Author's response to reviews

Title: Recurrent wheezing is associated with intestinal protozoan infections in Warao Amerindian children in Venezuela: a cross-sectional survey

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Author's response to reviews: see over
Dear Prof. Harris,

It is a great pleasure to submit to BMC Infectious Diseases our second revised manuscript entitled ‘Recurrent wheezing is associated with intestinal protozoan infections in Warao Amerindian children in Venezuela: a cross-sectional survey’.

We thank the associate editor for his constructive comments and suggestions. All suggestions made by the associate editor have been extensively discussed and, where possible, implemented in the revised manuscript. The explanatory details are listed below.

Thanks to the highly relevant suggestions of the associate editor, our revised manuscript has improved significantly. We are confident that the current manuscript meets the quality criteria of BMC Infectious Diseases, and that the subject will be appreciated by the Journal’s readership.

All authors have contributed significantly to the work. This manuscript has not been published and is not being considered for publication elsewhere. All authors have read and approved the manuscript. The order of authors listed in the manuscript has been approved by all authors.

We hope hearing from you at your earliest convenience.

Sincerely yours,

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Rebuttal

Comments on the manuscript of the Associate editor

- Page 5: ?.. consistent protective effect of infection with Ascaris lumbricoides, Trichuris trichiura, hookworm and Schistosomiasis. Should read Schistosoma sp instead of schistosomiasis. It is a disease not a parasite. Response: we agree with the associate editor. Schistosomiasis has been changed to Schistosoma sp.

- Page 6: ?Stool samples were preserved in sodium acetate-acetic-formalin (SAF) preservative [29] and stored at 4°C?? Why the samples are stored in 4C after SAF preservation? Response: SAF preserved samples are usually stored at room temperature, but 4 °C is also possible (Bussolati G, Annaratone L, Medico E, D'Armento G, Sapino A (2011) Formalin Fixation at Low Temperature Better Preserves Nucleic Acid Integrity. PloS ONE 6(6): e21043. doi:10.1371/journal.pone.0021043). In this study setting, in the Delta Amacuro in Venezuela, temperatures varied from 10 °C till 40 °C. Because storing at a stable temperature of 4 °C did not damage the stool samples and a top temperature of 40 °C maybe would, we chose to store the feces samples at 4 °C. We stored most of the samples (blood and urine samples for a bigger study) in a refrigerating box of 4 °C and because we travelled a lot, it was important for transfer to keep all the samples in a safe and stable place. To keep a stable temperature and for reasons of convenience we stored the SAF preserved samples at 4 °C.

- Page 6: ?Pure polymerase chain reaction (PCR) template preparation kit (Roche, Germany). Real time PCR of fecal samples was performed on the Roche LightCycler 480 system for detection of Dientamoeba fragilis, Giardia lamblia, Cryptosporidium parvum, Ascaris lumbricoides, Strongyloides stercoralis, Ancylostoma duodenale and Necator americanus. [30–32]? The authors should provide more details (rewrite this section) about their Real time PCR methods (DNA extraction, primers sequence, probe, cycle,?etc). The references provided are not accurate. > Reference 30: the authors used a single organism D. fragilis and used ABI 7500 Real Time detection system > Reference 31: The authors did not use the same PCR machine as described in this manuscript. They focused on E histolytica, Giardia and Cryptosporidium and they used iCycler Real Time PCR detection system. > Reference 32: the authors focused on soil helminth parasites and used Rotor Gene 6000 real-time PCR system (Rotorgene-Q).
Since each Real time PCR system is different, the authors should provide in details their approach.

Response: We agree that there could be differences between the used PCR systems named in the references. To provide more detailed information about our PCR and to clarify the literature sources, we changed the alinea ‘Fecal samples in ethanol were stored at -70°C until DNA isolation with the High Pure polymerase chain reaction (PCR) template preparation kit (Roche, Germany). Real time PCR of fecal samples was performed on the Roche LightCycler 480 system for detection of Dientamoeba fragilis, Giardia lamblia, Cryptosporidium parvum, Ascaris lumbricoides, Strongyloides stercoralis, Ancylostoma duodenale and Necator americanus. [27-29]’ in the methods section into ‘Fecal samples in ethanol were stored at -70°C until DNA isolation with the High Pure PCR Template Preparation Kit (Roche, Almere, The Netherlands). Real-Time PCR of fecal samples was performed on the Roche LightCycler 480 system using the Roche FastStart Kit. Detection of the protozoa Dientamoeba fragilis, Giardia lamblia and Cryptosporidium sp. was done in one multiplex PCR with Phocine Herpes Virus (PhHV) as an internal control using primers and probes described by Verweij et al. [27, 28] PCR on the nematodes Ascaris lumbricoides, Strongyloides stercoralis, Ancylostoma duodenale and Necator americanus was also performed in a multiplex PCR with primers and probes described by Basuni et al. [29]. The primers and probes sequences are listed in table 1, including the amount that was used in a single reaction. All PCRs were carried out in 25 µl reactions in the presence of 4 µg BSA fraction V (Sigma-Aldrich, Zwijndrecht, The Netherlands). After an initial activation step of 10 minutes at 95°C the reaction consisted of 45 cycles at 95°C for 10 seconds, 58°C for 20 seconds and 72°C for 20 seconds.’

Furthermore, we added an extra table to provide the exact information on primers and probes which we used during multiplex PCR on protozoa and nematodes (table 1).

• Page 6: ?The diagnosis of parasitic infections was based on both microscopy and PCR? Please delete this sentence. It was already mentioned above.
Response: We deleted the sentence which was mentioned above.

• Page 6: ?Definitions? The authors should be clearer. Definitions of what?
Response: We agree that it is a short term and a single word is not very transparent for the reader. These are the definitions of the study objectives atopic eczema, recurrent wheezing and malnutrition. We changed the word ‘definitions’ to ‘Definitions of atopic eczema, recurrent wheezing and malnutrition’.

• Page 7: ?Recurrent wheezing was only assessed in children aged 12 months and above?. Please give the number of children. It is not so obvious.
Response: n=123 children. We added this information in the methods section to this
Recurrent wheezing was only assessed in children aged 12 months and above (n=123).

- Page 7: ?From August to November 2012, 229 children 0 to 24 months of age were included?.
The number in Table 1 =228. Please consolidate number.
Response: Thank you for noticing this mistake. In the village Araguabisi the number of children included was 16 instead of 15. We changed this in table 1, now the total number of subject is 229 again. NB table 1 is now table 2, new table 1 is the PCR table.

- Page 8: ?Fecal samples were collected from 100 children (44%). Children from whom stool samples were obtained were more likely to suffer from stunting than children from whom stool samples were not obtained (56 % vs. 40%, p=0.013)?
Why stools samples were not taken from all children? Some children might be asymptomatic or might be in incubation time during the visits of the team. Please comment on this.
Response: We agree that we miss important information because we failed to collect stool samples from all participants. It is indeed possible that some children are symptomatic or in incubation time during our visits. The approach was to collect stool samples from all participants. Due to possible causes only 100 samples were collected. In every village we were staying a maximum of 5 days and after that we had to go to the next village. A lot of the parents with their children visited the research team by boat and some were not able to return for a stool sample the next days. Other parents did not understand the reason to collect a stool sample if their children weren’t visible ill or malnourished. In the discussion section we suggest this: ‘The prevalence of stunting was higher in children from whom stool samples were obtained than in children without a stool sample. A possible explanation for this finding is that parents of malnourished children were more likely to collect stool samples because of concerns about the state of health of their children.’

- Page 9: ?The prevalence of atopic eczema in the study population was 19% (n=43) and recurrent wheezing was observed in 23% (n=28).? Please give the total number of children with recurrent wheezing since this observation was only made on children >12 months?
Response: The total number of children with recurrent wheezing was 48 (21%). We changed the sentence in the methods section were we added this information. ‘The prevalence of recurrent wheezing in the last 12 months was 21% (n=48) and for children aged 12 months and above, it was 23% (n=28)’.

- Page 9: ?The overall gastrointestinal parasitic infection prevalence in children 0 to 2 years of age in our study was 70%.? This number might be lower if the stools samples were taken in all children (229 instead of 100). I am not totally convince with the approach taken by the authors, collecting only 100 stools samples.
Response: We agree that this number might be lower if we collected stool samples from
all participants. However, earlier performed research in 2012, in Warao children from 1 to 15 years, also give high prevalences of parasitic infections. The prevalence of helminth infections was 31% and the prevalence of protozoan infections was 65%. [22] Ideally, we would have collected stool samples from all participants. It was not our preconceived approach to collect only 100 samples. In the future, we recommend longer stays in villages or create more moments in time to collect feces.

- **Table 2 is not clear and difficult to understand. The authors should simplify it and add it in the manuscript instead of supplementary Table.**
  Response: We agree that table 2 is a large table with a lot of information. The analyses were performed on the children from who we have collected stool samples (n=100 for atopic eczema, n=61 for recurrent wheezing). We added table legends to make this more clear. For table 3a: *These analyses were performed on children from who we collected stool samples (n=100). And for table 3b: *These analyses were performed on children from who we collected stool samples (n=61). We simplified the table and added the new tables in the manuscript.
  NB: table 2a and 2b are now table 3a and 3b, because of a new added table on PCR primers and probes.

- **The authors should clarify the source of Figure 1.**
  Figure 1 is a self-made image and we used two images within these image to clarify atopic eczema and recurrent wheezing. (links below).

  The photo of the boy we took in the Delta Amacuro (photo of a young Warao boy) ourselves and we edited this photo. Since the authors from this websites of the images of atopic eczema and recurrent wheezing are untraceable (old website, not used website, not possible to call or e-mail), we could not get the official permission. Therefore, sadly, we made the decision to delete figure 1 from the manuscript.