Author’s response to reviews

Title: Performance of Commercial Dengue NS1 ELISA and Molecular Analysis of NS1 Gene of Dengue Viruses Obtained During Surveillance in Indonesia

Authors:

A Aryati (dr_aryati@yahoo.com)
Hidayat Trimarsanto (anto@eijkman.go.id)
Benediktus Yohan (yohan@eijkman.go.id)
Puspa Wardhani (puspa_pk@yahoo.co.id)
Sukmal Fahri (sukmalfahri@yahoo.co.id)
R. Tedjo Sasmono (sasmono@eijkman.go.id)

Version: 5
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Author's response to reviews: see over
Dear Editor,

We would like to submit our revised manuscript entitled “Performance of Commercial Dengue NS1 ELISA and Molecular Analysis of NS1 Gene of Dengue Viruses Obtained during Surveillance in Indonesia” for publication in BMC Infectious Diseases.

We have looked at the Reviewers’ comment and suggestions and found that the comments/suggestions were constructive and we deeply appreciate them. We have revised our manuscript accordingly, as well as provided responses to reviewers’ comments.

Following this cover letter, please find enclosed the point-by-point responses to the concerns according to three separate referees.

We hope that our revision is sufficient and our article could be considered for publication in BMC Infectious Diseases.

Sincerely yours,

R. Tedjo Sasmono
Corresponding Author
RESPONSE TO REFEREE 1

Reviewer's report
Title: Performance of Commercial Dengue NS1 ELISA and Molecular Analysis of NS1 Gene of Dengue Viruses Obtained during Dengue Surveillance in Indonesia
Version: 3 Date: 17 October 2013
Reviewer: David W Smith
Reviewer's report:
This is a very interesting and informative paper that presents a large and detailed analysis of dengue diagnostic assays in Indonesia. The data on NS1 nucleotide and amino acid sequences is an important contribution.

Major Compulsory Revisions

1. The diagnosis of dengue is complex, especially in endemic/epidemic areas where there is a mixture of primary and secondary infections occurring in populations with different background immunity. It would be better if the authors gave this greater emphasis as a possible cause for variable findings about NS1 and IgM sensitivity reported in different populations. The sometimes conflicting information in the literature makes it difficult for laboratories to develop confident interpretations of test results in possible secondary infections, and readers need to understand why these will take some time to resolve! The need for local studies is stated in the final sentence of the manuscript, but the reasons why this is necessary could be more strongly emphasised in the discussion.

Response:
We value the input from the reviewer and have emphasized this information in the Discussion section (page 13, line 292-295).

2. Page 6, Methods: It doesn’t say where the specimens were tested – in each of the centres or at a centralised laboratory? I can’t see where the specimen storage and transport methods are stated, which is particularly important if samples were sent to a central laboratory. If the samples were tested in the regional laboratories, how did they ensure uniformity of quality? Could this account for some of the geographical variation?

Response:
All specimens were tested in centralized laboratory. Specimen storage and transport have been included in the Method section (page 6, line 115-119). We have also discussed the possible contribution of sample storage and handling to the geographical variation (page 13, line 297-299).

3. Page 9: Neither the results nor the methods state the time after onset of illness for the collection of samples? Was this data collected? If it was, was there a broad distribution of times and, if so, how would that have affected the results? If not, the authors should discuss the possible impact of time of collection on their
Majority of samples were collected at day 3-5 of illness. However, not all data were available. Therefore, we were not able to analyze the effect of collection time on the sensitivity of the assays. It should be noted that in order to minimize the effect of collection time, we only used samples that were positive by dengue genome detection and/or virus isolation.

4. Page 6, line 126: The separation of primary and secondary dengue cases is critical in assessing dengue diagnostic tests. This line states that the separation of primary and secondary dengue was done “according to the manufacturer’s instructions”. I was unable to find this information through the product support pages. It would be better to actually state what the criteria were and give a link. Also there should be references that justify the use of this definition instead of the WHO laboratory criteria, and it should be included in the discussion.

Response:
Information on the determination of infection status was described in product insert (Cat No. E-DEN01D), in which Panbio Unit >22 for IgG is suggestive of secondary infection. We feel that inclusion of this information in the Discussion may cause very exhaustive discussion.

5. Page 9, line 200/201: The specificity analysis did not include patients with other flavivirus infections. Is there a reason for that such as unavailability of samples? As a minimum other publications looking at specificity across flaviviruses should be referenced to reassure the readers.

Response:
We did not include samples with other flavivirus infection because of unavailability of samples, since diagnosis of other flavivirus is not routinely performed in Indonesia. Unfortunately, we could not access confirmed cases of flavivirus infection in health institutions in Indonesia. We have included a reference in regard to NS1 assay specificity in the Discussion section (p13, line 284-288).

6. Page 10/11: Did the authors consider delays in specimen transport or variations in laboratory testing as possible causes of variations in sensitivity of the tests?

Response:
We have discussed this concern in the Discussion section (p13, line 297-299)

7. Page 15: The discussion of the NS1 Ag variation and test sensitivity is very interesting, but there doesn’t appear to be a clear statement about why they think the assay was much less sensitive for DENV-4 NS1. It doesn’t appear to be due to antigenic changes. There should be a definite statement that they don’t know
the cause for that and it may be due to virus characteristics that they haven’t noticed, higher rates of immune complex bound antigens, differences in test performance in the various laboratories, specimen handling and/or transport differences, etc.

Response:
We have included our statement that we do not have conclusive cause for the low sensitivity of the NS1 on DENV-4 (page 15, line 338-343).

Minor Essential Revisions

1. Page 4, line 79; “accurate diagnosis with the broad spectrum” would be clearer as “accurate diagnosis due to the broad spectrum” (corrected, line 79)

2. Page 4, line 80: “dengue diagnosis tools” should be “dengue diagnostic tools” (corrected, line 81)

3. Page 4, line 80/81: “NS1 antigen has become the basis for commercial diagnostic assays” is a bit unclear as IgM and IgG detection are the basis for many commercial diagnostic assays. If the intent is to say that laboratories are increasingly using NS1 detection as the preferred diagnostic test, which is true, then it should say that. (corrected, line 79-82)

4. Page 5, line 91: the phrase “geographical regions of the study site” doesn’t seem right. Should it be “study sites in different geographical regions”? (corrected, line 91)

5. Page 5, line 96: “used and becoming” should be “used and are becoming” (corrected, line 96)

6. Page 6, line 114: “written consents” should be “written consent” (corrected, line 115)

7. Page 6, line 122: Does this mean that the NS1 ELISA was repeated, or an antibody ELISA? Please be explicit. (corrected, line 125)

8. Page 7, line 141: Please specify the modifications (included, line 142)

8. Page 7, line 205: “assessed” should be “assess”. (corrected, line 199)

Discretionary Revisions

1. The outlier in NS1 performance was the DENV-4 positives, which drags down the overall sensitivity of the test. It is mentioned briefly in the discussion, but it would be better to include some data about how the NS1 test performed if DENV-4 was excluded.
Response:
We have presented the sensitivity data when DENV-4 was excluded in the Discussion section (page 14, line 328-329).

2. The sensitivity of the NS1 assay is discussed in several places. Did the authors look at the S/CO ratios? It would be useful to see comparative data showing the distribution of S/CO ratios for the different dengue serotypes

Response:
We did not look at the S/CO ratio. The absorbance of the samples actually can be measured and compared for each serotype. However, as majority of the positive samples give very high (maximum) A450 absorbance (which indicates saturated concentration of NS1), then we could not specifically assess the correlation between S/CO ratio and serotypes.

Level of interest: An article of importance in its field

Quality of written English: Needs some language corrections before being Published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I declare that I have no competing interests'
RESPONSE TO REFEREE 2

Reviewer's report
Title: Performance of Commercial Dengue NS1 ELISA and Molecular Analysis of NS1 Gene of Dengue Viruses Obtained during Dengue Surveillance in Indonesia
Version: 3
Date: 18 October 2013
Reviewer: SUNETHRA GUNASENA

Reviewer's report:
Discretionary Revisions / Minor Essential Revisions
1. Title
Comment: Change unnecessary upper case in the words
Length of the title suggest to be shorter
2. Abstract
Background
Line 26: Omit “various” at the beginning of the sentence
Para 2: Use the word assay instead of kit
3. Method
Line 36:
Suggest: 188 samples were confirmed for dengue by RT-PCR and/or virus isolation.
4. Results:
Need to change as per the text following corrections
5. Conclusions:
Need to change as per the text following corrections
6. Introduction
Para 1:
Line “undifferentiated fever (Dengue Fever, DF)” change to undifferentiated fever and dengue fever
Line 71: “with aid the of” replace with the word “by”
Para 2
Line 80: “the detection of virus NS1 antigen” change to “the detection of virus encoded NS1 antigen”
Line 82 to line 83:
change to “Assays have been developed to diagnosed DENV infections by the detection of NS1 protein in blood during the acute phase”
Para 3
Line 87: “accuracies” change to “accuracy”
Line 89 – 92:
change to “A multi-country evaluation study reported that the best performing NS1 assay had only a moderate sensitivity (median 64%, range 34-76%), with 100% specificity. The poor sensitivity of the evaluated assay has been related to geographical regions of study site suggesting the need for further assessment [10].”
Para 4
Line 90: “becoming” change to “has been”
Line 99: “isolated” change to “collected”
7. Materials and Methods
“Sample collection and dengue detection” change to “Sample collection and detection of dengue”

Para 1
Line 114 - 115: Change to “febrile patients with clinically suspected dengue based on WHO-SEARO 2011 guidelines were enrolled upon obtaining written consents”

Line 116: Omit “Detection of”

Line 118 - 122: change to “Dengue infection was confirmed with the results of conventional RT-PCR … as the gold standard”
Comment: Authors need to clarify of different molecular methods used

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)
Line 133 - 136: rewrite as “RNA extraction and PCR preparation/reaction procedures were performed at the Good Clinical Laboratory Practice (GCLP)-certified laboratory at the Eijkman Institute. Strict control measures were adopted to prevent cross contamination between samples”

8. Results
Sub topic, Sensitivity of NS1 and IgM tests “referred to” genome detection/virus isolation
Comment: Change to “Sensitivity of NS1 and IgM tests with reference to genome detection/virus isolation”

Line 193 to line 197: Change to “Of the samples tested 106 were positive for NS1 antigen and 188 samples were positive by dengue genome detection and / or by virus isolation”

Line 199 - 201: change to “None of the 43 confirmed non-dengue cases and 20 healthy individuals’ samples were positive by NS1 ELISA giving specificity of 100%”

Line 202 - 204: Change to “140 (73.7%) of samples detected positive by Panbio Dengue Duo IgM & IgG Capture ELISA. If both NS1 and IgM positive results were combined, the detection rate was increased to 89.4”.

Para 2
Line 229 - 230: Comment: Omit as it is given in the methodology

Response
All minor language corrections have been accommodated in the revised manuscript. We deeply appreciate for the detail proofreading of our manuscript. However, there were a few suggested corrections that also addressed by other reviewers, in which we may have to accommodate their suggestion as well.

Major compulsory revisions
1. Materials:
Line 122: “To confirm the accuracy of the detection results, ELISA was repeated on NS1-negative samples”
Comment: meaning not clear, did authors repeat NS1ELISA assay on negative samples?

Response: 
We have clarified this sentence in the Method section (page 6, line 124-125).

Line 123 - 126:
Comment: Authors have used molecular assays and / or virus isolation as the gold standard. Detection of IgM and IgG in a single sample is not confirmatory and cannot use as support the diagnosis in PCR positive samples. However it is useful in identifying primary infection from Secondary infections according to the manufacturer’s guidelines

Response
We have re-write the sentence accordingly (line 125-128).

2. Results:
Para 1: Re writing recommended as it has included methodology. This para should contain only results. Should not have repetition with methodology

Response
We have re-written the paragraph and omitted the methodology.

Line 197: Using the molecular detection results as the gold standard, the sensitivity of NS1 ELISA kits was assayed. For all Indonesian samples, the sensitivity of NS1 ELISA reached 56.4% (Table 1)
Comment 1: Did the authors used Dengue genome detection and / or only the genome detection as gold standard or only the dengue genome detection as mentioned in the methodology? Clarify

Response
We have clarified our statement (line 194). We used genome detection as gold standard.

Comment 2: Sensitivity of NS1 ELISA only the average value is valid as some centers has only few positive samples.
Sentence corrected as “Sensitivity of NS1 ELISA was 56.4% when genome detection and / or by virus isolation used as the gold standard”

Response
As above.

Line 201: As the IgG test could not be used to distinguish between current and
past dengue infection, in this study we did not assessed its performance
Comment: Did the manufacturer mention that an IgM result by itself is valid for this Duo test? Authors need to clarify

Response
The Dengue Duo IgM and IgG ELISA consist of two separate kits with their own controls. And according to the manufacturer, the result of IgM ELISA itself is valid for determination of dengue infection.

Sub topic: Comparison of the sensitivity of NS1 test and IgM test
Para1:
Comments: 1. Different geographical region cannot be compared to each other, in some regions there is not sufficient number of samples

Response
This study reflected actual condition in surveillance setting, in which we could not control the number of collected samples. We have re-analyzed the data and statistical significant remains the same even after omission of geographical regions with insufficient number of samples (Table 2, footnote “c” and “d”).

2. Performance of NS1 ELISA and IgM ELISA cannot be compared directly as these two markers detect diagnostic markers at two time frames (acute Vs convalescent) of the dengue illness. Performance should be checked independently against the duration of illness

Response
For the NS1 and IgM, since most samples were collected in acute phase, there should be overlapping presence of NS1 and IgM (Peeling, R. W. et al 2010, Nat Rev Microbiol).

Line 211:
Comment: Without the duration of illness these observations are not valid and may be incorrect. According to authors observations, IgM positivity in secondary infections is higher than in primary infections which is in contrast to published information

Response
We aware that published information observed the higher IgM detection in primary infection. Our result indicated the higher IgM detection in secondary detection. This observation is similar to the one obtained by Andries A-C et al, 2012, which has been referred in the manuscript. One likely explanation would be that the samples with primary infection were collected at the time when IgM was still low (in most of our samples were less than 5 day), however we cannot confirm this explanation since we do not have complete data on the duration of illness.
Sub topic: NS1 sensitivity in relation to geographical regions, virus serotypes, disease severity, and infection status

Line 216:
Comment: Not correct as in some regions only few samples included

Response
We have re-analyzed the data and statistical significant remains the same even after omission of geographical regions with insufficient number of samples (Table 2, footnote “c” and “d”).

Line 220 -227:
Comment: Better to give the result and then the explanation
A total of 23 out of 24 samples from Samarinda that were positive for DENV-4 by RT-PCR, which included two mixed infections with DENV-1 and -2 (data not shown) were negative for NS1 contributed to the low sensitivity of the NS1 ELISA in detecting DENV-4. Confirmation with IgM and IgG ELISA detected 20 of them positive for dengue

Response
We have corrected the sentence accordingly (line 215-218)

Line 230:
Comment: Statistical significance has been calculated comparing the two tests not the different component of the said variable. Authors should have separate tables giving the statistical significance of variables they mentioned

Response
We have re-phrased the paragraph to emphasize on the statistics of the tests that compared the variables (line 224-227).

Line 231 - 232:
Comment: Statement cannot be applied to DSS group as there not enough subjects in this group

Response
This statement can be applied in general to the disease severity, since when we regarded the DSS as DHF group, the statistics for disease severity remain insignificant.

Line 234 - 238:
Comment: Not valid for geographical regions as some regions sample size is not Sufficient

Response
We have re-analyzed the data and statistical significant remains the same even after omission of geographical regions with insufficient number of samples (Table 2, footnote “c” and “d”).

Sub topic“ IgM sensitivity in relation to geographical regions, virus serotypes, disease severity, and infection status

Line 242 - 245:
Comment: Not valid for geographical regions as some regions sample size is not Sufficient

Response
As above, we have re-analyzed the data and statistical significant remains the same even after omission of geographical regions with insufficient number of samples (Table 2, footnote “c” and “d”).

3. Discussion
Para 1
Line 274: word “kit” change to “assay” in all places

Response
We have revised accordingly.

Line 275 - 278:
Comment: Authors mentioned use of PCR and / or virus isolation as the gold standard in the methods and in results

Response
We have removed the sentence from the Discussion

Line 278 - 281:
Suggest: Samples positive by RT-PCR and/or virus isolation were subjected to NS1 gene sequencing and then analyzed according to the NS1 positivity by ELISA. Give the number of samples that were PCR positive but repeatedly negative by NS1 ELISA

Response
Number has been provided (line 271).

Line 283: Overall, our results revealed the sensitivity of the NS1 kit reached 56.4%.
Suggest: Our results detected overall sensitivity of 56.4% in the NS1 assay used.

Response
We have revised the sentence accordingly (line 273-274).
Suggest: Panbio Dengue Duo IgM & IgG ELISA assay detected IgM in 140 of the 188 samples tested with sensitivity of 73.7% 
Comment: Authors cannot claim “this data suggest the better detection rate of IgM than that of NS1” without comparing the duration of illness.

Response
We have clarified that the IgM assay does not necessarily have better sensitivity than NS1 assay (line 278-280).

Line 292 to Line 325:
Comment: Discussion should follow the correct statistical analysis of the variables. Statistical analysis performed comparing the two tests rather than the components of the variable

Response
We have re-phrased a few sentences to emphasize on the ANOVA test shown in Table 2 for comparing components of the variable.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests: Not applicable
RESPONSE TO REFEREE 3

Reviewer's report
Title: Performance of Commercial Dengue NS1 ELISA and Molecular Analysis of NS1 Gene of Dengue Viruses Obtained during Dengue Surveillance in Indonesia
Version: 3
Date: 26 October 2013
Reviewer: Linda Hueston

Reviewer's report:
Thank you for the opportunity of reviewing this very interesting article. Given the increasing availability of commercial NS1 antigen detection kits and IgM and IgG antibody kits for dengue fever across the globe there is a real and on-going need to assess not only the accuracy, sensitivity and specificity of such assays but to consider these in light of geography. It is extremely important to understand that how one approaches the laboratory diagnosis of dengue in Indonesia may well be different to how one approaches laboratory diagnosis in countries like Australia or the USA. Indeed the usefulness of these kits will vary among nations with high dengue burdens - it will also vary over time and this suggests that on-going evaluation is needed. The authors have attempted to do this and have largely succeeded in doing so.

One concern I have is that the authors only examined the use of the PanBio kits, therefore the question I have is do all NS1 antigen kits and Dengue IgG and IgM kits give the same results or would other kits have a better performance. Perhaps PanBio is the only kit available for purchase in Indonesia and if this is true then the authors results very valuable and would be of great interest to clinicians. If it is not true and other kits are available then knowing how other kits perform could be even more useful to clinicians. If the authors cannot add results using different manufacturers products then it is important that the authors make a statement to the effect that other products may perform differently.

Response
We only assessed the Panbio kit since this kit is widely available in Indonesia. There are several other kits such as the Platelia NS1 (Biorad), but not widely used nor available throughout Indonesia. When this revision is being written, publication on the performance of Platelia NS1 (Biorad) in Indonesia is available in PLOS One (Kosasih et al, Nov 19, 2013), in which the overall sensitivity was lower than Panbio kit.

There are numerous language corrections which need addressing and I have made some suggestions below. Also I would suggest the author's specify NS1 antigen when they are referring to the kits, they mention NS1 sometimes referring to antigen, sometimes referring to the proteins themselves. Also some kits for antibody detection use NS1 antigen rather than whole native antigens or recombinant antigens - this can affect the sensitivity and specificity of antibody assays. So some clarification of the type of antigen used in the kits under study would be helpful.

Response
We have revised and clarified the term according to the context of the sentences (NS1 antigen and NS1 protein specifically).

Line 36 - mention the total number of collected samples used.
Line 44 - should read "the sensitivity increased to 89.4%" and "NS1 antigen sensitivity varied when correlated...."
Line 54 - should read "..........the low sensitivity of NS1 antigen detection did not relate to NS1 genetic diversity."
Line 55 - should read "the performance of the NS1 antigen test............"
Line 56 - what do you mean by "infectious status of patients"? Do you mean primary or secondary infection, do you mean the time following onset of symptoms or do you mean severity of disease presentation. You need to explain this.
Line 61 - should read "..........with a large global burden"
Line 62 - should read "There are an estimated 50 million infections........"
Line 70 - should read"..........within each serotype"
Line 84 - should read "High level early viremia........"
Line 88 - should read "plasma/serum samples have been described"
Line 96 - should read "........ and are becoming the tool of choice....."
Line 108 should read "..........during dengue surveillance in 2010-2012.
Line 117 - Alere is the manufacturer not Inverness.
Line 141 - should read "Detection and serotyping were confirmed......"
Line 143/144 - should read "The Simplexa Dengue assay was performed according to the manufacturer's instructions."
Line 148 - you mention inoculating C6/36 monolayers but don't mention the size of the flasks used.
Line 164 - should read "........using the method described by the manufacturer"
Line 193/194 delete ".......for NS1 antigen"
Line 205 - change "assessed" for assess
Line 208 - do you mean the difference between NS1 antigen and IgM ELISA kit or do you mean an IgM test kit that uses NS1 antigen?
Line 232 - delete "Result of....." should read "Comparing the sensitivities......."
Line 242 - should read "IgM sensitivity appeared to be affected by geographical regions"
Line 304 - should read ".....NS1 antigen kit evaluated here showed higher sensitivity......."
Line 372 - should read "The non-structural (NS) proteins ........"}
Line 396 - should read "A previous study also observed........"

Response:
We appreciate the detail proofreading of our manuscript. All suggested language corrections have been accommodated.

Level of interest:An article of importance in its field
Quality of written English:Needs some language corrections before being published
Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I declare that I have no competing interests.