CONSTRUCTION AND EXPRESSION OF H5N1 INFLUENZA VIRUS HEMAGGLUTININ-SPECIFIC scFv-Fc MONOCLONAL ANTIBODIES IN HEK293T CELLS

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Abstract. Monoclonal antibody (MAb) is a key element in the development of rapid test kits for many infectious diseases. Our group previously developed two antigen-binding fragment (Fab) MAbs, H5Fab-6 and H5Fab-9, specific to hemagglutinin (H5 HA) of influenza A virus H5N1, but these Fabs do not have a constant fragment (Fc) portion with which to bind with gold particles in a strip test. In order to overcome this impediment, we joined a single-chain variable fragment (scFv) with an Fc region to produce a scFv-Fc MAb, which was expressed in mammalian HEK293T cells. Specificity and sensitivity of each generated scFv-Fc MAb for H5 HA was tested using western blotting and dot-enzyme-linked immunosorbent assay (dot-ELISA), respectively. Two scFv-Fcs (designated H5scFvFc-6 and H5scFvFc-9) were constructed and purified to near homogeneity with a yield of 12.87 mg/l and 33.56 mg/l, respectively. Western blotting indicated that both scFv-Fcs reacted as expected with H5 HA with a sensitivity of 60 pg of H5 HA. These scFv-Fc MAbs should prove useful in the development of antibody-based diagnostic tools.

Keywords: avian influenza virus, H5N1, HEK293T cell line, heterologous expression, scFv-Fc antibody

INTRODUCTION

Avian influenza viruses (AIVs) are single-stranded, negative-sense RNA influenza A viruses of the family Orthomyxoviridae (Nelson and Holmes, 2007; Yin *et al*, 2013), which are classified into subtypes according to their hemagglutinin (HA) and neuraminidase (NA) antigens (Webster *et al*, 1992).

In 1997, the first human case of highly pathogenic avian influenza (HPAI) H5N1 infection was reported in Hong Kong, and several HPAI H5N1 outbreaks since have occurred around the world, resulting in over a billion poultry deaths and causing more than 60% mortality in humans (de Jong *et al*, 1997; Poovorawan *et al*, 2013). Currently, many AIV subtypes can produce virulent disease and lead to severe pandemics in humans owing to presence in these viruses of mutations or adapta-

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tions allowing person-to-person transmission (Webster *et al*, 1992; Wong and Yuen, 2006). In response to grave concerns regarding control and surveillance of this deadly disease, various studies have been conducted with the aim of developing vaccines, antiviral drugs, therapeutic treatments, and diagnostic tests (Fang *et al*, 2007; Hao *et al*, 2009; Luo *et al*, 2009; Maneewatch *et al*, 2009; He *et al*, 2010; Pitaksajjakul *et al*, 2010; Zhang *et al*, 2010; Linke *et al*, 2011; Zhuang *et al*, 2011; Zhang *et al*, 2013).

In order to prevent an epidemic of HPAI, rapid diagnosis of this disease is very important. A diagnosis system is needed that has high sensitivity, specificity and rapid readout, especially one that can differentiate HPAI from low pathogenic avian influenza (LPAI) subtypes (Li *et al*, 2011). Notably, influenza virus HA is the major target for differentiating between HPAI and LPAI subtypes, and this protein also is useful for producing both therapeutic and diagnostic antibodies (Luo *et al*, 2009; He *et al*, 2010; Pitaksajjakul *et al*, 2010; Linke *et al*, 2011; Zhuang *et al*, 2011).

One of the rapid diagnostic tools that has good specificity and high sensitivity for detecting many types of infectious viruses especially at their early phase of infection is antibody-based immunochromatographic strip test (ICT). This method has numerous advantages, such as low cost per test, ease of use, rapid operation, suitability for both untrained and trained user (robustness), portability, and long storage shelf-life (Chen *et al*, 2008; Cui and Tong, 2008; Charlton *et al*, 2009). Studies on the development of effective ICTs for influenza virus have emphasized that a pair of monoclonal antibodies (Mabs) targeting HA protein is necessary for successful production of a universal rapid detection kit (He *et al*, 2010). The lowest reported detection limit for H5N1 influenza virus detection using strip ICT is 0.1-0.5 HA unit (Chen *et al*, 2008; He *et al*, 2010).

Recombinant antibody technology is required to prepare MAbs suitable for high-throughput production to meet the increasing demand for such diagnostic reagents; however, many of these recombinant MAbs, such as single chain variable fragment (scFv) and antigen binding fragment (Fab), may have low affinities or lack appropriate functions in the absence of additional engineered components (Jager *et al*, 2013). However, a scFv-Fc format has sufficient IgG-like properties for use in all current immuno-based assays (Kirsch *et al*, 2008; Thie *et al*, 2011; Pohl *et al*, 2012). Moreover, scFv-Fc can be heterologously expressed in both yeast and mammalian cells suitable for large-scale production with yields of 10-27 g/l (Jager *et al*, 2013). Hosts of choice in mammalian expression systems are human embryonic kidney (HEK) cell lines, including HEK293, HEK293T, and HEK293E cells, all of which have extensively been employed for transient antibody expression, providing an inexpensive and rapid production platform without requiring laborious generation of stable cell clones (Jager *et al*, 2013). Thus, transient antibody expression in HEK cells provides a suitable means for small-scale production of recombinant MAbs for the screening phase, and this procedure is able to be scaled-up to produce ≈ 1 g/l of antibodies (Zhang *et al*, 2011; Jager *et al*, 2013).

Fab MAbs specific to HA of H5N1 (H5 HA) previously developed at the Center of Excellence for Antibody Research, Faculty of Tropical Medicine, Mahidol University, Bangkok included one specific to influenza A and one to H5N1. However,

because Fabs do not contain the Fc portion required for conjugation with nanogold particles required in a strip ICT, these MAb will have to be constructed to contain an Fc region before they can be further developed for use in a diagnostic test.

Hence, in this study, we constructed two scFv-Fc MAbs from our previously generated Fabs that were selected from a chimeric/human Fab phage display library specific to H5 HA (Pitaksajjakul *et al*, 2010), expressed them in HEK293T, and following purification determined antibody yield, purity, binding specificity, and detection sensitivity.

MATERIALS AND METHODS

Cell line

HEK293T cells were used for the transient production of scFv-Fcs. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing glucose with L-glutamine (Hyclone, South Logan, UT) supplemented with 10% (v/v) fetal bovine serum (Hyclone).

Construction of scFv cloning plasmids and scFv-Fc expression vectors

Variable heavy chain (V_H) and variable light chain (V_L) genes of H5scFvFc-6 and H5scFvFc-9 MAbs were amplified from H5Fab-6 and H5Fab-9 MAbs-pComb3XSS plasmid, respectively using CSCVHo-FL and CSCG-B primers for amplification of V_H and CSCVK and CkJo-B primers for V_L (Andris-Widhopf *et al*, 2000). Gel-purified V_H and V_L amplicons were ligated using overlapping PCR method (Andris-Widhopf *et al*, 2000), subjected to additional gel-purification and inserted into pGEMT easy vector (to generates *scFv*-pGEMTs) (Promega, Madison, WI). Primers and PCR conditions used in the study are shown in Table 1. The *scFv*-pGEMTs plasmids then were used as template for each PCR with LS-scFv-Fc Fw and LS-scFv-Fc Rv primers to amplify scFv containing restriction sites. These scFv amplicons and pcFcB^{-}R^{-}, a pcDNA expression vector containing human Fc region, were digested with *Hin*dIII and *Bam*HI (New England BioLabs, Hitchin, UK), and followed by ligation with T4 ligase (New England BioLabs). All DNA constructs were verified by restriction enzyme mapping and DNA sequencing (Macrogen Korea, Seoul, Korea).

Expression and purification of H5scFvFc-6 and H5scFvFc-9 MAbs

Constructed plasmids of H5scFvFc-6 and H5scFvFc-9 MAbs were transfected into HEK293T cells, which were seeded at a concentration of 5×10^5 cells/ml one day before transfection in a T75 flask containing 15 ml of the above DMEM culture medium and incubated at 37°C under an atmosphere of 5% CO_2. FuGENE HD (Promega) was used as transfection agent at a ratio of 3:1 FuGENE:DNA following the manufacturer's recommendations. Culture supernatants (15 ml) of transfected HEK293T cells containing the expressed scFv-Fc MAbs were collected from day 2 to day 14, being replaced with 15 ml of new culture medium every 3 days. The scFv-Fc MAbs were purified from supernatants using Protein A affinity column (GE, Uppsala, Sweden). Protein concentration was measured using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

SDS-PAGE analysis of purified scFv-Fc MAbs

Five µl aliquots of purified scFv-Fc MAbs were mixed with sample buffer without β-mercaptoethanol, incubated at 95°C for 5 minutes, and analyzed by 10%

Table 1
Primer sequences and PCR conditions used in scFv-Fc construction.

Target	Primer	Sequence (5′-3′)	PCR components	Thermocycling condition
V_H	CSCVHo-FL	GGTCAGTCCTCTAGATCTTCCGGC GGTGGTGGCAGCTCCGGTGGTGGC GGTTCCGCCGTGACGTTGGACGAG	Final volume: 25 µl • 2.5 µl of 10X PCR buffer plus $MgCl_2$ • 0.5 µl of 10 mM dNTP mix • 15 pmol of each primer • 0.5 U Hi-fidelity DNA polymerase (Roche, Germany) • 50 ng of each Fab template.	•94°C, 5 minutes •30 cycles: 94°C for 15 s, 60°C for 15 s, and 72°C for 2 minutes •72°C, 10 minutes.
	CSCG-B	CTGGCCGGCCTGGCCACTAGTGGA GGAGACGATGACTTCGGTCC		
V_L	CSCVK	GTGGCCCAGGCGGCCCTGACTCAG CCGTCCTCGGTGTC	Final volume: 25 µl • 2.5 µl of 10X PCR buffer plus $MgCl_2$ • 0.5 µl of 10 mM dNTP mix • 15 pmol of each primer • 0.5 U Hi-fidelity DNA polymerase (Roche, Germany) • 50 ng of each Fab template.	•94°C, 5 minutes •30 cycles: 94°C for 15 s, 60°C for 15 s, and 72°C for 2 minutes •72°C, 10 minutes.
	CkJo-B	GGAAGATCTAGAGGACTGACCTAG GACGGTCAG		
scFv	CSC-F	GAGGAGGAGGAGGAGGAGGTGGCC CAGGCGGCCCTGACTCAG	First reaction: • 2.5 µl of 10X PCR buffer plus $MgCl_2$ • 0.5 µl of 10 mM dNTP mix • 0.5 U Hi-fidelity DNA polymerase (Roche, Germany) • 100 ng of purified V_L and V_H template • No addition of primers.	First round: •94°C for 5 minutes •20 cycles: 94°C for 15 s, 56°C for 15 s, and 72°C for 1 minute •4°C.
	CSC-B	GAGGAGGAGGAGGAGGAGGAGCTG GCCGGCCTGGCCACTAGTGGAGG		
	CSC-F	GAGGAGGAGGAGGAGGAGGTGGCC CAGGCGGCCCTGACTCAG	Second reaction: • 15 pmol of CSC-F and CSC-B primers • 0.5 µl of 10 mM dNTP.	Second round: •20 cycles: 94°C for 15 s, 56°C for 15 s, and 72°C for 2 minutes •72°C for 10 minutes.
	CSC-B	GAGGAGGAGGAGGAGGAGGAGCTG GCCGGCCTGGCCACTAGTGGAGG		
scFv-Fc	LS_scFV_Fc_F	GAGGAGGAGGAGAAGCTTGCCACC ATGAGCGTGCCTACCCAGGTGCTGGGC	Final volume: 25 µl • 15 pmol of both primers	•94°C for 5 minutes •30 cycles: 94°C for 15 s,

	CTGCTGCTG CTGTGGCTGACCGATGCC	• 25 ng of scFv plasmid	60°C for 15 s, and 72°C
	AGATGCGCCCTGACTCAGCCGTCCTCG	• 2.5 µl of 10X PCR buffer plus MgC_{12}	for 2 minutes
	GTGTC	• 0.5 µl of 10 mM dNTP mix	• 72°C, 10 minutes.
LS_scFV_Fc_R	GAGGAGGAGGAGGGATCCACCACCCGA	• 1.5 U Hi-fidelity DNA polymerase	
	GCCCCCGCCAGACCACCGCCTCTAGAGGA	(Roche, Germany).	
	GGAGACGATGACTTCGGTCC		
(Sequencing) M13-F	CGCCAGGGTTTTCCCAGTCACGAC		
CSCVHo-FL	GGTCAGTCCTCTAGATCTTCCGGC		
	GGTGGTGGCAGCTCCGGTGGTGGC		
	GGTTCCGCCGTGACCGTTGGACGAG		

SDS-PAGE at 100 V. Protein bands were stained with Coomassie brilliant blue R-250 dye (Thermo Scientific).

Dot blot analysis of expressed scFv-Fc MAbs

Three µl aliquot of culture fluid taken every 3rd day was dotted onto a polyvinylidene difluoride (PVDF) membrane (GE), treated with 5% skim milk at room temperature for 2 hours, then incubated with goat anti-human IgG (H+L) conjugated with horseradish peroxidase (HRP) (1:2,500 dilution) (KPL, Gaithersburg, MD) at room temperature for 1 hour, and stained with 3, 3′ diaminobenzidine (DAB) reagent set (KPL).

Western blot analysis of scFv-Fc MAbs specificity

Two scFv-Fc MAbs were tested for specific binding to recombinant H5 HA derived from H5N1 AIV strain A/Vietnam/1203/04 (from an insect cell expression system) (Stevens *et al*, 2006). H5 HA (500 ng) was mixed with sample buffer containing β-mercaptoethanol (Thermo Scientific), subjected to 10% SDS-PAGE as described above, transferred onto PVDF membrane, treated as described above before being incubated with each scFv-Fc MAb at 4°C overnight followed with incubation with HRP-conjugated goat anti-human IgG (H+L) (1:2,500 dilution at room temperature for 1 hour and visualized using DAB reagent set.

Dot-ELISA test of scFv-Fc MAbs sensitivity

A 10-fold serial dilution of H5 HA (from 20 to 2 pg/µl) was used to determine sensitivity of each scFv-Fc MAb. Three µl aliquots of H5 HA samples were dotted onto PVDF membrane strip, treated with 5% skim milk at room temperature for 2 hours, incubated with each prepared scFv-Fc MAb (500, 50, 5, 0.5, and 0.05 ng/ml) at 4°C overnight, then incubated with

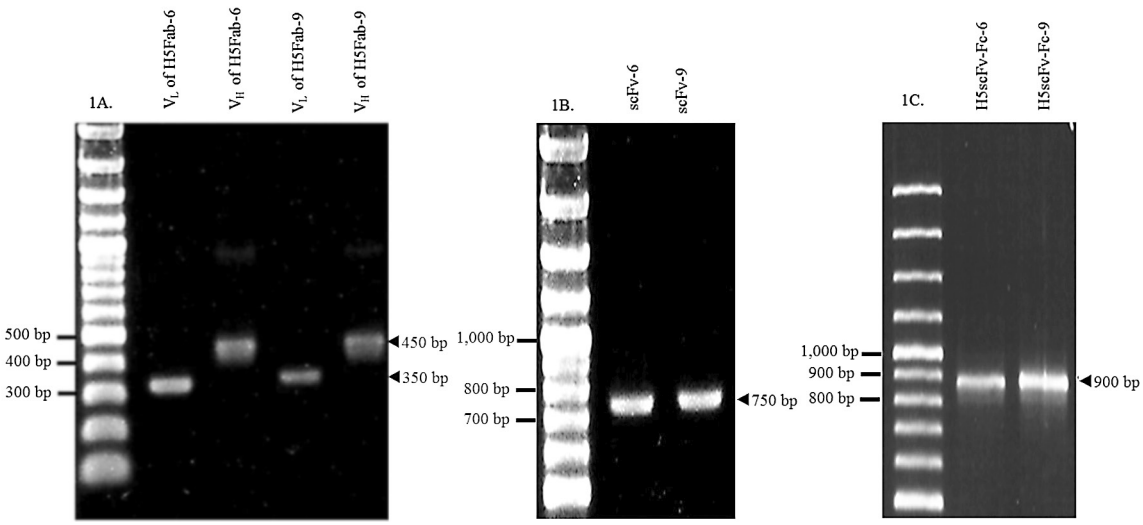

Fig 1–Electrophoretic profiles of V_H, V_L, scFv, and scFv-Fc amplicons. PCR conditions are described in Table 1. (A) Amplicons of H5Fab-6 and H5Fab-9 V_L and V_H. (B) Amplicons of scFv-6 and scFv-9. (C) Amplicons of H5scFv-Fc-6 and H5scFv-Fc-9.

HRP-conjugated goat anti-human IgG (H+L) (1:2,500 dilution) at room temperature for 1 hour and visualized using DAB reagent set.

RESULTS

Construction of scFv and scFv-Fc plasmids for scFv-Fc antibody expression

We engineered H5Fab-6 (H5N1-specific) and H5Fab-9 (influenza A-specific) Fab antibody fragments into the corresponding scFv-Fc molecules because adding an Fc portion to these MAbs would make them more useful for future applications, such as ICT development. V_H and V_L was amplified from Fab-pComb3XSS vector that encodes the respective Fab generating the expected amplicon of 400 and 350 bp, respectively (Fig 1A). These amplicons were then ligated by means of overlapping PCR and inserted into pGEMT to generate *scFv*-pGEMTs carrying the expected 750 bp fragment (Fig 1B). After sequence confirmation, *scFv* portion of *scFv*-pGEMT was amplified, restriction digestion and inserted into pcFcB$^-$R$^-$ carrying the expected 900 bp fragment (Fig 1C).

Transient expression of scFv-Fc IgG-like MAbs in HEK293T cells

In order to assess successful engineering of the scFv-Fc expression vectors, HEK293T cells were transfected with each constructed plasmid using FuGENE HD reagent and cultured for 14 days. Dot blot analysis of scFv-Fc MAb in culture medium every 3 days showed that scFv-Fc was produced in HEK293T cells from day 2 post-transfection (Fig 2). ScFv-Fc MAb in culture medium was purified using Protein A affinity chromatography and analyzed by SDS-PAGE, demonstrating production of scFv-Fc (230 kDa) with near homogeneity (Fig 3). The yield of H5scFvFc-6 and H5scFvFc-9 was 12.9 and 33.6 mg/l, respectively.

Specificity and sensitivity of scFv-Fc MAbs binding to H5 HA

In order to determine if these scFv-Fcs are suitable for diagnostic application, we tested their specific binding to the antigen,

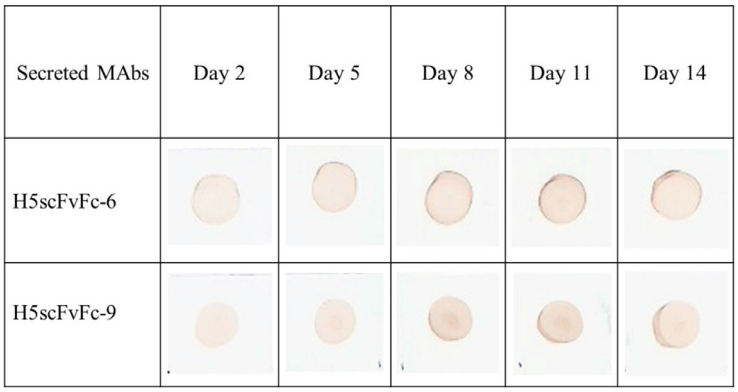

Fig 2–Heterologous expression of scFv-Fc MAbs by HEK293T cells. Cells were cultured in a T75 flask following FuGENE HD assisted transfection with H5scFvFc-6 or H5scFvFc-9. Western dot blot analysis was performed on culture fluid obtained from day 2 to day 14 post-transfection to determine amount of secreted antibody.

H5 HA, recognized by the original Fabs. Under sulfhydryl reducing conditions, both H5scFvFc-6 and H5scFvFc-9 reacted with the 50 kDa H5 HA antigen as demonstrated by western blotting (Fig 4).

Limit of H5 HA detection by these two antibodies was evaluated using dot-ELISA, which showed a detection limit of 60 pg for both scFv-Fc antibodies (500 ng/ml) (Fig 5).

DISCUSSION

The aim of this study was to develop new MAbs for detecting AIV H5N1, with the ultimate goal of applying these MAbs to the creation of a strip ICT kit. In the previous work, two groups of Fab MAbs specific to influenza virus HA were generated: one group specific to AIV H5N1 and the other cross-reactive with several influenza A subtypes, including H5N1, H1N1, H3N2, and H9N2 (Pitaksajjakul *et al*, 2010). In order to develop a new rapid detection strip ICT with high specificity and sensitivity from these two groups of Fab MAbs, each of which recognizes a different epitope from that of existing ICTs, we plan to use H5N1-specific H5Fab-6 as the immobilized antibody on the test line and cross-reactive H5Fab-9 as the antibody conjugated to gold particle (He *et al*, 2010). Preliminary studies indicated that these two Fab MAb molecules were too short to bind to gold particles in an ICT. In order to make use of the Fab portion that contains the binding region to HA, we needed to lengthen the Fab molecules. We chose the scFv-Fc format because it contains both an Fc region, capable of conjugating with gold particles, and scFv region for binding with HA. Additionally, the scFv-Fc antibody format can be used in many types of immunoassays, such as ELISA (Thie *et al*, 2011), immunoblot (Kirsch *et al*, 2008) and surface Plasmon resonance (Thie *et al*, 2011; Pohl *et al*, 2012).

H5scFvFc-6 and H5scFvFc-9 MAbs were constructed using two of the nine Fab MAb clones that were previously produced (Pitaksajjakul *et al*, 2010). H5Fab-6 is highly specific to H5N1 influenza virus, and H5Fab-9 cross-reacts with influenza A subtypes (Pitaksajjakul *et al*, 2010). Recombinant H5Fab-6 and H5Fab-9 plasmids were used as DNA templates to amplify V_H and V_L coding regions for construction of scFvs DNA fragments. Each scFv gene, encoding one V_H and one V_L chain of immunoglobulin connected by a peptide linker, was then fused with an IgG Fc gene

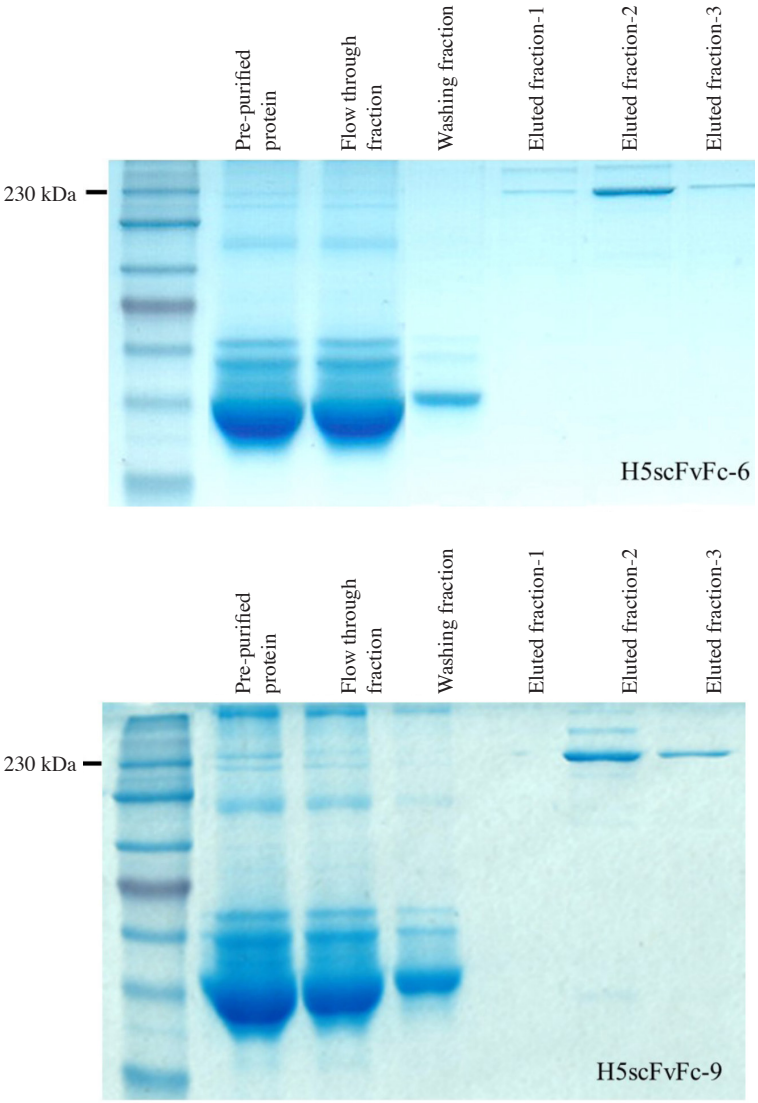

Fig 3–SDS-PAGE profiles of affinity purification of H5scFvFc-6 and H5scFvFc-9 MAbs. H5scFvFc-6 (upper panel) and H5scFvFc-9 (lower panel) fractions eluted from Protein A affinity column were resolved in a 10% SDS-PAGE and stained with Coomassie blue dye. Left lane, pre-stained protein size marker (10-230 kDa) (NEB).

to generate a scFv-Fc gene. This scFv-Fc coding construct was reported previously to have similar properties to those of native IgG (Shu *et al*, 1993; Moutel *et al*, 2009).

The scFv-Fc MAbs were expressed in a mammalian HEK293T cell line, widely used for heterologous protein expression (Ho and Pastan, 2009). HEK293T cell heterologous protein expression relies on transfection with DNA encoding the gene of interest (Schenk *et al*, 2007). In the small pilot scale of this study (15 ml in a T75 flask), the production yields were in the appropriate range for small-scale transient scFv-Fc expression (10-20 mg/l) (Jager *et al*, 2013), but the generation of a stable cell expression system, which can produce higher yields (Chusainow *et al*, 2009) is in progress. Furthermore, HEK293T cells can be very efficiently transfected with plasmids, and their ability to folding, modify and secrete heterologously expressed proteins (Schenk *et al*, 2007) make them well-suited for antibody production. The antibodies expressed by HEK293T cells generally contain post-translational modifications required for their function (Schenk *et al*, 2007). In addition, the use of a helper reagent during transfection, such as FuGENE HD or lipofectamine, is also important. In this study, FuGENE HD, a non-liposomal reagent that functions by complexing with nucleic acids, was used, allowing plasmids to overcome the electrostatic

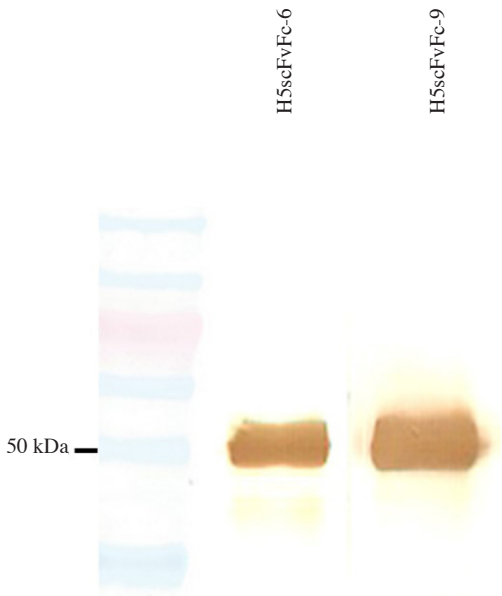

Fig 4–Specificity assay of H5scFvFc-6 and H5scFvFc-9 MAbs. Binding of H5scFvFc-6 and H5scFvFc-9 MAbs to 500 ng of H5 HA (50 kDa) was assessed by western blotting. Left lane, pre-stained protein size markers.

repulsion of the cell surface and to be efficiently taken up by cells (Chen *et al*, 2013). Our optimization experiments indicated that a ratio of FuGENE HD: scFv-Fc plasmid of 3:1 in a 24-well plate (data not shown) was optimal for expression, and this ratio will be employed in the scale-up experiments.

Western blotting assay performed with 500 ng of H5 HA confirmed the specificity of both scFv-Fc MAbs. Dot-ELISA showing a detection limit of both scFv-Fc MAbs at 20 pg/µl (60 pg) is similar to that of Fab MAbs (Pitaksajjakul *et al*, 2010). Given that both antibodies showed good specificity and high sensitivity with H5 HA, this combination of antibodies

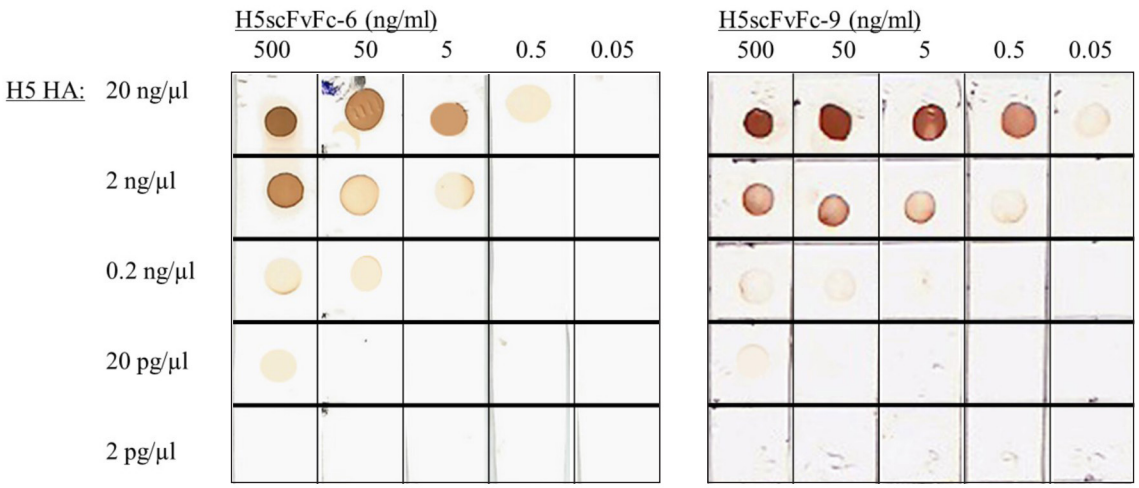

Fig 5–Sensitivity assay of H5scFvFc-6 and H5scFvFc-9 MAbs. Binding capacity of H5scFvFc-6 and H5scFvFc-9 to H5 HA (3 µl per spot) was determined by dot-ELISA. At 500 ng/ml, both scFv-Fcs showed a limit of detection of 60 pg of H5 HA antigen.

may be promising for use in the detection of H5N1 infection. While there are many studies on the development of scFv MAbs specific to H5N1 influenza virus hemagglutinin (Fang *et al*, 2007; Hao *et al*, 2009; Maneewatch *et al*, 2009; Zhang *et al*, 2010, 2011, 2013), this is the first study to develop scFv-Fc specific to HA of H5N1 virus.

In summary, two scFv-Fc MAbs were successfully constructed from Fab MAbs, and both are specific to H5 HA with the requisite sensitivity. The inclusion of the Fc portion in these scFv-Fc MAbs will allow binding of gold particles, a necessary property for the development of strip ICT. Future work will aim at producing a clinically useful strip ICT employing the constructed scFv-Fc MAbs.

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