Author's response to reviews

Title: Enzyme-linked immunoassay for dengue virus IgM and IgG antibodies in serum and filter paper blood

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Author's response to reviews: see over
General remarks
Reviewer 2 remarks that our response to the reviewer’s comments was not available, although it had been uploaded to the website. That is unfortunate because all questions had been carefully addressed in that document. In the following we will address the questions of the two reviewers point by point.

The objective of this study was to analyse the errors which may occur in the measurement of antibody concentrations with ELISA. We chose an approach which is common in the validation of measuring quantitative values, i.e. looking at systematic errors (inaccuracy) and random errors (imprecision). In addition we analysed the data in a qualitative fashion and for that reason we used a diagnostic classification scheme. We do not pretend that the used algorithm correctly classifies all possible situations, but in the following we will argue that it is a sensible approach. However, we would agree with the reviewers that presenting this algorithm in a manuscript, albeit only meant as an illustration of the methodology, may easily be misinterpreted by readers as the one and only algorithm for interpretation of dengue ELISA. Therefore, we now propose to omit the figure, and replace it by words in the text. We trust that this will bring more balance in the manuscript.

Major comments
Comments reviewer Maria Guzmán

1. We fully agree with the reviewer that the relationship of IgM/IgG for the classification of infection should be done on acute samples only. In the WHO guidelines (Dengue haemorrhagic fever, 2nd edition 1997) on page 44 this rule is applied for paired serum samples collected between days 2-14. In our study however the samples were collected on the first day of presenting to a health facility, (called T0, but on average this is day 2 in the disease) and three weeks later (T3). Koraka et al. (JCM 2001) showed a significant increase in dengue virus specific IgG ratio’s between paired serum samples collected in the acute (day 2-4) and convalescent phase (>15 days). The 4-fold rule holds true for PRNT, VNT, HAI and CF assays. The use of a four-fold increase in IgG ratio as an indicator for a recent dengue virus infection has been published by Cobelens et al. (Trop Med and International health, vol. 7 pp 331-338, 2002). We now refer to this method in our manuscript (see corrections page 9, last section). A gold standard technique like the PRNT would be desirable. However this test requires a lot of serum, because samples need to be tested against all the serotypes and geographic related flaviviruses. As suggested by the reviewer we have specified that the analysis was performed on OD values, corrected as index values (IV) instead of titres (page 9 and the first sentence of the section “data analysis”)

2. As suggested by the reviewer we have included a sentence explaining the correlation between the filter papers and serum (page 13, second section of “Comparison of serum and filter papers”)
3. As suggested by the reviewer we have removed paragraph 3 on page 17.

4. We agree with the reviewer indicating that several studies have shown a good correspondence (80%-96%) between IgM and IgG antibodies detected in whole blood on filter paper versus serum (Vazquez et al. Rev Panama Salud Public 1998; Punnarugsa et al, JCM 1991). In our study the agreement for dengue virus IgG antibodies measured in whole blood spot versus serum on t0 and t3 was comparable with other studies. In table 1, we added the kappa value as a measure of agreement. The values for IgM were considerably lower in filter paper compared to previous studies, especially on t0. Other studies using whole blood collected from filter paper have shown a good correlation between IgM concentrations measured in serum and in eluted filter paper blood (Vazquez et al Rev Panam Salud Pulica; Mubarak et al J Med Virol 2004) Bismas indicated in a study on the inter-test comparisons between filter paper absorbed blood eluate and serum for malaria serology a very good correlation for IgG antibodies, but a poor correlation for IgM specific antibodies (J Immunoassays Immunochem, 2004).

We have addressed our observations more specifically in the manuscript (page 13, second section of “Comparison of serum and filter papers”)

Comments to reviewer Jyh-Hsiung Huang.

General:

1. The reviewer indicates that we should not recommend this filter paper test for dengue seroprevalence studies. However for seroprevalence studies one only as to test for IgG specific antibodies. In this study the agreement between dengue virus IgG antibodies collected from eluate filterpaper samples and serum collected on T0 and T3 was rather good and comparable to previous publications for different viruses (Punnarugsa et al, JCM 1991; Rawatt et al, Indian J Med Res 2001). In addition, as longs as misclassification (in this case IgG positive and negative) is a random two directional process, the overall seroprevalence is accurate. We have addressed this in the last section on page 18, and our conclusion. The sentence on (previously) p10, line 4 did not refer to any basic principle, but to the fact the analysis of intra-individual variation cannot be done on IgM because that typically changes over time. This apparently confusing sentence deleted.

2. We agree with the reviewer that paired serum samples are required to confirm or refute an acute infection. Criteria to differentiate between primary and secondary infections using the MAC ELISA as described in the WHO guidelines (Dengue haemorrhagic fever, 2nd edition 1997) is also based on similar assays and applies IgM/IgG ratios. Several studies showed that also the Focus ELISA can be used to differentiate between a primary and a secondary infection (Koraka et al. JCM 2003, Koraka et al. J Med Virol 2004; Cobelens et al Trop Med Int Med 2002). We further refer to our previous answers and the general remarks to clarify why we propose to omit figure 1.
3. See 2.

4. The reviewer suggests that a two-fold increase would be significant when antibodies levels are expressed by ratio’s and that a four-fold increase should be used for antibody titres based on serial dilutions. We fully agree with the reviewer his comment. In our previous response we already discussed the issue of fourfold increase of titers and twofold increase of molar antibody concentrations and we explained that the classification of diagnoses was hardly affected when we changed the algorithm from two to four fold increments of antibody concentrations. Our decision to use the 4-fold increase was also based on a previous study published by Cobelens et al Trop Med & Int Med 2002 using a similar method. The change was already made in the previous revised version of the manuscript. Now we also included the reference of Cobelens et al.

5. We agree with the reviewer that the distinction between antibody levels from dengue virus and other closely related cross-reactive flaviviruses cannot be improved by increasing or decreasing of the cut off value. We only referred to a paper in which the authors played with different cut offs on the quantitative scale, in order to improve specificity, but we agree with the reviewer that our statement was confusing. This sentence was modified and explicitly refers to the previous studies (page 15 line 15)

6. We agree with the reviewer that in this particular study the ELISA for dengue virus specific antibody detection cannot be used for the diagnosis of dengue virus infection early in the disease. This is mainly due to the low correlation between IgM antibodies detected in serum versus filter paper blood (see comment 4 reviewer 1 MG). However, on this point we have to raise the point that the IgG results were not inaccurate but imprecise. The correlation between serum IgG and filter paper blood IgG was within the acceptable range as published in previous papers with a mainly random scatter of IgG values in plot 2 C. Therefore we can use this method for seroprevalence studies based on IgG antibody detection, taking cross-reactivity into account. We now have changed this sentence: “The results of measuring IgG in serum and filter papers eluates showed a rather good agreement with almost equal misclassification in both directions (Fig 1, panel C). Therefore, with sufficient sample sizes and while taking cross reactivity with other flaviviruses into account, dengue virus sero-prevalence studies, based on the detection of IgG antibodies eluated from filter papers, are a useful tool for epidemiologists.

Minor:

7. The reviewer indicates that a good surveillance system should be able to detect all clinical cases. We agree with the reviewer’s vision about the fact that good clinical definitions, also for uncomplicated dengue fever, are very important for surveillance systems but we also acknowledge the fact that many cases of dengue virus infection cannot be clinically distinguished from many other undifferentiated fevers. In our manuscript we did not issue an advice on how surveillance should be organised but describe the reality of many countries where
notification only includes cases when signs of hemorrhagic diathesis are found. Since serology is usually only ordered in the context of a clinical suspicion, serological tools will often only contribute to confirm these clinical findings. In some remote areas with limited facilities, a random sampling method using filter paper to collect blood is a useful tool to quickly find out if certain agents are present in the area. In order to avoid further confusion we omitted the sentence without losing continuity of the discussion.