Author's response to reviews

Title: Acute dengue virus 2 infection in Gabonese patients is associated with an early innate immune response, including strong interferon alpha production

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To the Editor

BMC Infectious Diseases

Dear Sir,

As advised in your last email (28th April 2010), please find enclosed our revised manuscript entitled *Acute dengue virus 2 infection in Gabonese patients is associated with an early innate immune response, including strong interferon alpha production* that we are re-submitting for publication in *BMC Infectious Diseases*.

Dengue virus (DENV) is transmitted by mosquitoes and generally causes an acute illness in humans. Until the 1960s, most cases occurred in south-east Asia but the virus has now spread to South Asia, South and Central America, the Caribbean and Africa. Human immune responses have mainly been explored in Asia and the Americas.

For the first time, we have measured plasma levels of 50 soluble factors (cytokines, chemokines and growth factors) implicated in immune response to viral diseases, in 38 African patients who developed acute dengue fever (DF) during the first Gabonese DENV-2 outbreak in 2007. Our results show that acute DENV infection elicits a strong and early innate immune response involving the production of IFN-α and numerous pro-inflammatory factors. Furthermore, using a cell-staining technique, we observed an adaptive immune response involving CD4+ and CD8+ T cell activation and IFN-γ and interleukin-7 production.

In view of the novelty of these observations, their scientific interest and their public health implications, we hope you will find them worthy of publication.

We look forward to receiving your comments and decision.

Sincerely yours,

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I. Response to the reviewer Beatriz Sierra (referee n°1)

Major compulsory revisions

1a – “This reviewer is not sure that the used controls are appropriate. Authors said that thirty control plasma samples were randomly selected among 4500 samples collected during a previous study in Gabon. First, authors should specify what was that previous study about, since those plasmas could have been gotten form people that were suffering diseases or other status that could be influencing in the immune response, and in consequence altering the levels of the immunologic studied parameters.”

As requested, controls have been better described in the sections “Methods” and “Results”. Thirty control plasma samples were randomly selected among 4349 samples collected from healthy individuals throughout Gabon during a previous study (Becquart et al, Plos one, 2010). These individuals were themselves randomly selected among the Gabonese population excluding children and elderly persons (more than 65 years)

1b – “Second, they should specify if those plasmas were tested negative for DENV and for anti-dengue antibodies, and even for anti-flavivirus antibodies.”

These control plasmas have been tested negative for DENV by qRT-PCR assay and for anti-DENV and West Nile virus antibodies by ELISA. This information has been added to the text. These samples have not been tested for yellow fever but the majority of people living in Gabon have been vaccinated against Yellow fever virus.

1c – “They should also verify that the plasma donors matched in the age average, the gender, and the ethnicity with the individuals included in the study.”

Ethnicity matched between the study and control populations (p<0.05). In contrast, control population (60% male; mean age 47 years, range 30-66 years) and patients (40% male; mean age 31 years, range 18-66 years) did not match in age and gender. However, all individuals of the study and control populations were adults (>18 years). Moreover, no significant difference in cytokines levels tested in the study was observed when older individuals were randomly removed from the control population. In the same way, levels of cytokines were not significantly different between males and females in the control population.

2. “Individuals included in the study should be tested for anti-flavivirus antibodies, since a past infection for a flavivirus could be modifying the antidengue response due to the crossreactive memory immune response”

Plasmas from study population have been tested negative for anti-West Nile virus antibodies by ELISA. This data has been included in the article.
Discretionary revisions

Authors studied concentrations of 50 cytokines, chemokines and growth factors in plasma, and also explored some markers by FACS in PBMC. PBMC may result in a "mixed responses" from all the different cells types that were present. It had being interesting to know which were the immunological cells contributing to the production of the different studied cytokines, chemokines and grow factors. Performing such experiments in T, and/or B and/or NK depleted PBMCs could add some valuable information in this respect. At least, authors could make the statistical analysis of the correlation between the percentage of CD3, CD4, CD69/HLA-DR and CD95 in PBMCs in the different donors with the levels of cytokines, chemokines and grow factors studied.

Identifying the immune cells contributing to the production of the soluble factors studied would be very interesting, but a larger size of our panel (more than 6 patients) would be necessary to correlate the percentage of CD3, CD4, CD69/HLA-DR and CD95 with the levels of soluble factors studied.
II. Response to the reviewer Celso Ramos (referee n°2)

Major compulsory revisions

1 – “The title only includes the possible role of INF-alpha as a modulator of immune response, others such as IFN-gamma are not considered in the discussion”

We focused on the role of the IFN-α because one of the most significant results of this study is the confirmation of the importance of IFN-α in modulating DENV infection in DENV-2-infected adults, as previously observed in vitro and in animal studies. We do not agree with the reviewer, we in fact discussed the higher levels of IFN-γ observed in the DENV-2 infected patients but it did not seem necessary to us to discuss it more in our manuscript because this point has already been well described in numerous previous studies (Kurane, 1991; Green, 1999; Chakravarti, 2006; Chen, 2006; Restrepo, 2008).

2 – “If the authors´ hypotesis is is correct, then the increase of IFN-alpha in patients plasma should be related to the viremia, however this information is missing”

As requested, we have included this data in the manuscript. No correlation was found between the cycle threshold (Ct) obtained by qRT-PCR and IFN-α levels ($r^2<0.4$). This absence of correlation between Ct and INF-α levels may be due to several factors: absence of significant difference in the clinical forms of DENV infection observed in the infected patients, consistent and high IFN-α levels among all DENV-2 infected patients and bias due the small size of the study population.

3 – “The effect of IFN-alpha on the activation of T lymphocytes (markers on CD4* and CD8*) can not be discussed since the limited numbers of patients included(N= 6) and healthy controls (n= 5)”

We agree that a larger size of panel is required to further explore the role of IFN-α in the activation of T lymphocytes during dengue infection. That is why we have not discussed the effect of IFN-α on the activation of T lymphocytes

4 – “Some results of figures do not correspond to those in result section and discussion”

We checked all the values of cytokines in the figures and all of them correspond to the text (Results and Discussion).

5 – “also it is not clear if the vertical bar on most figures is the standard deviation value”

The vertical bars on the figures show the standard deviation. Figures presentation has been modified and standard deviation values for controls have been included to each figure. Legends of figures have also been modified.
6 – “other results are misinterpreted.”

We carefully checked our manuscripts and we did not see what the reviewer means by “others results are misinterpreted”.

7 – “Data (%) on table 1 are not comparable when are discussed, and all data are not percentages (i.e. CD4+/Cd8+ is ratio)”

We have checked the values in the table and they are in agreement with our comments in the discussion section of the paper. We agree with the reviewer that all data in the Table 1 are not percentage. Therefore, presentation of table has been modified.

Minor essential revision

1 – “Authors mentioned the simultaneous outbreak of Chikungunya and dengue virus in Gabon in 2007, however they do not mention the number of cases of each, particularly those of dengue fever (only 54 cases positive for dengue virus detection are mentioned, however the question is if the 36 patients included in this study come from that group of patients”

During the outbreak, 773 blood samples were collected during the first week after symptom onset from febrile patients who visited healthcare centers in Libreville and other Gabonese towns. Among these patients, 275 (35.6%) and 54 (7.0%) were positive for chikungunya virus and DENV, respectively. Soluble factor concentrations were measured in 36 DENV-2-infected patients from which blood samples were collected between D0 and D11 after symptoms onset. The sections “Methods” and “Results” have been modified to include this data.

2 – “in addition serologic diagnostic assay (if applicable, for instance the detection of IgM, IgG and/or NS1) is not mentioned.”

Plasmas from study population have been tested negative for anti-West Nile virus antibodies by ELISA. Plasmas from control population have been tested negative for DENV by qRT-PCR assay and for anti-DENV and anti-West Nile virus antibodies by ELISA. This data has been included in the article.

3 – “Also it is not clear the occurrence of some cases of dengue hemorrhagic fever ( Why the authors mentioned the tourniquet test?).”

We agree with the reviewer. The use of the words “Tourniquet test” was a mistake and it has been removed from the article. No severe cases of dengue infection (DHF, DSS) were observed during the epidemic and among all DENV-2-infected patients enrolled in this study. We have modified this information in the section “Methods”.

4 – “The PBMC from patients and contros were matched by age and sex ?.”

PBMC from five randomly selected healthy volunteers (40% male; mean age 29 years, range 25-32 years) were also used as controls. The mean age and the male/female gender ratio
were not different between this group and the study population (p>0.7). Sections “Methods” and “Results” have been modified to include this information.