Author's response to reviews

Title: Children of Senegal River Basin show the highest prevalence of Blastocystis sp. ever observed worldwide

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Author's response to reviews: see over
Dear Editor,

You will find enclosed the revised version of our manuscript. All comments of reviewers were taken into account and/or discussed below. Changes and additions are shown in red in the text. We hope that these changes will allow the publication of this study in BMC Infectious Diseases.

With our best personal regards

Dr Eric Viscogliosi

Reviewer 1's report
Reviewer: Ken Boorom

Reviewer's report: The paper presents a prevalence study of Blastocystis infection in Senagelese children, with subtyping information, along with an analysis of potential relationships between infection, subtypes, and symptoms. As few studies exist of Blastocystis in hyper-endemic environments, especially with subtyping, the paper's topic is of interest.

A few items of concern appear below:
1. (SELECTION CRITERIA) The paper does not indicate the selection criteria used for the 93 children. Although this may appear in the clinical trials referenced, it should be included in this paper (example: "Children were recruited through healthcare providers, who were asked to provide written material to all healthcare seeking patients between January 1, 2012 and March 30, 2012"). Enough information should be provided to understand any possible influences the selection criteria might have on the study's results. For example, if patients are recruited from healthcare seeking patients, then patients who are more prone to illness, or patients from families with better access to healthcare might be over-represented.

   Additional information is provided in the revised version of the manuscript (lines 143-145) as follows: “…and were recruited on the basis of their age (6 to 10 years in October 2011) through village nurses, healthcare workers in the community and school directors. Date of birth was ascertained from vaccination cards or school register”.

2) (NEGATIVE CONTROLS) The study notes that 100% of the DNA samples negative by non-q PCR tested positive by qPCR, using more powerful amplification techniques. But the study does not describe the use of negative controls, preferably a sample known to be Blastocystis-negative which was subjected to the same DNA extraction method. The concern here is the possibility that the qPCR-positive results may be artifacts related to contamination, either from other samples, reagents from suppliers, or environmental sources (dust, etc.). qPCR is extraordinarily sensitive - for clinical work, Roche has required that PCR amplification be performed in a lab physically separated from the lab where DNA is extracted, for example. In one lab I've worked with, 10% of the samples were dedicated to negative controls (saline), and at one point, these began testing positive for Blastocystis, necessitating a shutdown of the facility for deep cleaning. Recent studies have found that reagents from suppliers have enough DNA contamination to test positive using highly sensitive amplification techniques (i.e. Smith Retrovirology 2010, 7:112, Contamination of clinical specimens with MLV...)
Details were not given in the first version of the manuscript concerning the various controls carried out in this study. Therefore, additional information is provided in the revised version (lines 186-190) as follows: “Due to the high sensitivity of this detection method, various controls were performed to prevent artifacts related to contamination though different sources: DNA extraction controls (isolation of DNAs without stool and from a Blastocystis sp.-negative stool) subsequently used in qPCR assays and negative (DNA matrix replaced by water) and positive (DNA obtained from a Blastocystis sp. ST4 culture) qPCR controls.” In addition, we confirm that PCR amplification was performed in a lab physically separated from the lab where DNA was extracted (information not indicated in the revised version of the text).

3. Lines 309-310 indicate that the prevalence of Blastocystis was higher in symptomatic children:

a. The authors should evaluate a 2x2 contingency matrix, preferably with Fisher's exact test, and report the p-value for this relationship.

Since all children were positive for Blastocystis, we could not evaluate a 2x2 contingency matrix.

b. The statement is confusing, as the paper indicated that 100% of the participants were infected.

We agree with reviewer 1 and reviewer 2 (see below) that the following sentence was confusing: “Interestingly, prevalence rate of Blastocystis sp. was significantly higher in the symptomatic children (54/93 or 58%) than in the asymptomatic children (39/93) as previously observed in a pediatric unit in Turkey [57]”. Consequently the sentence was modified in the revised version (lines 323-325) as follows: “Interestingly, the majority of children infected by Blastocystis sp. (54/93 or 58%) presented various gastrointestinal disorders as previously observed in a pediatric unit in Turkey [57]”.

4 (METHOD FOR DETERMINING SYMPTOMS): The authors should provide more information on the way symptom information was collected for those interested in repeating the study with other groups. For example, were children asked to list GI symptoms? Or were they asked about each symptom individually.

If a written form was used, please include a copy of the form.

Regarding this comment, two sentences have been added in the revised version (lines 145-148): “A standardized questionnaire was completed for each child (see supplementary material). In particular, children were asked about each gastrointestinal symptom individually including abdominal pain, diarrhea, and vomiting”. Effectively, a written form was used (in French) throughout the study. This form has been added as supplementary material in the revised version.

5) It is difficult to discern individual rows of data in Table 1 - extra spacing or borders between adjacent rows would be most helpful.

Table 1 was performed according to the instructions of the journal. Yet we presented the Table with a wider spacing between the lines for easy reading.
We agree with reviewer 1 concerning a part of the following statement: “Globally, no association between ST and symptom status was statistically significant from our present data suggesting rather the coexistence of pathogenic and nonpathogenic isolates of ST1, ST2, and ST3 among our Senegalese cohort”. Consequently the end of the sentence “suggesting rather the coexistence of pathogenic and nonpathogenic isolates of ST1, ST2, and ST3 among our Senegalese cohort” was removed in the revised version (line 347).

The request of the reviewer is not clear in this part of the text. We assume that the reviewer wishes to emphasize the zoonotic potential of the parasite. This point is already the subject of several references in the text (eg, references 7, 10, 14...). As the number of references is limited, we fail to see the interest of adding an additional reference for zoo keepers.

Line 85 - A prevalence of nn% was reported in Australian zoo handlers.

“The request of the reviewer is not clear in this part of the text. We assume that the reviewer wishes to emphasize the zoonotic potential of the parasite. This point is already the subject of several references in the text (eg, references 7, 10, 14...). As the number of references is limited, we fail to see the interest of adding an additional reference for zoo keepers.”

Line 87 - suggested --> suggests

“suggested” was changed in the text by “suggests”

Line 88 - Remove Thereby

“Thereby” was removed

Line 91 - Comma after handlers

Comma has been added

Line 93 - Suggest removing word "other"

“other” has been removed
Reviewer 2's report  
Reviewer: Kevin Shyong-Wei Tan  

Major comments:  
The epidemiological study by Safadi et al presents the first report of a 100% prevalence of the emerging parasite Blastocystis among 93 Senegalese children. The authors used a combination of PCR and the more sensitive qPCR to determine presence and subtype (genotype) of the infected sample. It was observed that the frequency of ST 1, 2 and 3 were typical of more surveys worldwide. Two cases of ST4 infection were detected. Overall, the study is well conducted and the novelty lies in the 100% prevalence, which is indeed the firstsuch record for Blastocystis. The manuscript is well written, the figures are of good quality and the results appropriately discussed. It would have been ideal if some form of viability assay was conducted concurrently with the survey (staining or culture for microscopic evidence of parasitosis, determining parasite density). This is to exclude spurious detection (false positives from ingested animal matter etc). Despite this, the study is a major finding and should be acceptable for publication after some minor revision.

Minor comments:  
Line 81: Change ‘protozoa’ to ‘protist’ for accuracy.

“protozoa” was changed in the text by “protist”

Line 309-311: Confusing. How can one comment on prevalence of Blastocystis in symptomatic vs asymptomatic groups if both are 100%?  

See above the response to the first reviewer  

Methods: Was ethical consent provided for sample collection? The details should be provided.

Details regarding ethical consent for sample collection were provided in the revised version of the manuscript (lines 137-141) as follows: “The present study was approved by the National Ethics Committee of the Ministry of Health of Senegal (September 2011; protocol number SEN11/43, clinicaltrials.gov: NCT01553552). Oral and written informed consents were obtained from the parents or the legal guardians of the children in accordance with the Code of Ethics of the World Medical association (Declaration of Helsinki).

Other authors suggest that besides subtype, parasite density may also factor in pathogenesis. The authors should include some mention of this in their discussion. Perhaps a more comprehensive survey including multiple parameters would shed more light on the pathogenesis topic.

As stated by reviewer 1, parasite density may also represent a factor of pathogenesis. In our study performed on the field, all samples of fresh stool were immediately added in STAR buffer for storage and transport to France preventing microscopic examination. However this factor remains widely debated in the literature and it is known that shedding of Blastocystis may be cyclical, yet it is unusual for more than one stool sample to be examined. This factor could hardly be taken into account in our study and therefore has not been discussed in the manuscript.
An interesting observation is the lack of substantial co-infection with multiple subtypes despite 100% prevalence. Can the authors speculate on the reason for this?

As stated in our manuscript, the prevalence of mixed ST infection was 8.6% (8/93 samples) in our Senegalese cohort. This value was roughly similar to that calculated (6%) from an average of all epidemiological studies published to date. It is therefore difficult to conclude that this value is underestimated even if it is our opinion. Indeed, this underestimation may be related to the method used to identify Blastocystis. Indeed, we are directly sequencing the qPCR product and the sequencing chromatogram obtained probably reflects the major ST in case of mixed ST infection. DNA from another ST can be present in trace amounts within the extracts, amplified but undetected by sequencing. As we mentioned in a previous study (Meloni et al. 2012), mixed ST infections can be identified by non-qPCR followed by cloning and sequencing of a large number of clones. Thereby, by sequencing 50 clones, three different STs were identified, each of them represented by various proportions of clones probably related to the number of parasites of each ST. However, this approach has not been used in the present study and all our comments on a possible underestimation of mixed ST infections remains totally speculative. Therefore, we fail to see the interest in developing this speculative issue in the revised version of the manuscript.

Additional modifications not proposed by the reviewers

Typographical errors were identified in the text and corrected
* “non specific” was changed in the text by “non-specific” (line 112 revised version)
* “shacking” was changed in the text by “shaking” (line 164 revised version)