Reviewer's report:

Title: Detection of a novel avian influenza A (H7N9) virus in humans by multiplex one-step real-time RT-PCR assay

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Reviewer: lunbiao cui

The manuscript by Fan et al. describes the implementation of a qRT-PCR method for the simultaneous detection of the universal influenza A virus (FluA), HA and NA genes of the novel H7N9 subtype that emerged recently in China. The authors also incorporated Rnase P genes in the assay to ensure the quality of the clinical samples. The performance of the assay is reported to be comparable to the WHO protocol for H7N9. Overall, the data are generally clear. However, the data presented on this manuscript has some drawbacks on the way the assay was validated for specificity, sensitivity and intra- inter laboratory variation.

Major comments:
1. The method presented here is not innovative, there have been some reports on the development of assays based on similar technologies and the application of other more innovative methods such as isothermal amplification. The overall contribution of this manuscript relies on the application of a multiplex assay for the simultaneous detection of universal FluA, HA, NA genes, and Rnase P genes in a single reaction.

2. Authors designed a new set of primers and probes to specifically detect the novel H7N9 virus by a multiplex real-time RT-PCR assay in one reaction tube. But no other H7 or N9 subtypes viruses were tested. Also, other respiratory viruses and avian influenza such as H5N1 and H9N2 should be included in assessing specificity.

3. There is no mention of number of replicates included for the determination of the detection limit of the assay. Data about intra- inter laboratory variation is very important for assessing the performance of the assay. This information should be included and the results of this determination should be reported with appropriate confidence limits.

4. Since either an unrelated H7 or N9 could be detected, authors need to make a recommendation about how to follow-up on samples which are only positive for one of the genes as occurred in WHO protocol.

Minor comments:
1. Overall, there are numerous grammatical and usage errors which must be corrected.

2. It is not clear that 130 samples mentioned in P125 included in 1011 samples
mentioned in P131?

3. Figure 1 is not necessary, it could be deleted.

4. Authors mentioned the sensitivities of the FluA, H7, and N9 primer and probe sets were all 5×10^-2 TCID50, and the detection limits for the RNA transcripts were all 1×102 copies. It should be provide the unit of TCID50, per ml or 200 ul?, 1×102 copies per action or per ul?

5. P120, 21 other respiratory pathogens or commensals were used to assess the specificity. Please specialize it, we only saw 13 pathogens.

6. Rnase P gene (RP) served as an internal control to monitor the quality of the clinical specimens. We would like to read the results of it in 1011 specimens. Also, author should give the recommendation when negative results occurred in the discussion section.

7. P164, tissue culture methods detecte 13 positive samples. How to identify flu subtypes? cytopathogenic effect, IFA, or PCR?

8. Author said tissue culture results were used as the true result for sensitivity and specificity calculations. It is difficult to understand “For both the tissue culture and multiplex assay, the sensitivities and specificities for the H7 and N9 genes were 100%”.

9. It is unacceptable for the data presented in Figure 2, such as patient 34, throat swab was positive first day while sputum or tracheal aspirates specimens was positive until 13 days. Does throat swab and sputum or tracheal aspirates specimens collected in the same day?

10. p194-195 repeated as in the introduction.

11. It is not true that one family cluster of avian influenza A (H7N9) virus infection was reported during the outbreak of this virus in China. Several cases have been reported.

**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:**

No to all of the above.