Author's response to reviews

Title: Development of a sensitive novel diagnostic kit for the highly pathogenic avian influenza A (H5N1) virus

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Author's response to reviews: see over
Dear Editor in chief,

RE: MS 1863308731255726

We hereby submit our revised manuscript entitled “Development of a sensitive novel diagnostic kit for the highly pathogenic avian influenza A (H5N1) virus”.

We appreciate very much for valuable comments by reviewers. We have modified the text, Fig. 2 and its legend. On the title page, I added my new affiliation and also corrected my address for the correspondence. I want to keep my previous e-mail address for the communication.

Please find below point-by-point responses to the concerns.

Sincerely,

Yasuko Tsunetsugu-Yokota

Reply to Reviewer 1

The manuscript by Yasuko etc. describes a diagnostic method for H5N1 influenza virus. 19 clinical specimens from 13 patients in Vietnam infected with clade 1.1 or clade 2.3.4 H5N1 HPAI virus was tested in this luminescence-linked enzyme immunoassay developed. Approximately 80% of clinical specimens were H5-positive using the POCube system. The author concluded that this novel H5/A kit using POCube is a rapid and sensitive screening test for H5N1 HPAI virus infection in humans. The kit is still equipment-dependent. The study may promote H5N1 diagnosis in humans. However, the current presentation of results is not complete. The missing or unclear information includes:

Specificity study: Sequence variance in H5 leads to different clades (clade 0-9) of H5N1 and new H5N1s are emerging every year. In this study, only four H5 strains were tested. It is not sufficient. No results were shown about tests with non-H5 AIVs, such as H1N1 and H3N2.

I agree that the specificity is a critical issue for the development of clinical diagnosis kit like ours. In fact, we extensively and carefully confirmed the specificity of the kit by testing it against various purified type A influenza viruses including H1N1pdm, which were available in our institute. They were all negative as described on page 13 - 14, lines 206-210. Furthermore, we tested clinical swab specimens taken from 15 seasonal influenza and 30 non-influenza patients and found no false positives of H5 (page 14, lines 210-212).

We are confident that our kit using anti-H5HA mAbs is highly specific for H5HA
detection. We have recently reported the epitope mapping study of OM-b mAb, one of kit components (Kobayashi-Ishihara et al., PLOS One, 2014, ref. No. 22). The OM-b mAb was highly specific to H5HA and broadly reactive to the Asian type H5HA. We added this information in the Results and Discussion (page 18, lines 273-277).

**Sensitivity study:** *For a clear understanding to readers, the sensitivity of the test should be compared to any conventional methods, such as Hemagglutination and TCID50. Table 1 is confusing. The results of different methods should be presented in the same table with details like sample volume used.*

We are sorry for confusing you.

In Table 1, we listed the purified H5N1 influenza virus strains used for the virus titration by our kit. We routinely measure the virus titer as TCID$_{50}$ at 50 µl. Just for the reference, the copy numbers of RNA per ml (we added to the table) by a real-time qRT-PCR method were shown. As you pointed out, either TCID$_{50}$ or hemagglutination is conventionally used for virus titration.

Here, we serially diluted these representative virus strain solutions to show the sensitivity of our kit. For clearer understanding to readers, we calculated the TCID$_{50}$ titer at each dilution of viruses according to the information in Table 1 and depicted in the revised Fig. 2. Accordingly, we modified the text (page 13, lines 200 - 205) and Fig. 2 legend.

**Cut-off value:** *Is this cut-off value fixed for all samples or adjusted before every batch of tests? If it is fixed, more negative samples in different conditions should be included to generate this cut-off value, such as bloody samples, non-H5 AIVs and other type of microorganism. If it is adjusted every time, this test may not be claimed as rapid test with 5-15 mins.*

Thank you for your valuable comment. The cut-off value is carefully pre-determined in each lot of H5/A kit and programmed to the IC card supplied with each lot. Then, the POCube machine gives you the result + or -. Usually, it is not necessary to determine the cut-off value every time as far as you use the same lot. However, we routinely measure the negative/positive controls when we start the machine to make sure of the kit reliability.

So far we observed no false positives in various solutions including clinical swab specimens of non-H5 influenza and non-influenza patients in Japan as described above. However, we will keep in check the reliability of the diagnosis made by our kit in future when it is available worldwide and used in various conditions.

**Statistical review:** *Yes, but I do not feel adequately qualified to assess the statistics.*

Because we determine positive/negative results based on a predetermined cut-off
index, further statistical analysis may not be applicable in this study.

Reply to Reviewer 2

1. This paper describes the rapid detection of avian influenza H5N1 using a chemiluminescent immunoassay with antibodies raised against the H5 protein and also against the influenza A pan specific nuclear protein. The method uses a commercially available luminometer and detection reagents with a panel of monoclonal antibodies previously described by the authors. The performance of the test is determined with both purified virus and clinical samples and compared with both RT-PCR and Immunochromatography. Unsurprisingly it performs better than the latter and not as well as the former, although it is considerably faster. The work appears to have carefully carried out and there are no flaws in either the method or the analysis.

2. Discretionary Revision: Table 4 is a summary of data largely already in Tables 2 and 3 and could be omitted

Thank you for your suggestion. However, some cases were not clearly positive (i.e., they were suspicious) due to discrepant results for the H5HA and type A. We think that summarizing the data in Table 4 will help the reader to better understand our argument. Therefore, please allow us to keep it.