Author's response to reviews

Title: Stability and Infectivity of Novel Pandemic Influenza A (H1N1) Virus In
blood-derived matrices under Different Storage Conditions

Authors:

Xue Wang (xue.wang@fda.hhs.gov)
Olga Zoueva (Olga.Zoueva@fda.hhs.gov)
Jiangqin Zhao (Jiangqin.Zhao@fda.hhs.gov)
Zhiping Ye (Zhiping.Ye@fda.hhs.gov)
Indira Hewlett (Indira.Hewlett@fda.hhs.gov)

Version: 2 Date: 26 October 2011

Author's response to reviews: see over
Response to Reviewers’ Questions

We have attempted to address the comments of the two Reviewers.

Reviewer #1:

- Major Compulsory Revisions
  Abstract – Conclusion
  The authors draw the conclusion, that these data have implications for storage, handling and transport of blood derived samples from influenza patients for epidemiological and laboratory investigations. At this point the authors have to take into consideration that the detection of influenza viruses in blood of humans with a fatal outcome is a really rare event and that it is not proven that this virus is infectious. From my knowledge there is no report of transmission of influenza viruses by blood or blood derived samples. This has to be pointed out by the authors.

  We agree.
  We have pointed out in Abstract-Conclusion: “It should be noted that there is little known about influenza viremia, and whether influenza viruses can be transmitted by blood or blood derived samples.”

  Abstract – Background
  The authors address the question whether virus in blood of infected patients is infectious. The described approach is useful to shown that cell culture virus remains infectivity after incubation in different matrixes. The question raises above cannot be answered by this manuscript.

  We agree.
  We have point out in Abstract-Background: “and may pose a safety concern for collection, handling and transport of specimens for epidemiological and public health investigations if infectious virus is present in samples.” And deleted the previous question of infectivity of viremia.

- Minor Essential Revisions
  Abstract – fourth paragraph (Conclusion)
  “These data indicate that influenza virus encapsidated RNA” is more precise. Influenza virus RNA wouldn’t be stable under these conditions.

  We agree
  We put “encapsidated” before RNA.

  Background third paragraph “In addition it is important to.” Here is something missing!

  We deleted that phrase.

  Background – third paragraph
  ..spiked varying amounts of pandemic influenza A(H1N1) virus in PBS…. (see above)

  We deleted the “RNA”: in the text.

Material
Preparation of virus dilutions in PBS, buffy coats or plasma. It’s really hard to understand following sentence: “10 µl of virus stock was 10 times diluted to 1 µl, or 0.1 µl of virus stock/ 10 µl of…. ” It would be nice to make the dilution more clear.

We agree.
We have changed clearly to: “10 µl of 3.55 × 10⁸ EID₅₀/ml virus stock was diluted to 3.55 × 10⁶, 3.55 × 10⁵, or 3.55 × 10⁴ EID₅₀ in 10 µl of phosphate buffered saline (PBS). ”

Real-time PCR
“The 25-µl PCR mixture consisted of 100 nM primer…” of each primer or of both? “The limit of detection was 1 fg of virus RNA per reaction with the TaqMan assay since the initial sample dilution was 1:10.” This data are not shown! A reference or the results has to be shown.

We agree.
We have put “each of” in the text. We show the data of “The limit of detection was 1 fg of …. ” in supplement.

Results
Viral RNA detection in virus spiked PBS, plasma or buffy coat held at room temperature up to 72 h. The dilution is “(10 µl of virus stock, 10 µl of PBS containing 1 µl of virus stock, or 0.1 µl of virus stock, mixed with 130 µl of buffy coats)” as previously mentioned in the material section. But why is the dilution mixed with 130 µl of buffy coats before adding the mixture to PBS, plasma or buffy coat.

We have fixed the problem and changed in the text “Ten fold dilutions of H1N1 virus in 10 µl of PBS starting with 3.55 × 10⁶ to 3.55 × 10⁴ EID₅₀ of H1N1 virus in 10 µl of PBS were mixed with 130 µl of PBS, plasma, or buffy coat separately and held for different periods of time at room temperature.”

The standard deviation and student’s T-test is missing in this graph taking into account that “The data were represented as an average of triple experiments” (see Materials and Methods). The standard deviation has to be added to figures 1, 2, 3 and 4. Otherwise it might by hard to draw any conclusions from these experiments.

We have added standard deviation in the figures.

From my point of view a positive control (untreated virus) is missing in all experiments. Furthermore the zero hour time point is shown in the graph, but there is no zero hour dot indicated. Was a sample used for RT-PCR after zero hours?

Generally, we use 10 µl of virus dilution in 130 µl of PBS to isolate virus RNA as controls. They are in Y-axis. We also put a sentence in Method Section as: “Virus stability and infectivity for time “0” indicated in the figures was tested immediately (within 10 min) after mixture of virus and the specific matrix.”

Furthermore “blood”, as mentioned in the figure, wasn’t used (also Fig. 3 and 4).

We have fixed this.

Fig 1 a and B
Why are the CT values for the PBS samples in A and B so different?
It is likely because they were done at different times and in different studies.

Viral RNA recovery in buffy coats stored at 4 °C. Change “degraded in buffy” to degraded in buffy …

We have corrected that.

**Reviewer #2:**
In general, the use of clinical samples would add more weight to their study. Presence of, e.g. influenza virus specific antibodies might change their results in any direction. Please comment on this briefly.

We agree that it would be ideal to test clinical samples but they are difficult to obtain and therefore we chose to evaluate spiked samples.

There is an apparent lack in statistical methods.

We added t-test.

1. Page 3: Assuming the likelihood, that influenza causes viremia rarely and only in severe cases the explanation to use of blood coat samples for epidemiological and public health investigations seems to be somehow contradictory. The authors might want to alleviate their indication to analyze blood samples and explain in more detail why blood samples were chosen opposed to widely used respiratory specimens.

After emergence of H5N1 there has been concern regarding blood safety, since this virus can be widely disseminated in the body and viremia has been reported. We therefore tested the potential safety concern by using samples spiked with virus.

Since viremia was also reported in influenza A/H5N1 the authors might want to include H5N1 virus as a second pathogen. Alternatively, please explain the rationale to use pandemic H1N1 only in more detail.

Both H5N1 and H1N1 can cause viremia, and H1N1 has spread worldwide. H5N1 has remained confined to Southwestern Asia. Since H5N1 is a highly pathogenic pandemic strain and is more restricted from a biosafety perspective in a laboratory setting, we chose to perform the proof-of-concept study using a less virulent strain such as H1N1.

2. Page 3: “In addition it is important to”. Please clarify important to what finding.

We deleted that.

3. Please rephrase “…varying amounts of influenza A (H1N1) virus RNA in PBS…” It is the virus but not the RNA, which was used for spiking experiments.

We deleted RNA.

4. Page 4. It would be informative for readers to get to know the EID50 of the virus stock used. Please consider to use virus titers instead of xx µl were spiked into a volume of xx µl.
We agree.
We have changed to EID 50 in xx µl.

5. Page 5: As mentioned above, please consider to use virus titers (EID50) in the results section throughout.

We agree.
We have changed to EID50 in whole text.

6. Page 5: Strikingly, RNA concentrations already differed at time point 0 by more than 1 log although one has to assume that identical virus concentrations were spiked into buffy coats to be analyzed at different temperatures over time. Please clarify this finding.

We agree.
We noted this. It is likely because they were done at different times and in different studies. There are more differences between matrices. Therefore we added “Virus stability and infectivity for time “0” indicated in figures was tested immediately (within 10 min) after mixture of virus and the specific matrix.”

7. Page 7. The discussion is rather cursory. The authors might want to put their results e.g. in the context of the stability of blood-borne viruses in different matrices. The authors are encouraged to go back to the literature and to mention other virus detection methods for influenza, i.e. rapid antigen tests or multiplex assays for simultaneous detection and typing. Alternatively, be less exclusively.

We have addressed this in the discussion and explained why we used RNA and infectivity assays to measure influenza virus in the samples. We added this in the discussion: “Virus culture is often used to confirm the presence of infectious virus in clinical samples. Rapid influenza antigen tests have also been used to detect virus, although their specificity has hampered widespread for influenza diagnosis [14]. Our study focused on the use of RT-PCR and infectivity as a measure of influenza virus stability in the blood matrices.”

8. Page 8. The authors are encouraged to comment on any inhibitory effects of the different matrices used, which can also contribute to significantly different viral RNA copy numbers.

We have added some discussion points about this issue. Such as, we put this in :”one possible reason may be due to virus adsorption to blood components such as platelets and red cells [16]. Higher temperature may cause more virions to associate with these blood derived components [7].”

9. Figure 1: Please consider to use the widely accepted term cycle threshold (Ct-value) on the y-axis to indicate the cycle, at which the fluorescence signal of real-time PCR increase above baseline. In addition, consider to use “buffy coat” instead of “blood” in the figure legends to avoid misunderstanding (whole blood, plasma or alike).

We have fixed them.

10. Figure 2: Please extend the y-axis to 40 in figure A in the same manner as for B and C.

We have changed that.
11. In general, the authors might want to use box plots to indicate the range of Ct-values, which were measured by real-time PCR in triplicates.

We did them in Fig. 3 B & C.

12. There are some typos to be corrected, e.g. page 5 “degradedin”, page 7 references “number versus names”

We have corrected them.