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Human Monoclonal Single Chain Antibodies (HuScFv) that Bind to the Polymerase Proteins of Influenza A Virus

Umaporn Thathaisong[1], Santi Maneewatch[2], Kasem Kulkeaw[1], Kanyarat Thueng-in[1], Ornutcha Poungpair[1], Potjanee Srimanote[1], Thaweesak Songserm[3], Pongsri Tongtawe[1], Pramuan Tapchaisri[1] and Wanpen Chaicumpa[2]

SUMMARY Current anti-influenza drugs target the viral neuraminidase or inhibit the function of the ion channel M2 protein. Not only is the supply of these drugs unlikely to meet the demand during a large influenza epidemic/ pandemic, but also has an emergence of drug resistant influenza virus variants been documented. Thus a new effective drug or antiviral alternative is required. The influenza virus RNA polymerase complex consists of nucleoproteins (NP) that bind to three polymerase subunits: two basic polymerases, PB1 and PB2, and an acidic polymerase (PA). These proteins play a pivotal role in the virus life cycle; thus they are potential targets for the development of new anti-influenza agents. In this study, we produced human monoclonal antibodies that bound to the influenza A polymerase proteins by using a human antibody phage display library. Complementary DNA was prepared from the total RNA of a highly pathogenic avian influenza (HPAI) virus: A/duck/Thailand/144/2005(H5N1). The cDNA synthesized from the total virus RNA was used as template for the amplification of the gene segments encoding the N-terminal halves of the PB1, PB2 and PA polymerase proteins which encompassed the biologically active portions of the respective proteins. The cDNA amplicons were individually cloned into appropriate vectors and the recombinant vectors were introduced into *Escherichia coli* bacteria. Transformed *E. coli* clones were selected, and induced to express the recombinant proteins. Individually purified proteins were used as antigens in bio-panning to select the phage clones displaying specific human monoclonal single chain variable fragments (HuScFv) from a human antibody phage display library constructed from Thai blood donors in our laboratory. The purified HuScFv that bound specifically to the recombinant polymerase proteins were prepared. The inhibitory effects on the biological functions of the respective polymerase proteins should be tested. We envisage the use of the HuScFv in their cell penetrating version (transbodies) as an alternative influenza therapeutic to current anti-virus drugs.

Influenza viruses are highly contagious respiratory pathogens causing disease in both humans and animals.[1] They are divided into three types: A, B, and C, based on their structural proteins (NP and M1), epidemiologic patterns, host range, and pathogenicity.[2] Influenza A viruses are further classified into subtypes according to the antigenic differences of two surface glycoproteins, *i.e.*, hemagglutinin (H or HA) and neuraminidase (N or NA). Currently there are 16 H and 9 N subtypes. The H1N1, H2N2, and H3N2 principally cause influenza in humans while the H5, H7 and H9 cause disease among avian. Human influenza caused by type A viruses may be sporadic, seasonal epidemic, or devastating pandemic.[3] The recent emergence of highly pathogenic avian influenza (HPAI) virus strains belonging to the

From the [1]Faculty of Allied Health Sciences, Thammasat University, Rangsit Campus, Pathumthani, [2]Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, [3]Faculty of Veterinary Medicine, Kasetsart University, Kam-paeng-saen Campus, Nakhon Pathom, Thailand.
Correspondence: Wanpen Chaicumpa
E-mail: tmwcc@mahidol.ac.th

H5N1 subtype and their association with high lethality among poultry,[4] their cross-species infectivity to mammals[5-8] and human,[9] as well as their continuous antigenic change[10] create a global threat of a new influenza pandemic that might be caused by either the highly pathogenic strains of avian influenza A viruses, H5N1 subtype, that have adapted to more efficient human-to-human transmission, or the emergence of a new influenza virus subtype through genetic reassortment.[11] Current influenza vaccines are likely to have low, if any, protective efficacy against the new virus. Furthermore, the supply of the only family of effective anti-influenza drugs, *i.e.* neuraminidase inhibitors, might not meet the high demand of a large outbreak/pandemic. The drug must be given to the patient as early as possible in the course of the infection in order to be effective, and drug resistant virus mutants have emerged.[12,13] Thus, for influenza pandemic preparedness, it is necessary that a vaccine that confers broad protection against heterologous influenza strains/subtypes and new anti-influenza drugs are developed. A therapeutic antibody that can interfere with the virus pathogenicity would be a viable alternative.

The influenza viral RNA dependent-RNA polymerase complex consists of nucleoprotein (NP) and three polymerase subunits: two basic polymerases (PB1 and PB2), and an acidic polymerase (PA). The PB1 contains RNA-dependent RNA polymerase motifs[14-15] and binds to the vRNA and cRNA promoters.[16-18] It is an endonuclease which functions in pirating the capped primers from host pre-mRNAs for viral RNA transcription.[19] The PB2 recognizes and binds the host cap acquired from the host mRNAs by the PB1.[20-22] The exact role of the PA is less understood, but evidence suggests that it is involved in viral transcription and replication.[23-27] The individual polymerase subunits interact as follows: the N-terminal region of the PB1 interacts with the C-terminal of the PA, while the PB1-C-terminal interacts with the N-terminal of the PB2 subunit. No direct interactions have been demonstrated between the PB2 and the PA.[32] Recently, it has been found that a peptide homologue to the PA-binding domain of the PB1 (N-terminal portion of PB1) blocks the polymerase activity of influenza A viruses.[33] Because of the essential role of the polymerase proteins in the influenza virus' infectious cycle, targeting the polymerase subunit component could provide a novel strategy for the development of antiviral compounds against the influenza A virus.

Thus this study aimed at producing a therapeutic human monoclonal antibody targeting these polymerase subunit components to increase the therapeutic arsenal against influenza A viruses.

MATERIALS AND METHODS

Virus

A highly pathogenic avian influenza (HPAI) H5N1 virus, A/duck/Thailand/144/2005 (H5N1), isolated from the lungs of an infected duck from Nakhon-pathom province, Thailand, in 2005, was propagated in embryonated eggs.

Production of recombinant N-terminal-PA, -PB1 and -PB2 of the influenza A virus

Amplification of DNA sequences encoding N-terminal halves of the PA, PB1 and PB2 (*designated* PA-5', PB1-5', *and* PB2-5' *sequences, respectively*)

The total RNA was extracted from the H5N1 virus using TRIzol® reagent (Invitrogen™ Life Technologies, CA, USA). The RNA was reverse transcribed to cDNA using a Revert Aid™ H Minus Strand cDNA synthesis kit (Fermentas Life Sciences, Vilnius, Lithuania) and uni12 primer. The quality of the cDNA was checked by PCR amplification of the H5 subtype gene segment by using as forward primer: 5'-ACACATGCYCARGACATACT-3' and as reverse primer: 5'-CTYTGRTTYAGTGTTGATGT-3'.

For amplification of the *PB1-5'*, *PB2-5'*, and *PA-5'* sequences, three pairs of nucleotide primers (Table 1) were designed from the avian influenza A (H5N1) sequences available in the GenBank database (accession no. AY646179.1, AY627897.1, AY651713.1, AY651715.1, AY651716.1, AY626147.1, AY626148.1, and AY551934.1). The PCR reaction mixture (25 µl) consisted of: 2 µl of cDNA, 0.5 µl of each primer (20 µM), 2.5 µl of 10x buffer with $MgSO_4$, 2 µl of 2.5 mM dNTPs, 0.2 µl of 5 units/µl pfu DNA polymerase (Fermentas), and ultra-pure distilled water (UDW). The PCR condition consisted of 1 cycle denaturing at 94ºC for 1 minute; 35 cycles of annealing

Table 1 Primer sequences used for the amplification of the *PA-5'*, *PB1-5'* and *PB2-5*' gene segments and the sizes of their PCR amplicons

Primer	Sequence	PCR product (bp)
PA-5':		
Forward	5'-AGGATCCAATGGAAGACTTTGTG-3'	720
Reverse	5'-AAAGCTTGCCGTTCGGTTCG-3'	
PB1-5':		
Forward	5'-AGGATCCAATGGATGTCAATC-3'	1,134
Reverse	5'-ACTCGAGAAGATCAATGTTTGCAAGC-3'	
PB2-5':		
Forward	5'-TGTCGACATGGAGAGAATAAAAG-3'	1,212
Reverse	5'-ACAAGCTTGAACACCATTGCTAC-3'	

Underlined alphabets are restriction sites of endonucleases

at 45°C for 1 minute, extension at 72°C for 1 minute, and denaturing at 94°C for 1 minute. The final extension was done at 72°C for 10 minutes. A Thermo-Cycler (Eppendorf, Germany) was used. The sizes of the PCR products were verified by a 1% agarose gel electrophoresis, ethidium bromide staining, and UV transillumination in comparison to the bands of the 1 kb DNA ladder (Fermentas) run concurrently in the same gel.

Cloning of the PA-5', PB1-5' and PB2-5'sequences into cloning vectors

The *PB1-5'*, *PB2*-5' and *PA-5'* amplicons were cloned into a pTZ57R/T vector (Fermentas) *via* the overhang T of the vector and the A of the DNA amplicons. The ligation mixture (30 µl) contained: 6 µl of 5x ligation buffer, 3 µl of the vector, 6 µl of the *PB1-5'*, *PB2*-5' or *PA-5'* sequences, 1 µl of T4 DNA ligase (5 units/µl) and UDW. The mixture was incubated at 22°C overnight. Individual ligation products were introduced into competent JM109 *E. coli* cells by using a chemical transformation method. White colonies of the transformed *E. coli* grown at 37°C overnight on a selective agar plate, *i.e.,* LB agar containing 100 µg ampicillin/ml, 100 mM IPTG, and 5% X-Gal were randomly picked and individually inoculated into 5 ml of LB broth containing 100 µg ampicillin/ml (LB-A) and incubated at 37°C with shaking at 250 rpm overnight. The cells were harvested by centrifugation at 4,000 x *g* for 5 minutes. The recombinant plasmids were extracted from the *E. coli* cell pellets for the DNA insert verification by using the alkaline lysis method. The verified recombinant plasmids were doubly digested with restriction endonuclease enzymes (Fermentas). The restriction enzymes and the respective buffers used were: *Bam*HI and *Xho*I, and *Bam*HI buffer for *PB1-5'*; *Sal*I and *Hin*dIII and 2x Tango buffer for *PB2-5'*; and *Bam*HI and *Hin*dIII, and *Bam*HI buffer for *PA*-5'. The digested product was subjected to a 1 % agarose gel electrophoresis, ethidium bromide staining, and UV transillumination in order to ensure that the recombinant plasmids contained DNA inserts of the correct sizes. Then the recombinant plasmids were extracted from the *E. coli* cells using a plasmid extraction kit (Qiagen, Germany) according to the manufacturer's instruction. The DNAs were sequenced and aligned with the relevant sequences, *i.e.,* N-terminal-PB1, N-terminal-PB2 and N-terminal-PA-coding sequences of the influenza A viruses available at the GenBank of the National Center for Biotechnology Information using the BLAST network services.

Cloning of the PB1-5', PB2-5' and PA-5'-coding sequences into protein expression vectors

The recombinant plasmids carrying *PB1-5*' sequences from the cloning vector were subcloned into a pET-20b(+) protein expression vector (Novagen, Germany) whereas the recombinant plasmids carrying *PB2-5'* and *PA-5'* sequences were individually subcloned into pQE30 and pQE31 protein ex-

pression vectors (Qiagen, Germany), respectively. BL21 (DE3) pLysS *E. coli* cells were used as protein expression hosts for the recombinant *PB1-5'*-pET-20b(+) vector, whereas M15 *E. coli* cells were used as protein expression hosts for the recombinant *PB2-5'*-pQE30 and *PA-5'*-pQE31 vectors.

Expression of the recombinant N-terminal-PB1, -PB2 and -PA

A single transformed *E. coli* colony carrying the *PB1-5'*, *PB2-5'* or *PA-5'* recombinant vector was inoculated into LB-A broth and the culture was incubated at 37°C with shaking at 250 rpm overnight. Five milliliters of the overnight *E. coli* culture were added into 500 ml of a fresh LB-A broth and incubated at 37°C with shaking until the A_{600nm} reached 0.6. IPTG was added to a final concentration of 1 mM and incubated further at 37°C for 3 hours. The bacterial cells were harvested by centrifugation at 4,000 x *g* at 4°C for 20 minutes. For the preparation of the N-terminal PA protein, bacterial cells carrying a recombinant *PA-5'*-pQE31 vector (in one gram of pellet) were resuspended in 5 ml of lysis buffer-1 (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). The cells were sonicated at 40% amplitude and 0.5 cycles in an ice bath. The cell debris was removed by centrifugation at 12,000 x *g* at 4°C for 20 minutes. The supernatant (cell lysate) was transferred to a new tube and the recombinant protein was purified using a Ni-NTA agarose (Qiagen, Germany). For preparing the N-terminal PB1 and PB2, bacterial cells carrying the respective recombinant plasmids (one gram) were resuspended in 5 ml of lysis buffer-2 (100 mM NaH_2PO_4, 10 mM Tris HCl, 8 M urea, pH 6.3). The bacterial lysates were prepared and purified as for the N-terminal PA protein.

Phage bio-panning for selecting phage clones that display HuScFv specific to the recombinant N-terminal polymerase proteins

Phage library displaying human single chain variable fragments

The M13 phage library displaying human single chain variable fragments (VH-peptide linker-VL; HuScFv) was constructed in our laboratory from peripheral blood B lymphocytes of 60 Thai blood donors using pCANTAB5E phagemids and M13KO7 helper phages. This large repertoire library had an antibody diversity of ~2.6 x 10^8. Before use in the bio-panning, the library was propagated in *E. coli* cells which were co-infected with the helper phages to yield a phage titer of ~6.5x 10^{12} cfu/ml.

Preparation of E. coli *transformants carrying recombinant* huscFv-*phagemids*

Purified recombinant N-terminal-PB1,-PB2 and -PA proteins were used as antigens in phage bio-panning to select phage clones displaying HuScFv that could bind to the respective proteins. In the bio-panning process, one microgram of each purified recombinant protein in a carbonate-bicarbonate buffer, pH 9.6 (coating buffer) was individually immobilized in wells of an ELISA plate (EIA/RIA 8 well strip-flat bottom, Corning, NY, USA). The plate was incubated at 37ºC overnight then the wells were washed with a washing buffer (PBS containing 0.1% Tween-20; PBST). Two hundred microliters of a blocking solution (5% skim milk in PBS) were added into each well and incubated at 25°C for 1 hour. The blocking solution was discarded and the wells were washed three times with PBST. The phage library (~3x 10^{11} phage particles) was added into each antigen coated well and the plate was incubated for 1 hour. During the incubation, the contents in each well were mixed every 15 minutes. After discarding the fluid from each well, all wells were extensively washed with PBST to remove unbound phages. Phages bound to the immobilized antigen were harvested by adding 50 µl 0.1 M HCl-glycine, pH 2.2 and the plate was incubated at 25ºC for 5 minutes. The fluid in each well was individually collected into a 1.5 ml-tube containing 3 µl of 2 M Tris-base. The fluid was then added into a tube containing 200 µl of HB2151 *E. coli* which had been grown to log-phase and the tube was incubated at 37ºC for 20 minutes. An aliquot of 125 µl was used to spread onto each 2x YT agar plate containing 100 µg/ml of ampicillin and 2% glucose (2x YT-AG) and the plates were incubated at 37ºC overnight.

Determination of *E. coli* colonies with *huscFv*-phagemids

For determining the transformed *E. coli* that carried the *huscFv*-phagemids, a small portion of the bacterial colonies on the 2x YT-AG plate were ran-

domly picked and a replica plate was made. Bacteria of the same colony remaining on the loop were inoculated into 100 µl of distilled water and used directly as DNA template for checking the presence of the *huscFv* by PCR. The PCR was carried out using a specific pair of primers: forward, 5'-CCATGATTACGCCAAGCTTTGGAGCC-3' and reverse, 5'-GCTAGATTTCAAAACAGCAGAAAGG-3' primers. The 25 µl PCR reaction mixture contained 1 µl of DNA template, 2.5 µl of 10x PCR buffer with KCl, 1.5 µl of $MgCl_2$, 2 µl of 25 µM dNTPs, 0.5 µl of each 25 µM primer, 0.2 µl of *Taq* DNA polymerase (5 units/µl) (Fermentas) and distilled water. The PCR conditions were: 1 cycle denaturing at 94ºC for 5 minutes, 30 cycles of annealing at 50ºC for 1 minute, extending at 72ºC for 1.5 minutes, and denaturing at 94ºC for 1 minute. Additional chain elongation was performed at 72ºC for 10 minutes. After amplification, the PCR products were analyzed for the presence of *huscFv* by a 1% agarose gel electrophoresis and ethidium bromide staining. The PCR products were also subjected to *Mva*I restriction endonuclease digestion for determining the restriction fragment length polymorphism (RFLP) of the *huscFv* sequences derived from individual phage clones.

Verification of the HuScFv binding activity to recombinant polymerase proteins

The HB2151 *E. coli* colonies that gave positive *huscFv* amplicons of the expected size (~1,000 bp) were picked from the replica 2x YT-AG plate and individually grown in 2x YT-A broth containing 1 mM IPTG. For verification of the ability to express HuScFv, the *E. coli* cells were suspended in PBS and subjected to sonication by using the ultrasonic-homogenizer LABSONIC® P (Satorius AG, Germany) at 40% amplitude for 0.5 cycles in an ice bath. The bacterial homogenate was centrifuged at 12,000 x *g* at 4ºC for 10 minutes; the supernatant (bacterial lysate) was collected. The lysate of each *E. coli* clone was subjected to an indirect ELISA (HuScFv-ELISA) and a dot-ELISA for detecting the binding specificities of the HuScFv to the respective recombinant polymerase proteins.

For the indirect ELISA, one microgram of individual recombinant protein was used to coat each well of an ELISA plate. After incubation and blocking the unoccupied sites in each well with 5% skim milk, 100 µl of individually transformed HB2151 *E. coli* lysates were incubated with the immobilized polymerase proteins in the wells using HuScFv specific to TlyA hemolysin of *Leptospira* spp. as irrelevant HuScFv control, lysate of normal HB2151 *E. coli* as negative HuScFv control, and PBS as blank. After incubation, the wells were washed with PBST and the HuScFv bound to the target proteins were detected by using mouse anti-E-Tag monoclonal antibody (Amersham, USA), goat anti-mouse immunoglobulin-HRP conjugate (Southern Biotech, AL, USA) and ABTS (2, 2 azino-bis [3-ethylbenzthiazoline-6-sulfonic acid]) substrate (ZYMED CA, USA) as detection reagents. The ELISA signal was measured at A_{405nm} using a Multiskan® *EX*, Labsystem ELISA reader.

For the dot-ELISA, 3 µl aliquots of individual recombinant proteins were applied onto a NC membrane and air-dried. Empty sites on the NC were blocked using 5% skim milk in PBS and washed. The NC was cut into squares, each square containing one protein dot, which were incubated individually with HuScFv prepared from different clones of the transformed HB2151 *E. coli* at 25ºC for 1 hour with gentle agitation at 4ºC overnight. The recombinant protein-HuScFv reaction was revealed by using the mouse anti-E-Tag monoclonal antibody, goat anti-mouse immunoglobulin-AP conjugate and BCIP/ NBT phosphatase substrate. The results were observed visually.

RFLP patterns of the *huscFv* sequences

The RFLP of the *huscFv* sequences encoding the specific HuScFv to the three recombinant proteins were studied by digesting the individual *huscFv* amplicons with *Mva*I restriction endonuclease. The digestion mixture consisted of 2 µl of the *huscFv* PCR product, 2.5 µl of 10x buffer, 0.5 µl of *Mva*I (10 units/µl) and 20 µl of UDW. The mixture was incubated at 37ºC for 6 hours. The digested product was subjected to electrophoresis in a 12% polyacrylamide gel in 0.5% TBE buffer. The DNA fragments in the gel were visualized under a UV transilluminator (Biodoc-It™ Imaging system, UVP Transilluminator, Cambridge, UK) after staining with ethidium bromide. The diversity of the DNA banding patterns of the *huscFv* from different clones was observed.

RESULTS

Cloning of the *PB1-5'*, *PB2-5'* and *PA-5'* sequences

Complementary DNA was produced from the total RNA extracted from the influenza A virus subtype H5N1 [A/duck/Thailand/144/2005(H5N1)] by RT-PCR using a ThermoScript™ Revers Transcriptase (Invitrogen). The quality of the cDNA was checked by PCR using specific primers for detecting hemagglutinin 5 gene segments. The PCR amplicon of the hemagglutinin gene segments was seen as a band of about 545 bp (Fig. 1A) implying good quality of the cDNA preparation. The cDNA was used as a template for PCR amplification of the 5'-halves of the PB1, PB2, and PA-coding sequences using specific primers for individual genes. The *PB1-5'*, *PB2-5'* and *PA-5'* amplicons were seen as bands of 1,134, 1,212 and 720 bp, respectively (Fig. 1B) which were the expected sizes of the respective gene segments. A BLAST search across multiple nucleotide sequences of the database showed that our cloned *PB1-5'* and *PB2-5'* sequences recovered from the transformed-JM109 *E. coli* that harbored the recombinant pTZ57R/T cloning vectors with the *PB1-5'* and *PB2*-5' inserts had 100% identity to the *PB1* (*e.g.*, accession numbers EF112238.1, EF112245.1, EF112246.1) and *PB2* (*e.g.*, accession no. EF-112217.1, EF112219.1, EF112220.1) sequences of influenza A viruses of the database. Nevertheless, there was only 99% identity between our cloned *PA-5'* sequences to the *PA* sequences of the database (*e.g.*, accession no EF112270.1, EF112288.1 and EF112289.1).

Expression of the recombinant N-terminal-PB1, -PB2, and -PA polymerase proteins

Fig. 2 illustrates recombinant N-terminal-PB1 (Fig. 2A), -PB2 (Fig 2B), and -PA (Fig. 2C) proteins which were individually prepared and purified from homogenates of the respective transformed *E. coli* clones. The purified proteins were subjected to SDS-PAGE and Western blotting using mouse anti-His, goat anti-mouse-immunoglobulin-HRP and peroxidase substrate to reveal and verify the correct sizes of the recombinant polymerase proteins, *i.e.*, *Mr* of 43, 41, and 26 for PB1, PB2 and PA, respectively (Fig. 2D).

Phage bio-panning for selecting phage clones that displayed HuScFv specific to the N-terminal polymerase proteins

The recombinant polymerase proteins were used as antigens in phage bio-panning to select phage clones that displayed HuScFv to each protein.

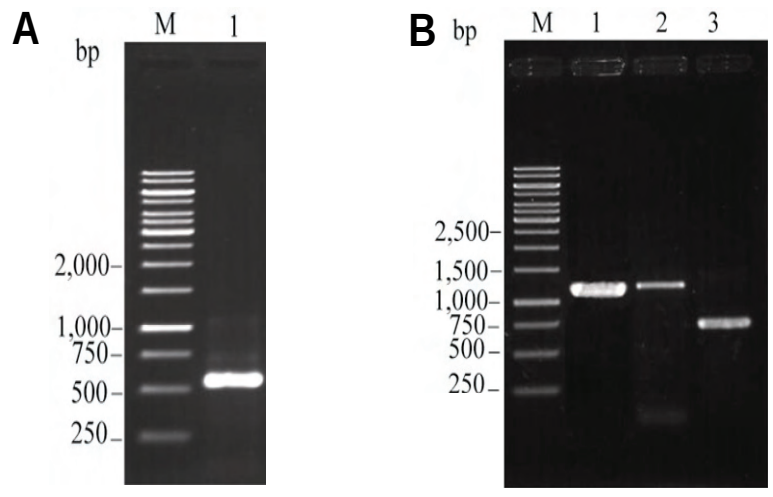

Fig. 1 (A) Amplicon of H5 gene segments and (B) amplicons of *PA-5', PB1-5'* and *PB2-5'* sequences (lanes 1-3, respectively) that were amplified using cDNA prepared from total RNA of the influenza virus, A/duck/-Thailand/2005(H5N1) as template. Lanes M, 1 kb DNA ladder. Numbers at the left of A and B are DNA sizes in base pairs (bp).

From 10 randomly selected transformed *E. coli* clones derived from phage bio-panning with recombinant N-terminal-PB1 and -PA, all of them, *i.e.*, clones b1-1 to b1-10 and clones a1-a10, revealed the *huscFv* sequences (left and right blocks of Fig. 3A, respectively). Only 7 of the 10 selected clones derived from phage bio-panning with the recombinant N-terminal-PB2 were positive for *huscFv* segments, *i.e.*, clones b2-1, b2-3 to b2-6, b2-9 and b2-10 (middle block of Fig. 3A). Among the 10 *huscFv* positive clones derived from bio-panning with the N-terminal-PB1 protein, 8 clones could express HuScFv, *i.e.*, clones b1-1, b1-4 to b1-10 (left block of Fig. 3B). The HuScFv could be expressed from all of the 7 *huscFv* positive clones that were derived from bio-panning with the N-terminal-PB2 (middle block of Fig. 3B), while only 8 of the 10 *huscFv* positive clones from bio-panning with the N-terminal-PA could express the HuScFv (right block of Fig. 3B).

Binding specificity of the HuScFv to the homologous recombinant polymerase proteins

HuScFv prepared from *huscFv* positive *E. coli* clones were tested by indirect ELISA for their

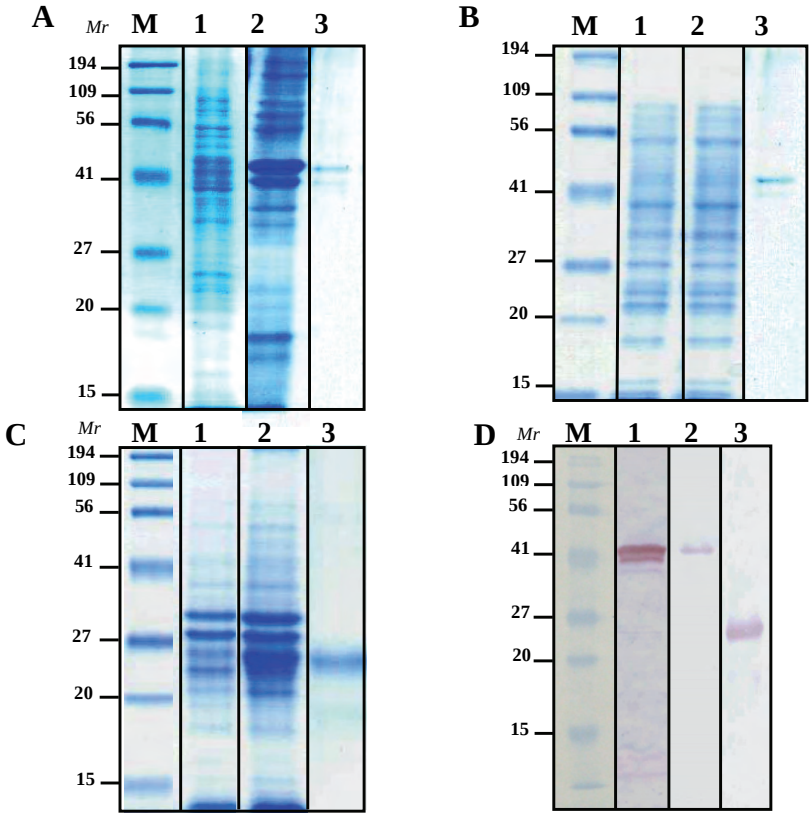

Fig. 2 Results of SDS-PAGE and Western blot analysis of the recombinant N-terminal-PB1, -PB2, and -PA. (A), SDS-PAGE-separated patterns of a selected transformed *E. coli* clone that carries recombinant *PB1-5'*-plasmids, grown under non-induced (lane 1) and IPTG-induced (lane 2) protein expression conditions; lane 3, doublet bands of purified recombinant N-terminal-PB1 protein. (B), SDS-PAGE-separated patterns of a selected transformed *E. coli* clone that carries recombinant *PB2-5'*-plasmids, grown under non-induced (lane 1) and IPTG-induced (lane 2) protein expression conditions; lane 3, doublet bands of purified recombinant N-terminal-PB2 protein. (C), SDS-PAGE-separated patterns of a selected transformed *E. coli* clone that carries recombinant *PA-5'*-plasmids, grown under non-induced (lane 1) and IPTG-induced (lane 2) protein expression conditions; lane 3, diffuse band of purified recombinant N-terminal PA protein. (D), Western blot patterns of SDS-PAGE separated-recombinant N-terminal PB1 (*Mr* ~41), -PB2 (*Mr* ~43), and -PA (*Mr* ~26), probed with mouse anti-E-Tag monoclonal antibody. Numbers on the left of all blocks are relative molecular masses (*Mr*).

binding activity to the homologous proteins. Fig. 4 shows the indirect ELISA results of HuScFv from transformed HB2151 *E. coli* that gave positive binding to the homologous recombinant N-terminal-PB1, -PB2, and -PA proteins. HuScFv prepared from 6 of the 8 HuScFv expressing clones, *i.e.*, clones b1-1, b1-5 to b1-8, and b1-10, could bind to the recombinant N-terminal-PB1 with significant ELISA OD (two times higher than OD of BSA which was used as the control) (Fig. 4A). HuScFv of 4 out of the 7 clones, *i.e.*, clones b2-1, b2-4, b2-9 and b2-10 bound to the recombinant N-terminal-PB2 (Fig. 4B). HuScFv of all of the 8 HuScFv expressing *E. coli* clones derived from bio-panning with the N-terminal-PA could bind to the homologous protein (Fig. 4C).

Fig. 5 shows dot-ELISA results of the indirect ELISA positive clones which were completely conform to the indirect ELISA results.

RFLP of the *huscFv* sequences of which the expressed HuScFv could bind to the homologous recombinant polymerase proteins

Fig. 6 shows *Mva*I-cut-DNA banding patterns of the *huscFv* sequences of the 6, 4, and 8 transformed HB2151 *E. coli* clones that expressed HuScFv bound to the recombinant PB1, PB2 and PA proteins, respectively. Four different RFLP patterns were found among the *huscFv* sequences of the 6 *E. coli* clones that produced HuScFv to the recombinant PB1 (lanes 1-6). The *huscFv* sequences from the 4 clones that expressed HuScFv to the recombinant PB2 had all different RFLP patterns (lanes 7-10). The *huscFv* of the 8 clones that expressed HuScFv to the recombinant PA had 7 different RFLP patterns (lanes 11-18).

DISCUSSION

Because of the pivotal role of the poly-

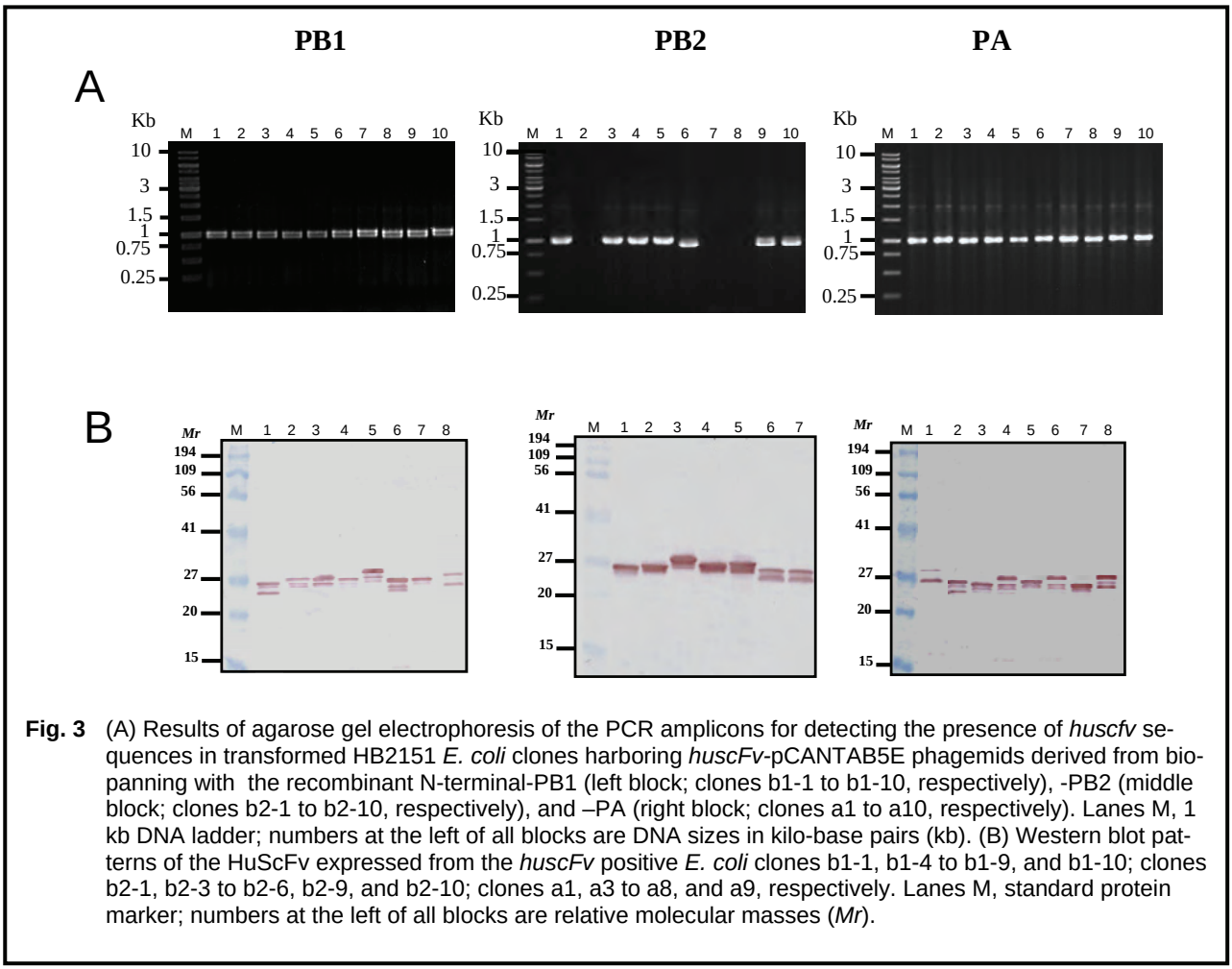

Fig. 3 (A) Results of agarose gel electrophoresis of the PCR amplicons for detecting the presence of *huscfv* sequences in transformed HB2151 *E. coli* clones harboring *huscFv*-pCANTAB5E phagemids derived from bio-panning with the recombinant N-terminal-PB1 (left block; clones b1-1 to b1-10, respectively), -PB2 (middle block; clones b2-1 to b2-10, respectively), and –PA (right block; clones a1 to a10, respectively). Lanes M, 1 kb DNA ladder; numbers at the left of all blocks are DNA sizes in kilo-base pairs (kb). (B) Western blot patterns of the HuScFv expressed from the *huscFv* positive *E. coli* clones b1-1, b1-4 to b1-9, and b1-10; clones b2-1, b2-3 to b2-6, b2-9, and b2-10; clones a1, a3 to a8, and a9, respectively. Lanes M, standard protein marker; numbers at the left of all blocks are relative molecular masses (*Mr*).

merase proteins in the infectious cycle of the influenza virus, we produced human monoclonal antibodies in the form of single chain variable antibody fragments (HuScFv) that specifically bound to the polymerase proteins by using a human antibody phage display library. The ultimate purpose is to develop cell penetrating antibodies that could interfere with the functions of the intracellular polymerase subunits in the virus transcription process. Because the native polymerase proteins are difficult to prepare directly from the virus, we produced recombinant N-terminal polymerase PA, PB1 and PB2 proteins of the influenza A virus, H5N1 subtype, instead of using the native counterparts. The recombinant proteins were used in phage bio-panning for selecting the phage clones that displayed HuScFv specific to the polymerase proteins on their surface. The selected phage clones were individually propagated in bacteria; the bacteria were grown, induced to express the HuScFv, and the HuScFv were purified. We have chosen the N-terminal portions of the three polymerase subunits as the HuScFv targets for several reasons: the recombinant N-terminal PA protein (amino acids 1-240) that we produced encompassed the nuclear localization signal (NLS) region-I which is located at amino acid residues 124-139 and a large part of the NLS region-II which is located at residues 186-247 of the native PA.[28,34] The recombinant N-terminal PB1 (amino acids 1-378) contains the peptides that bind to 3' and 5' of the vRNA at amino acid positions 1-83 and to the PA in the polymerase complex at amino acid residues 1-25. It contains also two NLS at amino acid residues 180-195 and 202-252. The recombinant N-terminal PB2 (amino acids 1-404) contains the viral nucleoprotein (NP) binding site at amino acids 1-269, the PB1 binding site at amino acids 206-258, and the cap binding site at amino acids 242-282. Antibodies that interfere with the biological functions located in the N-terminal portions of these polymerase proteins would inhibit the virus transcription and replication. Besides, producing the full length of the functional recombinant polymerase proteins is definitely not an easy task. To our knowledge, there is no report in the literature of a successful production of the full length recombinant polymerase proteins of the influenza virus using the prokaryotic expression system. Some attempts have been made to amplify the protein coding sequences,[35,36] but not the recombinant protein expression.

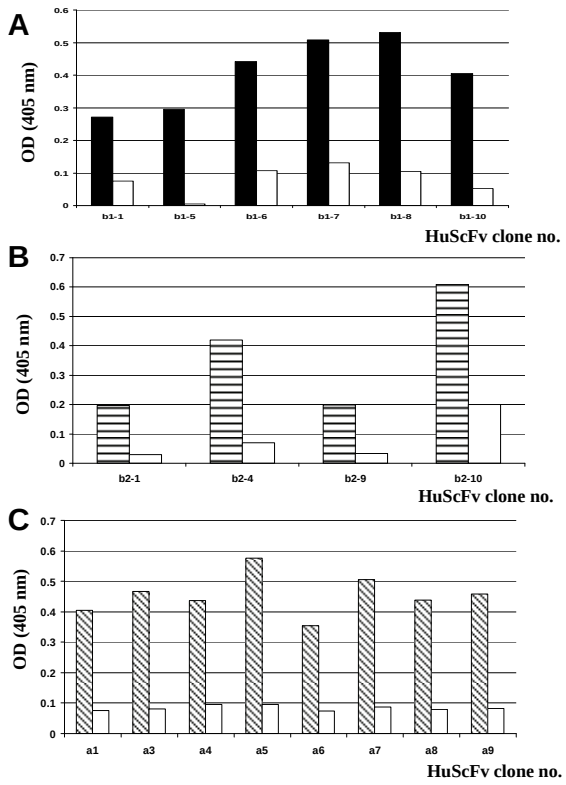

Fig. 4 Results of the HuScFv-ELISA for the detection of the binding of HuScFv expressed from the transformed HB2151 *E. coli* clones to the recombinant N-terminal-PB1 (A), -PB2 (B), and -PA (C). Only the HuScFv from the *E. coli* clones that were ELISA positive (OD at A_{405nm} two times higher than the OD at A_{405nm} of the BSA control) are shown.

The *PB1-5'*, *PB2-5'* and *PA-5'* sequences were successfully amplified from the cDNA. Their amplicons were seen as bands of about 1,134, 1,212 and 720 bp, respectively. Sequence analysis of the amplified *PB1-5'*, *PB2-5'* and *PA-5'* sequences showed that there were 99-100% identity of nucleotide sequences to those of the sequences deposited in the database. It has been previously reported that there was 98.8-100% identity of the PB1, PB2 and PA nucleotide sequences among the H5N1 virus strains isolated from Thailand during 2004-2005. Nucleotide sequences of 97.2-97.7% homology were found for the PB1, PB2 and PA coding sequences of the H5N1 strains isolated in Thailand in 2005 and China in 2005.[37]

The respective gene sequences were also successfully cloned into *E. coli* expression hosts. In the prokaryotic expression system, pET-20b(+), pQE30 and pQE31 protein expression vectors are commonly used, because they were specially constructed for the purpose of producing a fusion protein in which a 6x His tag is added spontaneously to the protein sequence. The 6x His tag is relatively small and does not interfere with the structure or function of the purified protein. The advantage of using this tag is the ability of the tag to bind to metal-chelating surfaces; therefore, the recombinant fusion protein can be simply purified and detected by using a nickel-affinity column and anti-His antibody, respectively. The pET-20b(+) vector contains the 6x His tag at the C-terminal of the inserted fragment. The advantage of this vector is to ensure that only full-length target proteins are purified and detected by the anti-His antibody. Although the pET-20b(+) vectors can be used for expression of all three recombinant polymerase proteins, we have explored the possibility of using the pQE expression vectors, *i.e.*, the pQE30 and pQE31, for the recombinant PB2 and PA polymerase protein expression. The pQE expression vectors contain the 6x His tag at the N-terminal of the inserted fragment. The recombinant proteins could also be purified using the anti-His antibody. Both pET-20b(+) and pQE protein expression vectors contain strong promoters, bacteriophage T7 and T5 promoters, respectively.

PB1-5' segments were subcloned into a pET-20b(+) protein expression vector and the *E. coli* BL21 (DE3) pLysS cell was used as expression host. The *PB2-5'* and *PA-5'* segments were subcloned into pQE30 and pQE31 protein expression vectors, respectively, and M15 *E. coli* cells were used as expression host. Recombinant N-terminal-PB1, -PB2, and -PA proteins have an approximate *Mr* of 43, 41, and 26, respectively. The recombinant N-terminal-PB1 and -PB2 were detected in the insoluble cytoplasmic fraction while the recombinant N-terminal-PA protein was found in both soluble and insoluble cytoplasmic fractions but the amount in the latter was more. To obtain the soluble target proteins, the conditions of protein expression were modified by

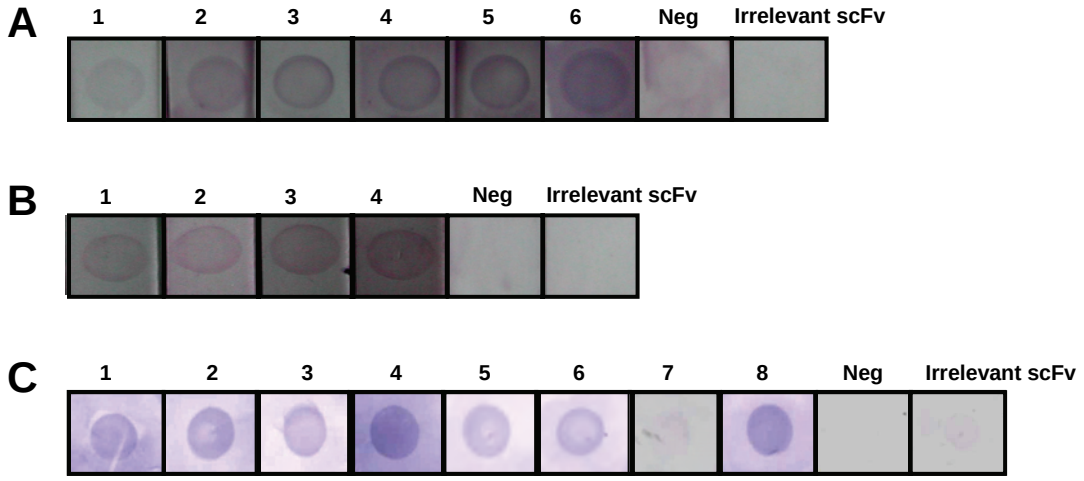

Fig. 5 Results of the dot-ELISA for the detection of the binding of the HuScFv to the respective homologous recombinant N-terminal PB1, -PB2, and –PA. Row A, all NC squares were dotted with recombinant N-terminal PB1; the antigen dotted NC were individually reacted with HuScFv from clones b1-1, b1-5 to b1-8, and b1-10 (blocks 1-6). Row B, all NC squares were dotted with recombinant N-terminal PB2; the antigen dotted NC were individually reacted with HuScFv from clones b2-1, b2-4, b2-9, and b2-10 (blocks 1-4). Row C, all NC squares were dotted with recombinant N-terminal PA; the antigen dotted NC were individually reacted with HuScFv from clones a1, and a3-a9 (blocks 1-8). Neg, PBS (blank) was used instead of HuScFv. Irrelevant scFv, HuScFv specific to *Leptospira* spp. TlyA hemolysin was used as the negative HuScFv control.

decreasing the concentration of IPTG (0.5-0.2 mM) and the bacterial culture temperature (30°C). The amount of soluble recombinant PA protein was slightly increased (data not shown). However, the recombinant PB1 and PB2 proteins were still found in the insoluble cytoplasmic fraction. All recombinant proteins were used for selecting phage clones displaying HuScFv to the epitopes on the molecules with the awareness that the important epitopes may not be exposed on the surface and could not be recognized by the HuScFv on the phage particles.

His-tagged recombinant N-terminal-PB1, -PB2 and -PA proteins were purified by a one-step purification process using a nickel column. His-tagged protein was individually captured by the column while other *E. coli* contaminants were washed out by a wash buffer. Bound recombinant proteins were then eluted out with an appropriate eluting buffer. The technique is convenient and the amount of target proteins was adequate for our experimental use. Nevertheless, for larger quantities, other expression systems such as yeast or insect cell expression systems might have to be used.

The recombinant proteins were individually used as antigens in the phage bio-panning for the selection of the phage clones displaying HuScFv to the protein epitopes. A single round of the bio-panning was used instead of the repeated rounds of the process commonly used by other investigators.[38] The repeated bio-panning (~2-4 cycles) eliminates the non-specific phage clones but tends to generate complete and partial deletions of the *huscFv* gene insert from the phage genome rendering unproductive clones. From the single round bio-panning in this study, 70-100 % of HB2151 *E. coli* clones transformed with individual *huScFv*-phagemids containing the *huscFv* gene insert (Fig. 3A), and 80-100 % of the representative *huscFv* positive clones that were screened could produce HuScFv (Fig. 3B), indicating that some *huscFv*-phagemids carrying *E. coli* could not express HuScFv under the conditions used in this study. One reason for this could be that the *huScFv* contains a stop codon within the nucleotide sequence.

The *huscFv* sequences of the selected phage clones were determined by observing the multiplicity of the DNA banding patterns by RFLP. An RFLP pattern multiplicity of 80-100% was found for the *huscFv* of *E. coli* clones that could produce the HuScFv (Fig. 6). This implies that the epitope diversity of the HuScFv might be high, *i.e.*, the HuScFv produced from different *E. coli* clones may bind to different epitopes on the recombinant polymerase

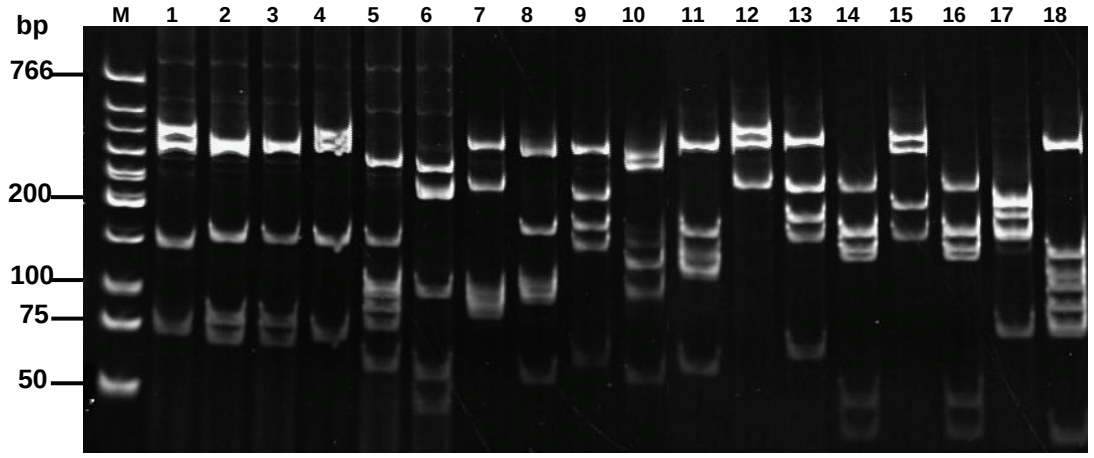

Fig. 6 DNA banding patterns for determining RFLP of *Mva*I-cut *huscFv* amplified from recombinant pCANTABE5 phagemids transformed HB2151 *E. coli* clones. Lanes 1-6, DNA banding patterns clones b1-1, b1-5 to b1-8, and b1-10, respectively; lanes 7-10, DNA banding patterns clones b2-1, b2-4, b2-9, and b2-10, respectively; lanes 11-18, DNA banding patterns clones a1, and a3-a9, respectively. Lane M, Low molecular size DNA ladder. Numbers at the left are DNA sizes in base pairs (bp).

proteins or the HuScFv of phage clones with different RFLP banding patterns might have different binding affinities to the same epitope.

The expressed HuScFv were found to bind specifically to their homologous proteins when tested by indirect ELISA and dot-ELISA *in vitro* (Fig. 4 and 5). Nevertheless, the ELISA OD and the intensity of the color spots of the dot-ELISA were different among clones of the same protein specificity. A possible reason is that although the *E. coli* transformants were grown under the same condition they did not divide and perhaps did not produce the HuScFv at the same rate. That would render variations in both the numbers of *E. coli* cells as well as their produced HuScFv molecules.

Summarizing, human monoclonal antibodies to the N-terminal-PB1, -PB2 and -PA polymerase proteins of influenza A virus H5N1 subtype were successfully produced in the form of ScFv using a human antibody phage display library. The next step is to test the inhibitory activities of the herein produced specific HuScFv on the polymerase biological functions and to identify their epitope specificity.

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