Chicken single-chain antibody fragments directed against recombinant VP7 of bluetongue virus

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VP7, the major structural core protein of bluetongue virus, is conserved among the 24 BTV serotypes. The gene encoding VP7 of serotype 4 was expressed in *E. coli*. A semi-synthetic chicken antibody library was screened with the resulting protein. Six single-chain antibody fragments (scFvs) were isolated. Immune sera blocked the binding of four of the six scFvs in ELISA. These scFvs recognised recombinant VP7 coated directly onto a plastic surface. Their behaviour therefore differs from that of scFv F10 (Fehrsen et al., 2005) which was selected earlier on directly immobilised bluetongue virus and which binds to VP7 only when it is captured by an immobilised immunoglobulin directed against bluetongue virus.

Keywords: Bluetongue virus; ELISA; expression; scFvs; VP7

Introduction

Bluetongue virus (BTV) belongs to the genus *Orbivirus* of the family *Reoviridae* (Verwoerd, 1970; Borden, Shope & Murphy, 1971). Its genome consists of 10 dsRNA segments coding for three non-structural and seven structural proteins. The structural proteins VP2 and VP5 form the outer capsid while VP1, VP3, VP4, VP6 and VP7 are found in the viral core (Verwoerd, Els, De Villiers & Huismans, 1972). The major core protein, VP7, is highly conserved and can be used in enzyme-linked immunosorbent assays (ELISA) to detect all 24 BTV serotypes (Oldfield, Adachi, Urakawa, Hirasawa & Roy, 1990; Eaton, Gould, Hyatt, Coupar, Martin & White, 1991; Kowalik & Li, 1991; Nagesha, Wang, Shiell, Beddome, White & Irving, 2001). ELISAs based on murine monoclonal antibodies (mAbs) (Köhler & Milstein, 1975; Appleton & Letchworth, 1983) can detect BTV antigens as well as virus-specific antibodies (Lunt, White & Blacksell, 1988; Afshar, Heckert, Dulac, Trotter & Myers, 1995). The Office International des Epizootic (OIE) recommends using competitive

ELISAs to detect serogroup-specific antibodies due to their specificity and sensitivity (Afshar, Eaton, Wright, Pearson, Anderson, Jeggo, & Trotter, 1992). Recombinant phage-displayed mAbs are an alternative to the use of classical murine mAbs (McCafferty, Griffiths, Winter & Chiswell, 1990). The advantage of recombinant antibody fragments is that they can be identified by their DNA sequences and therefore can easily be standardised worldwide in any laboratory. They are also a renewable source of reagents. Indeed, the traditional mouse mAb 20E9, approved for BTV diagnosis by the OIE (OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2004), has been expressed as an scFv (Nagesha et al., 2001). The *Nkuku*® library (Van Wyngaardt, Malatji, Mashau, Fehrsen, Jordaan, Miltiadou & Du Plessis, 2004) has been used as a source of chicken-derived BTV-specific recombinant antibody fragments. One such scFv (F10) could detect antibodies to all 24 BTV serotypes in an inhibition ELISA (Fehrsen, Van Wyngaardt, Mashau, Potgieter, Chaudhary, Gupta, Jordaan & Du Plessis, 2005). ScFv F10 binds only to VP7 when captured by anti-BTV immunoglobulins and not to the protein immobilised directly onto plastic surfaces. This property of F10 not only requires an extra ELISA step and but also depends on polyclonal immunoglobulins of limited supply.

Accordingly, the aim of this study was to express the gene coding for VP7 of BTV serotype 4 in *E.coli,* select VP7-specific phage-displayed scFvs from a large semi-synthetic chicken antibody library, the *Nkuku*® library (van Wyngaardt et al., 2004) on VP7 directly absorbed to a plastic surface as in ELISA and to evaluate them as immunodiagnostic reagents in an inhibition ELISA.

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Materials and Methods

Expression and purification of VP7

The gene encoding VP7 of BTV serotype 4 (VP7st4) cloned into the pET-32a(+) vector (Novagen, USA) was provided by Dr. C. Potgieter, Virology Section, Onderstepoort Veterinary Institute. Plasmid DNA (~1 ng,) was mixed with 50 µl competent *E. coli* Origami™ (DE3) pLysS cells (Novagen, USA) and incubated on ice for 30 min. The transformation mixture was heat-shocked at 42°C for 30 s and immediately transferred to ice. Luria-Bertani (LB) medium (200 µl) was added and incubated with shaking at 800 rpm and 28°C for 1 h (Thermomixer Comfort, Merck Chemicals). The transformants were then selected by an overnight incubation at 30°C on LB plates supplemented with 50 µg/ml carbenicillin, 12.5 µg/ml tetracycline and 25 µg/ml kanamycin. The next day a single colony was inoculated into LB medium and grown overnight at 220 rpm and 30°C. Luria Bertani medium supplemented with 50 µg/ml carbenicillin was inoculated with the overnight culture (1:50 dilution) and grown at 220 rpm and 37°C until the optical density at 600 nm reached 0.9. Protein expression was induced by adding isopropyl ß-D-thiogalactopyranoside (IPTG; Calbiochem, USA) to a final concentration of 1 mM. An uninduced control was also prepared. The cultures were then grown for a further 4 h at 30°C. The induced and uninduced cells were harvested by centrifugation at 4000 × g for 30 min and 4°C. Cells not used immediately were stored at -20 °C. Transfected Origami™ cells, as well as cells not containing the VP7 gene, were treated with Bugbuster® Protein Extraction Reagent (Novagen, USA) following manufacturer's instructions to obtain VP7 and a negative control for the ELISAs and SDS-PAGE analysis. The insoluble inclusion bodies containing VP7 were partially purified by means of series of wash steps according to the Bugbuster® protocol. After centrifugation at 13000 x g for 30 min at room temperature, the partially purified VP7 inclusion body pellet was

solubilised for 1 h at 4°C on a rotating mixer in a solution that consisted of 8 M urea (pH 8.0), 50 mM NaH_2PO_4 and 300 mM NaCl. The protein was dialysed exhaustively (three buffer changes over 36 hours) against phosphate buffered saline (PBS) at 4°C to remove the urea. The protein concentration was determined spectrophotometrically at 280 nm (NanoDrop® ND-1000 spectrophotometer; OD 1=1mg/ml protein) and aliquots of VP7 were stored at -70°C until used.

SDS-PAGE and immunoblot

The protein samples were mixed 1:1 with an SDS-PAGE loading buffer (1.5 M Tris pH 6.8, 20% SDS, 30% glycerol, 1% 2-mercaptoethanol and 0.03 mM bromophenol blue), denatured at 100°C for 5 min and separated on a 10% discontinuous gel at 100 V and 400 mA for 1 h. The proteins were transferred onto a PVDF membrane (Invitrogen, USA) at 100 V and 400 mA in Towbin buffer (25 mM Tris and 192 mM glycine). The membrane was blocked for 1 h at room temperature on a rocker in 2% (w/v) fat-free milk powder (MP) in PBS followed by a wash cycle that consisted of three 5 min washes with 0.05% (v/v) Tween-20 in PBS (PBS/T). For detection of the histidine tag, the blot was incubated for 1 h at room temperature with an anti-histidine tag murine mAb (Novagen, USA) diluted 1:500 in 2% MP in PBS/T. The wash cycle was repeated followed by an 1 h incubation at room temperature with polyclonal rabbit anti-mouse IgG conjugated to horseradish peroxidase (P0260; Dakocytomation, Denmark) diluted 1:1000 in 2% MP in PBS/T. After a final wash cycle, a substrate solution (0.06 g 4-chloro-1-naphthol dissolved in 10 ml ice-cold methanol before mixing with 60 µl hydrogen peroxide (30% v/v) in 100 ml PBS) was added.

Direct ELISA

The recombinant VP7st4 was tested to determine whether it can react with polyclonal rabbit anti-BTV serum. Vp7 was coated at 10 µg/ml in PBS overnight at 4°C and the

wells were blocked with 2% MP/PBS for 1 h. A rabbit anti-BTV as well as a pre-immune serum diluted 1:100 2% MP/PBST were added and incubated for 1 h. After washing, polyclonal swine anti-rabbit immunoglobulins conjugated to HRP (Dakocytomation, Denmark) diluted 1:1000 in MP/PBS/0.05%T was added. In addition, VP7-specific scFv F10 was also tested for binding to directly immobilised bacterially expressed VP7. This was done essentially as described previously (Van Wyngaardt et al., 2004). *O*-phenylenediamine dihydrochloride was added to each well. The signal was measured spectrophotometrically at 492 nm after the reaction was stopped with 2 N H_2SO_4.

Selection of scFvs against VP7 from the Nkuku® library

The panning was performed as described previously (Van Wyngaardt et al., 2004) except for the following additional antigen coating and phage elution strategies. In addition to direct coating on Polysorp plates (Nunc™, Denmark), His-Grab Ni^{2+} chelate-coated plates (Pierce) were also used to trap the antigen via its poly-histidine tails. Phage-displayed scFvs that bound to VP7 immobilised on Ni^{2+} chelate-coated plates via poly-histidine tails were released in two steps in the first round, first by treatment with a mixture of equal volumes of undiluted hyperimmune rabbit and guinea pig sera for 20 minutes at room temperature and then with 100 mM triethylamine, pH 12 (TEA). In the following rounds each phage pool was then eluted with only the serum or TEA. Enrichment was monitored by titration of the input and output phages from each selection round as well as a polyclonal phage ELISA of the unpanned *Nkuku*® library and the outputs of the consecutive selection rounds. Single bacterial colonies from selection round three and four titre plates were picked randomly. The phagemids were rescued by the addition of M13KO7 helper phage and the phage-displayed scFvs were tested in a monoclonal phage ELISA.

Polyclonal and monoclonal Phage ELISAs

These ELISAs were performed according to methods described previously (Van Wyngaardt et al., 2004) but Polysorp immunoplates (Nunc^{TM}, Denmark) were coated overnight at 4 °C with recombinant VP7 (40 µg/ml) in PBS. Origami^{TM} cellular proteins in PBS (40 µg/ml) and 2% w/v MP in PBS were used as negative control antigens to verify the VP7 specificity of the selected phage-displayed scFvs.

Soluble scFv ELISA

Antigen coating was done as described for the phage ELISA and all other steps were performed as described previously (Van Wyngaardt et al., 2004).

DNA sequencing

The DNA inserts of several positive clones tested in monoclonal scFv ELISA format from selection round three and four were sequenced (ABI 3100 Genetic Analyzer) as described previously (Van Wyngaardt et al., 2004). The sequences obtained were analysed using BioEdit version 7.0.4 (Hall, 1999).

Large scale growth of soluble scFvs

Overnight bacterial cultures of the positive clones were diluted 1:100 in 50 ml 2×TY medium that contained 100 µg/ml ampicillin and 2% glucose and shaken at 37°C for 2 h until the OD_{600nm} reached 0.9. The bacterial cells were centrifuged at 2000 × g for 20 min at room temperature. The supernatants were removed followed by a brief re-centrifugation to remove the remaining supernatant. The bacterial pellets were resuspended in 10 ml 2×TY containing 100 µg/ml ampicillin and 1 mM IPTG. ScFv TD12 was resuspended in 2 ml since the yield of this scFv was low when grown in a 10 ml volume. After an overnight incubation at 220 rpm and 30°C, the cells were removed by centrifugation for 20 min at 2000 × g at room temperature. Sucrose to a

final concentration of 2% was added to the scFv-containing supernatants and stored at -20°C until used.

Stability testing of scFvs

The stability of the scFvs stored under different conditions was investigated. The selected scFvs were stored at 4°C and room temperature for nine days. Aliquots were taken daily and stored at -20°C. After the final aliquot was taken and frozen, all samples were thawed and tested in an ELISA as described for the monoclonal scFv ELISA (Van Wyngaardt et al., 2004). In addition, all scFvs were stored at -20°C to test their ability to withstand several freeze/thaw cycles. Each scFv was thawed and frozen daily for nine consecutive days. Samples were taken daily and stored at -20°C until tested. After nine days all samples were thawed and tested in an ELISA.

Inhibition ELISA

Inhibition ELISAs were performed as described previously (Fehrsen et al., 2005). The recombinant VP7 was coated at 40 µg/ml on a Polysorp ELISA plate (Nunc^{TM}, Denmark) and blocked with MP in PBS. The plate was incubated with a hyperimmune guinea pig anti-BTV-4 serum or a negative guinea pig serum diluted 1:10 in MP/PBS/0.05%T. Anti-BTV-4 sheep serum, anti-BTV-10 chicken IgY (20 µg/ml) and hyperimmune rabbit anti-BTV-4 sera were also tested. The scFvs diluted in 2% MP/PBS were added to the wells and detected with a 1:1000 dilution of anti-c-*myc* mouse mAb 9E10 conjugated to horse radish peroxidase (Roche). The supernatants containing the scFvs were titrated to determine suitable dilutions for use in the inhibition ELISA.

Results

Expression of the gene coding for VP7

In order to obtain a target protein for panning, the cloned gene coding for VP7 of BTV serotype 4 was expressed in *E. coli* Origami™ cells. Using this system, a 50 kDa protein was produced which corresponds to the expected 37 kDa viral protein fused to C- and N-terminal his-tags as well as a thioredoxin tag. SDS-PAGE analysis (Figure 1A) revealed that this protein was mainly present in an insoluble form (Figure 1A; lane 3). Immunoblotting using an anti-polyhistidine antibody confirmed that the histidine tags were present on the 50 kDa fusion protein (Figure 1B; lane 1). The protein was purified from the inclusion bodies but further attempts at purification using Ni-NTA affinity columns resulted in unacceptable losses (Figure 2; lanes 5 to 7). The protein was therefore used without further purification but was solubilised with urea and then dialysed against PBS to remove the urea which could potentially interfere with subsequent panning and ELISA procedures. A polyclonal rabbit serum raised against whole BTV reacted with the bacterially expressed VP7 when coated on the surface of an ELISA plate while scFv F10 did not react with this protein (Figure 3).

Selection of phage-displayed scFvs against VP7

The *Nkuku*® library, a large semi-synthetic phage display library based on chicken immunoglobulin genes, was panned by exposing the recombinant antibody repertoire to VP7 using two different antigen coating and phage elution strategies. In one the protein was coated directly on the surface of Polysorp microtitre plates and in the other it was trapped via its poly-histidine tags on Ni^{2+} chelate-coated plates. Phage-displayed scFvs that bound to VP7 coated directly on microtitre plates were eluted with triethylamine at pH 9.5, whereas those that bound to antigen immobilised on

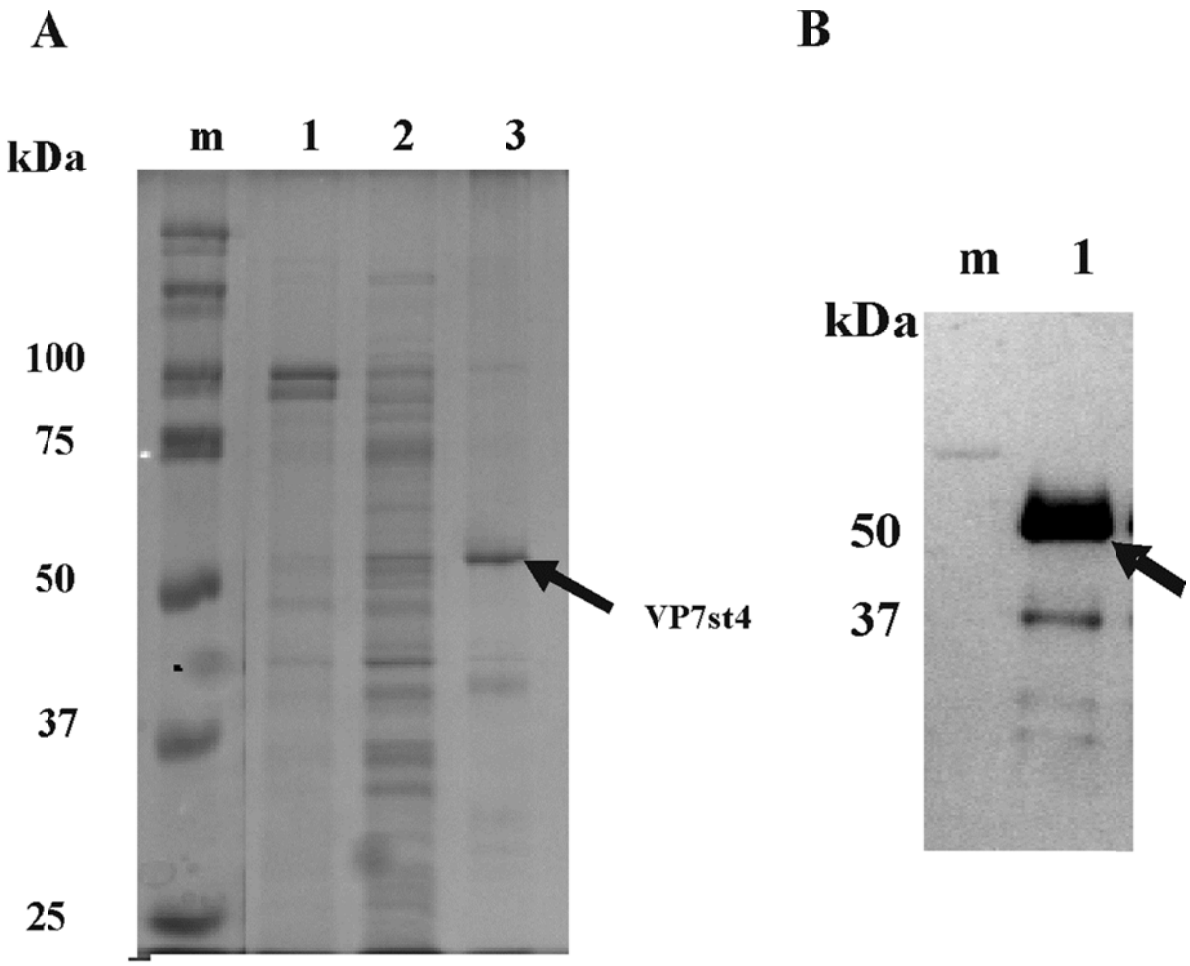

Figure 1. (A) SDS-PAGE of VP7 expressed in *E. coli*. Lane m: marker; lane 1: uninduced insoluble Bugbuster® treated bacterial pellet; lane 2: induced soluble proteins of Bugbuster® treated bacterial pellet; and lane 3: induced insoluble Bugbuster® treated bacterial pellet. Equal amounts of samples were loaded. (B) Immunoblot of VP7 detected using an anti-histidine tag antibody. Lane m: marker, lane 1: solubilised VP7 (arrow).

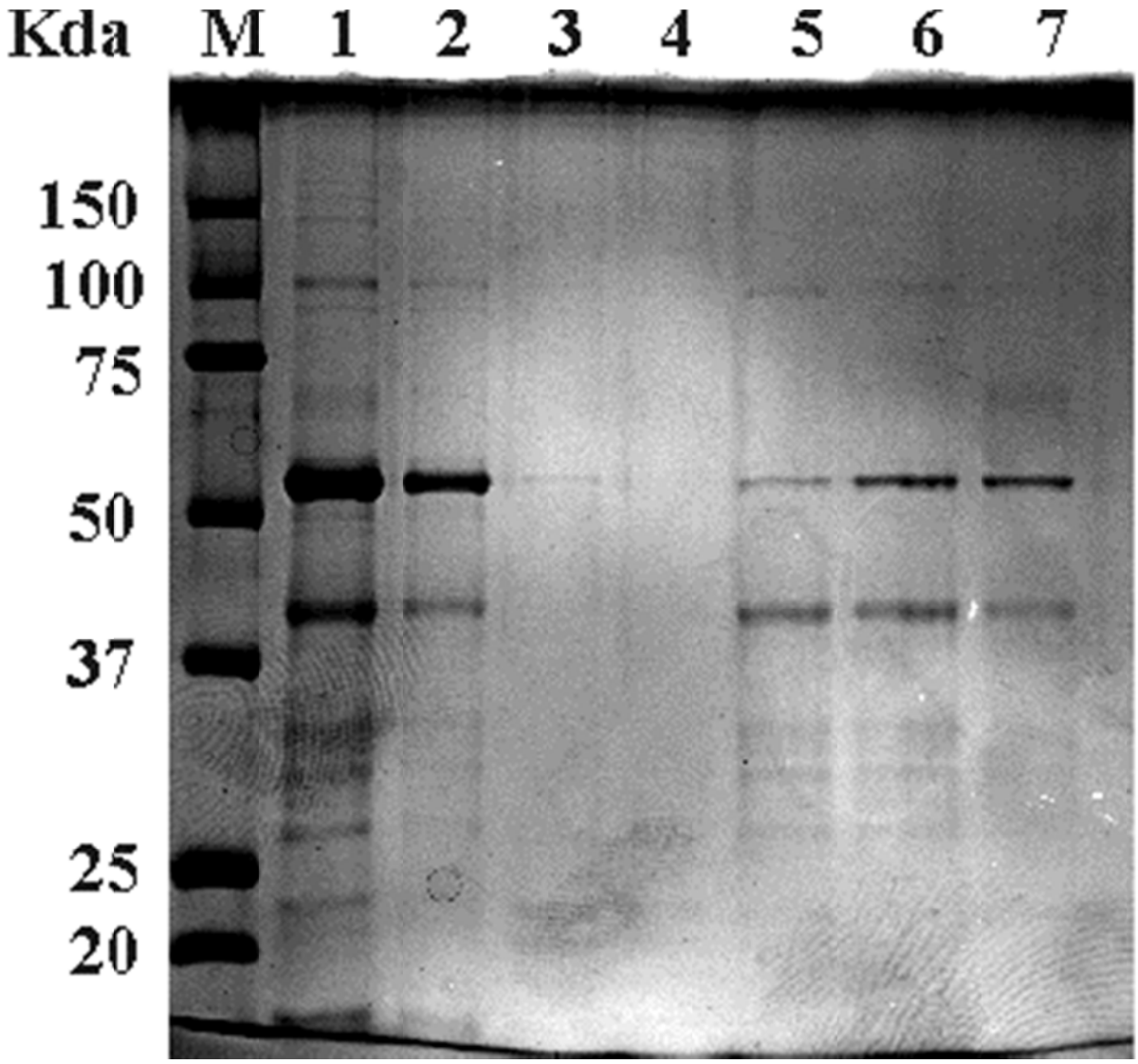

Figure 2. SDS-PAGE of recombinant VP7 purified by nickel chelate affinity chromatography. Lane m: marker; lane 1: solubilised protein; lane 2: flow-through from the Ni-NTA column; lanes 3-4: column wash fractions; and lanes 5-7: fractions eluted with 8 M urea in 500 mM NaH_2PO_4 and 50 mM Tris-Cl at pH 4.5. Equal volumes of each sample were loaded.

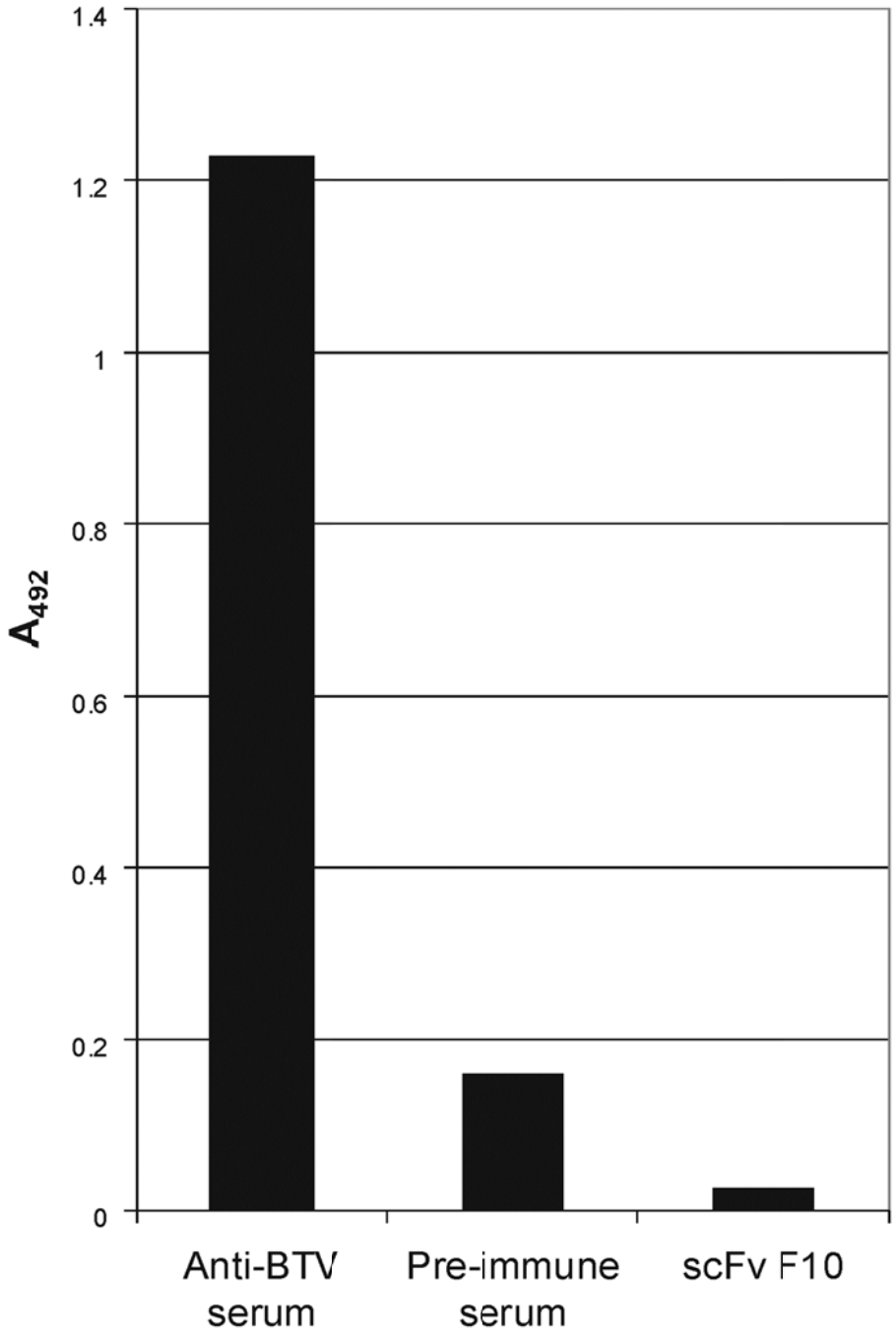

Figure 3. Binding of anti-BTV rabbit serum, pre-immune rabbit serum and scFv F10 to directly immobilised, bacterially expressed VP7.

Ni^{2+} chelate-coated plates were first released by competition with hyperimmune rabbit and guinea pig sera in the first round. This was done in an attempt to enhance the selective isolation of scFvs that perform well in antibody inhibition assays. This competitive elution was followed by a high pH treatment. The eluted phages were kept separate and released with either serum or high pH in following rounds. Phage pools obtained after each round of selection were tested in ELISA for antigen binding (Figure 4). An aliquot of the *Nkuku*® library prior to panning was used as an un-enriched negative control. It produced an absorbance signal at 492 nm of below 0.3. After four rounds of selection, the phage scFvs released by competition with immune serum produced an ELISA signal of 1.25, while those eluted at high pH from both Polysorp and Ni^{2+} chelate-coated plates yielded ELISA signals of 2.5 and 3.6. This indicated that phage pools were enriched for scFvs that bound to VP7 during each consecutive selection round (Figure 4).

Single phage clones which had been randomly picked from the titre plates after the third and fourth selection rounds from the three different elution strategies (see above) were tested in ELISA to identify individual VP7 specific binders. The scFvs were expressed in phage-displayed and soluble formats. *E.coli* Origami™ proteins and milk powder were included as negative controls. Clones expressing soluble scFvs with ELISA signals at least double that of the negative controls were considered as positive and their DNA inserts were sequenced. Forty-three sequences were analysed in this way, yielding six unique binders (Figure 5). Two (H2 and A1) resulted from high pH elution from VP7 adsorbed directly to Polysorp immunoplates while three (TC9, TD12 and TA8) resulted from the elution at high pH from His-Grab plates. One scFv (SA12; Figure 6), released from VP7 trapped on His-Grab wells using only hyper-immune serum after four selection rounds gave an ELISA signal of

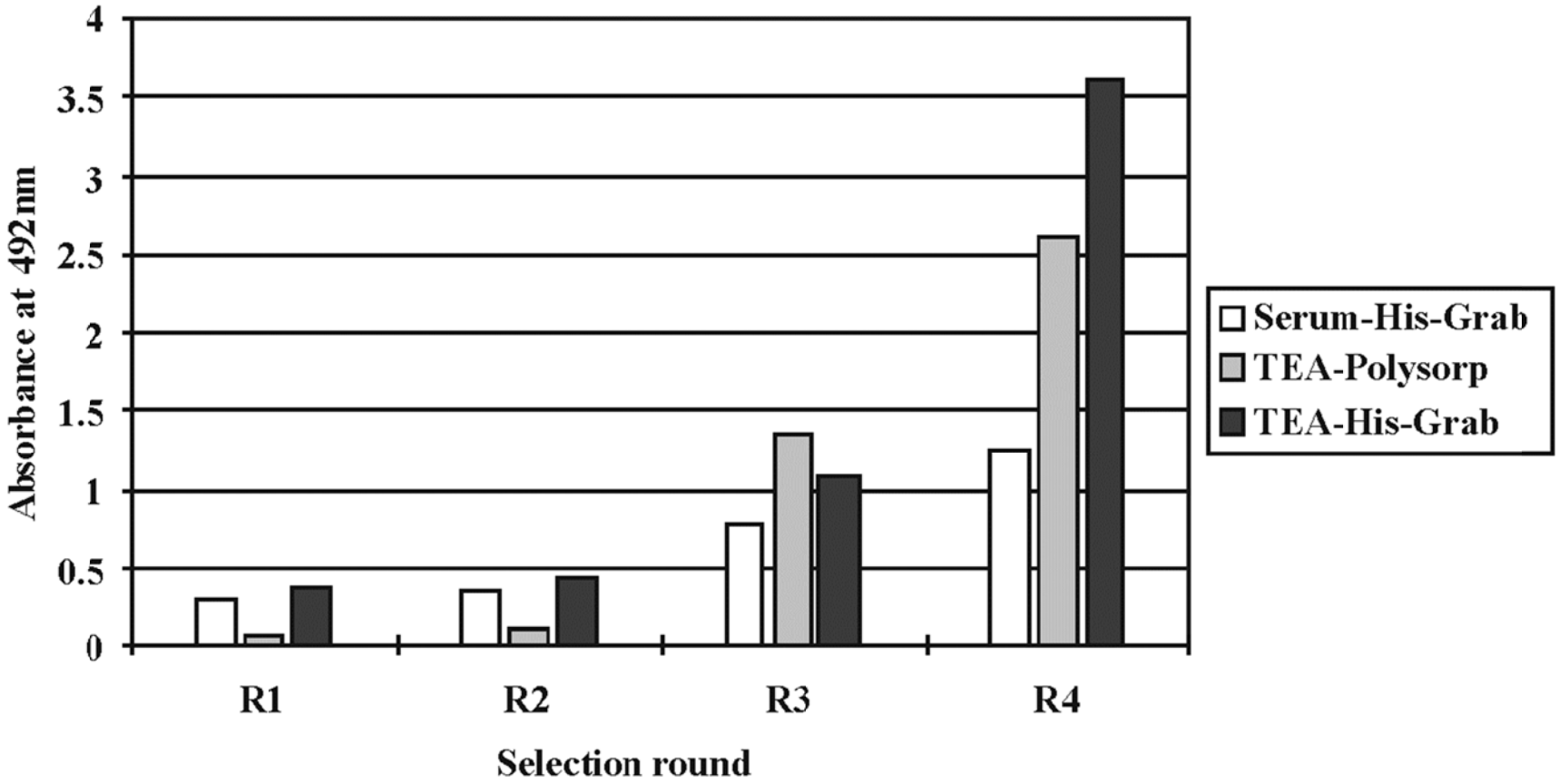

Figure 4. ELISA of phage antibody pools after each round of selection on VP7st4. Phages were released from Polysorp ELISA plates using TEA and from His-Grab plates using a mixture of hyperimmune guinea pig serum and rabbit serum followed by TEA. R1 to R4 represents selection rounds one to four.

Variable heavy chain complementarity determining regions

	1	2	3
F10	SSYQMF	AITNDGGGTNYGSAVK	DVYGNSYGFGVDSI-----
A1	SNAMGW	DIDNTGSRTAYGAAVK	DYDSGCGASICGIYAGSID
H2	.YT.Q.	G.G.D..I.Y.AP...	MVSDNNNTP.D--------
TA8	.YD.A.	G.YSG.GV.Y.AP...	TSCA.GYSCWYTDAGSID-
TC9	.YD...	G.GS...Y.G.....	A.GGCTSTWCWGYAGGID-
TD12	.YD.A.	S.SSD..D....S...	TNGPNNID----------
SA12	.FN...	E.SS..TT.F.....	AGCNSYSCDPYVGSID---

Variable light chain complementarity determining regions

	1	2	3
F10	SGGGSYAGSYYYG-	DNTNRPS	GYDSSTKAGI
A1	GGSNNYG-------	NNDRRPS	RDSSYVGI--
H2	...YSYG-------	EST....	T..INRY.GI
TA8	..GSSSYYGSYYYG	D.TN...	I.DSIGI---
TC9	.S.GSAYG------	G.TK...	YEG.Y.G.--
TD12	...YRYG-------	D.NQ...	Y..SGGNVGI
SA12	..DSSYG-------	ESTK...	E.IINI----

Figure 5. Deduced protein sequence showing only the CDRs of the scFvs A1, H2, TA8, TC9, TD12 and SA12. The CDR of the previously identified scFv F10 (Fehrsen *et al*. 2005) is included for comparison. "." represents identical amino acids and "-" represents gaps in the alignment. Genbank accession numbers: A1, JF266699; H2, JF266700; SA12, JF266701; TA8, JF266702; TC9, JF266703; TD12 JF266704.

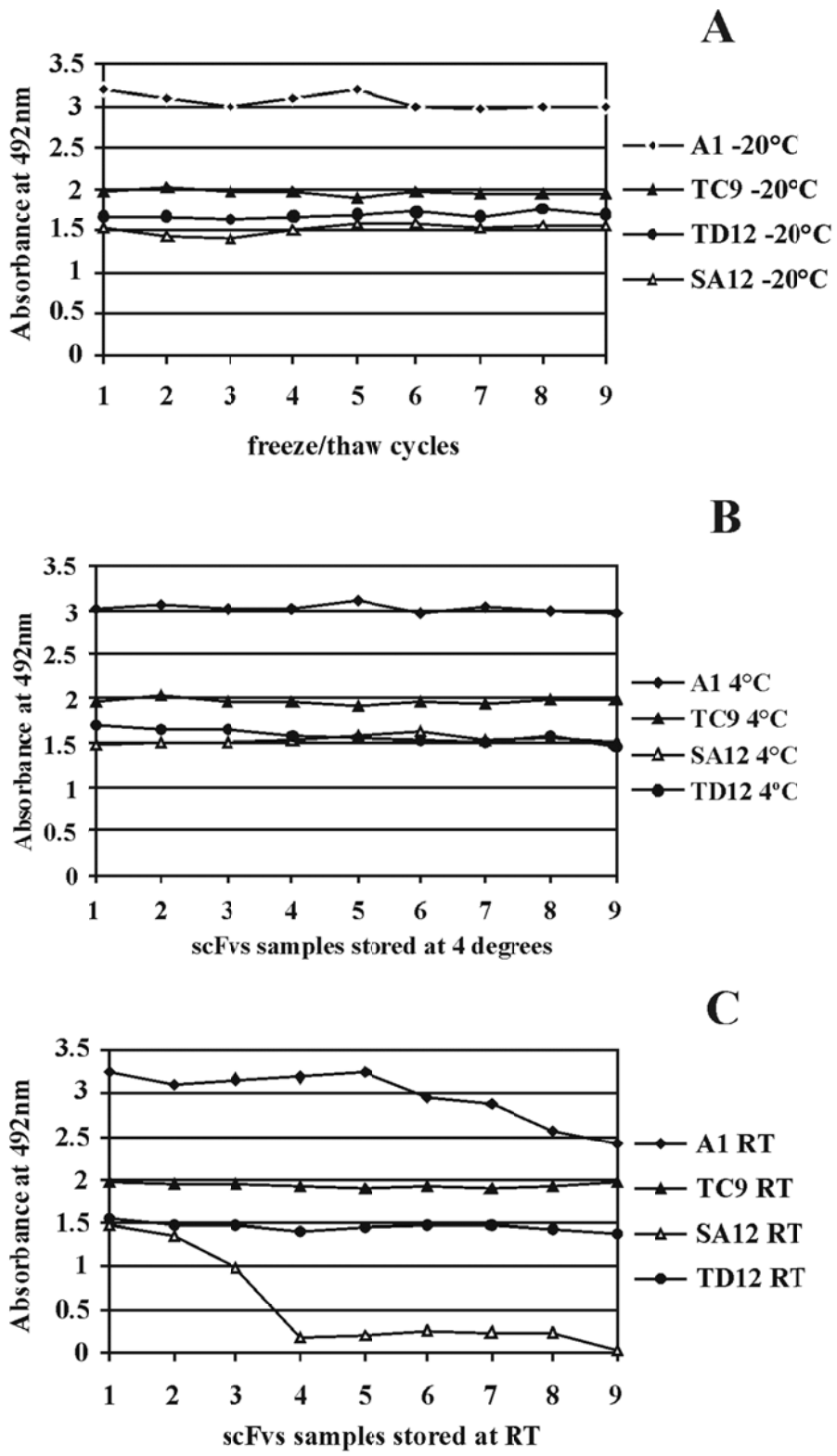

Figure 6. ELISA signals of soluble scFvs A1, H2, TC9, TD12 and SA12 after nine freeze/thaw cycles from -20 ˚C (A) and when stored at 4 ˚C (B) and room temperature (C) for nine days.

1.45. After TEA elution both immobilisation strategies yielded soluble scFvs that reacted strongly with VP7 with ELISA signals of 3.0 or higher (Figure 6).

Stability of scFvs

To be useful, antibodies need to be stable. To see whether the VP7-binding scFvs could withstand adverse storage conditions, samples were stored at room temperature, 4°C and -20°C for nine days. Nine consecutive freeze/thaw cycles did not reduce the ELISA signals of the VP7-specific scFvs A1, TC9, TD12 and SA12 (Figure 6A) and neither did storage at 4°C over the same period (Figure 6B). When stored at room temperature the absorbance signal for scFv A1 dropped gradually by 20% from day five to day nine and scFv SA12 showed a rapid decrease in signal of at least 87% over the first four days (Figure 6C).

Inhibition ELISA

Homologous anti-BTV-4 guinea pig serum and heterologous chicken anti-BTV-10 IgY were tested in an inhibition ELISA to determine whether the binding of the VP7-specific scFvs could be inhibited by BTV-specific antibodies present in the sera. Soluble fragments A1, TC9, TD12 and SA12 were tested at pre-optimised dilutions but H2 and TA8 were omitted due to inconsistent results or low ELISA signals. Using hyperimmune anti-BTV-4 guinea pig serum, the ELISA signals were reduced from 9% for TD12 up to 30% for A1 (Figure 7A) while with the anti-BTV-10 chicken IgY the signals were reduced from 51% for TC9 up to 86% for SA12 (Figure 7B). An anti-BTV-10 hyperimmune rabbit serum and an anti-BTV-4 sheep serum had no effect on the binding of the scFvs to VP7 (results not shown).

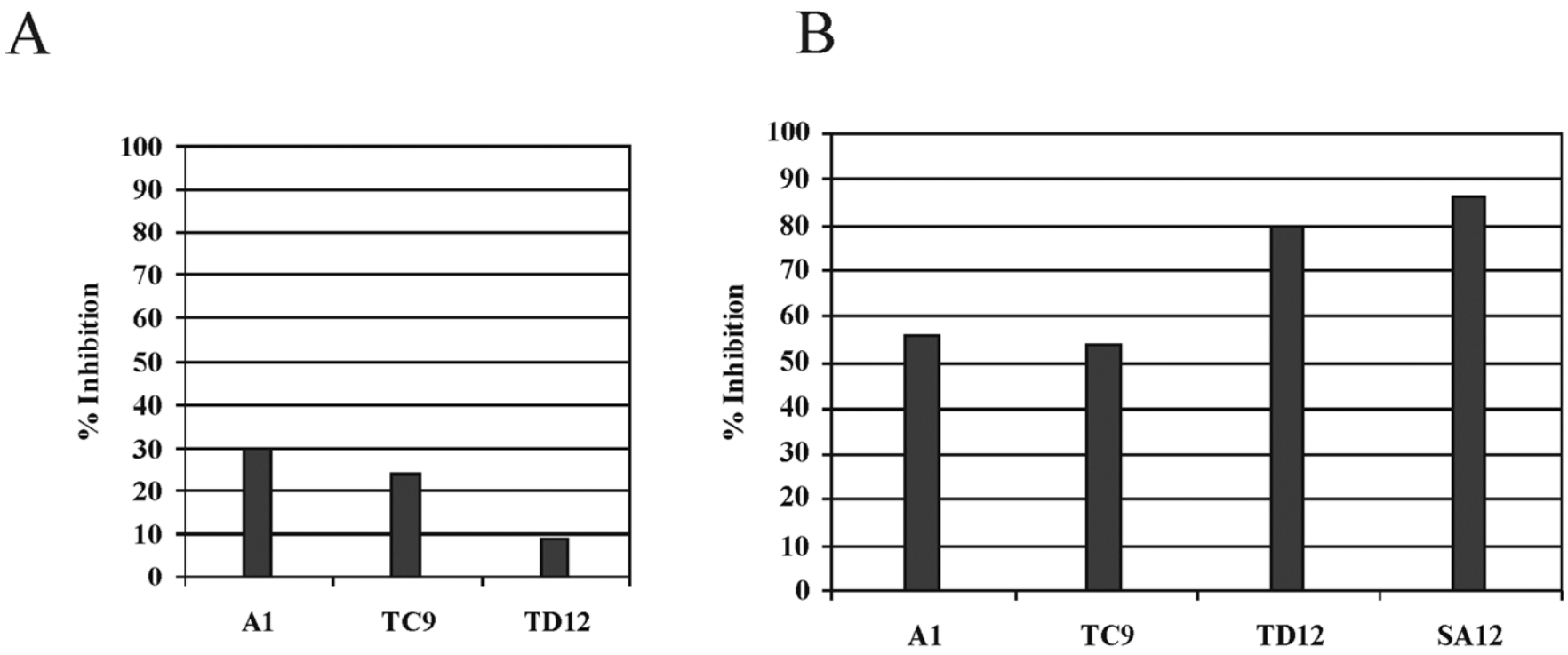

Figure 7. ELISA showing the inhibition of the binding of scFvs to recombinant VP7. (A) Using anti-BTV-4 guinea pig serum; scFvs A1, TC9 and TD12 diluted 1:3, 1:64 and 1:16 respectively. (B) Using anti-BTV-10 chicken IgY; scFvs A1 and TC9 were diluted 1:32 and TD12 and SA12 were diluted 1:16.

Discussion

A recombinant single-chain antibody fragment against VP7 of BTV had been obtained previously by screening the large semi-synthetic *Nkuku*® phage display library based on chicken immunoglobulin genes on purified BTV particles (Fehrsen et al., 2005). The specificity of this scFv (F10) was established by its binding to trapped VP7 in an ELISA where it was also found that this scFv binds only to VP7 captured by anti-BTV immunoglobulins and not to the protein immobilised directly onto plastic surfaces. Here the same antibody library was screened on recombinant VP7 with the aim of isolating recombinant scFvs that can recognise this antigen when it is coated directly onto a plastic surface. All the scFvs described here can therefore be used in ELISAs that require one step less than the ELISA based on scFv F10 (Fehrsen et al., 2005) and also eliminates the need to prepare anti-BTV trapping IgG. The binding of scFv F10 to VP7 was inhibited by anti-BTV sera raised against 24 serotypes (Fehrsen et al., 2005). In contrast, the newly identified scFvs were able to recognise the recombinant VP7 when it was directly immobilised on ELISA plate wells but their binding could not be inhibited by immune sera as readily as scFv F10. Proteins produced by expressing foreign genes in *E. coli* can potentially be obtained rapidly and at low cost. Those secreted into the periplasm are often soluble, stable and folded in a native form (Courtney, Buchwalder, Tessier, Jaye, Benavente, Balland, Kohli, Lathe, Tolstoshev & Lecocq, 1984; reviewed by Marston, 1986; Cabilly, 1989). Some, however, accumulate in the reducing environment of the cytoplasm, usually aggregating into an insoluble mass due to inefficient folding into the correct conformation (Goeddel, Kleid, Bolivar, Heyneker, Yansura, Crea, Hirose, Kraszewski, Itakura & Riggs, 1979; De Boer, Comstock & Vasser, 1983; Georgiou, Telford, Shuler & Wilson, 1986; reviewed by Marston, 1986). The resulting inclusion bodies can be obtained in a relatively pure form since they are dense and thus easily

collected by centrifugation at low speed. Accordingly, VP7 was purified from such insoluble inclusion bodies which mainly contained the protein (Figure 1A, lane 3). Additional bands on the immunoblot (Figure 1B) indicated that there were also histidine-rich proteins smaller than the full length VP7 with C- and N- terminal histidine tags and a thioredoxin tag. These bands could have represented degraded or partially translated VP7. The purified recombinant protein was insoluble but it could be solubilised in a denaturing buffer and then dialysed against PBS without it precipitating. When coated directly on a plastic surface, it was recognised by BTV-specific antibodies in a polyclonal rabbit serum which proved that some of the native viral epitopes were present. Based on this finding it was decided to proceed with selection for VP7-specific scFvs.

Six scFvs that recognised the recombinant VP7 were selected from the *Nkuku*® library by following two different selection strategies. Two scFvs (A1 and H2) were selected by panning on the denatured recombinant VP7 coated directly on Polysorp immunoplate wells while four scFvs (SA12, TA8, TC9 and TD12) were selected on histidine tagged VP7 captured on Ni^{2+} chelate-coated wells. Their deduced amino acid sequences revealed that they were different from each other and also from the scFv F10 which is specific for baculovirus expressed VP7 of BTV-1 (Figure 5; Fehrsen et al., 2005). The larger number of scFvs obtained using the second strategy suggests that epitopes on captured VP7 were more accessible than on the directly immobilised VP7. It is also possible that immobilisation of VP7 via histidine tags could have reduced or eliminated the protein distortion caused by direct immobilisation on plastic surfaces (Kennel, 1982) and thereby contributed to the preservation of native epitope structure.

Hyperimmune anti-BTV serum from sheep, the natural host of BTV, was tested for an inhibitory effect on the binding of the scFvs to recombinant VP7. Such inhibition would serve to identify scFvs suitable for future use in immunodiagnostic inhibition ELISAs. Since no inhibition was obtained with the sheep serum, hyperimmune guinea pig and rabbit sera as well as anti-BTV chicken IgY were also tested as an attempt to elucidate the lack of inhibition observed. Maximum inhibition (86%) was obtained using anti-BTV-10 chicken IgY (Figure 7B) while guinea pig serum provided only a 30% inhibition and rabbit serum showed, just like sheep serum, no inhibition. Differences in the immune systems of above-mentioned four species may account for this variation in inhibition. In addition to this, all the polyclonal sera and the chicken IgY used were raised against whole virus while the scFvs in this study were selected on denatured recombinant VP7. If the BTV-specific antibody repertoires in the sheep and rabbit sera were largely dependent on native VP7 integrity for epitope recognition, it is conceivable that the use of denatured VP7 in inhibition ELISAs could have diminished or even eliminated the inhibitory effect of the latter two sera.

In contrast to the insoluble VP7 expressed by the full length gene in prokaryotes, the cloning of only a predicted antigenic region of VP7 yielded soluble protein (Wang, Scanlon, Kattenberg, Mecham & Eaton, 1994; Reddy, Tiwari, Kataris & Rai, 2006; Pathak, Biswas, Tembhurne, Hosamani, Bhanuprakash, Prasad, Singh, Rasool & Mondal, 2008). This approach could result in more epitopes on the recombinant VP7 that retain their native structure (Pathak et al., 2008) and may extend the use of the assays described here to the detection of antibodies in sheep serum. These findings suggest that a combined strategy based on the selection of scFvs on antigenic regions of VP7 combined with elution by BTV-specific sheep sera

be more successful in the search for scFvs that can detect BTV-specific antibodies in the natural host.

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