Characterization of mAb6-9-1 monoclonal antibody against hemagglutinin of avian influenza virus H5N1 and its engineered derivative, single-chain variable fragment antibody

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Hemagglutinin (HA), as a major surface antigen of influenza virus, is widely used as a target for production of neutralizing antibodies. Monoclonal antibody, mAb6-9-1, directed against HA of highly pathogenic avian influenza virus A/swan/Poland/305-135V08/2006(H5N1) was purified from mouse hybridoma cells culture and characterized. The antigenic specificity of mAb6-9-1 was verified by testing its cross-reactivity with several variants of HA. The mimotopes recognized by mAb6-9-1 were selected from two types of phage display peptide libraries. The comparative structural model of the HA variant used for antibody generation was developed to further facilitate epitope mapping. Based on the sequences of the affinity- selected polypeptides and the structural model of HA the epitope was located to the region near the receptor binding site (RBS). Such localization of the epitope recognized by mAb6-9-1 is in concordance with its moderate hemagglutination inhibiting activity and its antigenic specificity. Additionally, total RNA isolated from the hybridoma cell line secreting mAb6-9-1 was used for obtaining two variants of cDNA encoding recombinant single-chain variable fragment (scFv) antibody. To ensure high production level and solubility in bacterial expression system, the scFv fragments were produced as chimeric proteins in fusion with thioredoxin or displayed on a phage surface after cloning into the phagemid vector. Specificity and affinity of the recombinant soluble and phage-bound scFv were assayed by suitable variants of ELISA test. The observed differences in specificity were discussed.

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Abbreviations: CDR, complementarity determining region; HA, hemagglutinin; HI, hemagglutination inhibition test; HRP, Horseradish Peroxidase; mAb, monoclonal antibody; PBS, phosphate-buffered saline; pfu, plaque forming unit; scFv, single-chain variable fragment antibody; RBS, receptor binding site; VH, variable region of the heavy chain; VL, variable region of the light chain

INTRODUCTION

Antibodies are used in many research applications, as well as in various diagnostic assays and medical treatments. Hybridoma technology enables production of huge amounts of monoclonal antibodies (mAb) against the target antigen but it is a laborious, time consuming and expensive technique. Growing demand for recombinant antibodies and their derivatives prompted the development of various expression platforms. Up to date many attempts of their production in mammalian cells (Ho *et al.*, 2006), insect cells (Choo *et al.*, 2002), plants (Dobhal *et al.*, 2013), yeast or bacteria (Rouet *et al.*, 2012) have been reported. The derivatives of antibodies such as the antigen-binding fragment (Fab) or the single chain variable fragment (scFv) are of particular interest. Their reduced structures and molecular weights facilitate expression and purification process. The scFv fragment consists of variable regions of heavy (VH) and light (VL) chains of immunoglobulin connected by a flexible linker (Kalenik *et al.*, 2014). The scFv can be modified by conjugation to drugs or toxins and may penetrate the blood and tissue more rapidly than a complete antibody (Cao *et al.*, 2012a; Oriuchi *et al.*, 2005). Easiness of modification gives many possibilities of direct or indirect detection of this protein and facilitates manipulations of its activity (affinity to antigens). These properties are especially important for such targets as rapidly evolving surface antigens of influenza virus.

The influenza viruses belong to *Orthomyxoviridae* family. Their genome consists of a single- stranded negative sense RNA. Due to the antigenic drift and shift, resulting frequently in novel mutants and re-assortants that are able to cross the species barriers, the influenza viruses are still a serious global problem for humans and their livestock, especially for poultry and pigs. Highly pathogenic avian H5N1 influenza viruses are responsible for serious economic losses to the poultry industry and have caused numerous threats to public health. Since 1996 when H5N1 virus was isolated for the first time in China it has spread rapidly and continued to evolve, which resulted in a periodic emergence of new phylogenetic groups in several regions of the world. Currently, H5N1 viruses are divided into multiple clades and sub-clades based on antigenic variation (Smith *et al.*, 2012; Smith *et al.*, 2015; Li *et al.*, 2013). Hemagglutinin (HA), a strong surface antigen, is synthesized as a precursor, HA0 molecule, which is cleaved into two subunits, HA1 and HA2 (Szewczyk *et al.*, 2014). The HA1 subunit encompasses the membrane-distal globular head containing the receptor binding site (RBS) and the vestigial esterase domain. The HA2 subunit encompasses the stalk region (Velkov, 2013). The main functions of HA during virus infection are attachment of the virus to the host cell re-

ceptor and mediation of the fusion of the virus envelope with the endosomal membrane after endocytosis. This glycoprotein is a major target for anti-virus antibodies and a subject of high antigenic variability (antigenic drift). Identification and characterization of its antigenic epitopes is needed for understanding of the molecular mechanisms of antigenic drift and viral immune escape, which in turn might result in better selection of the vaccine strains. The reactivity of selected monoclonal antibodies with escape mutants of H5 HA were intensively studied but the map of H5 antigenic epitopes is still incomplete. Most of the characterized mAbs against HA from H5N1 virus recognize the epitopes localized in the globular head of HA, most of them overlap the RBS and are isolate-specific, (e.g. Cao *et al.*, 2012b; Du *et al.*, 2013; Wu *et al.*, 2014), while antibodies binding to the stem region have often a broad activity (Tan *et al.*, 2015; Zuo *et al.*, 2015). Extensive review of H5 HA antigenic sites was provided by (Velkov *et al.*, 2013) who described the anti-HA antibodies divided into 3 groups: (i) RBS selective, (ii) non-RBS, membrane-distal globular domain selective and (iii) HA2 selective.

The mAb6-9-1 is a monoclonal mouse antibody secreted by the hybridoma cell line 6-9-1, which was generated by us from immune cells of a mouse immunized with the recombinant H5 HA from A/swan/Poland/305-135V08/2006 (H5N1). We described usage of Fab (antigen-binding fragment) fragment of mAb6-9-1 in electrochemical immunosensor detecting HA from H5N1 viruses (Jarocka *et al.*, 2014). It was applied for detection of HA (17-530 aa) from A/Bar-headed Goose/Qinghai/12/05(H5N1) and HA1 subunit of HA from two strains of H5N1 viruses: A/swan/Poland/305-135V08/2006 and A/Vietnam/1194/2004 (Jarocka *et al.*, 2014). The strongest binding was observed for the long HA from A/Bar-headed Goose/Qinghai/12/05(H5N1) with the detection limit of 2.2 pg/mL. The successful application of the derivatives of this antibody prompted us to perform its broaden characterization, especially mapping of an epitope recognized by mAb6-9-1 on the structural model of the HA from A/swan/Poland/305-135V08/2006 (H5N1). Moreover, we described cloning, expression and characterization of recombinant scFv fragments derived from the mAb6-9-1. The specificity of two variants (VLVH and VHVL) of scFv produced in fusion with thioredoxin were evaluated by indirect and sandwich ELISA assays and confirmed with the scFv variant produced by the phage display method.

MATERIALS AND METHODS

Hybridoma propagation and monoclonal antibody purification. Mouse hybridoma cell line 6-9-1 that secreted monoclonal antibody against hemagglutinin from A/swan/Poland/305-135V08/2006 (H5N1) was generated from splenocytes obtained after immunization of mice with the recombinant H5 HA. The hybridoma cells were maintained in RPMI-1640 medium (20 mM L-glutamine, 10% fetal bovine serum, 5% CO_2, 37°C). The mAb6-9-1 was purified from culture supernatant using protein G (ThermoScientific).

Comparative modeling of HA from A/swan/Poland/305-135V08/2006 (H5N1). Structures suitable for modeling were chosen from a default BLAST search against the PDB database (accessed February 5th, 2015), with respect to the serotype (H5), X-ray resolution, and sequence length. Two structures emerged through these criteria: 2ibx (A/Vietnam/1194/2004 (H5N1)) and 2fk0

(A/Vietnam/1203/2004 (H5N1)), both having over 90% sequence identity. Preparation of X-ray structures was done in Chimera (v1.11) and consisted of deletion of non-standard residues and HOH molecules, N- and C-terminal fix and incomplete side chains repair. Both structures were aligned with MatchMaker (Meng *et al.*, 2006) and the most similar chains were used as templates; 2IBX_A, 2IBX_B, 2FK0_K, 2FK0_L. Modeler software (Sali & Blundell, 1993) was used throughout the modeling process. Ten different models were generated and manually evaluated with respect to hydrogen bond patterns, AA contact and clashes and Ramachandran plot. The best model was subjected to a minimization procedure using AMBER force field (ff14SB) and a steepest descent procedure to relieve steric hindrances (15Å box, TIP3PBOX solvent model and Cl⁻ to neutralize the structure).

Epitopes mapping. Epitope recognition by purified mAb6-9-1 was determined using two Phage Display Peptide Libraries, Ph.D.™-C7C and Ph.D.™-12 (New England Biolabs) according to the manufacturer's protocol (Ph.D Phage Display Libraries, version 1.2, New England Biolabs). Briefly, the 96-well plates (Maxisorb Nunc) were coated with purified mAb6-9-1 (7.5 µg/well) and incubated at 4°C overnight. After washing with TBST (TBS + 0.1% Tween-20) and blocking with BSA solution (5 µg/ml), the titrated phage peptide library (Ph.D.™-C7C or Ph.D.™-12) was added to each well (10^7–10^8 plaque forming units (pfu) in 100 µl) and incubated for 60 minutes at room temperature. Unbound phages were discarded, plates washed 10 times with TBST and bound phages were eluted with 0.2 M glycine-HCl, (pH 2.2), 1 mg/ml BSA. In a parallel experiment the OET protein (see below, Antigens and antibodies used in ELISA) at concentration 0.8 mg/ml was used for phage elution. Eluted phages were amplified and taken to the next panning rounds. After three rounds of panning several phage clones were randomly selected, subjected to phage ELISA, (performed according to the protocol described in Ph.D Phage Display Libraries, New England Biolabs) to confirm their binding capacity with mAb6-9-1. DNA isolated from individual phage clones was sequenced and amino acid sequence of the corresponding peptides determined. The Pepitope server (http://pepitope.tau.ac.il/) (Mayrose *et al.*, 2007a) was used to map the selected peptides onto the 3D model of HA from A/swan/Poland/305-135V08/2006 (H5N1). Using the consensus methodology, both PepSurf (Mayrose *et al.*, 2007b) and Mapitope algorithms (Bublil *et al.*, 2007) were executed and their results combined into a single prediction including only residues predicted to be a part of the epitope in both algorithms. Mapping results were compared with the surface of the H5 HA structural model using UCSF Chimera (v1.11).

Synthesis of the scFv cDNA. Primers. Sequences of primers used in the experiment of scFv synthesis and cloning are listed in the Supplementary file Table S1 at www.actabp.pl.

Synthesis of VH and VL regions. Sequences encoding the variable regions of light (VL) and heavy (VH) chains were obtained according to the method and procedure described by (Ladiges & Osman, 2001). The main steps of procedure are described compendiously below. Total RNA was isolated from the hybridoma cell line 6-9-1 using RNeasy Plus Micro Kit according to the manufacturer's instructions (Qiagen). 6.5 µg of isolated RNA and oligo (dT)15 primer were used to synthesize blunt-ended cDNA using the cDNA Synthesis System (Roche). Next, the PCR OG/GO linker was ligated to

the cDNA by T4 DNA ligase (Invitrogen). The resulting cDNA was used to amplify VH and VL fragments using Easy-A High-Fidelity PCR Master Mix (Agilent Technologies) supplemented with the sets of primers, OG and IgGkapp or OG and IgG1HC1 for VL and VH synthesis, respectively. IgGkapp primer corresponded to the constant region of the light chain CL whereas IgG1HCl corresponded to the CH1 gene (sequences are provided in Supplementary file 1: Table S1 at www.actabp.pl). The PCR products were cloned into the pGEM-T easy vector (Promega). Several clones were sequenced and analyzed using vbase2 on-line program (http://www.vbase2.org/vbdnaplot.php) to determine sequences of CDRs of VH and VL.

Assembling of scFv. Selected functional clones of light and heavy chain of mouse IgG were used to synthesize scFv construct. First, to obtain scFv in VH-VL orientation the variable fragments of heavy and light chains were amplified using two pairs of primers either VHNdeF and scFvVHR or scFvVKF and VkNotR. Then VH and VL fragments were assembled into scFv by overlapping PCR reaction using external primers VHNdeF and VkNotR. The same method was applied to synthesize scFv in opposite orientation (VL-VH) using specific primers: NdeVLF, linkVLR and linkVHF, VHrevNot. The PCR reactions were performed using *Pfu* Polymerase (Fermentas). Finally, PCR products were digested with NdeI and NotI restriction enzymes and ligated into the modified pET26b expression vector (Novagen) carrying the sequence encoding His- and Strep-tags previously cloned by us.

scFv expression in *E. coli*. The scFv constructs were re-cloned from the pET26b into the modified pET201 vector carrying the sequence encoding thioredoxin which resulted in obtaining two recombinant plasmids: pET201/TVHVL and pET201/TVLVH that were used for scFv overexpression in *E. coli* Origami (DE3) strain. For an efficient production of recombinant TscFvs several different conditions of temperature (37°C, 30°C, 25°C, 20°C), IPTG concentration (10 mM, 1 mM, 0.1 mM, 0.05 mM, 0.01 mM) and time of induction (between 1 h–48 h) were tested. The TscFv expressed by *E. coli* were purified on a gravity flow Ni-TED 1000 column (Marcherey-Nagel) in denaturing conditions according to the procedure described in manufacture's manual (Marcherey-Nagel). The first and the second elution fractions were mixed and dialyzed against stepwise decreasing concentration of urea in the range between 6.0–0 M. Each step lasted at least 3 hours. Purity of recombinant proteins was evaluated with SDS PAGE.

Phage displayed scFv (PhscFv). The cDNA fragment encoding scFv in VL-VH orientation (without thioredoxin) was amplified by Easy A polymerase (Easy-A High-Fidelity PCR Master Mix, Agilent Technologies) with the RRPvuF and VHrevNot primers (sequences are provided in Supplementary file 1: Table S1 at www.actabp.pl) using recombinant plasmid pET26b carrying scFv cDNA as a template. The obtained PCR product was digested with PvuII and NotI and cloned into pSEX81 phagemid vector (Progen). Small-Scale Phage Rescue was conducted according to the published protocol 154.3.4 by (Dorsam et al., 2002) with minor changes (first centrifugation was prolonged to 25 min and 5 h incubation in 37°C was changed to 18 h in 30°C). The obtained phages (PhVLVH) were titrated according to the online protocol 'Helper Phage Production' (Stockinger Lab website: http://www.oardc.ohio-state.edu/stockingerlab/Protocols/HelperPhage.pdf) and used in Phage ELISA. The ELISA was conducted according to (Dor-

sam et al., 2002) with minor modifications. Briefly, MaxiSorp plates were coated with 500 ng of the antigen and incubated overnight at 4°C, washed with PBS, blocked (2 h, 2% skim milk in PBS), incubated with 107-108 pfu for 2 h, washed and incubated with anti-M13 antibodies conjugated with HRP (GE Healthcare).

Sequencing of mAb6-9-1. The amino acid sequence of heavy and light chains of mAb6-9-1 was estimated by mass spectrometry of trypsin-digested mAb fragments. The analysis was performed in Mass Spectrometry Lab at the Institute of Biochemistry and Biophysics Polish Academy of Sciences, Warsaw.

Hemagglutination inhibition test. HI tests were performed as described previously (Stachyra et al., 2014; Stachyra et al., 2016) using inactivated viruses as antigens, either the highly pathogenic A/Turkey/Poland/35/2007 (H5N1) (National Veterinary Research Institute, Pulawy, Poland) or the low pathogenic A/chicken/Belgium/150/1999 (H5N2) (DG Deventer, Netherlands). HI titer was defined as the reciprocal of the highest dilution of sera that completely inhibited hemagglutination.

Antigens and antibodies used in ELISA. The following antigens corresponding to HA from A/swan/Poland/305-135V08/2006 (H5N1) were used: (1) HA/Nde, a short variant containing region of 17-340 aa with His-tag at N- terminus produced (in our laboratory) in *E. coli*; (2) OET, a long variant containing region of 17-530 aa derived from baculovirus system (Oxford Expression Technologies, UK); The following recombinant HA antigens were purchased from Immune Technology Corporation (all of them are 6x His-tagged and derived from 293 cell culture): Qinghai – 17–530 aa from A/Bar-headed Goose/Qinghai/12/05 (H5N1); Vietnam – 17–530 aa from A/VietNam/1203/2004 (H5N1); Anhui – 18–530 aa from A/Anhui/1/2005 (H5N1); Cambodia – 24–341 aa from A/Cambodia/R0405050/2007 (H5N1); Hubei – 18–530 aa from A/Hubei/1/2010 (H5N1); Germany – 17–342 aa from A/Turkey/Germany-MV/R2472/2014 (H5N8); Wuhan – 17–529 aa from A/Wuhan/359-95(H3N2); Netherlands – 17–527 aa from A/chicken/Netherlands/1/03 (H7N7); New Caledonia – 18-530 aa from A/New Caledonia/20/99 (H1N1).

Antibodies. IgY 745 – anty-H5 HA (H5N1) polyclonal IgY was purified in our laboratory from eggs collected from laying hens immunized with DNA vaccine based on pCI vector with cloned cDNA sequence encoding 1-568 aa of HA from A/swan/Poland/305-135V08/2006 (H5N1) (Stachyra et al., 2014). IgY was purified using Pierce™ Chicken IgY purification kit (ThermoFisher). Anti-M13 HRP-conjugated monoclonal antibody (HRP/Anti-M13 Monoclonal Conjugate, GE Healthcare); anti-Strep-tag II monoclonal antibody (StrepMAB- Classic, IBA, GmbH); anti – chicken IgY (Goat anti-Chicken IgY Fc Secondary Antibody, HRP conjugate, Thermo Scientific); anti-mouse HRP antibody (Anti-Mouse IgG (γ-chain specific) – Peroxidase antibody produced in goat, Sigma Aldrich) were purchased as indicated.

Indirect and sandwich ELISA. Indirect ELISA. The 96-well MaxiSorp plates (Nunc, Denmark) were coated overnight at 4°C with 300 ng of the respective antigens. Following 4x washing with PBST buffer (0.1 M PBS with 0.05% Tween-20) plates were blocked with 2% BSA in PBS for 1.5 h. Next, plates were incubated with purified antibody (mAb6-9-1 or TscFv) overnight at 4°C and after washing incubated (1.5 h, 37°C) with the anti-strep-tag antibody, washed and incubated (1 h, 37°C) with anti-mouse HRP conjugated IgG. In the case of mAb6-9-1 the step with anti-strep incubation was omit-

ted. Following incubation with TMB (3,3',5,5' tetramethylbenzidine, Sigma-Aldrich) absorbance at 450 nm was measured. Denatured (reduced) antigens were obtained after incubation with 10 mM DTT for 1 h at 37°C according to (Wu *et al.*, 2014).

Sandwich ELISA. The same plates as in Indirect ELISA were coated with 500 ng of the TscFv. Washing and blocking conditions were as above. Next, plates were incubated with antigens for 1.5 h at room temperature and after washing incubated (1.5 h) with IgY745 anti- H5 polyclonal IgY at various dilutions in the range 1:4 000 to 1:16 000, washed again and incubated (45 min, room temperature) with HRP-conjugated goat anti-chicken IgY Fc (Thermo Scientific) 1:12 000. Following incubation with TMB absorbance at 450 nm was measured.

RESULTS AND DISCUSSION

Prediction of the epitope recognized by mAb6-9-1

In order to facilitate mapping of the antigenic sites recognized by mAb 6-9-1 the structural model of the HA variant (A/swan/Poland/305-135V08/2006 (H5N1)) used for antibody generation was created (Fig. 1a) as described in Materials and Methods.

Two commercial phage display peptide libraries were used for selection of peptides that were reactive against mAb6-9-1. In both of them, the library of random peptides (linear dodeca- or loop-constrained heptapeptides in Ph.D.™-12 Phage Display Peptide Library or Ph.D.™-C7C Phage Display Peptide Library, respectively) are fused to a minor coat protein pIII of the M13
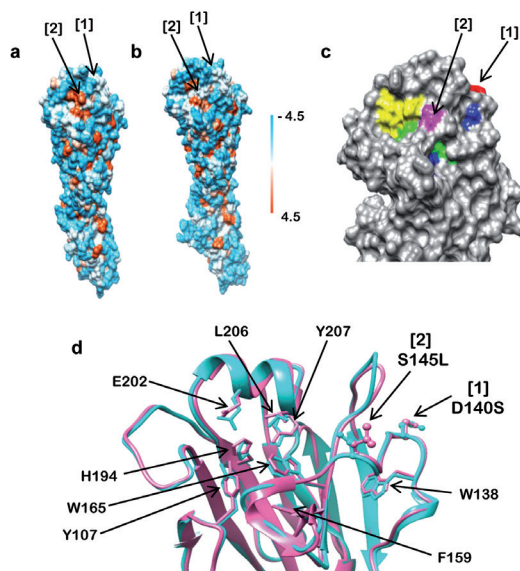

Figure 1. Model of three-dimensional structure of HA from A/swan/Poland/305-135V08/2006 (H5N1) (a) and A/Vietnam/1194/2004 (H5N1) (b).
Both proteins are represented as Connolly surface, colored by amino acids hydrophobicity based on Kyte-Doolittle scale (blue – hydrophilic, red – hydrophobic) (Kyte & Doolittle, 1982). **(c)** enlarged head of HA from A/swan/Poland. Yellow surface represents the six amino acids building the RBS interface (Y107, W165, H195, E202, L206, and Y207), dark blue depicts the four residues of the mAb6-9-1 epitope (W138, D140, F159 and W165). W165 is shared between these two groups and is colored green. **(d)** ribbon diagram of overlapped structures of the part of HA head from Poland (cyan color) and Vietnam (pink color). Two residues defining the binding specificity of mAb6-9-1 are marked on each structure, [1] D140 (Poland)/S (Vietnam), [2] S145 (Poland)/L (Vietnam).

phage. After three rounds of panning several potentially positive clones (phage-displayed peptides) were selected for further analysis. First, they were subjected to Phage ELISA to verify their binding capacity with mAb6-9-1, then phage DNA was isolated and sequenced to reveal the predicted amino acid sequence of the mimotopes (polypeptides displayed on the surface of the phages). Phage clones selected from the Ph.D-12 Library were examined in three concentrations: 10^9, 10^8 and 10^7 pfu/well, respectively. The absorbance value (OD450) two-fold higher than the value of the negative control was considered positive. The first concentration (10^9 pfu/well) was too high, since it resulted in positive signal for all selected clones. The lower concentrations allowed to select 7 clones having the highest reactivity among 21 phages examined (Fig. 2). Six of them expressed the same peptide sequence, VHWDFRQWWQPS, while one different, FPSDWWSQAWSM.

Similar analyses were performed for phage clones selected from the Ph.D-7C Library and resulted in selection of four mimotopes (ETDTLTQ, KTFLSSH, PHKPAMN and KPYTFVG) which occurred with the similar frequency.

The Pepitope server was used for epitope mapping using these affinity-selected peptides and the structural model of HA from the A/Swan/Poland/305-135V08/2006 (H5N1). The combined algorithm (consensus results of Mapitope and Pepsurf algorithms) selected one cluster of four HA residues (W138, D140, F159 and W165) from 6 mimotopes that are shown on the structural model (Fig. 1c–d). The Pepitope analysis with only one, (FPSDWWSQAWSM) identified a larger cluster of 8 HA residues (K135, S136, W138, D140, A143, V147, S148 and W165) as an epitope. Both clusters shared three residues (W138, D140 and W165). All of them were localized on the globular head of the HA1 subunit in the proximity of the RBS. Moreover amino acids W138 and D140 were localized within the antigenic epitope called region A, while W165 in the region B. Antigenic epitopes (A–E) were proposed for H5N1
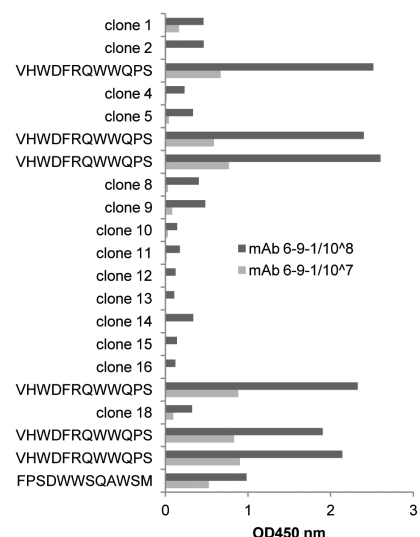
Figure 2. Reactivity of individual phage clones with mAb6-9-1 in Phage ELISA.
Plates were coated with mAb6-9-1 or BSA and to each well 10^8 pfu and 10^7 pfu were added, plates were read at 450 nm. For each phage clone signal obtained with target protein (mAb6-9-1) was compared with that obtained without target protein (e.g. BSA); presented on the horizontal axis OD value is reduced by the background (OD value for BSA). For the positive phage clones the amino acid sequences of the dodecapeptides are provided.

Table 1 The sequences of complementarity determining regions (CDRs) of scFv6-9-1.

	CDR1	CDR2	CDR3
Light chain	SSVNY	YTS	QQFTSSPWT
Heavy chain	GYSITSDYA	ISYSGST	ARSGISYYFGTDY

Amino acids complementarity which determines CDRs of the VH and VL domains were estimated according to the international ImMunoGeneTics information system® (IMGT, http://www.imgt.org)

highly pathogenic avian influenza viruses based on the classification of the antigenic domains of H3 (from H3N2 virus) (Peng et al., 2014). Both epitopes, A and B, contrary to the epitopes C–E, contribute strongly to the antigenic variation of the HPAI (highly pathogenic avian influenza) H5N1 viruses (Peng et al., 2014).

The conclusions from epitope mapping were verified by both, the Hemagglutination Inhibition test and ELISA assay. In the HI test ability of mAb6-9-1 to inhibit binding of H5N1 (by RBS) to receptors on erythrocytes surface were analyzed. The HI titer was moderately positive, equal to 32, for the homologous virus, A/Turkey/Poland/35/2007(H5N1), while for a nonhomologous virus A/chicken/Belgium/150/1999(H5N2) (DG Deventer, Netherlands) it reached only 8, which was considered as negative. The HI results suggest that the epitope recognized by mAb6-9-1 might be localized nearby the RBS. Similarity between both antigens used in HI test was 86%, besides HA cleavage sites the differences were localized around RBS and the mapped epitope (aa positions: 135, 140, 154, 100, 124, 129, 140, 142, 154, 170, 172, 184, 205, Fig. 3) which may explain the low HI titer obtained for the nonhomologous antigen.

With ELISA assay we analyzed binding of mAb6-9-1 to various HA antigens from different clades of H5N1 viruses as well as H1, H3 and H7 HA from several selected strains (Fig. 4a). The results showed that mAb6-9-1 can recognize both, a short (HA/Nde) and a long (OET) variants of H5 HA from A/swan/Poland/305-135V08/2006 (H5N1), clade 2.2 as well as H5 HA from A/Bar-headed Goose/Qinghai/12/05 (H5N1), clade 2.2 and H5 HA from A/Anhui/1/2005 (H5N1), but not the H5 HA from A/Hubei/1/2010 (H5N1), clade 2.3.2.1, A/Cambodia/R0405050/2007 (H5N1), clade 1 nor A/Turkey/Germany- MV/R2472/2014 (H5N8). The data is in concordance with observation that the most variable inter-clade positions are largely located on the globular head proximal to the RBS (Velkov et al., 2013). We also found that mAb6-9-1 does not recognize the H3 HA from A/Wuhan/359/95 (H3N2), the H1 HA from A/New Caledonia/20/99 (H1N1) nor the H7 HA from A/chicken/Netherlands/1/03 (H7N7) (Fig. 4a).

Reactivity with HA/Nde antigen containing only the HA1 part of H5 HA confirmed that the epitope rec-
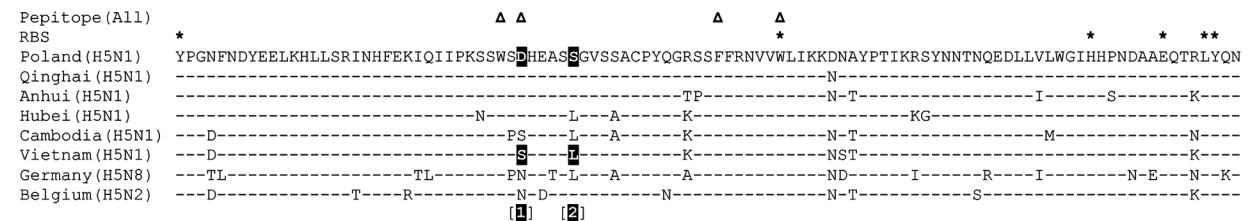
ognized by mAb6-9-1is located within HA1 subunit (Fig. 4b). Moreover, ELISA confirmed that mAb6-9-1 binds probably a conformational epitope because denaturation of the antigens (reduction of disulfide bonds with 10 mM DTT) resulted in a complete loss of mAb6-9-1 binding (Fig. 4b).

Comparison of the 3D structures of H5 HA proteins from A/swan/Poland/305-135V08/2006 (H5N1) and from A/Vietnam/1194/2004 (H5N1) (Fig. 1a, b and d) and an alignment of amino acid sequences of the H5 HA antigens tested by ELISA and in HI tests (Fig. 3) clearly showed that two amino acids, D140 and S145 in the proximity of the predicted epitope might decide about antigen binding specificity of mAb6-9-1. None of the analyzed HA proteins with the residue other than aspartic acid in the position [1] i.e. D140 and other than serine in the position [2] i.e. S145 was recognized by mAb6-9-1.

## Construction and expression of the scFv – mAb6-9-1 derivative

The cDNA fragments encoding variable regions of heavy and light chains of mAb6-9-1 were cloned into the pGEM Teasy vector and sequenced. Each of four selected clones of VH carried the same (functional) sequence. Similarly, one functional sequence was found among thirty analyzed clones carrying VL, however, majority of them were classified as nonfunctional due to an internal stop codon in CDR3. Taking into account the nature of hybridoma cells such finding is not surprising and was reported previously (Toleikis & Frenzel, 2012). The predicted amino acid sequences of the CDRs in both variable chains are shown in Table 1. Sequences encoding VL and VH were assembled in two possible orientations (VL-VH and VH-VL) with the flexible linker (Gly4Ser)3 into scFv and cloned into the modified pET201vector downstream the sequence encoding thioredoxin (Supplementary file Fig. S1 at www.actabp.pl). The choice of thioredoxin as the fusion partner was based on the literature data indicating that the formation of disulfide was essential for many scFvs described (Jurado et al., 2006; Sonoda et al., 2010).

Finally, two recombinant variants of scFv in fusion with thioredoxin (TscFv: TVHVL and TVLVH) were

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Pepitope(All)                                 Δ Δ              Δ    Δ
RBS             *                                           *                                *       *   **
Poland(H5N1)    YPGNFNDYEELKHLLSRINHFEKIQIIPKSSWSDHEASSGVSSACPYQGRSSFFRNVVWLIKKDNAYPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQTRLYQN
Qinghai(H5N1)   ------------------------------------------------------------N-----------------------------------------
Anhui(H5N1)     -------------------------------------------TP-----------N-T-----------------I------S-------K----
Hubei(H5N1)     ----------------------------N--------L---A-------K---------------KG---------------------------
Cambodia(H5N1)  ---D-----------------------PS----L---A------K-----------N-T-----------------M-------------N----
Vietnam(H5N1)   ---D----------------------S------D----------K-----------NST----------------------------K----
Germany(H5N8)   ---TL----------------TL------PN--T-L---A------A---------ND------I------R----I--------N-E---N--K-
Belgium(H5N2)   ---D------------T----R---------N-D----------N-------------N-T----------S-----------------K----
                                           [1]   [2]
```

Figure 3. Alignment of the fragments of HA antigens in the region adjacent to the mapped epitope of mAb6-9-1.
The residues corresponding to those forming the RBS according to (Al-Majhdi, 2007) and those identified by the Pepitope software (W138, D140, F159, W165) as the potential target of mAb6-9-1 are marked above the alignment by asterisks and triangles, respectively. The positions that seem to define binding specificity of mAb6-9-1 are marked as white letters on a black background and additionally identified as [1] and [2] below the alignment. Belgium (H5N2) was used only in HI test. For the full names of the viruses refer to the text.
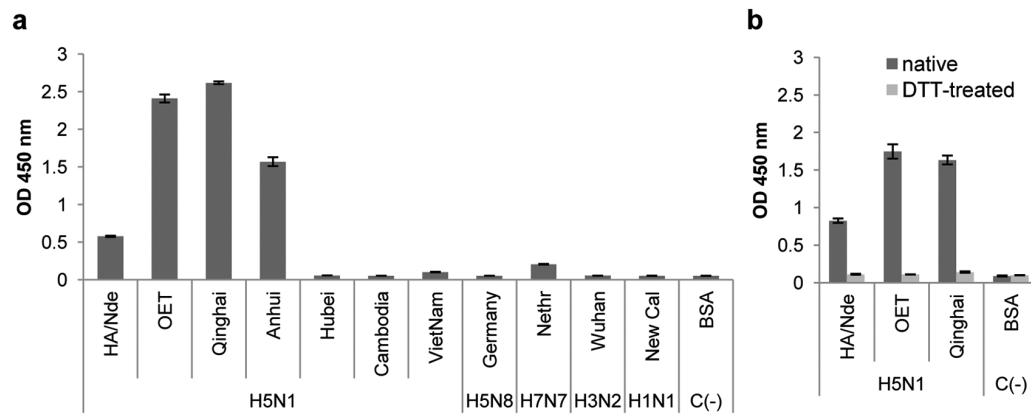
Figure 4. Reactivity of mAb6-9-1.
(**a**) Indirect ELISA with HA antigens in the native form. (**b**) Indirect ELISA with the selected HA antigens in the native and the reduced forms. C(-), negative control. The plates were coated with 300 ng of HA from the indicated viruses. Purified mAb6-9-1 and the anti-mouse IgG conjugated to HRP were used as primary and secondary antibody. The full names of the viruses are provided in the text.
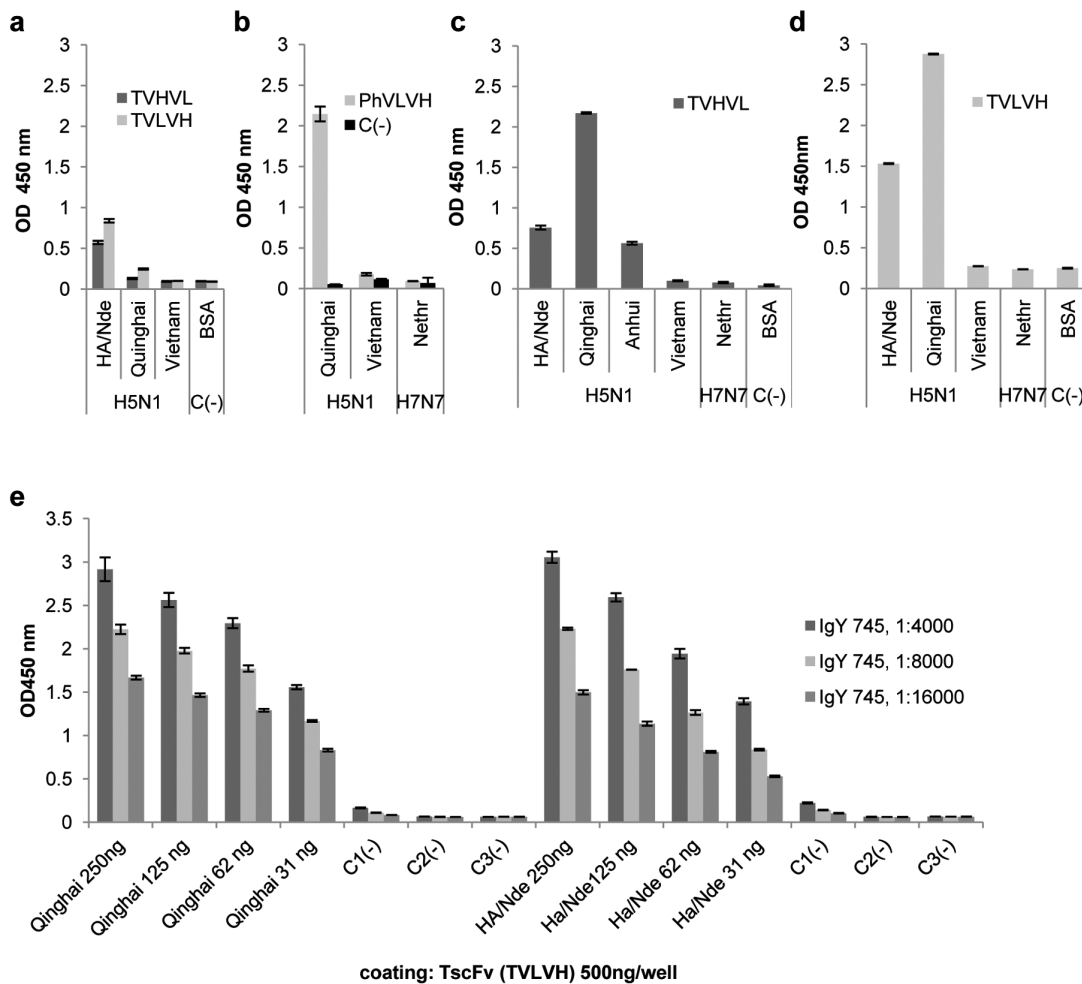
Figure 5. Verification of antigenic specificity of scFv.
(**a**) Indirect ELISA using TscFv. The plates were coated with 300 ng of the indicated HA antigens or BSA as a negative control, recombinant TscFv (TVHVL, TVLVH) were used as primary antibody, while anti-strep IgG served as secondary antibody. (**b**) Phage ELISA using phage displayed scFv (PhscFv). Plates were coated with 500 ng of the indicated antigens, incubated with phages (PhVLVH or the negative control) and with anti-M13 IgG as secondary antibody. (**c**) and (**d**) sandwich ELISA using TVHVL and TVLVH, respectively. The plates were coated with 500 ng of TVHVL or TVLVH, incubated with the indicated HA antigens (250-31 ng/well) or BSA (c-), negative control, next incubated with anti-H5 HA polyclonal chicken IgY and with anti-chicken IgG-HRP. (**e**) sandwich ELISA using TVLVH. The plates were coated with 500 ng/well of TVLVH, concentrations of HA antigens and dilutions of anti-H5 HA polyclonal chicken IgY were as indicated. C1(-), C2(-) and C3(-) various negative controls: ELISA performed without HA antigen, without IgY 745, and without antigen and IgY745, respectively.

produced in *E. coli* and purified on Ni-TDA agarose. SDS-PAGE analysis indicated the high purity of TscFvs obtained and their expected molecular weights to be about 42 kDa (Supplementary file Fig. S1b at www.act-abp.pl).

Antigen binding specificity of the recombinant scFv 6-9-1

Irrespectively of the TscFv variant, in the indirect ELISA the strongest binding was observed with HA/Nde – short H5 HA (17-340aa) from A/swan/Poland/305-135V08/2006 (H5N1) (Fig. 5a). Surprisingly, the TscFv failed to recognize H5 HA from A/Bar-headed Goose/Qinghai/12/05 (H5N1), the protein previously very well recognized by mAb6-9-1 (Fig. 4a and 5a). Higher plasticity of scFvs in comparison to the full-size antibodies may affect their specificity and affinity (Shepelyakovskaya *et al.*, 2011). To verify if this change of specificity was due to incorrect scFv folding a phage displayed variant of scFv (PhVLVH) was obtained. Published data showed that the conversion of scFv from phage-bound to soluble form may results in change of specificity because the phage pIII protein may provide structural support for antigen- binding site (Kaku *et al.*, 2012). The results of phage ELISA (Fig. 5b) showed that indeed PhVLVH was able to specifically recognize the HA antigen from A/Bar-headed Goose/Qinghai/12/05 (H5N1). These results strongly support the conclusion that the cloned cDNA encoding the variable regions of heavy and light chains correspond to the heavy and light chains of mAb6-9-1. Moreover, comparison of the amino acids sequences determined by mass spectroscopy of trypsin-digested mAb6-9-1 with CDRs sequences of VH and VL predicted from nucleotide sequences of cloned scFv showed 87% and 100% identity, respectively (Supplementary file Fig. S2). The alteration of antigen specificity observed by indirect ELISA prompted us to search for a better method of testing the activity of TscFv.

We performed sandwich ELISA using TscFvs (TVH-VL and TVLVH, 500ng/well) as capture antibodies, anti-H5 HA IgY 745 as detection antibody and various HA antigens. TscFvs were capable of targeting not only the short fragment of H5 HA (HA/Nde) but also the long fragment of H5 HA from A/Bar-headed Goose/Qinghai/12/05 (H5N1) (Fig. 5c–d).

The H5 HA from A/Anhui/1/2005 (H5N1; clade 2.3.4) was also weakly recognized by TscFv, while binding to H5 HA from A/Vietnam/1203/2004 (H5N1; clade 1) and to H7 HA from A/chicken/Netherlands/1/03 (H7N7) was not detected (Fig. 5c–d). Obtained results showed that binding capacity of TscFv to the long H5 HA version may be stronger than to the short H5 HA (HA/Nde) (Fig. 5c–d). This observation was confirmed in detailed analysis when serial two fold dilutions of both HA antigens in the range of 250–30 ng/well and anti-H5 HA IgY (1:4000–1:16000) were applied (Fig. 5e). All data from sandwich ELISA were in accordance with the results obtained for mAb6-9-1 (Fig. 4a).

The observed discrepancies of reactivity of TscFv toward the long version of H5 HA, in two kinds of ELISA, indirect and sandwich (Fig. 5a and 5c, d, e), may be explained by technical inconsistencies. The lack of binding to the long antigen was observed in the indirect assay, when HA antigens were used to coat the plates and the TscFv bound to the HA antigen was detected by the anti-Strep antibody. However, the binding with the same antigen was observed in the sandwich ELISA,

when TscFv was used to coat the plates and HA antigens bound to TscFv were recognized by the specific polyclonal anti-HA IgY antibody, detected subsequently by secondary anti-chicken IgY antibody conjugated with HRP. Due to the high plasticity of scFv, in the indirect ELISA, the Strep-tag was probably hidden in the conformational structure of TscFv and was not accessible for the anti-Strep antibody.

In conclusion, epitope recognized by the full-length monoclonal antibody was mapped in the proximity of the RBS. This assumption is supported by the results of HI assay. Unfortunately, contrary to mAb6-9-1, TscFv did not demonstrate HI activity (not shown). However, such result does not exclude that scFv recognized the same epitope as mAb. To prevent hemagglutination, antibody must bind to or near RBS and cause steric hindrance (Ascione *et al.*, 2009; Zhang *et al.*, 2013). It is more likely that the reduced size of scFv is not an effective barrier between virus and the red cell. Both antibodies display narrow specificity to H5 HA from H5N1 influenza viruses, mostly from clade 2.2 and might be used effectively in immunosensors specifically detecting these type of viruses (Jarocka *et al.*, 2014; Jarocka *et al.*, 2016).

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