Arch Virol (2006) 151: 1551–1566
DOI 10.1007/s00705-006-0729-8

Archives of Virology
Printed in Austria

Chicken recombinant antibodies specific for very virulent infectious bursal disease virus

S. I. Sapats[1], **L. Trinidad**[1], **G. Gould**[1], **H. G. Heine**[1], **T. P. van den Berg**[2], **N. Eterradossi**[3], **D. Jackwood**[4], **L. Parede**[5], **D. Toquin**[3], and **J. Ignjatovic**[1]

[1]CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Australia
[2]Avian Virology & Immunology, Veterinary and Agrochemical Research Centre VAR-CODA-CERVA, Brussels, Belgium
[3]Agence Française de Sécurité Sanitaire des Aliments, VIPAC, Ploufragan, France
[4]Food Animal Health Research Program, The Ohio State University/OARDC Wooster, Ohio, U.S.A.
[5]Balai Penelitian Veteriner, Jl. RE Martadinata 30, Bogor 16114, Indonesia

Received November 4, 2005; accepted January 16, 2006
Published online March 3, 2006

Summary. A phage-displayed single chain variable fragment (scFv) antibody library was constructed from the immune spleen cells of chickens immunized with very virulent infectious bursal disease virus (vvIBDV) strain CS89. A library consisting of around 9.2×10^7 clones was subjected to 3 rounds of panning against captured CS89 virus. Analysis of individual clones by nucleotide sequencing revealed at least 22 unique scFv antibodies binding to vvIBDV in ELISA. Testing of the scFv antibody panel in ELISA against classical, variant or vaccine strains and a wide variety of vvIBDV isolates from the UK, China, France, Belgium, Africa, Brazil, Indonesia and the Netherlands identified one antibody, termed chicken recombinant antibody 88 (CRAb 88) that was specific for vvIBDV. CRAb 88 was capable of recognizing all vvIBDV strains tested regardless of their country of origin and showed no reactivity with classical, variant or vaccine strains, lending support to the use of this scFv as a powerful diagnostic tool for the differentiation of vvIBDV strains. Immunoprecipitation studies revealed that CRAb 88 was directed towards a highly conformational epitope located within the major neutralizing protein VP2. Sequence analysis of the hypervariable region of VP2 of the IBDV strains tested indicate that Ile(256) and Ile(294) may play roles in binding of

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide database and have been assigned accession numbers AY255642 to AY255663.

CRAb 88. This is the first reagent of its type capable of positively distinguishing vvIBDV from other IBDV strains.

Introduction

Infectious bursal disease virus (IBDV) causes a highly contagious immunosuppressive disease in chickens resulting in considerable economic losses to the poultry industry. IBDV contains a double stranded bi-segmented RNA genome contained within a non-enveloped capsid. The genome encodes five viral proteins, two of which, VP2 and VP3, make up the capsid of the virion [37]. The VP2 protein is the host protective antigen and is responsible for eliciting neutralizing antibodies [37]. Hence, most methods of differentiation focus on the VP2 protein, specifically a hypervariable region (HVR) located between amino acid residues 211 and 340 [4]. Recent studies using X-ray crystallography to elucidate the atomic structure of IBDV have indicated that certain residues within the HVR region of VP2 occur in the most exposed regions of the virus and may therefore be involved in binding to cell-surface receptors [12].

For around 30 years classical IBDV strains predominated throughout the world and were effectively controlled by vaccination. The sudden appearance of antigenic variants in the USA and very virulent (vv) strains in Europe in the late 80s necessitated the introduction of new vaccines based upon variants and less attenuated "hot" strains, respectively [37]. vvIBDV strains quickly spread to other regions of the world causing mortalities as high as 80% in fully susceptible chicks. Australia is free of vvIBDV, however, both classical and variant strains have been identified in Australia that are distinct from overseas strains and of low pathogenicity [34]. The emergence or accidental incursion of vvIBDV strains into Australia would have a significant impact on the local poultry industry and necessitate urgent control measures. Hence, a rapid and inexpensive diagnostic test was desired by the local poultry industry that can quickly distinguish vv strains from other IBDV strains in the case of an exotic incursion.

Currently, the only method to accurately determine a vv phenotype is based upon testing of isolates in chickens, however this process is expensive and time-consuming. Other methods have been introduced in an attempt to predict a vv phenotype, which include both molecular and immunological techniques. Nucleotide sequencing of the HVR of VP2 has been the most frequently used method to predict a strain's phenotype, and more recently, restriction fragment length polymorphism (RFLP) [26]. Several different restriction enzymes have been used for RFLP analysis of IBDV strains. In particular, the presence of unique *Bsp*MI and *Ssp*I sites have been correlated with vvIBDV strains, which correspond to amino acid substitutions 222(Pro $\rightarrow$ Ala) and 294(Leu $\rightarrow$ Ile), respectively [26, 29, 41]. A number of exceptions have been described that lack a *Bsp*MI or *Ssp*I restriction site due to either the presence of a silent mutation or an amino acid substitution [10, 27, 32, 42]. In addition, a number of variant strains have been isolated that contain an *Ssp*I site [2, 28]. The use of a vvIBDV-specific monoclonal antibody (MAb) could offer some significant advantages as its epitope recognition

is directly related to the encoding amino acids and would not be affected by such silent mutations. In addition, MAbs offer a fast and cheap method of differentiation without the need for sophisticated apparatus associated with nucleotide sequence determination and/or RFLP. To date, no MAb specific for vvIBDV has been isolated, although a panel of mouse MAbs have been developed that are able to differentiate between classical and vvIBDV strains based upon the lack of reactivity of two MAbs with vvIBDV [14, 15, 17, 38].

An alternative method of generating antibodies by recombinant means involves the use of a phage display system to screen antibody libraries. In this system, it is possible to use the white blood cells of an immune host for the PCR of the antibody heavy- and light-chain variable genes (V_H and V_L). These genes are then joined via a flexible synthetic linker by cloning into a specially prepared phagemid vector producing single-chain variable fragments (scFv) [24]. By making fusion proteins between the scFvs and the filamentous bacteriophage M13 gene III coat protein, scFvs can be displayed on the phage surface while the coding sequence of the displayed scFv is contained within the bacteriophage. Panning against the immunizing antigen to select for specific binders then identifies the scFvs of interest. We have previously shown that scFv isolated from chickens immunised with IBDV had the potential to differentiate between antigenically and genetically closely related strains [35]. We therefore attempted to select from a vvIBDV immune library a scFv that was specific for vvIBDV.

Methods

Virus strains

Twenty-five Australian IBDV strains used have been previously described [25, 34]. Other IBDV strains were obtained from the following sources: CS89, F52/70 and 1/68 from Dr N. Chettle, Veterinary Laboratories Agency, Weybridge, UK; 849VB from Dr Thierry van den Berg, Veterinary and Agrochemical Research Centre, Brussels, Belgium; Var E, GLS5, and STC from Dr J. J. Giambrone, Auburn University, Alabama, USA. Testing of selected CRAbs against a wider panel of IBDV strains not available in our laboratory (Table 2) was performed at Agence Française de Sécurité Sanitaire des Aliments, Ploufragan, France, and Food Animal Health Research Program, Ohio State University, Wooster, Ohio, USA.

Experimental infection of chickens and purification of mRNA for cDNA synthesis

Ten three-week-old specific-pathogen-free (SPF) chickens were infected by intra-ocular administration of vvIBDV strain CS89 as previously described [35]. Spleens from six immune chickens collected at 4 weeks after infection were pooled and the white blood cells isolated by centrifugation as previously described [35]. The mRNA from 1.2×10^9 white blood cells was extracted using a QuickPrep mRNA purification kit (Amersham Pharmacia Biotech), and approximately 6 μg of mRNA was randomly primed to produce complementary DNA (cDNA) [35].

Construction of a scFv antibody expression library

A recombinant antibody library was constructed from the lymphocyte cDNA by sequential ligation of PCR-amplified V_H and V_L gene fragments into the phagemid vector

pCANTAB-link at either side of a flexible linker sequence $(Gly_4Ser)^3$ as described previously [35]. Briefly, V_H and V_L chains were amplified using primer combinations HF-*Sfi*/HR-*Xba* and LF-*Sal*/LR-*Not* to amplify a 390-bp and a 350-bp fragment, respectively. The V_H and V_L PCR fragments were gel purified and digested with *Asc*I/*Xba*I and *Sal*I/*Not*I, respectively, and individually cloned into the *Asc*I/*Xba*I or *Sal*I/*Not*I of pCANTAB-link, creating two plasmid libraries, pCANTAB-link-H (7.1×10^5 clones) and pCANTAB-link-L (8.6×10^5 clones), respectively. The resulting libraries were propagated in *E. coli* DH5α cells [33] and the complementary V_H and V_L fragments ligated into the corresponding *Sal*I/*Not*I and *Asc*I/*Xba*I site of the pCANTAB-link-H and pCANTAB-link-L library. The DNA was electroporated into *E. coli* TG1 cells (Amersham Pharmacia Biotech) and grown overnight at 30 °C on SOB plates containing 100 μg/ml ampicillin and 2% glucose [33]. The final library containing both the V_H and the V_L regions yielded approximately 9.2×10^7 clones. The bacterial cells were scraped into 2xYT medium [33] and a 10-ml aliquot infected with 4×10^{10} p.f.u. of helper phage M13KO7 (Amersham Pharmacia Biotech). The culture was incubated overnight with shaking at 37 °C, the cells pelleted and the supernatant containing phage filtered through a 0.45-μm filter. The phage were concentrated using polyethylene glycol (PEG) precipitation and resuspended in 1/100 volume 2xYT media ready for panning.

Selection and screening for phage-displayed scFv antibodies

Maxisorp Immunotubes (Nunc) were coated overnight at room temperature with 3 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), 3 ml of CS89 virus diluted (1/100) in PBS containing 5% foetal calf serum was added, and the assay was incubated at 37 °C for 1 h. Tubes were blocked for 1 h with PBS containing 5% skim milk, and three rounds of panning were carried out [35]. Individual colonies obtained from the third round of panning were propagated and infected with M13KO7 helper phage [35].

Expression of phage-displayed antibodies

Overnight cultures of individual colonies were diluted 1/10 using 10 ml of 2xYT containing 100 μg/ml ampicillin and 2% glucose and 2.5×10^{10} p.f.u. of M13KO7 helper phage (Amersham Pharmacia Biotech). Cultures were shaken at 150 rpm for 2 h, 37 °C, and then centrifuged at 1000 *g* for 15 min. The bacterial pellets were resuspended in 10 ml of 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 was removed for analysis in ELISA.

Expression of soluble antibodies

Each phage expressing scFv was used to infect a non-suppressor strain of E. coli (HB2151) (Amersham Pharmacia Biotech) [35]. Expression of soluble scFv (SAb) was achieved by the addition of 1 mM isopropylthio-β-D-galactoside (IPTG) to the growth medium and subsequent shaking at 30 °C for 6 h. Cells were pelleted and the SAbs extracted from the periplasm using mild osmotic shock [35].

Antigen specificity of selected CRAbs

Antigen specificity of selected CRAbs was determined in an antigen ELISA using both phage-expressed (CRAb-phage) and soluble antibodies (SAbs) [35]. In brief, polystyrene ELISA plates were coated with rabbit anti-IBDV serum and test IBDV antigen (added at saturation 1/20). After incubation of 1 h at 37 °C, either phage-expressed CRAbs (diluted with 1/5

volume 10% skim milk) or CRAbs expressed as soluble antibodies were added at a dilution of 1/10 using 100 µl/well. Phage-expressed antibodies were detected using anti-M13-IgG HRP (Amersham Pharmacia Biotech) whereas SAbs were detected using mouse-anti-E tag antibodies (Amersham Pharmacia Biotech), followed by the addition of anti-mouse IgG-HRP. Antigen-antibody complexes were detected by the addition of 2,2′azino-bis (3-ethybenz-thiazoline-6-sulfonic acid) (ABTS) (Sigma Chemical Corporation), and the absorbance at 405 nm was determined.

Sequencing of scFv

Nucleotide sequences and deduced protein sequences of scFv constructs were determined as previously described [35].

Immunoprecipitation of IBDV proteins using soluble antibodies

IBDV strain CS89 was purified on a sucrose cushion as previously described [11] and dialysed extensively against PBS. IBDV proteins were solubilized using n-octyl-β-D-glucopyranoside (Sigma Chemical Corporation) at a final concentration of 0.02% and sonicated on setting 4.5 for 10-second pulses 10 times with 5-second pauses in between. The insoluble material was pelleted by centrifugation at 150,000 *g* for 2 h at 4 °C. The supernatant containing soluble proteins was dialysed overnight against PBS at 4 °C.

Approximately 10 µl of each SAb was added to 30 µl of the solubilized CS89 protein preparation. After a 1-h incubation at room temperature, 2 µl α-E-Tag Antibody (Amersham Pharmacia Biotech, diluted 1/10 in PBS) was added to each SAb mixture, and the reaction was incubated for 1 h at room temperature. Approximately 50 µl of a protein A-Sepharose preparation (100 mg Protein A-Sepharose swelled in 1 ml dH_2O) (Amersham Pharmacia Biotech) was added to each sample followed by a 1-h incubation at room temperature with gentle intermittent mixing. Samples were centrifuged at 10,000 *g* for 2 min to pellet the immunoprecipitated material. The pelleted material was washed 3× with 1 ml PBS and finally resuspended in 50 µl 2× SDS-PAGE sample buffer, boiled for 5 min, spun at 10,000 *g* for 2 min to pellet the Sepharose beads, and the supernatant containing the immunoprecipitated proteins was analysed by western blot.

Approximately 20 µl of the immunoprecipitated proteins was separated on a 12% SDS-PAGE gel and transferred to a supported nitrocellulose membrane (Osmonics) [33]. The membrane was blocked with 5% skim milk/PBST, and then probed with either MAb 9-6 directed against the VP2 protein (1/10 in blocking buffer), or MAb 17–80 directed against VP3 (1/20 in blocking buffer) [20], followed by goat α-mouse IgG-HRP conjugate (Sigma Chemical Corporation). The enhanced chemi-luminescent (ECL) western blotting detection system (Amersham Biosciences) was used to develop the blot.

Results

Isolation and identification of IBDV-specific scFv antibodies

A scFv antibody library of 9.2×10^7 clones was prepared to select for clones specific for vvIBDV. IBDV-specific phage clones were selected by successive rounds of panning against vvIBDV strain CS89. After the third round of panning, 400 individual clones were propagated and screened for binding to CS89 and Australian strain 002/73 in ELISA in order to isolate clones specific for vvIBDV. Of the 400 clones tested, 14 reacted with only CS89, while the majority of the

Table 1. ELISA reactivity of selected CRAbs generated against CS89

CRAb[b]	Specificity[c]	Australian IBDV[a]						Other IBDV[a]				Neg
		Classical				Variant		Classical		vv CS89	Variant Var E	
		002/73	06/95	K-2	M-4	02/95	08/95	F52/70	1/68			
154	VP2	++++[d]	++++	++++	++++	++++	++++	++++	++++	++++	+++	−
66	VP2	++++	++++	++++	++++	++++	++++	++++	++++	++++	++	−
176	VP2	++++	++++	++++	++++	++++	+++	++++	++++	++++	+	−
149	VP2	++++	++++	++++	++++	++++	+++	++++	++++	+++	+	−
151	VP2	++++	++++	++++	++++	+++	++	+++	+++	+++	+	−
174	VP2	+++	++++	++++	+++	++	++	+++	+++	+++	−	−
19	VP2	++++	++++	++++	+++	+++	−	+++	+++	++	−	−
27	VP2	+	+++	+	++	+++	+++	+++	+++	++++	−	−
45	VP3	++++	++++	−	++++	++++	+++	++++	++	++++	++	−
62	VP3	+++	++++	−	++	++++	++	+++	+	++++	+	−
18	VP2	++++	++++	++++	−	−	−	++++	++++	++++	−	−
26	VP2	++++	++++	++++	−	−	−	++++	+++	++++	−	−
37	VP2	+++	++++	++++	−	−	−	++++	++++	++++	−	−
32	VP2	+	+++	++	−	−	−	+++	+++	++++	−	−
35	VP2	−	−	−	+	++	++	−	−	++	−	−
38	VP2	−	+++	−	−	−	−	++++	++++	+++	−	−
29	VP2	−	−	−	−	−	−	++++	++++	++++	−	−
28	VP2	−	−	−	−	−	−	++++	+++	++++	−	−
39	VP2	−	−	−	−	−	−	++	++	++++	−	−
30	VP2	−	−	−	−	−	−	++	+	++++	−	−
50	VP2	−	−	−	−	−	−	+	+	++++	−	−
88	VP2	−		−	−	−	−	−	−	++++	−	−
9-6[e]	VP2	++++	++++	++++	++++	+++	++	++++	++++	++++	−	−

[a]IBDV strains, as bursal homogenate, used at saturation (1/20)
[b]Chicken recombinant antibodies tested as phage-displayed antibodies. Phage diluted with 1/5 volume of 10% skim milk
[c]Specificity of CRAbs as determined by immunoprecipitation of solubilized CS89 proteins
[d] ++++, +++, ++, + and − = Correspond to absorbances of >1.1, 0.7–1.1, 0.3–0.07, 0.1–0.3 and <0.1 respectively
[e]Conventional monoclonal antibody against IBDV used according to Fahey et al. [20]

Table 2. Reactivity of CRAb 88 and CRAb 154 in ELISA with other IBDV strains

Pathotype	Country of origin	IBDV strain	Accession no.	Absorbance with CRAb	
				154	88
classical	Germany	Cu-1wt	AF362747	2.15	0.09
classical	UK	F52/70	D00869	2.04	0.11
classical	UK	1/68	Unpublished	1.26	0.08
classical	USA	STC	D00499	1.15	0.10
vaccine/CEF adapted		Cu-1M	AF362771	1.81	0.15
vaccine/CEF adapted		Bursine 2	Y14960	1.91	0.14
vaccine/CEF adapted		D78	Y14962	2.09	0.15
vaccine/CEF adapted		CT	Y14961	2.11	0.15
vvIBDV	Brazil	99009	AJ878902	2.18	1.90
vvIBDV	Senegal	99006	AJ878887	2.04	1.86
vvIBDV	Africa	88180	AJ001941	2.12	0.55
vvIBDV	UK	UK661	X92760	2.14	1.82
vvIBDV	Netherlands	DV86	D16630	2.15	1.86
vvIBDV	Belgium	849VB	AY321949	2.09	1.79
vvIBDV	France	96108	AJ001948	1.82	1.54
vvIBDV	France	91184	AJ001943	2.13	1.80
vvIBDV	France	94432	Y14955	2.00	1.14
vvIBDV	France	91168	Y14957	2.11	1.85
vvIBDV	France	89163	Y14956	2.19	1.80
vvIBDV	France	92309	AJ001945	2.14	1.82
vvIBDV	France	95072/8	AJ001947	2.11	1.85
vvIBDV	France	91247	AJ001944	2.12	1.83
vvIBDV	China	Sichuang	Unpublished	2.11	1.81
vvIBDV	China	Hunan	Unpublished	2.14	1.89
vvIBDV	Indonesia	Indo 1	AF508738	1.53	1.12
vvIBDV	Indonesia	Indo 5	AF508742	1.92	1.88
vvIBDV	Indonesia	Indo 6	AF508743	1.48	1.25
vvIBDV	Indonesia	Indo 8	AF508745	1.80	1.39
attenuated vvIBDV	Indonesia	Indo 11	AF508748	1.99	0.09
variant	USA	GLS5	M97346	2.14	0.07
variant	USA	VarA	AF293792	0.58	0.08
variant	USA	VarE	D10056	1.58	0.07
field strain	USA	H2	AF281234	0.67	0.08
field strain	USA	Q2	AF281240	0.74	0.09
field strain	USA	S1	AF281237	0.83	0.06
field strain	USA	T1	AF281238	0.62	0.07
field strain	USA	V1	AF281235	0.50	0.07
field strain	USA	1610	AF281222	0.69	0.07
field strain	USA	1174	AF281220	0.63	0.06
field strain	USA	1568	AF281221	0.79	0.07
field strain	Puerto Rico	586	AF305739	1.09	0.07
serotype II	USA	OH	U30818	0.08	0.07

clones (63%) reacted with both viruses. All 14 CS89-specific clones and 180 cross-reactive clones, randomly selected, were tested in ELISA for their binding to a panel of Australian and other representative IBDV strains (Table 1). All 194 CRAbs reacted with strain CS89, which had been used as the immunizing antigen and for panning, but showed differences in reactivity to other IBDV strains. In total, 22 different antigen-binding patterns were observed; for each antigen-binding pattern a representative CRAb was randomly chosen and is shown in Table 1. CRAbs 154, 66, 176, 149 and 151 recognized all IBDV strains, including US VarE, however, CRAb 154 showed the highest level of reactivity with US Var E. CRAbs 29, 28, 39, 30 and 50 reacted only with overseas strains, while CRAb 88 reacted only with vvIBDV CS89.

Testing of additional IBDV strains with CRAb 88 and CRAb 154

As the specificity of CRAb 88 was assessed in our laboratory using a limited number of IBDV strains, we confirmed its specificity using a wider panel of other strains including a large number of vvIBDV strains that have been isolated and characterised to date. Specificity of CRAb 88 and CRAb 154 was assessed both as phage (Table 2) and soluble antibody (results not shown), both yielding similar results in ELISA. As shown in Table 2, CRAb 88-phage reacted with all vvIBDV strains regardless of their country of origin. A slightly lower OD value was obtained with the African isolate 88180, and CRAb 88 did not react with an attenuated vvIBDV Indonesian isolate Indo11 [31]. No reactivity was detected with any of the classical or US variant strains. In addition, no reactivity was detected with any of the 4 vaccine strains tested. CRAb 154-phage reacted with all IBDV strains tested, including four tissue-culture-propagated IBDV strains, and did not react with the serotype II OH strain.

DNA sequence analysis of scFv clones

An alignment of the deduced amino acid sequences of the 22 unique IBDV-specific CRAbs is shown in Fig. 1. Three complementarity-determining regions (CDRs) and four framework (FW) regions were identified in each of the V_H and V_L fragments. Although all CRAbs were unique in both their heavy- and light-chain sequences, a number of similarities were observed between particular CRAbs. A number of heavy-chain sequences were similar, and these included the following groups of CRAbs: (28 and 88), (30 and 39), (27 and 35), (18 and 37), (45 and 62), (154, 149, 176 and 151). There appeared to be less similarity between the light chains with only 2 CRAbs (32 and 66) being almost identical.

Immunoprecipitation with SAbs

In western blotting, none of the CRAbs tested reacted with denatured CS89 viral proteins, suggesting that the epitopes recognized by CRAbs are conformation-dependent (results not shown). Therefore CS89 proteins were solubilized using a

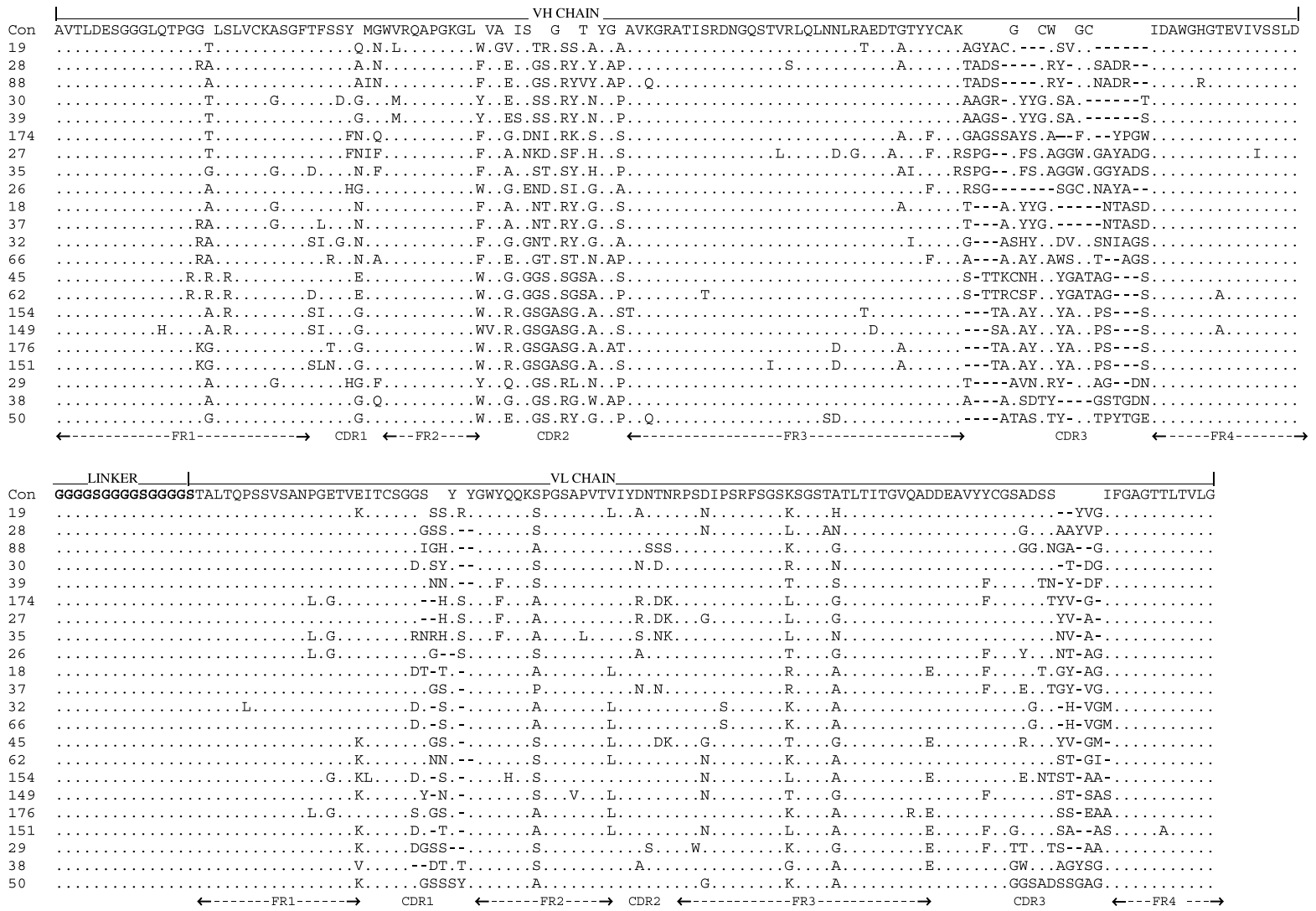

Fig. 1. Alignment of the deduced amino acid sequences of the variable regions of the heavy- and light-chain genes of 22 selected scFv clones. Only those amino acids that differ from the consensus sequence are shown, while identical amino acids are shown by dots. Gaps introduced into the alignment are 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

non-ionic detergent to determine whether selected CRAbs were directed towards the VP2 or VP3 protein. Two previously characterized MAbs, one specific for VP2 (MAb 9-6) and the other specific for VP3 (MAb 17–80), were used to identify proteins immunoprecipitated by each of the CRAbs, which were used as SAbs. The majority of CRAbs, including CRAb 88, immunoprecipitated the VP2 protein (∼40 kDa), while CRAb 45 and CRAb 62 immunoprecipitated the VP3 protein (∼30 kDa) (Table 1). Interestingly, one particular strain (K-2) was not recognized by these 2 VP3-specific CRAbs, suggesting that the VP3 protein may display a degree of variability. A representative blot of proteins immunoprecipitated by CRAbs 28, 32, 45, 88, 154 and 176 is shown in Fig. 2. Bands at approximately 47 kDa and 23 kDa represent H- and L-chain proteins, respectively, of the secondary antibody (α-E-Tag) used in the immunoprecipitation of IBDV proteins. A negative control antibody CRAb 0, obtained from an un-panned library [35], was also included.

Comparison of VP2 sequences of IBDV strains

A comparison of available VP2 sequences of the HVR of classical strain F52/70 to that of vvIBDV strains tested with CRAb 88 (Table 2) identified four amino acids which may potentially influence the binding CRAb 88, including Ala(222),

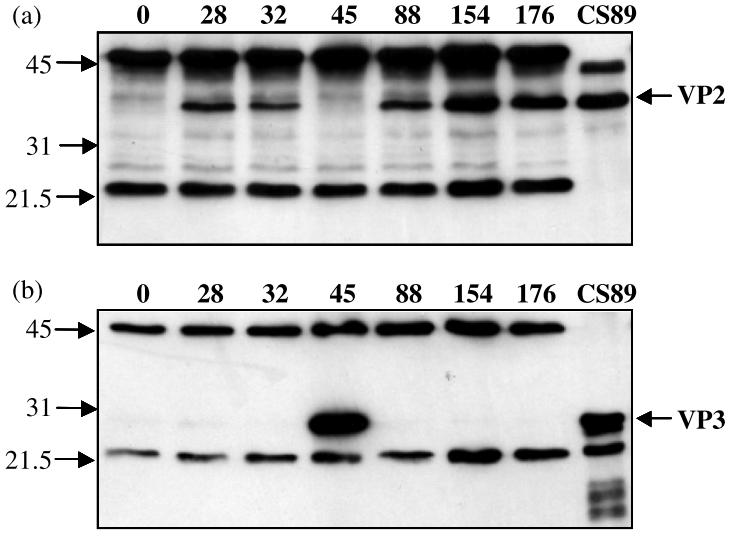

Fig. 2. Western blot analysis of CRAbs to determine their protein specificity. CRAbs expressed as soluble antibodies (SAbs) were mixed with a solubilized CS89 virus, and the precipitated proteins were analysed by SDS-PAGE followed by western blot analysis using two monoclonal antibodies for detection, Mab 9-6, specific for VP2 (**a**) and Mab17–80, specific for VP3 (**b**). A negative control CRAb antibody (CRAb 0) obtained from an unpanned library (Sapats et al. [35]), was also included. Molecular mass of protein markers is shown in kDa. Bands at approximately 47 kDa and 23 kDa represent H- and L-chain proteins respectively, of the secondary antibody (α-E-Tag) used in the immunoprecipitation of IBDV proteins

		Hydrophilic peak A									Hydrophilic peak B	
Pathotype	Strain	213	215	222	256	272	279	294	299	300	321	324
vvIBDV	UK661	D	Q	A	I	I	D	I	S	E	A	Q
	DV86	D	Q	A	I	I	D	I	S	E	A	Q
	89163	D	Q	A	I	I	D	I	S	E	A	Q
	91247	D	Q	A	I	I	D	I	S	E	A	Q
	96108	D	Q	A	I	I	D	I	S	E	A	Q
	Indo 1	D	Q	A	I	I	D	I	S	E	A	Q
	849VB	D	Q	A	I	I	D	I	N	E	A	Q
atypical vvIBDV	Indo 5	D	Q	S	I	I	D	I	S	E	A	Q
	Indo 6	D	Q	S	I	I	D	I	S	E	A	Q
	Indo 8	D	Q	S	I	I	D	I	S	E	A	Q
	88180	D	Q	Q	I	I	D	L	N	Q	A	Q
vvIBDV with additional changes	95072/8	N	H	A	I	I	D	I	S	E	A	Q
	92309	N	Q	A	I	I	D	I	S	E	A	Q
	91168	D	Q	A	I	I	D	I	S	E	A	L
	94432	D	Q	A	I	I	D	I	S	E	V	Q
attenuated vvIBDV	Indo 11	D	Q	A	I	T	N	I	S	E	A	Q
classical	F52/70	D	Q	P	V	I	D	L	N	E	A	Q

Fig. 3. Summary of amino acid residues found in HVR of VP2 of strains tested with CRAb 88, including vv strains and classical strain F52/70. Numbering of amino acid residues is according to Bayliss et al. [4]. Residues observed predominantly in vv strains are shown shaded black, while residues that are common to most IBDV strains are shown shaded in grey

Ile(256), Ile(294) and Ser(299) (Fig. 3). A number of atypical vv strains were identified that bound equally well to CRAb 88 but contained a number of substitutions, including Ala → Ser(222) (Indo 5, 6 and 8) and Ser → Asp(299) (849VB). The African isolate 88180, previously identified as showing a lower level of reactivity with CRAb 88, appeared to be the least similar to other vvIBDV strains tested in that it contained three substitutions including Ala → Pro(222), Ile → Val(294) and Ser → Asp(299). Four French isolates (92309, 95072/8, 91168 and 94432) contained Ala(222), Ile(256), Ile(294) and Ser(299) but had additional amino acid substitutions within the first or second hydrophilic regions (A and B) of VP2. An attenuated vvIBDV strain from Indonesia (Indo 11) that failed to react with CRAb 88 contained Ala(222), Ile(256), Ile(294) and Ser(299) as well as 2 additional amino acid substitutions at positions Thr(272) and Asp(279).

Discussion

Despite numerous attempts by various research groups to isolate a MAb specific for vvIBDV, none have been successful. The difficulty in generating reagents specific for vvIBDV using conventional hybridoma technology has presumably been due to the fact that vvIBDV strains are antigenically very similar to classical virulent strains when compared by serum neutralization tests [21]. Therefore, it was decided not to inactivate the virus to avoid any risk of modifying or degrading critical epitopes, but, instead, to inoculate with live virus and pool the spleens of several surviving birds. This approach probably had an additional advantage by decreasing the background through the destruction of B cell precursors while expanding the IBDV-specific mature repertoire of B cells. Indeed, using phage display technology, we were not only able to isolate a scFv that is specific

for vvIBDV strains from a library containing 9.2×10^7 scFv clones, but also a large number (194) of IBDV-specific scFvs were selected that displayed 22 different antigen binding patterns in ELISA with Australian and other IBDV strains. Five scFv groups represented by CRAbs 29, 28, 39, 30 and 50 did not react with any of Australian IBDV strains, indicating that antigenic differences exist between Australian and other classical IBDV strains isolated in other countries. Although it has been recognised that Australian and other classical IBDV strains differ genetically [34], antigenic differences between these strains have not been previously recognized. It must be emphasised that the significance of these antigenic differences detected by scFvs remains unknown. These antigenic differences may not be important, in particular for protective immunity, since a live IBDV vaccine based on Australian strain V877 has been used to vaccinate against classical and vvIBDV strains (Phil Lehrbach, Fort Dodge Australia, personal communication). Although the majority (16/22) of isolated scFvs were cross-reactive, most likely due to the dominance of these epitopes, only five scFvs were broadly cross-reactive, with observable differences particularly with US variant E. Only CRAb 154 reacted strongly with all US variants, and as such is a valuable diagnostic reagent. Only one other MAb, MAb 29, has been identified to date that cross-reacts with all IBDV strains [36]. We were previously unable to isolate any scFv that cross-reacted with variant E from a library raised against Australian classical strain 002/73 [35] with the exception of a VP3-specific scFv (CRAb 20). The rare isolation of scFv that cross-reacts with all IBDV strains confirmed the unique antigenicity of USA variants [36].

The majority (20/22) of CRAbs including CRAb 88 were directed against the VP2 protein. Immuno-precipitation was the only method by which it was possible to determine this specificity, due to the highly conformational nature of antigenic binding domains within the VP2 protein [1, 5, 22]. The fact that CRAb 88 recognized a highly conformational epitope within VP2 enabled us to speculate which amino acids within the VP2 protein may be important in influencing the binding of CRAb 88. Initial testing of CRAb 88 showed that it could clearly discriminate between vv strain CS89 and classical virulent strain F52/70, which differ by only five amino acid residues in VP2 [9]. Four of these sequence changes occurred within the hypervariable region (HVR) of VP2 at Ala(222), Ile(256), Ile(294) and Ser(299), and one at Leu(451), close to the VP2/VP3 cleavage region. The four amino acid residues in the HVR may play a critical role in defining a conformational epitope recognized by CRAb 88. The high specificity of CRAb 88 for vvIBDV strains regardless of their country of origin was confirmed in further testing with an additional 38 IBDV strains including 17 vvIBDV strains. A slightly lower level of reactivity was observed with one highly pathogenic African isolate (88180). Sequence analysis of this isolate revealed that it had four amino acid substitutions including Glu(222), Leu(294), Asp(299), and Glu(300) but retained Ile(256). Three other strains had amino acid substitutions including Indo 5, 6 and 8 [Ser(222)]. However, both of these viruses retained Ile(256) and Ile(294) and reacted strongly with CRAb 88. Therefore, the lowered level of reactivity seen with 88180 may have been due to the substitution of Leu for Ile at residue

294. These conclusions would support the hypothesis that CRAb 88 recognizes a conformational epitope in which Ile(256) and Ile(294) may play a critical role. However, it must be remembered that the isolation of strains such as African isolate 88180 is rare, with the vast majority of vvIBDV (∼98%) having both Ile(256) and Ile(294) [3, 32]. In other studies related to Western African countries, strains with a typical vvIBDV-like VP2 variable domain were found in Nigeria in 1988 and between 1995–2000 [30, 40] as well as in Senegal in 1998 [18].

A number of strains tested with CRAb 88 contained the putative markers for vvIBDV, i.e. Ala(222), Ile(256), Ile(294) and Ser(299), but also had a number of other unique amino acid substitutions [23]. Four of the French vvIBDV isolates contained amino acid substitutions within the 2 major hydrophilic domains (A and B) previously identified within the HVR of VP2 [13, 16, 17, 19]. None of these amino acid substitutions appeared to influence the binding of CRAb 88. Therefore, it appears that CRAb 88 can tolerate a number of amino acid substitutions within either of these hydrophilic regions. This is an important finding as changes within these regions are known to influence the antigenicity of IBDV strains [22]. Furthermore, it is these regions of the genome that are most likely to undergo genetic changes in order to overcome ever-increasing vaccination regimes. Interestingly, CRAb 88 failed to react with Indonesian isolate Indo11. This isolate, however, differed from other vvIBDV by having two amino acid changes within the HVR of VP2: Ile → Thr(272) and Asp → Asn(279) and did not cause either clinical disease or mortality characteristic of vvIBDV [31]. Therefore we could speculate that the inability of CRAb 88 to bind to Indo11 could indicate that CRAb 88 may not only be specific for vvIBDV but could also be an indicator of virulence of naturally occurring strains. The variable region within VP2 has been shown to play a role in virulence [8, 39]. However, it does not appear to be the sole determinant of virulence, and other regions of the genome may be involved [6, 7, 38]. More research is needed to asses which amino acid residues are directly involved or influence the binding of CRAb 88. In this instance, the use of a reverse genetics system [39] could be used to sequentially mutate amino acids of interest within VP2.

Acknowledgements

We would like to thank the Chicken Meat and Egg Programmes of the Rural Industries Research and Development Corporation for their financial assistance.

References

1. Azad AA, Jagadish MN, Brown MA, Hudson PJ (1987) Deletion mapping and expression in Escherichia coli of the large genomic segment of a birnavirus. Virology 161: 145–152
2. Banda A, Villegas P, El-Attrache J (2003) Molecular characterization of infectious bursal disease virus from commercial poultry in the United States and Latin America. Avian Dis 47: 87–95
3. Banda A, Villegas P (2004) Genetic Characterization of very virulent infectious bursal disease viruses from Latin America. Avian Dis 48: 540–549

4. Bayliss CD, Spies U, Shaw K, Peters RW, Papageorgiou A, Muller H, Boursnell ME (1990) A comparison of the sequences of segment A of four infectious bursal disease virus strains and identification of a variable region in VP2. J Gen Virol 71: 1303–1312
5. Becht H, Muller H, Muller HK (1988) Comparative studies on structural and antigenic properties of two serotypes of infectious bursal disease virus. J Gen Virol 69: 631–640
6. Boot HJ, ter Huurne AA, Hoekman AJ, Peeters PP, Gielkens AL (2000) Rescue of very virulent and mosaic infectious bursal disease virus from cloned cDNA:VP2 is not the sole determinant of the very virulent phenotype. J Virol 74: 6701–6711
7. Boot HJ, Hoekman AJW, Gielkens ALJ (2005) The enhanced virulence of very virulent infectious bursal disease virus is partly determined by its B-segment. Arch Virol 150: 137–144
8. Brandt M, Yao K, Liu MH, Heckert RA, Vakharia VN (2001) Molecular determinants of virulence, cell tropism, and pathogenic phenotype of infectious bursal disease virus. J Virol 75: 11974–11982
9. Brown MD, Skinner MA (1996) Coding sequences of both genome segments of a European 'very virulent' infectious bursal disease virus. Virus Res 40: 1–15
10. Cao YC, Yeung WS, Law M, Bi YZ, Leung FC, Lim BL (1998) Molecular characterization of seven Chinese isolates of infectious bursal disease virus: classical, very virulent, and variant strains. Avian Dis 42: 340–351
11. Cho BR, McDonald TL (1980) Infectious bursal disease virus: further characterization with evidence for a single-stranded RNA virus. Avian Dis 24: 423–434
12. Coulibaly F, Chevalier C, Gutsche I, Pous J, Navaza J, Bressanelli S, Delmas B, Rey FA (2005) The birnavirus crystal structure reveals structural relationships among icosahedral viruses. Cell 120: 761–772
13. Dormitorio TV, Giambrone JJ, Duck LW (1997) Sequence comparisons of the variable VP2 region of eight infectious bursal disease virus isolates. Avian Dis 41: 36–44
14. Eterradossi N, Toquin D, Rivallan G, Guittet M (1997) Modified activity of a VP2-located neutralizing epitope on various vaccine, pathogenic and hypervirulent strains of infectious bursal disease virus. Arch Virol 142: 255–270
15. Eterradossi N, Rivallan G, Toquin D, Guittet M (1997) Limited antigenic variation among recent infectious bursal disease virus isolates from France. Arch Virol 142: 2079–2087
16. Eterradossi N, Arnauld C, Toquin D, Rivallan G (1998) Critical amino acid changes in VP2 variable domain are associated with typical and atypical antigenicity in very virulent infectious bursal disease viruses. Arch Virol 143: 1627–1636
17. Eterradossi N, Arnauld C, Tekaia F, Toquin D, Coq HL, Rivallan G, Guittet M, Domenech J, Van den Berg TP, Skinner MA (1999) Antigenic and genetic relationships between European very virulent infectious bursal disease viruses and an early West African isolate. Avian Pathol 28: 36–46
18. Eterradossi N, Cardinale E, Rivallan G, Arnauld C, Tekaia F, Toquin D, Domenech J, Le Coq H, Guittet (1999) Caractersation antigenique et genomique de souches ivoiriennes et senegalaises du virus de la maladie de Gumbora (IBDV). Comptes-rendus des Themes Jourees de la Recherche Avicole, Saint-Malo, France, 23–25 Mar 229–232
19. Eterradossi N, Gauthier C, Reda I, Comte S, Rivallan G, Toquin D, de Boisseson C, Lamande J, Jestin V, Morin Y, Cazaban C, Borne P (2004) Extensive antigenic changes in an atypical isolate of very virulent infectious bursal disease virus and experimental clinical control of this virus with an antigenically classical live vaccine. Avian Pathol 33: 423–431
20. Fahey KJ, McWaters P, Brown MA, Erny K, Murphy VJ, Hewish DR (1991) Virus-neutralizing and passively protective monoclonal antibodies to infectious bursal disease virus of chickens. Avian Dis 35: 365–373

21. Goddard RD, Wyeth PJ, Varney WC (1994) Vaccination of commercial layer chicks against infectious bursal disease with maternally derived antibodies. Vet Rec 135: 273–274
22. Heine HG, Haritou M, Failla P, Fahey K, Azad A (1991) Sequence analysis and expression of the host-protective immunogen VP2 of a variant strain of infectious bursal disease virus which can circumvent vaccination with standard type I strains. J Gen Virol 72: 1835–1843
23. Hoque MM, Omar AR, Hair-Bejo M, Aini I (2002) Sequence and phylogenetic analysis of the VP2 gene of very virulent infectious bursal disease virus isolates. J Biochem Mol Bio Biophys 6: 93–99
24. Hust M, Dubel S (2005) Phage display vectors for in vitro generation of human antibody fragments. Methods Mol Biol 295: 71–96
25. Ignjatovic J, Sapats S (2001) Confirmation of the existence of two distinct genetic groups of infectious bursal disease virus in Australia. Aust Vet J 80: 689–694
26. Jackwood DJ, Sommer SE (1999) Restriction fragment length polymorphisms in the VP2 gene of infectious bursal disease viruses from outside the United States. Avian Dis 43: 310–314
27. Liu J, Zhou J, Kwang J (2002) Antigenic and molecular characterization of recent infectious bursal disease virus isolates in China. Virus Genes 24: 135–147
28. Majo N, El-Attrache J, Banda A, Villegas P, Ramis A, Pages A, Ikuta N (2002) Molecular characterization of Spanish infectious bursal disease virus field isolates. Avian Dis 46: 859–868
29. Meir R, Jackwood DJ, Weisman Y (2001) Molecular typing of infectious bursal disease virus of Israeli field and vaccine strains by the reverse transcription/polymerase chain reaction/restriction fragment length polymorphism assay. Avian Dis 45: 223–228
30. Owoade AA, Mulders MN, Kohnen J, Ammerlaan W, Muller CP (2004) High sequence diversity in infectious bursal disease virus serotype 1 in poultry and turkey suggests West-African origin of very virulent strains. Arch Virol 149: 653–672
31. Parede LH, Sapats S, Gould G, Rudd M, Lowther S, Ignjatovic J (2003) Characterization of infectious bursal disease virus isolates from Indonesia indicates the existence of very virulent strains with unique genetic changes. Avian Pathol 32: 511–518
32. Rudd MF, Heine HG, Sapats SI, Parede L, Ignjatovic J (2002) Characterisation of an Indonesian very virulent strain of infectious bursal disease virus. Arch Virol 147: 1303–1322
33. Sambrook J, Russell DW (2001) In: Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
34. Sapats SI, Ignjatovic J (2000) Antigenic and sequence heterogeneity of infectious bursal disease strains isolated in Australia. Arch Virol 145: 773–785
35. Sapats SI, Heine HG, Trinidad L, Gould GJ, Foord AJ, Doolan SG, Prowse S, Ignjatovic J (2003) Generation of chicken single chain antibody variable fragments (scFv) that differentiate and neutralize infectious bursal disease virus (IBDV). Arch Virol 148: 497–515
36. Snyder DB, Vakharia VN, Savage PK (1992) Naturally occurring-neutralizing monoclonal antibody escape variants define the epidemiology of infectious bursal disease virus in the United States. Arch Virol 127: 89–101
37. van den Berg TP (2000) Acute infectious bursal disease in poultry: a review. Avian Pathol 29: 175–194
38. van den Berg TP, Morales D, Eterradossi N, Rivallan G, Toquin D, Raue R, Zierenberg K, Zhang MF, Zhu YP, Wang CQ, Zheng HJ, Wang X, Chen GC, Lim BL, Muller H (2004) Assessment of genetic, antigenic and pathotypic criteria for the characterization of IBDV strains. Avian Pathol 33: 470–476

39. van Loon AA, de Haas N, Zeyda I, Mundt E (2002) Alteration of amino acids in VP2 of very virulent infectious bursal disease virus results in tissue culture adaptation and attenuation in chickens. J Gen Virol 83: 121–129
40. Zierenberg K, Nieper H, van den Berg TP, Ezeokoli CD, Voß M, Muller H (2000) The VP2 variable region of African and German isolates of infectious bursal disease virus: comparison with very virulent, "classical" virulent, and attenuated tissue culture-adapted strains. Arch Virol 145: 113–125
41. Zierenberg K, Raue R, Műller H (2001) Rapid identification of "very virulent" strains of infectious bursal disease virus by reverse transcription-polymerase chain reaction combined with restriction enzyme analysis. Avian Pathol 30: 55–62
42. Zorman-Rojs O, Barlic-Maganja D, Mitevski D, Lübke W, Mundt E (2003) Very virulent infectious bursal disease virus in Southeastern Europe. Avian Dis 47: 186–192

Author's address: Dr. I. Sandra Sapats, CSIRO Livestock Industries, Australian Animal Health Laboratory, Private Bag 24, Geelong, VIC 3220, Australia; e-mail: sandra.sapats@csiro.au