Selection and characterization of scFv antibody against nucleocapsid protein of Porcine reproductive and respiratory syndrome virus

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is a widespread infectious agent in pigs. Nucleocapsid (N) protein of PRRSV has been identified as the most immunodominant viral protein. The main goal of the work was the selection and characterization of a single-chain antibody fragments (scFv) antibody specific to the N protein. Specific scFv antibody clone D5 was selected from the Tomlinson phagemid library and purified by immobilized metal affinity chromatography from the periplasmatic space of *E. coli* cells. The antibody was then characterized by sequencing and the ability to recognize the native virus N protein by Western blot and competitive ELISA. Pepscan analysis identified the position of the binding epitope between amino acids 62–84 of the N protein. Our study could help to improve the diagnostics and prevention of PRRSV in Central Europe.

N protein, expression, binding epitope, immunoblot, ELISA

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure in sows, respiratory distress in piglets and growing pigs. The causative agent of PRRS is an enveloped, positive-stranded RNA virus classified to the genus *Porartevirus*, family *Arterividae* in the order *Nidovirales*. Two previously recognized genotypes were recently classified as independent virus species. Former European virus genotype (Genotype I) is now designated as *Porcine reproductive and respiratory syndrome virus 1* (Adams et al. 2017). The viral genomic RNA is coding nine open reading frames (ORFs): ORF1 (1a–1b, encoding a replicase polyprotein), and ORF 2 to 7 (encoding six structural proteins). Minor membrane-associated glycoproteins (GP2, GP3, and GP4) are the products of ORFs 2 to 4. Open reading frames 5 to 7 are coding major structural proteins - GP5, M, and N proteins (Meulenberg et al. 1997; Ostrowski et al. 2002; Johnson et al. 2011). The nucleocapsid N protein (ORF7) of the virus is a small basic protein with molecular mass of 15 kDa. The expression is localized in the nucleus (Rowland et al. 1999) of infected cells and represents 20–40% of the protein content of the virion (de Vries et al. 1992; Faaberg and Plagemann 1995; Godeny et al. 1995; Bautista et al. 1996). The N specific antibodies are produced within the first few days following infection and represent a vast majority of the total antibody response against the virus (Urniza et al. 1997). For these reasons, N protein is the main antigen in currently used serological tests (Meulenberg et al. 1995; Cho et al. 1996; Denac et al. 1997; Seuberlich et al. 2002; Ferrin et al. 2004). Monoclonal antibodies (mAbs) are useful components of diagnostic tests ensuring adequate sensitivity and particularly their specificity (Yoon et al. 1995; Dea et al. 2000; Ren et al. 2010; Van Breedam et al. 2011). However, the development of reliable mAbs is labour-intensive and time-consuming. Protein engineering has made it possible to manipulate recombinant antibodies and to construct antibodies which retain complete antigen binding function (Muller et

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al. 1997). Single-chain antibody fragments (scFv) consist of variable regions of heavy and light chains with a connecting flexible linker (Bird et al. 1988). In contrast to the natural antibody secreted to an extracellular environment, scFv can be easily expressed in a functional form in *Escherichia coli*, allowing protein engineering to improve the properties of scFv such as the increase of affinity and alternation of specificity (Munro and Pelham 1987; Bird et al. 1988).

The aim of this study was the selection and characterization of recombinant scFv antibodies directed against the N protein of the PRRS virus which could later be used in the development of diagnostic methods or possibly in studies of the life cycle of the virus.

Materials and Methods

Expression of recombinant N protein

Expression and purification of recombinant N protein of PRRS 1 virus was performed according to a previously published procedure (Janková and Celer 2012). The appropriate virus gene was PCR amplified and cloned into pENTR-TM/SD/D-TOPO® Vector (Invitrogen. Thermo Fisher Scientific, Waltham, USA) and subsequently subcloned into pDestTM17 Gateway® Vector (Invitrogen) by site-specific recombination. The expression of recombinant protein was performed in BL21 (DE3) pLysS *E. coli* strain (Stratagen, La Jilla CA). The resulting recombinant N protein was purified by immobilized-metal affinity chromatography (IMAC) using a polyhistidine tag under denaturing conditions using a NGC Chromatography System (Bio-Rad, Hercules, California, USA).

Selection of N specific recombinant phages

Human Tomlinson I+J scFv phage library (Laboratory of Molecular Medicine, MRC, Cambridge) was used. The library is in a phagemid format that allows scFv to be expressed either on the surface of a bacteriophage, or in an alternative *E. coli* producing a soluble scFv. The diversity of libraries (I and J) is estimated to 1.47×10^8 for I and 1.37×10^8 for J. The soluble scFv fragments produced bear c-myc and poly-histidine tags to facilitate detection and purification. The panning procedure and the selection of monoclonal scFv antibodies was already described (Celer et al. 2003) with minor modifications.

The immunotube (NUNC, ThermoFisher Scientific, Waltham, USA) was coated with 100 µg/ml of recombinant N protein (PRRSV1) overnight at 4 °C. After blocking with 2% skimmed milk, 10^{12} recombinant phages were added and incubated for 2 h at room temperature (RT). After washing with phosphate buffered saline (PBS) containing 0.1% Tween 20, bound phages were eluted by 100 mM trietylamine and immediately neutralized with 1M Tris pH 7.4. Exponentially growing TG1 *E. coli* cells (MRC, Cambridge, UK) were then used to amplify eluted phages. TG1 cells were infected with 250 µl of eluted phages for 30 min at 37 °C without shaking. Fifty µl of infected cells were plated on tryptone yeast extract (TYE) agars supplemented with 100 µg/ml ampicillin and 1% glucose and incubated overnight at 37 °C. The TYE medium was added (1–2 ml) to the cells grown on the plate. Fifty µl of loosened cells were resuspended in the 50 ml TYE medium containing 100 µg/ml ampicillin, 1% glucose and were shaken until OD_{600} reached 0.4 (spectrophotometer: SmartSpec 3000, Bio Rad, CA, USA). Ten ml of grown bacteria were infected with helper phage KM13 (MRC, Cambridge, UK) (at a final concentration of 5×10^{10}) and were left for 30 min at 37 °C without shaking and then spun at 3,300 *g* for 10 min. The bacterial pellet was resuspended in 50 ml of TYE medium containing 100 µg/ml ampicillin, 25 µg/ml kanamycin and 1% glucose and grown overnight at 30 °C. The night culture (40 ml) was spun at 10,800 *g* for 30 min and the supernatant was used in precipitation of scFv phage with 8 ml 20% polyethylene glycol 6000, 2.5 M NaCl (PEG/NaCl) for 1 h at 4 °C. The mixture was centrifuged at 3,300 *g* for 30 min and the pellet containing the scFv phage was resuspended in 2 ml of PBS. The scFv phage obtained was used for the next round of selection. In total, five rounds of panning were carried out.

Production of soluble scFv antibodies

Selected recombinant phages were used to infect HB2151 *E. coli* cells (MRC, Cambridge, UK). Exponentially grown HB2151 cells (OD_{600} = 0.4) were infected by selected N specific phages for 30 min at 37 °C. Then the mixture was spotted on TYE agar supplemented with 100 µg/ml ampicillin, 1% glucose and incubated overnight at 37 °C. Individual colonies were inoculated in 2 × tryptone yeast (TY) medium supplemented with 100 µg/ml ampicillin and 0.1% glucose and expression of recombinant antibodies was induced by 1 mM IPTG (isopropyl β-D-thiogalactoside). Production of soluble N specific scFvs was tested by ELISA (Crowther 2009).

Soluble monoclonal scFv antibodies were purified from the periplasmatic space by osmotic shock. The bacterial pellet was lysed in sucrose buffer (pH = 7.5) (200 mM Tris HCl, 20% sucrose, 1 mM EDTA) for 10 min at RT, then chilled milliQ water was added for additional 5 min. Cell debris was removed by centrifugation at 8,000 *g* for 30 min and the supernatant was dialyzed at 4 °C for 16 h to remove EDTA. Then 0.1 M DTT and benzonase were added and incubated for 1 h at RT. Recombinant scFv was purified by IMAC using a polyhistidine tag using the NGC Chromatography System (Bio-Rad, Hercules, USA).

Sequencing

Positive phagemid clones were characterized by sequencing. Phagemid DNA was extracted and heavy chain variable (VH) and light chain variable (VL) region was PCR amplified using vector specific primers, gel purified amplicons were sequenced (Macrogen Europe, Amsterdam, The Netherlands). The sequences obtained were analysed by Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the complementarity determining regions (CDRs) in heavy chain (VH) and light chain (VL) variable region. Multiple sequence alignment was performed using Geneious v.10 software (Biomatters Ltd, Auckland, New Zealand).

Competitive ELISA

To analyse the specificity of the scFv antibody to PRRS N protein, competitive ELISA was performed. The 96 well flexible assay plate was coated with 100 mg/ml of the recombinant N protein overnight at 4 °C. Porcine sera (PRRS specific antibodies determined by IDEXX ELISA (Idexx Laboratories, Inc., Maine, US) were diluted 1:100 (determined by checkerboard titration). After blocking with 2% bovine serum albumin (BSA, Sigma Aldrich, Darmstadt, Germany) 50 µl of 1:1, the mixture containing swine serum and our scFv antibody (D5) (diluted 1:100) was added and incubated at RT for 1 h. After washing with T-PBS (PBS, 0.1% Tween 20), 100 µl of anti-c-myc-peroxidase (Merck, Darmstadt, Germany) diluted 1:30 000 (2% BSA in PBS) were added and incubated for 1 h at RT. Plates were then washed three times with T-PBS and the reaction was developed using 3,3´5,5´-tetramethylbenzidine (Test-Line, Brno, Czech Republic). Colour development was stopped with 1N H_2SO_4 and the optical density was read at 450 nm (Infinite M200 PRO, Tecan, Austria).

Identification of antibody binding site

To identify the position of D5 binding epitope, N protein was synthesized as six aa20-mers (Genecust, Ellange, Luxembourg) (Table 1). The binding site was then identified by peptide ELISA. Ten wells of a 96-well plate were coated overnight with 5 ng per well of each of the peptides diluted in carbonate buffer (pH 7.4) at 4 °C. The plate was then blocked by 2% BSA in PBS, and 50 µl of D5 antibody clone (1:100) were added to the wells and incubated for 1 h at RT. Irrelevant scFv antibody served as a negative control. After washing with T-PBS, 100 µl of anti-c-myc-peroxidase (diluted 1:5000 in PBS with 2% BSA) were added and incubated for 1 h at RT. After washing, the reaction was developed using 3,3´5,5´-tetramethylbenzidine (Test-Line, Brno, Czech Republic). Colour development was stopped with 1N H_2SO_4 and the optical density was read at 450 nm (Infinite M200 PRO, Tecan, Austria).

Table 1. Sequence and position of peptides covering the whole length of the N virus protein. The amino acid sequence and position in the N protein is indicated.

Peptide	aa sequence	Position
Peptide 1	MAGKNQSQKKKKSTAPMGNG	1-20
Peptide 2	QPVNQLCQLLGAMIKSQRQQ	21-40
Peptide 3	PRGGQAKKKKPEKPHFPLAAED	41-61
Peptide 4	DIRHHLTQTERSLCLQSIQTAF	62-84
Peptide 5	NQGAGTASLSSSGKVSFQVEFM	85-107
Peptide 6	LPVAHTVRLIRVTSTSASQGAS	108-130

Results

Selection of scFv antibodies

Five rounds of scFv antibodies selection were performed. After each selection round, a titre of the eluted phages was determined to evaluate the efficiency of selection (Plate VI, Fig. 1). The number of selected binders increased approximately 100 × between the first and fourth selection rounds. The increasing number of N specific binders in the total phage population was confirmed by polyclonal scFv-phage ELISA performed after each selection round (data not shown). To identify individual binders, scFv phages eluted in the third and fourth selection rounds were used to infect TG1 *E. coli*. Randomly selected colonies were tested for their ability to bind PRRS N protein in ELISA test and immunoblot. In total, fourth clones were identified as N protein specific binders (Plate VI, Fig. 2).

Production of soluble scFv antibodies

The specific binders identified were then used to infect nonsuppressor strain *E. coli* HB2151 and tested for the expression of soluble scFv molecules. Only two clones were able to express whole length soluble scFv molecules. For further analysis, these two clones (labelled as B4 and D5) were expressed and purified from bacterial periplasm by IMAC. The expression level of the B4 clone was substantially lower than that of D5. For this

reason, further experiments were performed with the D5 clone only. The yield of purified soluble form of D5 scFv reached 1 mg per 1 litre of bacterial suspension. The soluble D5, containing polyhistidine tag, was purified from bacterial periplasm by osmotic shock and the purity of the scFv obtained was checked on 12% SDS-PAGE gel (Plate VII, Fig. 3).

Sequencing

For further characterization, both clones (B4 and D5) were sequenced. The VH and VL regions, polyhistidine tag and the linker are shown and compared in Fig. 4 (Plate VII).

Identification of the D5 binding site

To identify the binding epitope of D5 scFv antibody, N protein was synthesized as six independent peptide fragments. The reactivity of D5 scFv clone with individual peptides in ELISA test is shown in Fig. 5 (Plate VIII). The D5 scFv displayed the strongest reactivity with peptide 4 spanning aa 62-aa84.

Competitive ELISA

The specificity of soluble D5 scFv to N protein was estimated by competitive ELISA test on the panel of PRRS positive and negative swine sera. The dilution of swine sera and scFv antibody was determined by checkerboard titration. The result of swine sera testing is shown in Fig. 6 (Plate VIII). The PRRS positive sera (sera 1–10) gave significantly different reactivity compared to negative sera (11–20).

Discussion

Porcine reproductive and respiratory syndrome virus is one of the most important viral pathogens affecting the swine industry. The development of new sensitive and specific diagnostic tests is therefore crucial to prevent the spread of the virus among host animals and for the introduction of successful control programs. Monoclonal antibodies (mAbs) are useful components of diagnostic tests ensuring adequate sensitivity and particularly their specificity (Yoon et al 1995; Dea et al 2000; Ren et al 2010; Van Breedam et al 2011). However, the development of reliable mAbs is labour-intensive and time-consuming, and the binding affinity of mAbs can easily be affected by amino acid changes in the target structure. This drawback can partially be overcome by scFv antibodies which can be selected from a scFv library and following characterization can easily be produced and purified in an *E. coli* expression system in large quantities. The whole procedure is fast and can be achieved in several weeks (Nieba et al 1997; Klimka et al 2000; Watkins and Ouwehand 2000).

The goal of our work was the development of a soluble scFv antibody against N protein PRRSV which could be used in the development of improved diagnostic methods, and for studying the role of N protein in the virus life cycle. Recombinant virus N protein has already been developed in our laboratory (Janková and Celer 2012) and used as antigen for scFv selection.

The Tomlinson semi-synthetic libraries were chosen for recombinant phage selection. Selection revealed a pattern of phage recovery that was consistent with the progressive enrichment of target-specific clones. In percentage terms, recoveries rose steadily from round 1 to round 4. Since recovery data provide only a preliminary indication of the progress of selection, monoclonal phage ELISA was a critical benchmark for these experiments. This clearly revealed that clones in rounds 3 and 4 showed high reactivity with N protein. The input of recombinant phage particles used in each round of selection was 10^{12}. In the fourth round of selection the proportion of N protein specific phages to the whole phage population was approximately $1{:}10^{4.5}$ in comparison with the second round in which this

proportion was estimated to $1:10^{6.5}$. The overall enrichment of specific phages was then of one hundred times in four selection rounds. Similar enrichment was also obtained in our previous experiments (Celer et al 2003; Blazek et al. 2004). Finally, two clones labelled as D5 and B4 were chosen for further characterization by sequencing and were expressed as soluble scFv fragments.

In prokaryotic cells, recombinant proteins cannot form disulphide bonds in the reducing environment of the cytoplasm (Pollitt and Zalkin 1983; Derman and Beckwith 1991). On the other hand, the oxidative environment of periplasmatic space in Gram-negative bacteria is suitable for expression of disulphide-bonded proteins (de Marco 2009). The expression of soluble scFv fragments was thus performed from the periplasmatic space of HB2151 *E. coli* cells. Expression of scFv was checked by picking at random a number of eluted clones and infecting them into *E. coli* HB2151. The Tomlinson phagemid library is known to contain a high percentage of scFv clones containing stop codon leading to premature termination of scFv translation (Cárcamo et al 1998; Barderas et al 2006). Supernatants from induced cultures were screened by immunoblot and only those producing full length scFv molecules were used in further experiments. Normally, conventional *E. coli* strains produce target scFv in the inactive form due to the reducing environment of the cytoplasm. Expression of scFv molecule in periplasm in the majority of cases leads to the expression of soluble molecule with no need for refolding and easy purification (Yang et al 2010). A large amount of IMAC purified D5 (1 mg) was obtained from 1 litre of culture medium.

The full-length expression was only observed in B4 and B5 clones, but only the D5 clone expressed a sufficient amount of recombinant scFv molecule. The failure to express the B4 clone was most likely due to factors such as stability of variable domains and their folding process, which also influenced affinity to the antigen (Kipriyanov et al 1997; Ewert et al 2003). The primary sequence of particular antibody can to some extent also influence expression of recombinant proteins (Nieba et al 1997).

The affinity constant of selected scFv antibodies can be largely influenced by concentration of the antigen used (Li et al 2000). Lower antigen concentration favours antibodies with a higher affinity constant, but these conditions diminish the number of selected clones. In our experiments 100 mg/ml of recombinant N protein was used in the first selection round. Selection efficiency achieved in our experiment is shown in Fig. 1.

To evaluate the reactivity of the D5 antibody with native N virus protein, the procedure for indirect competitive ELISA was applied on a set of porcine sera with known PRRS status. As expected, PRRS positive sera displayed considerably lower absorbance data when compared with PRRS negative sera. This clearly proved that the D5 scFv antibody was competing with a serum antibody directed against the same epitope as scFv antibody did. At the same time competitive ELISA demonstrated that developed D5 scFv could be useful in the development of serological diagnostic tests.

To better characterize the D5 antibody, pepscan analysis was performed. For this purpose, N protein was synthesized as six peptide fragments. The D5 antibody displayed strong reactivity with the fragment labelled as peptide no.4 covering amino acids 62–84. Localization of a discontinuous epitope in the same hydrophilic region, (amino acids 55–66) has already been described (Rodriguez et al 1997). The reactivity of the D5 antibody with denatured antigen as well as with native virus antigen suggests that the D5 antibody recognizes continuous linear epitope.

Our goal was to improve the diagnostics and prevention of PRRSV in Central Europe. This was achieved by selection and characterization of a soluble scFv which has the potential to simplify the production of specific reagents at a large scale. Competitive ELISA in combination with peptide and indirect ELISA was used as a tool for characterization of epitopes of the N protein.

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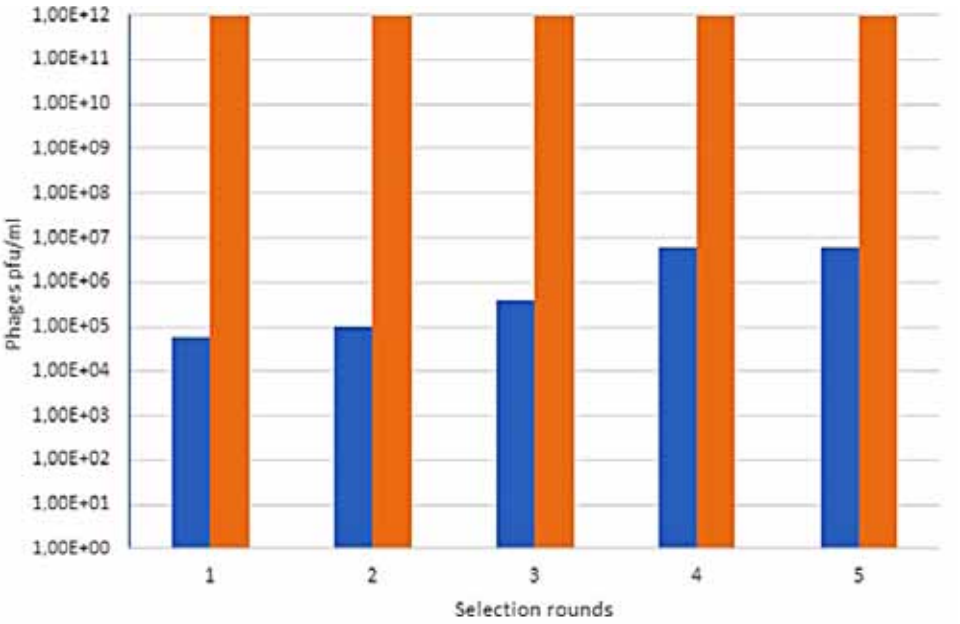

Fig. 1. Number of eluted phages. The blue columns show the number of eluted scFv-phages in each selection round. The orange columns indicate the number of input phages in each selection round.

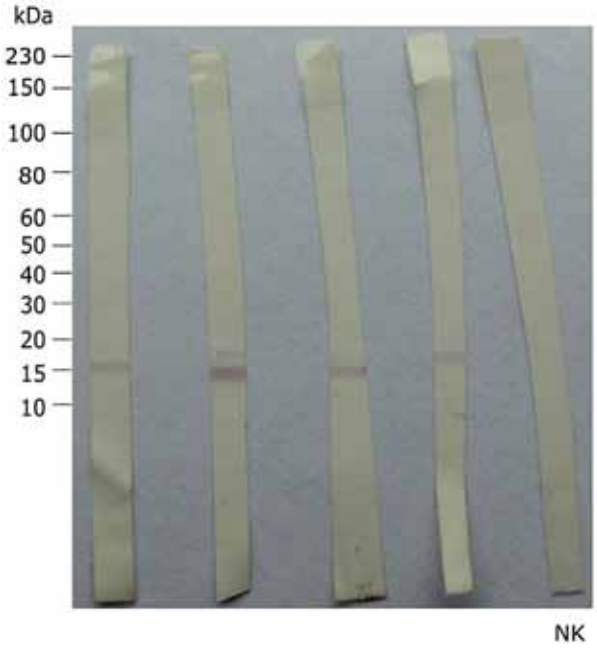

Fig. 2. Specific binding of N specific phage clones to purified recombinant N protein. Colour reaction with 15kDa protein band indicates a positive reaction (Lanes 1–4). ScFv antibody selected against irrelevant protein was used as negative control (NK). Molecular mass ladder is indicated on the left.

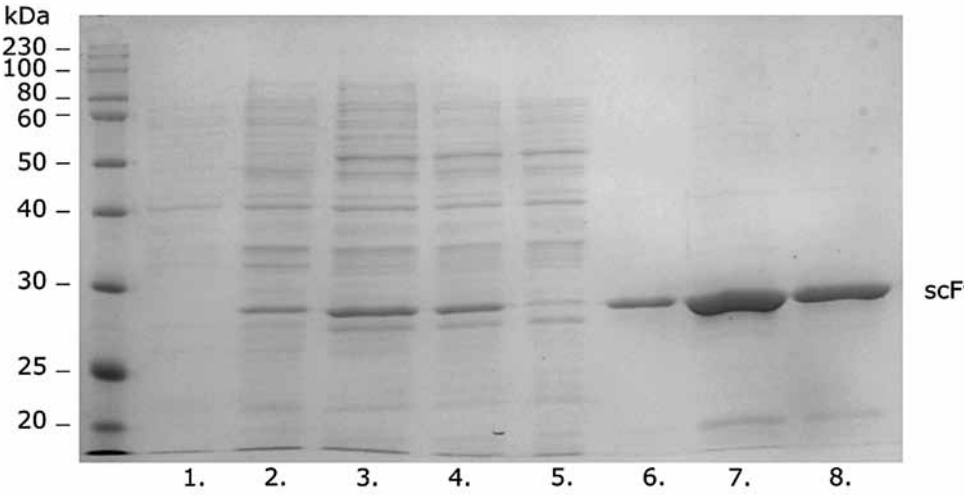

Fig. 3. Purification of the D5 scFv clone. Coomassie blue stained 12% SDS-PAGE shows the level of purity of D5 scFv clone. Lane 1 - cell lysate before induction; lane 2 - cell lysate after induction; lane 3 - cell lysate following dialysis; lane 4 - cell lysate after incubation with 0.1M DTT and benzonase; lane 5 - cell lysate after incubation with agarose beads; lanes 6 to 8 - fractions of purified D5 scFv.

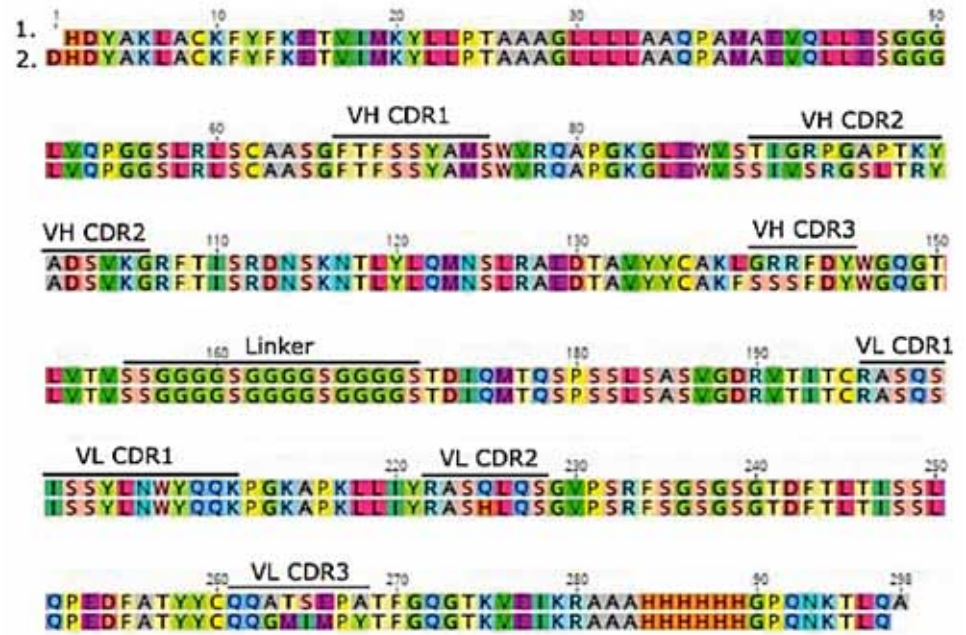

Fig. 4. Comparison of amino acid sequences of two identified clones: D5 (1) and B4 (2). CD regions of VH and VL chains are underlined.

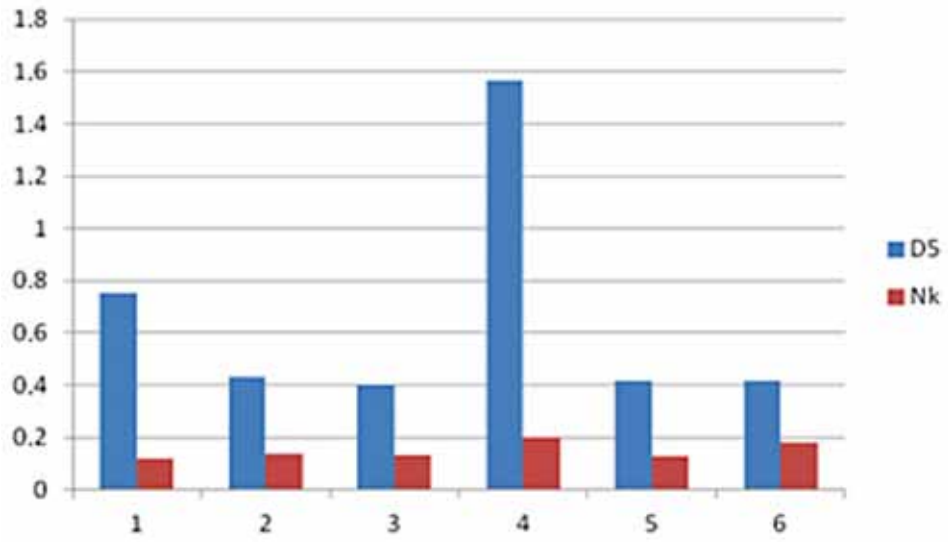

Fig. 5. Reactivity of D5 scFv with N protein fragments. Identification of protein fragments is shown in Table 1. Irrelevant scFv antibody was used as negative control.

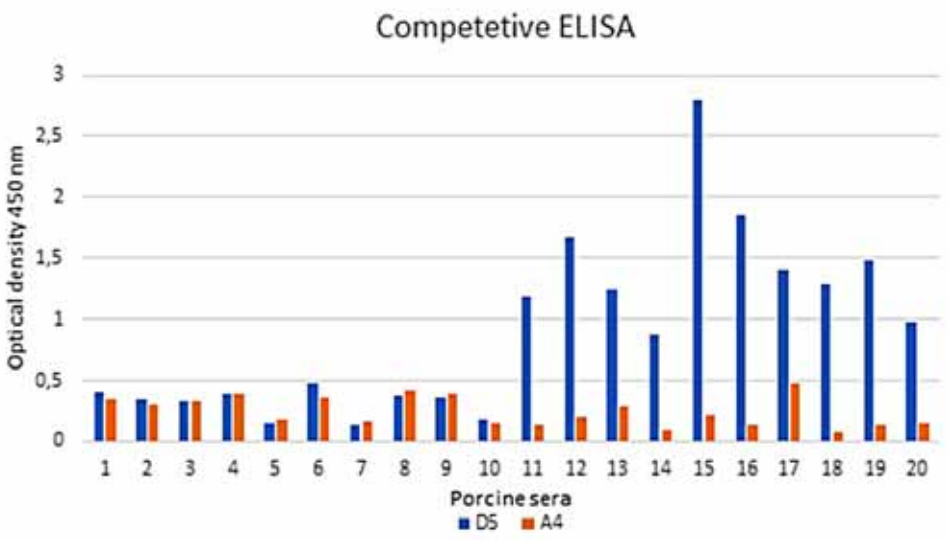

Fig. 6. Competitive N-protein ELISA test. PRRS positive (samples 1–10) and PRRS negative (samples 11–20) porcine sera were tested using D5 scFv clone (blue columns) and irrelevant scFv clone (orange columns).