Author's response to reviews

Title: High number of diarrhoeal co-infections in travellers to Benin, West Africa

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Dear Nathaniel Nazareno,

Thank you for your kind reply. Please find enclosed our point-by-point response to the reviewer’s comments and two revised versions of the manuscript, one with changes indicated, the other revised and clean. The reviewer requested for determination of bfpA among the EPEC strains. These experiments were carried out by MSc Sointu Mero who also added the data in the manuscript and, accordingly, she has now been listed as one of the authors.

I hope you will find the paper now suitable for publication in the BMC Infectious Diseases. Naturally, we will be happy to assist you with any further changes, if required.

Kind regards,

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Reviewers' comments:

Reviewer 1

The manuscript seeks to elucidate the etiology of travelers’s diarrhea in visitors to the republic of Benin. This is an important question since very little is known about diarrheal etiology in West African countries, particularly francophone ones. Moreover, travelers’ diarrhea studies indirectly provide insight into potential causes of life-threatening infantile diarrhea, the so –called ‘rest of the hippopotamus’(1).

Major compulsory revisions

The paper’s main flaw is the small sample size and in particular the small number of pre-travel samples that were evaluated. It is admittedly hard to collect the samples the investigators need and the population of travelers from which the sample was recruited was small. The investigators however need to clearly acknowledge these limitations (perhaps include a limitations para in the discussion). On the other hand, they also need to make sure that the abstract indicates that the design and analysis is based on pre- and post- samples. I was very negatively pre-disposed to the article until I discovered half way through that pre- samples were collected. And although these samples were few, the absence of pathogens in them provides significant value to interpreting the data obtained.

RESPONSE:

Data on pre-samples was added to the abstract as follows:

“All 18 pre-travel samples proved negative for bacterial pathogens. Of the 39/45 (87%) travellers having had TD, EPEC was detected in post-travel samples in 30 (77%) cases, ....”

A paragraph concerning the low number of travellers has been added to the discussion on page 16:

“The major limitation of the present investigation was its low number of travellers. Nonetheless it represents one of the few TD studies carried out among a large group of travelers visiting the same place and residing in similar conditions for exactly the same period of time. Despite the homogeneous circumstances, the variety of pathogens proved large, the findings varying considerably between individual travellers.”
Another thing the authors may wish to point out is the bias the absence of pre-travel samples introduced. Individuals who did not submit a pre-travel sample are more likely to submit a post-travel one if their diarrhea is particularly disturbing, thus increasing the proportion of exceptionally virulent pathogens recovered.

**RESPONSE:** We have modified the chapter on page 13 in the discussion to point out more clearly the fact that those volunteers who only provided post-travel stool samples appeared more likely to have TD, and even the selection of pathogens may be more virulent than in those recruited prospectively:

“Although Sub-Saharan Africa is generally presented as an area with a 20 – 60% risk for TD [17-20], 87% of our travellers reported having contracted the disease. However, part of our group were recruited at the airport only after the journey. All these volunteers had TD and, in fact, their symptoms appeared somewhat more severe than those of the prospectively recruited TD patients (data not shown); the symptoms presumably encouraged them to take part in the study. The actual proportion of patients with TD should, therefore, be evaluated from those who agreed to participate at baseline: 60% (12/20) of them had TD. Interestingly, the proportion of cases with ETEC appeared higher among those recruited at the airport (67%) than those enrolled prospectively (33%), correlating with the exceptionally virulent nature of ETEC and severity of symptoms.”

The questionnaire and pre-travel advice seems to be rather unsuited to a West African country. West African diets rarely include true ‘salads’ or undercooked meats. Salads are available in major cities but these are largely tourist fare. However there are foods that are high risk for diarrhea, which tend to be warm foods that are not terminally cooked (for example boiled and then pounded root vegetables). Travelers should have been advice to avoid these and told what they were. I see no indication from the paper that this was included in the advice and this may in fact account for the high rate of infection.

**RESPONSE:** The travellers were provided health care advice both in an information session arranged for the whole group and in individual health care appointments. All participants were also handed in writing the general instructions given to all travellers on how to prevent TD. These leaflets include those listed by the reviewer as well as the other information provided to all travellers in well-equipped travel medicine appointments. The first chapter of the methods section has now been modified to point out this more clearly:

“...They were each given questionnaires and test tubes for stool samples either at an information session or a health care appointment before the journey; they also received brochures on pertinent health issues, including detailed instructions on preventive measures against TD. ...“
As the authors indicate, this study revealed EPEC as an important cause of travellers’ diarrhea, something that does not feature in the published literature. Were these atypical or typical EPEC (bfp results)? Would the authors be able to conduct PCR for lineage-specific EPEC virulence genes such as espC or specific bfpA/per alleles?

RESPONSE: The analyses have now been carried out and the manuscript has been revised in the methods on page 9 as follows:

“We also investigated the bundle-forming pilus structural gene (bfpA) linked to the virulence of typical EPEC. The gene was amplified using the primers F_bfpA_001 CTGTCTTTGATTGAATCTGCAATGG and R_bfpA_001 CTGAAATAGCATTCTGTGACTTATTGG. The detection was performed with the Stratagene MxPro 3005P instrument (Agilent Technologies, Inc., CA) utilizing SYBR Green chemistry (Thermo Fisher Scientific, Finland) by a standard two-step protocol with melting curve analysis. Briefly, initial denaturation time of 15 min was followed by 45 cycles of denaturation at 94°C for 1 minute, and annealing/extension at 60°C for 1 min. The PCR amplicon was confirmed correct by Sanger sequencing and characteristic melting temperature.”

and in the results on page 11 as follows:

“11/34 (32%) of EPEC strains proved bfpA-positive.”

and in the discussion on page 15 as follows:

“In our patients, of whom all but one were adults, 32% of the EPEC strains proved bfpA-positive, and were thus considered to represent typical EPEC serotypes [25, 27]. No difference was seen in the occurrence of bfpA between those with and those without symptoms.”

Discretionary: The data demonstrate that diagnosis for diarrheagenic E. coli is important for traveller’s diarrhea and should also be available in West African countries since it is possible that these pathogens account for a large proportion of infantile diarrheas as well. The authors may also want to compare their data with that of the GEMS study (2), which did not include Benin and was focused on infantile diarrhea, because their data suggests that a separate study of infantile diarrhea in Benin is warranted.

RESPONSE: We thank the reviewer for an important point of view. This has now been added to the discussion on page 15 as follows:

“Moreover, little is known about the aetiology of childhood diarrhoea in Benin; no data from there was included even in a recent report centring on Africa and Asia [27]. Molecular biology methods are not currently feasible for routine diagnostics in developing countries, yet periodic microbial surveillance for diarrhoea is considered a necessity today [27,28].”
Reviewer: Thomas Löscher

Reviewer's report:
In this study, samples from the first or second stool passed after returning home were investigated in 45 Finnish travelers of a group travel to Benin, West Africa, with a multiplex RT-PCR assay for major bacterial enteropathogens. In 39 of 45 travelers having had traveler’s diarrhea (TD) during their stay, bacterial pathogens were detected with enteropathogenic Escherichia coli being most common (30 travelers), followed by enteroaggregative E. coli (EAEC, 23), enterotoxigenic E. coli (ETEC, 22), Shigella or enteroinvasive E. coli (EIEC; 7), enterohaemorrhagic E. coli (EHEC, 2), and Salmonella (1) In 31 (79%) of the TD cases two or more bacterial pathogens were identified. Yersinia, Campylobacter, and Vibrio cholera were not detected. It is conclude that PCR diagnostics reveal in most patients a multitude of pathogens, and that the role of each pathogen should be re-evaluated.

Although there are few data using modern PCR diagnostics in travelers returning from a West African destination with or without a history of TD there are considerable limitations with this study:

The number of travelers investigated is rather small (45),

RESPONSE: The number of volunteers has been brought up as a major limitation of the study (see above, reply to the first comment of reviewer 1).

only bacterial pathogens have been investigated,

RESPONSE: In fact, these travellers were also investigated for three parasites in their stools, applying a modern qPCR method, and none of them had any. Unfortunately, the qPCR method has not been published as yet, and our collaborators are not willing to present pertinent data in advance. They agreed that this can be mentioned in the discussion as “data not shown”, which has now been added to page 15 as follows:

“It should be pointed out that the stools of our volunteers were also explored for parasites with a newly developed qPCR method which has not been published as yet. The analysis showed that none of the volunteers had Giardia lamblia, Cryptosporidium parvum, or Entamoeba histolytica in their post-travel specimens (Juha Kirveskari, personal communication).”

and samples have not been collected at the time of TD but only after return and after a variable interval between TD episodes and return (intervals not indicated). Therefore, the (often multiple) bacterial pathogens found in the stool may not represent the etiological agents of a previous TD episode but rather a change of the intestinal bacterial flora when travelling from a low to a high
incidence area for diarrheal disease. This has already been shown in other investigations for EAEC, EPEC, ETEC, and salmonella. To adjust for such an effect, an adequate control group of travelers without TD episode(s) should have been included. However, only 6 travelers without TD had been investigated (and showed similar frequencies of various E. coli pathotypes).

RESPONSE:
We thank the reviewer for remarking on this important point. Since 37/39 (95%) of the travellers with TD were still symptomatic at the time of the post-travel stool sample, we considered the pathogens detected in their stool samples relevant. We have now added to the Results on page 10 the following:

“37/39 volunteers (95%) had ongoing symptoms at the time of sampling.”

Whether a person gets symptoms depends on a number of factors: the nature of the pathogen, the bacterial dose obtained, the infective dose, the colonization resistance provided by each individual’s intestinal microbiota, various intestinal immune mechanisms including pre-existing (specific and cross-reactive) antibodies etc. A couple of examples:

- Practically all travellers get symptoms when infected with Campylobacter, while inhabitants in areas endemic for it may remain fully asymptomatic (own unpublished data from Guinea-Bissau).
- Diarrheaogenic E. coli strains have been reported in asymptomatic travellers in several studies.
- The infective dose varies according to host: e.g. the infective dose of Salmonella Typhimurium is 10 000 times smaller in animals recently treated with antimicrobials.

One of the main findings of this investigation was the multitude of pathogens detected in the post-travel specimens. Likewise, some other studies conducted by modern molecular biology methods have revealed an abundance of pathogens in the stool samples of asymptomatic travellers. We emphasize the need for larger subject populations, since an asymptomatic control group is actually missing in most studies addressing the aetiology of TD. The present study provides data from a large group of subjects travelling together, one of our points being that they were all exposed to similar circumstances while visiting the same destination for the same duration. Unfortunately, as the volume of volunteers with TD was exceptionally high, the number of asymptomatic travellers remained small. Even if they had several pathogens, the selection appeared to favour less virulent strains (see also response to question 1 of Reviewer 1). Indeed, a larger group of volunteers will be required to confirm this.