher in countries that consume more dairy products than in countries with lower dairy product consumption. The statistics on correlation of beef consumption also indicate an increased risk although less definitive in that in some studies beef, pork, and lamb were lumped together as “red meat.” A discussion of the beef consumption issue has been added in the last paragraph of the discussion.
(ii) identification of BLV in blood components may or may not indicate a transmission route (but is a strong possibility - exosomes in the blood may also be a possible transmission route).

Thank you for bringing up the important point about the exosomes. We have added a sentence about their possible in transmission in the first paragraph of the discussion section.

This study adds to the evidence that BLV infections in cattle are a likely risk to humans. Perhaps the authors should boldly state this. See last sentences of the Conclusion section for a somewhat bolder statement.

Given the high quality of this manuscript it is pedantic to suggest some improvements in the wording of several sentences. However the following may be helpful:

The following suggestions have been incorporated into the manuscript.

Line 26 - add the word "current" to research - "The purpose of this current research was to determine if BLV is present in human blood" This was added.

Line 28 - add "96 self selected male and female human subjects." There were 96 females but no males. The recruitment flyers were placed in a waiting room for women undergoing female specific surgery.

Line 33 - under Conclusion add "... lead to leukemia and other cancers."

I was unable to find a line 33 under “Conclusions,: but we added in line 461 of conclusions “explain why breast and other cancer types have an array of somatic cell mutations”

Line 51 - add "In cattle, BLV is found..." This was added.

Line 55 - add "... human females from Columbia, Australia and Argentina ..." Done.

Line 120 expand acronym UCB to University of California at Berkeley. Done.
Maria Carolina Ceriani (Reviewer 2): To the authors, I highly appreciate your contribution to this study. The manuscript aimed to determine the presence of BLV in human blood. BLV as a \( \delta \)-retrovirus is closely related to the human T-lymphotropic virus type 1 (HTLV-1) and both known as direct oncoviruses. Detection of BLV in human blood would be of great importance and may help improve preventive strategies, and to induce governments to implement measures in an effort to eradicate the virus mostly from those herds that are heavily infected.

However, there are some major and minor comments I want to address.

Lane 33: you seem to be convinced that BLV might lead to leukemia in humans, but all the reported studies in the literature are only referred to the association of BLV and the development of breast cancer. Do you have any certainty of this fact? Have you ever made any test? I strongly recommend for publishing to include some testing of human lymphomas to see if you can detect BLV. In the first paragraph of the discussion, we have added a reference to a published abstract describing a small survey of human cancers in various organs. For lymphomas, 1/15 was positive for BLV.

Lane 52: reference [4] does not mention that mammary epithelial cells exfoliate into milk.

This was an editing mistake. The correct reference was deleted by mistake and it has now been added..

Lane 55: you mention three different research groups, but with the exception of the paper from Mesa and col, the other groups have one author in common, which could mean that they are not really different groups.

They are actually 3 different research groups: Columbia, Argentina, and USA.
Although I was a collaborator on the work with the Argentinian group I consider it an independent research group that initiated the study and a member of that lab came to use our in situ PCR machine, which is no longer manufactured and only used refurbished machines are available. Also, members of other research groups learned from us how to do the in situ PCR technique. The other publications with my name on them all originated in my laboratory and were carried out there. I changed the wording to “5 separate investigations.”

Lane 84: I don’t believe PBMC are viable after 14 days in the refrigerator. Not more than 2-3 days is recommended. You don’t show in the results the percentage of hemolysis, but after 14 days it is expected that the blood should be completely hemolysed. Hemolysis is a term usually used to refer to the rupture of erythrocytes (red blood cells). The buffy coat on the specimens we used was clearly identifiable on a centrifuged blood specimen as a grayish white area above the larger red area occupied by erythrocytes. The amount of DNA we obtained from the leukocytes in the buffy coat was sufficient to run the assays. We have modified the text (last 6 lines of the paragraph with subheading “DNA extraction”) to make it clear that all samples were tested for DNA quality using the DNA of the GAPDH housekeeping gene present in all human cells as an indicator. Since erythrocytes in peripheral blood have no nuclei, if they did hemolyze it would not be relevant. We gave a subjective hemolysis judgement just in case hemolysis of the erythrocytes could serve as a proxy indicator of blood sample quality. However, since hemolysis of erythrocytes in the specimen was minimal and was not related to outcomes of our study it probably was not relevant and is not a good indicator of leukocyte quality. We changed the text in the paragraph Under the “blood processing” section to help clarify these issues.

In Results section, you mention the frequency of amplification for one, two or three genes, but you amplified four genes. The env gene was amplified only on samples that were positive for any of the other 3 genes. Its purpose was only to rule out contamination by the positive control cell line which has a unique signature mutation. Did you have any sample that amplified all the four tested genes simultaneously? The different genome regions were never amplified simultaneously because the required reaction conditions (temperature and cycling times) were different for each region because the primers were different.

Lane 139: "...it the most highly conserved region and has the highest degree of sequence variation." Doesn’t it sound like a discrepancy? If it is highly conserved, it is expected to have very little variations. Thank you for pointing out this possibly confusing wording. The text has been changed to clarify that “conserved” means the genome was not deleted.
Lane 162: Did you check all the positive blood samples for the mutation? Because later, you mention that env gene is usually deleted in clonal cells. Yes, all BLV samples were tested for env to rule out contamination from the control cell line. Env was present in all of these samples probably because the subjects were assumed to be free of leukemia or lymphomas because they responded to recruitment flyers in the waiting room for female related surgeries.

Lane 183-188: the primers sequences to amplify env gene, are included in Table 1. That means that env was amplified to check the sequence in several samples. How many of the tested samples were positive for env? Or it was deleted? The sequence of only two LTR is shown in the manuscript.

As the text says, only sequences that differed from the reference were shown and all others that are not shown are identical to the reference sequence. See paragraph 3 of the discussion.

Lane 218: what do you mean by "well-matched for age". It means that the age distribution of the two groups (BLV-positive and BLV-negative) was similar in distribution. The wording of the text has been changed (fourth paragraph of the discussion) and the term well-matched deleted because intentional matching for age was not part of the experimental design. Thank you for mentioning this. It needed to be corrected.

Lane 223: if you isolate PBMC, and perform genomic DNA extraction, there is not doubt that the viral genome is integrated. You need to demonstrate that they can produce infective particles released to the supernatant, as PBMC from cows.

The first paragraph of the discussion addresses this question and explain why integration is not necessary to detect the virus in cells.
Lane 235: On what assumption can you speculate that human to human transmission is feasible? We are assuming that If human to human transmission were possible it would probably be via the routes that transmission occurs in cattle: through blood and milk. In humans that would mean breast milk transmission to babies and blood transmission through blood contaminated IV needles, dialysis equipment, tattoo needles, etc., blood transfusions, perinatal exposure of baby to infected mother’s blood. However, it is too early to speculate on this and arouse concerns and fears that may be unfounded.

Reviewer reports: