**Author’s response to reviews**

**Title:** Molecular prevalence of intestinal parasites infections in children with diarrhea in Franceville, southeast of Gabon.

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**Author’s response to reviews:**

Franceville, April 3, 2020

BMC Infectious Diseases
To the Editor-in-Chief

Dear Editor,

We would like to submit the revised manuscript entitled: “Molecular prevalence of intestinal parasites infections in children with diarrhea in Franceville, southeast of Gabon”, by OYEGUE-LIABAGUI SANDRINE LYDIE et al., which we would like to present for an original research. The manuscript number for the first revision was ID: INFD-D-19-02707R1.

Indeed, after its first revision on March 17th, 2020, we received the comments of reviewers on March 24th, 2020.

We have corrected the manuscript according to these comments and improved its quality. We have used the ‘tracked changes’ function as recommended, so that the editors and referees can see the changes we have made.

We acknowledged all the comments of the reviewers and incorporated their suggestions. See from page 2 all our responses to the reviewers’ comments.
All authors have seen and approved the manuscript and have taken due care to ensure both the integrity of this work and conformity with national and institutional policies.

Thank you in advance for taking the time to review this revised manuscript.

Sincerely yours,

Sandrine Lydie OYEGUE-LIABAGUI, corresponding author
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RESPONSE TO REVIEWER’S COMMENTS

Reviewer 1 (José Guillermo Esteban)
Review Comments:

1. Perhaps, the most relevant aspect in the present study is the concept of diarrhea, which is usually defined as the decrease of the consistency and, at the same time, an increase of the frequency of evacuations. Diarrhea is actually a sign that reveals the physio-pathological alteration of one or several functions of the intestine (secretion, digestion, absorption or motility) and, finally, is an indicator of an intestinal disorder related to the transport of water and electrolytes. Perhaps, the concept of diarrhea should be defined, as it may refer to "acute" (not exceeding a duration of two weeks), "persistent" (a duration of two to four weeks) or "chronic" (when ongoing for more than four weeks). Infections that often provoke acute diarrhea, just as is the case with chronic diarrhea, are due to intestinal parasites.

Correct. Following this wise suggestion, we added the following sentences in the introduction: “The World Health Organization (WHO) and United Nations International Children Emergency Fund define diarrhea as more than three loose or watery stools during a 24h period. A duration of 14 days is the proposed criterion for acute diarrhea or persistent diarrhea (1).” See the “Introduction” section: page 2 lines 3-4.

2. It remains unclear what is really meant by "direct microscopic examination", as there is nothing in the Ms that could indicate how the microscopic study of the intestinal parasite infections in the children affected by diarrhea in Franceville was carried out.

Following this good suggestion, for a better understanding, in “Materials and methods”, we changed the description of “Direct microscopic examination” with:

“Direct examination
Macroscopic examination was performed on each stool sample to note the appearance, consistency, color and possible presence of blood, mucus and adult forms of parasites. Direct microscopic examination after staining was carried out using the Kop-Color kit, according to the
manufacturer’s instructions (Para-Selles/Kop-Color II Fumouze®; Fumouze Diagnostics; Levallois-Perret, France). The Kop-Color II kit is a differential staining process of parasitic elements using a mixture of staining agents one of which is Lugol. Briefly, after homogenizing the stool, a volume of stool equivalent to the size of a pea was placed in a hemolysis tube containing 1 ml of diluent (physiological water). The mixture is triturated and stirred to obtain a homogeneous suspension. 10 µL of KOP-COLOR II were placed on a slide and 1 drop or 25 µL of the stools suspension was added. The mixture thus obtained was well mixed. A coverglass was placed over the stool suspension and then examined using a microscope with white light (blue filter). Parasitic elements appear in yellow, yellow-orange or brownish-yellow on a more or less dark blue background.” See the “Materials and Methods: in direct microscopic examination” section: page 5 lines 11-24.

3. When the authors refer to "PCR detection", 18 parasite species are being mentioned, which are listed in Table 1. I do not understand why they are listed in that order as no particular criteria become evident. At least, initially protozoans (i.e. amoeba, flagellates, coccidia, ciliates, microsporidia and chromista), trematodes, cestodes, and nematodes. Moreover, the same species do not appear in Table 1, only E. dispar, without nuancing E. histolytica; neither do S. haematobium/mansonii appear.

We took into account this suggestion and changed the order of the parasites listed, protozoans are mentioned first and helminths after: “Cryptosporidium hominis/parvum, Cystoisospora belli, Entamoeba hartmani, Entamoeba histolytica/dispar, Dientamoeba fragilis, Trichomonas intestinalis, Blastocystis hominis Enterocytozoon bieneusi, Encephalitozoon intestinalis, Ancylostoma duodenale, Necator americanus, Ascaris lumbricoides, Enterobius vermicularis, Strongyloëides stercoralis, Trichuris trichiura, Schistosoma mansoni, intercalatum, haematobium, Hymenolepis sp”. See the “Materials and Methods: PCR detection” section: page 5 lines 26-31 and Table 1.

Also, rather surprising is the fact that neither in the Ms, nor in Table 1, the search for Giardia is mentioned as it is one of the most frequent protozoan parasites in low-income countries.

Correct. Giardia was selected for analysis in our samples when the study was designed. But unfortunately, we had not obtained the primers at the time of the analyzes. The search for Giardia in our stool samples is ongoing and will be the subject of a future article.

4. The PCR of the parasites is another relevant point. Yet, one thing is what the authors put, another thing is explain more about the technique in question.

We improved the description of the PCR technique: “The identification of parasitic species was carried out using conventional PCR or multiplex amplification. Briefly, five microliters of DNA were amplified with a 1X Taq polymerase buffer (Invitrogen. Alphalab; Saint Laurent d’Aigouze, France), 0.8 µM of each primer, 0.2 mM dNTP (Invitrogen), 1.5 mM MgCl2, and 0.024 U of Taq DNA polymerase (Invitrogen. Alphalab; Saint Laurent d’Aigouze, France), for Cryptosporidium hominis/parvum, Cystoisospora belli, Entamoeba hartmani, Entamoeba histolytica/dispar, Dientamoeba fragilis, Trichomonas intestinalis, Blastocystis hominis Enterocytozoon bieneusi, Encephalitozoon intestinalis, Ancylostoma duodenale, Necator
amERICANUS, Enterobius Vermicularis, StrongyloëIDES STERCORALIS, Trichuris Trichiura, Schistosoma mansoni, intercalatum, haematobium, Hymenolepis sp. For Ascaris lumbricoïdes, five microliters of DNA were amplified with a 1X Taq polymerase buffer, 0.2 μM of each primer, 0.2 mM dNTP, 1.5 mM MgCl2, and 0.024 U of Taq DNA polymerase. Specific cycling programs for each species are described in the corresponding references mentioned in Table 1. PCR products were detected by 2% agarose gel electrophoresis stained with GelRed® (Invitrogen. Interchim; Montluçon, France). See the “Materials and Methods: PCR detection” section: page 6 lines 2-12.

5. With regard to the "study population" in the Results section, it would be more convenient to highlight the increase in the creatinine level, which is known to be generated from creatinine; and which is known to be a waste product of the metabolism, being filtered by the kidneys in order to be eliminated through urine. Thus, measuring it in serum is indicative of the kidney function. Perhaps, the question to be posed is: what does this parameter contribute in the context of intestinal parasitism? As several etiological agents (bacteria, viruses and parasites) are known to be able to alter this parameter.

We followed this suggestion and changed the previous sentences in the “Discussion” section (In our study, creatinine levels are significantly higher in infected diarrheal children than in uninfected diarrheal children. Similarly, in a validation study, it was shown that the uretic/creatinine ratio is a predictive factor in the evolution of hemolytic uremia associated with diarrheal syndromes) to: “Indeed, a previous study determined the proportion of morbidity attributed to S. haematobium infection and showed that, based on the results of prevalence ratios and attributable fractions, urine albumin-to-creatinine ratio (UACR) was identified as the most reliable tool for detecting schistosome-related morbidity, followed by dipsticks, visual urine inspection, questionnaires and finally clinical examination. In addition, prevalence of albuminuria determined using UACR was positively associated with the presence of microhaematuria and proteinuria detected by dipsticks. Their finding suggests that these indicators used in combination can be a better predictor of the presence of urinary tract morbidity due to S. haematobium infection in children than the use of a single test parameter, which would thereby facilitate effective and timely interventions.” See the “Discussion” section: page 10 lines 2-11.

6. Also, the fact that 60% of the 100 children studies presented with fever is relevant. The question becomes rather evident: were bacterial and virus infections ruled out? This point is of utmost importance in the context of the study carried out, as these etiological agents are usually present with fever.

Correct. The study on viruses, namely rotavirus A, adenovirus, bocavirus, astrovirus, saprovirus, norovirus type 1 and norovirus type 2, has already been carried out on these samples and data analysis is underway. We also plan to look for bacteria in these same samples.

7. With regard to "prevalence of intestinal parasites", the authors indicate that 81% of the children did not present parasites after the direct examination of the feces!!! This is rather surprising as there are no data concerning the microscopic vision of the direct examination. In fact, the eggs of Hymenolepis should be visible as the authors were certainly faced with H. nana
which is transmitted by eggs, being quite normal in children below the age of five. Indeed, in the Discussion, they refer to H. nana as the most prevalent pathogen. Also, it is not clear why they mention this species in the Discussion.

Indeed. For a better understanding, we changed this sentence “The majority of children (81%) showed no parasite after direct examination of intestinal parasites in diarrheal stool” to: “Microscopic examination of stool samples did not lead to the detection of parasites among the 81 participants examined. However, the PCR test detected at least one intestinal parasite in 61 stools samples with 61% of global prevalence”. See the “Result: Prevalence of intestinal parasites” section: page 7 lines 11-13.

We also changed this sentence “More than half of the study population tested positive for at least one parasite (61)” to: “The global prevalence of intestinal parasitic infection was 61%.” See the “Discussion” section: page 11 line 7.

Moreover, the primers available to us do not specifically identify the Hymenolepis nana species, only Hymenolepis in general. Our apologies, Hymenolepis diminuta is very rare in humans, which is why we referred to Hymenolepis nana in the discussion. We changed Hymenolepis nana to “Hymenolepis sp”. See the “Discussion” section: page 10 line 16.

8. Moreover, at this level of prevalence of intestinal parasites, it would be important to get to know the total prevalence of each species detected as the results referring to parasite prevalences do not seem to coincide. Another thing is the detection of multiparasitism (= polyparasitism), which evidences the deficiencies of personal sanitary conditions as well as of the environmental conditions.

The global prevalence of each species detected do coincide with the results of parasite prevalence, however, only the highest prevalences were cited in the text: “Cryptosporidium hominis/parvum: 19% (19/100); Encephalitozoon intestinalis: 15% (15/100); Hymenolepis sp: 31% (31/100). The prevalence of other species was 2 and 4% for Enterocytozoon bieneusi (2/100), Dientamoeba fragilis (4/100), Trichuris trichiura (4/100), respectively, as shown in Figure 1.

9. Another thing is the detection of multiparasitism (= polyparasitism), which evidences the deficiencies of personal sanitary conditions as well as of the environmental conditions.

Correct. In the “Discussion” section, we added the following sentence: “Indeed, generally inadequate personal hygiene, unsafe water supplies, low levels of parental education and environmental conditions have been associated with polyparasitism”. See the “Discussion” section: page 11 lines 23-25.

10. Concerning the "distribution of intestinal parasitic infections in relation to age and sex", it can be observed that children below age 4 are those most parasitized (52 children) vs older children (above age 4) who do practically not present any parasites (9 children). Also, it should be mentioned that the authors indicate that the prevalence of infectious parasites was higher in the older children. Frankly, this does not go together with the data presented in the tables.
In the “Discussion” section, we changed “the prevalence of parasitic infections was higher among older children” to: “the prevalence of parasitic infections was higher among young children”. See the “Discussion” section: page 11 line 31.

11. Finally, concerning the conclusions, it is surprising that the authors conclude that STHs and intestinal protozoan parasite prevalences are very high in the children. This is rather surprising with regard to Trichuris, in which the result is not as relevant as the authors present it.

That is correct. We changed the following sentence “The prevalence of intestinal parasitic infections, including soil-transmitted helminthes (STHs) and protozoa parasites were very high in children in our study population.” to: “Intestinal parasitic infections with Cryptosporidium hominis/parvum, Encephalitozoon intestinalis and Hymenolepis sp, were more prevalent in children in our study population.” See the “Conclusion” section: page 12 lines 8-10.

12. Concerning the English language, there are several errors. The Ms should be revised by a native speaker of English.

Our apologies, our manuscript has been revised by a native English speaker.

Reviewer 2 (Graciela Teresa Navone)
Review Comments:
The present study describes the prevalence of IPIs and associated parasites in children with diarrhea living in Franceville, southeast of Gabon. The diagnosis is mainly based on PCR technique. They also describe the clinical status and blood parameters of the children analyzed. Then, they study the association of intestinal infections with the age, and the seasonal variation of the parasite prevalence.
The following observations were included in the attached PDF MS

1. Abstract
   (page 2)
   Line 40: "helminths" is more frequently used word than "helminthes".

   Correct. We changed "helminthes" to: "helminths" throughout the article.

   According to this suggestion, for a better understanding, we changed this sentence “Polyinfection rate was 19.7%, such as ten children with double infections (83.3%) and two children with triple infections (16.7%)” to: The polyparasitism rate was 19.7%, with 83.3% double infections and 16.7% triple infections”. See the “Abstract” section: page 2 lines 17-18.

2. Introduction
   The main objective is not clear. The authors said "By employing PCR, the aim of the current study was to assess the prevalence of IPIs and associated parasites in children with diarrhea living in Franceville, southeast of Gabon". But, they calculated only the prevalence of parasites of which they have primers available.
The authors calculated the prevalence of intestinal parasites by PCR, but also studies the association with the age, the seasonal variation of the prevalence and analyze blood samples and other clinical parameters. So, it is recommendable to also specify the following objectives: Seasonal, age and clinical parameter association of the intestinal parasite infections in the "Introduction".

Correct. We changed the main objective to: “The main objective of this study was to assess the prevalence of eighteen IPIs by microscopic and molecular diagnosis and their distribution according to age and seasonality, in children with diarrhea living in Franceville, south-east of Gabon”. See the “Introduction” section: page 4 lines 21-23.

3. (page 3)
Line 55: Blastocystis organisms isolated from humans have commonly been referred to as B. hominis. However, because of extensive genetic diversity (even among organisms isolated from humans) and low host specificity, the designation Blastocystis sp. is considered more appropriate. Indeed, the work cited mentions this parasite as "Blastocystis spp."

Correct. We changed Blastocystis hominis to: “Blastocystis sp”. See the “Introduction” section: page 3 lines 27-33 and page 4 line 2.

4. Materials and methods
(Page 4)
Line 45: I don’t understand the needed to include the time of sample collection. It is more important to specify the number of collected samples per child.

Indeed, we removed the time of sample collection and added: “and two samples, among which one blood sample and one stool sample, were collected per child”. See the “Materials and Methods: study area and participants” section: page 4 lines 30-31.

5. (Page 5)
Lines 47-57: The parasites analyzed could be listed following a criterion, such as protozoan first, helminths secondly. It would be interested to specify why these parasites were selected.

Correct. We changed the order of the parasites listed, protozoan first and helminths second: “Cryptosporidium hominis/parvum, Cystoisospora belli, Entamoeba hartmani, Entamoeba histolytica/dispar, Dientamoeba fragilis, Trichomonas intestinalis, Blastocystis hominis Enterocytozoon bieneusi, Encephalitozoon intestinalis, Ancylostoma duodenale, Necator americanus, Ascaris lumbricoides, Enterobius vermicularis, Strongyloides stercoralis, Trichuris trichiura, Schistosoma mansoni, intercalatum, haematobium, Hymenolepis sp”. See the “Materials and Methods: PCR detection” section: page 5 lines 26-31 and Table 1.

This study is part of a project on pathogens associated with diarrhea in children in urban and rural areas in Gabon whose main objective is to inventory all pathogens including viruses, bacteria and parasites in the same samples. Moreover, the results presented here are only part of the expected results produced during this project.
6. Line 60: It is recommendable to specify the concentration, units or quantity of reagents employed, and the specific cycling programs for each gene. In this way, your protocol could be replicated by other researchers. For example, as a supplementary material or including (in the table 1) the reference of works where the primers were obtained.

Correct, we added the concentration and units of reagents employed in the text: “The identification of parasitic species was carried out using conventional PCR or multiplex amplification. Briefly, five microliters of DNA were amplified with a 1X Taq polymerase buffer (Invitrogen), 0.8 μM of each primer, 0.2 mM dNTP (Invitrogen. Alphalab; Saint-Laurent d’Aigouze, France), 1.5 mM MgCl2, 0.5 μg/μL, and 0.024 U of Taq DNA polymerase (Invitrogen. Alphalab; Saint-Laurent d’Aigouze, France), for Cryptosporidium hominis/parvum, Cystoisospora belli, Entamoeba hartmani, Entamoeba histolytica/dispar, Dientamoeba fragilis, Trichomonas intestinalis, Blastocystis hominis Enterocytozoon bieneusi, Encephalitozoon intestinalis, Ancylostoma duodenale, Necator americanus, , Enterobius vermicularis, Strongyloides stercoralis, Trichuris trichiura, Schistosoma mansoni, intercalatum, haematobium, Hymenolepis sp. For Ascaris lumbricoides, five microliters of DNA were amplified with a 1X Taq polymerase buffer, 0.2 μM of each primer, 0.2 mM dNTP , 1.5 mM MgCl2, 0.5 μg/μl, and 0.024 U of Taq DNA polymerase. Specific cycling programs for each species are described in the corresponding references mentioned in Table 1. PCR products were detected by 2% agarose gel electrophoresis stained with GelRed® (Invitrogen. Interchim; Montluçon, France).” See the “Materials and Methods: PCR detection” section: page 6 lines 2-12.

Furthermore, the references of the studies in which the primers were obtained were included in Table 1.

7. Results
(page 6)
Line 29: It is recommendable to specify the age groups in years.

Given that we have children under one year old in our participants, we prefer to indicate age groups in months in order to have the exact age of each child.

8. (page 7)
Line 40: "Children under 6 months of age were infected with 3 parasitic species of which Encephalitozoon intestinalis, Hymenolepis sp, and Trichuris trichiura". It is better replacing "of which" by "which are".

Correct. We replaced “of which” by “which are”. See the “Results: Distribution of intestinal parasite infections by age and gender” section: page 8 line 2.

9. Line 44: It is better "24-month age group".

Given that the age groups consist of children included in a very specific interval, we considered it more interesting to indicate the minimum and maximum limits of the interval of each age group.
10. (page 8)
Line 20: The samples were collected between November 2016 and August 2017, so it is not clear how it was studied in a year period. The same number of samples must be analyzed in each month to be comparable. The authors studies 100 individuals, but during a long period of time. It is necessary to specify how many samples were analyzed in each period.
The goal of collecting stool samples over a year was to look at the temporal distribution of the parasites. In fact, a study assessing the prevalence of rotaviruses in diarrheal stools in children under 5 years of age and conducted in three localities in Gabon, had shown a temporal distribution of rotaviruses, with high prevalence between the months of February and April which correspond to the long rainy season (SE Lekana-Douki et al. 2015). Also, we asked ourselves the question of knowing if this temporal distribution was also found with the intestinal parasites or not.

Correct. We took into account this good suggestion and added the number of samples analyzed in each period: “The highest prevalence of parasitic infections was observed during the short dry season (December to January) and the long dry season (May to September), (76.9 %: n = 10/13 and 64.3 %: n =18/28) for the short dry season and the long dry season, respectively). Moreover, the lowest prevalence of parasitic infections was seen from February to April (long rainy season) and in November (during the short rainy season), (58.5 %: n = 31/53) and 33.3 %: n = 2/6) for the long rainy season and the month of November, respectively). The distribution of intestinal parasites was also analyzed monthly. The distribution of Hymenolepis sp was consistently found throughout the year except in July with the highest prevalence observed in February which corresponds to the long rainy season (35.5%: n = 11/31). The same is true for Encephalitozoon intestinalis, Cryptosporidium hominis/parvum, Trichuris trichiura and Cryptosporidium hominis/parvum whose prevalence peaked during the long rainy season and the month of May (long dry season). For Dientamoeba fragilis, the highest prevalence was observed in April and Enterocytozoon bieneusi was found in May and June only. The distribution of parasitic species by month was significantly different (p = 0.0003).” See the “Results: Distribution of intestinal parasites species by season” section: page 8 lines 25-30 and 32.

11. Discussion:
(page 9)
Line 12: change "intestinal parasites infections" by "intestinal parasite infections".

Correct. We changed "intestinal parasites infections" by "intestinal parasite infections". See the “Discussion” section: page 9 lines 18-19.

12. (page 10)
Line 45: The authors said "Unfortunately, The existence of parasites in direct examination was not seen in our study". This is better replacing it by "Parasites were not seen in our study"

Correct. We replaced "Unfortunately, the existence of parasites in direct examination was not seen in our study" by: “Parasites were not seen in our study by direct examination”. See the “Discussion” section: page 11 line 14.
Declaration of competing interests:
We declare that we have no competing interests.