Author's response to reviews

Title: Prevalence and Genetic Characterization of Cryptosporidium, Enterocytozoon, Giardia and Cyclospora in Diarrheal Outpatients in China

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Author’s response to reviews: see over
1. Cover Letter:

Dear Editor,

We would like to thank you and all the reviewers for the positive and constructive comments, and we address your comments point by point in the file of response which had just uploaded.

We would like to submit the enclosed manuscript “Prevalence and Genetic Characterization of Cryptosporidium, Enterocytozoon, Giardia and Cyclospora in Diarrheal Outpatients in China”, which we wish to be considered for publication in BMC infectious disease. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication. We know that your journal has high publication standards, so we had this manuscript copyedited by one or more of the highly qualified native English speaking editors at a professional English editing service.

Cryptosporidiosis, microsporidiosis, giardiasis and cyclosporiasis are emerging infectious diseases. Cryptosporidium spp., Enterocytozoon spp., Giardia spp. and Cyclospora spp. are globally distributed diarrhea-causing intestinal protozoan parasites of humans, livestock, companion animals and wildlife. Thus far, no study has confirmed whether Cryptosporidium andersoni, Enterocytozoon spp. and Giardia spp. assemblage C could infect human outpatients on a large scale. In this study, we investigated fecal specimens from 252 diarrhea patients in a pediatric clinic (169) and an intestinal clinic (83) of a hospital in Pudong, Shanghai, China, from October 2012...
to March 2013. Cryptosporidium spp. and E. bieneusi were detected in 13.49% of the 252 diarrhea patients, whereas the Giardia infection rate was 6.75%. Our data indicated the first outbreaks of cryptosporidiosis caused by Cryptosporidium andersoni and of microsporidiosis caused by E. bieneusi in China. Thus, Cryptosporidium andersoni should be considered the fourth major Cryptosporidium species infecting humans in the world.

We believe that our findings could be of interest to the readers of BMC infectious disease. We hope that the editorial board will agree on the interest of this study.

Your kind consideration would be greatly appreciated.

Sincerely,

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2. Response:

Dear editor,

We would like to thank you and all the reviewers for the positive and constructive comments, and we address your comments point by point below:
Reviewer 1: Bonnie Meatherall

COMMENT 1: Background, paragraph 2: about using *Enterocytozoon* species and Microsporidia.
RESPONSE: In paragraph 2 of the background section, we have used the common term microsporidia instead of *Enterocytozoon* spp. to make it clearer to the reader (p. 4, line 69).

COMMENT 2: Methods, paragraph 2: about the age range of participating patients in the methods or in results section.
RESPONSE: Following to your suggestion, we have now described the age range in the results section as: “No age-associated differences in the patients involved (ranging from 1 month to 77 years) was found in our study” (p. 6, line 102; p8, lines 164-165).

COMMENT 3: The sentence “In future studies, case tracking by hospitals will be improved with the cooperation of patients.” is unclear.
RESPONSE: By this we meant that in order to better understand the source of infection, we will attempt to seek the cooperation of patients involved in future studies to investigate their habits, such as contact with animals, drinking water and water conditions. We rewrote this sentence in the revised manuscript (p. 12, lines 241-243).

COMMENT 4: Discussion, paragraph 4: The first sentence “…all positive specimens belonged to assemblage C…” should changed.
RESPONSE: We appreciate your proposal about the description and we have corrected it to “Genotyping results of *G intestinalis* indicated that all but one positive
specimens belonged to assemblage C (p. 11, line 228).

**COMMENT 5:** Figure 1 legend: The word “human” should be plural (“humans”). Was there a $\chi^2$ analysis for the giardia seasonality data set that could be included in the legend?

**RESPONSE:** We have corrected “human” to “humans” in the caption for Figure 1. We have supplemented the data of the chi-square analysis for the Giardia seasonality in Figure 1 and its caption (p. 23, line 442, 445-447).

**COMMENT 6:** Figure 2: Why were these age groups chosen to represent the age variable? Were they based on data point distribution or is there clinical relevance to these categories?

**RESPONSE:** This study was a cross-sectional investigation. We analyzed the differences among these age groups as described in other publications. However, considering your question and other references (Maha et al. 2013), we amended the age groups, given that children aged younger than 5 years (especially 0-2 years) are especially vulnerable to cryptosporidiosis. Finally, we divided the patients into three groups (shown in the revised manuscript).

**COMMENT 7:** Methods, paragraph 5: I think more information is required regarding the statistical methods used beyond the software package used for the analysis. How was the sample size determined? Which statistical techniques were used for univariate and multivariate analysis? How were multivariate models built? What P-value is considered significant in this analysis? How was age handled as a variable?

**RESPONSE:** This was a cross-sectional investigation and fecal samples were collected from patients who satisfied the enrolled criteria. Specimens were collected from patients with fecal excretion heavier than 200g and with no less than three events of diarrhea per day. The stools consistency was usually thin and mixed with mucus or blood. (p. 6, lines 103-106). A chi-square test was used to analyze the data, and $P < 0.05$ was considered to be statistically significant (p. 8, lines 148-150).
COMMENT 8: Minimal discussion regarding limitations. For example, no data on whether patients in the clinic were immunosuppressed or whether patients had direct contact with animals/livestock. Another limitation is that only two seasons (winter and spring) were studied.

RESPONSE: We appreciate your proposal, but in some cases the information were very difficult to obtain. However, we will try to obtain more information. Given that most previous studies focused on summer or autumn, when people are more vulnerable to diarrhea, the occurrence of these parasites in winter and spring has been mostly neglected. We therefore mainly focused on these two seasons. We believe that the findings in our study were very important to supplement those from closely related studies.

Reviewer 2: Stephen Vaughan

COMMENT 1: Provide Definition of Diarrhea

RESPONSE: We agree with the suggestion of the reviewer and have provided a more detailed definition of diarrhea in the revised manuscript (p. 6, lines 103-106).

COMMENT 2 and 3: Why were Wet Prep/AFB staining/EIA Crypto/Giardia/Entamoeba + other bacterial causes of diarrhea (C. diff/E. coli/Shigella/Salmonella/Yersinia/Camphylobacter/Cholera) not included? Biggest concern with this paper is that there is no testing versus Gold Standard (other tests as outlined above) and their was not an extensive search for other (traditionally more commonly) causes of diarrhea, therefore we can not be sure that the patients Diarrhea was caused by the identified organisms.

RESPONSE: A large number of samples were examined microscopically in our previous studies, using modified acid-fast staining, auramine-phenol stain or Lugol's iodine solution, but the positive rate was very low. The most common concentration methods include Sheather’s (1923) sucrose flotation method and formol-ether
(formol-ethylacetate) concentration. Although some investigators find Sheather’s method to be superior (Ma and Soave, 1983), it is rarely used in the analysis of human stool samples due to their high fat content. PCR-based methods are more sensitive than conventional and immunological assays for detecting oocysts in feces. Identifying the parasite species infecting humans by molecular methods is important in determining the epidemiology of a disease and likely transmission routes (Fayer and Xiao et al, 2007).

Our main targets in the present study were parasites. We will consider the variety of other pathogens you referred in future studies.

COMMENT 4: The finding of C. andersoni in stool does not confirm it is the cause of diarrhea, but rather that it was ingested (and the authors state that it is in Shanghai tap water…therefore it must be passed in stools.
RESPONSE: Although a previously study by Feng mentioned that tap water in Shanghai was contaminated with C. andersoni, people with diarrhea may drink the contaminated water. However, we cannot exclude the possibility of other infection routes. We also cannot confirm it was the cause of diarrhea, although it would be of great significance and can provide more comprehensive treatment for clinicians.

COMMENT 5: Abstract: (Background Line 2) spelling of Wildlife (not widelife)
RESPONSE: This has been corrected in the revised manuscript (p. 2, line 27).

COMMENT 6: Background: 1st Paragraph last sentence (provide reference).
RESPONSE: We have added the references to the revised manuscript (p. 4, line 64).

COMMENT 7: Discussion: Last paragraph 2nd sentence (provide reference of aerosol transmission).
RESPONSE: We have added the references to the revised manuscript (p. 12, line 236).
Reviewer 3: Wilson Chan

COMMENT 1: The most major concern that exists is whether or not the detection of Cryptosporidium, Enterocytozoon, and Giardia actually represent etiologic agents of the patients' diarrhea. The study is concerning as there has been no testing of healthy, case-matched controls; as such, it is impossible to evaluate whether the findings are associated with disease. For example, as the authors themselves point out, there is ample C. andersoni in source and tap water in Shanghai; it may well be that even healthy people have detectable DNA of C. andersoni in their stool as a result of water ingestion.

RESPONSE: We appreciate the positive comments about the manuscript. We did not conclude that the three parasites were the etiologic agents of the patients’ diarrhea in our study. However, we speculate that they might be associated with diarrhea and we aimed to provide some information for clinicians. Our sequencing analysis indicated that C. andersoni isolated from human patients had high similarity with isolates from water or infected cattle samples (GenBank numbers KF271452-KF271485), and might be associated with disease. We thus cannot draw the conclusion that C. andersoni was the etiologic agents of the patients' diarrhea. We will therefore try to obtain more specimens from patients and healthy humans in the same area in future studies to investigate and trace the source, which might further improve the quality of that study.

COMMENT 2: Furthermore, it would be helpful to correlate the PCR results with standard diagnosis of Cryptosporidium, Enterocytozoon, Giardia, and Cyclospora. While PCR is a more sensitive method, correlation with standard parasitological methods would strengthen the validity of the PCR results.

RESPONSE: A large number of samples were examined microscopically in our previous studies, using modified acid-fast staining, auramine-phenol stain or Lugol's iodine solution, but the positive rate was very low. The most common concentration
methods include Sheather’s (1923) sucrose flotation method and formol-ether (formol-ethylacetate) concentration. Although some investigators find Sheather’s method to be superior (Ma and Soave, 1983), it is rarely used in analysis of human stool samples due to their high fat content. PCR-based methods are more sensitive than conventional and immunological assays for detecting oocysts in feces. Identifying the parasite species infecting humans by molecular methods is important in determining the epidemiology of a disease and likely transmission routes (Fayer and Xiao et al, 2007).

Our main targets in the present study were parasites. We will consider the variety of other pathogens you referred in future studies.

**COMMENT 3:** It is also not clear if the PCR testing occurred over the entire study collection period, or was performed in one batch after all 252 specimens were collected. Especially in the latter case, it would be important to have controls during the testing to rule out cross-contamination. This was likely done, but explicitly outlining this would again strengthen the validity of the results.

**RESPONSE:** In the course of our study, we followed the former method you referred to. PCR tests were done after the weekly collection of specimens. In our lab, sample packaging, DNA extraction, PCR preparation, PCR testing and results observation were performed in a separate room to avoid cross-contamination. In addition, a positive control (C. bailyei) and negative control were added to each PCR, and positive samples were confirmed by two-directional PCR and sequencing (p. 7, lines 141-143). In addition, our lab has obtained certification from the Chinese National Accreditation Service for Conformity Assessment (CNAS), and also obtained certification of international mutual recognition (ilac-MRA).

**COMMENT 4:** Minor typographical and language errors, but these are easily remedied.

**RESPONSE:** We have corrected typographical and language errors in the manuscript with the assistance of a professional editing service.
COMMENT 5: In the background section, a citation to support the statement in lines 60-62 would be important.

RESPONSE: We have added the reference to the revised manuscript (p. 4, line 64).