Author's response to reviews

Title: Prevalence and molecular characterisation of human adenovirus in diarrhoeic children in Tanzania; a case control study

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Author's response to reviews: see over
## Reviewer’s responses

**Reviewer 1** (Thaweesak Chieochansin)

<table>
<thead>
<tr>
<th>Reviewers Comment</th>
<th>Authors response</th>
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| **Major Compulsory Revisions:**
| Overall: The contents in the text were confusing, did not well organizing, and fragmented. The main problem was the objective in the study was not focusing along the text. The authors should keep in mind all the time when building up the manuscript. | -We have rewritten the manuscript as suggested by the reviewer. Please see responses from other comments. |
| -What was the main objective(s) in this manuscript? | -The main objectives of the manuscript were “to determine the prevalence of adenovirus in diarrhoeic and non-diarrhoeic children. The study also performed molecular characterization of the detected adenovirus. Furthermore we evaluated the association between adenovirus with clinical characteristics, HIV status and seasonality”. These objectives are stated in the last paragraph of introduction, on page 5;line 101-105. |
| -Did the objective(s) was/were The prevalence study?, | -As stated above, determining the prevalence of adenovirus in diarrhoeic and non-diarrhoeic children was one of the objectives of the study. |
| -The molecular epidemiology study?, and/or The association study? | -Yes, we also did molecular characterization of the detected adenovirus. This was also one of the objectives of the study. |
| -And what was a proposing for HIV patient that including in the study? | -Because adenoviruses have been increasingly recognized as a major problem in immunocompromised hosts including HIV. It can cause infections with fatal outcome (Leen and Rooney, 2004; Kroes et al., 2007). In order to better understand the causes of diarrhoea in HIV infected individuals, we also searched for an association between adenovirus and HIV infection. This information is important for control |
and preventive measures. In addition this information is scarce in the African region.

**Title of manuscript:**

- Could change to relate the texts that write in the article.

- The authors should decide which point of the research they want to point out.

- It will be the same criticize as the objective that mention above. If the objective is clearer, the rest of the manuscript, even the title, would be better.

- We have changed the title now it reads “Prevalence and molecular characterization of human adenovirus in diarrhoeic children in Tanzania; a case control study.”

- We have mentioned the objectives of the study in the section of introduction, on page 5; line 101-105

- As stated above, the objectives of the study have been stated at the end of the section of the introduction

**Abstract and Key word:**

- If the authors want to address HIV patient in the manuscript, the stronger information, reference, and objective would be more specific too.

(Personal opinion I still not quite understand the propose for adding HIV patient into this study)

- We appreciate this comment. However, the authors are of the opinion that, due to the high prevalence of HIV in Sub-Saharan Africa, research on infectious diseases in this part of the world should generally consider the aspect of HIV-co infection as a potential factor influencing other infectious diseases, regardless of whether there is a previously known association.

- The information on the association between adenovirus and HIV infection has been added in the introduction on page 4, line 87-89 with references.

- It is also stated in the objectives of the manuscript at the end of introduction on page 5.

**Introduction:**

- The authors should provide more details especially the information that related to the study. The aim of introduction part is to provide reader of basic and relate knowledge to this study. Therefore, if the authors had been already decided what story that going to tell the reader, the introduction should relate to the following story.

The un-necessary information should be omitted

- We have re written the introduction part in order to be clearer. The introduction contains the burden due diarrhoea disease in general; the types of diseases which human adenovirus can cause; types and species of adenovirus responsible for different types of infections; the role of human adenovirus in
immunocompromised hosts including HIV infected individuals; the reported prevalence of diarrhea attributable to adenovirus; and available data of prevalence of adenovirus in the study setting.

### Material and Methods:

- Even the diarrhea patient was used as the previously study; the brief detail should be shown in the text.

- Moreover, another group of samples had been collected for this study, for clearly and easily understanding, all details of samples MUST BE shown.

- It should be great if compare the details in two groups of samples in table.

- The details of the study population have been added in the material and methods section, subsection study population, on page 5-6; line 110-116

- This study is not another group of samples, see table1. The samples are the same as the previous study (Moyo S et al 2014, PLoS One. 2014 May 20;9(5):e97562).

- As stated above the samples are the same as the previous study.

- What were the inclusion and exclusion criteria of diarrhea and none-diarrhea patient?

- Or did the patients were out- or in- patient?

- We have added a subsection of inclusion and exclusion criteria in the material and methods section, on page 6; line 127-136.

- All cases were inpatients and controls were either inpatients (HBC) or outpatients (CBC). This has been stated in the manuscript section of material and methods, on page 6; line 117-126.

- These children were admitted due to other infections apart from diarrhea in a different paediatric ward. They had malaria, septicaemia, pneumonia etc. These infections were not recorded. However when we analyzed the difference of prevalence of adenovirus in the two control groups, there was no significant difference found. This is mentioned in the result section on page 9-10; line 213-215.

3. For the non-diarrhea group, “...admitted to hospital due to diseases other than diarrhoea (n=235)”, what was a disease that identified in this group of patient? Because it should be affect the result interpretation and discussion.

4. In Data collection “...some of the clinical information...” What data(s) was collected? These SOME data(s) could be collected in all patients. Or...
just some of patients?  
-And the quality of data is homogeneous in all of individual.  
-And why collect only SOME data?  
-Data from all participants was collected in the same manner, using two designed questionnaires, one for diarrhoeic and another for non-diarrhoeic children.  
-The word some has been deleted.

5. In Data collection “... HIV status was obtained from patient files.” Why this information was so importance? And was it ethically right?  
-Adenovirus causes infections in immunocompromised individuals including HIV, therefore we think it was important to look at the association between HIV and adenovirus infection. We obtained ethical approval for testing and accessing HIV results. Furthermore written consent was obtained from the mother/guardian of the child.

6. For Adenovirus detection, Why using ELISA for detection method? As I mention before now a day more than 51 genotypes of HAdV had been classified. The ELISA kits that used in this study were designed to detect only 51 genotypes. As my experience, the ELISA is very limit to detected HAdV  
-It is true that there are published more than 51 genotypes, but most of these new genotypes have not been cultured and there is a lot of discussion whether these new genotypes are genuine genotypes or the result of recombination’s between known genotypes. At present time only type 1 to 51 are available as reference strains at ATCC, and hence the ELISA could not be validated against the new genotypes. This does not exclude the possibility that the ELISA kit is able to detect the new genotypes.

Thank you for the comment, yes PCR is a more sensitive technique compared to EIA. However, PCR is relatively more expensive and, in epidemiological studies which involve large number of samples, EIA could be a better alternative. This is especially true in developing countries. We therefore opted to use EIA for detection.  
- It is true that there are published more than 51genotypes, but most of these new genotypes have not been cultured and there is a lot of discussion whether these new genotypes are genuine genotypes or the result of recombination’s between known genotypes. At present time only type 1 to 51 are available as reference strains at ATCC, and hence the ELISA could not be validated against the new genotypes. This does not exclude the possibility that the ELISA kit is able to detect the new genotypes.
7. For Real time PCR for adenovirus genotyping, Why did not used this technique for virus detection in all samples? Because this technique is more sensitive and specific for HAdV

-Thank you for the comment. as stated above, we opted to use EIA because it was less costly compared to PCR without losing much sensitivity, taking into consideration the large number of samples.

8. For Real time PCR for adenovirus genotyping, “...In some cases when a larger PCR-product (322 bp) was needed...” Why the longer PCR-product was needed in some cases?

-Since the target of the real-time PCR from the conserved region of the hexon-coding gene of adenoviruses was rather small, a longer sequence was needed in some few cases to be able to differentiate between genotypes when using BLAST for identification.

9. For Sequencing of PCR amplicons, The region for HAdV genotyping is fiber gene. Why the author did not sequence the fiber gene instead of hexon gene for genotyping?

-We amplified the hexon gene and this was then followed by the sequencing and genotyping of the hexon gene. We chose this method because it is widely used. Hexon gene amplification and genotyping have been used in many studies see references below:


Result:

1. The data in “Prevalence of adenovirus” and “Distribution of adenovirus infection by age” should be summarize and put in the table. And these two parts should be combined into only one part. Then summarized the table into text that will make this part is less confusing

-We have combined the two sections as suggested. Some of the text has been deleted to avoid confusion, on page 9-10; line 210-224. The result of the distribution of adenovirus by age is already on
2. For “Association between demographic/clinical characteristics...” why HIV so importance? And what about the common clinical signs of HAdV infection like watery diarrhea, vomit, etc.?

-As stated earlier, HIV was one of the clinical characteristics of the patient included. In addition, we have also analyzed other clinical signs such as watery diarrhea, persistent diarrhea, dehydration status and nutritional status as seen in table 1.

3. For “Seasonality of adenovirus infection”, “We divided the months of the study according to the season of the year...” What were the criteria that used for your season divided? Rain level? Temperature? Humidity? And the reference(s) was needed.

-We divided the rain months according to the seasons known in Tanzania.
-We have also added the reference in the result section on page 11 (Camberlin P et al 2003)

4. The phylogenetic tree analysis of HAdV should be constructed. Because the authors would be more understanding and discussing about molecular distribution of this virus.

-We have now constructed the phylogenetic tree; it is included as figure 2.

Discussion:
The following are the comment base on contents in the manuscript, however, the overall of details in this discussion part is fragmented mis-pointed

1. “...Enzyme Immune Assay (EIA) able to detect 51 types...” Please discuss more about sensitivity, specificity, and limitation of this kit

-We have added more discussion explaining the limitation of EIA which is less sensitive compared to PCR for detection of adenovirus, on page 12, line 270-275.

2. “...Comparing adenovirus prevalence in this study with reports from other African countries, we observe large variations. Studies detecting HAdV by PCR, which is known to be more sensitive, have reported higher prevalences [18, 19, 24] compared to studies employing EIA [15, 25]...” WHAT is a point that the authors going to tell from this sentence?

-AND WHY PCR base method did not used for the detection in this study?

-We have now added the limitation of EIA for detection of adenovirus compared to PCR, and therefore we further gave examples of differences observed in the prevalence of adenovirus depending on the method which was used. The statement has now been paraphrased to be clearer. Page 12; line 274-279.

-PCR was not used for reasons mentioned above.

3. “...prolonged shedding of adenovirus in stool after previous infection of more than one month prior to the study. Alternatively, it could be asymptomatic adenovirus infections in children who may have acquired immunity from previous infections.” WHAT is the evidence(s) and reference(s) that make the authors conclude like this? IS it reasonable?

-We thank the reviewer for the comment. However, this is the discussion part, and we are discussing\raise possible explanations of our findings. We think this is a plausible hypothesis that needs further research. We have
4. “...We found that the majority of HAdV infected diarrhoeic children were dehydrated. This concurs with reports from other developing countries [26] suggesting that adenovirus can cause severe diarrhoea.” There are so many studies that already proof that HAdV can cause acute watery diarrhea. Please search more details and re-write this paragraph

- we have removed the word suggesting as it is already known that adenovirus cause acute watery diarrhoea. The statement now reads “This concurs with reports from other developing countries confirming that adenovirus cause severe diarrhoea” this is on page 12; line 286-287.

5. “Sequence analysis showed... This observation is consistent with...adenovirus type 40 has been observed” This paragraph is totally confusing. Please re-arrange and re-write this paragraph

- We have now rearranged and rewritten this paragraph to be more clear. Page 13; line 288-293.

6. “...Hence findings of this study partly support the theory of prolonged shedding of these human adenovirus species in faeces.” THIS is totally mis-conclusion

- Thank you for the comment. However, in this section In this section we are discussing our findings proposing different explanations. However we have changed the word “partly” to “could” page 13; line 305. We have provided references suggesting that adenovirus species which are non-enteric can cause prolonged shedding of the virus for months.

7. For HIV point, it is still not clear what the point that the authors want to mention is? It make HIV is another story inside your manuscript. Therefore if the authors want to incorporate HIV into the study, the authors should make the clear objective at the beginning.

- Adenovirus is increasingly known as a causative agent of infection in immunocompromised individuals including HIV. In the African region only one study has been done to look at HIV infection and adenovirus, Magwalivha M et al 2010. In Tanzania there are no studies which have looked at the association between adenovirus and HIV. We therefore think it was important to look at this association in this study. We have added some background information in the introduction as suggested and association with HIV-status is part of the objective.

**Conclusion:**

“...indicate prolonged excretion of adenovirus in stool, or acquired immunity from previous exposure from non-diarrhoeic children.” This is over conclusion.

- Since this is a hypothesis given in the discussion, we have deleted this statement from the section of conclusion.
### Minor Essential Revisions:

<table>
<thead>
<tr>
<th><strong>Abstract:</strong></th>
<th>-Currently the total number of words in the abstract is 324, this is within journal guidelines which allows up to 350 words. We have summarized the objectives and the main findings of the study.</th>
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<tbody>
<tr>
<td>1. Too long and too much information. The abstract should be summarize and highlight the study.</td>
<td>-As stated above, we have deleted this statement from the section of conclusion both in the abstract and after discussion.</td>
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<tr>
<td>2. “...prolonged viral shedding in stool, or acquired immunity from previous exposure for the non-diarrhoeic children.” Is it over conclusion?</td>
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| 3. “This first report on molecular epidemiology of HAdV in Tanzania observed diversity of HAdV types that circulate in the study setting.” Are the authors sure about this sentence? | This is the first molecular characterization study on human adenovirus, in Tanzania. Other studies which were conducted did not do molecular characterization eg.  

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<td>1. Please provided the reference for “Human adenovirus (HAdV) causes acute diarrhoea sporadically, as well as in outbreaks”.</td>
<td>-We have now updated the information to read, “to date there are more than 60 types identified” page 4;line 79.</td>
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<td>2. “To date there are 52 types of adenoviruses identified...” Please check the up to date data. I think it more than 52 type of HAdV had been classified already</td>
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### Material and Methods:

1. “...(UNG activation),...(polymerase activation),...(denaturation),...(annealing) and ...(extension)...”
   - Could be deleted if desired.
   - Thank you for the suggestion, however, we opted not to delete the statement, we think it will be useful for some readers.

2. In real time PCR condition, the fluorescent detection step should be addressed. (Normally the detection should be after each of extension step.)
   - We have added the following statement explaining when fluorescence was measured. “Fluorescence emission was set to be measured at the end of extension step”. This is on page 8; line 180-181.

### Result:

1. All P-value in the text “P” should be italic “P”
   - We have changed “P” to italic “P”

2. For “Molecular epidemiology”, “...BLAST...” should be also mention in material and method section
   - We have added this information as suggested by the reviewer in the material and methods subsection *Sequencing of PCR amplicons*
   - Nucleotide sequences were analysed using BLAST service at NCBI. The results were compared to known adenovirus sequences in the GenBank” on page 8-9; line 187-190.

3. For “Molecular epidemiology”, “...The proportions of enteric adenoviruses (type 40 and 41) were not significantly different in diarrhoeic and non-diarrhoeic children (50%, 12/24 vs. 46%, 6/13, P=0.82).”
   - How about other genotypes result rather than F40,41?
   - We have done analysis for genotypes other than enteric adenovirus as suggested, the following statement has been added in results section of molecular epidemiology “similarly the proportion of non-enteric adenovirus did not differ between diarrhoeic and non-diarrhoeic children (50%, 12/24 vs. 53.85%, 7/13, P= 0.82)” on page 11; line 257-259.

### Discussion:

1. “The molecular epidemiology of human adenovirus species and types from Tanzania is described for the first time in the present study.” Be careful about over claim
   - As stated above, this is the first report of molecular characterization of human adenovirus in Tanzania.

2. “...EIA method specific to enteric adenovirus 40/41 which was used in the previous study.” Please add the reference Moyo S et al 2007.
| reference |
|------------------|----------------------------------|
| 3. “…In order to detect serotype drift in the study setting, future studies are needed over a prolonged period of time, as reported elsewhere [9,10, 29].” What is the point of this sentence? Please re-write. | -We have re written the paragraph as suggested by the reviewer. Please see page 13; line 293-298 |

<table>
<thead>
<tr>
<th>Level of interest:</th>
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<tbody>
<tr>
<td>An article of limited interest</td>
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<th>Quality of written English:</th>
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<tbody>
<tr>
<td>Needs some language corrections before being published</td>
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<tr>
<td>-We have done language correction as suggested.</td>
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<tr>
<th>Statistical review:</th>
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<tr>
<td>Yes, and I have assessed the statistics in my report</td>
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<tr>
<td>-We have done further statistical testing such as comparison of adenovirus genotypes other than enteric adenovirus in cases and controls, as suggested by the reviewer.</td>
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<tr>
<td>Points</td>
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<tr>
<td>1-Is the question posed by the authors well defined?</td>
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<tr>
<td>2- Are the methods appropriate and well described?</td>
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<td>3- Are the data sound?</td>
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<tr>
<td>4- Do the figures appear to be genuine, i.e. without evidence of manipulation?</td>
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<td>5- Does the manuscript adhere to the relevant standards for reporting and data deposition?</td>
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<td>6- Are the discussion and conclusions well balanced and adequately supported by the data?</td>
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<td>7- Are limitations of the work clearly stated</td>
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<tr>
<td>8- Do the authors clearly acknowledge any work upon which they are building, both published and unpublished?</td>
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<tr>
<td>9- Do the title and abstract accurately convey what has been found?</td>
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<tr>
<td>10- Is the writing acceptable?</td>
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