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Hemagglutinin pseudotyped lentiviral particles: Characterization of a new method for avian H5N1 influenza sero-diagnosis

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Abstract

Background: Highly pathogenic avian influenza (HPAI) H5N1 has spread globally in birds and infected over 270 humans with an apparently high mortality rate. Serologic studies to determine the extent of asymptomatic H5N1 infection in humans and other mammals and to investigate the immunogenicity of current H5N1 vaccine candidates have been hampered by the biosafety requirements needed for H5N1 micro-neutralization tests.
Objective: Development of a serodiagnostic tool for highly pathogenic influenza that reproduces H5N1 biology but can be used with less biohazard.
Study Design: We have generated and evaluated H5 hemagglutinin pseudotyped lentiviral particles encoding the luciferase reporter (H5pp).
Results: H5pp entry into target cells depends on α2-3 cell surface sialic acids and requires low pH for membrane fusion. H5pp infectivity is specifically neutralized by sera from patients and animals infected with H5N1 and correlates well with conventional microneutralization test.
Conclusions: H5pp reproduce H5N1 influenza virus entry into target cells and potentially provides a high-throughput and safe method for sero-epidemiology.
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Keywords: Influenza; Pseudotyped lentiviral particles; H5N1; Serodiagnostic

1. Introduction

The global spread of highly pathogenic avian influenza A (HPAI) H5N1 viruses in poultry and its transmission to humans poses a pandemic threat. Since 2003 there have been 270 human cases with over 160 deaths (http://www.who.int/csr/disease/avian_influenza/country). Binding of influenza virus to cellular receptors is determined by the viral hemagglutinin. The HA1 subunit of hemagglutinin binds to terminal sialic acids of glycoproteins and glycolipids at the cell surface (Skehel and Wiley, 2000; Skehel and Wiley, 2002). Avian influenza viruses preferentially bind α2-3-linked sialic acids (SA) (Skehel et al., 1982; Skehel et al., 1983, Russell et al., 2006) while human influenza viruses preferentially recognize α2-6-linked SA. Subsequent virus

Abbreviations: HI, hemagglutination inhibition; HA, hemagglutinin; H5, hemagglutinin of H5N1; H5pp, H5 pseudotyped viral particles; AMLV, amphotrophic envelope of the murine leukemia virus; sNA, soluble recombinant neuraminidase from *Vibrio cholerae*; SNA, *Sambucus Nigra* (elderberry) Bark lectin; MAA, *Maackia amurensis* lectin II; HPAI, highly pathogenic avian influenza; SA, sialic acids

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entry and uncoating is dependent on low pH (Skehel and Wiley, 2000). During the final stage of the virus life cycle the HA binds to the SA receptor requiring the enzymatic activity of the neuraminidase for the release of the viruses from the cell surface (Dong et al., 1992).

Twelve percent of household contacts of confirmed H5N1 patients during the H5N1 outbreak in Hong Kong in 1997 had neutralizing antibodies against the H5N1 (Katz et al., 1999). However, seroepidemiologic studies during the recent H5N1 outbreaks indicate a low transmission rate of H5N1 virus to humans in spite of extensive exposure to infected poultry (Vong et al., 2006). At present, microneutralization tests confirmed by Western blot assays are the gold standard for detection of anti-H5 specific antibodies in humans (Rowe et al., 1999). However, H5N1 microneutralization tests require BSL-3 containment which precludes such studies in many affected countries. The conventional haemagglutination inhibition (HI) test is not suitable for serodiagnosis of avian H5N1 infections in humans (Rowe et al., 1999). The modified HI test using horse erythrocytes (Stephenson et al., 2003) is currently under evaluation but is not a functional assay for inhibition of entry and is not amenable to high throughput testing.

Pseudotyped lentiviral particles expressing heterologous viral glycoproteins have been described for several viruses including Hepatitis C (Bartosch et al., 2003), SARS (Simmons et al., 2004) and the avian influenza virus H7N7 (Duisit et al., 2002). The principle of production is schematically presented in Fig. 1A. H5 pseudotyped particles (H5pp) are capable only of a single-round infection and do not produce progeny virus, and can therefore be produced under lower biosafety requirements than the wild-type virus. Here we describe a system to produce lentiviral particles pseudotyped with the H5 hemagglutinin envelope (H5pp) isolated from a patient in Cambodia in 2005. H5pp show similar entry characteristics with respect to receptor usage, pH requirement and neutralization compared to the wild type H5N1. Our data indicate that H5pp will be a useful in serodiagnostic assays and analysis of cellular pathways of H5N1 entry.

2. Experimental procedures

2.1. Production and purification of H5pp

The pseudotyped particles production and sucrose gradient purification were performed as described previously (Lozach et al., 2004). Briefly, HEK293T cells were transfected with pNL Luc E^- R^- and pCDNA-synH5 (for luciferase) or pcHMWS-EGFP, pCDNA-synH5 and pCMV-dR8.91 (for eGFP) and grown in the presence of soluble *Vibrio cholerae* neuraminidase (sNA) (6.2 mU/ml; Roche). Supernatant was harvested 24 h post-transfection, filtered and concentrated and particles were titrated in an infectivity assay using luciferase and the HIV p24 as readout. For sucrose purification, 200 ng of p24 equivalent of H5pp were centrifuged overnight over a 20–60% discontinuous sucrose gradient. Fractions of 600 μl were taken and pelleted in the

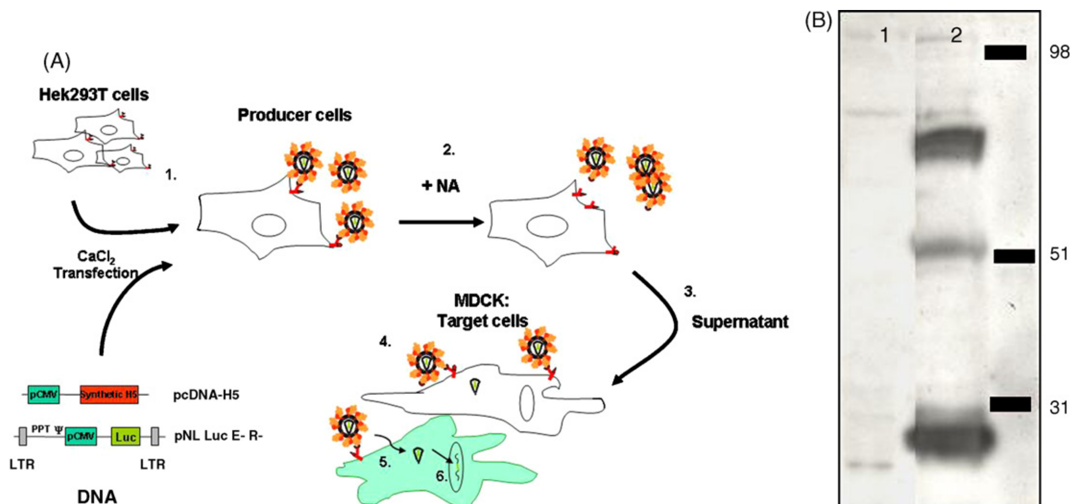

Fig. 1. (A) Production of lentiviral particles pseudotypes with a synthetic H5 envelope protein. 1. HEK293 T cells ("producer cells") are transfected with provirus expressing the luciferase or eGFP reporter gene driven by the CMV promoter and a plasmid coding for the synthetic H5 gene. Double transfected cells secrete pseudotyped viruses containing the proviral RNA surrounded by the HIV core and a lipid bilayer containing the H5 hemagglutinin. 2. After transfection, sNA was added to the medium to release the particles that were bound to the SA present on the cell surface of the producer cells. 3. The supernatant containing the secreted pseudotyped viruses is harvested and incubated with the target cells. 4. The H5 pp interact with the SA receptor followed by endocytosis and HA2-mediated fusion in the endosome. 5. After endocytosis, the HIV nucleopcapsid is released into the cytoplasm. 6. The viral mRNA is reverse transcribed into DNA and imported into the nucleus where it will be integrated into the DNA of the host cell. Reporter gene expression can be detected by a luciferase assay or FACS analysis for the luciferase or GFP gene, respectively. (B) Expression of H5 in producer cells. A Western blot was performed on cell lysates from the HEK293T producer cells. The blot was then stained with a human sera against H5N1 (TH001). Lane 1: Non-transfected HEK293T cells; lane 2: HEK293T cells transfected with pNL Luc E^- R^- and pCDNA-synH5.

presence of 0.05% BSA. The pellet was dissolved in 75 μl DMEM; 1/10 was used to infect MDCK cells and 1/5 was used to perform a Western blot as described (Lozach et al., 2004). Immunostaining was performed with an anti-H5N1 duck serum, a human anti-H5 serum (TH001) or an anti-p24 (Abcam, ab9044).

2.2. Neutralization assay

MDCK cells (4000 cells/well) were seeded in 96-well plates in 100 μl of DMEM. 10^4 RLU of H5pp were incubated with twofold serial dilutions of serum (starting dilution 1:20) for 1 h at 37 °C (CO_2 incubator) in 60 μl total DMEM. Subsequently, 100 μl of fresh medium was added and 140 μl of the virus–antibody mixture was transferred to the cells. The luciferase assay was performed 48 h later by direct addition of Steady-Glow Luciferase substrate (Promega). Sera were scored positive when they inhibited the H5pp infectivity $\geq$80% at serum dilutions $\geq$1/20.

2.3. Lectin staining

Huh7 cells were treated with 0.025 U/ml sNA (Roche) for the indicated periods of times. They were harvested by Accutase (Sigma) treatment, washed and distributed over a 96-well plate. Cells were incubated with Fluorescein SNA (Vector laboratories, 10 μg/ml), Biotinylated MAA II (Vector laboratories, 20 μg/ml) or PBS containing 1% BSA (1 h, 4 °C) and washed three times. When needed, secondary staining was performed with Streptavidin-FITC (BD, 25 μg/ml) prior to analysis by FACScan.

2.4. Sera

Human sera from confirmed H5N1 patients were provided by Institut Pasteur Cambodia and the University of Hong Kong. Sera from H5N1 vaccinated volunteers, collected at the 28 day post second dose of the vaccine (Treanor et al., 2006) were provided by the National Institute of Allergy and Infectious Diseases (Rockville, MD). Avian sera were provided by Institut Pasteur Cambodia and by Robert Webster (St. Jude Children's Research Hospital, Memphis, TN). Serostatus for mammalian sera was confirmed by microneutralization tests and for avian sera by haemagglutination inhibition tests (WHO, 2002; OIE, 2004).

3. Results

3.1. Production of infectious lentiviral H5 pseudotyped particles (H5pp)

The HA of A/Cambodia/408008/05 (H5N1) virus was used. Sequence analysis confirmed that this is a clade 1 H5N1 virus with no known mutations relevant for receptor binding including E190 and G225, suggesting that the H5 protein retained the binding characteristics for α2,3-linked SA. The HA sequence was codon-optimized (Geneart, Germany), cloned into a eukaryotic expression vector and protein expression verified after transfection into HEK293T cells by Western blot (Fig. 1B). Three main bands were seen which is consistent with the expected molecular weight for the uncleaved HA0 and the cleaved subunits HA1 and HA2.

H5pp were generated as described in Fig. 1A. Fig. 2A shows that H5 pseudotyped but not non-enveloped particles (data not shown) yielded a luciferase signal in MDCK cells. Addition of sNA during the production of H5pp increased the infectivity by more than 1 log (Fig. 2A). FACS and microscopy analysis show that H5pp infectivity could also be observed when using a different reporter gene (eGFP) (Fig. 2B). Electron microscopy confirmed that viral particles with an average size of 130 nm were generated in transfected cells and could be visualized at the plasma membrane (Fig. 2C).

To confirm that the luciferase signal was due to H5pp, concentrated supernatants of producer cell lines were separated over a sucrose gradient and fractions tested for infectivity in MDCK cells and for the presence of HA and p24 antigens. The peak of infectivity, H5 and p24 protein detection was detected in the same fractions 10–16 (Fig. 3A, B and C).

In addition to MDCK cells, Huh7, 293T, BHK, Vero, HeLa and J774 cells were also susceptible to H5pp infection (Fig. 4A) which is in accordance with previous reports (Matlin et al., 1981; Kaverin and Klenk, 1995; Kaverin and Webster, 1995; Govorkova et al., 1996; Schultz-Cherry et al., 1998; Kuiken et al., 2003; Rimmelzwaan et al., 2003).

3.2. H5pp reproduce key steps of H5N1 entry mechanism

Removal of the sialic acids from the cell surface by preincubation of cells with sNA treatment, decreased infection (Fig. 4A). *Sambucus Nigra* lectin (SNA) was used to label α2-6-linked SA and *Maackia amurensis* lectin II (MAA) to detect α2-3-linked SA. sNA treatment decreased the expression of both α2-3-linked and α2-6-linked SA, but with different kinetics. Treatment for 30 min diminished the expression of α2-3-linked SA (Fig. 4B grey bars), while the α2-6-linked SA were not affected until more prolonged incubation times (Fig. 4B black bars). The reduction of the infection upon sNA treatment closely correlates with the expression level of α2-3-linked SA. A 30 min pre-incubation period with sNA resulted in a decrease in infection (Fig. 4B white bars) while longer incubation times did not further decrease the levels of infection.

Our results show that infectivity by H5pp, but not AMLVpp was greatly reduced by NH_4Cl treatment which inhibits acidification of the endosomes in a dose-dependent manner (Fig. 5). Similar results were obtained with Bafilomycin A, another inhibitor of endosomal acidification (data not shown). Altogether our results show that H5pp uses α2-3-linked SA as receptor and infect cells in a pH-dependent mechanism.

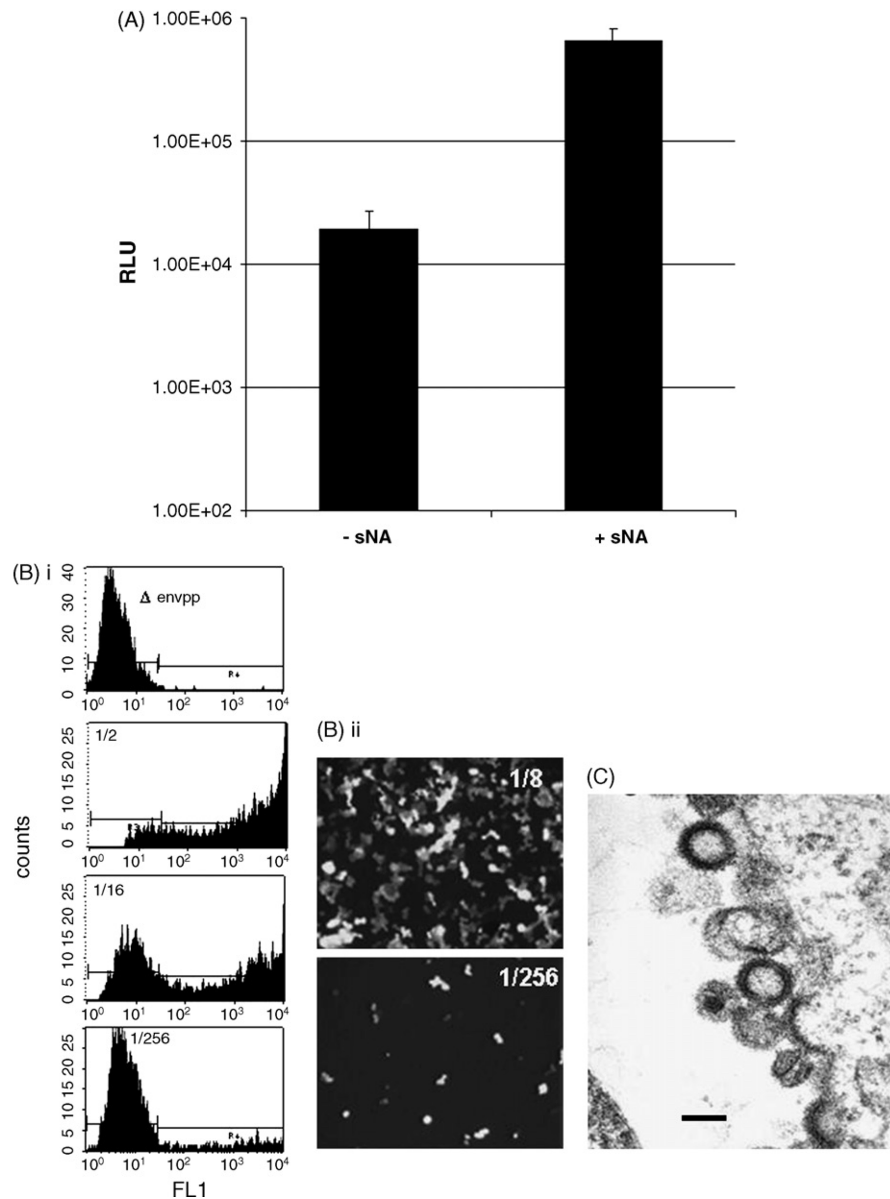

Fig. 2. (A) The yield of H5 pp in the supernatant is increased in the presence of neuraminidase (sNA). sNA was added to the HEK293T producer cell line after transfection. 24 h later, the supernantant was harvested, filtered and incubated with Huh7 cells. The yield is expressed in relative luciferase units (RLU). (B) (i) FACS analysis of MDCK cells infected with H5pp expressing the eGFP reporter gene. Supernatant of the producer cells were harvested and concentrated 100 times over a sucrose cushion. MDCK were incubated with Δenvpp or different dilutions of H5pp. Incubation of the MDCK cells with a 2-fold, 16-fold or 256-fold dilutions of the H5pp resulted in 92.3%, 55.16% or 7% eGFP-positive cells, respectively; (ii) fluorescence images of infected cells. (C) EM image of 293T producer cells with budding H5pp. The bar represents 120 nm.

3.3. H5pp are neutralized by patient sera

Initial neutralization experiments showed that a convalescent serum from a H5N1 infected patient (TH001) reduced H5pp infectivity to background levels while infectivity of VSV-Gpp was not affected (Fig. 6A). A serum taken from a another H5N1 patient (p0302295) at day 10 after the onset of disease was less potent in neutralizing H5pp but still inhibited infectivity of H5pp. We extended these findings by analyzing the neutralizing capacity of additional human and avian sera in the H5pp neutralizing assay (Fig. 6 and Tables 1A and 1B).

15/15 sera from confirmed H5N1 patients, 5/5 post-H5N1 human vaccinee sera and 2/227 human sera from healthy controls inhibited H5pp infectivity (sensitivity 100%; specificity 99%). The two false positive human sera were from persons >65 years of age. Two other human sera (#260, elderly and #195 child) had indeterminate neutralizing activity. 12/12 confirmed H5 seropositive avian sera and 0/41 seronegative avian sera neutralized H5pp. 8/10 sera from H5 vaccinated chicken and ducks tested positive. The two negative sera were also negative in conventional microneutralization tests.

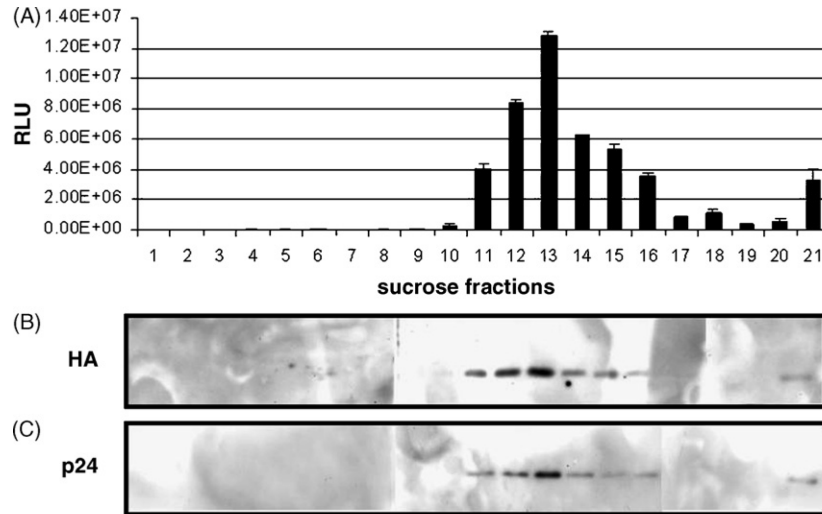

Fig. 3. Secreted infectious particles containing H5 protein and p24 proteins can be purified on a sucrose gradient. Concentrated supernatant was loaded on a 20–60% sucrose gradient. Fractions were analyzed for (A) infectivity (luciferase activity); (B) HA expression; and (C) p24 expression. Infectivity, HA and P24 are detected in the same fractions 10 to 16.

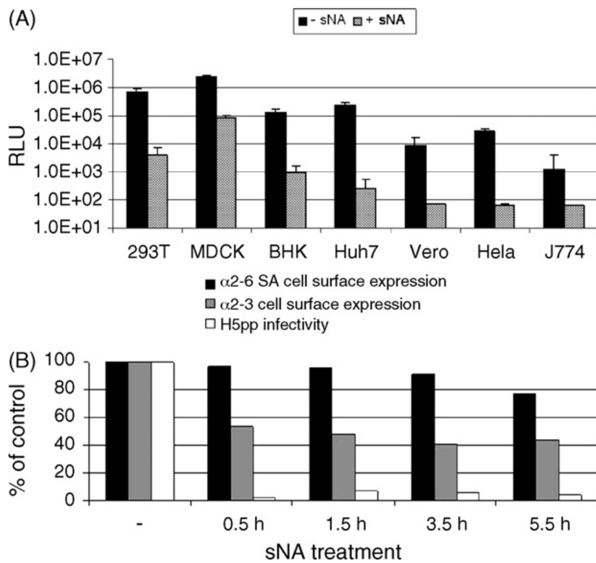

Fig. 4. (A) Different cell types are permissive for H5pp and the entry is dependent on sialic acid. Cells were pretreated with medium containing 0.025 U/ml sNA for 1 h at 37 °C. Then, the cells were incubated with concentrated H5pp in the presence or absence of sNA. Infection is expressed in relative luciferase units (RLU). (B) Infection with H5pp depends on expression of α2-3-linked sialic acids. Huh7 cells were pre-incubated for various time periods with sNA (0.025 U/ml) prior to infection with H5pp. At the time of infection, SNA and MAA staining was performed to determine α2-6-linked and α2-3-linked sialic acid (SA) expression, respectively. All data are expressed in percentage compared to the no treatment control, which is considered 100%.

4. Discussion

We have developed lentiviral vectors expressing H5 hemagglutinin (H5pp). H5pp entry requires α2,3-linked SA and low endosomal pH and can be abrogated by sera containing H5N1-specific antibodies. Selective removal of cell surface α2,3-linked SA (Ada et al., 1961; Tomlinson et al., 1992) reduces H5pp infection highlighting that H5pp are an ideal tool to study influenza cell tropism such as the role of critical HA residues in receptor usage. H5pp encode a reporter gene which allows for high throughput analyses under lower biosafety requirements than wild type H5N1.

Using a novel H5pp neutralization assay, we detected neutralizing antibodies in sera from previously confirmed avian and human H5N1 cases. We have observed a tight correlation

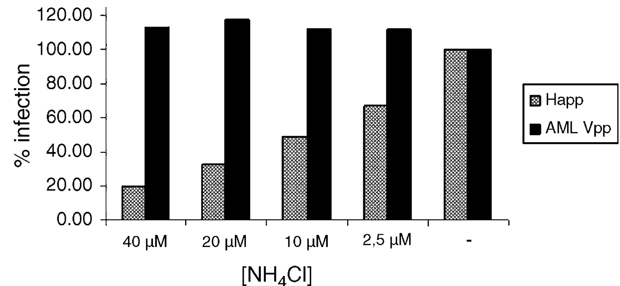

Fig. 5. H5pp entry is pH-dependent. Cells were pre-treated for 30 min with medium containing the indicated concentrations of NH_4Cl prior to infection with H5pp or AMLVpp in the presence of NH_4Cl. Infectivity was measured by luciferase expression and are expressed as present of control (untreated cells).

Table 1A
Overview of the human sera tested in the H5pp neutralization test

H5N1 Status	# Positive / # Tested
H5N1 confirmed patients	15/15
H5N1 vaccinated volunteers	5/5
Healthy controls – ≤18 years	0/106
Healthy controls – young adults	0/20
Healthy controls – ≥65 years	2/121

The criterion for H5pp positive is ≥80% reduction of mfectivity at a serum dilution of ≥1/20.

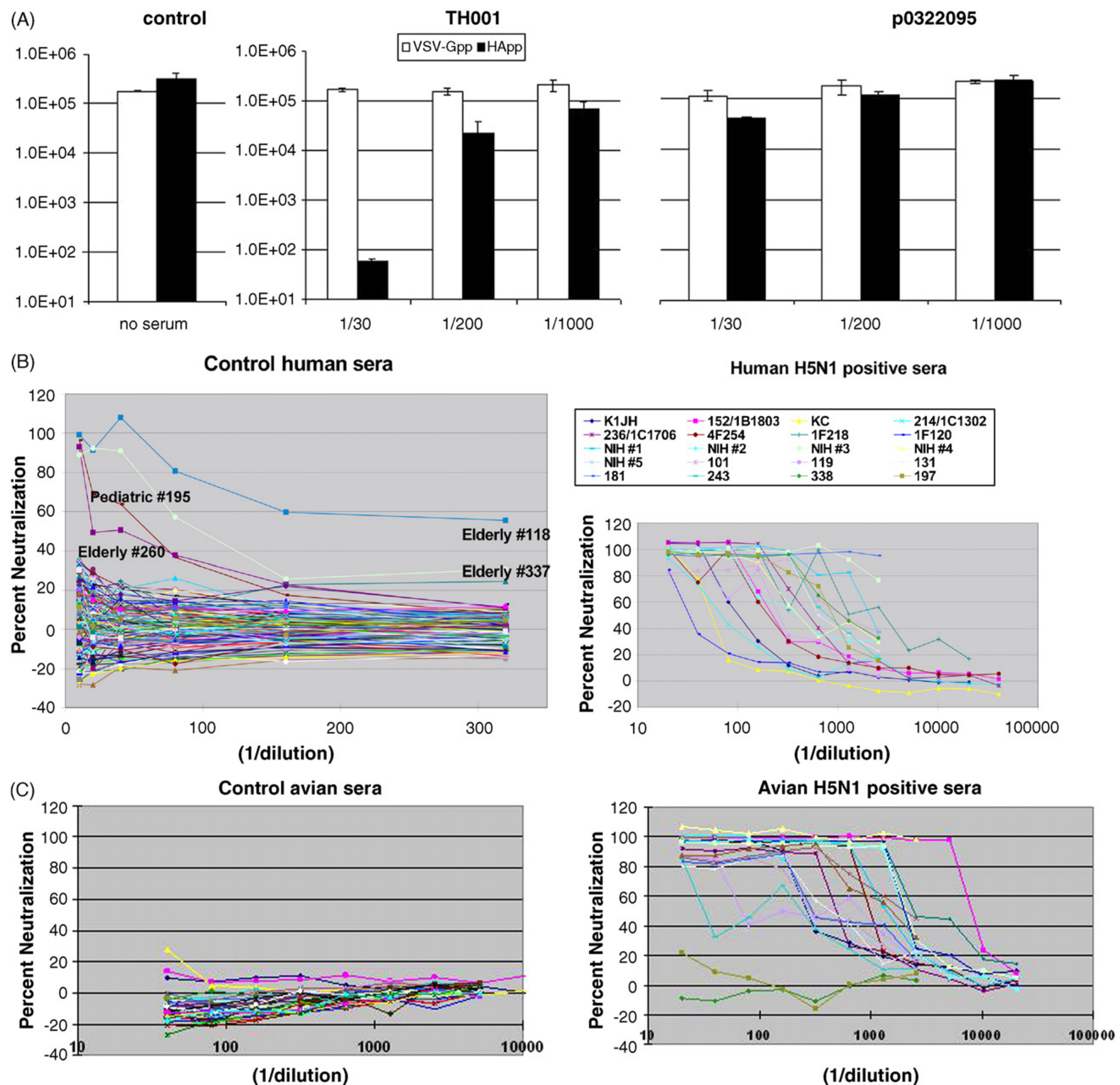

Fig. 6. (A) Infection with H5pp, but not VSV-G are neutralized by pre-incubation of H5pp with sera from H5N1 infected patients. Infectivity of pseudotyped viral particles incubated with indicated dilutions of the sera for 1 h at 37 °C is visualized. The yield is expressed in relative luciferase units (RLU). (B) Sera from H5N1 infected patients and vaccinated persons neutralize the infection of H5pp. Infectivity of pseudotyped viral particles incubated with indicated dilutions of the sera for 1 h at 37 °C is visualized. The luciferase expression is presented as percent neutralization compared to no serum control (= 0% neutralization). NIH #1-#5 are sera from H5N1 vaccinated persons. (C) Sera from H5N1 infected and vaccinated poultry neutralize the infection of H5pp. Infectivity of pseudotyped viral particles incubated with indicated dilutions of the sera for 1 h at 37 °C is visualized. The luciferase expression is presented as percentage neutralization compared to no serum control (= 0% neutralization).

Table 1B
Overview of the avian sera tested in the H5pp neutralization test

H5N1 status	# Positive / # Tested
H5N1 positive sera[a]	12/12
H5N1 negative sera[a]	0/41
H5N1 vaccinated chicken or ducks	8/10[b]

The criterion for H5pp positive is ≥80% reduction of mfectivity at a serum dilution of ≥1/20.

[a] Based on hemagglutination inhibition and micro-neutralization test.

[b] The two negative sera were also negative on micro-neutralization tests.

of the H5pp neutralization assay with characterized positive and negative human and avian (Tables 1A and 1B). The two false positive sera were detected in the H5pp assay came from elderly persons and it was previously reported that false positive results in the H5 microneutraliztion test are also more common in the elderly (Rowe et al., 1999). While a more comprehensive clinical and epidemiological evaluation is needed, our results indicate the H5pp neutralization assay is a novel approach that can be used for large-scale H5 seroprevalence

studies on human and animal sera. The advantages over existing techniques for detection of H5N1 neutralizing antibodies include easy and flexible read-out, handling under BSL2 condition and the requirement of small quantities of serum. The system is suited for automated high throughput screening in a 96-well plate or 384-well plate format. The H5pp particles can be produced from synthetic codon optimized DNA without access to the live H5N1 virus or cloned viral genes. The flexibility of the system encourages the development of a multiplex assay where different subtypes or clades of influenza viruses can be screened simultaneously. In conclusion, the neutralization assay presented here might prove a valuable tool in global efforts to increase the surveillance of influenza viruses in both animals and humans.

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