Author's response to reviews

Title: Diagnosis and subtype analysis of Blastocystis spp. in 442 patients in a hospital setting The Netherlands.

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Version: 2  Date: 19 June 2013

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

please find below our response to the reviewers. We do excuse for being late with our resubmission, especially the submission of sequences to Genbank (as requested by several reviewers) took more time than anticipated.

In our point by point reply to the reviewers comments, the comments are displayed in Arial font, our reply in times new roman font.

We thank all reviewers for their comments and hope we have addressed them satisfactorily.

Yours sincerely,
On behalf of all authors,

A. Bart
Editor's Requirement:

--> Please clarify the ethical approval sought for your work and include this within the methods section.

We clarified this issue in the methods section: for use of anonymized samples for improving diagnostic techniques no ethical clearance is necessary in our country. There is an opt-out system for patients that do not want their anonymized samples to be used in this way.
Reviewer's report #1

Minor compulsory Revisions:
1. Some of the citations are out of date. For example there have been 14 Blastocystis subtypes for some time. ST14 was published six months ago and available 3 months before that (Fayer et al., Parasitology Research 2012; page 5 line 17). Also page 13 line 17 fails to cite recent European studies by Poirier in France (2011), Forsell in Sweden (2012) and Alfellani in the UK (2013) as well as other Danish studies that have a significantly higher ST4 prevalence than detected here. A more complete discussion of relative prevalence related to geography would also be helpful.

We updated the references and corrected the omission of ST14.

2. Blastocystis is not always italicised; spp. is not always followed by a period/full stop; Microsporidia should not be italicized.

These errors were corrected throughout the manuscript.

3. Page 5 line 3 – here ‘faecal’ is used but on line 8 ‘fecal’ is used. Except for ‘Triple Faeces Test’ ‘faec’ is only used in ‘defaecation’ – please be consistent in usage.

We have tried to consistently use British English spelling.

4. Line 22 – there is no information on how the amplicons were purified, whether they were sequenced using the amplification primer(s), and whether one or both strands were sequenced.

We have revised the methods section (p7 l22-23) to include this information.

5. References – in addition to the ones that seem to be missing mentioned earlier, reference 1, 17 and 23 are incomplete (no volume/pages), reference 12 lacks the final part of the citation (should be Euro Surveill. 16(24):pii=19891 ), reference 14 has an author name incomplete (Delgado-Viscogliosi), in reference 18 Blastocystis is not capitalized, and references 6 and 17 have the doi information included but none of the others do.

We have manually checked all references that were imported via Reference Manager program.

6. Finally, in Table 1, the Total in the final column should be 4 not 1.

Corrected

Discretionary Revisions:
1. Page 3 line 9 – I think this is slightly misleading. As written it implies that the microscopy performed on the three samples was the same, which it was not, and seems to indicate that PCR was performed on a fourth sample. I believe it would be better worded as follows: Advanced microscopy on two samples and sequence confirmed PCR on a third sample from the same individual were used...
Corrected according to the referees suggestion

2. Line 12 – I believe it would be clearer if it said: …gold standard of combined sequence-confirmed PCR and positive advanced microscopy…

Corrected

3. Page 6 line 24 – how was ‘2 clear vacuolar forms’ chosen as the criterion?

We included 2 clear vacuolar forms as the criterion to circumvent ambiguity in microscopical diagnosis. In specimen with such low numbers, a second observation is routinely checked by a second microscopist in our laboratory.

4. Page 7 line 9 – it would be worth specifying here that DNA was extracted and tested for ALL day 2 samples from each patient, not just microscopy positive ones. This eventually becomes clear from the text but it would be simpler to specify here.

Corrected.

5. Page 8 line 2 – how many sequences appeared to be mixed and were these all mixed Blastocystis subtypes or Blastocystis plus a non-specific sequence? Some comment on whether intra-subtype variation was seen would be useful. Are the sequences going to be deposited in GenBank?

We deposited the sequences to Genbank and included Genbank accession numbers.

6. Page 9 line 12ff – sample 2 was also examined by microscopy after FECT. It would be useful to state how often Blastocystis was detected since FECT has been reported to be less sensitive for Blastocystis detection. If possible also report on whether subtypes were equally affected. Also, do the authors have any information on infection intensity being correlated with subtype? On page 10 line 18 it is mentioned that this information was recorded for at least two samples. The literature suggests that intensity may be correlated with symptoms, although I am not convinced, and if the authors have information that could contribute to resolving this issue it would be welcome.

FECT is in our experience not a good method to detect Blastocystis, as many others have shown previously. We did not observe a significant difference in Blastocystis numbers according to genotype, which is why we did not include this information.

7. Page 10 line 13ff – there are other possible explanations for the differential sensitivity of microscopy and PCR on some samples. 1. The microscopy +ve cells detected may not actually have been Blastocystis; 2. Since microscopy and PCR were performed on different samples, the PCR –ve sample may actually also have been microscopy –ve; 3. Evidence suggests that certain Blastocystis PCR primers do not amplify all subtypes equally.
We included the first and third alternative explanations for the microscopy positive, PCR negative samples. The second alternative explanation is unlikely, as for all protozoa studied the DNA signal detected by PCR is fairly constant over time, and less prone to intermittent shedding than the intact parasites observed by microscopy.

8. Line 22 – which subtype was identified from the weak band?

ST2, this is mentioned in the third footnote to table 1.

9. Page 11 line 6 – was there any correlation between subtype and age?

There was no significant correlation between subtype and age.

10. Page 13 lines 18-19 – I do not understand the link being made between different relative prevalence and different transmission cycles. Are the authors suggesting zoonotic transmission of ST3, for example? Alternatively prevalence could be linked to variable cyst numbers produced by different subtypes, or differential survival of subtypes in the environment, or different infectious doses being needed. None of these are really to do with the transmission cycle.

Different relative prevalence may be due to different exposition to spores, which in turn may be due to different transmission cycles, including zoonotic transmission as the referee suggests. We have clarified this by adding a sentence summing up different variables that could influence different relative prevalences.

11. Lines 19-20 – is there any information on these patients that might link them to birds?

If this information exists, we cannot retrace it, as samples and patient information were anonymized to meet ethical and legal requirements with regard to this research. A link to birds was not extracted from the patient data before anonymization.

**Reviewer's report #2**

**Title:** Diagnosis and subtype analysis of Blastocystis spp. in 442 patients in a hospital setting The Netherlands.

**Version:** 1  **Date:** 23 March 2013

**Reviewer:** Eric Viscogliosi

**Reviewer's report:**

Blastocystis is an anaerobic parasite that inhabits the intestinal tract of humans and a wide range of animals. To date, Blastocystis is the most common intestinal parasite found in human feces and its prevalence is by far higher than those of other intestinal protozoan parasites such as Giardia, Entamoeba and Cryptosporidium. Its prevalence usually exceeds 5% in the general population of developed countries, can reach 30 to 60 % in developing countries, and largely exceeds 40% in individuals with chronic gastrointestinal illness such as irritable bowel syndrome (IBS). This parasite is also frequently found in immunocompromised individuals and a higher risk of Blastocystis infection has been found in humans with close animal contact emphasizing its zoonotic potential. It also exhibits a large genetic diversity that could be correlated with the differential pathogenic power of the 13 subtypes (STs) identified so far.
Accumulating recent studies shed new light on the pathogenic power of this parasite suggesting that Blastocystis infection is associated with a variety of gastrointestinal disorders, may play a significant role in IBS, and may be linked with skin disorders.

Therefore, the exploration of the genetic diversity of Blastocystis isolates in human populations is of special interest for understanding the biology and assessing the public health significance of this parasite. It also permits characterization of the transmission dynamics and pathogenicity of the different Blastocystis STs. To my knowledge, the authors have performed in the present study including a large number of patients the first epidemiological survey of Blastocystis isolates in The Netherlands. They show the high prevalence of the parasite in their hospital-populations of patients (24%) and acquired the first data regarding the prevalence of different STs. As in most countries around the world, ST3 was predominant followed by STs 1, 2 and 4. These data have been compared to those obtained from other hospital populations in different countries. In addition, the authors have shown the high sensitivity of stool examination with microscopy which is almost identical to that of PCR if performed by technicians having extensive experience in parasitological diagnosis. Interestingly, the authors propose a "standardized" protocol for routine microscopic examination of stools with the use of the Triple Faeces Test.

This work is an interesting and useful contribution to our understanding of genetic diversity of Blastocystis. This study also proposes a protocol to improve the detection of this parasite by microscopic observation. Globally the manuscript is clearly and concisely written and no superfluous tables are present. Thus I think that the paper should be considered for publication but I would like to see some minor essential revisions addressed first:

Minor essential revisions

1). In the background part of the manuscript, the authors should include more recent publications in the field. For instance, regarding the association between Blastocystis infection and IBS, the reviews by Boorom et al. (2008), Yakoob et al. (2010), Dogruman-Al et al. (2010) and Poirier et al. (2012) should be included. This is also the case for the study by Tan et al. (2009) regarding Blastocystis infection in immunocompromised patients. Concerning the genetic diversity of Blastocystis, the reference Parkar et al. (2010) should be definitely added. Concerning the transmission of the parasite, the studies by Eroglu et al. (2010) and Leelayoova et al. (2008) should be added. In addition, the authors indicate in the text that "recent studies suggest certain molecular methods to have highest sensitivities". However recent studies describing the development of real-time PCR assays for sensitive and specific detection of Blastocystis by Stensvold et al. (2012) and Poirier et al. (2011) are not mentioned. This part of the manuscript must be reconsidered.

We tried to avoid excessive referencing, but as the reviewer suggested we included the references mentioned and rephrased part of the introduction as the referee suggested (see also response to reviewer #1).

2) It seems that the sequences obtained in this study were not submitted to the Genbank since accession numbers are not mentioned in the text. GenBank accession numbers have to be supplied because the corresponding sequences could be used in further epidemiological or phylogenetic studies.
We submitted the sequences to Genbank (see previous reviewer’s comments).

3) By experience I remain skeptical regarding the determination of predominant ST for sequences containing a mixed signal due to co-infecions. Is that the authors are confident of the sequences obtained in these cases? What is the percentage of cases of co-infection? Is that the sequences obtained in the case of co-infections were included in the statistical analysis? They should be logically excluded from these analyzes. Globally, the authors should clarify these points.

Only in fifteen samples a higher background was observed in one or both sequence traces of these samples, resulting in base-calling quality scores of < phred30. These samples may have contained mixed sequences, but only in few of these cases a second ST was likely. From data on mixed templates we estimate the ratio between the subtypes in these cases to be 1:4 to 1:8. This is a different situation from a 1:1 ratio, where it is indeed very difficult to discern which subtypes are present, and if so, which one is the dominant one. As an illustration, we include a sequence trace where a possible mixed sequence is present (green bases denote phred quality values <30). We are confident that the reviewer will agree that in such mixed sequences, assigning the dominant ST is possible.

4) Table 1: the percentages of each ST should be included in this table in brackets next to the corresponding number of isolates.

Corrected.
Reviewer's report #4
Title: Diagnosis and subtype analysis of Blastocystis spp. in 442 patients in a hospital setting The Netherlands.
Version: 1 Date: 3 April 2013
Reviewer: Hisao Yoshikawa

Reviewer's report:
This manuscript reports on the subtyping of human Blastocystis detected from clinical fecal samples in Netherlands. For the detection of Blastocystis, the authors had applied SAF preservation for the examination of light microscopy and PCR subtyping with eluted DNA by the MagNA pure LC isolation kit (Roche). However, the previous study showed microscopic diagnosis of SAF-fixed stool samples for Blastocystis resulted in poor diagnosis among all five European reference laboratories (Utzinger et al. 2010 Clin. Microbiol. Infect. 16:267).

Conversely, MagNA pure LC isolation kit also reported as low rate of detectability of Blastocystis at DNA level compared to other kits (Yoshikawa et al. 2012 Parasitol. Res. 109:1045) and three Blastocystis-positive samples among 21 fecal samples could not detected by PCR with DNA samples extracted by MagNA pure LC isolation kit (Morris et al. 2009 Parasitol. Res. 104:341). These results indicate that SAF preservation and MagNA pure LC isolation kit are limited for detection of Blastocystis.

Therefore, this study could not detect real prevalence of Blastocystis in the human fecal samples in Netherlands based on SAF-preserved samples and PCR subtyping with the extracted with MagNA pure LC isolation kit. It is also difficult to evaluate the results of relationship between specific subtype(s) and patient history based on the inappropriate methodologies.

The authors should use more sensitive methodologies for the diagnosis of human Blastocystis, such as short-term in vitro culture or more sensitive kit for extraction of the DNA from fecal samples.

We most strongly disagree with this referee. Concerning microscopy: the referee cites a study from Utzinger et al. In this study, stool samples were used that were fixed >4 h past production, i.e. too late, which is exactly why we mention the time to fixation in p12 l21-24. Moreover, not all laboratories routinely screen for Blastocystis, which may also have influenced the results in that study for this species. The fact that five other laboratories have differences in their diagnostic yields for microscopy of Blastocystis, without defining a gold standard by an independent method, just proves that microscopy between these five laboratories is different. It is not a valid argument to suggest that our study used “inappropriate methodologies”.

Concerning DNA isolation and PCR: the referee cites two studies that allegedly show limited yield for Blastocystis DNA with the Roche MagNA Pure LC DNA Isolation Kit. The study by Yoshikawa states a PCR yield of 10% for the MagNA Pure LC DNA Isolation Kit (5/50 culture positive samples were positive by their PCR with their application of the Roche DNA isolation). Obviously, this is in sharp contrast to our finding of >20% in our population. In our opinion the study referred to is flawed, possibly by incompatibility of PCR reagents and DNA extraction methods.

It is well known in the molecular diagnosis field that suppliers of DNA extraction and PCR reagents tend to optimize their PCR reagents for their own DNA extraction methods. Especially in silica-based DNA extraction protocols pH plays a major role in elution
efficiency, and we have experienced that the eluate from the MagNA Pure LC DNA Isolation Kit has too high a pH for efficient DNA amplification using PCR reagents from certain other suppliers. Unfortunately the study by Yoshikawa et al does not state which PCR buffer system is used, but from the described Taq polymerase used, it is clear that it is not a Roche buffer system. In general, investigators that in a comparative study of 5 DNA isolation methods fail to amplify spiked samples after extraction with 2 kits and find extraction yields of 10%, 48% and 94% for the other kits should be very critical of the read-out method used, i.e. the PCR in this case. Systematic use of an internal control (as in our study) could overcome such problems. The inhibition control used by Yoshikawa et al was only applied to “some” samples, and was an external control, using DNA isolated with DNAzol, which in fact may have lowered the pH of MagNA Pure LC DNA Isolation Kit-extracted samples sufficiently to obtain PCR amplification.

In addition, inappropriate storage and dilution conditions and inappropriate sensitivity of the used 18S rDNA PCR may have played a role. The latter is evident from the part of the study that shows that a different PCR on certain DNA isolations is positive while the 18S rDNA PCR is negative. As indicated in our Methods section, we stored samples in with Stool Transport and Recovery (STAR) buffer, used a 10 minute prelysis step (as recommended in the revised protocol of November 2011 of the manufacturer), and used 20 ul fecal suspension as input material. The study referred to by the reviewer apparently uses the equivalent of 200 ug washed feces stored at -20C without a special conservation buffer, and lysis conditions are not evident from that methods section. Since in DNA extraction and PCRs on fecal material the adage “less is more” holds true, these might be additional factors in the abominable recovery obtained for most kits tested in the study by Yoshikawa et al.

The other study the reviewer refers to, Morris et al. 2009 Parasitol. Res. 104:341, does not exist. Probably the reviewer means Jones et al Parasitol Res. 2009 Jan;104(2):341-5? Indeed there are three patients in that study that had a “physician diagnosis of symptomatic Blastocystis infection” that have negative PCRs after DNA extraction with the MagNA Pure LC DNA Isolation Kit. However, as there was no independent other method used on the same sample, this is not a reason to dismiss the MagNA Pure LC DNA Isolation Kit as inappropriate for Blastocystis DNA extraction.

In conclusion, the studies cited by the reviewer in our opinion do not convincingly show that the methods used by us, if applied by experienced users, are inappropriate. In contrast, the agreement between the methods used by us on such a large number of samples indicates the opposite.

Level of interest: An article of insufficient interest to warrant publication in a scientific/medical journal

Quality of written English: Not suitable for publication unless extensively edited

We think that the incorporation of the remarks and suggestions of reviewer #1, who is a native speaker, addresses this issue satisfactorily.
Reviewer's report #3
Title: Diagnosis and subtype analysis of Blastocystis spp. in 442 patients in a hospital setting The Netherlands.
Version: 1 Date: 31 March 2013
Reviewer: Ken Boorom

Reviewer's report:
The paper is a diagnostic study of Blastocystis infection performed at a clinical laboratory in The Netherlands. It is noteworthy for the use of multiple detection methods, along with sequence-based subtyping of Blastocystis samples.

SUGGESTION #1:
I would strongly urge the authors to include supplementary data with their study, for example, the sequences obtained from the 442 patients, along with the gender/age and results from each of the diagnostic tests. This would be a useful resource for the Blastocystis research community. I don't believe any study exists with sequence data for 442 patients.

If patient confidentiality is an issue, the ages could be reported as binned data (i.e. 30-34, 35-40, etc.)

We have submitted the sequences to Genbank. The discrepancies between the different tests can be extracted from footnotes to Table 1. We have not included the data on patient age as these are confounded by the association with travel: we observed a higher prevalence in travelers, which results in a higher prevalence in the age groups in which travelers are overrepresented (30-40 in our population).

Content Issues

Issue #1: The study compares the frequency of detection of Blastocystis in patients to prevalence numbers from other studies
The abstract notes that "(24.2%) patients were diagnosed with Blastocystis spp. infection, which is higher than most 14 previously reported prevalences in industrialized countries."

The patients studied in this report do not represent a random sample of the population. Such patients are (a) seeking healthcare and (b) have been seen by a physician at the tropical medicine department, who presumably has exercised judgement in ordering a test which, based on the patient's history, is likely to yield a positive result.

It is not uncommon to find Blastocystis infection in 30-40% in IBS patients (i.e. Yakoob/Haider studies from Pakistan) or even higher rates in screened cases (i.e. Stensvold's studies from Denmark) Population-based studies from the same countries will show Blastocystis infection at a much lower rate.

The problem arises when the prevalence number is quoted out of context, for example, "Blastocystis infection is rampant in the Netherlands, as 24.2% of the population is infected" or "Blastocystis can not be a problem in the Netherlands, as 24.2% of the population is infected."

The use of a different term, such as frequency of detection, instead of prevalence can help make the distinction. Alternately, emphasizing this point in the abstract and avoiding comparison of the frequency of detection with prevalence numbers would be appropriate.
This is a valid comment by the reviewer. We added “in a university hospital” to the description of the population in the abstract and used frequency of detection rather than prevalence in the results paragraph. We added “in our patient population” in the discussion paragraph of the abstract.

Page 5
Line 4: I suggest including the note that patient selection influences the frequency of detection, if population studies from Japan will be quoted next to the frequency of detection of Blastocystis in patients referred for testing.

We added “especially the patient population studied,” in the next sentence.

Line 6: Suggest changing “prevalence” to “reported prevalence” The prevalence does not change based on the detection mechanism.

Corrected

Issue #2:
Page 5, Line 17:
Host genetic factors have been found to influence expression of symptoms in Blastocystis infection. A study by Olivio-Diaz is attached.
It may be worth noting that three labs (i.e. Long, Tan, Kumar) have identified up-regulation of IL-8 in the pathogenesis of Blastocystis infection, and the Olivio-Diaz study found that patients with a genetic predisposition to high IL-8 production show symptoms more often.
So far, this is the only model that would explain the finding that in families where multiple members are infected with the identical strain of Blastocystis, not all members show symptoms (unpublished data, and Nagel, 2012)

we included this possibility with appropriate reference and thank the reviewer for pointing out this study to us.

English Usage Issues
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All pages
I believe all instances of "sequence confirmed PCR" to "sequence-confirmed PCR"

Corrected

There are a few instances where "Blastocystis" is not italicized in the main text. The instances of Blastocystis and Blastocystis hominis in the index should be italicized.
(If you are using Microsoft Word, there is a feature under the Replace command that allows all instances of Blastocystis to be changed to italicized Blastocystis)

Corrected (see reviewer 1)

Page 3
All of the above corrected

Page 5
Line 16: "moment of infection" to "length of infection"
Corrected to “duration of infection”

Page 5
Line 23 "describe" to "described" (should be consistent with use of the present or past tense)
Corrected to present tense throughout the sentence

Page 7
Line 21 "size separated" to "size-separated"
Page 8
Line 3 Remove comma, "obtained, were" to "obtained were"
Page 9
Line 4 Add comma, "January 2009 442 TFT sets" to "January 2009, 442 TFT sets"
Line 13 Add comma "In 90 patients both" to "In 90 patients, both"
Line 14 Change comma to period. "Blastocystis, in 10 patients" to "Blastocystis. In 10 patients..."

All of the above corrected

**Level of interest:** An article of importance in its field
**Quality of written English:** Needs some language corrections before being published
**Statistical review:** No, the manuscript does not need to be seen by a statistician.
**Declaration of competing interests:**
I declare that I have no competing interests.