Author's response to reviews

Title: Type specific Real time PCR for detection of human herpes virus 6 in schizophrenia and bipolar patients: a case control study

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Author's response to reviews:

Dear Reviewer

Dr Keely Cheslack-Postava,

Thanks so much for very useful and valuable comments.

1. Line 64-66. Reference #6 is a study on detection of HHV6 in CSF from children admitted to the ER with encephalitis symptoms. A more appropriate reference for the percent of the general population infected should be substituted.

Reference #6 was substituted and the percentage was changed according to the new reference.

2. Line 70-71. More explanation and reference(s) should be given for the statement “Nowadays the number of disease associated with HHV-6 infection is increasing” (And it should read “diseases”)

More explanation with 3 references was added.

3. Methods. Season of birth is mentioned in the results but not the methods. Also, the reason for discussing season of birth should be clarified.

Presumably, this would affect viral exposure prenatailly or soon after birth; however, this study measured viral exposure in adulthood.

In line 83-84, it was mentioned that demographic data was collected which season of birth is one of those data and we did not write them one by one.

In Lines 197-200 we explained that because in some studies there were
relationship between SC and BD with season of birth then we decided to investigate about that.

4. Line 171-173. This statement is not supported by any references or the Current study.
   Reference was added.

5. Lines 207-208. It is not clear how the results of this study lead to these Recommendations.
   We changed the conclusion.

6. Very few subjects overall (N=3) were positive, therefore, it is difficult to draw conclusions from this study and it was clearly underpowered. The authors should discuss what they hypothesize to be the relevant time window of exposure to the virus with regard to disease status, and how this relates to what was measured in this cross-sectional study. Perhaps a different time point in life or disease history is more relevant for risk of SCZ/BD?
   We changed the conclusion.

7. Table 2. Please clarify whether tests of significance refer to all cases vs. controls or to SC vs. BD vs. controls.
   Significance refer to all cases vs controls were clarified.

8. Line 144-145. “One hundred copies of viral DNA were detected in the positive samples.” This statement should be explained.
   Because we did quantitative Real time PCR then we should do and mention the viral copy number of the positive samples.

9. Minor grammatical editing/proofreading is needed throughout. Examples:
   line 68 should read “…the cause of the common …”
   line 116-117 “The positive control was” or “The positive controls were”
   line 168 “and causes”
   All edited.

10. Methods: What were the specific DSM-IV diagnostic codes included under
each of the diagnoses?
The psychiatrist diagnosis was according to DSM-IV criteria. We don’t have the details of the patient’s signs and symptoms.

Dear Reviewer
Dr Zongchang Li,
Thanks so much for very useful and valuable comments.

Given rare detection rate of HHV-6 virus in the PBMCs (1.5%) , a false Negative result may be caused due to the small sample size included in this study. The conclusion was not adequately supported by the data and a larger sample size was suggested.

For detection of HHV-6 DNA we did all the tests twice to reduce false positive results. The conclusion was corrected.

In the laboratory analysis section of the methods (Lines 110-126), were all qPCR assays were carried out in biological replicates? In addition, it stated that “The assay was validated by using a 10-fold dilution series of the positive control” , the number of serial dilution points of standard curves was not reported. This information is important to assess the quality of the assays, please add it.

In laboratory analysis we added the standard curve to show the number of serial dilutons and also we added that we did quantitative PCR.

How were control subjects recruited? If they were being evaluated clinically? If did they have other medical disorders , but just not a history of psychiatric disorders.

All controls were recruited after sex and age adjustment with the patients and all filled up the questioners. The controls with history of any illness were excluded from the study.

In the DNA extraction section of the methods, the author stated “The concentration of extracted DNA was assessed by optical density (OD) 260/280 ratios”. There was a error of this description. The 260/280 ratios was calculated evaluate the DNA purity and the concentration of DNA sample was determined by measuring absorbance at 260 nm.

In the DNA extraction section the absorbance at 260 nm was corrected.
Dear Reviewer
Glenn Konopaske,
Thanks so much for very useful and valuable comments.

Major compulsory revisions:
1) In the second paragraph of the discussion, the authors state that SITH-1 "causes" mood disorders. Mood disorders are multifactorial. SITH-1 can contribute to the risk of mood disorders, but is unlikely to be the sole cause. It was corrected.

2) In the third paragraph of the discussion, the authors state that the study Did not find an association between HHV-6 and "psychiatric disorders." This Is untrue, the study did not find an association between HHV-6 and Schizophrenia or bipolar disorder. It was corrected.

3) Since there are three groups, independent t-test are inappropriate for the continuous variables. An ANOVA followed by post-hoc comparisons controlling for multiple comparisons (e.g., SNK or Dunnett's) should be done. Actually we compared all cases with controls, then we had two groups for comparison and we added under the table.

Minor essential revisions
1) In the first paragraph of the results section, a p-value should be given for inter-group differences in proportions of HHV-6A and -6B positivity. In rare detection rates usually we don’t report P-value but if you recommend to add it we can add: that P-value is 1 when we want to compare one HHV-6B positive sample in the patients, with the HCs and P-value is 0.293 when we want to compare two HHV-6A positive samples in HCs, with the patients.

2) The following sentence in the second paragraph of the discussion is Awkward and should be re-phrased: "Nowadays disease association of this virus is increasing..." It was corrected.

3) The following sentence in the conclusion is unintelligible and should be re-phrased: "Huge challenges need to find the pathogenesis..." It was corrected.
4) In the Beta-globin PCR portion of the methods section, the authors state that beta-globin PCR is used to indicate "perfect" nucleic acid extraction. No extraction is perfect. Beta-globin PCR is used to indicate the quality of the extraction and the sample.

Discretionary revisions
1) Although it is explained in the discussion, it should be made clear why HHV-6A/B was studied in these patients.

2) Were the diagnoses of schizophrenia and bipolar disorder confirmed by SCID? If not, why not?

3) In the first sentence of the statistical analyses section, the authors refer to qualitative variables. These should be termed categorical variables.

4) In the discussion the authors use words such as "proven" and "cause". Such definitive terms should be avoid. Instead phrases such as "associated with", "increases the risk of", "appears to" or "suggests that" would be more appropriate.

Dear Reviewer

Duncan Sinclair,

Thanks so much for very useful and valuable comments.

1) As mentioned by a previous reviewer, the low number of individuals in whom HHV-6A/B-positive PBMCs were detected makes interpretation of any disease association difficult. This does not necessarily mean the study should not be published. However, the issue should be discussed thoroughly. In particular:

a) The number of positive individuals should be provided in the abstract, and the lack of statistical power to identify diagnostic differences mentioned in the discussion.

b) The rationale for detection of viral DNA in PBMCs rather than whole blood or
plasma should be provided in the manuscript, rather than in responses to the previous reviewer. It should also be described more clearly. Does HHV-6 DNA in PBMCs reflect persistent, low level active infection in tissue, even in the absence of HHV-6 DNA in plasma (this appears to be what is suggested)? If so, references would be helpful, particularly given that the study by Nitsche at colleagues (ref. 24) suggests that HHV-6 DNA measurement in PBMCs is less sensitive than in plasma, especially for HHV-6A since HHV-6A was undetectable in PBMCs but detectable in plasma.

It was added to discussion.

c) In general, explanation of the methods for investigating HHV-6 infection should be provided, along with clarification of which methods are specific for active infection or viral particles, and discussion of the different incidences of HHV-6 prevalence by different methods.

It was added to discussion.

2) Better citations across the manuscript are required. Without having checked all references in the manuscript, it appears some do not support the claims being made or are not peer-reviewed. For example:

a) Neither of the claims in the sentence ‘Human herpes virus 6 infects most infants less than 12 months of age and about 90% of the population is seropositive’ are supported by the provided reference (ref. 6), which is entirely inappropriate. This sentence must be removed or appropriate references for both claims provided.

Reference No.6 was omitted and appropriate reference was added.

b) By my reading, ref. 24 is not cited accurately or faithfully- 4% of patients (not healthy controls) were HHV-6A positive in PBMCs, and it is not mentioned that 88% percent of patients were HHV-6B positive in PBMCs (inconsistent with this study)

Reference No.24 was omitted.

c) References 14 and 22 are not peer-reviewed manuscripts, and for ref. 22 a link to a PDF of an abstract from an undisclosed conference is provided, which may not be appropriate

Reference 14 was replaced by an appropriate reference.

Reference 22 does not have a peer-reviewed manuscript, but because it has interesting findings, we added to the study.

d) Numerous relevant papers, for example (Achour et al., 2007; Caserta et al., 2010; Levy et al., 1990; Okuno et al., 1989; Tanaka-Taya et al., 1996, see below) are not cited.

Reference Achour et al, Caserta et al and Tanaka-Taya et al, were added to the study.

We did not add Levy et al, and Okuno T et al, references because they mainly
talk about the seroprevalence of HHV-6 but we did not assess the seroprevalence of this virus.

3) It would be helpful to include a figure with the assay standard curves, to indicate the reliability/sensitivity of the assays.

Standard curve figure was added.