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Hemagglutinin and neuraminidase matching patterns of two influenza A virus strains related to the 1918 and 2009 global pandemics

Yonghui Zhang\textsuperscript{a,b,1}, Xiaojing Lin\textsuperscript{a,1}, Fengwei Zhang\textsuperscript{a,c}, Jia Wu\textsuperscript{a,c}, Wenjie Tan\textsuperscript{a}, Shengli Bia, Jianfang Zhou\textsuperscript{a}, Yuelong Shua, Yue Wang\textsuperscript{a,*}

\textsuperscript{a}State Key Laboratory for Molecular Virology and Genetic Engineering, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Yingxin Street 100, Xuanwu District, Beijing 100052, PR China
\textsuperscript{b}Institute of Pathogens and Vectors, Dali University, Dali 671003, Yunnan Province, PR China
\textsuperscript{c}Wenzhou Medical College, Wenzhou 325000, Zhejiang Province, PR China

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\textbf{A B S T R A C T}

The current pandemic influenza A (H1N1) virus has revealed a complicated reassortment of various influenza A viruses. The biological study of these viruses, especially of the viral envelope proteins hemagglutinin (HA) and neuraminidase (NA), is urgently needed for the control and prevention of H1N1 viruses. We have generated H1N1-2009 and H1N1-1918 pseudotyped particles (pp) with high infectivity. Combinations of HA1918 + NA2009 and HA2009 + NA1918 also formed infectious H1N1pps, among which the HA2009 + NA1918 combination resulted in the most highly infectious pp. Our study demonstrated that some reassortments of H1N1 viruses may hold the potential to produce higher infectivity than do their ancestors.

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\textbf{Introduction}

Influenza A virus, which belongs in the family Orthomyxoviridae, regularly causes epidemics, with sudden attacks in humans and animals, including the H2N2 Asian influenza of 1957 with more than 1 million deaths and the H3N2 Hong Kong influenza of 1968 with 0.5 million deaths [1–3]. Influenza A virus possesses eight negative single-stranded RNA segments that encode eleven proteins (HA, NA, NP, M1, M2, NS1, NEP, PA, PB1, PB1-F2, PB2) and is subtyped by 16 HA and nine NA envelope proteins [1]. A novel influenza A (H1N1) virus first isolated in North America has been a major global focus since April 2009, spreading to 74 countries, infecting more than 35,000 people, and causing 163 related deaths to date [4]. Genetic study has suggested that this new H1N1 virus contains a unique combination of gene segments from both North American and Eurasian swine lineages, of which PB2, PA, PB1, HA, NP, and NS are derived from North American swine lineage (triple reassortant), and the NA and M are derived from Eurasian swine lineage [5]. Simply put, this new H1N1 virus exhibits an obviously complicated gene segment reassortment of previous prevalent virus strains [6–9].

The trend of this H1N1 virus pandemic raises the memory of another H1N1 virus strain in human history, namely, the catastrophic “Spanish" influenza virus of 1918 that killed more than 50 million people worldwide [1,10]. Although this virus finally disappeared from detection, it is possible that this strain still remains in the earth, and any possible reassortment of two or more catastrophic H1N1 viruses may be a great challenge to human survival [9]. Although varieties of influenza A virus display a strong ability for reassortment of their gene segments [5,11], it is not clear whether the reassortment between H1N1 2009 and H1N1 1918 viruses will occur. Meanwhile, investigation of the biological aspects of these strains is urgently needed for influenza A virus control and prevention. In this study, we established H1N1-2009pp and H1N1-1918pp using the HAs and NAs cloned from the H1N1 viruses of 2009 and 1918. We then characterized the HA and NA matching patterns of these two influenza A virus strains to provide important biological information about these H1N1 viruses.

\textbf{Materials and methods}

\textit{Cell culture.} Human lung carcinoma cells (A549) and human embryonic kidney cells (293T) (American Type Culture Collection, Manassas, VA) were grown in Dulbecco’s modified essential medium (DMEM, Invitrogen, CA) supplemented with 10% fetal bovine serum.

\textit{Plasmid construction.} To produce the H1N1-1918pp and H1N1-2009pp and to study the HA and NA matching pattern, cDNA fragments encoding the full length HA of the A/South Carolina/1/1918 (GenBank Accession No. AF117241) and A/California/05/2009

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(GenBank Accession No. FJ966952) strains and the full length NA of the A/Brevig_Mission/1/1918 (GenBank Accession No. AF250356) and A/Ohio/07/2009 (GenBank Accession No. FJ969534) strains were synthesized (Luihetong Inc., Beijing, China) and were named HA1918, HA2009, NA1918 and NA2009, respectively.

**H1N1pp production.** H1N1-1918pp and H1N1-2009pp were produced by transfecting 293T cells with four plasmids: the HA expression plasmid; NA expression plasmid; Gag-pol encoding plasmid; and reporter plasmid. At 72 h post-transfection, H1N1pp was harvested from the supernatant of the transfected cells by filtration through a 45-μm Durapore PVDF membrane filter (Millipore, Ireland) and used for further assays.

**Infection assay.** The pp infection assay was performed as reported previously [12,13]. The naive H1N1 virus HA0 needs to be matured by TPCK-trypsin cleavage to form its functional subunits HA1 and HA2 [14]; hence, to explore the best conditions for pp infection, pps were pre-treated with TPCK-trypsin at a final concentration of 0, 2, 40, 80, and 160 μg/ml at 37 °C for 1 h before infection. A549 cells (5–10 × 10^3) were seeded in a 96-well plate one day prior to infection. The pps were diluted 1:1 in a total volume of 100 μl DMEM. For the infection process, culture medium in the 96-well plate was replaced with the diluted pps, incubated for 4 h, and then replaced with DMEM supplemented with 3% FBS. At 72 h post-infection, the infected cells were rinsed twice with phosphate-buffered saline (PBS), and GFP reporter positive cells were counted by FACS (BD, FACSAria Franklin lakes NJ).

**Hemagglutinin assay.** To determine the hemagglutination activity of the pps, a hemagglutination assay was performed. The pp samples were diluted serially in PBS (by 2-fold from 1 to 1/16) in 96-well plates with 50 μl per well. Then, 50-μl aliquots of 1% chicken red blood cells were added to each well and left for 30 min, and the hemagglutination reaction was observed.

**Data analysis.** Significant differences were evaluated using a two-tailed Fisher’s exact test (release 12.1; SPSS Inc., Chicago, IL). Differences were considered significant at p < 0.05.

**Results**

**Alignments of 1918 and 2009 influenza A (H1N1) virus HA and NA amino acid sequences**

To standardize the research, amino acid sequences of the HA from A/South Carolina/1/1918 (GenBank Accession No. AF117241) and A/California/05/2009 (GenBank Accession No. FJ966952) strains and the NA from A/Brevig_Mission/1/1918 (GenBank Accession No. AF250356) and A/Ohio/07/2009 (GenBank Accession No. FJ969534) strains were aligned using the HA and NA from 1918 as the standards. Amino acids identical to the 1918 strain are represented by ".". The amino acid marked in red represents the cleavage site of the HA precursor linking the functional HA1 and HA2 domains (Fig. 1), which is an arginine at amino acid position 344 [15]. Of the 569 amino acids in HA, HA2009 presents 79 (13.88%) amino acids different from those of the 1918 strain. Of the 469 amino acids in NA, 59 (12.85%) amino acids of NA2009 are different from those of the 1918 strain.

**Generation of H1N1-1918pp and H1N1-2009pp**

To establish functional H1N1-2009pp, plasmids for HA2009, NA2009, gag-pol, and GFP reporter were co-transfected into 293T cells, and pp were harvested at 72 h post-transfection. Prior to the infection assay, pp samples were treated with TPCK-trypsin at concentrations of 0, 2, 40, 80, and 400 μg/ml. As shown in Fig. 2A, H1N1-2009pp could not infect A549 cells when pp samples were treated with 0 or 2 μg/ml TPCK-trypsin (Fig. 2A). Interest-

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**Fig. 1.** Amino acid sequence alignment of HAs and NAs. The H1N1 1918 strain was used as a standard. Amino acids identical to the 1918 strain are replaced by ".". The amino acid marked in red represents the cleavage site (R344) of the HA precursor linking the functional HA1 and HA2 domains. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)
...H1N1 virus strain as well. Similarly, to examine whether the HAs and NAs of 1918 and 2009 H1N1 virus strains may form infectious pps, raising the alarm for the possible reassortment of the two catastrophic H1N1 virus strains.

**Hemagglutination ability of H1N1pps**

To further confirm the HA function of the H1N1pps, pp samples generated from the four combinations HA2009 + NA2009, HA1918 + NA1918, HA2009 + NA1918, and HA1918 + NA2009 were subjected to the hemagglutination assay. The hemagglutination ability of HA1918 + NA1918 was 8-fold weaker than that of the other three H1N1pp combinations, all of which were positive at a dilution of 1/16 (Fig. 3B). These results are consistent with the infectious ability observed for the H1N1pp of HA1918 + NA1918 that displayed relatively weak infectivity to A549 cells.

**Discussion**

In this study, we successfully generated two pseudotyped particles, H1N1-2009pp and H1N1-1918pp. Similar to all pps we have made, including hepatitis C virus [12,13], vesicular stomatitis virus, severe acute respiratory syndrome coronavirus, and Rabies virus (Wang, unpublished data), the H1N1-2009pp and H1N1-1918pp hold similar biological characteristics with their ancestor viruses. Our work will not only benefit the knowledge on influenza A viruses, but it also will advance the exploration of neutralizing antibodies to H1N1 virus.

One important task for influenza A virus control and prevention is to survey the possible reassortment of two or more catastrophic viruses [9]. Methods based on bioinformatics cannot provide direct solid data to support its analysis, and owing to biosafety limits, it is not suitable to perform reassortments using wild-type viruses. Consequently, our pp system provides a safe and convenient approach to investigating possible reassortments. Our data demonstrated that the combination of HA2009 + NA1918 resulted in infectivity greater than that of the naive combinations HA2009 + NA2009 and HA1918 + NA1918. This suggests that the H1N1-2009 virus may not only recombine with H1N1-1918, but it may also produce a more infectious new virus, raising an alarm for the possible reassortment of the two catastrophic H1N1 virus strains.

Our data revealed that the infectivity of the H1N1pps depends on a certain concentration of TPCK-trypsin, suggesting that the HA precursor is matured by trypsin digestion. Our coworkers' data demonstrated that the infectivity of the wild-type H1N1-2009 virus also depended on trypsin, but the concentration was only 2 µg/ml (Zhou, unpublished data). This difference might be explained by the fact that the pps in our study were harvested in DMEM supplemented with as much as 10% fetal bovine serum.

Due to the lack of serum from a recovered H1N1 infected patient, we have not performed neutralizing antibody experiments for this report; the confirmation and exploration of neutralizing antibodies are scheduled in our following research. In conclusion, we have developed a solid H1N1pp platform for studying this...
virus, including its receptors, the functional analysis of HA and NA, neutralizing antibodies, anti-H1N1 drug development, diagnosis of H1N1 virus, and vaccine design.

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Fig. 3. HAs and NAs matching patterns and hemagglutination assay results. (A) HAs and NAs matching patterns, in which HA1918 + NA2009 is significantly higher than are HA2009 + NA2009 (p < 0.05) and HA1918 + NA1918 (p < 0.05). (B) Hemagglutination assay of H1N1pps, in which HA1918 + NA1918 is positive at a dilution of 1/8, while others are positive up to 1/16 dilution. The data in A and B represent the average of three independent experiments.