Novel Phage Display-Derived H5N1-Specific scFvs with Potential Use in Rapid Avian Flu Diagnosis

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Introduction

The highly pathogenic avian influenza A (HPAI) viruses of the H5N1 subtype infect poultry and have also been spreading to humans. Although new antiviral drugs and vaccinations can be effective, rapid detection would be more efficient to control the outbreak of infections. In this study, a phage-display library was applied to select antibody fragments for HPAI strain A/Hubei/1/2010. As a result, three clones were selected and sequenced. A hemagglutinin inhibition assay of the three scFvs revealed that none exhibited hemagglutination inhibition activity towards the H5N1 virus, yet they showed a higher binding affinity for several HPAI H5N1 strains compared with other influenza viruses. An ELISA confirmed that the HA protein was the target of the scFvs, and the results of a protein structure simulation showed that all the selected scFvs bound to the HA2 subunit of the HA protein. In conclusion, the three selected scFVs could be useful for developing a specific detection tool for the surveillance of HPAI epidemic strains.

Keywords: H5N1 virus, antibody, phage library, diagnosis
control a large outbreak [30]. The commonly used diagnostic tests for the avian flu virus are based on two main techniques: real-time polymerase chain reaction (PCR) tests with biorecognition of nucleic acid probes, and enzyme-linked immunosorbent assay (ELISA) tests with specific antibodies [4, 27]. In contrast to standard viral diagnostic tests, which are usually time-consuming and labor-intensive, real-time PCR and ELISA tests allow virus typing within 3–4 h and can be performed automatically. Until now, real-time PCR is the most sensitive technique for detecting viruses in respiratory specimens [11]. However, real-time PCR diagnostic tests require costly equipment and skilled personnel, thereby hindering the application of these tests in developing countries where they are needed the most. In contrast, diagnostic tests employing ELISA are easy to use and have a fast turnaround time (30–60 min). Moreover, ELISA-based antibody-antigen immune-binding is more useful as a point-of-care test than PCR-based tests [27].

The human monoclonal single-chain fragment variable (scFv) phage display library is a powerful tool both for studying the immune response of a virus of interest and for screening antibodies specific to the target virus [7, 15]. The scFv fragment combined with heavy chain (VH) and variable light chain (VL) domains is displayed outside the phage surface, and the scFv binding specific to the target is biopanned from the phage display library [16]. Several studies have already developed antibodies from a scFv library that bind specifically to the spring viraemia of carp virus (SVCV), H5N1 M2 protein, and other viruses [19, 33]. However, scFv library technology could also be applied to the development of an influenza diagnostic tool, as scFv antibodies can bind to a variety of antigens, such as haptens, proteins, and whole pathogens, plus they could be used in ELISA tests [1, 20].

Accordingly, this study applied an scFv phage display library to select antibody fragments against the epidemic H5N1 strain A/Hubei/1/2010 isolated recently in China, for which no specific diagnostic antibodies have yet been reported. The antibodies selected via phage display screening were further evaluated for their specificities to the H5N1 virus using ELISA tests and for their binding affinity to HA using noncompetitive ELISA. Thereafter, functional studies of the selected scFvs were performed using influenza hemagglutination inhibition (HI) assays to evaluate their roles in the inhibition of hemagglutinin agglutination. A final investigation using a protein structure simulation assay revealed that the target epitopes of the selected scFvs were present in the hemagglutinin (HA) protein of the A/Hubei/1/2010 virus.

Materials and Methods

Phage Library, HA Protein, and H5N1 Virus

The Human Single Fold Tomlison scFv Libraries I+J were purchased from the MRC HGMP Resource Centre, Cambridge, UK. The horse radish peroxidase (HRP)-conjugated Protein A was purchased from GE Healthcare Life Science (China). The four inactivated HPAI H5N1 strains (A/Hubei/1/2010, A/Anhui/1/2005, A/Guangdong-Shenzhen/1/2011, and A/Chicken/Hong Kong/AP156/2008), pandemic H1N1 (A/Sichuan/SWL1/2009), seasonal H1N1 (A/Tianjinjingjin/15/2009), seasonal H3N2 (A/Fujian tongan/196/2009), and influenza B-Yamagata (B/Sichuananyue/139/2011) were obtained from the Chinese Center for Disease Control and Prevention (China CDC), and all were inactivated using beta-propiolactone. Enterovirus 71 and Dengue virus I were prepared at the authors’ institute and inactivated using formaldehyde. The recombinant HA (HA1+HA2) [34, 35], NA [14], and NP [18, 31] proteins were obtained from the College of Veterinary Medicine at Huazhong Agricultural University and were expressed in E. coli BL21 with pGEX-KG (GE Healthcare Life Science, China) as the vector and purified using Glutathione Sepharose Affinity Chromatography.

Phage Display Biopanning

The phage display biopanning was performed according to the manufacturer’s instructions with a minor modification to ensure scFv peptides specific to the epidemic strain A/Hubei/1/2010 were chosen as the target for the library screening. In the first round of biopanning, 96-well plates (Nunc-Nalgene, USA) were coated with the inactivated H5N1 viruses at a hemagglutination titer of 1:32 in a 0.1 M NaHCO3 buffer overnight at 4°C. The next day, the plates were blocked for 2 h at 4°C using 3% bovine serum albumin (BSA) diluted in phosphate-buffered saline (PBS). Following three washes with PBS, the immobilized inactivated viruses were incubated with the Tomlison I+J phage libraries (500 µl/well) at room temperature for 2 h with gentle shaking. Thereafter, any unbound phages were removed by washing 10 times with 0.01% (v/v) Tween 20 in PBS in the first round of panning. The bound phages were then eluted by adding 50 µl of an elution buffer (50 µl of 10 mg/ml trypsin + 450 µl of PBS), followed by 10 min of gentle shaking at room temperature. The eluted phage clones were then titered in E. coli TG1 and subjected to further rounds of panning. The second and third rounds of panning were performed under similar conditions, except that the hemagglutination titer of the target antigen was decreased to 1:4 and the unbound phages were removed by washing 20 times with 0.01% (v/v) Tween 20 in PBS.

Selection of Phage Clones with Specificity for H5N1 Virus Using ELISA

In the third round of selection, 96 of the phage clones grown on TYE plates (15 g bacto-agar, 8 g NaCl, 10 g tryptone, 5 g yeast extract in 1 L; Sigma) were selected to be amplified and evaluated
for their antigen-binding specificity using an ELISA assay. First, 96-well microtiter plates (Nunc Maxisorp plate; Gibco BRL) were coated with the inactivated H5N1 virus at a hemagglutination titer of 1:32 in a 0.1 M NaHCO₃ (pH 8.6) buffer overnight at 4°C. The supernatant (50 µl) of the selected phage clones was then poured into the virus-coated wells. The ELISA protocol was optimized by testing different blocking conditions using 2%, 3%, and 5% dried skimmed milk powder in PBS as the blocking buffer; different washing conditions using Tween 20 concentrations of 0.1%, 0.3%, and 0.5% in PBS as the washing buffer; and different secondary antibody concentrations of 1:1,000, 1:2,500, and 1:5,000 HRP-anti-M13 for detection. The specific affinities of the selected phage clones to the H5N1 virus were evaluated using a panel of different influenza viruses that included Enterovirus 71, Dengue virus I, pandemic H1N1 (A/Sichuan/SWL1/2009), seasonal H1N1 (A/Tianjinjingnan/15/2009), seasonal H3N2 (A/Fujian tongan/196/2009), influenza B-Yamagata (B/Sichuananyue/139/2011), HPAI A/H5N1 A/Hanoi/1/2005, A/Guangdong-Shenzhen/1/2011, A/Hubei/1/2010, and A/Chicken/Hong Kong/AP156/2008. The inactivated viruses were diluted in 0.1 M NaHCO₃ (pH 8.6) to a final concentration of 10 µg/well separately and coated onto 96-well microtiter plates overnight at 4°C. The subsequent ELISA steps were performed using the above-mentioned optimizations.

**Binding Character of scFvs to H5N1 Virus**

To identify the proteins recognized by the scFvs, the binding activities of the prokaryotic-expressed HA, NA, and NP of the H5N1 virus to the scFvs were detected using an ELISA. The wells were coated with 1 µg/ml HA, NA, and NP as immobilized antigens. Next, 50 µl of 10 µg/ml scFvs as the first antibodies and 1:2,000 diluted HRP-Protein A as the second antibody were added sequentially. Finally, the OD₄₅₀ nm was detected.

The binding affinity of HA to the scFvs was determined using a noncompetitive ELISA. The antigen concentration, coating time, and incubation time of the antigen with the antibody were optimized by testing different antigen concentrations: 5, 2.5, 1.25, 0.625, and 0.3125 µg/ml in a 0.1 M NaHCO₃ buffer (pH 8.6); different coating times: 12, 24, 36, and 48 h at 4°C in a humidity box; and different incubation times: 1, 2, 4, 8, and 16 h at room temperature. The [scFv] OD₅₀% was then measured by adding different scFv concentrations of 10, 5, 2.5, 1.25, 0.625, and 0.3125 µg/ml to the immobilized antigen using the optimized ELISA conditions. Finally, the affinity constant K(aff) was calculated using the formula K(aff) = (n - 1)/(2n[scFv] - [scFv]), derived from the law of mass action, where n = [Ag]/[Ag'] [2, 23].

**Sequence Determination of Selected Phage Clones**

Seven positive phage clones were subjected to further sequence verification after precipitation with PEG-NaCl. The sense primer 5’-CAG GAA ACA GCT ATG AC-3’ and reverse primer 5’-CGA CCC GCC ACC GCC GCT G-3’ were used to amplify and sequence the variable heavy (V₅) fragment from the phage clones; the sense primer 5’-CAT CTG TAG GAG ACA GAG TC-3’ and reverse primer 5’-CTG TGC GGG CCC ATT CA-3’ were used for the variable κ (Vk) fragment; and the sense primer 5’-CAG GAA ACA GCT ATG AC-3’ and reverse primer 5’-CTG TGC GGG CCC ATT CA-3’ were used for the full length of the exogenous sequence. The PCR conditions were as follows: 95°C for 10 min; 30 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min (for the whole sequence, the extension time was extended to 2 min); and the final extension time was 7 min at 72°C [3]. The amplified fragments that were the right size, as determined by electrophoresis, were subjected to further DNA sequencing. Consequently, the fragments from three phages (A1, A10, and F11) were found to match those of the expected scFv. The corresponding amino acid sequences of the three phages were determined based on the resulting DNA sequences.

**HI Assay**

A 1% (v/v) horse red blood cell (RBC) solution was prepared by washing and mixing with PBS containing 0.5% BSA. The inactivated H5N1 virus was diluted to 4 HAU/25 µl for use in the following assays. The scFv was purified with a PEG/NaCl solution, whereas the purified wild-type phage and control (positive and negative) serum were treated with the receptor destroying enzyme (RDE II) produced from Vibrio cholerae serovar 558 Ogawa (Denka Seiken Co. Ltd) in a 1:4 volume; 25 µl of each test sample was then diluted into two-fold serial dilutions using 25 µl of PBS in 96-well V-bottom microtiter plates (Gibco BRL). Next, 25 µl of the 4 HAU virus was added to the wells and incubated at room temperature for 20 min. Thereafter, 50 µl of the 1% horse RBC solution was added and the plates were incubated further at room temperature for 1 h, following which the agglutination titers were determined. The HI assay results were expressed as the reciprocal of the highest dilution of the tested sample for which hemagglutination was inhibited. All the assays were performed in triplicate. Each assay included specific positive antisera and non-immunized sheep serum controls. The purified wild-type phage was used as the negative control.

**Protein Structure Simulation Analysis of scFv-HA Complex**

The structures of the scFv molecules (A1, A10, F11) and HA protein from HPAI strain A/Hubei/1/2010 were predicted using the I-TASSER program with an average error of 0.08, is a reliable tool for predicting protein structures, and has already been successfully applied by many research groups [36]. All the structures predicted by the I-TASSER program were then visualized using UCSF Chimera 1.7 [22] and Discovery Studio Visualizer 3.5 (http://accelrys.com/products/discovery-studio/visualizaton-download). The docking simulation server Zdock (http://zdock.umassmed.edu/) was used to study the complex structures between each scFv and viral HA protein. All of the above-mentioned complex structures were downloaded from the Zdock server and visualized using the Discovery Studio Visualizer 3.5. The resulting files were then transformed into PDB files with a Chimera-readable structure and
visualized in UCSF Chimera 1.7. Finally, the results were exported and saved as PNG files.

**Binding Site Alignment of H5, H3, and H1 Strains**

The hemagglutinin sequences of the Influenza A virus mentioned in Fig. 3, including HPAI H5N1 strains A/Hubei/1/2010, A/Anhui/1/2005, A/Guangdong-Shenzhen/1/2011, pandemic H1N1 A/Sichuan/SWL1/2009, seasonal H1N1 A/Tianjingjingnan/15/2009, and seasonal H3N2 strain A/Fujian tongan/196/2009, were downloaded from the flu sequence database of the China CDC (http://1.202.129.144:8050/epiflu-portal/). The HA sequence for Influenza B and A/Chicken/Hong Kong/AP156/2008(H5N1) were not included in this alignment owing to a significant sequence discrepancy and unavailability in the online database, respectively. All the downloaded amino acid sequences were subjected to MEGA5 and the alignments conducted using MUSCLE methods.

**Results**

**Identification of scFvs Specific to HPAI H5N1 by Screening Phage Display Library**

To identify the molecules with a high affinity to the inactivated H5N1 virus, three rounds of biopanning were performed to enrich the positive phage clones. The titer of the phage eluate from the first round ($2.3 \times 10^4$ CFU/ml) increased in the third round to $4.0 \times 10^6$ CFU/ml. After three rounds of panning, 96 individual phage clones were selected for further functional studies. The binding ability of the selected phages to the immobilized HPAI H5N1 viral particles was determined using an ELISA. Among the 96 clones, seven showed a high affinity to H5N1 virus A/Hubei/1/2010 (Fig. 1).

The inserted antibody fragments of these seven strains were then subjected to a PCR with primers specific for the light and heavy chains of the antibody molecules. The lengths of the amplification products of the seven phage clones were 527, 368, and 935 bp, which coincided with the expected lengths of the light chain, heavy chain, and whole scFv of the antibody molecule, respectively (data not shown). Thus, the sequencing results showed that the seven clones only contained three different sequences: A1, A10, and F11, respectively, which were then studied further. The amino acid sequences of A1, A10, and F11 were predicted using MEGA4 and showed several mutated sites in the scFv molecules, namely, at positions 50–53 in CDR2 and 91–96 in CDR3 of the kappa chain, and at positions 50–58 in CDR2 and 95–98 in CDR3 of the heavy chain (Fig. 2).
number of amino acid residues was determined from the Kabat sequence database. As shown in Fig. 2, the complimentary domain regions (CDRs) of the scFv molecules were rich in serine, threonine, and proline, which have polar side chains. Moreover, the CDR of each scFv was rich in specific amino acid(s), such as glycine and arginine in A1, glycine and histidine in A10, and tyrosine in F11. These amino acid discrepancies may influence the activity of the binding region in a sequence-dependent manner.

The specific affinity of the three selected clones to the H5N1 virus is depicted in Fig. 3. The scFV fragments showed a low binding activity with Enterovirus 71, Dengue virus I, pandemic H1N1 (A/Sichuan/SWL1/2009), seasonal H1N1 (A/Tianjintongnan/15/2009), seasonal H3N2 (A/Fujian tongnan/196/2009), and the influenza B-Yamagata (B/Sichuananyue/139/2011), whereas they showed a high affinity to HPAI A/H5N1 A/Guangdong-Shenzhen/1/2010, A/Chicken/HongKong/AP156/2008, A/Anhui/1/2005, and A/Hubei/1/2010. Therefore, these results indicate that the three identified scFvs may recognize the common epitopes of the H5N1 strains.

**Hemagglutination Inhibition Activity of Three Selected scFvs Against H5N1 Strains**

The hemagglutination inhibition of the three scFv fragments with a specific binding affinity to H5N1 was investigated using HI assays against a panel of H5N1 strains, including A/Guangdong-Shenzhen/1/2011, A/Chicken/HongKong/AP156/2008, A/Anhui/1/2005, and A/Hubei/1/2010. The HAI titers of the three scFv fragments to the four H5N1 viruses were <1:10. However, despite their specific binding ability to the inactivated H5N1 viruses observed in the ELISA tests, none of the scFv fragments from the selected phage clones was able to block the binding of HA with horse RBCs.

**Binding Characteristics of scFvs to HA Protein**

It has already been reported that most antibodies with hemagglutination inhibition activity bind with the globular head of the HA protein and block the HA binding to its receptor [5, 17]. Thus, to investigate why the selected scFv fragments with a high affinity to H5N1 showed no hemagglutination inhibition activity, it was first checked whether the scFvs could recognize viral HA. To this end, an ELISA was conducted using prokaryotically expressed viral HA, Neuraminidase (NA), and nucleoprotein (NP) proteins as the antigens. All three scFvs (A1, A10, and F11) showed an affinity to the immobilized HA, but not to the immobilized NA and NP, indicating that their binding to the H5N1 virus was due to their specificity for HA (Fig. 4).

The binding affinities of HA to the scFvs were measured using a non-competitive ELISA. The use of serial dilutions of HA at 5, 2.5, 1.25, 0.625, and 0.3125 μg/ml for binding to the three scFvs resulted in a sigmoid curve for the OD when using an ELISA. Moreover, HA concentrations of 2.5, 1.25, and 0.625 μg/ml were used to detect the antigen-antibody reaction curve. The optimized coating time was 24 h at 4°C in a humidity box, while the optimized incubation time was 1 h at 37°C.
time was 16 h at room temperature. When using a coating concentration of 2.5 µg/ml, the OD50% for [F11], [A1], and [A10] was 26.7 × 10^{-12}, 220 × 10^{-12}, and 235 × 10^{-12} mol/L, respectively; with a coating concentration of 1.25 µg/ml, the OD50% for [F11], [A1], and [A10] was 208.7 × 10^{-12}, 400 × 10^{-12}, and 415 × 10^{-12} mol/L, respectively; and with a coating concentration of 0.625 µg/ml, the OD50% for [F11], [A1], and [A10] was 420 × 10^{-12}, 220 × 10^{-12}, and 235 × 10^{-12} mol/L, respectively. After calculation, the medium K_{aff} for F11, A1, and A10 was 2.59 × 10^7, 1.002 × 10^7, and 0.987 × 10^7 L/mol, respectively. Thus, the three scFVs could be useful for developing a specific detection tool for surveillance of the HPAI epidemic strain.

**Structures of Complex Formed by Binding of Selected scFvs to Viral HA**

The structures of the scFv molecules (A1, A10, F11) and HA protein from the HPAI strain A/Hubei/1/2010 were predicted using the protein structure simulation program I-TASSER [36] and visualized with UCSF Chimera 1.7 [22] and Discovery Studio Visualizer 3.5. According to the predicted structures, the four CDRs of A1, A10, and F11 formed a loop at the surface of the molecule [20, 22], and all these loops were plaited to form a pocket-like structure that possibly contained the potential interaction sites with the HA protein (Fig. 5B). For a further study of the sites on the scFv molecules involved with binding to the HA protein, the structures of each scFv and the viral HA were entered into the online protein docking simulation server Zdock. As shown in Fig. 5C, instead of binding with the globular head (receptor-binding subdomain, labeled in green in Fig. 5A), A10 and F11 were both bound with helix A in the HA2 subunit through their loop structure in the CDR (Figs. 5A [red] and 5C [green]). Moreover, interaction with the N-terminal alpha-helix of the HA2 subunit (Leu532-Gly550) was observed in the structure of the A1-HA complex. Since the HA2 subunit is relatively conserved compared with the HA1 subunit and is not a receptor binding site [12, 17], it is possible that the broad H5N1 recognition abilities and non-blocking hemagglutination activity of the three scFvs were due to their binding to the HA2 subunit.

To understand the structural basis for the discriminative binding activity among the different influenza subtypes shown in Fig. 3, sequence alignments were also performed for the binding sites of HPAI H5N1 strains A/Hubei/1/2010, A/Anhui/1/2005, A/Guangdong-Shenzhen/1/2011, pandemic H1N1 A/Sichuan/SWL1/2009, seasonal H1N1 A/Tianjingjingnan/15/2009, and H3N2 A/Fujian tongan/196/2009. For the binding sites of the A10 molecules (Glu430-Met447) and F11 (Leu446-Asp465), the signature M447L variant was identified among the amino acid sequences of H5 and two other subtypes. For most of the scFv interactions, since hydrogen bonds were the major force maintaining the antibody and antigen recognition, the sulphydryl in the 447th methionine with hydrogen bonds forming the potentials in the binding region was speculated to affect the A10/F11 and HA interaction based on newly formed hydrogen bonds (Figs. 6A and 6B). For the interacting sites of the A1 molecules (Leu532-Gly550) (Fig. 6C), several amino acid variants, such as A539S and M/S547A, were detected among the H3 subtypes and two other subtypes, plus there was a high similarity in the amino acid sequences between the H5 and H1 subtypes, except for an S533A substitution. Possibly, the substitution of alanine was due to the lack of hydroxyl in the serine for forming a hydrogen bond to stabilize the interaction of the A1-HA complex.

**Discussion**

Using sequential biopanning to screen a specific phage display library (Human Single Fold Tomlison I+J scFv Libraries), this study identified three unique scFvs that exhibited a specific binding affinity to several strains of HPAI A/H5N1, including A/Hubei/1/2010, A/Guangdong-Shenzhen/1/2011, A/Chicken/HongKong/AP156/2008, and A/Anhui/1/2005, representing the main strains causing H5N1 infection in China. All three fragments included mutations at similar positions, indicating that they probably bind to very similar epitopes and are highly specific to HPAI A/H5N1 strains [9, 26].

Despite showing a binding affinity to HA from the H5N1 virus, none of the selected scFv fragments showed any hemagglutination inhibition activity in HI assays. Previous studies have shown that H5N1 antibodies inhibiting viral-induced hemagglutination often target the receptor-binding subdomain (RBD) in the HA1 subunit [17]. Thus, since the target of the current biopanning process was the whole inactivated HPAI A/H5N1 virus, it is possible that the scFv fragments selected in this study did not target HA, the well-defined antigen most H5N1 antibodies recognize. Hence, the specificity and binding affinity of the three scFvs for the HA protein were studied. All three scFvs (A1, A10, and F11) displayed high affinity for the HA protein, but not for NA and NP, which prompted a further investigation of whether the selected scFv fragments targeted other regions of the HA protein, apart from the
**Fig. 5.** Predicted structure of HA protein and scFv-HA complexes.

(A) Schematic of HA protein of HAPI virus of H5N1 subtype (cited from DuBois et al. [8]). The HA protein of H5N1 is composed of a HA0 precursor, which is cleaved into two subunits, HA1 and HA2 (in red). HA1 consists of an F' fusion subdomain (in blue) and receptor-binding domain (RBD) comprised of a single polypeptide with a receptor-binding subdomain (green) and vestigial esterase subdomain (yellow). (B) Schematic of positive antibody A10. The four CDRs are labeled red. Since the structures of the three scFv molecules are very similar, the A10 molecule is shown as a representative of the other two molecules. (C) Simulated structures of three scFv-HA complexes. The HA1 and HA2 subunits of the HA protein are labeled in orange and green, respectively, and the scFv molecules A1, A10, and F11 are labeled in white, blue, and purple, respectively. The left panel includes separate images of each antibody-HA complex. The middle panel shows the integrated binding of all three scFvs. The right panel shows a closer view of the binding site of each complex from a different angle.
known receptor-binding subdomain to which most H5N1 antibodies bind.

Thus, the next step involved determining the binding sites of the selected scFvs and HA using a bioinformatic approach. The HA protein of H5N1 is composed of a HA0 precursor, which is cleaved into two subunits, HA1 and HA2. H5N1 antibodies against HA are often specific to the globular head (receptor-binding subdomain) in the HA1 subunit [32]. The interaction between antibodies and the globular head region physically blocks the interaction of HA with receptors on the target cells [7]. Moreover, epitopes of H5N1 antibodies have been identified in the 140 site antigenic loop of the HA1 subunit [21], and other H5N1 antibodies targeting similar regions have also recently been reported [13]. Thus, to identify the target sites of the three selected scFv fragments, the structures of A1, A10, and F11 were predicted using a structure protein simulation program [36], and then docked with the HA protein using the protein docking simulation server Zdock. In contrast to other H5N1 antibodies that bind to the globular head in the HA1 subunit, A1, A10, and F11 all chelated to the HA2 subunit, which is a relatively conserved sequence compared with the HA1 subunit: A1 bound with the N-terminal alpha helix, whereas A10 and F11 bound with helix A. It is possible that antibodies specifically targeting helix A in virus-induced cell agglutination have a neutralizing activity [24], yet A1, A10, and F11, as well as most of the antibodies against the HA2 subunit, showed no inhibitory effect as regards blocking the agglutination of horse RBCs in HI tests [24]. Therefore, this affinity to the HA2 subunit may explain the inability of the three selected scFvs to prevent the cell agglutination triggered by the HA protein.

Although HA2-specific antibodies lack an RBD-binding ability, they show a specificity toward a broad collection of H5N1 viruses, and the results obtained for the scFv fragments identified in this study were consistent with these earlier findings [10]. The selected scFvs showed a high affinity for all the HPAI A/H5N1 strains tested using an ELISA, but no affinity for the other seasonal epidemic

Fig. 6. Binding site alignment of A10 (A), F11 (B), and A1 (C) among different strains of influenza A virus. The hemagglutinin sequences of the influenza A virus mentioned in Fig. 3, including HPAI H5N1 strains A/Hubei/1/2010, A/Anhui/1/2005, A/Guangdong-Shenzhen/1/2011, pandemic H1N1 A/Sichuan/SWL1/2009, seasonal H1N1 A/Tianjingjingnan/15/2009, and seasonal H3N2 A/Fujian tongan/196/2009, were all downloaded from the flu sequence database of the China CDC. The alignments were accomplished in MEGA5 using MUSCLE methods.

flu strains in China or other types of virus, such as the control viruses, Enterovirus 71, Dengue virus I, pandemic H1N1 (A/Sichuan/SWL1/2009), seasonal H1N1 (A/Tianjingjingnan/15/2009), seasonal H3N2 (A/Fujian tongan/196/2009), and influenza B-Yamagata (B/Sichuananyue/139/2011) subtypes. Therefore, these results indicate that the three scFv fragments were highly specific to the H5 subclade of the influenza A virus.

In addition, further alignments of the scFv binding sites of the above-mentioned influenza A strains suggested that amino acid variants with hydrogen bond forming potentials in the relevant sites may contribute to the distinct binding activity through such noncovalent interactions.

Clinical tests are crucial for the proper control of H5N1 infection, as there are no obvious clinical signs or symptoms for the early diagnosis of HPAI A/H5N1 infection, and it is difficult to distinguish the infections caused by H5N1 from those caused by other respiratory pathogens, such as adenovirus, rhinovirus, and human metapneumovirus. Although several commercial kits are currently available for the diagnosis of H5N1, their accuracy and specificity remain under debate [4]. Hence, antibodies highly specific to H5 subclade strains are ideal candidates for the development of new diagnostic kits. Moreover, the selected molecules specific to the HA2 subunit of the H5N1 virus identified in this study can be further developed for use in clinical diagnostic tests for humans and for surveillance of H5N1 infections in birds and the external environment.

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