Short Communication

H7N9 influenza viruses interact preferentially with α2,3-linked sialic acids and bind weakly to α2,6-linked sialic acids

Irene Ramos,[1] Florian Krammer,[1] Rong Hai,[1] Domingo Aguilera,[1] Dabeiba Bernal-Rubio,[1] John Steel,[4] Adolfo García-Sastre[1,2,3] and Ana Fernandez-Sesma[1,3]

Correspondence
Irene Ramos
irene.ramos-lopez@mssm.edu
Ana Fernandez-Sesma
ana.sesma@mssm.edu

[1]Department of Microbiology, Icahn School of Medicine at Mount Sinai, 1468 Madison Avenue New York, NY 10029, USA

[2]Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, 1468 Madison Avenue New York, NY 10029, USA

[3]Division of Infectious Diseases, Department of Medicine, Icahn School of Medicine at Mount Sinai, 1468 Madison Avenue New York, NY 10029, USA

[4]Department of Microbiology and Immunology, Emory University School of Medicine, GA 30322, USA

The recent human outbreak of H7N9 avian influenza A virus has caused worldwide concerns. Receptor binding specificity is critical for viral pathogenicity, and still not thoroughly studied for this emerging virus. Here, we evaluated the receptor specificity of the haemagglutinin (HA) of two human H7N9 isolates (A/Shanghai/1/13 and A/Anhui/1/13) through a solid-phase binding assay and a flow cytometry-based assay. In addition, we compared it with those from several HAs from human and avian influenza viruses. We observed that the HAs from the novel H7 isolates strongly interacted with α2,3-linked sialic acids. Importantly, they also showed low levels of binding to α2,6-linked sialic acids, but significantly higher than other avian H7s.

As of 7 June 2013, a total of 132 laboratory-confirmed cases of human infection with avian influenza A virus (IAV) H7N9, including 32 deaths have been reported to the World Health Organization (WHO, 2013). Although the number of cases greatly increased during the month of April 2013, there is still no evidence of human-to-human transmission so far (WHO, 2013). Thus, the increased cases in humans suggested better transmission from avian sources to humans than in the case of other avian viruses.

Some receptor restrictions for avian influenza viruses in human airways account for the reduced ability of avian strains to establish infections in humans (Kuiken *et al.*, 2006; Matrosovich *et al.*, 2004; Neumann & Kawaoka, 2006; Nicholls *et al.*, 2007; Peiris *et al.*, 2007; van Riel *et al.*, 2006). The capacity of the IAV to infect birds or humans is partially defined by the binding specificity of the haemagglutinin (HA), the major glycoprotein on the influenza virus surface. In general, HAs from human strains preferentially bind sialic acids attached through an α2,6 linkage to the terminal galactose (SAα2,6) of the oligosaccharides on the cell surface. These types of linkages are predominant in the respiratory epithelia in the upper respiratory tract of humans and other mammals such as ferrets (Shinya *et al.*, 2006). In contrast, the HA of avian

strains bind preferentially to α2,3-linked sialic acids (SAα2,3), which are abundant in the avian intestinal tract and are also present in the human lower respiratory tract (Nicholls *et al.*, 2007; Pillai & Lee, 2010). Binding to SAα2,6 receptors is one of the requirements for efficient replication in the human upper respiratory tract which facilitates respiratory droplet-based transmission. Efficient human-to-human transmission of emerging influenza viruses by respiratory droplets is a prerequisite for rapid spread throughout the human population, and animal viruses with this ability pose a major pandemic threat potential. Analysis of the first three available HA sequences from the novel Chinese H7N9 viruses (A/Shanghai/1/13, A/Shanghai/2/13 and A/Anhui1/13) indicated the presence of amino acid residues in the receptor binding site (RBS) that are associated with enhanced binding of H5 and H7 HAs to SAα2,6. The A/Shanghai/1/13 HA possesses an A138S (H3 numbering) mutation (Table 1), which has been reported to confer SAα2,6 binding ability to an Indonesian H5N1 swine isolate (Nidom *et al.*, 2010). Usually, alanine is conserved in this position in avian viruses and the presence of a serine could indicate adaptation to the human host in this virus (Kageyama *et al.*, 2013; Nidom *et al.*, 2010). However, this mutation is

Table 1. Mutations associated with a change in receptor specificity of novel Chinese H7N9 human and avian isolates and amino acids in those corresponding positions in the two H7s (in bold) included in the receptor binding characterization

Strain name	aa 138	N158 glycosylation site	aa 186	aa 226
A/Shanghai/1/2013	S	No	G	Q
A/Shanghai/2/2013*	A	No	V	L
A/Shanghai/3/2013	A	No	V	L
A/Shanghai/4/2013*	A	No	V	L
A/Shanghai/4664T/2013	A	No	V	L
A/Anhui/1/2013	A	No	V	L
A/Fujian/1/2013*	A	No	V	L
A/Hangzhou/1/2013	A	No	V	I
A/Hangzhou/2/2013	A	No	V	L
A/Hangzhou/3/2013	A	No	V	L
A/Zhejiang/1/2013†	A	No	V	I
A/Zhejiang/2/2013*	A	No	V	L
A/Zhejiang/DTID-ZJU01/2013*	A	No	V	L
A/pigeon/Shanghai/S1069/2013	A	No	V	L
A/chicken/Shanghai/S1053/2013*	A	No	V	L
A/chicken/Jiangsu/K27/2013‡	A	No	V	L
A/chicken/Jiangsu/K89/2013*	A	No	V	L
A/chicken/Zhejiang/DTID-ZJU0/2013	A	No	V	Q
A/environment/Shanghai/S1088/2013*	A	No	V	L
A/environment/Hangzhou/34/2013	A	No	V	L

*Identical to A/Anhui/1/13.
†Identical to A/Hangzhou/1/13.
‡Identical to A/Hangzhou/3/13.

only present in the A/Shanghai/1/13 isolate and was not detected in any of the later H7N9 isolates [Global Initiative on Sharing All Influenza Data (GISAID) database, 16 May 2013]. HAs of A/Anhui/1/13 and A/Shanghai/2/13 possess two mutations that are also associated with increased binding to SAα2,6. G186V as well as Q226L are described to enhance SAα2,6 binding in H7 IAV (Belser et al., 2008; Srinivasan et al., 2013; Yang et al., 2010). L226 in combination with S228 plays a synergic role on SAα2,6 binding of human H3N2 isolates and introduction of these two mutations into H7 HAs led to increased SAα2,6 binding as well (Srinivasan et al., 2013). However, all novel Chinese H7N9 isolates so far possess a G228. It is of note that two human isolates have acquired an isoleucine in position 226 whereas A/Shanghai/1/13 and a chicken isolate show the avian prototypic glutamine at this position (Table 1). G186V and Q226L are found in almost all Chinese H7N9 isolates so far (Table 1), including viruses of human, chicken, pigeon and environmental origin (Table 1). The presence of abundant SAα2,6 receptors has been demonstrated in chickens and quail (Guo et al., 2007; Wan & Perez, 2006), and avian viruses with increased binding to SAα2,6 have been isolated from chickens (Matrosovich et al., 2001; Pawar et al., 2012; Watanabe et al., 2011). Therefore, the G186V and Q226L mutations could represent adaptations to chicken (or quail) rather than adaptions to the human host. Further, an H7 isolate possessing an E186 and exhibiting binding to SAα2,6 was

unable to transmit via respiratory droplets in the ferret model (Belser et al., 2008). In addition to the mentioned mutations all Chinese H7N9 isolates possess the T160A substitution which leads to the loss of a glycosylation site and has been associated with increased SAα2,6 binding activity in an H5N1 isolate (Gao et al., 2013; Kageyama et al., 2013; Wang et al., 2010). However, this mutation is present in most recent H7 isolates from both the American as well as the Eurasian lineage, and many of these HAs do not exhibit binding to SAα2,6 (Figs 1 and 2). A recent transmission study with A/Shanghai/2/13 (HA identical to A/Anhui/1/13) indeed showed very limited transmission from ferret to ferret via aerosol droplets (one animal out of three) and no transmission from pigs to ferrets in the same setting (Zhu et al., 2013).

In order to shed light on the receptor binding specificity of the novel Chinese H7N9 isolates, we aimed to investigate the binding of A/Shanghai/1/13 and A/Anhui/1/13 HA to SAα2,3 and SAα2,6 substrates. The amino acid sequence of the HA of A/Shanghai/1/13 is so far unique; the sequence of the A/Anhui/1/13 HA, however, is identical to the sequence of A/Shanghai/2/13 and to that of many other isolates (Table 1). The study was performed using a solid-phase binding assay as well as a flow cytometry-based assay as previously described (Ramos et al., 2011).

For the solid-phase binding assay, we used recombinant HAs derived from H7N9 strains, and European or North

American lineage H7 viruses (A/mallard/Netherlands/12/2000 H7N3, A/rhea/North Carolina/39482/1993 H7N1 and A/chicken/Jalisco/12283/2012 H7N3), as well as human H5N1, H1N1 and H3N2 strains (Fig. 1a). In order to generate the recombinant HAs, we cloned the HA sequence in frame into a modified pFastBac baculo transfer vector (Invitrogen) that features a C-terminal T4 trimerization domain and a hexahistidine tag and expressed recombinant protein in the baculovirus expression system as described previously (Goff *et al.*, 2013; Krammer *et al.*, 2012). For the solid-phase binding assay, 96-well ELISA plates were coated with the specific HA at 20 µg ml^{-1}. After blocking and washing, we added the polyacrylamide (PAA)–biotin-conjugated glycans Neu5Acα2,3Galβ1,4GlcNAc-PAA (3′SLN-PAA) and Neu5Acα2,6Galβ1,4GlcNAc-PAA (6′SLN-PAA), provided by the Consortium of Functional Glycomics, at the specified concentrations. Then, we incubated the plates with streptavidin–HRP (R&D Systems), developed the assay with the substrate *o*-phenylenediamine (Invitrogen) and determined the absorbance at 450 nm with a microplate reader (BioTek).

As shown in Fig. 1(b), all the H7 tested, including those corresponding to either human or avian strains, showed clear preference for binding to SAα2,3, as indicated by the high levels of absorbance upon incubation with 3′SLN-PAA. In the case of H5/Vietnam/04, as previously described by us and others (Ramos *et al.*, 2011; Stevens *et al.*, 2006; Yamada *et al.*, 2006), we also observed a strong binding preference for SAα2,3 residues. In contrast, the H1 and H3 HAs from human viruses, as expected, showed preferential binding to 6′SLN-PAA. When we compared the relative binding for SAα2,3 of the several H7s included in this study, we observed that the HA corresponding to those viruses isolated from human cases of avian influenza (H7/Shanghai/13, H7/Anhui/13) bound with less affinity to 3′SLN-PAA than the avian isolates tested (H7/mallard/00, H7/rhea/93 or H7/chicken/12) (Fig. 1c). Interestingly, the H7 from human H7N9 isolates showed low levels of binding to the SAα2,6 substrate, which were significantly higher than the ones detected in the case of the H7N1 and H7N3 avian isolates (Fig. 1c).

In order to confirm these results, we studied the glycan binding properties of these two H7N9 viruses using a flow cytometry binding assay as described previously (Ramos *et al.*, 2011). Briefly, Madin–Darby canine kidney (MDCK) epithelial cells were infected at an m.o.i. of 5 with the recombinant viruses indicated in Fig. 2(a). At 20 h post-infection, the cells were harvested, washed with cold PBS, and incubated with 20 µg ml^{-1} of the biotinylated glycans 3′SLN-PAA or 6′SLN-PAA, and anti-M2 antibody E10 (Mount Sinai Hybridoma Shared Research Facility) (Tan *et al.*, 2012) for 1 h at 4 °C. The cells were then washed with PBS containing 1% BSA and incubated with streptavidin–FITC (Jackson Immunoresearch) and secondary anti-mouse rhodamine antibody (Jackson Immunoresearch). GS4071 (the free carboxylate of oseltamivir) was added during both incubations to avoid cleavage of the sialic acids of the synthetic polymers. Flow cytometry was

performed using a FACScan flow cytometer (Becton Dickinson) and analysed with FlowJo software.

The recombinant viruses were generated by reverse genetics as described previously (Fodor *et al.*, 1999). They expressed the HA and neuraminidase (NA) or only HA (as specified in Fig. 2a) from the indicated viruses and the rest of the genes from A/Puerto Rico/8/1934 (PR8). First, we analysed the percentage of infected cells by gating the population of cells positive for anti-M2 staining (Fig. 2b). We observed a high percentage of infected cells in all cases, ranging between 62.95 and 95.72% of the total cells. Next, we evaluated the proportion of cells that bound to the SAα2,3 or SAα2,6 biotinylated glycans within the population of M2-positive cells, which express also the HA of interest on their surface. Additionally, we evaluated the mean fluorescence intensity (MFI) of the 3′/6′SLN-PAA positive population (Fig. 2b). In this case, we observed that the H7/Shanghai/13 HA bound similarly to the HA from H7N1 and H7N3 avian isolates (H7/rhea/93 and H7/chicken/12) and the H5 from H5/Vietnam/04 to the SAα2,3 substrate (values >92% binding cells and MFI >131.01). In all these cases, virtually no binding to the SAα2,6 substrate (<2% binding cells) was detected. However, whilst H7/Anhui/13 HA also showed strong binding to the SAα2,3 glycan (82% binding cells, MFI 131.01), we could observe some binding to SAα2,6 (12.20% binding cells, MFI 52.83), which was higher than for the rest of IAVs included in the assay. Importantly, although these data clearly indicated some affinity for the human-like receptor SAα2,6 for H7/Anhui/13 HA, the interacting capacity was significantly lower than the H1N1 human isolates H1/Brisbane/07 and H1/California/09 (Fig. 2b).
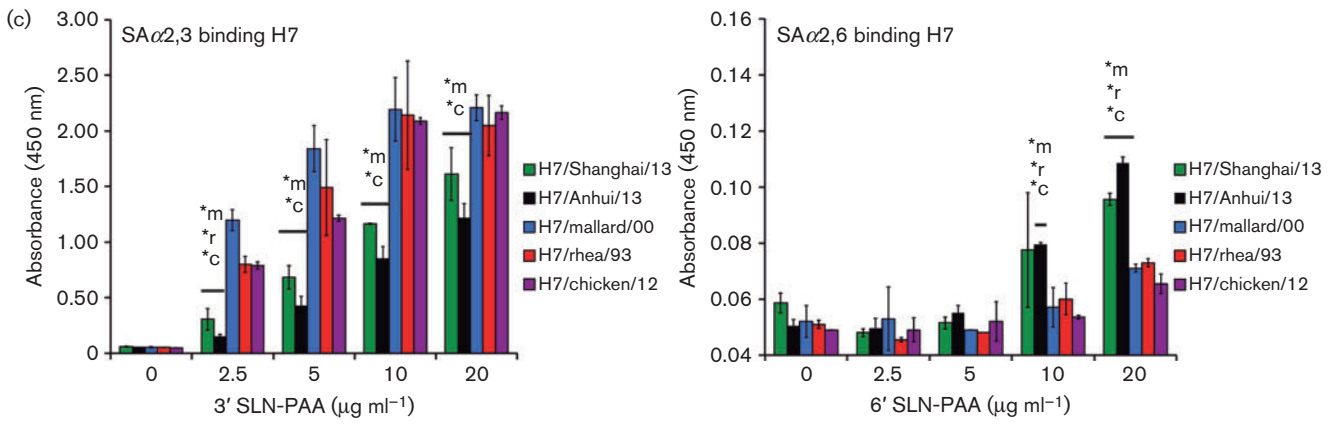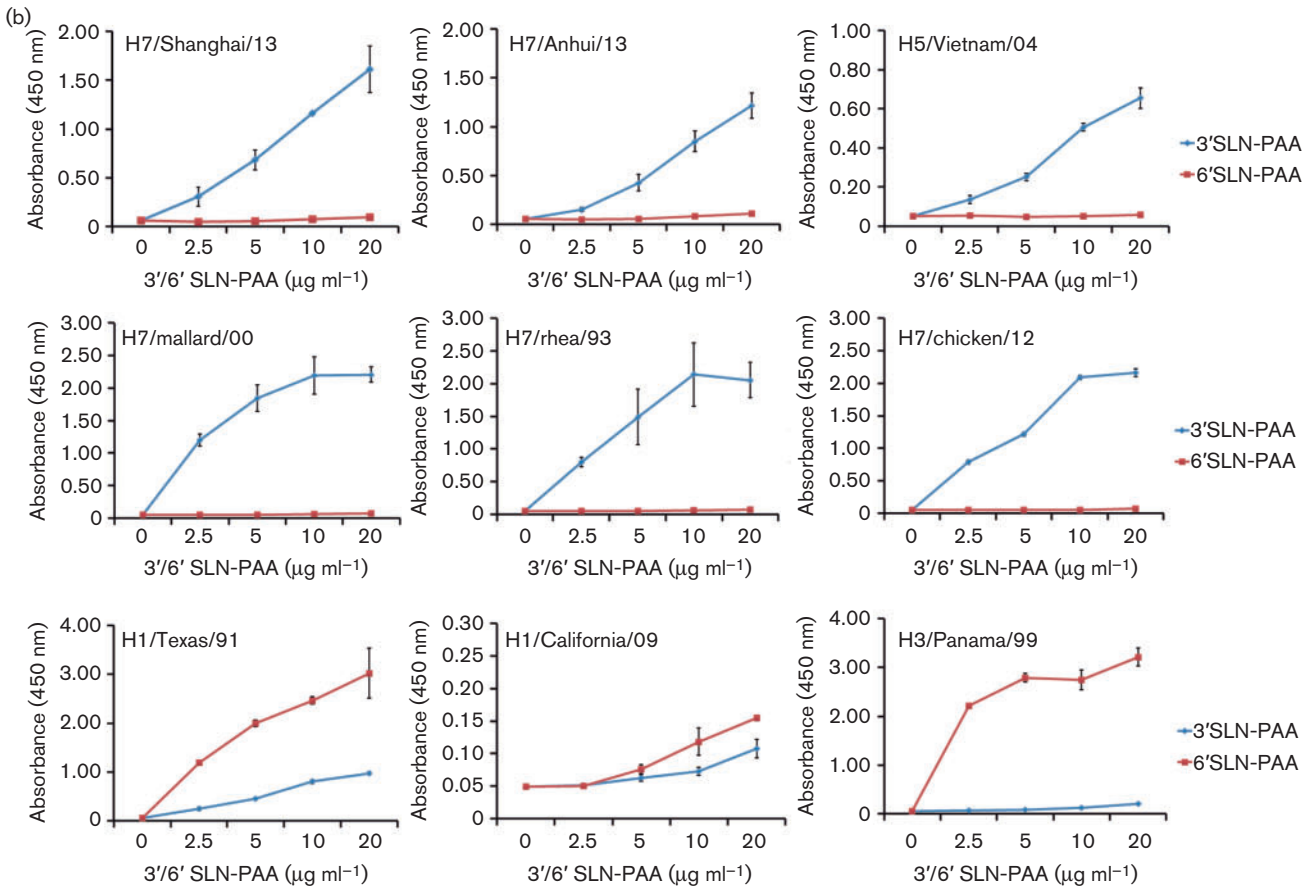
Overall, the data from both assays indicated that, whilst strong affinity for SAα2,3 residues is maintained in the novel H7N9 IAV, mutations A138S in the HA of A/Shanghai/1/13, and G186V and Q226L in HA of A/Anhui/1/13 might be responsible for the increased interaction for SAα2,6 as compared with other H7 avian viruses, facilitating adaptation of this novel strain to the human host. Surprisingly, while using the solid-phase binding assay, we detected some levels of binding to the SAα2,6 glycan for H7/Shanghai/13 HA, but this was not the case when we used the flow cytometry-based assay. These differences could be due to the different setting in which the HA is allowed to interact with the sialylated substrate, since in one case recombinant HA was used and in the other case HA was expressed on the surface of infected cells. Therefore, the use of both systems combined indicates that, whilst the HA from both H7N9 isolates weakly interact with SAα2,6, this interaction is slightly stronger for H7/Anhui/13. Of interest, mutations G186V and Q226L in HA of H7/Anhui/13 are also present in most of the H7N9 viruses isolated from human, avian or environmental sources (Table 1; sequences were obtained from the GISAID).

These data are in accordance with two recent reports that evaluated the receptor binding of the HA from the human
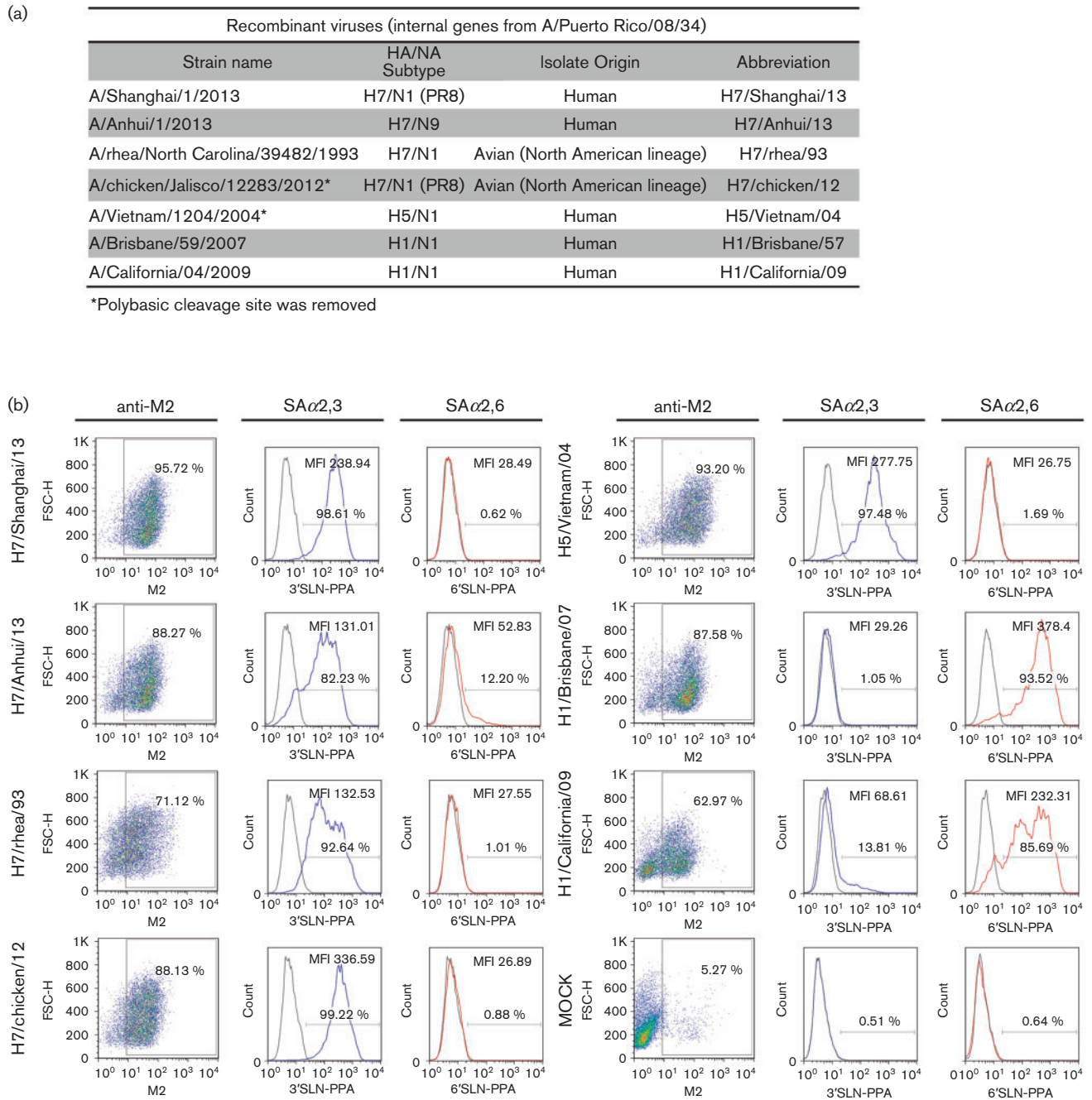
(a)

Recombinant HA proteins			
Strain name	HA Subtype	Isolate Origin	Abbreviation
A/Shanghai/1/2013	H7	Human	H7/Shanghai/13
A/Anhui/1/2013	H7	Human	H7/Anhui/13
A/mallard/Netherlands/12/2000	H7	Avian (Eurasian lineage)	H7/mallard/00
A/rhea/North Carolina/39482/1993	H7	Avian (North American lineage)	H7/rhea/93
A/chicken/Jalisco/12283/2012*	H7	Avian (North American lineage)	H7/chicken/12
A/Vietnam/1204/2004*	H5	Human	H7/Vietnam/04
A/Texas/36/1991	H1	Human	H1/Texas/91
A/California/04/2009	H1	Human	H1/California/09
A/Panama/2007/1999	H3	Human	H3/Panama/99

*Polybasic cleavage site was removed

(b)

(c)

(a)

Recombinant viruses (internal genes from A/Puerto Rico/08/34)			
Strain name	HA/NA Subtype	Isolate Origin	Abbreviation
A/Shanghai/1/2013	H7/N1 (PR8)	Human	H7/Shanghai/13
A/Anhui/1/2013	H7/N9	Human	H7/Anhui/13
A/rhea/North Carolina/39482/1993	H7/N1	Avian (North American lineage)	H7/rhea/93
A/chicken/Jalisco/12283/2012*	H7/N1 (PR8)	Avian (North American lineage)	H7/chicken/12
A/Vietnam/1204/2004*	H5/N1	Human	H5/Vietnam/04
A/Brisbane/59/2007	H1/N1	Human	H1/Brisbane/57
A/California/04/2009	H1/N1	Human	H1/California/09

*Polybasic cleavage site was removed

(b)

Fig. 2. Receptor binding specificity of HAs using a flow cytometry-based binding assay. (a) Recombinant influenza A viruses included in the assay. (b) Percentages of infected cells (anti-M2 antibody-positive cells) are shown in the dot plots. Positive M2 cells were gated (except in the mock-infected samples, where we gated the total cell population) and the percentage of cells binding to 3′SLN-PAA or to 6′SLN-PAA, as well as the mean of fluorescence intensity (MFI) of the binding cells, was calculated (indicated in each histogram).

H7N9 to the same sialyl-glycans using two different assays from the ones used in our study (Xiong *et al.*, 2013; Zhou *et al.*, 2013). Xiong *et al.* (2013) compared the receptor specificity of A/Anhui/1/13 and an avian H7N3 using biolayer interferometry. Similarly to our observations, they reported that the human H7N9 showed a lower avidity for SAα2,3 and a higher avidity for SAα2,6 than the avian H7N3, whilst retaining the SAα2,3 binding preference. Zhou *et al.* (2013) obtained results consistent with ours using a similar experimental setting to the first one used in this study. They used a solid-phase binding assay, where they coated ELISA plates with different concentrations of the sialyl-glycans and detected virus binding with anti-HA antibodies. Using this assay, they also reported binding of the H7N9 viruses to SAα2,6 (which is higher in the case of A/Anhui/1/13 than A/Shanghai/1/13), but in both cases this binding to SAα2,6 was lower than the strong binding reported for both viruses to SAα2,3 substrates.

Further analysis of the interaction of the H7N9 virus with sialyl-receptors using glycan arrays has also been recently reported (Belser *et al.*, 2013; Watanabe *et al.*, 2013). Whilst our studies as well as the ones reported by Xiong *et al.* (2013) and Zhou *et al.* (2013) provide quantitative information using a limited number of polymers, glycan arrays are a powerful technology to elucidate the qualitative range of glycans that interact with the influenza virus HA. Using these assays, Belser *et al.* (2013) showed that A/Shanghai/1/13 bound to a broader array of SAα2,3 than SAα2,6, whilst A/Anhui/1/13 showed mixed binding to SAα2,3 and SAα2,6 glycans. Watanabe *et al.* (2013), however, found mixed binding to SAα2,3 and SAα2,6 in the case of A/Shanghai/1/13, and a clear reduction in binding to SAα2,3 and an increased interaction with the same type of SAα2,6 using a human H1N1 virus. Interestingly, when they performed this assay in the presence of a neuraminidase inhibitor, they observed a clear increase in the number of SAα2,3 glycans, indicating that their previous observations might reflect the combined activities of the HA and NA proteins.

Our data are also consistent with a recent report that showed weak binding of A/Anhui/1/13 HA to tissue samples from the human respiratory tract (Tharakaraman *et al.*, 2013). Additionally, transmission experiments in ferrets and pigs, two of the most established models to evaluate possible adaptation to airborne transmission in humans, demonstrated limited aerosol transmission in ferrets and no transmission in pigs (Watanabe *et al.*, 2013; Zhu *et al.*, 2013).

In summary, we analysed the receptor binding specificity of the novel H7N9 influenza virus from two human isolates. Whilst these H7 HAs showed weak binding for SAα2,6 as compared with H7 HAs from other non-H7N9 avian isolates that do not possess the mutations associated with a change in receptor specificity (Table 1), they bound strongly to SAα2,3. This low but significant binding affinity to SAα2,6 might have facilitated avian-to-human trans-mission of the virus (Han *et al.*, 2013). However, based on

data from other H7 strains (Belser *et al.*, 2008) that exhibit a similar SAα2,6 binding activity and from transmission studies conducted with the novel H7N9 strains in ferrets (Zhu *et al.*, 2013), we would argue that this virus is not able to transmit readily from human to human via aerosol transmission. This notion is supported by recent data from Chinese laboratories, which tested 20 739 human cases of upper respiratory tract infection in March and April 2013 for the presence of H7N9 and found only six positives, suggesting that, so far, there is no spread of this novel virus in the local population (Xu *et al.*, 2013).

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