Reviewer 2

Comments and Suggestions for Authors

Major remarks

Figure 2. What exactly is the origin of the PB1, PA and NP subunits of the polymerase, IND5 or CK2A? (see also remark for lines 427-443 and to Fig 8).

Response:

In Figure 4A, there is some ambiguity about the values and the yscale. To remove that ambiguity, and for the sake of readability, values (not their log10) should be reported, and the log10y scale should be emphasized by its subdivisions [29] between powers of 10. Same remark for Fig 6C.

**Response: Fig. 4A and Fig. 6C have been modified in the revised version.**

Line28687. Between “We tested…” and “Our results show…”, a sentence should explain the rationale of the test being used (what is the rationale of adding increasing amounts of NEP?). This would also help in understanding the data (lines 288-92). Same remark for the PB2-NP interaction (lines 292 95). In brief, how NEP and NP could be expected to modulate the activity of the viral polymerase? Further, these possible interactions with NEP and NP are not discussed at all (neither here nor in the discussion section). 28687

**Response: We have modified text and included additional reference to state the rationale of this experiment.**

Line 327 and Table 1. Contradictory data. “mice infected with 10^3 PFU of the avian isolate CK2A showed no disease symptoms”, while according to Table 1, the MLD50 of CK2A is 2.2 x 10^3 PFU. Same inconsistency for CK2A-526R (all mice survived with an inoculum of 10^3, but MLD50 is 2.2x 10^3). Moreover, according to the legend to Table 1, MLD50 were calculated after inoculating mice with 10^2 to 10^6 PFU. With such a protocol, it is impossible to reliably calculate an MLD50 below 10^3.

**Response The challenge dose for different groups of mice is from 1-106 pfu per mice (as shown in methods). For CK2A and CK2A-K526R groups, no mice died when the dose is equal or lower than 10^3 pfu. Therefore, the MLD50 of some groups is lower. Table 1 legend has a typo, challenge dose should to 1-106 pfu. We have made correction in the revised version.**

Fig 7E is somewhat misleading, in that one may understand that there is a competition between four viruses (526 R or K and 627 E or K). It would be better to put the name of the two viruses in front of the nucleotide sequence (CK2A-526R in front of AGA [R] ..GAG [E], and CK2A-627K in front of the line below. The data show that the latter “wins” the competition. The legend also is misleading. Line 417-18 should read “..mixed populations of [526R-627E] and [526K-627K]”.Line 383… rather “Comparisons of the two CK2A-derived viruses (i.e. [526R-627E] and [526K-627K]) through either virus growth kinetics or competition assays suggested that the E627K substitution had a greater positive effect on the replication potential in A549 cells than the K526R substitution”.

**Response: We have modified Fig. 7E and revised text to address these concerns**

Lines 427-443, 117-119 and 109-110. The available nucleotide sequences of the CK2A virus are only partial, precluding full-length alignments. Nevertheless, comparison of the available PB2 sequences shows another substitution (G669V) between IND5 and CK2A. Furthermore, comparison of the other polymerase subunit sequences reveals at least 2 substitutions between the PB1s and 4 substitutions between the PAs. But in fact sometimes it is difficult to find the precise information  about the composition of the polymerase complex in the minireplicon assays (what is the origin of the PB1, PA and NP subunits, IND5 or CK2A?).

**Response: CK2A was used because it was isolated during 2003 before H5N1 human cases emerged in Indonesia. Residues selected for testing in this study on available sequences. We understand these residues tested can not fully represent all the possibilities. Further study based on analyses of more sequences of all PB2 with K526R substitution will provide more information for understand role of other adaptive mutation associated with K526R \.**

More specifically for lines 427-443 and Fig. 8: since there are other substitutions (not only in PB2, but also in PB1 and PA) between IND5 and CK2A that could subtly alter the activity of the polymerase, it is necessary to know the relative efficiency of the two polymerase complexes (IND5 and CK2A). To this end, Graphs A and B in Fig. 8 should be combined in a unique graph, with the 100% value assigned to IND5-wt (otherwise, the activity of the wt-CK2A RNP relative to that of IND5 could be added in Fig 8A). Maybe a logarithmic y-scale (log2 or log10) could help to clearly see the differences between the conditions.

Lines 441-43. It is somewhat excessive to assign the difference of polymerase activity solely to the two PB2 substitutions R288Q and K526R, since (i) all the substitutions were not assayed and (ii) other substitutions in PB1 and PA may also play a role.

**Response: We agree with this reviewer that there are other possibilities in PB1 and PA of both strains. Fig. 8 tests if R288Q is associated with K526R for enhanced polymerase activity in Indonesia H5N1 virus. We consider it is easier for reader to understand acquired mutation,R288Q, would enhance PB2 K526R RNP polymerase activity in the A/CK/Indonesia/2A/03 background (PB2-526K) while reversed mutation, Q288R, would negatively affect RNP polymerase activity in the A/Indonesia/05/03 background (PB2-526R).**

Lines 535-39. Perhaps a sentence could be added with the meaning that there is more than one unique pathway of adaptation to mammals for the viral polymerase, since at least two substitutions [PB2-K526R and PB2-E627K] have now been identified that prime these viruses for infecting human; with the added information that K526R does not negatively impact the viral replication in avian cells.   
Perhaps the authors should try to map the substitutions in the structure of the viral polymerase (cf Pflug 2014, Pflug 2018), at least to the main structural domains of PB2.

**Response: We have modified text in the revised version to state multiple adaptive strategies are utilized by different avian influenza virus to gain cross species replication.**   
   
Minor remarksLine 21. ..human infection, we showed that: (1)…Lines 43-44. Please update the figures (as to September 2017, there were 860 lab-confirmed infections including 454 deaths).Line 208. Somewhat ambiguous. Maybe rather “…the later-emerging avian H5N1 viruses which carry 526R PB2 (in blue)….Line 285….that 627K and 526R substitutions in PB2Line 366… E627K substitution was [] found in one of five mice…(the meaning of “only” is ambiguous here).Line 482. Initiated by the [] emergence…(sudden may be excessive: cf ref 44, which should be added here with 42-43).Line 533. …primarily..

**Response: Corrections have been made in the revised version**