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# Generation of chicken single chain antibody variable fragments (scFv) that differentiate and neutralize infectious bursal disease virus (IBDV)

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**Summary.** Phage-displayed recombinant antibody libraries derived from splenic mRNA of chickens immunized with an Australian strain of infectious bursal disease virus (IBDV) were constructed as single chain variable fragments (scFv) by either overlap extension polymerase chain reaction (PCR) or sequential ligation of the individual heavy ($V_H$) and light ($V_L$) chain variable gene segments. Sequential cloning of the individual $V_H$ and $V_L$ genes into a newly constructed pCANTAB-link vector containing the synthetic linker sequence $(Gly_4Ser)_3$ was more efficient than cloning by overlap extension PCR, increasing the library size 500 fold. Eighteen IBDV specific antibodies with unique scFv sequences were identified after panning the library against the immunizing antigen. Eight of the clones contained an identical $V_H$ gene but unique $V_L$ genes. In ELISA analysis using a panel of Australian and overseas IBDV strains, one scFv antibody was able to detect all strains, whilst 3 others could discriminate between Australian and overseas strains, classical and variant strains and Australian field strains and vaccine strains. In addition, some scFvs showed significant neutralization titres *in vitro*. This report shows that generation of chicken antibodies *in vitro* by recombinant means has considerable potential for producing antibodies of diverse specificity and neutralizing capacity.

## Introduction

Recombinant antibody libraries of high diversity are technically easier to generate from chickens than from other mammalian species such as mice, due to the peculiar mechanism of immunoglobulin gene diversification in birds [20, 24, 28]. Chickens possess only one functional immunoglobulin heavy chain variable region ($V_H$)

gene and one light chain variable region ($V_L$) gene from which diversity is created by gene conversions involving immunoglobulin gene rearrangements and recombination with a number of non-functional pseudogenes. Approximately 25 $V_L$ pseudogenes upstream of the $V_L$ gene and up to 100 $V_H$ pseudogenes upstream of the $V_H$ gene contain sequences similar to the single $V_L$ or $V_H$ genes, but lack other functional elements including the promoter region. Single primers designed around the conserved regions flanking the unique functional $V_H$ and $V_L$ genes can therefore be used in the polymerase chain reaction (PCR) to amplify the complete spectrum of rearranged variable fragments and enable the cloning of highly diverse chicken immunoglobulin repertoires. Chickens can mount an antibody response against a diverse range of molecules as the high frequency gene conversion mechanism operates continuously during B cell proliferation in the bursa and persists after the B cells have left the bursa. Multiple conversion events that increase the antibody diversity can occur during the lifetime of the B cell. Several research groups have constructed chicken recombinant antibodies against a variety of proteins [2, 6, 35]. Chicken recombinant antibodies, like mouse or human derived antibodies, can be expressed in various forms (Fab or scFv) or systems, of which the phage display system is the most widely used [34].

*Infectious bursal disease virus* (IBDV) is a double stranded RNA virus belonging to the family *Birnaviridae* [32]. It causes a highly contagious immunosuppressive disease in chickens by depleting B cell populations within the bursa of Fabricius. A large number of IBDV strains have been identified throughout the world and grouped according to their relative pathogenicity and antigenicity. Three major groups have been identified: classical virulent, very virulent (vvIBDV) and antigenic variants. The ability to differentiate various strains, such as vaccine strains, antigenic variants and vvIBDV, is of major concern to poultry industries worldwide, as effective control relies on the identification of the type of IBDV causing the disease and subsequent administration of appropriate vaccine strains.

Although conventional monoclonal antibodies have been generated against IBDV [7, 8, 27], they tend to be limited in their ability to differentiate various strains. The use of a recombinant DNA techniques and phage display technology to construct and express chicken antibodies offers a number of advantages. The first and most obvious advantage is obtaining antibodies from the natural host, thereby enabling the selection of antibodies that might not be produced in a heterologous host such as a mouse. Unlike conventional monoclonal antibodies, phage-displayed recombinant antibodies can be selected during the panning process allowing rapid isolation of highly specific antibodies.

In this study, we constructed highly diverse recombinant antibody libraries from the spleens of immunized chickens. A range of IBDV-specific scFvs were isolated from the libraries; 15 were able to neutralize virus *in vitro*, one was specific for all Australian strains and one differentiated between Australian vaccine strains and field strains.

## Materials and methods

### *Virus strains*

Australian IBDV strain 002/73 isolated by Firth (1974) was used for immunization of chickens and as a capture antigen in ELISA. Additional Australian IBDV strains used included six classical field strains (K-2, R-1, N1/99, N2/99, A-1, Y5-3) and five variant field strains (01/94, 03/95, 04/95, 08/95 and H-1) [26; Ignjatovic and Sapats, submitted]. Overseas strains used were classical strains 52/70 and 1/68, as well as vvIBDV strain CS89 (obtained from Dr N. Chettle, Central Veterinary Institute, Weybridge, UK) and VarE (obtained from Dr J. J. Giambrone, Auburn University, Alabama, U.S.A.).

### *Immunization of chickens*

Four three-week-old specific pathogen-free (SPF) chickens were immunized by intra-ocular administration of strain 002/73 as previously described [26]. Chickens were boosted two weeks later by intra-muscular injection with 0.5 ml of formalin-inactivated preparation of 002/73 emulsified with an equal volume of Freund's incomplete adjuvant. Four weeks post secondary vaccination, chickens were euthanised and spleens removed for purification of lymphocytes. Titres of immune sera were determined by ELISA prior to collection of spleen cells.

### *Preparation of mRNA and cDNA*

Spleens from hyper-immune chickens (ELISA antibody titres $\geq$200,000) were pooled and minced through a stainless steel mesh screen with cold PBS and layered onto an equal volume of Histopaque (Sigma Chemical Co., St. Louis, MO, U.S.A.). White blood cells were separated by centrifugation for 20 min at 700 *g* and mRNA extracted from $2.4 \times 10^9$ cells using QuickPrep mRNA purification kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Approximately 8 $\mu$g of mRNA was randomly primed in a total volume of 100 $\mu$l to produce complementary DNA (cDNA) using a cDNA synthesis kit (Boehringer Mannheim, Indianapolis, U.S.A.).

### *PCR amplification of heavy and light chain variable fragment genes*

PCR reactions were set up in a total volume of 100 $\mu$l containing 5 $\mu$l of cDNA, 10 $\mu$l of 10$\times$ cloned *Pfu* DNA polymerase reaction buffer (Stratagene, La Jolla, U.S.A.), 1 $\mu$l dNTPs (25 mM each), 2 $\mu$l (6 ng) of each forward and reverse primer (Table 1). After chilling the tubes on ice, 2 $\mu$l of cloned *PfuTurbo* was added (Stratagene) and reactions were cycled 30 times for 45 sec at 94 °C, 45 sec at 50 °C and 2 min at 72 °C. In the final cycle, the extension time at 72 °C was increased to 10 min. Amplifications were repeated five times and the final $V_H$ and $V_L$ products were pooled respectively. $V_L$ and $V_H$ products of approximately 350 and 390 bp were gel purified using a gel extraction kit (Qiagen, Hilden, Germany).

### *Cloning procedure 1: assembly of $V_H$, $V_L$ and $(Gly_4Ser)_3$ linker by overlap extension PCR*

$V_H$ and $V_L$ chains were amplified as described using primers HF & HR for the $V_H$ region or LF & LR for the $V_L$ region (Table 1). Gel-purified $V_H$ and $V_L$ chains were connected via a synthetic linker $(Gly_4Ser)_3$ using overlap extension PCR. Purified $V_H$ and $V_L$ (100 ng each) were used in PCR as described above containing 2 $\mu$l (20 ng) of each linker primer,

**Table 1.** Sequences of oligonucleotides used for construction of libraries and improved phagemid vectors

| Primer | Nucleotide sequence (5′ to 3′) |
|---|---|
| | *Antibody library construction primers*: |
| HF[a] | GCCGTGACGTTGGAC |
| HR[a] | GAACCGCCTCCACCGGAGGA GACGATGACT TCGG* |
| LF[a] | CGGTGGCGGATCGGCGCTGACTCAGCC |
| LR[a] | ACCTAGGACGGTCAGGG* |
| Link1 | GGTGGAGGCGGTTCAGGCGGAGGTGGCTCT |
| Link2 | CGATCCGCCACCGCCAGAGCCACCTCCGCCTGA* |
| HF-*Sfi* | ATGTCTATGGCCCAGCCGGCCGTGACGTTGGACG |
| LR-*Not* | AGTTACTGGAGCGGCCGCACCTAGGACGGTCAGGG* |
| LF-*Sal* | GGCGGTGGCGGGTCGACAGCGCTGACTCAGCCGTCCTCG |
| HR-*Xba* | GAACCGCCTCCACCATCTAGAGAGGAGACGATGACTTCGG* |
| HF-*Asc* | TTAGCTGGGCGCGCCGTGACGTTGGACGAGTC |
| | *Vector modification primers*: |
| B94 | CGGCCATGGGGCGCGCCGTCTAGAGCTAAGATATCGC |
| B95 | GGCCGCGATATCTTAGCTCTAGACGGCGCGCCCCATGGCCGGCT* |
| B99 | CGCGCCACTGCAGCTCTAGATCCCGGGTCGACAGATATCAGTGC |
| B100 | GGCCGCACTGATATCTGTCGACCCGGGATCTAGAGCTGCAGTGG* |
| B107 | TAACTAATTCTAGATGGTGGAGGCGGTTCAGGCGGAGGTGGCTCT |
| B108 | TATAGATTATGTCGACCCGCCACCGCCAGAGCCACCTCCGCCT* |
| | *DNA sequencing primers*: |
| Seq1 | GGTTCAGGCGGAGGTGGCTCTGG |
| Seq2 | AGAGCCACCTCCGCCTGAACC* |

*Denotes anti-sense primer

[a]Primer sequence derived from previously published data [35]

Restriction enzyme sites used for cloning shown underlined

Link1 & Link2 (Table 1). After 7 rounds of amplification (94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min), 2 μl (200 ng) of each primer HF-*Sfi* and LR-*Not* (Table 1) were added and an additional 24 cycles was performed. Approximately 500 ng of the 750 bp scFv overlap extension product was gel purified and digested sequentially with *Sfi*I and *Not*I according to the manufacturers instructions (New England Biolabs, MA, U.S.A.). The *Sfi*I/*Not*I digested scFvs were further purified using a PCR purification kit (Qiagen).

Approximately 300 ng of the digested scFv fragments were ligated with 500 ng of *Sfi*I/*Not*I digested pCANTAB 5E vector (Amersham Pharmacia Biotech) in a total volume 100 μl using the rapid DNA ligation Kit (Boehringer Mannheim). Contaminating salts were removed from the ligated DNA using the Rapid PCR purification kit (Boehringer Mannheim). DNA was electroporated into electrocompetent *Escherichia coli* (*E. coli*) TG1 cells (Amersham Pharmacia Biotech) with a transformation efficiency of approximately $1 \times 10^9/\mu g$ pUC 19 DNA. An aliquot was taken to determine the library size on SOB plates containing 100 μg/ml ampicillin and 2% glucose (SOB-AG) [25]. The remaining culture was plated out onto 50 SOB-AG plates and incubated overnight at 30 °C. The resulting lawn of bacterial cells was scraped into 20 ml 2xYT and a 1 ml aliquot diluted with 9 ml 2xYT containing 50 μg/ml

ampicillin and 2% (w/v) glucose (2xYT-AG) [25] and infected with $6 \times 10^{10}$ plaque forming units (pfu) of M13KO7 (Amersham Pharmacia Biotech). After 2 h of shaking at 37 °C the cells were collected by centrifugation and resuspended in 10 ml 2xYT containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. The culture was incubated overnight with shaking at 37 °C. Cells were pelleted at 1400 *g* for 15 min and the supernatant, containing phage, was filtered through a 0.45 μm filter. Phage particles were concentrated from the supernatant by polyethylene glycol precipitation (PEG) [10]. The concentration of infectious phage particles was determined by infecting log phase *E. coli* strain TG1 with serially diluted phage, incubating at 37 °C for 30 min and plating on SOB-AG plates.

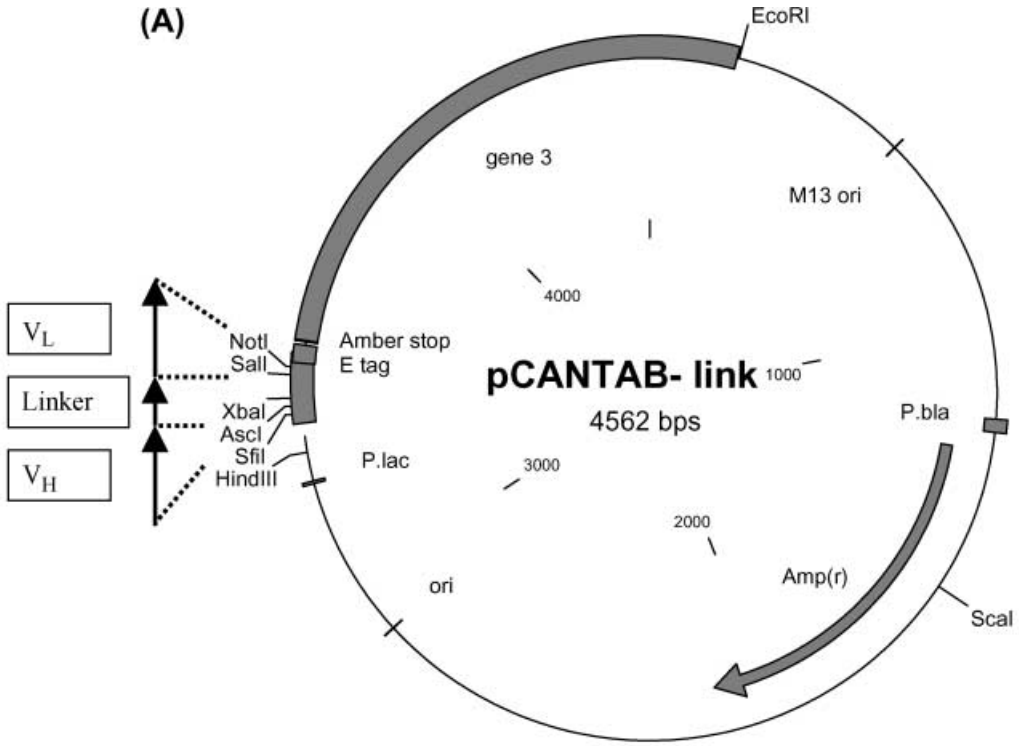
### *Cloning procedure 2: direct subcloning of $V_H$ and $V_L$ genes into the modified phagemid vector pCANTAB-link*

The pCANTAB 5E vector was modified in order to acquire additional restriction enzyme sites for direct subcloning of individual $V_H$ and $V_L$ fragments. Additional unique restriction sites (*Nco*I, *Asc*I, *Xba*I and *Eco*RV) were inserted between the *Sfi*I and *Not*I sites of the pCANTAB 5E vector by ligation of two partially overlapping oligonucleotides (B94 and B95) (Table 1). The resulting vector was named pCANTAB-*Asc*I. Two partially overlapping oligonucleotides (B99 and B100) (Table 1) were then inserted between the *Asc*I and *Not*I sites of pCANTAB-*Asc*I to introduce additional unique *Sma*I and *Pst*I restriction enzyme sites. The resulting vector was named pCANTAB-*Sma*I. An oligonucleotide encoding the polypeptide linker $(Gly_4Ser)_3$ was constructed by annealing two overlapping oligonucleotides (B107 and B108) (Table 1) and filling in with Klenow polymerase. The resulting linker fragment was digested with *Xba*I and *Sal*I to generate the appropriate overhanging ends, and inserted between the *Xba*I and *Sal*I restriction sites of pCANTAB-*Sma*I, yielding the phagemid vector pCANTAB-link (Fig. 1).

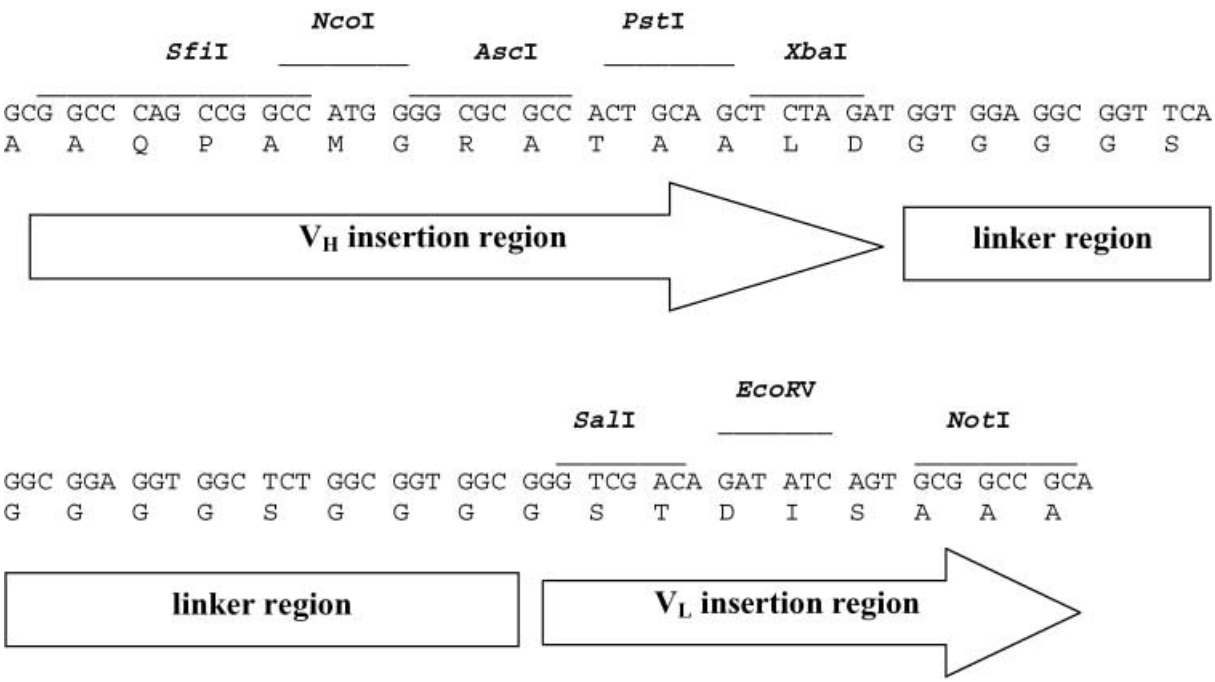
$V_H$ and $V_L$ chains were amplified as described previously using modified primers HF-*Asc*/HR-*Xba* and LF-*Sal*/LR-*Not* (Table 1). $V_H$ and $V_L$ fragments were gel purified and digested with *Asc*I/*Xba*I and *Sal*I/*Not*I, respectively (New England Biolabs). The pCANTAB-link vector was digested with either *Asc*I/*Xba*I (for cloning of $V_H$) or *Sal*I/*Not*I (for cloning of $V_L$) and the $V_H$ and $V_L$ chains cloned respectively, creating 2 intermediate plasmids pCANTAB-link-H and pCANTAB-link-L. After propagation of these plasmids in *E. coli* DH5α cells [25], purified $V_L$ genes were cloned into the *Sal*I/*Not*I site of pCANTAB-link-H and purified $V_H$ genes were cloned into the *Asc*I/*Xba*I site of pCANTAB-link-L. The ligated DNA was electroporated into electrocompetent *E. coli* TG1 cells and recombinant phage produced as described above.

### *Selection procedure for phage antibodies*

Maxisorp Immunotubes (Nunc, Denmark) were coated overnight at room temperature with 4 ml of rabbit anti-IBDV IgG diluted in 50 mM sodium carbonate buffer, pH 9.6. After washing with PBS containing 0.1% Tween 20 (PBS-T), 4 ml of 002/73 virus diluted in PBS containing 5% foetal calf serum was added at 37 °C for 1 h. Tubes were blocked for 1 h with PBS containing 5% skim milk and panning was carried out according to instructions provided with the pCANTAB 5E expression module (Amersham Pharmacia Biotech), with some modifications. Briefly, 8 ml of the PEG precipitated phage were mixed with 7 ml of 5% skim milk, and 3 ml aliquots were poured onto the blocked tubes. After a 2 h incubation at 37 °C the tubes were washed 20 times with PBS, followed by 20 washes with PBS-T. Log phase *E. coli* TG1 cells (4ml) were added to the tubes and incubated at 37 °C to allow infection by phage. Aliquots were plated onto SOB-AG to determine the titre of phage. The remaining culture was infected with helper phage M13K07 as described previously and subjected to an




**(B) Cloning regions for $V_H$ and $V_L$ in pCANTAB-link**



additional 2 rounds of panning. In later experiments the number of washes between rounds 1, 2 and 3 of panning was changed to 5, 10 and 20 washes with PBS-T, respectively.

*Expression of phage-displayed antibodies*

Individual colonies were inoculated into 500 μl of 2xYT-AG media and grown at 30 °C overnight with shaking at 250 rpm. Aliquots of 50 μl were transferred to 500 μl of 2xYT-AG containing M13KO7 helper phage. Cultures were shaken at 37 °C for 2 h at 150 rpm and then centrifuged at 1000 *g* for 15 min. The bacterial pellets were resuspended in 2xYT containing 100 μg/ml ampicillin and 50 μg/ml kanamycin and grown at 30 °C overnight with shaking at 250 rpm. Cells were pelleted and the phage-containing supernatant (∼ 400 μl) removed for analysis in ELISA. For large scale screening against IBDV strains, cultures were scaled up to 10 ml.

*Expression of soluble antibodies*

ELISA positive phage were used to infect a non-suppressor strain of *E. coli* (HB2151) according to the instructions provided by Amersham Pharmacia Biotech with some minor modifications. Briefly, 2 μl of phage supernatant was used to infect 100 μl of log phase *E. coli* HB2151 cells and the cultures were plated out onto SOB-AG containing 100 μg/ml naladixic acid (SOB-AGN). Single colonies were inoculated into 2xYT-AG and cultures were grown shaking overnight at 30 °C. Aliquots of overnight culture were diluted 1/25 into fresh SB medium [3] containing 100 μg/ml ampicillin and 2% (w/v) glucose (SB-AG) and incubated shaking at 30 °C for 2.5 h. Cells were pelleted and resuspended in fresh SB-A medium containing 1 mM of isopropylthio-β-D-galactoside (IPTG) and shaken for 6 h at 30 °C to induce expression of soluble scFv protein (Sab). Cells were pelleted and the periplasm (containing soluble antibodies) extracted using mild osmotic shock [1]. For neutralization studies, large scale stocks of soluble antibodies were produced and dialysed extensively against PBS.

*Characterisation of scFvs by sandwich ELISA*

*Phage ELISA*: For detection of binding to IBDV, phage were subjected to ELISA in which IBDV antigen was captured onto microtitre plates (Disposable Products, SA) in the same manner as described for panning, but in 100 μl volumes. IBDV antigen was used at saturation (1/20), phage were diluted with 1/5 volume 10% skim milk and 100 μl aliquots tested in ELISA. Bound phage were detected using anti-M13 HRP conjugate (Amersham Pharmacia Biotech) and absorbances at 405 nm were recorded after the addition of AZINO-bis 3-Ethylbenz 2,2′-thiazoline-6-sulfonic acid (ABTS) (Sigma). Control monoclonal antibody (Mab) 9–6 specific for IBDV has been previously described [8].

**Fig. 1.** Cloning and expression vector pCANTAB-link. **A** phagemid map and **B** DNA sequence of multiple cloning region. The phagemid contains a plasmid origin of replication (*ori*) and a phage origin of replication (*M13 ori*). The ampicillin-resistance gene (*Ampr*) is expressed from the beta-lactamase promoter (*P.bla*). The flexible linker sequence $(Gly_4Ser)_3$ is flanked by multiple cloning regions for the insertion of $V_H$ and $V_L$ gene fragments behind the lac promoter (*P.lac*) downstream of the leader peptide of the M13 gene 3 but upstream of the E-tag peptide. The *Sfi*I and *Not*I sites are in identical position and reading frame to those in the parent vector pCANTAB 5E. An amber translational stop codon between the E-tag and the remaining M13 gene 3 fragment enables expression of phage-displayed or soluble scFv depending on *E. coli* host used (suppressor or non-suppressor strain, respectively)

*Sab ELISA*: IBDV antigen was captured onto microtitre plates (Disposable Products) as for the phage ELISA. Sabs were diluted 1/10 in 2% skim milk and 100 µl aliquots tested in ELISA. Bound Sabs were detected using anti-E tag monoclonal antibody (Amersham Pharmacia Biotech), and by goat-anti-mouse IgG-HRP conjugate (Bio-Rad, Richmond, CA, U.S.A.). For estimation of relative Sab yields, $\log_2$ dilutions of Sab were directly coated onto microtitre plates and E tag was detected as previously described. Absorbencies were recorded as for the phage ELISA.

### *Virus neutralization assay*

$\text{Log}_2$ dilutions of Sabs were incubated with 32–50 median tissue culture infective doses of IBDV classical strain GT101 [8] for 1 h at 37 °C. Antibody-virus mixture was then added to a freshly prepared suspension of chicken embryo fibroblasts (quadruplicate samples for each dilution) in 96 well tissue culture plates and incubated at 37 °C in the presence of 3% $CO_2$ [8]. Cell monolayers were examined visually for cytopathic effect 4–5 days later and complete absence of cytopathic effect in all replicates was recorded as virus neutralization.

### *DNA sequence determination and analysis*

Phagemid DNA was isolated using a Qiagen Miniprep kit. Each scFv construct was sequenced using the S1 and S6 sequencing primers (Amersham Pharmacia Biotech) that were complementary to the vector sequence. Two additional primers, Seq1 and Seq2 (Table 1), were used which were complementary to the linker sequence. Nucleotide sequences were determined using the Big Dye Terminator Ready Reaction Kit in conjunction with the 377 XL automated DNA sequencer (Applied Biosystems, U.S.A.). Resulting sequences were aligned using the CLUSTAL-X (version 1.81) multiple sequence alignment program [29]. DNA sequence analyses were performed using programs on the Internet accessed via the Australian National Genomic Information Service (ANGIS). The nucleotide sequence data reported in this paper has been submitted to the GenBank nucleotide database and has been assigned accession numbers AF506494–AF506512.

## Results

### *Selection of antibodies from scFv libraries constructed by overlap extension PCR and cloning in pCANTAB 5E*

Initially, scFv fragments from immune chickens were cloned into the pCANTAB 5E vector. Although high yields of chicken immunoglobulin $V_H$ and $V_L$ fragments were obtained by PCR amplification, the subsequent joining of $V_H$ and $V_L$ via a linker sequence using overlap extension PCR produced a library containing only $1.5 \times 10^5$ clones. The library was subjected to two different panning procedures. In the first procedure, phage were panned three times against IBDV strain 002/73, washing 40 times after each panning. After each panning step, 88 clones were screened for binding in an ELISA against 002/73. After the first, second and third panning, 2%, 28% and 81% of clones, respectively, were ELISA positive. All clones isolated from the third round of panning were identical by DNA sequencing and were designated chicken recombinant antibody 34 (CRAb34). One ELISA negative scFv, designated CRAb0, was chosen from the original unpanned library to serve as a negative control. Since the first panning procedure identified only one IBDV specific scFv, an attempt was made to re-pan the library

using less stringent conditions. In the second panning procedure the number of washes between pannings was reduced to 5, 10 and 20 after the first, second and third panning steps, respectively. Despite a reduction in the number of washing steps it was not possible to isolate additional antigen positive CRAbs other than those identical to CRAb34, indicating that the library was limited in terms of diversity.

### *Construction of phagemid vector pCANTAB-LINK*

In an attempt to increase the size and diversity of the library, we developed a new vector that enabled the sequential ligation of $V_H$ and $V_L$ PCR fragments either side of a flexible linker region $(Gly_4Ser)_3$. Unique restriction sites and a linker region were introduced into the pCANTAB 5E vector in several steps, resulting in the final vector, pCANTAB-link (Fig. 1). It encodes a flexible linker region $(Gly_4Ser)_3$, which allows construction of functional scFv antibodies by sequential insertion of $V_H$ and $V_L$ fragments on either side of a linker region. This eliminated the need to connect $V_H$ and $V_L$ at the PCR level.

### *Selection of antibodies from scFv libraries constructed by sequential cloning in pCANTAB-link*

The same batch of chicken lymphocyte mRNA used for construction of the pCANTAB 5E library was used for the construction of the scFv library in the pCANTAB-link vector. Our choice of restriction fragments was *Asc*I-*Xba*I for the ligation of $V_H$ fragments upstream of the linker region and *Sal*I-*Not*I for ligation of $V_L$ fragments downstream of the linker as these sites were absent in chicken immunoglobulin variable fragments published in GenBank. Two separate intermediate libraries were constructed, one containing the $V_H$ genes and the other containing the $V_L$ genes. Cloning either the $V_H$ or $V_L$ first made no difference to the diversity of the library, $V_H$ and $V_L$ libraries yielding approximately $2.1 \times 10^4$ and $8.2 \times 10^5$ clones respectively. The final library containing both the $V_H$ and the $V_L$ yielded approximately $7.5 \times 10^7$ clones, and upon superinfection with helper phage gave rise to approximately $4.7 \times 10^{12}$ phage particles. Phage were subjected to three rounds of panning with 5, 10 and 20 washes after each round, respectively. The binding capacity of (combined) phage was examined after each round of panning using ELISA. With each successive round of panning, an increase in the ELISA absorbance was observed indicating an enrichment of IBDV specific clones (results not shown). Three hundred individual clones obtained from the third panning of the pCANTAB-link library were screened for binding to 002/73 in ELISA. Of the 300 clones tested, 70% reacted positively in an ELISA. One hundred ELISA positive clones were randomly chosen and tested for their binding to a panel of Australian and overseas IBDV strains, first as phage displayed antibodies and subsequently as soluble antibodies. Representative clones for the various ELISA patterns were randomly chosen for DNA sequence analysis.

### *DNA sequence analysis of scFv clones*

Sequencing of CRAb34 and one representative clone from the observed ELISA patterns described above, identified 18 unique CRAbs. An alignment of the deduced amino acid sequences of the 18 IBDV specific CRAbs, as well as the control CRAb0, is shown in Fig. 2. Three complementarity determining regions (CDRs) and four framework (FW) regions were identified in each of the $V_H$ and $V_L$ fragments. Antibodies constructed in pCANTAB-link (i.e. all antibodies except for CRAb34 and CRAb0) contain additional amino acids either side of the linker, (LD5′ and T3′) due to the introduction of extra restriction sites to facilitate cloning into the vector. Apart from this minor difference, eight of the CRAbs (3, 5, 15, 24, 33, 34, 83 and 96) shared identical $V_H$ sequences, however their $V_L$ sequences were unique. Of the clones with a different $V_H$ region, CRAb23 was most similar to CRAb34 in the sequence of the CDR3 region of the $V_H$ chain with only 2 amino acid substitutions. The CDR3 region in $V_H$ of CRAb11 was unusually longer than those in all other CRAbs.

### *Characterization of scFv clones by ELISA against different IBDV strains*

The reactivity of both phage-displayed and soluble antibodies was assessed by ELISA against a panel of IBDV strains including Australian vaccine strain (002/73) as well as classical and variant field isolates and foreign IBDV strains including classical strains (52/70, 1/68), antigenic variant (VarE) and a very virulent strain (CS89) (Table 2). All CRAbs reacted well with strain 002/73 that had been used for immunization and as an antigen for panning, but reactivity with other IBDV strains varied between different CRAbs and also between phage-displayed and soluble antibodies. Overall the phage-displayed form of the CRAbs could be grouped into 6 ELISA binding patterns (Table 2) when assayed at saturation concentration for antibodies and antigen. The phage-displayed form of CRAb20 (CRAb20p) was the only CRAb capable of recognizing all IBDV strains including VarE. CRAbs 12 and 21 were cross-reactive with all strains with the exception of VarE. CRAbs 3, 5, 8, 15, 22, 23, 24, 33, 52, 72, 83 and 96 reacted with most strains excluding VarE and 2 Australian variants (04/95 and 08/95). CRAb11 bound to all Australian strains, whilst showing no binding to any overseas strains. CRAb7p bound to all strains excluding both the VarE and Australian variants. CRAb34p showed the highest binding to Australian vaccine strain 002/73 and reduced binding to other Australian field strains. Major differences in ELISA patterns were observed between some phage-displayed and solubly expressed CRAbs (Table 2). This is exemplified by the phage-expressed form of CRAb34 (CRAb34p) that reacted predominantly with only the two Australian vaccine strains (002/73 and V877), thus enabling their differentiation from field strains. In contrast, when expressed in a soluble form, CRAb34 showed broader specificity and reacted with several other strains, albeit to a lesser degree (Fig. 3). In order to determine if reactivity patterns were due to a concentration factor, a titration of CRAb34 expressed as a soluble

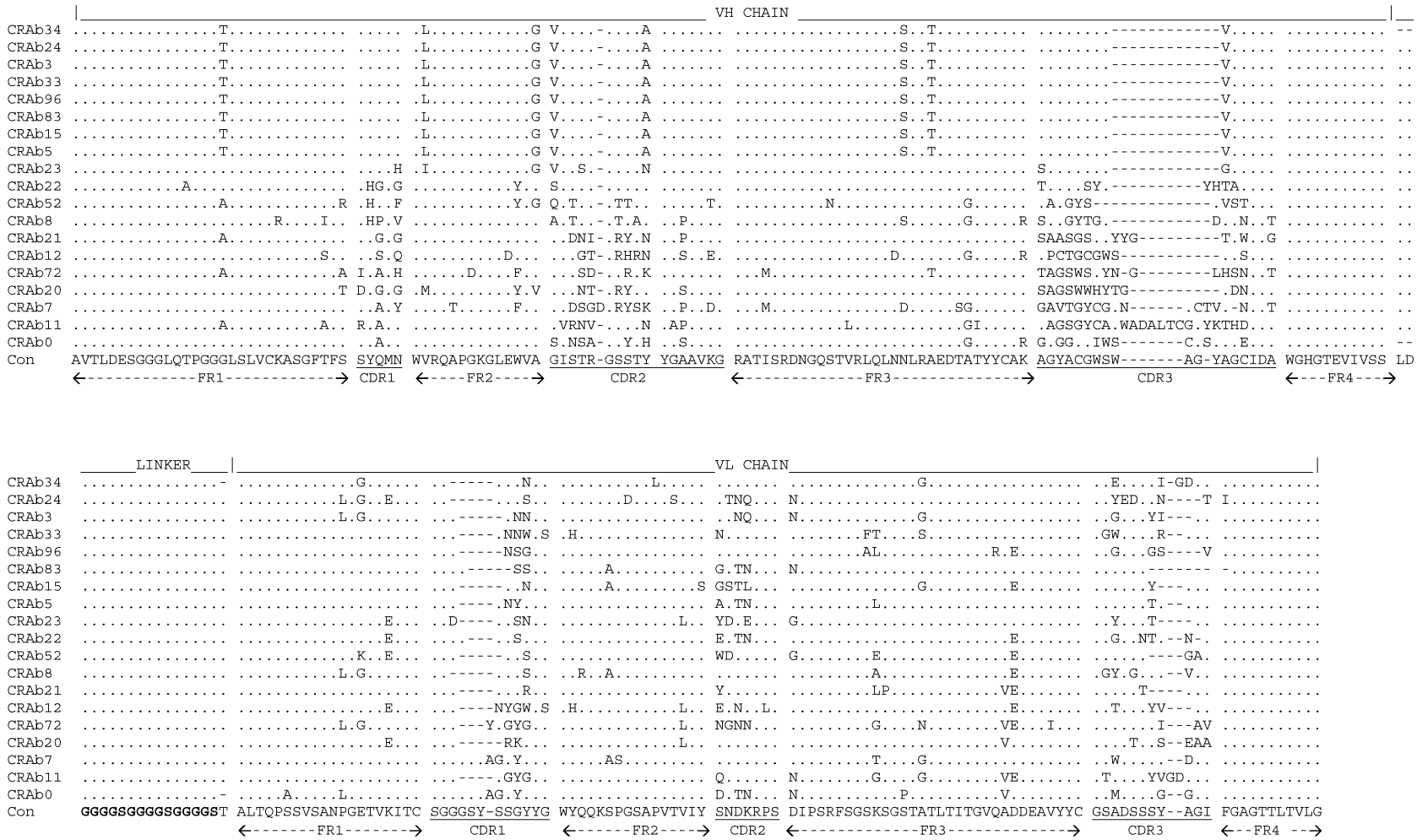



**Fig. 2.** Alignment of the deduced amino acid sequences of the variable regions of the heavy and light chain genes of scFv clones. Only those amino acids that differ from the consensus sequence are shown, while identical amino acids are shown by dots and gaps introduced during alignment are shown by dashes. Complementarity determining regions (*CDR1-3*) and conserved framework regions (*FR1-4*) are shown beneath the consensus sequence. The linker sequence $(Gly_4Ser)_3$ is shown in bold

**Table 2.** ELISA binding pattern of chicken recombinant antibodies with different IBDV strains

| | Australian IBDV strains[a] | | | | | | | | | | | | Foreign IBDV strains[a] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | classical | | | | | | | variant | | | | | classical | | var | vv | |
| CRAb[b] | 002/73 | K-2 | R-1 | N1/99 | N2/99 | A-1 | Y5-3 | H-1 | 01/94 | 03/95 | 04/95 | 08/95 | 52/70 | 1/68 | VarE | CS89 | Neg |
| 20p | +++[c] | +++ | ++ | +++ | ++ | ++ | +++ | ++ | ++ | ++ | + | ++ | ++++ | +++ | ++ | ++++[d] | - |
| 20s | ++++ | ++++ | +++ | + | + | + | + | + | + | +++ | + | - | +++ | ++++ | - | ++++ | - |
| 12p | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | - | ++++[e] | - |
| 12s | ++++ | ++++ | ++ | ++++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ | ++ | - | ++ | - |
| 21p | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | ++ | - |
| 21s | +++ | +++ | + | ++ | +++ | +++ | ++ | - | - | - | - | - | + | + | - | + | - |
| 3p * | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | - | - | ++++ | ++++ | - | ++++[f] | - |
| 3s * | ++++ | +++ | ++ | +++ | ++ | +++ | +++ | ++ | ++ | ++ | + | + | + | + | - | ++ | - |
| 5p * | ++++ | ++++ | ++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ | - | - | ++ | +++ | - | ++ | - |
| 5s * | ++++ | ++++ | +++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | + | - | +++ | ++++ | - | ++++ | - |
| 8p | ++++ | ++ | ++ | ++ | +++ | +++ | +++ | ++++ | +++ | +++ | - | - | - | - | - | - | - |
| 8s | ++++ | ++++ | +++ | ++++ | ++++ | ++++ | +++ | +++ | +++ | +++ | - | - | ++ | +++ | - | +++ | - |
| 15p * | ++++ | ++++ | ++++ | ++++ | +++ | ++++ | ++++ | ++++ | ++++ | ++++ | - | - | ++++ | ++++ | - | ++++ | - |
| 15s * | ++++ | ++++ | ++ | ++++ | +++ | +++ | ++++ | +++ | +++ | +++ | - | - | ++ | +++ | - | ++ | - |
| 22p | ++++ | +++ | +++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | - | - | +++ | ++++ | - | +++ | - |
| 22s | ++++ | +++ | ++ | +++ | +++ | ++ | +++ | +++ | ++ | +++ | - | - | ++ | ++ | - | ++ | - |
| 23p | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | - | - | +++ | +++ | - | +++ | - |
| 23s | ++++ | ++ | + | ++ | ++ | ++ | ++ | + | + | + | - | - | + | + | - | ++ | - |
| 24p * | ++++ | +++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | - | - | +++ | ++ | - | ++ | - |
| 24s * | ++++ | ++++ | +++ | ++++ | +++ | ++++ | +++ | +++ | +++ | +++ | - | - | +++ | +++ | - | ++ | - |
| 33p * | ++++ | +++ | ++++ | +++ | ++++ | +++ | ++++ | +++ | +++ | +++ | - | - | ++++ | +++ | - | ++++ | - |
| 33s * | ++++ | +++ | ++ | +++ | +++ | +++ | +++ | +++ | ++ | +++ | - | - | - | - | - | - | - |
| 52p | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | - | - | ++ | ++ | - | ++ | - |
| 52s | ++++ | ++ | + | ++ | ++ | ++ | ++ | +++ | ++ | ++ | - | - | - | - | - | + | - |
| 72p | +++ | +++ | +++ | + | +++ | ++ | + | ++ | + | ++ | - | ++ | ++ | ++ | - | ++ | - |
| 72s | ++++ | ++++ | ++ | +++ | +++ | ++++ | +++ | +++ | +++ | +++ | + | - | - | - | - | - | - |
| 83p * | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | - | - | ++++ | ++++ | - | ++++ | - |
| 83s * | ++++ | ++++ | +++ | ++++ | +++ | +++ | ++++ | +++ | +++ | +++ | - | - | ++ | +++ | - | ++ | - |
| 96p * | +++ | ++++ | +++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | +++ | - | - | +++ | +++ | - | +++ | - |
| 96s * | ++++ | ++++ | ++ | ++++ | +++ | +++ | ++++ | +++ | ++ | +++ | - | - | ++ | +++ | - | ++ | - |
| 11p | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++ | ++ | ++ | ++ | ++[g] | - | - | - | - | - |
| 11s | ++++ | ++++ | +++ | ++++ | ++++ | ++++ | +++ | ++++ | ++ | ++++ | ++ | ++ | - | - | - | - | - |
| 7p | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | - | - | - | - | - | ++ | ++ | - | ++[h] | - |
| 7s | ++++ | +++ | +++ | ++++ | ++++ | ++++ | ++ | - | - | - | - | - | - | - | - | + | - |
| 34p * | +++[i] | + | - | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| 34s * | ++++ | +++ | ++ | +++ | + | ++ | +++ | ++ | + | ++ | - | - | - | + | - | - | - |
| 0p | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 0s | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Mab 9-6 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++ | ++++ | + | + | ++++ | ++++ | - | ++++ | - |

[a]IBDV strains, as bursal homogenate, used at saturation (1/20)

[b]Chicken recombinant antibodies used as phage displayed (p) or soluble (s) antibody. Sab diluted 1/10 with 2% skim milk, phage diluted with 1/5 volume 10% skim milk. Asterisk * denotes clones that have identical $V_H$ sequence

[c]++++, +++, ++, + and − correspond to absorbances of >1.1, 0.7–1.1, 0.3–0.7, 0.1–0.3 and <0.1, respectively

[d−i]ELISA groups based on phage antigen binding patterns. Each group represented by one CRAb in shading. e.g. CRAb20 (binding to all strains), CRAb12 (binding to all strains except Var E), CRAb3 (binding to most strains, except Var E, and Australian variants 04/95 and 08/95), CRAb11 (specific for all Australian strains), CRAb7 (specific for all classical strains) and CRAb34 (greater binding to vaccine strains)

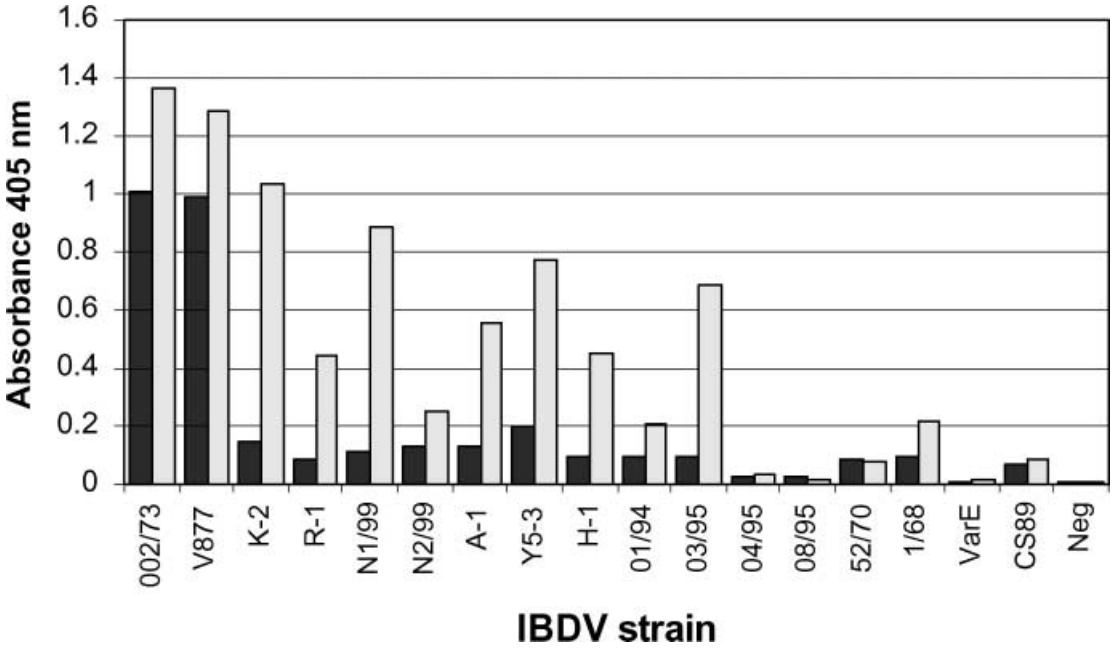



**Fig. 3** Binding of phage-displayed (*p*) and soluble (*s*) forms of CRAb34 with different IBDV strains in sandwich ELISA. IBDV strains, used in excess (1/20), were immobilized to the wells of the microtitre plate using rabbit anti-IBDV IgG. CRAb34p was diluted with 1/5 volume 10% skim milk, while CRAb34s was diluted 1/10 using 2% skim milk. Absorbances above 0.2 were considered positive. ■ CRAb34p, ■ CRAb34s

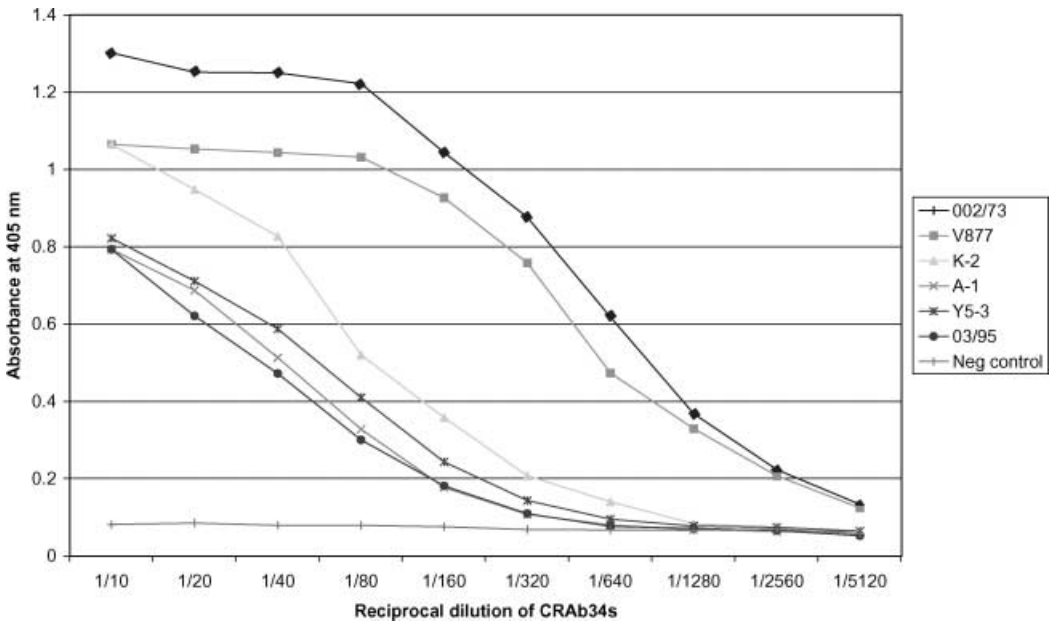



**Fig. 4.** Titration of CRAb34 soluble antibody against IBDV strains. Specificity of binding depended on concentration of the soluble antibody. CRAb34s was titrated against a fixed concentration of viral antigen (1/20) immobilized to the wells of the microtitre plate using rabbit anti-IBDV IgG. Absorbances above 0.2 were considered positive

antibody was performed with different strains (Fig. 4). The level of reactivity was clearly dependant on the relative antibody concentration; at high dilutions (1/320) CRAb34s was able to discriminate vaccine strains from other strains. All strains were used at saturation conditions and reacted strongly with the control Mab 9–6 to a similar degree (results not shown).

**Table 3.** Neutralization and ELISA titres of chicken recombinant antibodies

| CRAB | Reciprocal titres[a] | | | |
|---|---|---|---|---|
| | | ELISA of Sab | | ELISA of phage Ag binding[e] |
| | VN[b] | Ag binding[c] | E tag[d] | |
| 20 | 12,800 | 10,240 | 2,560 | 128 |
| 5* | 6,400 | 10,240 | 2,560 | 64 |
| 11 | 6,400 | 5,120 | 2,560 | 64 |
| 8 | 3,200 | 20,480 | 5,120 | 32 |
| 24* | 2,560 | 2,560 | 640 | 64 |
| 15* | 1,024 | 81,920 | 5,120 | 128 |
| 83* | 800 | 5,120 | 1,280 | 256 |
| 72 | 800 | 80 | 640 | 16 |
| 22 | 160 | 40,960 | 5,120 | 64 |
| 23 | 160 | 40,960 | 5,120 | 128 |
| 96* | 64 | 10,240 | 2,560 | 32 |
| 52 | 32 | 10,240 | 2,560 | 128 |
| 33* | 16 | 1,280 | 1,280 | 128 |
| 34* | 16 | 640 | 320 | 32 |
| 21 | 8 | 1,280 | 2,560 | 16 |
| 7 | 0 | 10,240 | 2,560 | 128 |
| 12 | 0 | 20,480 | 5,120 | 256 |
| 3* | 0 | 10,240 | 2,560 | 128 |
| 0 | 0 | 0 | 1,280 | 0 |
| Mab 9-6[f] | 12,800 | NA[g] | NA | NA |
| Anti-002/73[h] | 128,000 | NA | NA | NA |

*Asterisk denotes chicken recombinant antibodies that have identical $V_H$ sequence

[a]Reciprocal of the highest dilution that neutralized virus cytopathic effect or giving positive (greater than double background) reaction in ELISA

[b]$Log_2$ dilutions of soluble antibody incubated with 32–50 median tissue culture infective doses of IBDV strain GT101

[c]$Log_2$ dilutions of soluble antibody reacted with excess of 002/73 antigen (1/20) captured by rabbit anti-IBDV IgG in ELISA wells and bound antibody detected using anti-E tag monoclonal antibody and anti-mouse IgG-HRP

[d]$Log_2$ dilutions of soluble antibody bound directly to ELISA wells and E tag detected using anti-E tag monoclonal antibody and anti-mouse IgG-HRP

[e]$Log_2$ dilution of phage antibody reacted with excess of 002/73 antigen captured by rabbit anti-IBDV IgG in ELISA wells and detected using anti-M13 IgG-HRP

[f]Mouse monoclonal antibody against 002/73 strain

[g]*NA*, not applicable

[h]Chick hyperimmune sera

*Neutralization of IBDV infectivity by CRAbs in vitro*

All 18 CRAbs were assessed for their ability to neutralize the tissue culture adapted IBDV strain GT101 derived by passaging of 002/73 in chicken embryo fibroblasts (Table 3). Fifteen of the soluble scFvs were able to neutralize 32 $TCID_{50}$ of GT101. Ten scFvs had titres higher than 160. Five antibodies showed low neutralizing titres of between 8–64, whilst 3 scFvs (CRAbs 3, 7 and 12) showed no neutralizing properties. In general, the neutralization titres of the scFvs were lower than that of either immune sera (128,000) or Mab 9–6 (12,800). The one exception was CRAb20 with a neutralization titre equal to that of Mab 9–6 (12,800). Significant differences in neutralization titres were observed between CRAbs bearing the same $V_H$ sequence. This was best exemplified by CRAb5 and CRAb3, which had neutralization titres of 6,400 and 0, respectively.

The neutralization titres for GT101 did not correlate with the ELISA antigen binding titres for phage displayed and soluble CRAbs against 002/73 (Table 3). For example CRAb20 with the highest neutralization titre of 12,800 and CRAb52 with a low neutralization titre of 32 had identical binding and E tag titres in ELISA of 10,240 and 2,560, respectively. In general the phage antibody titres for antigen binding in ELISA were significantly lower than the soluble antibody titres, however the titres of soluble antibody were highly variable and dependent upon the particular CRAb. No correlation between phage and soluble antibody binding titres was observed.

## Discussion

This is the first report of genetically engineered chicken antibodies against IBDV. We generated scFv with variable specificity and neutralization capacity from immunized chickens. The method used for cloning of $V_H$ and $V_L$ fragments was found to greatly influence the quality of the library, in terms of both size and diversity. Assembly of $V_H$, $V_L$ and linker fragments by overlap extension PCR using a commercially available vector yielded a library containing only $5 \times 10^5$ clones. Despite the use of different panning strategies, only one functional scFv (CRAb34) was obtained from this library. Overlap extension PCR has previously been identified as being the most inefficient and problematic stage in constructing scFv libraries (16). To overcome this obstacle we constructed a new phagemid vector, pCANTAB-link, that encoded a polypeptide linker region $(Gly_4Ser)_3$ flanked by two multiple cloning regions. $V_H$ or $V_L$ fragments obtained by PCR could then be directly ligated into unique restrictions sites upstream (*Sfi*I, *Nco*I, *Asc*I, *Pst*I and *Xba*I) or downstream (*Sal*I, *Eco*RV and *Not*I) of the linker. This eliminated the need for PCR assembly, and significantly increased the efficiency of generating scFv libraries. Using pCANTAB-link, we not only achieved a 500-fold increase in library size, but were also able to isolate an additional 17 unique CRAbs. The presence of unique restriction sites between the linker and the variable fragments should facilitate simple downstream modifications of libraries/clones, such as shuffling $V_H$ and $V_L$ fragments and exchanging linkers for example for

the generation of diabodies and triabodies [31]. In addition, this vector may be used for the construction of scFv libraries from any species.

Analysis of the nucleotide sequences revealed that all 18 scFvs possessed unique $V_L$ sequences, however 8 shared identical $V_H$ sequences. Seven of these scFvs with identical $V_H$ sequence showed similar ELISA patterns, binding to the majority of Australian and overseas IBDV strains. However, CRAb34 bound predominantly to only Australian vaccine strains despite having an identical $V_H$ chain. The importance of the $V_H$ region in defining antigen binding, and particularly the CDR3 region, has been well documented [12, 13, 19, 21], however our results with CRAb34 would suggest that the $V_L$ may be important in dictating the fine antigen specificity as has been previously found with other scFvs [11, 14].

From our panel of 18 scFvs we identified several with unique specificities that might be useful for diagnostic applications. Previously Australian IBDV strains could not be differentiated from overseas strains with existing monoclonal antibodies [26]. CRAb11 was capable of differentiating all Australian IBDV strains from overseas strains in ELISA, while CRAb20 detected all IBDV strains including VarE. CRAb7 differentiated all classical from variant strains. In addition, CRAb34 displayed on the phage surface or as a diluted Sab could differentiate Australian vaccine strains from genetically and antigenically closely related field strains [26]. This demonstrates the power of phage display technology in generating highly specific recombinant antibodies capable of detecting minor antigenic differences between closely related epitopes. If validated with larger representative panels of Australian and overseas strains, these CRAbs could be used in diagnostic laboratories throughout Australia. Antibody maturation by mutagenesis or chain shuffling followed by subtractive panning could be employed to further improve the strain differentiation capacity of CRAbs.

ELISA reactivity of phage-displayed and soluble CRAbs revealed a number of differences. Overall, scFv expressed on phage showed broader antigenic specificity in comparison to the same scFv expressed as a Sab. This may be caused by a co-operative effect due to the capacity of each phage particle to display more than one antibody fragment, especially when binding to a large multivalent antigen such as a virion [23]. Furthermore, the secondary antibody (anti-M13 HRP conjugate) that binds to the abundant M13 coat protein on the bacteriophage in large numbers, could result in an amplification of the ELISA signal [33]. It is interesting to note however, that the reverse situation was true for CRAb34 in that the soluble form appeared to have broader specificity than the phage-displayed scFv. Differences in reactivity between phage expressed scFvs and soluble scFvs have been reported [33], while others have found no differences [22, 30]. Clearly, different antibodies behave differently in different expression systems.

The yields of Sabs were highly variable and dependent upon the particular CRAb. The proteolytic activity of *E. coli* periplasmic proteases can affect the expression efficiency of many heterologous proteins such as antibody fragments that are transported to the host periplasm [18]. In addition, production of recombinant proteins in periplasm of *E. coli* can be limited by folding problems leading to periplasmic aggregates [4].

Although recombinant chicken antibodies have been previously generated against egg white lysozyme, bovine serum albumin, bovine thyroglobulin [6] and murine serum albumin [35], none have been shown to posses functional biological activity such as neutralization. A number of scFvs have been shown to possess neutralizing capacity against viruses such as Tick-Borne encephalitis virus [15], rabies virus [22], vesicular stomatitis virus [17], canine parvovirus [36] and viral haemorrhagic septicaemia virus [5]. However, these have been derived from mouse hybridoma cell lines and not directly from the immunized host.

The majority of our antibodies showed neutralization capacity against IBDV strain GT101 *in vitro* and some displayed high neutralization titres. Interestingly, the 8 scFvs that had identical $V_H$ sequences, showed significant differences in neutralization titres despite 7 of these having similar ELISA reactivity. Our data would suggest that $V_L$ may play an important role in defining the neutralization capacity of scFvs. Different $V_L$ sequences could affect neutralization by influencing factors such as affinity, expression levels, protein folding and stability. Using single amino acid substitutions in scFvs, it has been shown that the antigen-antibody association rate constant had no relationship with the neutralising activity, whereas a low dissociation rate constant appeared to be related to neutralizing activity [5]. It has been suggested that scFvs may cover the virion surface thereby masking ligands involved in receptor docking, or alternatively induce conformational changes within the virion coat protein that hinder molecular events critical for viral infectivity [17].

In conclusion, we have used phage display to generate a panel of chicken recombinant antibodies specific for IBDV. The relative ease of generating diverse recombinant antibodies that can detect minor antigenic differences between viral strains illustrates the power of this technology for producing diagnostic or potentially therapeutic reagents.

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