Author's response to reviews

Title: Serum Immunoglobulin G, M and A Response to Cryptosporidium parvum in Cryptosporidium-HIV Co-infected Patients

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Author's response to reviews: see over
To,
The Editor
BMC Infectious Diseases

Subject: Revised manuscript for publication in BMC infectious Diseases

Dear Sir,

Please find the revised manuscript entitled ‘Serum Immunoglobulin G, M and A Response to Cryptosporidium parvum in Cryptosporidium-HIV Co-infected Patients’ and pointwise reply to the reviewer’s comments. Ethical approval for the study was granted by the Ethical Committee of the Post Graduate Institute of Medical Education and Research, Chandigarh, India. This is included in the ‘methods’ section of manuscript under subheading 'Ethical Clearance' on page 8 lines 13-14.

Reply to Reviewer’s comments

We are thankful to the reviewers for their critical suggestions. We have modified the manuscript as per the suggestion of the reviewers.

Reviewer 1.

1. The subjects studied for antibody response in the present study were earlier examined for prevalence of Cryptosporidium (as seen by staining methods, antigen detection and nested PCR. (Kaushik et al ref 20) and the patients in gp I and III were found positive for Cryptosporidium parvum by nested PCR (secondary primers specific for species parvum). This is now included in lines 2-7 on page 6.

2. We admit that the limitation of the present study is that we have used crude soluble antigen of Cryptosporidium parvum, which may contain a variety of proteins and non-protein components, both with and without antigenic properties. However, we have included positive and negative controls with each plate in the assay to avoid any false results. Further, we suggest that more studies with use of purified antigenic fractions are required. 'Limitations of our study' section is now added in the last paragraph in the discussion on page 16, lines 6-12.

3. The difference in seropositivity in HIV infected as well as healthy subjects in reports from different geographical areas may be attributed to the duration of illness and type of antigen used to study the antibody response. No reports are available regarding prevalence of anti-Cryptosporidium antibodies in India for comparison. However, varying rates of Cryptosporidium positivity in both HIV (4.6-12%) and non-HIV (.06-13%) subjects are reported from different geographical locations in India (Kaushik et al, 2008 ref 20). This is now included in discussion on page 14, lines 14-20

The cut off absorbance value (Optical Density) for each dilution was determined by the mean absorbance of the 5 negative control sera (apparently healthy individuals, excluded for HIV seropositivity, Cryptosporidium and intestinal parasitic infections by stool examination) plus 2 Standard Deviation (S.D). The test sera giving absorbances that were equal and above the cut off O.D were considered ELISA positive at that dilution under the test. This is now included page7, lines 10-15 and page 8, lines 6-10. Positive and negative controls were included with each batch. The cut off value
was calculated to be 0.109 for IgG, 0.824 for IgM and 0.796 for IgA ELISA. This is now included in lines 16-17 on page 9 of manuscript.

4. The optimum dilutions of the antigen, serum and anti-human horse redish peroxidase (HRP) conjugate (Sigma-Aldrich, USA) were determined by checkerboard titration with known positive (pooled sera from 5 patients found positive for Cryptosporidium by staining techniques and confirmed with antigen detection and PCR) and 5 negative control sera (apparently healthy individuals, excluded for HIV seropositivity, Cryptosporidium and intestinal parasitic infections by stool examination). However, the results may vary due to difference in the reagents and experimental conditions used in different laboratories.

5. The sera were collected within 15 days of onset of infection as available. This is now included in 'Methods' section on page 6 line 20-22. However, sera were not available for the follow-up studies and the kinetics of antibody response was not studied.

6. Present study did not show any correlation between the antibody response and history of diarrhoea. In contrast to our study, Frost et al, 2005 (ref 31) reported that in HIV positive individuals, a strong serological response to the 27-kDa antigen group was associated with a reduced risk of diarrhea. This is now included on page 15 line 18-2. However, regarding role of antibodies in protection from diarrhoea there are controversial reports. This is discussed on page 15. Besides a number of factors are suggested to contribute into pathogenesis of and protection from cryptosporidiosis including innate and cellular immune response. Therefore, the antibody response may not be the only factor in protection and further studies in more number of patients are required in this area to find out. This discussion is now included in lines 4-9 on page 16 of the manuscript.

Reviewer 2.

1. In the present study, the person performing the serologic assays was not blinded to the clinical status of the patients. This is now included in the 'methods' section under subheading 'subjects' on page 6 lines 22-23.

2. In the present study, 20 subjects studied for antibody response in group IV were earlier examined for prevalence of Cryptosporidium (as seen by staining methods, antigen detection and nested PCR. Please refer to our earlier study Kaushik et al ref no. 20). For determining the specificity of ELISA, 25 patients with other parasitic infections (4 each with toxoplasmosis and amoebiasis, 2 with ascariasis and 5 each with malaria, hydatid and neurocysticercosis, respectively (Group V) were also included in the study. This is mentioned on lines on page 6 lines 2-16 in the Materials and Methods section.

The cut off absorbance value (Optical Density) for each dilution was determined by the mean absorbance of the 5 negative control sera (apparently healthy individuals, excluded for HIV seropositivity, Cryptosporidium and intestinal parasitic infections by stool examination) plus 2 Standard Deviation (S.D). The test sera giving absorbances that were equal to and above the cut off O.D were considered ELISA positive at that dilution under the test. This is now included page 7, lines 10-15 and page 8, lines 6-10. However, these sera were not tested with Western blotting for anti-Cryptosporidium antibodies.

3. We are in agreement with the reviewer that antibodies have a controversial role in protection from Cryptosporidium and that successful production of antibodies in these patients as shown in our study
may not be necessarily associated with protection. To highlight the same, we have mentioned previous studies in support or against role of antibodies in protection. The interpretation of study by Chappel et al, 1999 (ref 30) is now included on page15 lines 14-18.

However, the mechanism of diarrhoea in cryptosporidiosis is not well-understood and is suggested to be due to disrupted mucosal architecture and intestinal dysfunction resulting from the infection and the host response to the infection besides other factors (ref 33, 34) suggesting that antibody responses may not be the only factors playing significant role in protection from symptomatic cryptosporidiosis, and other innate and cellular immune responses may also be contributing in the protection. This is now included in the discussion on page 16 lines 4-9.

4. Tables 3 and 4 are now deleted from the manuscript and the tables and are now briefly summarized in results and discussion.

Thanks and regards,

Kirti Kaushik