Recombinant influenza virus with a pandemic H2N2 polymerase complex has a higher adaptive potential than one with seasonal H2N2 polymerase complex

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The reassortment of influenza viral gene segments plays a key role in the genesis of pandemic strains. All of the last three pandemic viruses contained reassorted polymerase complexes with subunits derived from animal viruses, suggesting that the acquisition of a reassorted polymerase complex might have a role in generating these pandemic viruses. Here, we studied polymerase activities of the pandemic H2N2, seasonal H2N2 and pandemic H3N2 viruses. We observed that the viral ribonucleoprotein (vRNP) of pandemic H2N2 virus has a highly robust activity. The polymerase activity of seasonal H2N2 viruses, however, was much reduced. We further identified three mutations (PB2-I114V, PB1-S261N and PA-D383N) responsible for the reduced activity. To determine the potential impact of viral polymerase activity on the viral life cycle, recombinant H3N2 viruses carrying pandemic and seasonal H2N2 vRNP were studied in cell cultures supplemented with oseltamivir carboxylate and tested for their abilities to develop adaptive or resistant mutations. It was found that the recombinant virus with pandemic H2N2 vRNP was more capable of restoring the viral fitness than the one with seasonal vRNP. These results suggest that a robust vRNP is advantageous to influenza virus to cope with a new selection pressure.

INTRODUCTION

Influenza A virus has a segmented genome, which allows exchange of gene segments between viruses in co-infected cells (Palese & Shaw, 2007). This virological feature plays an important role in the genesis of pandemic influenza viruses. Sequence analysis studies have indicated that at least the last three influenza pandemic strains acquired surface glycoprotein gene segments of animal origins by gene reassortments. The 1968 pandemic H3N2 virus obtained an avian viral haemagglutinin (HA) gene, whereas the 1957 pandemic H2N2 virus had both HA and neuraminidase (NA) genes of avian virus origin (Kawaoka et al., 1989; Webster & Laver, 1972). The pandemic H1N1/2009 virus acquired HA and NA genes from the classical swine and Eurasian swine viruses, respectively, via multiple rounds of reassortment in pigs (Neumann et al., 2009; Smith et al., 2009). The acquisition of these novel surface glycoprotein gene segments allowed the pandemic strains to escape from pre-existing herd immunity in humans, thereby facilitating transmission between humans. In addition, other critical mutations, such as those that can change the HA receptor-binding specificity from α2,3-linked to α2,6-linked sialic acid, were also detected in these surface glycoproteins.

Apart from acquiring novel surface glycoprotein gene segments, the last three influenza pandemic viruses also possessed reassorted polymerase complexes with at least one subunit of animal origin. Both 1957 and 1968 pandemic viruses had an avian PB1 segment, whereas the 2009 pandemic virus had avian PB2 and PA gene segments circulating in swine influenza viruses (Kawaoka et al., 1989; Smith et al., 2009; Webby et al., 2000). Although there is some evidence suggesting that reassorted polymerase complexes with avian PB1 might have enhanced polymerase activity in human cells (Chen et al., 2008; Li et al., 2009; Naffakh et al., 2000; Wendel et al., 2015), the evolutionary advantages of having a reassorted polymerase complex in a pandemic virus are not clear. For example, it is not known whether a reassorted polymerase might have a role in facilitating the genesis or transmission of pandemic influenza virus. On the other hand, it is not known whether a pandemic virus modulates its polymerase activity after its circulation in humans.
H2N2 influenza virus has had a very short history in humans. The pandemic began in 1957 and the virus was extinct in humans by 1968. Its circulation period in humans was thus far the shortest among all seasonal influenza subtypes. The rapid extinction of H2N2 virus in humans suggested that the virus might have features that do not favour its circulation in humans. This has prompted us to study a number of human H2N2 viruses isolated at different time points. Specifically, it is of interest to study whether the polymerase activities of pandemic and seasonal H2N2 viruses have any implications for the evolution of these human viruses. Here, the polymerase activities of pandemic and seasonal H2N2 viruses have been studied. In addition, the role of viral polymerase activity for a virus to cope with a new selection pressure has been investigated in vitro.

RESULTS

Polymerase activities of pandemic H2N2 and H3N2 viruses are higher than the seasonal H2N2 viruses

To determine the possible change of viral polymerase activities during its circulation in humans, viral ribonucleoproteins (vRNPs) derived from a pandemic H2N2 virus (JP/57), various seasonal H2N2 viruses (JP/62, NL/63, CB/64, PD/65 and TK/67; see Methods for abbreviations) and a pandemic H3N2 virus (HK/68) were studied using a luciferase reporter assay for influenza viral polymerase activity (Fig. 1a). It was observed that the vRNP of the pandemic H2N2 virus (JP/57) had the highest polymerase activity among the tested strains. By contrast, all the tested seasonal H2N2 vRNPs (JP/62, NL/63, CB/64, PD/65 and TK/67) were found to have much reduced polymerase activities. In particular, activities of NL/63 (23.3 ± 7.0 %), CB/64 (8.4 ± 1.7 %), PD/65 (29.2 ± 7.3 %) and TK/67 (19.0 ± 2.2 %) vRNPs were below 30 % of the JP/57 vRNP. Interestingly, although the pandemic H3N2 vRNP (HK/68) was found to be less active than the pandemic H2N2 vRNP (JP/57) in the assay, its activity was more robust than many seasonal H2N2 viruses. Overall, our results suggest that the viral polymerase activity of the pandemic H2N2 virus declined after its introduction in humans.

Reduced polymerase activity of seasonal H2N2 virus is due to mutations in PB2, PB1 and PA, but not NP segments

To determine which of the vRNP subunits is responsible for the reduced polymerase activity as observed in seasonal H2N2 viruses, viral ribonucleoprotein gene segments (PB2, PB1 and PA) from the above seasonal strains were used to replace the corresponding segments of pandemic H2N2 virus individually (Fig. 1b–d). In comparison with polymerase activity of pandemic H2N2 vRNP (JP/57), the use of PB2 segment derived from seasonal H2N2 viruses isolated after 1962 or pandemic H3N2 virus reduced the activity (Fig. 1b). Strikingly, replacing the PA of pandemic H2N2 virus with any PA derived from seasonal H2N2 and pandemic H3N2 viruses dramatically reduced the polymerase activities, by more than 50 % (Fig. 1c). By contrast, the effect of substituting PB1 on viral polymerase activity was found to be less prominent. The use of PB1 derived from H2N2 viruses isolated in or after 1963 only slightly reduced the viral polymerase activity. Interestingly, the use of PB1 from HK/68 H3N2 virus did not have such inhibitory effect and the resultant recombinant vRNP had an activity slightly higher than the WT (Fig. 1d).

The effect of substituting NP on the H2N2 viral polymerase activity was also studied (Fig. 1e). None of these recombinant vRNPs was found to have significantly reduced activity, indicating that the reduced polymerase activity observed among seasonal H2N2 viruses was unlikely to have been caused by mutations in the NP segment.

The above results suggest that mutations found in seasonal PB2, PB1 and PA, but not NP, might account for the reduced polymerase activities of seasonal H2N2 viruses. To identify the mutations responsible for the reduction, we analysed protein sequences of the studied H2N2 viruses and identified amino acid polymorphisms shared by the less robust vRNPs (PB2: I67V, I114V and T676I; PB1: S261N, D464N, F596S and I667T; PA: I62M, K142N, S277F, D383N and V557M). These mutations were then individually introduced into the pandemic H2N2 vRNP and tested for the effects on polymerase activity (Fig. 2). Among these mutations, only PB2-I114V, PB1-S261N and PA-D383N mutations could significantly reduce the polymerase activity (P<0.05; Fig. 2a–c). To further determine whether these three point mutations could have negative synergistic effects on the viral polymerase activity, double or triple mutants containing these mutations were tested (Fig. 2d). All of the double mutants reduced polymerase activities more than the corresponding single mutants did, whereas the triple mutant containing PB2-I114V, PB1-S261N and PA-D383N mutations reduced activity the most. Thus, these three mutations can synergistically reduce the viral polymerase activity. It is worth noting that the seasonal H2N2 viruses (NL/63, CB/64, PD/65 and TK/67) carry these three mutations, which means they have the lowest polymerase activities among the tested strains (Fig. 1a, Table 1).

Growth kinetics of recombinant viruses carrying H2N2 vRNPs

In order to study the impact of reduced polymerase activity on virus replication, recombinant viruses encoding vRNPs with the most robust (pandemic H2N2, JP/57) and the most attenuated (seasonal H2N2, CB/64) polymerase activities were generated. To avoid creating H2N2 viruses that might be of great pandemic concern, all recombinant viruses were generated in a human H3N2 background.
Fig. 1. Polymerase activities of recombinant H2N2 and H3N2 vRNPs. (a) Viral RNPs of pandemic/seasonal H2N2 and pandemic H3N2 strains were reconstituted in 293T cells. (b–e) Recombinant vRNPs in a JP/57 background with PB2 (b), PA (c), PB1 (d) or NP (e) substitutions were reconstituted. Polymerase activities were determined by luciferase reporter assay and were normalized by GFP expression. The normalized results are expressed as mean relative polymerase activity relative to WT JP/57 vRNP. ‘Mock’ represents the negative control without vRNP complexes. Error bars represent 1 SD (n=3; *P<0.05, compared with WT JP/57 vRNP by t-test).
Fig. 2. Polymerase activities of recombinant vRNPs with mutations in different polymerase subunits. JP/57 WT (WT) vRNP and recombinant vRNPs with PB2 (a), PB1 (b), PA (c) or multiple (d) mutations were reconstituted in 293T cells. Polymerase activities were determined by luciferase reporter assay and were normalized to GFP expression. The normalized results are expressed as mean relative polymerase activity relative to the WT polymerase activity level. ‘Mock’ represents the negative control without vRNP complexes. Error bars represent 1 SD (n=3; *P<0.05, activities lower than the WT; #P<0.05, compared with the corresponding single mutants; ##P<0.05, compared with the double mutants, by t-test; *data used in (b) and (d) are derived from the same experiment.

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**Table 1.** Correlation between polymerase polymorphisms at PB2-114, PB1-261 and PA-383 and viral polymerase activity

<table>
<thead>
<tr>
<th>Polymorphic site</th>
<th>Virus</th>
<th>PB2-114</th>
<th>PB1-261</th>
<th>PA-383</th>
<th>Polymerase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JP/57</td>
<td>I</td>
<td>S</td>
<td>D</td>
<td>++ +</td>
</tr>
<tr>
<td></td>
<td>JP/62</td>
<td>I</td>
<td>S</td>
<td>N</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>NL/63</td>
<td>V</td>
<td>N</td>
<td>N</td>
<td>+</td>
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<tr>
<td></td>
<td>CB/64</td>
<td>V</td>
<td>N</td>
<td>N</td>
<td>+</td>
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<td></td>
<td>PD/65</td>
<td>V</td>
<td>N</td>
<td>N</td>
<td>+</td>
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<td></td>
<td>TK/67</td>
<td>V</td>
<td>N</td>
<td>N</td>
<td>+</td>
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<tr>
<td></td>
<td>HK/68</td>
<td>V</td>
<td>S</td>
<td>N</td>
<td>+ +</td>
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(HK/68). Madin–Darby canine kidney (MDCK) cells were infected by these recombinant viruses at an m.o.i. of 0.01 and the progeny viruses were harvested at 12, 24, 48 and 72 h post-infection (Fig. 3). It is of note that the recombinant virus with JP/57 vRNP had a significantly higher virus titre than the one with CB/64 vRNP at early post-infection time points (>1 log), suggesting that viral polymerase activity is a rate-limiting factor for progeny virus production at this stage. Nonetheless, both recombinant viruses achieved robust virus replication in cell culture and were able to yield similar maximum virus titres (>10⁷ p.f.u. ml⁻¹) at the late post-infection time points. In addition, the replication kinetics and progeny virus titres of these viruses were confirmed to be comparable with the WT HK/68 virus.

![Fig. 3. Viral growth kinetics of recombinant viruses with different vRNPs. MDCK cells were infected with WT HK/68 virus and recombinant HK/68 viruses with JP/57 and CB/64 vRNPs at m.o.i. 0.01. Progeny viruses were harvested at 12, 24, 48 and 72 h post-infection and the viral titres were determined by standard plaque assay. Error bars represent 1 SD (n=3; *P<0.01, by t-test).](http://jgv.microbiologyresearch.org)

**Table 2.** HA and NA gene mutations found in recombinant viruses with different vRNPs after serial passage

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. passages to develop oseltamivir resistance</th>
<th>Mutations identified at passage 6 with oseltamivir</th>
</tr>
</thead>
<tbody>
<tr>
<td>JP/57 vRNP-HK/68-A</td>
<td>5</td>
<td>HA1-N137D, NA-R292K</td>
</tr>
<tr>
<td>JP/57 vRNP-HK/68-B</td>
<td>5</td>
<td>HA1-G218R, HA2-T156A</td>
</tr>
<tr>
<td>JP/57 vRNP-HK/68-C</td>
<td>3</td>
<td>HA1-G135E, HA1-N248T</td>
</tr>
<tr>
<td>CB/64 vRNP-HK/68-A</td>
<td>NA</td>
<td>HA1-G158E*</td>
</tr>
<tr>
<td>CB/64 vRNP-HK/68-B</td>
<td>NA</td>
<td>HA1-G158E*</td>
</tr>
<tr>
<td>CB/64 vRNP-HK/68-C</td>
<td>NA</td>
<td>HA1-G158E*</td>
</tr>
</tbody>
</table>

NA, Not applicable.

*Mutation also identified in virus passaged in DMSO control.*

**Correlation of polymerase activity and adaptation under selection pressure of oseltamivir carboxylate**

We have previously demonstrated that viruses with robust polymerase activities are more capable of generating adaptive mutations under new selection pressure (Li et al., 2009). It was of interest to determine whether recombinant viruses with pandemic (JP/57) and seasonal (CB/64) vRNPs behave differently under a new selection pressure. Recombinant viruses carrying JP/57 or CB/64 vRNP were serially passaged in the presence of 5 μM oseltamivir carboxylate, a level that could reduce the progeny virus titres by at least 2 logs. The recombinant viruses were also serially passaged in DMSO as internal negative controls. Each of the recombinant viruses was serially passaged in independent triplicates.

Viral RNA of the progeny viruses after the sixth serial passage were extracted and checked for potential adaptive mutations. Previous studies indicated that mutations in HA and NA genes might confer resistance to oseltamivir carboxylate (Ginting et al., 2012; Gubareva et al., 2002; Pizzorno et al., 2014; Tai et al., 1998) and, therefore, we sequenced the HA and NA genes from the passaged viruses. For the recombinant virus with JP/57 vRNP, two unique mutations were found in each of the triplicates passaged in the presence of oseltamivir carboxylate (Table 2). The viruses from the sixth passage were confirmed to be insensitive or less sensitive to the oseltamivir carboxylate treatment (Fig. 4; and see below). Retrospective analyses of samples passaged earlier indicated that these mutations occurred in the third to the fifth passages (Table 2). In addition, all polymerase genes obtained from the sixth passage were studied by dideoxy sequencing and the consensus sequences of these passaged viruses were found to be identical to the WT sequences. Furthermore, none of these mutations was detected in the control experiments (DMSO control). For the recombinant viruses with CB/64 vRNP, each of the triplicates developed a G158E mutation in the HA1 region. This mutation, however, was also...
identified in the corresponding control experiments, suggesting that this mutation is not related to oseltamivir resistance. As the passaged recombinant viruses with CB/64 vRNP were still found to be sensitive to oseltamivir carboxylate (Fig. 4), no further characterization of these mutants was conducted.

Six putative adaptive mutations were identified in the passaged recombinant virus with JP/57 vRNP (Table 2). These mutations included G135E, N137D, G218R and N248T of HA1, T156A of HA2 and R292K of NA. With the exception of NA-R292K mutation, which is known to confer oseltamivir resistance (Tai et al., 1998), roles of the identified HA mutations in imparting oseltamivir resistance are not entirely clear. In order to investigate whether these HA mutations were responsible for oseltamivir resistance, these mutations were individually introduced into the HA gene of HK/68 virus. The WT virus and its HA point mutants were cultured in the presence of oseltamivir carboxylate or DMSO. Recombinant virus with a G135E, N137D, G218R or N248T mutation in HA1 yielded a viral titre similar to the one observed from its corresponding DMSO control (<1 log difference) (Fig. 5). These results suggested that each of these HA point mutations is individually associated with oseltamivir resistance. By contrast, the HA2-T156A mutant was still found to be sensitive to the oseltamivir carboxylate treatment.

The above single HA mutant viruses were also tested for NA activities using a standard NA inhibition assay (Yen et al., 2013). The enzymic kinetics of these NAs were similar to the WT (data not shown). These viruses were also found to have similar susceptibilities to oseltamivir carboxylate (Table 3), indicating that the oseltamivir resistance of these HA mutants was not because of altered NA activities.

**DISCUSSION**

The RNA-dependent RNA polymerase of influenza virus is responsible for transcription and replication of its own viral genes (Huang et al., 1990; Palese & Shaw, 2007). In this study, polymerase activities of pandemic H2N2, seasonal H2N2 and pandemic H3N2 viruses were examined, with polymerase activity of pandemic H2N2 virus being the highest among these vRNPs. Importantly, the viral polymerase activity of human H2N2 virus declined dramatically after its introduction in humans. Further characterization of these vRNPs indicated that the reduced viral polymerase activity was mainly due to three different mutations in the complex (PB2-I114V, PB1-S261N and PA-D383N). A pandemic H2N2 vRNP carrying these three mutations was found to have much reduced polymerase activity (Fig. 2d). In fact, the polymerase activities of our studied vRNPs correlated with the polymorphisms found in these positions (Fig. 1a; Table 1).

**Table 3.** IC$_{50}$ values of oseltamivir carboxylate for the HA mutant viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean IC$_{50}$ (nM) (95 % CI)</th>
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<tbody>
<tr>
<td>WT</td>
<td>0.82 (0.634–1.016)</td>
</tr>
<tr>
<td>G135E (HA1)</td>
<td>0.82 (0.670–0.998)</td>
</tr>
<tr>
<td>N137D (HA1)</td>
<td>0.89 (0.757–1.040)</td>
</tr>
<tr>
<td>G218R (HA1)</td>
<td>0.92 (0.456–1.835)</td>
</tr>
<tr>
<td>N248T (HA1)</td>
<td>0.83 (0.745–0.933)</td>
</tr>
<tr>
<td>T156A (HA2)</td>
<td>0.79 (0.736–0.849)</td>
</tr>
</tbody>
</table>

CI, Confidence interval.
Among these three viral polymerase subunits, replacing the pandemic H2N2 PA with a seasonal H2N2 PA resulted in the largest reduction in polymerase activity. Chimeric vRNPs of this kind were consistently found to be much less active than the pandemic H2N2 vRNP (Fig. 1c). This inhibitory effect was primarily due to the PA-D383N mutation, which is located at the PA-arch domain for DNA binding and has previously been reported to have an inhibitory effect that is primarily due to the PA-D383N mutation, which is located at the PA-arch domain for DNA binding and has previously been reported to have an inhibitory effect that is primarily due to the PA-D383N mutation, which is located at the PA-arch domain for DNA binding and has previously been reported to have an inhibitory effect. Further tests will be needed to determine whether the PA-383D/N polymorphism might affect the interaction between vRNA and viral polymerase complex. The PB1-261S/N polymorphism was previously suggested to have some roles in host adaptation (Kimble et al., 2016). However, little is known about the role of the PB2-114 residue in the viral life cycle. This residue is located at the N2 domain of PB2 and this subdomain has been reported to interact with the other two viral polymerase subunits (Pflug et al., 2014).

We also observed that there are some mutations that can enhance viral polymerase activity (e.g. PB2-T676I, PB1-P596S and PA-I62M; Fig. 2). These results also indicate that seasonal H2N2 vRNP might generate compensatory mutations to counteract the effects caused by a mutation. This might also explain why all the studied seasonal H2N2 polymerase complexes have polymerase activities that are higher than the one observed from the triple mutant. The roles of these residues in viral RNA synthesis are not fully understood, except the PB2-T676I mutation has been suggested to have effects on control of the host range (Neumann et al., 2014). We also tried to map all the studied point mutations to the crystal structure of a bat influenza A polymerase complex. These residues, together with those that can reduce viral polymerase activity, fall into different domains, suggesting that all of these mutations are not involved in a single specific viral RNA production event. It is more likely that these mutations modulate the overall viral polymerase activity through regulating different viral RNA transcription and replication events. In addition, it is possible that some of these positions, including the three most critical ones reported in this study, might modulate the viral polymerase activity by controlling the interactions between viral polymerase subunits and host factors. Besides, we also cannot exclude the possibility that some of our studied viral polymerases might have unique mutations that have subtle effects on the polymerase, thereby leading to some minor variations of their activities (Fig. 1a: NL/63, CB/64, PD/65 and TK/67).

Recently, Wendel et al. (2015) demonstrated that the viral polymerase activity of a seasonal H2N2 vRNP can be enhanced by replacing its PB1 with an HK/68 PB1. Our findings also support this, but our results further suggest that there are some mutations in the PB2 of the HK/68 virus that also might contribute to the enhanced viral polymerase activity of pandemic H3N2 virus (Fig. 1b). Further characterization of the PB2 of the HK/68 virus is needed. Nonetheless, it is tempting to speculate that the reassorted vRNPs found in the pandemic H3N2 virus might help to ‘restore’ the highly reduced viral polymerase activity of seasonal H2N2 virus, thereby enhancing the fitness of the pandemic H3N2 virus in humans.

PB2-627 and PB2-701 positions are well-known markers for host adaptations. The viruses tested in this study all have the same amino acid residue at each of these positions (PB2-627K and PB2-701D). As polymorphisms at these two positions would have strong effects on viral polymerase activity in mammalian cells, it will be of interest to test whether mutations at these positions and those reported in this study would have any synergistic effect on viral polymerase activity in future.

We have previously observed that a virus with a robust viral polymerase activity might have a better chance to adapt to new selection pressures (Li et al., 2009). Here, we tested recombinant viruses that carry a pandemic (JP/57) or seasonal (CB/64) H2N2 vRNP under the selection pressure imposed by oseltamivir carboxylate. In this model, mutations that confer oseltamivir resistance were more readily generated by the recombinant virus with JP/57 vRNP. By contrast, the recombinant virus with CB/64 vRNP was still susceptible to the drug after serial passage (Fig. 4). Among the resistance mutations generated by the JP/57 vRNP mutant, the NA-R292K mutation is a well-known marker for NA inhibitor resistance (Tai et al., 1998). Of the four resistance mutations found in the HA1 region, three (G135E, N137D and G218R) are located at positions that control receptor-binding specificity, and the G218R mutation is also known to reduce sialic acid binding affinity (Daniels et al., 1987; Laeeq et al., 1997; Takahashi et al., 2009). On the other hand, one of the passaged JP/57 vRNP mutants (HA1-N248T) acquired a potential glycosylation site at amino acid position 246. An H3N2 virus with a glycan at this position was shown to have reduced binding affinity to guinea pig and chicken red blood cells (Abe et al., 2004). These results suggested that some of our escape mutants might acquire oseltamivir resistance by reducing receptor-binding affinity. It is possible that, by reducing the sialic acid affinity, the newly generated virions of these mutants might more readily dissociate from infected cells. Further work, however, is required to test this hypothesis. Nonetheless, our results demonstrated that, apart from introducing NA mutations to alter its affinity to oseltamivir carboxylate, the recombinant virus with JP/57 vRNP can also generate escape mutants by introducing HA mutations. More importantly, our results suggest that a virus with a robust activity might be more capable of generating adaptive mutants.

Our results indicate that a virus with a robust vRNP might have evolutionary advantages under a new selection pressure. It is not known whether this is due to enhanced transcription and replication activity or altered polymerase fidelity. Further research is required to delineate the correlation between polymerase activity and adaptation.
However, it is tempting to speculate that the robust viral polymerase activity observed in pandemic H2N2 virus might facilitate the emergence of this pandemic strain in humans. It is possible that a robust vRNP might help the pandemic H2N2 virus to generate adaptive mutations for host adaptation and/or for human-to-human transmission. It is also interesting to note that there was a decline of viral polymerase activity in seasonal H2N2 viruses. The reason for the reduced activities is not known. However, human H2N2 viruses circulated only in humans for a very short period of time. Based on the results from this study, we speculate that the reduced polymerase activity of seasonal H2N2 virus might fail to support the virus to cope with new selection pressures (e.g. herd immunity) during its circulation in humans, resulting in extinction of H2N2 virus in humans.

METHODS

Cell cultures and viruses. Human embryonic kidney (293T) cells and MDCK cells were used in this study. The cells were maintained in minimal essential medium (MEM) supplemented with 10 % FBS and 1 % penicillin/streptomycin (Life Technologies). H2N2 influenza A viruses, comprising A/Japan/305/1957 (JP/57), A/Japan/170/1962 (JP/62), A/Netherlands/56/1963 (NL/63), A/Cottbus/1/1964 (CB/64), A/Potsdam/2/1965 (PD/65) and A/Tokyo/3/1967 (TK/67) and an H3N2 virus, A/Hong Kong/1/68 (HK/68), were used in this study. Recombinant H3N2 viruses carrying various H2N2 vRNPs in an HK/68 background were generated by reverse genetics as described by Hoffmann et al. (2000).

Luciferase reporter assay. Luciferase reporter assays were used to determine polymerease activities of recombinant vRNPs in 293T cells as described by Chin et al. (2014). In brief, plasmid mixtures including pcDNA3 plasmids expressing PB2, PB1, PA and NP, together with pPoll-Luc-NS and pMax-GFP plasmids, were transfected into 293T cells, and transfected cells were incubated at 37 °C for 48 h. Culture medium was then removed and transfected cells were lysed using Steady-Glo luciferase assay substrate solution (Promega) for 5 min. Luminescence and GFP signals were measured by a luminometer (PerkinElmer). All luminescence signals were normalized to GFP signals observed from the same wells.

Viral growth kinetics assay. Confluent MDCK cells in a 24-well plate were infected with recombinant viruses (m.o.i. 0.01). After 1 h of adsorption at 37 °C, inocula were removed and infected cells were washed with 0.9 % acidic NaCl solution (pH 2.0) to inactive unbound viruses (Yen et al., 2005). Infected cells were washed with PBS and were then replenished with virus culture medium (MEM with 1 % penicillin/streptomycin and 1 μg TPCk-treated trypsin ml⁻¹). Infected cells were incubated at 37 °C and culture supernatants were collected at various time points. Viral titres of harvested samples were determined by standard plaque assays.

Generation of adaptive mutants with oseltamivir carboxylate. Recombinant viruses were serially passaged six times in MDCK cells to determine genetic stability. MDCK cells were infected with recombinant viruses at m.o.i. 0.01 in each passage. After 1 h of viral adsorption at 37 °C, inoculum was removed and infected cells were replenished with virus culture medium in the presence of 5 μM oseltamivir carboxylate or 0.5 % DMSO as control. Infected cells were incubated at 37 °C for 48 h. Progeny viruses generated from each passage were harvested and titrated by standard plaque assays. Progeny viruses with known titres were then diluted and used to infect the cells in the following passage. Viral HA and NA gene sequences in the harvested samples were determined by standard dideoxy sequencing.

Enzyme-based NA inhibition assay and NA kinetics. NA inhibition assay was conducted essentially as described previously (Yen et al., 2013). Sensitivities of recombinant viruses to oseltamivir carboxylate were determined by an enzyme-based assay using a fluorogenic substrate [2'-3'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid] at a final concentration of 167 μM. The reaction mixture was incubated at 37 °C for 30 min and the reaction product (4-methylumbelliferone) was detected using a FLUOstar OPTIMA microplate reader (BMG Labtech; excitation 355 nm, emission 460 nm). The IC₅₀ for each virus was determined by plotting fluorescence as a function of the compound concentration followed by variable-slope dose–response curve fitting (GraphPad Prism).

Statistical analysis. Unless otherwise specified, all data represent the mean ± sd of triplicate experiments. Data were analysed by Student’s t-test. P values less than 0.05 were considered to be statistically significant.

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H2N2 polymerase activity and its impacts on adaptation


