Peer Review File

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Reviewer A:

This was a well written paper that investigated the pathogenicity of novel H7N9 viral isolates identified from a human patient who succumbed to a lethal infection. The H7N9 viruses have circulated more frequently in birds with little evidence of transfer to humans. However the H7N9 virus since 2013 has been responsible for 5 waves of infections in China which was the motivation for the authors to investigate the biological relevance of sequence variations in key target protein such as the HA which is required for virus binding and uptake in to target cells.

I would ask the authors to address a few minor points.

Methods:

Comment 1: Line 137 Mouse experiments. There would appear to be a discrepancy in the mouse numbers used in the experiment. The authors state that they used a total of 30 mice (Line 138) - but if there were 5 mice per group and 5 different viral doses used and two different viruses used + 2 more control groups of 5mice per group then that would be a total of 60 mice. Please comment and amend if necessary.

Reply 1: Thank you for your suggestion. We have recalculated the number of mice and the exact number used in this study was 55. (Line 151-152)

Comment 2: There is no mention in the methods of how the MLD50 was calculated.

Reply 2: We agree with the reviewer and have addressed the MLD50 method in the Method. (Line 157-158)

Comment 3: Line 219 The authors state that the QY WT MLD50 was less than 10 PFU but I could not ascertain how they could say that based on the data shown in Figure 3.

Reply 3: Since 10PFU of QY WT was still lethal to the group of mice, the MLD50 should be below 10.

Comment 4: Line 225-227. The mice receiving the QY HAdelta virus did not experience weight loss. But did the mice show seroconversion to the modified virus? I thought that would be important to show because previous studies with the virus were restricted to just tissue culture assays. However in vivo the immune system if it could recognize the virus should develop Abs to the virus to neutralize and clear the infection. Although the QY HAdelta virus may not be lethal- was it capable of causing a detectable humoral response in vivo?

Reply 4: We would like to thank the respected Reviewer A for his/her useful suggestion. We did not do this assay in this manuscript but have planned to carry out this assay to detect the humoral response of mice in the future study.

Discussion:

Comment 5: Line 253- THe Discussion is not the place where you introduce new data. I could not see anywhere before the Discussion that the data provided in Supplementary Table 1 was

described. The authors should have described this work in he Results- and discuss its relevance if required in the Discussion.
Reply 5: We agree with the reviewer and have described in the "Molecular characterization" part of the Result.

Comment 6: Line 254 Authors state "..H7 surface genes can recombine with various NA subtypes...." This is a poorly worded sentence. IAV viruses undergo genetic reassortment and not recombination these are two completely distinct mechanisms of genetic variance.
Reply 6: We agree with the reviewer, deleted inappropriate words, and rewrote the statement. (Line 281-284)

Comment 7: Line 269- I the word Pekin correct or should this be Peking?
Reply 7: Thank you for your suggestion. We have corrected it. (Line 296)

Comment 8: Line 296 -298 The authors need to rewrite this sentence as it is not very clear. What do they mean by mammalian biomarkers appearing in the virus? This does not make sense.
Reply 8: We agree with the reviewer and rewrote the statement. (Line 323-325)

Comment 9: Line 317 - typo on the word acqTusition- I thik this should be "acquisition"
Reply 9: Thank you for your suggestion. We have corrected it in the manuscript. (Line 344)

Refereces:
Comment 10: Line 353 missing page numbers
Comment 11: Line 361- issue number and page range is not shown for the reference
Comment 12: Lines 382, 387, 391, are missing page numbers
Comment 13: Line 395 - there is no volume or page numbers shown for the reference

Reply 10-13: For Q10-13, we have changed the citation style to the JTD, so all the missing page numbers appeared.

Reviewer B:
In this manuscript the authors have characterized a H7N9 virus isolate (QY) from a fatal human case of infection in 2017. The QY virus contains a polybasic cleavage (PBC) site between HA1 and HA2 and the authors demonstrate in a series of in vitro and in vivo assays that it can replicate without the addition of exogenous trypsin in cell culture. Furthermore, QY has the well described and characteristic highly pathogenic phenotype in intranasally infected mice that is typically shown by H5 and H7 subtype viruses with a PBC. They further show by replacing the stretch of polybasic residues in HA with a conventional HA cleavage site (termed QY HAdelta), that is only sensitive to serine proteases like trypsin, the capacity to replicate without exogenous trypsin and the highly pathogenic phenotype are lost. Finally, a limited set of experiments in which QY HAdelta is rescued into the genetic background of a low pathogenicity H7N9 isolate (ZJ1) or QY show that viruses with the QY HAdelta replicate in some cells or eggs better than the equivalent viruses with ZJ1. This finding is taken to suggest that 2017 viruses are better evolved for more efficient replication in avian and mammalian species cells than 2013 viruses. Overall, the

experiments are well executed and in general the conclusions made are supported by the data. However, the findings of the study simply reiterate the well known concepts of modulation of virulence of H7 subtype viruses by the nature of the HA cleavage site without presenting any novel findings. These specific details, including the LD50 of H7N9 viruses with a PBC in mice have been published already. The only point of study that might be considered novel is the result suggesting that other amino acid changes in the HA of the QY strain could be contributing to increased fitness in mammalian and avian cells. However, this latter analysis is very preliminary and under-developed to allow for any meaningful conclusion other than to show that there is a difference in the ability of the two virus HAs (QY and ZJ1) to support replication. There are a number of additional points for consideration that are listed below.

Comment 1: In the description of the mouse experiments on page 6, it is stated that 30 mice were divided into groups of 5 for inoculation with 5 different dilutions of either the QY WT or QY HAdelta viruses with one group left for the control virus. For two viruses and 5 dilutions of each virus a total of 50 mice would be required. In addition to the control group of 5 mice, this should be a total of 55 mice so this discrepancy should be explained or corrected.

Reply 1: Thank you for your suggestion. We have recalculated the number of mice and the exact number used in this study was 55. (Line 151-152)

Comment 2: Line 174, the NA mutation that confers resistance to oseltamivir is first termed “NA-292”. It would be best to follow a more conventional notation to immediately describe the mutation that is present, such as stating “showed that the R292K (N2 numbering) mutation in NA that is known to confer oseltamivir resistance...”

Reply 2: Thank you for your suggestion. We have corrected it through the manuscript. (Line 191)

Comment 3: For the first appearance of the term “QY HAdelta” in the results it would be helpful to describe the virus so that it is not necessary to look for it in the Methods section.

Reply 3: Thank you for your suggestion. We have described term of the QY HA delta in the first paragraph of the results. (Line 181)

Comment 4: Line 195. It is stated “The number of plaques formed was comparable in the presence of TPCK-trypsin”, but this statement is incorrect as it does not describe which virus is being discussed. It is true for QY WT but not for QY HAdelta. Please rewrite to indicate that it is specific only for QY WT.

Reply 4: We appreciated reviewer's constructive comments in the manuscript. We have rewritten this section which made a correct statement. (Line 211-214)

Comment 5: Similarly, the sentence on line 195 is also not correct as it does not specifically indicate which virus is being described and from the context of the other sentences it cannot be inferred which virus is discussed. Again, the statement about plaque size is true for QY WT but not for QY HAdelta because the latter does not form plaques without trypsin (at least not to any appreciable extent). Please specify that the statement is about QY WT.

Reply 5: We would like to thank the respected Reviewer A for his/her useful suggestion. We have rewritten this section based on the reviewer's comments.

Comment 6: Sentence on lines 209-211. It is stated that because QY HAdelta grew more quickly in eggs than QY WT that the polybasic motif might not be stable. It is not clear what is mean by this, and there is no analysis to suggest that this sequence is not stable in the context of growth in eggs such as sequence analysis showing reversion to the trypsin-only cleavage motif or other changes. What is more likely is that the PBC does not contribute the efficient replication of the virus in eggs and in some way might actually be detrimental. It should be noted that highly pathogenic avian viruses do not replicate well in eggs because they kill the embryo, so they may grow much less efficiently than low pathogenicity viruses. This is more likely to explain the observations that the suggestion that the PBC is not stable.
Reply 6: We answered comments 6 and 7 together, see comment 7.

Comment 7: Related to comment 6, it is suggested on lines 211-212 that the HA PBC may have been acquired to facilitate adaptation in humans and other mammals (apparently since it apparently does not help replication in chicken eggs). It may be worthwhile considering that the virus is still clearly an avian virus with primary spread in wild and domestic bird populations and that acquisition of a PBC by H7 subtype viruses is relatively frequently associated outbreaks in birds. Humans and other mammals remain largely dead-end hosts so are much less likely to act efficiently as adaptation hosts.
Reply 7: For comments 6 and 7, we would like to thank the respected reviewer for his/her useful comments 6 and 7. We have revised this part as reviewer's suggestion. (Line 227-233)

Comment 8: The analysis of the data presented in Lines 229-236 is over simplistic and insufficient to justify the suggestion that 2017 viruses are better adapted than 2013 isolates in both eggs and mammalian hosts. The suggestion is based on the analysis of only two viruses without consideration of any unique or specific differences in either virus that might set them apart from the population of viruses that are present in 2013 or 2017. To be meaningful this analysis would have to look at the differences in HA that are contributing to better growth of QY HAdelta containing viruses and determine whether these are representative of the evolution of the 2017 virus population as a whole compared to viruses isolated in 2013. As it stands the analysis can only say that the ZJ1 HA behaves differently than QY HAdelta, but not whether this is representative of viruses isolated between 2013 and 2017. This data should either be removed or this part of the study developed to support the suggestion that is made.
Reply 8: We would like to thank the respected reviewer for his/her useful comments on this part of results. We rewrote this section and did not make a conclusion based on both 2013 and 2017 H7N9 strains. We just made a statement that both isolates demonstrated the growth difference in the manuscript. (Line 251, 256-258)

Comment 9: Line 240, it is not correct to call a virus highly pathogenic in cells. Pathogenicity refers to the capacity of a virus to cause disease. A virus may be able to replicate without trypsin which is indicative of a virus that is highly pathogenic in avian hosts. This sentence should be rewritten to reflect these distinctions.
Reply 9: We agree the reviewer's advice. This sentence was revised to " The virus carries multiple basic amino acids in the connecting peptide of the hemagglutinin, making it lethal to mice in a low viral titer (less than 10 PFU)." (Line 263)

Comment 10: Line 255-256. It is stated that H7 subtype viruses infected only poultry with the exception of the H7N7 virus outbreak in the Netherlands in 2003, where human to human transmission was observed. This is not true, there are many documented cases of replication of H7 subtype viruses in humans prior to 2013. Furthermore, this is distinct from the ability of a virus to transmit from human to human. This section should be rewritten to correct the statement about infection in humans with H7 subtype viruses.
Reply 10: Again, we really appreciated reviewer's constructive comments on this part. We revised this part in the manuscript. (Line 281-284)

Comment 11: Figure 2 legend. It is stated that all infections were done with 1ug/ml TPCK-trypsin in the legend while the methods section states that infections in A549 and DF1 cells were done with 0.5μg/ml TPCK-trypsin, so this discrepancy should be resolved.
Reply 11: Thank you for your suggestion. We have corrected the mistake in the manuscript. (Figure 2)

Reviewer C:
The manuscript describe an increased virulence of an H7N9 human isolate with polybasic HA cleavage site in mice. The role of the polybasic cleavage was shown by using reverse genetic viruses in genetic background of the same H7N9 virus. The role of polybasic cleavage site in mice was previously shown in a publication by Sun et al. using reverse genetic viruses in the genetic background of the PR8 strain. This manuscript therefore adds to the completeness of evidence for the role of the polybasic cleavage on the virulence of this virus in mammal.
Comment 1: It should be also noted that a recent publication by Chan et al. described increased virulence of an avian H7N9 isolate with polybasic cleavage site. This publication should be cited and discussed.
Reply 1: We would like to thank the respected reviewer for his/her useful comments. We have cited this paper and discuss the ex vivo data in the manuscript. (Line 267-270)

Reviewer D:
The present manuscript investigated replication and pathogenesis of a human H7N9 influenza A virus isolate (QY) from a fatal infection in different cell lines, embryonated chicken eggs and in mice in vivo. The QY isolate contains a polybasic HA cleavage site. The authors show that the polybasic cleavage site facilitates trypsin-independent replication of the virus in different cell lines, whereas a mutant with the polybasic cleavage site deleted replicated strictly trypsin-dependent. Furthermore, the authors show that the polybasic cleavage site confers high pathogenicity to the QY isolate in mice. The authors also report that the HA of QY contains further mutations in addition to the polybasic cleavage site in comparison to a previous human H7N9 isolate (ZJI). Interestingly, by using recombinant viruses possessing the QY HA with deletion of the polybasic cleavage site in the backbone of the remaining genes of isolate ZJI the authors show that the HA of QY confers enhanced replication in different cell lines and embryonated chicken eggs.
The data are very interesting and relevant and add to the complex role of HA in influenza A virus

pathogenicity in mammalian hosts. However, the manuscript would benefit from some additional data and revisions in the text.

Major comments:

Comment 1: Page 7, molecular characterization: It would be much easier for the reader to show the different cleavage site sequences of the QY and ZJI H7N9 isolates and of the HAdelta mutant in a table. Line 161: The two motifs are not clear to the reader. Are they present in recent H7N9 isolates? It seems that a „P" residue is misisng in both sequences? PEVPKRKRTAR and PEVPKGKRIAR?

Reply 1: Thank you for your suggestion. We have corrected them in both sequences. (Line 177-178)

Comment 2: Figure 1: The plaques of QY WT are much larger in diameter in the presence of trypsin, indicating that the HA is not cleaved very efficiently by furin or other endogenous proprotein convertases in MDCK cells. This might be due to the minimal furin recognition motif of the cleavage site: R-X-X-R lacking a basic amino acid in position P2. The authors may add a comment on that in the manuscript. It would be important to compare the growth of QY WT and HAdelta in the absence and presence of trypsin in the different cell lines in Figure 1, to investigate this in more detail. Maybe the data shown in figures 1 and 2 can be combined.

Reply 2: We would like to thank the respected reviewer for his/her useful comments. Figure 1 was focused on the QY strain which was trypsin independent or dependent; however, Figure 2 compared the growth difference between WT and deletion strain of QY H7N9. Therefore, we described the data in two figures.

Comment 3: Figure 2: The authors state that the QY HAdelta mutant surpasses the QY WT virus in DF-1 cells at later time points post infection and conclude from this that the polybasic cleavage site might not be stable in chicken or other avian hosts. To my opinion this cannot be concluded from the data. The data rather show that QY WT replicates efficiently in DF-1 and MDCK cells and has reached its peak titer already at 48 h post infection; HA delta grows slower compared to QY WT.

Reply 3: We would like to thank the respected reviewer for his/her useful comments. We have rewritten this part of the result. (Line 227-233)

Comment 4: Figure 3: The data are interesting; however, the authors should show virus titers (lung) in addition to survival and body weight loss data. Did QY WT infection spread to other organs?

Reply 4: We would like to thank the respected reviewer for his/her useful comments. Actually, we did not do this assay in this study. However, we plan to carry out a molecular evolutionary study of the HA segment of H7N9 from 2013 to 2017 to see how HA change affects the virus growth in vivo and in vitro. We will determine the viral lung titers of RG H7N9 with various HA genotypes in this study.

Minor comment:

Comment 5: Introduction, page 4, line 68: „During the fifth wave of the pandemic …" H7N9

did not cause a pandemic to date. The authors should use „epidemics“ or „H7N9 outbreaks“ instead.

Reply 5: Thank you for your suggestion. We have corrected it. (Line 82)