Monoclonal antibodies isolated from human B cells neutralize a broad range of H1 subtype influenza A viruses including swine-origin Influenza virus (S-OIV)

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ABSTRACT

The new H1N1 swine-origin influenza virus (S-OIV) strain is a global health problem. The elucidation of the virus–host relationship is crucial for the control of the new infection. Two human monoclonal antibody Fab fragments (HMabs) neutralizing the novel H1N1 influenza strain at very low concentrations were cloned before the emergence of S-OIV from a patient who had a broad-range H1N1 serum neutralizing activity. The two HMabs neutralized all tested H1N1 strains, including S-OIV and a swine strain with IC50 ranging from 2 to 7 μg/ml. Data demonstrate that infection with previously circulating H1N1 strains can elicit antibodies neutralizing S-OIV. Finally, the human genes coding for the neutralizing HMabs could be used for generating full human monoclonal IgGs that can be safely administered being potentially useful in the prophylaxis and the treatment of this human infection.

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Introduction

Recently, a new strain of human H1N1 influenza A virus (Swine-origin influenza virus; S-OIV) was identified (Fraser et al., 2009a). As of September 11th, 2009, the virus has spread to many countries with more than 277,607 cases and 3205 deaths. These figures, together with the fact that the new virus has been assessed as having pandemic potential, make this new infection a serious concern for the global health(Fraser et al., 2009b; Trifonov et al., 2009b). The elucidation of the interplay between the new influenza virus and the human host, mainly the identification of S-OIV antigenic regions that are able to elicit broad-range and neutralizing antibodies, is crucial for the understanding of the epidemiology of this disease.

Indeed, human monoclonal antibodies with broad-range neutralizing activity against S-OIV can be key reagents in this scenario, as they can be used to probe in vitro the vaccine candidates and providing useful information for understanding data generated by preliminary in vivo studies(Parren et al., 1996). Furthermore, human monoclonal antibodies neutralizing influenza viruses are molecules potentially useful in prophylaxis and therapy (Simmons et al., 2007; Trifonov et al., 2009b). Even if antibody-based prophylaxis and therapy of influenza is at present an unexplored option, polyclonal and monoclonal human antibodies are effectively used in a number of different viral infections (Sawyer, 2000). It has been demonstrated that anti-influenza mouse and human monoclonal antibodies are effective in prophylaxis and therapy in mice (Palladino et al., 1995; Renegar et al., 2004; Smirnov et al., 2000), and passive immunization by vertical transmission of anti-influenza antibodies is protective against infection both in animal models and in human subjects (Luke et al., 2006; Martinez, Tsibane, and Basler, 2009; Prabakaran et al., 2009; Prabhu et al., 2009; Puck et al., 1980; Reuman et al., 1983; Simmons et al., 2007; Sui et al., 2009; Sun et al., 2009; Sweet et al., 1987a, 1987b; Throsby et al., 2006; Yoshida et al., 2009; Yu et al., 2008). All these observations indicate that passive antibody administration could be a potentially useful adjunctive treatment and preventive option in the S-OIV infection.

In this paper, we describe the molecular cloning, the binding characteristics, and the broad-range neutralizing activity of two human monoclonal antibody Fab fragments (HMab) directed against S-OIV hemagglutinin (S-HA). These antibodies, cloned from a patient before the emergence of the S-OIV strain, demonstrate that exposure to previous H1N1 strains elicits the production of S-OIV-neutralizing antibodies. The data presented here document not only that previous influenza H1N1 infections can provide a certain degree of protection, but also that some epidemiological features of the S-OIV infection as well as some unexplained results of the preliminary in vivo vaccine trials can be clarified at the molecular level.

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Results

Two distinct HMabs, named PN-SIA28 and PN-SIA49, were cloned from a patient exposed to previously circulating H1N1 influenza A strains, with a negative clinical history for influenza infection in the last years and with a detectable serum neutralizing activity against a 1934 influenza A isolate.

![Image](image_url)

Fig. 1. Immunofluorescence on A/Milan/UHSR1/2009-infected MDCK cells or HA-transfected cells. MDCK cells stained after 8 h of infection with HMabs PN-SIA28 (a) and PN-SIA49 (b, c [100 magnification]), mouse anti-NP1 (d), the negative control Fab509 (e) and a double stain with HMabs PN-SIA28 and PN-SIA49 of mock infected cells (f). HEK293T cells transfected with recombinant A/Puerto Rico/8/1934 HA expression vector and stained with positive control M145 Monoclonal antibody (g) HMabs PN-SIA28 (h) and PN-SIA49 (i). HEK293T cells transfected with recombinant A/Milan/UHSR1/2009 (S-OIV) HA expression vector and stained with positive control M145 Monoclonal antibody (l), HMabs PN-SIA28 (m) and PN-SIA49 (n). HEK293T cells transfected with recombinant A/South Carolina/1/18 HA expression vector and stained with positive control M145 Monoclonal antibody (o), HMabs PN-SIA28 (p) and PN-SIA49 (q). Mock-transfected HEK293T cells stained with control M145 monoclonal antibody (r). HMabs PN-SIA28 (s) and PN-SIA49 (t).
immunofluorescence staining on infected cells, both HMsabs featured a clear cytoplasmic pattern with plasma membrane reinforcement (Fig. 1) against S-OIV and all tested influenza A strains. No differences were observed among cells infected with the different isolates. Notably, cells transfected with the recombinant hemagglutinin (HA) genes of A/ Puerto Rico/8/1934, A/Milan/UHSR1/2009 and A/South Carolina/1/18 were recognized by both HMbA (Fig. 1), thus suggesting that both PN-SIA28 and PN-SIA49 bind to extremely conserved antigenic epitopes that are present in all H1 HA's we analyzed.

The epitope recognized by both antibodies is conformational as evaluated by Western blot analysis and present only in the HA0 form of hemagglutinin. In fact, no binding was observed under denaturing conditions and nor HA1 or HA2 subunits were separately recognized by PN-SIA28 and PN-SIA49 (Fig. 2).

PN-SIA28 and PN-SIA49 showed a strong neutralization activity against S-OIV and all other H1N1 strains used in the study. When neutralization activity was evaluated with immunofluorescence reduction assay PN-SIA49 featured IC50 (Fab concentration giving 50% of neutralization) ranging from 2.1 µg/ml against A/H1N1/PR/8/34 to 6.9 µg/ml against A/NC/20/99. PN-SIA49 neutralized S-OIV strain (A/Milan/UHSR1/2009) with an IC50 of 2.8 µg/ml. PN-SIA28 was demonstrated to have an IC50 ranging from 3.0 µg/ml against A/H1N1/WS/33 to 7.0 µg/ml against A/H1N1/NC/20/99. PN-SIA28 neutralized S-OIV strain with an IC50 of 4.0 µg/ml. No neutralizing activity was demonstrated for both Mabs against the B/Lee/40 influenza B strain nor against A/Victoria/3/75 H3N2 strain. (Table 1, Fig. 3). Similar results were obtained with plaque reduction assay (Fig. 4). When both Mabs were tested against H5N1 influenza viruses, no reductions of EID50 values were observed compared with untreated viral inoculums (A/turkey/Turkey/1/2005: negative control: 105.5 EID50/100µl, PN-SIA28: 106.82 EID50/100µl, PN-SIA49: 107.6 EID50/100µl; A/chicken/Egypt/A6/2008: negative control: 106.24 EID50/100µl, PN-SIA28: 106.7 EID50/100µl, PN-SIA49: 107.2 EID50/100µl). DNA sequence of the light and heavy variable regions demonstrated that the two HMsabs are different. Genbank accession numbers of the heavy and light sequences of PN-SIA28 and PN-SIA49 are GQ867592 and GQ867595; GQ867593 and GQ867594, respectively. PN-SIA28 and PN-SIA49 VH genes were V3-30 and V3-23, respectively. Both antibodies were mutated with respect to the germline encoded VH gene sequences (94.62% and 92.36% identity, respectively) (Fig. 5).

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>IC50 PN-SIA28</th>
<th>IC50 PN-SIA49</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/PR/8/34</td>
<td>4.1 µg/ml</td>
<td>2.1 µg/ml</td>
</tr>
<tr>
<td>A/WS/33</td>
<td>2.9 µg/ml</td>
<td>4.5 µg/ml</td>
</tr>
<tr>
<td>A/Mal/302/54</td>
<td>3.4 µg/ml</td>
<td>2.7 µg/ml</td>
</tr>
<tr>
<td>A/NC/20/99</td>
<td>7.0 µg/ml</td>
<td>6.9 µg/ml</td>
</tr>
<tr>
<td>A/swine/Parma/1/97</td>
<td>3.9 µg/ml</td>
<td>2.3 µg/ml</td>
</tr>
<tr>
<td>A/Milan/UHSR1/2009 (S-OIV)</td>
<td>4.0 µg/ml</td>
<td>2.8 µg/ml</td>
</tr>
</tbody>
</table>

### Discussion

Pandemic influenza viruses have emerged at least three times in the last 100 years with the most severe being the 1918 H1N1 Spanish influenza. The risk of an influenza pandemic caused by the newly emerged S-OIV strain is real, and the consequences of this event are difficult to estimate but could be catastrophic. Even if the pathogenic potential of the novel strains appears lower than that of the 1918 H1N1 Influenza virus, the possibility of a further evolution of the pathogen cannot be excluded (Lipsitch et al., 2009). The understanding of the role played by immunity elicited by previously circulating viruses, a better knowledge of the virus–host interplay, and more importantly, the availability of adjunctive novel tools for preventing and treating this disease may be crucial in contrasting a possible pandemic.

The molecular cloning of an unexposed patient of human monoclonal antibodies, endowed with strong neutralizing activity against S-OIV and against a broad range of human and swine H1N1 isolates, is the clear demonstration that some conserved antigenic determinants present in a wide range of H1N1 viruses are also present in the S-OIV strain. The two Mabs are displaced in competition experiments by the mouse Mab C179 (Takara), and they compete the one with the other (data not shown). This indicates that the two epitopes recognized by the two HMsabs described in this paper are at least overlapping and situated in the stem region of the HA molecule.

The presence of antibodies neutralizing a wide range of H1N1 influenza isolates (including S-OIV) in the antibody repertoire elicited by infection with previously circulating strains can explain the milder clinical syndrome and the lower rate of this infection observed in older subjects (Trifonov et al., 2009a, 2009b). Itoh et al. (2009) have shown that only sera of those born before 1918 have some neutralizing activity against S-OIV, suggesting a close serological relation between the novel strain with the 1918 pandemic strain but not with the antigenically divergent human H1N1 viruses circulating in the 1920s to 1950s and since 1977. This is apparently in contrast with our observation, as our antibodies are derived from the repertoire of a patient born well after 1918. However, neutralization assays performed with polyclonal sera cannot fully dissect the humoral immune response and it is likely that only the minority of antibodies elicited after the infection is endowed with cross-neutralizing activity, being the majority directed against more antigenic and variable viral antigens.

Furthermore, recently published data on the preliminary vaccine trials (Galli et al., 2009; Hancock et al., 2009) noted an unanticipated robust immune response to the H1N1 vaccine after a single dose administration even in patients with no measurable antibodies against S-OIV at the baseline. This unexpected brisk response to the vaccination can be explained by an immunotypic similarity not yet recognized between the 2009 H1N1 virus and recent seasonal strains. The data presented in this paper are providing a molecular evidence supporting this hypothesis, being important in the current time-limited run for the quest of an effective vaccination strategy and schedule.

Finally, these HMsabs have the potential of being useful as adjunctive tools in treatment and prophylaxis in the unfortunate case of a S-OIV pandemic. In a mouse model of H5N1 influenza infection, neutralizing human monoclonal antibodies were proven to
Fig. 4. Plaque inhibition assay. Sigmoidal dose-response curve fit nonlinear regression is reported for PN-SIA28 and PN-SIA49 against all viral strains studied in this paper.
be extremely efficient in conferring a significant immunity to mice, causing a milder disease and strongly reducing virus dissemination (Simmons et al., 2007). Therapy and prophylaxis of S-OIV infection by administration of human monoclonal antibodies is a plausible strategy. Several reports related to the 1918 “Spanish” pandemic indicated as early treatment with blood product from recovered patients was associated with a strong reduction in mortality, which was more evident in the case of an early treatment (Luke et al., 2006), with the speculation that the administration of neutralizing antibodies present in the blood-derived products favorably modified the virus–host balance causing a milder disease and preventing the development of respiratory complications (Luke et al., 2006). Administration of human monoclonal antibodies neutralizing S-OIV has the potential of having the same effect. The IC50 values of PN-SIA28 and PN-SIA49 Fab are in the micromolar range (2.1–7.0 μg/ml), comparable to those described previously by Throsby et al. (IC50 ranging from 0.55 to 15 μg/ml) (Throsby et al., 2008) or remarkably lower than those described by Sui (IC50 in the order of 10 μg/ml) (Sui et al., 2009). It should be noted that both works described the neutralizing activities of recombinant full IgGs while our HMabs are in the Fab form, and usually the neutralization capacity of Fabs strongly increases once expressed in IgG form (Lamarre and Talbot, 1995).

Finally, even if most of the currently circulating S-OIV strains are sensitive to mono-therapy with Oseltamivir and Zanamivir, in the absence of additional drugs they will probably give rise to resistant strains, as recently observed in these pandemics in Denmark, Japan, and Hong Kong (http://www.who.int/csr/disease/swineflu/notes/h1n1_antiviral_resistance_20090708/en/index.html) and as already happened with the vast majority of seasonal H1N1 that have now become resistant (Shetty, 2009). In the current situation, not knowing in advance the efficacy and of an anti-S-OIV vaccine, having an alternative path open to the scientific community could be important. Human monoclonal antibody mass production is feasible, scalable, the final product is free of adventitious agents and it is utilizing well-known procedures that are used for many drugs. Finally, given the fact that these antibodies are of human origin, the risk of unwanted effects is minimal.

In summary, the human monoclonal antibodies neutralizing the pandemic H1N1 S-OIV strain can be useful tools for the understanding of the disease, for the understanding of vaccine trial data in the shortest possible time, and can constitute the basis, alone or in a combination with other monoclonal antibodies, for a new class of drugs to be used in the treatment and in the prophylaxis of this disease.

Materials and methods

This work was approved by the San Raffaele ethical committee, and all human samples were collected after informed consent was obtained.

Human monoclonal antibodies

A patient, of the age of 55, with a negative clinical history of influenza virus in the past 10 years, and with a serum neutralizing titer (IC50 > 1:20) against a reference H1N1 1934 strain (A/PR/8/34), was used as a source of lymphocytes 3 weeks after a routine influenza seasonal vaccination and after written informed consent was given. This procedure was performed well before the emergence of the novel S-OIV strain. Fifteen B-cell lines producing antibodies reacting in immunofluorescence with influenza A-infected cells were obtained by Epstein-Barr Virus (EBV) transformation (Cole et al., 1984), and subsequently cDNA coding for Fab fragments were PCR amplified and cloned in appropriate bacterial expression vector (Burioni et al., 1997, 1998a) for avoiding instability of antibody...
production of EBV-transformed cell lines and for DNA sequencing. Transformed bacteria were used for production of the recombinant Fabs (Burioni et al., 1994; Perotti et al., 2008), which were purified as previously described (Burioni et al., 1998b). Only two of the Fabs were demonstrated to be endowed with neutralizing activity. An anti-hepatitis C virus Fab, named e509 (Burioni et al., 1998b), produced and purified with an identical procedure, was used as a negative control in all experiments. Mutations identified by comparing each sequence with germline sequences (International ImMunoGeneTics database at http://imgt.cines.fr/) were defined on the basis of nucleotide changes in the VH segment.

Isolation and identification of influenza A/Milan/UHSR1/2009 strain

All experiments were conducted in the BL53 laboratory of the Università Vita-Salute San Raffaele. The swine origin influenza virus (S-OIV) used in this study was isolated from the pharyngeal swab of a 26-year-old Italian patient of our hospital referring fever and malaise after a recent travel to the United States. The swab was seeded on 80% confluent MDCK (Madin-Darby canine kidney) cells (ATCC no. CCL-34TM). The cells were infected in modified Eagle medium (MEM, Gibco) with the addition of 2 μg/ml of trypsin. After 1 h, 10% fetal bovine serum (GIBCO), 50 μg/ml of penicillin (GIBCO), 100 μg/ml of streptomycin (GIBCO) and of l-glutamine (2 mM) (EuroClone) were added and cells were incubated at 35 °C, in 5% CO2 atmosphere for 5 days. Identification was performed directly on patient’s swab sample and on culture supernatant by whole length amplification and sequencing of the S-HA gene by using a previously described RT-PCR protocol (Puthavathana et al., 2005) with minor modifications (Raek et al., 2009). Primer forward was Bm-HA-1-Fw 5′-TAT TCG TCT CAG GGA GCA AAA GCA GGG G-3′, primer reverse was Bm-NS-890-Rev 5′-ATA TCG TCT CTT ATT AGT AGA AAC AAG GGT GTT TT-3′. The reaction was performed using the SuperScript III One-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen) and the following thermal profile: 30 min 50 °C; 10 min 94 °C; 30 s 94 °C, 1 min 53 °C, 1 min 72 °C (45 cycles). Sequencing was performed by using BigDye Terminators 3.1 with the automatic sequencer AbiPrism3130 (Applied Biosystems); the primers used in the amplification are: F1 5′-TAG GAA ACC CAG AAT GGC-3′; F2 5′-TAC TGG ACC TTG CTA GAA-3′; F3 5′-TCT ATT TGG AGC CAT TGC-3′.

Other influenza strains of human, swine or animal origin used in this study

The following reference strains were used: A/Puerto Rico/8/34 (A/PR/8/34); A/Wilson-Smith/33 (A/WS/33); A/Malaya/302/54 (A/Mal/302/54); A/New Caledonia/20/99 (A/NC/20/99). A swine strain (A/swine/Parma/1/97) was kindly provided by the Istituto Zooprofylattico di Brescia, Brescia, Italy. The influenza B strain B/Lee/40 and the influenza A/Victoria/3/75 were also tested in all studies. The avian A/chicken/Egypt/Ab/2008 and A/turkey/Turkey/1/2005 influenza A (H5N1) strains were also tested. All viruses, but A/swine/Parma/1/97, were cultured on MDCK cells propagated in MEM (GIBCO), supplemented with 10% fetal bovine serum (GIBCO), 50 μg/ml of penicillin (Gibco), 100 μg/ml of streptomycin (Gibco) and of l-glutamine (2 mM) (EuroClone). The A/swine/PR/1/97 isolate was analogously grown on a different, more permissive cell line (NSK-newborn swine kidney), kindly provided by the Istituto Zooprofylattico di Brescia. The cells were infected with each strain at 80% confluence, after addition to the medium of 1 μg/ml of trypsin. After the infection, the cells were incubated at 35 °C, in 5% CO2 atmosphere.

Immunofluorescence assays

Seven hours after infection, the MDCK cells were trypsinized, washed twice in PBS and spotted on a slide by cytocentrifugation (2 × 106 cells in each spot). The cells were then fixed and permeabilized by cold methanol/acetic (−20 °C) for 10 min at room temperature. After three washes in PBS, the cells were incubated for 30 min at 37 °C with each one of the HMabs (10 μg/ml). The cells were then washed again in PBS and incubated for 30 min at 37 °C with a fluoresceinated anti-human Fab monoclonal diluted in Evans Blue. Uninfected cells were used as control. Commercially available anti-NP protein mouse monoclonal (Anti-influenza A group, Argene, cod 11-030) was used as an infection control and as negative control e509Fab was used on infected cells. The same protocol was used to stain HEK 293T cells 48 h after transfection, but Hoechst (SIGMA) was used for counterstaining. M145 Monoclonal Anti-Human Influenza A (clone C179, Takara) directed against a conformational epitope of the viral hemagglutinin was used as positive control in transfection experiments.

Transfection of 293T cells with A/Puerto Rico/8/1934, A/South Carolina/1/18 and A/Milan/UHSR1/2009 strain hemagglutinin genes

The full-length HA gene of A/Puerto Rico/8/1934 was amplified with the above-described protocol and directly cloned into the pcDNA3.1 expression vector (Invitrogen). In particular, the HA gene was amplified by using the following pair of primers APrB34 CAC CAT GAA GGC AAA CCT GCT GGT ATG TG and APrB34 asbestos C ATG GCA TAT TCT GCA CTG CAA AGA TCC ATT AGA and directly cloned following manufacturer’s instructions. Both the A/Milan/UHSR1/2009 and the A/South Carolina/1/18 HA genes were artificially synthesized (Geneart, AG, Germany) flanking the full-length HA sequence with the recognition sites of Hind III and AgeI restriction enzymes at 5′ and 3′ extremities, respectively. Each artificially synthesized HA gene was subcloned from the shuttle vector supplied by the manufacturer (Geneart, AG, Germany) into the pcDNA3.1 expression vector after digestion with Hind III and AgeI enzymes. HEK 293T cells were transfected with lipofectamine 2000 reagent (Invitrogen) with the recombinant HA expression vectors following the manufacturer’s instructions. Forty-eight hours after transfection, cells were collected, washed twice with PBS and spotted on a slide by cytocentrifugation. Immunofluorescence was performed as described above.

Western blot

A Western blot assay was performed on lysates obtained from HEK293T cells transfected or mock transfected with A/Milan/UHSR1/2009 (S-OIV) HA expression vector. Ten microliters of lysates obtained by mechanical homogenization in NativePAGE™ Sample Buffer (Invitrogen), were loaded on a 10% polyacrylamide gel. After running, samples were transferred on a nitrocellulose membrane (Hybond-ECL; Amersham Bioscience) overnight at 30 V and 90 mA. The membrane was then blocked with PBS–milk (5%) for 1 h and then washed three times with PBS–TWEEN20 (0.1%). Each HMab (5 μg/ml) was then added together with the serum from a convalescent patient (obtained 30 days after infection) and a commercially available monoclonal antibody as positive controls (Anti-H1, GeneTex, GTX28262). All antibodies were incubated for 1 h at room temperature. After washing with PBS–TWEEN 20 (0.1%), secondary antibodies were added for 1 h and after an additional washing step, the substrate solution was added (SuperSignal® West Pico Chemiluminescent Substrate, PIERCE) and incubated for 2 min.

Virus neutralization assays

Fluorescence inhibition assay

Each viral isolate was titrated by the limiting dilution method, and the viral titer calculated by the Reed-Muench formula. 100 TCID50 of each isolate were preincubated 1 h at 37 °C with scalar concentrations of each human Fab (from 0.125 to 20 μg/ml). 250 μl of each pre-
incubated solutions were then added on MDCK cells and incubated 1 h at 37 °C in presence of 5% CO2. After the adsorption, the medium was replaced with serum-free medium with trypsin (1 μg/ml) and trypsin (1 μg/ml) were gently added on each well, and the plates were incubated 48 h at 35 °C in presence of 5% CO2. After 48 h the agarose medium was removed from each well and 1 ml of 70% methanol-crystal violet 1% was added to each well at room temperature. Finally, the wells were washed with tap water and dried to allow the count of PFU. The neutralization was determined counting the PFU reduction in presence of antibodies, in comparison with PFU observed in the infection control.

**Plaque inhibition assay**

Each viral isolate was titrated by the limiting dilution method, and the viral titer was calculated either as plaque forming units (PFU) on six-well flat-bottomed plates either as TCID50. 100 TCID50 of each isolate were preincubated 1 h at 37 °C with different concentrations of the human Fabs. The same controls used in IF assay were also used in the plaque reduction assay. One milliliter of each neutralization mix was added on MDCK monolayer and the infection was performed 1 h at 35 °C in presence of 5% CO2. After the adsorption, the medium was removed and the monolayer washed twice with sterile PBS. Two milliliters of MEM-agarose 0.8% supplemented with antibiotics (penicillin 50 μg/ml) (GIBCO), streptomycin (100 μg/ml) (GIBCO) and of-glutamine (2 mM) and trypsin (1 μg/ml) were gently added on each well, and the plates were incubated 48 h at 34 °C in presence of 5% CO2. After 48 h the agarose medium was removed from each well and 1 ml of 70% methanol-crystal violet 1% was added to each well at room temperature. Finally, the wells were washed with tap water and dried to allow the count of PFU. The neutralization was determined counting the PFU reduction in presence of antibodies, in comparison with PFU observed in the infection control.

**EID50 (embryonated infectious dose) reduction assay for HSN1 influenza strains**

Serial 10-fold dilutions (10^−3 to 10^−8) of Highly Pathogenic Avian Influenza (HPAI) HSN1 viruses (A/chicken/Egypt/A6/2008 and A/turkey/Turkey/1/2005) were prepared in PBS and each dilution was mixed with equal volume of HMAbs PN-SIA28 or PN-SIA49 (50 μg/ml) or negative control (PBS). After incubation for 1 h at 37 °C, the mixture was inoculated into the allantoic cavities of 10-day-old embryonated hen eggs (five eggs for each viral dilution).

After 72 h of incubation at 37 °C, the eggs were checked for vitality, chilled and allantoic fluids were tested individually for HA activity. The EID50 per 100 μl was calculated using the method of Reed-Muench.

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**References**


