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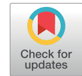
Characterization of pseudoparticles paired with hemagglutinin and neuraminidase from highly pathogenic H5N1 influenza and avian influenza A (H7N9) viruses



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ABSTRACT

The reassortment of two highly pathogenic avian influenza (HPAI) H5N1 and H7N9 viruses presents a potential challenge to human health. The hemagglutinins (HAs) and neuraminidases (NAs) of these simultaneously circulating avian influenza viruses were evaluated using the pseudoparticle (pp) system. Native and mismatched virus pps were generated to investigate their biological characteristics. The HAs and NAs of the two viruses reassorted successfully to generate infectious viral particles. H7 was demonstrated to have the ability to reassort with NA from the H5N1 viruses, resulting in the generation of virions that were highly infectious to bronchial epithelial cells. Although the Anhui H5 + Anhui N9 combination showed an moderate infectivity to the four cell lines, it was most sensitive to oseltamivir. The H7 in the pps was found to be predominantly HA0. Further, H5 in the pps primarily presented as HA1, owing to the particular mechanisms underlying its maturation. All NAs predominantly existed in monomer form. In our study, HAs/NAs, in all combinations, were functional and able to perform their corresponding function in the viral life cycle. Our data suggest that HAs/NAs from the (HPAI) H5N1 and H7N9 viruses are capable of assembly into infectious virions, posing a threat topublic health.

1. Introduction

Avian influenza viruses (AIVs), which belong to the Orthomyxoviridae family, contain eight gene segments and encode at least ten proteins: PB1, PB2, PA, HA, NP, NA, M1, M2, NS1, and NS2 (Sonnberg et al., 2013). AIVs are generally considered species-specific, with infection chiefly limited to birds (Su et al., 2015). In the last century, influenza A viruses with only three HA (H1, H2, and H3) and two NA (N1 and N2) serotypes have adapted to humans, resulting in influenza pandemics (McAuley et al., 2017; Sanejouand, 2017; Stevens et al., 2006; Viboud et al., 2016). The highly pathogenic avian influenza (HPAI) H5N1 virus has rapidly evolved and spread to new geographic regions since the isolation and identification of the strain A/Goose/Guangdong/1/1996 (GsGD) in 1996 (Poovorawan et al., 2013), and has caused 860 laboratory-confirmed cases of human infection and 454 deaths (WHO, 2018). In February 2013, a novel avian influenza H7N9 virus emerged in China, and has caused sporadic human infections (Gao et al., 2013). A total of 1623 infections in humans and 620 fatal cases have been identified since February 2013 (FAO, 2018). An increase in human infections with avian influenza A(H7N9) virus has been reported by China since October 2016 (WHO, 2017).

The AIVs genome contains eight negative single-stranded RNA segments, whose reassortment may theoretically produce additional novel viruses (Neumann et al., 2009; Palese and Shaw, 2007). HA and NA are surface antigenic proteins that play a major role in the host humoral immune response against these viruses (Nguyen et al., 2016). Influenza A viruses are believed to enter host cells via HA binding to sialic acid receptors on the cell surface; the binding affinity of HA to sialic acid is believed to account in part for the host specificity of several influenza A viral subtypes (Byrd-Leotis et al., 2017; Palese and Shaw, 2007). It's well known that human viruses preferentially recognize sialic acid linked to galactose by a-2,6 linkages, while avian viruses tend to bind to a-2,3 linkages (Byrd-Leotis et al., 2017). Many animals, including swine, chickens, and humans, have both a-2,3 and a-2,6 linkages on their epithelial cells, which may serve as a "mixing vessel" for the genesis of new viral types through co-infection (Neumann et al., 2009; Palese and Shaw, 2007). Genetic studies of the 2009 H1N1 strain have suggested that the 2009 H1N1 virus carries a complex genetic reassortment of previously prevalent viral strains (Garten et al., 2009; Smith et al., 2009).

AIVs subtypes, including the HPAI H5N1 and the avian influenza A H7N9 subtype, are catastrophic pathogens for humans (WHO, 2018;

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FAO, 2018). HPAI H5N1 and H7N9, which are both derived from an avian source, have caused a large number of infections in humans. These two viruses exist side by side, and are therefore potentially capable of reassortment with each other to generate an infectious “new virus”. Although the general mechanisms by which new influenza viruses emerge are understood, knowledge of how these viruses reassort and acquire human pandemic potential is limited; further, our molecular understanding of the virus and host factors involved in successful transmission and spread is rudimentary. Thus, research on possible reassortment among the HPAI H5N1 and H7N9 viruses is urgently necessary, particularly with regard to therapeutic targets and preventive strategy development. In this study, the HA and NA matching patterns and biological properties of two pathogenic avian strains (HPAI H5N1 and the novel H7N9 strain) were studied using a newly established relative influenza virus pp system. Our study provides important insights into the biological properties of these viruses.

2. Materials and methods

2.1. Ethics statement

All experiments with virus pseudoparticles were performed in an enhanced biosafety level 2 containment laboratory approved by the Centers for Disease Control and Prevention of Zhejiang Province, China. The pp system used in this study belong to Lentiviral Lentiviral packaging system. In this study, reassortment on two envelop proteins from two viruses does’t generate a real self-replicating virion, which is defined as an advantage of the pp system.

2.2. Cell culture

For pseudotyping, producer cells 293 T/17 human embryo kidney cells (Number CRL-11268) and five types of target cell: two alveolar derived human cell lines-A549 cells (Number CRM-CCL-185) and HCC827 cells (Number CRL-286); two human bronchus normal cells-BEAS-2B cells (Number CRL-9609) and HBE cells (Number CRL-2078); and MDCK (Number CCL-34) were all obtained from the American Type Culture Collection (ATCC; Manassas, VA) and grown in Dulbecco’s modified essential medium (DMEM; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum.

2.3. Plasmid construction

The cDNA fragments encoding full-length HA proteins from A/Anhui/1/2005 (#DQ371928) and A/Anhui/1/2013 (#EPI439507) influenza viruses, and the full-length NA proteins from A/Anhui/1/2005 (#EU128239) and A/Anhui/1/2013 (#EPI439509) influenza viruses were cloned as reported previously (Lin et al., 2009; Wu et al., 2010). HAs and NAs from HPAI H5N1 and Anhui H7N9 were designated Anhui H5 and Anhui N1; Anhui H7 and Anhui N9, respectively. For detection of Anhui N1 and Anhui N9, a 6 × His tag was added by PCR at the C-terminal. Adding His tag to the NAs didn’t impact particle formation and NA function (data not shown), as reported previously (Wu et al., 2010).

2.4. Pp Production and quantification

As reported previously (Lin et al., 2009; Wu et al., 2010), pps were produced by co-transfecting 293 T/17 cells with four plasmids: a GagPol-encoding plasmid (Fig. 1A), a cytomegalovirus (CMV)–green fluorescent protein (GFP) reporter plasmid (Fig. 1B), and an HA/NA expression plasmid (Fig. 1C). At 72 h post-transfection, pps were harvested by passage through a 0.45-μm Durapore polyvinylidene fluoride (PVDF) membrane filter (Millipore, New Bedford, Massachusetts, USA) (Fig. 1D). For quantification, purified pps were treated with 0.24 U/mL DNase and RNase at 37 °C for 1 h to eliminate any contaminating DNA and RNA, and then frozen at −70 °C to inactivate the DNase and RNase. The pps were then treated with proteinase K (Beyotime Biotechnology, Nantong, Jiangsu, China) at 50 °C for 30 min to digest the envelope proteins and release the CMV–GFP RNA. The proteinase K was inactivated at 100 °C for 3 min. The amount of CMV–GFP RNA in each pp was quantified by real-time quantitative reverse-transcription (qRT)-PCR using the forward primer 5’-AACAAAAGCTGGAGCTCGTTTAA-3’, the reverse primer 5’-GGGTCTCCTCAGAGTGATTGACTAC-3’, and the probe 5’- FAM-CCCCCAAATGAAAGACCCCCGAG-TAM -3’, where FAM is the fluorescent dye 6-carboxy-fluorescein and TAM is a non-fluorescent quencher. The assay was carried out on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with a Luna Universal Probe One-Step RT-qPCR Kit (NEB, USA). Pps were normalized for RNA copy number before infectivity, hemagglutination, and NA activity assays, and western blot analysis of the HA and NA expression and incorporation.

2.5. Infection assay

The pps infection assay was performed as reported previously (Lin et al., 2009; Wu et al., 2010). The HA0s of naive H7N9 virus and the chimeric combinations virus Anhui H7 + Anhui N1 require maturation via TPCK trypsin cleavage to enable formation of its functional subunits HA1 and HA2. Briefly, target cells (5–10 × 103) were seeded in a 96-well plate one day prior to infection. At 72 h post-infection, the infected cells were rinsed twice with PBS and digested into cell suspension with 0.25% EDTA-trypsin. The number of GFP reporter-positive cells was determined by FACS (BD FACSAria, BD Biosciences, Franklin Lakes, NJ).

2.6. Hemagglutination assay

To determine the hemagglutinating activity of the pps, a hemagglutination assay was performed (n = 3). Briefly, 50 μL of PBS (pH = 7.2) was added to all wells of the micro titer plates. In the first column, 50 μL of the pp sample was mixed and serially diluted by transferring 50 μL from the first well to the successive well until the penultimate column; in the last column (the control), virus was not added. Guinea pig (0.75%), turkey (0.5%), chicken (0.5%), horse (1%), sheep (1%), and human (O-type, 1%) working RBC suspensions were washed three times and prepared in phosphate-buffered saline (PBS, pH 7.2). Then, 50 μL of the RBC suspension was added to the corresponding wells on the plate. The plate was incubated at room temperature. Titers were recorded after 30 min for avian RBCs, 45 min for guinea pig, and one hour for horse, sheep, and human O-type RBCs. Hemagglutination units were expressed as the reciprocal of the maximum dilution of virus that resulted in complete agglutination.

2.7. NA activity detection

To detect the level of NA activity in each HA and NA combination, an NA activity assay was performed using the Neuraminidase Assay kit (Beyotime Biotechnology), according to the manufacturer’s instructions. Briefly, 10 μL of pps were added to the reaction buffer; then, MilliQ H2O was added with controls set in parallel. The fluorescence substrate was added, and the fluorescence intensity was measured by excitation at 322 nm and recording the emission at 450 nm using a Synergy H4 Hybrid Microplate Reader (Bio-TEK, Winooski, Vermont, USA).

2.8. HA-Receptor binding assay

The receptor binding preference was analyzed by a solid-phase direct binding assay as previously described (Chandrasekaran et al., 2008), using a sialylglycopolymer containing N-acetylneuraminic acid linked to galactose through either an α2,3 or an α2,6 bond (Neu5Acα2-

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