Arch Virol (2002) 147: 1303–1322
DOI 10.1007/s00705-002-0817-3

Archives of Virology
Printed in Austria

Characterisation of an Indonesian very virulent strain of infectious bursal disease virus

M. F. Rudd[1], **H. G. Heine**[1], **S. I. Sapats**[1], **L. Parede**[2], and **J. Ignjatovic**[1]

[1]CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Australia
[2]Research Institute for Veterinary Science, Bogor, Indonesia

Received January 11, 2002; accepted February 26, 2002
Published online May 24, 2002

Summary. An Indonesian very virulent (vv) strain of infectious bursal disease virus (IBDV), designated Tasik94, was characterised both in vivo and at the molecular level. Inoculation of Tasik94 into 5-week-old specific-pathogen-free (SPF) chickens resulted in 100% morbidity and 45% mortality. The complete nucleotide and predicted amino acid sequences of genomic segments A and B were determined. Across each of the three deduced open reading frames (ORFs), Tasik94 shared the greatest nucleotide homology to Dutch vv strain D6948. Phylogenetic analyses were performed using 15 full-length polyprotein sequences and a total of 105 VP2 hypervariable region sequences from geographically and pathogenically diverse strains. In each case, Tasik94 grouped closely with vv strains, particularly those from Europe. The deduced VP1, VP2, VP3, VP4 and VP5 protein sequences of Tasik94 were aligned with those from published strains and putative virulence determinants were identified in VP2, VP3 and VP4. Alignment of additional protein sequences across the VP2 hypervariable region confirmed that residues Ile[242], Ile[256] and Ile[294] were highly-conserved amongst vv strains, and may account for their enhanced virulence.

Introduction

Infectious bursal disease virus (IBDV), initially described in the 1960's [12], causes a highly contagious immunosuppressive disease of young chickens that destroys maturing surface IgM-bearing lymphocytes in the bursa of Fabricius [for reviews see 35, 42, 62]. Two distinct serotypes of IBDV have been identified. Serotype 1 viruses are pathogenic to chickens and differ markedly in their virulence [67], whereas serotype 2 viruses, isolated from both turkeys and chickens, are apathogenic [24, 32].

Prior to 1987, IBDV was controlled by vaccination of breeder hens and layer pullets with live attenuated virus followed by killed virus vaccines. Since 1987, a pathotypic variant, termed very virulent IBDV (vvIBDV), has emerged. These strains are capable of breaking through the maternal antibody-derived protection of chicks much earlier than classical strains and causing high flock mortalities. Very virulent IBDV has spread rapidly throughout Europe [11, 18, 63], Asia [9, 10, 30, 43, 60], Africa [73], and South America [15]. In contrast to antigenic variants isolated in the United States [55], these vvIBDV have remained antigenically similar to the classical viruses.

IBDV is a member of the genus *Avibirnavirus* in the family *Birnaviridae* [28]. Members of this family contain a genome composed of two segments of double-stranded RNA (dsRNA), designated A and B [16, 36]. The dsRNA genome is enclosed within a non-enveloped icosahedral capsid approximately 60 nm in diameter [6]. The larger segment A (∼3.4 kb) contains two open reading frames (ORFs) of 3,039 bp [22] and 438 bp, which partially overlap at the 5′ end of the genome [25]. The larger ORF encodes a 110 kDa precursor polyprotein (NH_2-VP2-VP4-VP3-COOH), which is autocatalytically cleaved by the *cis*-acting viral protease VP4 into three proteins designated precursor VP2 (pVP2) (48 kDa), VP3 (32 kDa), and VP4 (24 kDa) [4, 22]. The pVP2 is further processed into VP2 (38 kDa) during maturation of the viral particle [26]. VP2, the major structural protein of the viral capsid, carries highly conformational epitopes responsible for the induction of neutralising antibodies that confer protective immunity [1, 3, 19, 21]. VP3 is the second structural protein of the viral capsid, recognised by non-neutralising antibodies that often cross-react with both serotypes [3, 44].

The smaller ORF of segment A encodes VP5 (17 kDa) [2, 58], a 145 amino acid non-structural protein of unknown function [38]. VP5 has been shown not to be essential for viral replication and infection [39, 71], but plays an important role in the release of viral progeny from infected cells [31]. The smaller segment B (∼2.8 kb) encodes VP1 (90 kDa), an RNA-dependant RNA polymerase (RdRp) [33, 34, 57] with capping enzyme activities [56].

Several attempts to elucidate the residue(s) responsible for the increased pathogenicity of vvIBDV have identified conserved amino acid substitutions throughout both genome segments [8, 47, 69, 72]. More recently, the development and application of a reverse genetics system for IBDV [41] has shown that neither the non-coding regions (NCRs) [53], nor residues within VP1 [5] or the N terminus of VP2 [54] are responsible for the increased pathogenicity of vvIBDV. These results suggest that virulence determinants reside within the VP2, VP4 and/or VP3 protein(s).

This paper describes the complete genomic coding sequence of the first reported Indonesian vvIBDV strain Tasik94, the sixth full-length vvIBDV sequence published to date. In addition, this paper reports comprehensive phylogenetic analyses and protein alignments across VP1, VP5, VP2-VP4-VP3 and the VP2 hypervariable region of attenuated, classical, variant and vvIBDV strains, and the subsequent designation of VP2 residues Ile[242], Ile[256], and Ile[294] as potential virulence determinants.

Materials and methods

Virus propagation and titration

The vvIBDV strain Tasik94 was isolated in 1994 from a field outbreak in unvaccinated layer hens in West Java, Indonesia. Virus identity was confirmed using a modified IBDV antigen-capture ELISA (AC-ELISA) [20]. The virus was passaged once in three-week-old specific-pathogen-free (SPF) chickens by intraocular inoculation. Infected bursa were harvested three days post-inoculation (pi), homogenised as a 10% (w/v) suspension in sterile phosphate-buffered saline, and clarified by centrifugation for 10 min at 3,000 × g. Virus stock was serially diluted and inoculated into nine-day-old embryonating SPF eggs by the chorioallantoic membrane (CAM) route. Virus titre, calculated according to the method of Reed and Muench [49], was $10^{3.5}$ mean embryo lethal dose (ELD_{50}) per 0.2 ml.

Antigenic analysis

Bursal homogenate from experimentally infected birds was tested in an antigen ELISA using monoclonal antibodies 17–82, 9–6, 39A and 44–18 as described previously [20, 51]. Briefly, polystyrene microtiter plates were coated overnight at room temperature with rabbit α-IBDV IgG diluted in carbonate-bicarbonate buffer, pH 9.6. After washing, 1:10-diluted bursal homogenate was added and incubated for 1 h at room temperature. Monoclonal antibodies were added, followed by addition of anti-mouse IgG conjugated with horse raddish peroxidase (HRP). Following addition of the substrate, 5-aminosalicylic acid (Merck), absorbance was measured at 450 nm.

Experimental infection

Two groups of five-week-old SPF chickens were housed in separate rooms. Twenty chickens were inoculated intraocularly with $10^{3.5}$ ELD_{50} of Tasik94 virus, twenty chickens served as uninoculated controls. Birds were monitored daily for clinical disease, those deemed terminally ill were euthanased. Birds exhibited clinical signs typical of a vvIBDV infection, including severe depression, loss of appetite, dehydration and watery diarrhoea. Clinical signs became apparent two days pi, and surviving birds recovered by day six pi. Under these conditions, Tasik94 caused 100% morbidity and 45% mortality, confirming its designation as a vv strain [11].

RNA isolation

Total RNA was isolated using a modified proteinase K digestion method described previously [13]. Briefly, 0.5 g of bursa was homogenised in 5 ml of Tris-EDTA buffer. The homogenate was freeze-thawed three times to release intracellular virus. Proteinase K (100 μg/ml) and sodium dodecyl sulfate (1%) were added and incubated for 2 h at 60 °C. Sodium acetate (0.3 M) was added and incubated for 30 min at 4 °C. Nucleic acids were phenol:chloroform (1:1) extracted, ethanol precipitated and resuspended in 150 μl of diethyl pyrocarbonate-treated (DEPC) water.

Double-stranded RNA (including viral dsRNA) was purified using the lithium chloride differential precipitation method [14]. Briefly, single-stranded RNA was precipitated with 2 M lithium chloride and pelleted. The supernatant was transferred to a fresh tube and dsRNA was precipitated with 4 M lithium chloride and pelleted. The dsRNA pellet was washed with 70% ethanol and resuspended in 20 μl of DEPC water.

Table 1. Oligonucleotides used to amplify genomic segments A and B of Tasik94

Primer	Location	Sequence (5′ → 3′)
09	(A) 743–780	GTCTACACCATAACTGCCGCAGATGATTACCAATTCTC
10	(A) 1160–1189*	GGTGCCCTCCGCCCCGTCACACTAGTAGCC
50	(A) 1–17	GGATACGATCGGTCTGA
51	(A) 791–807*	CCTTGGACGCTTGTTTG
52	(A) 714–733	GTGGGGTAACAATCACACTG
53	(A) 1668–1683*	TGTGCACCGCGGAGTA
54	(A) 1595–1614	TACGAGGTAGTCGCGAATCT
55	(A) 2512–2529*	GACTTGCTGCCTGCTTGT
56	(A) 2415–2432	TGTGGCTGGAAGAGAATG
57	(A) 3247–3261*	AGGGGACCCGCGAAC
66	(B) 1–18	GGATACGATGGGTCTGAC
67	(B) 678–695*	ATCCTTGACGGCACCCTT
68	(B) 662–680	GCATAGCCCAGCTACTTGA
69	(B) 1369–1384*	GGGCAATGTTCATCGC
70	(B) 1232–1252	CGTGGTACTCAATTGACCTAG
71	(B) 2113–2131*	CGGTGTTGACTGGTCTGTT
72	(B) 1972–1988	CCGCTCGATGAGTTCCT
73	(B) 2813–2827*	GGGGGCCCCCGCAGG

Oligonucleotides were delineated from the consensus sequence of strains OKYM [69], UK661 [8], Cu-1 [58], and P2 [40], with the exception of oligonucleotides 09 and 10, which were based on primers N531 and N533 [21]. The positions of the primers designed to amplify segment (A) or (B) correlate to the numbering according to Bayliss et al. [2], and Mundt and Müller [40]

*Denotes antisense orientation

First-strand cDNA synthesis

Complementary DNA (cDNA) was synthesised using avian myeblastosis virus (AMV) reverse transcriptase (RT) (Promega). Briefly, 0.5 μg of purified dsRNA was mixed with 2 μl of dimethyl sulfoxide, incubated for 5 min at 100 °C then chilled on ice. One hundred pg of gene-specific primer (Table 1) was added, mixed, and incubated for 10 min at 42 °C then chilled on ice. To this was added 5 μl of AMV reaction buffer, 1 μl of 10 mM dNTPs, 2 μl of 25 mM magnesium chloride, 20 units (U) RNasin, 20 U AMV-RT enzyme and DEPC water to a final volume of 25 μl. Reactions were incubated for 1 h at 42 °C.

Polymerase chain reaction (PCR)

The cDNA templates were amplified using high-fidelity *Pyrococcus furiosus* Pfx polymerase (Life Technologies) in a DNA thermal cycler (Perkin Elmer Cetus). Briefly, 2.5 μl (10%) of each cDNA reaction mix was mixed with 100 pg of both primers, 5 μl Pfx amplification buffer, 1 μl of 10 mM dNTPs, 2 μl of 25 mM magnesium sulfate, 10 μl of PCR enhancer solution, 1 U Pfx DNA polymerase enzyme and DEPC water to 50 μl. After denaturing the template DNA for 5 min at 94 °C, amplification consisted of 35 cycles of denaturation (1 min at 94 °C), primer annealing (1 min at 55 °C), and primer extension (2 min at 68 °C), with a final single extension step of 7 min at 68 °C. Results were visualised on 1% agarose gel with ethidium bromide staining.

Cloning and sequencing

PCR products and *Sma*I-digested pUC19 plasmid [70] were purified by agarose gel electrophoresis and extracted using a QIAquick Gel Extraction Kit (Qiagen). Ligation was performed using T4 DNA Ligase (Promega) at 16 °C overnight, and ligation products were transformed into electrocompetent E. coli. DH5α cells (Clontech). Plasmids from transformants were isolated with a Miniprep Plasmid Purification Kit (Qiagen) and screened for the presence of desired insert using restriction digests. Plasmid DNA was sequenced using an ABI PRISM 377 DNA sequencer (PE Applied Biosystems) with a BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kit (ABI PRISM, PE Applied Biosystems). Each nucleotide was determined by sequencing a minimum of four individual clones in both orientations using plasmid-specific forward and reverse primers.

Sequence analysis

Sequence data were analysed with Sequencing Analysis 3.3 software (PE Applied Biosystems) and full-length genome sequences were assembled using SeqMan 4.00 in the Lasergene software package (DNASTAR Inc., Madison, WI, USA). Complete sequences were deposited in the GenBank database and the allocated accession numbers are shown in Table 2. Nucleotide sequences corresponding to the complete VP5, polyprotein and VP1 coding regions (Table 2), and a 435 bp region of the VP2 hypervariable region were used to study the relationship of Tasik94 to other IBDV strains. The alignments were performed using the neighbour-joining

Table 2. IBDV strains used for alignment of ORFs of segment A and/or segment B

Strain	Accession #		Classification	Origin	Reference
	Segment A	Segment B			
Tasik94	AF322444	AF322445	Very virulent	Indonesia	This report
D6948	AF240686	AF240687	Very virulent	Netherlands	[5]
HK46	AF092943	AF092944	Very virulent	Hong Kong	[29]
IBDVks[a]	L42284[c]	–	Very virulent	Israeli	[47]
IL3[a]	–	AF083093	Very virulent	Israeli	[72]
KK1	AF165150	–	Very virulent	South Korea	[27]
KSH	AF165151	–	Very virulent	South Korea	[27]
OKYM	D49706	D49707	Very virulent	Japan	[69]
UK661	X92760	X92761	Very virulent	United Kingdom	[8]
002/73	X03993	M19336	Classical	Australia	[22]
F52/70	D00869	–	Classical	United Kingdom	[2]
STC	D00499	–	Classical	United States	[25]
GLS	M97346	–	Antigenic variant	United States	[61]
2512[b]	–	AF083092	Attenuated	United States	[72]
Cu-1	X16107	–	Attenuated	Germany	[58]
P2	X84034	X84035	Attenuated	Germany	[40]
PBG98	D00868[c]	–	Attenuated	United Kingdom	[2]

[a]Correspond to the same strain, however segments sequenced by different groups
[b]Referred to as Winterfield 2512, an attenuated United States vaccine strain
[c]Did not include complete VP5 sequence

(NJ) method implemented within Clustal X Version 1.62b [59], with 1,000 bootstrapping replicates. The dendrograms were plotted with Treeview version 1.5 [46].

Results and discussion

Sequence analysis of Tasik94

RT-PCR amplification of segments A and B yielded 8 overlapping PCR products of 807, 969, 934, 846 and 695, 722, 899, 855 bp, respectively. PCR products were cloned and sequenced, covering 3,229 of 3,261 bp (99.0%) of segment A, and 2,794 of 2,827 bp (98.8%) of segment B. In addition, direct sequencing of RT-PCR products was performed to confirm that the consensus sequence was representative of the viral population. In total, 65 nucleotides located at the extreme 5′ and 3′ termini of segments A and B of Tasik94 were not determined, corresponding to those nucleotides 'masked' by terminal primers 50, 57, 66 and 73 (Table 1), which were based on the consensus sequence of published serotype I strains.

A single large ORF identified in segment B encoded the VP1 protein. The VP1 coding region was 2,637 nucleotides (879 amino acids) in length, as was the case with most aligned strains except 002/73, 2512 and P2. The Australian strain 002/73 was one residue shorter, containing a deletion at #105, while attenuated strains 2512 and P2 were also 878 amino acids in length, both having a stop codon at #879 rather than #880. Across the VP1 protein, Tasik94 shared the highest and lowest nucleotide homology to Dutch vv strain D6948 (99.7%) and Australian mildly virulent strain 002/73 (89.2%), respectively.

Within segment A, two separate and partially overlapping ORFs were identified, encoding VP5 and the polyprotein. The VP5 protein was 447 nucleotides (149 amino acids) in length, as was the case with aligned strains D6948, HK46, UK661, and GLS. The polyprotein was 3,036 nucleotides (1,012 amino acids) in length, consistent with the length of other serotype I strains aligned. If the polyprotein cleavage sites were assumed to be ^{511}LA▼A^{513} (VP2/VP4) and ^{754}MA▼A^{756} (VP4/VP3) [50], the processed proteins VP2, VP4 and VP3 were 512, 243 and 257 amino acids, respectively. Across viral proteins VP2, VP4 and VP3, Tasik94 showed the highest nucleotide homology to strain D6948 (99.3%, 99.7%, and 99.6%), and the lowest homology to strain 002/73 (91.9%, 89.9% and 93.3%), respectively. The average nucleotide homology of Tasik94 to the aligned strains across the 3 proteins varied by only 1.2%, ranging from 96.0% (VP4) to 97.2% (VP2).

Phylogenetic analyses

To determine the relatedness of Tasik94 to published serotype I strains of IBDV, a total of 15 nucleotide sequences, corresponding to the VP2-VP4-VP3 ORF (Table 2), were aligned in Clustal X and used to generate a bootstrapped phylogenetic tree (Fig. 1). The vv strains formed two distinct phylogenetic groups. Group (i) contained six vv strains, including Tasik94, which appeared most closely related to European strains D6948 and UK661. Group (ii) contained the South Korean

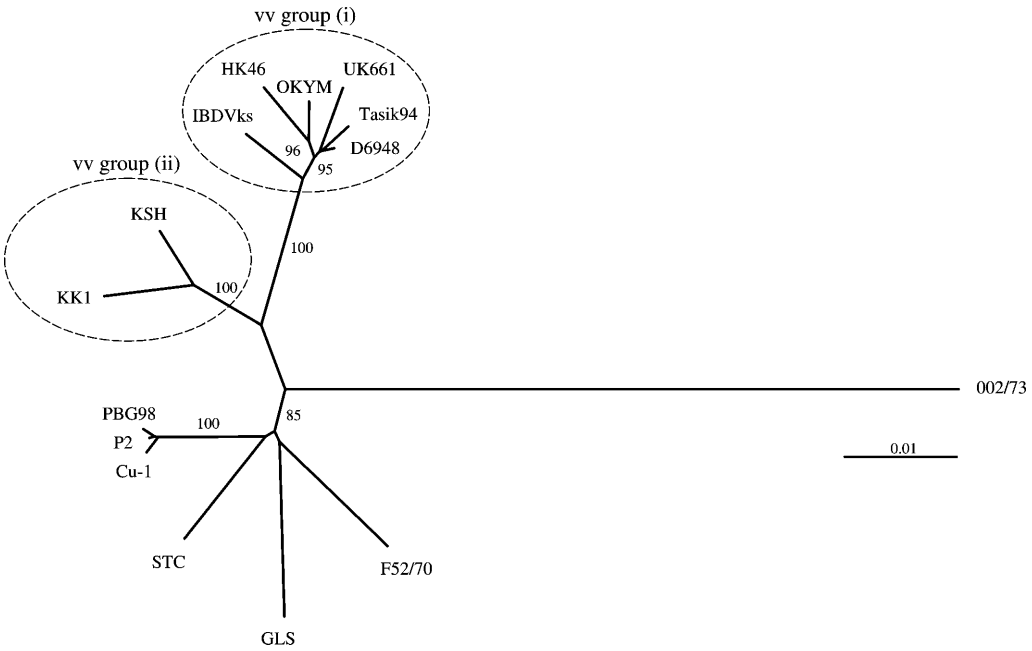


Fig. 1. Phylogenetic relationship of 15 published full-length serotype I IBDV strains based on nucleotide sequences encoding the full-length VP2-VP4-VP3 polyprotein. Branch lengths are proportional to the estimated genetic distances. Bar units correspond to the number of nucleotide substitutions per site. Bootstrap probabilities (expressed as a percentage of 1,000 re-samplings) greater than 80 are given beside the nodes. Two distinct groups of vv viruses are circled with dashed lines

strains KK1 and KSH, both of which were reported to induce high mortality rates in SPF birds [27]. Interestingly, although KK1 and KSH showed a high nucleotide homology to the majority of vv strains across the VP2 protein, they shared almost 100% nucleotide identity with non-vv strains across VP4, VP3 and VP5, possibly as a result of recombination event(s) within segment A. European attenuated strains PBG98, P2 and Cu-1 formed a tight cluster, which grouped loosely with classical strains STC and F52/70, and antigenic variant GLS. Australian strain 002/73 was highly divergent. Phylogenetic trees were also constructed using available nucleotide sequences for both VP5 and VP1 (data not shown). In both cases, although the spatial arrangement of the trees varied, Tasik94 grouped within the distinct cluster of vv strains.

A second phylogenetic tree (Fig. 2) was constructed using 435 nucleotides, spanning the VP2 hypervariable region (*Acc*I-*Spe*I fragment) of 105 serotype I strains. The tree was divided into four distinct groups. Group (i) contained 59 vv strains, all of which were closely related. Tasik94 grouped closely with Nigerian and European vv strains, particularly the Russian strain Tula94 and UK strains 74-89A and CS89. Group (ii) contained a total of 32 strains which were further divided into sub-groups (a) and (b). Group (ii)(a) contained 15 classical, non-attenuated


89224 (fr)
D6948 (ne)
MYGA-97 (cu)
TN1-93 (in)
DV86 (ne)
89163 (fr)
91168 (fr)
K280-89 (sa)
KK1 (sk)
K406-89 (eg)
HK46 (hk)
XJ-9 (ch)
90-11 (ja)
Ehime91 (ja)
OKYM (ja)
JK1-97 (in)
IBDVks (is)
C4-2 (ch)
HD96 (ch)
JS-18 (ch)
K357-88 (ge)
B2-28 (ch)
KSH (sk)
G9201 (ch)
G9303 (ch)
F9502 (ch)
UP1-99 (in)
KT1-98 (in)
AP1-93 (in)
RJ1-94 (in)
WB1-93 (in)
TP1-96 (in)
CH2-97 (in)
UP1-97 (in)
UP2-97 (in)
CH1-97 (in)
i
CS89 (uk)
74-89A (uk)
Tula94 (ru)
Tasik94 (id)
N4 (ni)
N13 (ni)
N14 (ni)
N7 (ni)
N8 (ni)
N6 (ni)
N9 (ni)
HR1-96 (in)
95072-2 (fr)
UK661 (uk)
91247 (fr)
JY86 (uk)
94432 (fr)
95072-8 (fr)
96108 (fr)
91184 (fr)
92309 (fr)
AH-2 (ch)
D11-2 (ch)
88180 (ic)
F52/70 (uk)
HKL6 (hk)
BV3 (us)
GZ911 (ch)
Univax (us)
CEF94 (ne)
HZ96 (ch)
Tri-bio (in)
P2 (ge)
PBG98 (uk)
Soroa (cu)
J1 (ja)
Cu-1 (ge)
D78 (ne)
Ts (ch)
EM3 (fr)
HN3 (ch)
BJ-1 (ch)
ii(b)
MISS (us)
GBF-1 (ja)
K (ja)
STC (us)
3212 (us)
GLS (us)
GZ902 (ch)
Variant A (us)
Ark (us)
Variant E (us)
E/DEL (us)
Ga (us)
U28 (us)
ii(a)
CJ801 (ch)
P3009 (ta)
K310 (sk)
01/94 (au)
03/95 (au)
02/95 (au)
08/95 (au)
04/95 (au)
iv
06/95 (au)
002/73 (au)
V877/K (au)
Bursavac live (au)
V877 (au)
GT101 (au)
iii
0.1

and antigenic variant strains, while group (ii)(b) contained 16 attenuated strains and the atypical Hong Kong strain HKL6. Groups (iii) and (iv) contained a total of 11 Australian strains, which were clearly distinct from strains circulating in any other country. Group (iii) contained vaccine strains 002/73, V877, V877/K (V877 inactivated), Bursavac live (V877 adapted to embryonating eggs) [22, 48], New South Wales field isolate 06/95 [51], and chicken embryo fibroblast (CEF)-adapted strain GT101. Group (iv) contained five antigenic variant strains [51]. Three strains did not fall into any of the aforementioned groups, these being vv strain 88180, classical strain F52/70, and atypical South Korean strain K310.

Identification of putative virulence determinants

To identify putative virulence determinants, the deduced amino acid sequence of the VP1, VP5 and VP2-VP4-VP3 proteins of published serotype I strains for which the complete sequence has been determined (Table 2) were aligned. Residues that were highly conserved amongst vv strains are summarised in Fig. 3.

Fig. 2. Phylogenetic relationship of 105 serotype I IBDV strains based on nucleotide sequences encoding the 435 bp VP2 hypervariable region. Strain names are listed followed by the database accession number in brackets: 89224 (AJ001942), MYGA-97 (AJ238647), TN1-93 (AJ404327), DV86 (Z25482), 89163 (Y14956), 91168 (Y14957), K280-89 (AF159217), K406-89 (AF159218), XJ-9 (AF155123), Ehime91 (AB024076), JK1-97 (AJ249520), C4-2 (AF076223), HD96 (AF076226), JS-18 (AF076227), K357-88 (AF159216), B2-28 (AF076225), UP1-99 (AJ277801), KT1-98 (AJ249521), AP1-93 (AJ245884), RJ1-94 (Y18682), WB1-93 (Y18650), TP1-96 (AJ249522), CH2-97 (AJ245885), UP1-97 (Y18612), UP2-97 (AJ249524), CH1-97 (AJ245886), CS89 (Z25481), 74-89A (Z25481), Tula94 (X89570), N4 (AF159207), N13 (AF159214), N14 (AF159215), N7 (AF159209), N8 (AF159210), N6 (AF159208), N9 (AF159211), HR1-96 (AJ249518), 95072-2 (AJ001946), 91247 (AJ001944), JY86 (Z25480), 94432 (Y14955), 95072-8 (AJ001947), 96108 (AJ001948), 91184 (AJ001943), 92309 (AJ001945), AH-2 (AF076224), D11-2 (AF076228), 88180 (AJ001941), HKL6 (AF051839), BV3 (AF076235), Univax (AF076236), CEF94 (AF194428), HZ96 (AF121256), Tri-bio (AJ249523), Soroa (AF140705), J1 (D16677), D78 (Y14962), Ts (AF076230), EM3 (Y14963), HN3 (AF076229), BJ-1 (AF076231), Miss (AF076234), GBF-1 (D16828), K (D16678), 3212 (AF091097), Variant A (M64285), Ark (AF076232), Variant E (D10065), E/DEL (X54858), Ga (AF076233), U28 (AF091099), P3009 (AF109154), K310 (AF165149), 01/94 (AF148076), 03/95 (AF148078), 02/95 (AF148077), 08/95 (AF148081), 04/95 (AF148079), 06/95 (AF148080), V877K (AF069578), Bursavac live (AF148075), V877 (AF069577), GT101 (AF069579). The VP2 hypervariable region sequences of the following strains were included manually: 90–11 [43], G9201, G9303, F9502, GZ911, GZ902, and CJ801 [30]. Branch lengths are proportional to the estimated genetic distances. Bar units correspond to the number of nucleotide substitutions per site. Indonesian strain Tasik94 is highlighted by an arrow (←). *fr* (France), *ne* (Netherlands), *cu* (Cuba), *sk* (South Korea), *eg* (Egypt), *hk* (Hong Kong), *ja* (Japan), *in* (India), *ch* (China), *is* (Israel), *ru* (Russia), *id* (Indonesia), *uk* (United Kingdom), *ge* (Germany), *sa* (South Africa), *ni* (Nigeria), *ic* (Ivory Coast), *us* (United States), *ta* (Taiwan), *au* (Australia)

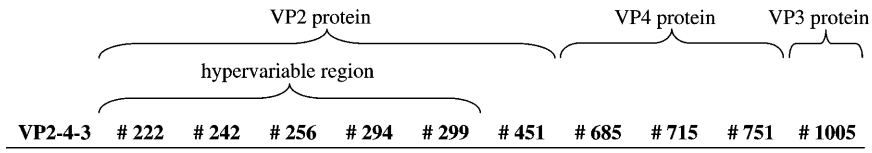
a

VP1	# 146	# 147	# 242	# 390	# 393	# 562	# 687	# 695
Tasik94	D	N	E	M	D	P	P	R
D6948	D	N	E	M	D	P	P	R
HK46	D	N	E	M	D	P	P	R
IL3	D	N	E	M	D	P	P	R
OKYM	D	N	E	M	V	P	P	R
UK661	D	N	E	M	D	P	P	R
002/73	E	S	D	L	E	S	R	K
2512	E	G	D	L	E	S	S	K
P2	E	G	D	L	E	S	S	K

b

VP5	# 1-4	# 18	# 49	# 78	# 137
Tasik94	MLSL	E	R	F	W
D6948	MLSL	E	R	F	W
HK46	MLSL	E	R	F	W
KK1	- - - -	K	G	I	R
KSH	- - - -	K	G	I	R
OKYM	- - - L	E	R	F	W
UK661	MLSL	K	R	L	W
002/73	- - - -	K	G	I	W
F52/70	- - - -	K	G	I	R
STC	- - - -	K	G	I	R
GLS	MLSL	K	G	I	R
Cu-1	- - - -	K	G	I	R
P2	- - - -	K	G	I	R

c



VP2-4-3	# 222	# 242	# 256	# 294	# 299	# 451	# 685	# 715	# 751	# 1005
Tasik94	S	I	I	I	S	L	N	S	D	A
D6948	A	I	I	I	S	L	N	S	D	A
HK46	A	I	I	I	S	L	N	S	D	A
IBDVks	A	I	I	I	S	I	N	S	D	A
KK1	A	I	I	I	S	L	K	P	H	T
KSH	A	I	I	I	S	L	K	P	H	T
OKYM	A	I	I	I	S	L	N	S	D	A
UK661	A	I	I	I	S	L	N	S	D	A
002/73	P	V	V	L	S	I	K	P	H	T
F52/70	P	I	V	L	N	I	K	P	H	T
STC	P	V	V	L	N	I	K	P	H	T
GLS	T	V	V	L	N	I	K	P	H	T
Cu-1	P	V	V	L	N	I	K	P	H	T
P2	P	V	V	L	N	I	K	P	H	T
PBG98	P	V	V	L	N	I	K	P	H	T

VP1 protein

Across VP1, Tasik94 contained an identical protein sequence to that of D6948 (Netherlands). IL3 differed from Tasik94 by 1 residue (0.11%), HK46 by 2, UK661 by 8 and OKYM by 13 residues (1.48%). A total of 8 residues, previously reported by Brown et al. [8], were found exclusively in vv strains at positions 146, 147, 242, 390, 393, 562, 687 and 695 (Fig. 3a). All 8 residues were located outside the putative RdRp and GTP-binding (GTP) motifs [17]. The significance of these residues as virulence markers is dubious, since an attenuated derivative of IL3, designated IL4 [72], contained the same residues as IL3 at all 8 positions. Additionally, Boot et al. [5] found that a genetically reassorted virus containing vv segment A and attenuated serotype II segment B exhibited similar pathology to wild type and recombinant vvIBDV, suggesting that the amino acid substitutions in VP1 do not influence pathogenicity.

VP5 protein

The Tasik94 VP5 protein was 149 amino acids in length, as was the case with strains D6948, HK46, UK661 and GLS, whilst the remaining strains were 145 residues long, lacking the first 4 residues (MLSL) (Fig. 3b). Whether the first methionine at position 85 is actually used as the start codon for the production of VP5 in IBDV strains containing the 4-residue-N-terminal extension is still unclear [5]. Recently, Weber et al. [66] used infectious pancreatic necrosis virus (IPNV) mutants derived by reverse genetics to show that the initiation of translation of IPNV VP5 occurs at the second in-frame start codon.

Tasik94 VP5 had an identical amino acid sequence to that of HK46 (Hong Kong) and OKYM (Japan). UK661 differed from Tasik94 by 2 residues (1.34%), while D6948 differed by a 2-residue deletion at position 70–71. Strains KSH and KK1 differed by 5 (3.36%) and 7 (4.70%) residues, respectively. Within VP5, only 4 residues were relatively conserved among vv strains (Fig. 3b). Two of these residues; Arg[49] and Phe/Leu[78], were exclusive to vv strains with the exception of KK1 and KSH, which we have shown to be highly divergent from the majority of vv strains. Residue Glu[18] was shared by four vv strains (Tasik94, D6948, HK46 and OKYM), with KK1, KSH and UK661 sharing Lys at this position with classical strains. Residue Trp[137], previously identified by Boot et al. [5] as being exclusive to vvIBDV, was shared by Australian strain 002/73, with vv strains KK1 and KSH sharing an Arg[137] with the remaining strains.

VP5 is not required for viral replication and infection [39, 71], but plays an important role in the release of viral progeny from infected cells [31]. Alignment results revealed no vv-exclusive residues within VP5, suggesting that virulence determinants are located within the larger ORF of segment A. This conclusion was

Fig. 3. Summary of amino acid residues highly conserved amongst vv strains across (**a**) VP1, (**b**) VP5, and (**c**) VP2-4-3 proteins. Residues observed predominantly in vv strains are shaded black. The dashed line represents the distinction between vv (above) and non-vv strains (below)

supported by Schröder et al. [54], who used reverse genetics and the construction of chimeric viruses to prove that VP5 is not responsible for the different pathotypes of serotypes I and II.

VP2-VP4-VP3 polyprotein

Full-length VP2-VP4-VP3 protein alignments revealed that Tasik94 contained a unique Ser residue within VP2 at position 222 (Fig. 3c). In addition to this substitution, strain D6948 differed from Tasik94 by 1 residue (0.20%), HK46 and OKYM by 2, IBDVks and UK661 by 6, KK1 by 17 and KSH by 18 (1.86%). Four residues; Asn[685], Ser[715] and Asp[751] (VP2), and Ala[1005] (VP3), were exclusive to vv strains with the exception of KK1 and KSH (Fig. 3c). The substitution at 751 was immediately upstream of the proposed VP4/VP3 cleavage site (^{754}MA▼A^{756}), which may influence polyprotein cleavage efficiency and/or protease activity. The importance of these residues as virulence markers is difficult

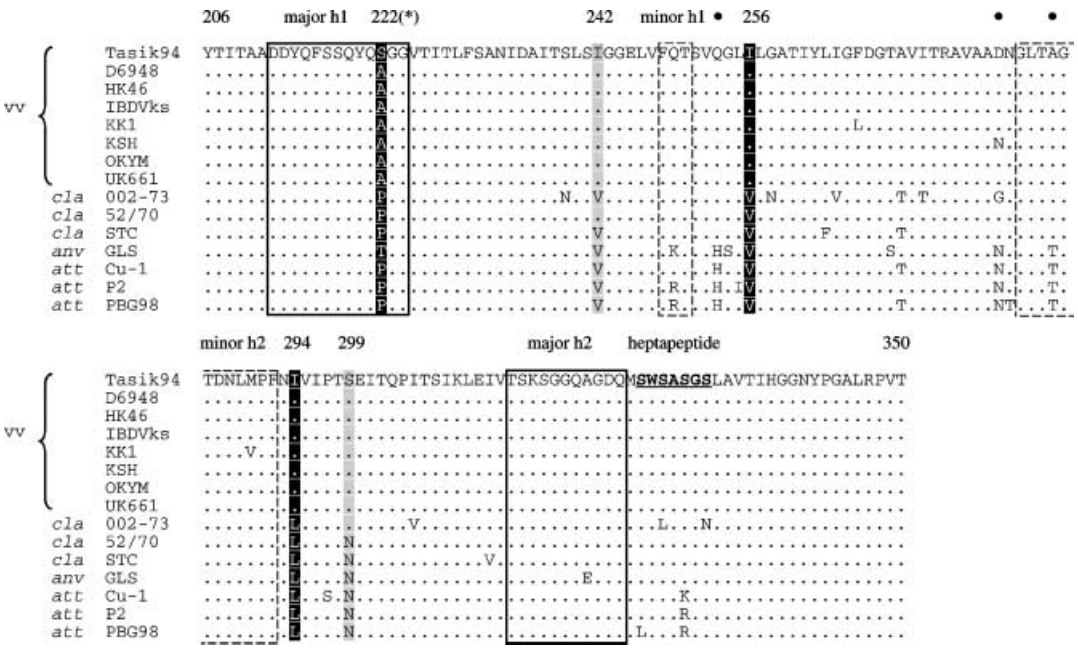


Fig. 4. Alignment of amino acids corresponding to the VP2 hypervariable region of 15 published full-length serotype I IBDV strains. The deduced amino acid sequence of Indonesian strain Tasik94 is shown, spanning residues 206–350. Differences from the Tasik94 sequence are shown, identical amino acids are marked as a dot. Major hydrophilic domains [1] are boxed with solid lines, minor hydrophilic domains [64] are boxed with dashed lines. The serine-rich heptapeptide (hp) [21] is shown in bold, underlined text. Residues exclusive to all aligned vv strains are darkly shaded (positions 222, 256 and 294). Residues exclusive to the majority of aligned vv strains are lightly shaded (positions 242 and 299). Residue Ser[222] found exclusively in Tasik94 is marked with an asterisk (∗). Residues responsible for the replication of IBDV in cell culture (positions 253, 279 and 284) [29, 37] are marked with a filled circle (●). *vv* (very virulent), *cla* (classical), *anv* (antigenic variant), *att* (attenuated)

to ascertain, due to the variable nature of the sequence data for strains KK1 and KSH, and the shortage of published sequence across this region.

Three residues; Ser/Ala[222], Ile[256] and Ile[294], all within the hypervariable region of VP2, were found exclusively in all vv strains for which the full-length polyprotein sequence was available (Fig. 3c, Fig. 4). This finding was significant since the variable domain, located between residues 206–350 [2, 25], has long been suspected of containing IBDV virulence determinants. In addition, residue 222 is located within the first hydrophilic peak, a region that has been linked with the emergence of antigenic variants [21, 45, 52, 61]. Also of interest were 3 additional residues within VP2; Ile[242], Ser[299] and Leu[451]. Residues Ile[242] and Ser[299] were unique to vv strains with the exception of F52/70 and 002/73, respectively (Fig. 4), while residue Leu[451] was exclusive to vv strains with the exception of IBDVks.

VP2 hypervariable region

The significance of residues Ser/Ala[222], Ile[242], Ile[256], Ile[294] and Ser[299] as putative virulence determinants was assessed by aligning VP2 hypervariable residues 206–350 of an additional 90 serotype I IBDV strains. In total, the alignment consisted of 105 serotype I sequences; 60 very virulent, 6 classical, 15 antigenic variant, 22 attenuated and 2 atypical strains from 20 different countries. Results of the alignment are summarised in Fig. 5. Of the 60 vv strains aligned, 57 (95.0%) contained residues Ala[222], Ile[242], Ile[256], Ile[294] and Ser[299], the 3 exceptions being Tasik94, D11-2, and 88180. To confirm that the Tasik94 Ser[222] residue was correct, separate RT-PCR products (generated independently by different operators) were sequenced directly. Results indicate that not only is the Ser[222] residue correct, but also appears to be conserved in the majority of Indonesian vv strains sequenced to date (Ignjatovic et al., manuscript in preparation). This suggests that substitutions between amino acids with similar physiochemical properties are tolerated at position 222 without significantly influencing pathogenicity, since both Tasik94 and atypical vv strain 88180 [18] vary from the majority of vv strains at residue 222, yet still induce 45% mortality in SPF chickens.

The amino acid substitution A222S did not appear to alter the antigenicity of Tasik94 compared with other vvIBDV strains. Tasik94 reacted with monoclonal antibodies 17–82, 9–6, 39A and 44–18, directed against conformational epitopes in the VP2 hypervariable region, as did vv strains DV86 and CS89 (data not shown).

The nucleotide substitution at position 795 which brought about the unique Ser[222] residue in Tasik94 resulted in a loss of the *Bsp*MI restriction site, which has been used previously to differentiate between vv and classical strains [74]. To confirm this, RT-PCR products of 447 bp were amplified using primers 09 and 10 (Table 1). Direct sequencing and restriction digestion analysis (not shown) confirmed that the *Bsp*MI site was absent in Tasik94. The *Ssp*I restriction site at position 1,011, which has been identified as unique to vvIBDV strains [30], was

Classification	VP2 residue #					Occurrence	Strain
	222	242	256	294	299		
60 very virulent	A	I	I	I	S	**57 x**	
	S	I	I	I	S	1 x	Tasik94
	A	I	T	I	S	1 x	D11-2
	Q	I	I	L	N	1 x	88180
28 classical, attenuated & vaccine	P	V	V	L	N	**18 x**	
	P	I	V	L	N	1 x	F52/70
	P	V	V	L	D	1 x	BJ-1
	T	I	V	L	N	1 x	CJ801
	S	I	A	L	N	1 x	P3009
	P	V	V	L	S	5 x	Aust. strains
	P	V	A	L	S	1 x	
15 antigenic variants	T	V	V	L	N	**6 x**	
	P	V	V	L	N	2 x	3212, Miss
	Q	V	V	L	N	2 x	GZ902, Variant A
	T	V	A	L	S	1 x	Aust. strains
	A	V	A	L	N	1 x	
	V	V	A	L	S	1 x	
	A	V	A	L	S	2 x	
2 atypical	S	V	I	L	N	1 x	HKL6
	S	V	V	L	N	1 x	K310
%	95.2	**97.1**	**98.1**	**99.0**	89.5		

Fig. 5. Summary of amino acids found within the hypervariable region of VP2 at positions 222, 242, 256, 294 and 299 of 105 serotype I IBDV strains. Residues observed predominantly in vv strains are shaded black. The percentage (%) figure is a measure of how exclusive each residue is amongst vv strains; it is calculated using the formula: [(# of times observed in vv strains) + (# of times not observed in non-vv strains)] divided by total # strains (105) × 100%

present in Tasik94. Restriction fragment length polymorphism (RFLP) analysis of the VP2 hypervariable region is undoubtedly useful in the rapid diagnosis and categorisation of IBDV isolates. However, the observation that an Indonesian vv strain lacks this *Bsp*MI cleavage site reveals limitations in the technique, and highlights the importance of direct RT-PCR sequencing for the unequivocal classification of new isolates.

D11-2, a vvIBDV from China [9], was the only strain of 105 to contain residue Thr[256]. This same substitution I256T was observed by Yamaguchi et al. [68] after attenuation of vv strain OKYM in cell culture. Strain 88180 varied from the majority of vv strains at three of the five residues, correlating with phylogenetic analyses suggesting it is the most divergent of the vv strains, showing greater similarity to antigenic variants GZ902 and Variant A.

Of the 45 non-vv strains, a total of 10 Australian strains shared Ser[299] with vv strains (Fig. 5). In addition, 3 of those 10 Australian strains also shared Ala[222] with vv strains. Australian strains have evolved independently from vv strains and are only mildly pathogenic, suggesting that neither Ala[222] nor Ser[299] are solely responsible for the vv phenotype, a contention supported by the fact that substitutions A222S in Tasik94 and A222Q in 88180 [18] do not result in a reduction in virulence. Across positions 242, 256 and 294, the number of non-vv

strains sharing a vv-specific residue was much lower, ranging from 3 at #242 down to 0 at #294 (Fig. 5). The finding that residues Ile[242], Ile[256] and Ile[294] are both highly conserved and highly exclusive to vv strains supports their designation as putative virulence determinants.

This contrasts with the findings of Boot et al. [5] however, who found that VP2 is not solely responsible for the vv phenotype. They found that a mosaic virus, in which the pVP2 region of CEF-adapted IBDV strain CEF94 was replaced with the corresponding region of vv strain D6948, resulted in a significant increase in the extent of bursal damage, however did not induce morbidity or mortality in SPF chickens.

One possible explanation is that specific substitutions in VP2 are required in conjunction with substitutions within VP4 and/or VP3 for realisation of the vv phenotype. In this report, VP4 and VP3 protein alignments revealed a total of four non-conservative substitutions found exclusively among vv strains with the exception of highly divergent vv strains KK1 and KSH. Three of these were located within VP4 at Asn[685], Ser[715] and Asp[751], and one within VP3 at Ala[1005]. Of particular interest is the substitution H751D, which is located only 4 residues upstream from the proposed VP4/VP3 cleavage site (^{754}MA▼A^{756}), potentially altering the three-dimensional structure surrounding the cleavage site. Such an explanation infers that the requirements for a vv phenotype are multigenic, whereby specific substitutions within VP2 are required for increased pathogenicity, while concomitant substitutions within VP4 upregulate the autocatalytic cleavage of the polyprotein required for increased virulence.

Recently, van Loon et al. [65] demonstrated the importance of VP2 in both the cellular tropism and pathogenicity of IBDV. They found that alteration of only two specific amino acids (Q253H and A284T) within the VP2 protein of UK661 resulted in both adaptation to tissue culture and attenuation in SPF chickens. These findings corresponded with those of Brandt et al. [7], who suggested that VP2 residues Gln[253], Asp[279] and Ala[284] are molecular determinants for the virulence, cell tropism and pathogenic phenotype of virulent IBDV. However, our alignment results suggest that the virulence of serotype I IBDV strains cannot be attributed solely to residues 253 and 284, since bursal-derived strains 002/73, F52/70 and UK661, which each contain Gln[253] and Ala[284], induce 0%, 10% and 80% mortality in SPF birds, respectively. We propose that while residues Gln[253] and Ala[284] appear to be crucial for the pathogenic phenotype of IBDV, i.e. the ability to inflict bursal damage in vivo, additional residues Ile[242], Ile[256] and Ile[294] are involved in determining the virulence, or degree of pathogenicity, of these viruses. Consequently, vv strains would be characterised by the VP2 genotype Ile[242], Gln[253], Ile[256], Ala[284] and Ile[294], compared with that of classical virulent strains; Val[242], Gln[253], Val[256], Ala[284], Leu[294] and attenuated strains Val[242], His[253], Val[256], Thr[284], Leu[294].

In summary, in vivo studies, sequencing and phylogenetic analyses confirmed the designation of Tasik94 as a vv strain of IBDV, the first such reported strain in Indonesia. Furthermore, alignments across each of the viral ORFs suggest that VP2 residues Ile[242], Ile[256] and Ile[294] could be responsible for the increased

virulence of vv strains. Work is currently underway to determine the biological significance of these residues through the generation of recombinant viruses and subsequent in vivo assessment.

Acknowledgments

We thank Dianne Tilley, Tony Pye and Dr. Deborah Middleton for their assistance. M. F. Rudd was the recipient of a Ph.D. scholarship from the Rural Industries Research and Development Corporation (RIRDC) Chicken Meat and Egg Programs, Project No. CSA-4J.

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. Bayliss CD, Spies U, Shaw K, Peters RW, Papageorgiou A, Müller H, Boursnell MEG (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
3. Becht H, Müller 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
4. Birghan C, Mundt E, Gorbalenya A (2000) A non-canonical lon proteinase lacking the ATPase domain employs the ser-Lys catalytic dyad to exercise broad control over the life cycle of a double-stranded RNA virus. EMBO J 19: 114–123
5. Boot HJ, ter Huurne AA, Hoekman AJ, Peeters BP, 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
6. Bottcher B, Kiselev NA, Stel'Mashchuk VY, Perevozchikova NA, Borisov AV, Crowther RA (1997) Three-dimensional structure of infectious bursal disease virus determined by electron cryomicroscopy. J Virol 71: 325–330
7. Brandt M, Yao K, Liu M, Heckert RA, Vakharia VN (2001) Molecular determinants of virulence, cell tropism, and pathogenic phenotype of infectious bursal disease virus. J Virol 75: 11974–11982
8. 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
9. 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
10. Chen HY, Zhou Q, Zhang MF, Giambrone JJ (1998) Sequence analysis of the VP2 hypervariable region of nine infectious bursal disease virus isolates from mainland China. Avian Dis 42: 762–769
11. Chettle NJ, Stuart JC, Wyeth PJ (1989) Outbreak of virulent infectious bursal disease in East Anglia. Vet Rec 125: 271–272
12. Cosgrove AS (1962) An apparently new disease of chickens – avian nephrosis. Avian Dis 6: 385–389
13. Davis RW, Thomas M, Cameron J, St John TP, Scherer S, Padgett RA (1980) Rapid DNA isolations for enzymatic and hybridization analysis. Methods Enzymol 65: 404–411
14. Diaz-Ruiz JR, Kaper JM (1978) Isolation of viral double-stranded RNAs using a LiCl fractionation procedure. Prep Biochem 8: 1–17
15. Di Fabio J, Rossini LI, Eterradossi N, Toquin MD, Gardin Y (1999) European-like pathogenic infectious bursal disease viruses in Brazil. Vet Rec 145: 203–204

16. Dobos P, Hill BJ, Hallett R, Kells DT, Becht H, Teninges D (1979) Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. J Virol 32: 593–605
17. Duncan R, Mason CL, Nagy E, Leong JA, Dobos P (1991) Sequence analysis of infectious pancreatic necrosis virus genome segment B and its encoded VP1 protein: a putative RNA-dependent RNA polymerase lacking the Gly-Asp-Asp motif. Virology 181: 541–552
18. Eterradossi N, Arnauld C, Tekaia F, Toquin D, Le Coq H, 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
19. Fahey KJ, Erny K, Crooks J (1989) A conformational immunogen on VP-2 of infectious bursal disease virus that induces virus-neutralizing antibodies that passively protect chickens. J Gen Virol 70: 1473–1481
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. 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
22. Hudson PJ, McKern NM, Power BE, Azad AA (1986) Genomic structure of the large RNA segment of infectious bursal disease virus. Nucleic Acids Res 14: 5001–5012
23. Islam MR, Zierenberg K, Eterradossi N, Toquin D, Rivallan G, Müller H (2001) Molecular and antigenic characterization of Bangladeshi isolates of infectious bursal disease virus demonstrate their similarities with recent European, Asian and African very virulent strains. J Vet Med B Infect Dis Vet Public Health 48: 211–221
24. Ismail NM, Saif YM, Moorhead PD (1988) Lack of pathogenicity of five serotype 2 infectious bursal disease viruses in chickens. Avian Dis 32: 757–759
25. Kibenge FS, Jackwood DJ, Mercado CC (1990) Nucleotide sequence analysis of genome segment A of infectious bursal disease virus. J Gen Virol 71: 569–577
26. Kibenge FS, Qian B, Cleghorn JR, Martin CK (1997) Infectious bursal disease virus polyprotein processing does not involve cellular proteases. Arch Virol 142: 2401–2419
27. Kwon HM, Kim DK, Hahn TW, Han JH, Jackwood DJ (2000) Sequence of precursor polyprotein gene (segment A) of infectious bursal disease viruses isolated in Korea. Avian Dis 44: 691–696
28. Leong JC, Brown D, Dobos P, Kibenge FSB, Ludert JE, Müller H, Mundt E, Nicholson B (2000) Family *Birnaviridae.* In: van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringle CR, Wickner RB (ed) Virus Taxonomy; Classification and Nomenclature of Viruses. Academic Press, London, 481–490
29. Lim BL, Cao Y, Yu T, Mo CW (1999) Adaptation of very virulent infectious bursal disease virus to chicken embryonic fibroblasts by site-directed mutagenesis of residues 279 and 284 of viral coat protein VP2. J Virol 73: 2854–2862
30. Lin Z, Kato A, Otaki Y, Nakamura T, Sasmaz E, Ueda S (1993) Sequence comparisons of a highly virulent infectious bursal disease virus prevalent in Japan. Avian Dis 37: 315–323
31. Lombardo E, Maraver A, Espinosa I, Fernandez-Arias A, Rodriguez JF (2000) VP5, the nonstructural polypeptide of infectious bursal disease virus, accumulates within the host plasma membrane and induces cell lysis. Virology 277: 345–357
32. McFerran JB, McNulty MS, Killop ER, Connor TJ, McCracken RM, Collins PS, Allan GM (1980) Isolation and serological studies with infectious bursal disease viruses

from fowl, turkeys and ducks: Demonstration of a second serotype. Avian Pathol 9: 395–404
33. Morgan MM, Macreadie IG, Harley VR, Hudson PJ, Azad AA (1988) Sequence of the small double-stranded RNA genomic segment of infectious bursal disease virus and its deduced 90-kDa product. Virology 163: 240–242
34. Müller H, Nitschke R (1987) The two segments of the infectious bursal disease virus genome are circularized by a 90,000-Da protein. Virology 159: 174–177
35. Müller H, Schnitzler D, Bernstein F, Becht H, Cornelissen D, Lutticken DH (1992) Infectious bursal disease of poultry: antigenic structure of the virus and control. Vet Microbiol 33: 175–183
36. Müller H, Scholtissek C, Becht H (1979) The genome of infectious bursal disease virus consists of two segments of double-stranded RNA. J Virol 31: 584–589
37. Mundt E (1999) Tissue culture infectivity of different strains of infectious bursal disease virus is determined by distinct amino acids in VP2. J Gen Virol 80: 2067–2076
38. Mundt E, Beyer J, Müller H (1995) Identification of a novel viral protein in infectious bursal disease virus-infected cells. J Gen Virol 76: 437–443
39. Mundt E, Köllner B, Kretzschmar D (1997) VP5 of infectious bursal disease virus is not essential for viral replication in cell culture. J Virol 71: 5647–5651
40. Mundt E, Müller H (1995) Complete nucleotide sequences of 5′- and 3′-noncoding regions of both genome segments of different strains of infectious bursal disease virus. Virology 209: 10–18
41. Mundt E, Vakharia VN (1996) Synthetic transcripts of double-stranded Birnavirus genome are infectious. Proc Natl Acad Sci USA 93: 11131–11136
42. Nagarajan MM, Kibenge FSB (1997) Infectious bursal disease virus: a review of molecular basis for variations in antigenicity and virulence. Can J Vet Res 61: 81–88
43. Nunoya T, Otaki Y, Tajima M, Hiraga M, Saito T (1992) Occurrence of acute infectious bursal disease with high mortality in Japan and pathogenicity of field isolates in specific-pathogen-free chickens. Avian Dis 36: 597–609
44. Öppling V, Müller H, Becht H (1991) Heterogeneity of the antigenic site responsible for the induction of neutralizing antibodies in infectious bursal disease virus. Arch Virol 119: 211–223
45. Öppling V, Müller H, Becht H (1991) The structural polypeptide VP3 of infectious bursal disease virus carries group- and serotype-specific epitopes. J Gen Virol 72: 2275–2278
46. Page RD (1996) TreeView: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12: 357–358
47. Pitcovski J, Goldberg D, Levi BZ, Di-Castro D, Azriel A, Krispel S, Maray T, Shaaltiel Y (1998) Coding region of segment A sequence of a very virulent isolate of IBDV–comparison with isolates from different countries and virulence. Avian Dis 42: 497–506
48. Proffitt JM, Bastin DA, Lehrbach PR (1999) Sequence analysis of Australian infectious bursal disease viruses. Aust Vet J 77: 186–188
49. Reed LJ, Muench H (1938) A simple method for estimating fifty per cent endpoints. Am J Hyg 27: 493–497
50. Sanchez AB, Rodriguez JF (1999) Proteolytic processing in infectious bursal disease virus: identification of the polyprotein cleavage sites by site-directed mutagenesis. Virology 262: 190–199
51. Sapats SI, Ignjatovic J (2000) Antigenic and sequence heterogeneity of infectious bursal disease virus strains isolated in Australia. Arch Virol 145: 773–785
52. Schnitzler D, Bernstein F, Müller H, Becht H (1993) The genetic basis for the antigenicity of the VP2 protein of the infectious bursal disease virus. J Gen Virol 74: 1563–1571

53. Schröder A, van Loon AAWM, Goovaerts D, Mundt E (2000) Chimeras in noncoding regions between serotypes I and II of segment A of infectious bursal disease virus are viable and show pathogenic phenotype in chickens. J Gen Virol 81: 533–540
54. Schröder A, van Loon AAWM, Goovaerts D, Teifke JP, Mundt E (2001) VP5 and the N terminus of VP2 are not responsible for the different pathotype of serotype I and II infectious bursal disease virus. J Gen Virol 82: 159–169
55. Snyder DB, Lana DP, Savage PK, Yancey FS, Mengel SA, Marquardt WW (1988) Differentiation of infectious bursal disease viruses directly from infected tissues with neutralizing monoclonal antibodies: evidence of a major antigenic shift in recent field isolates. Avian Dis 32: 535–539
56. Spies U, Müller H (1990) Demonstration of enzyme activities required for cap structure formation in infectious bursal disease virus, a member of the birnavirus group. J Gen Virol 71: 977–981
57. Spies U, Müller H, Becht H (1987) Properties of RNA polymerase activity associated with infectious bursal disease virus and characterization of its reaction products. Virus Res 8: 127–140
58. Spies U, Müller H, Becht H (1989) Nucleotide sequence of infectious bursal disease virus genome segment A delineates two major open reading frames. Nucleic Acids Res 17: 7982
59. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876–4882
60. To H, Yamaguchi T, Nguyen NT, Nguyen OT, Nguyen SV, Agus S, Kim HJ, Fukushi H, Hirai K (1999) Sequence comparison of the VP2 variable region of infectious bursal disease virus isolates from Vietnam. J Vet Med Sci 61: 429–432
61. Vakharia VN, He J, Ahamed B, Snyder DB (1994) Molecular basis of antigenic variation in infectious bursal disease virus. Virus Res 31: 265–273
62. van den Berg TP (2000) Acute infectious bursal disease in poultry: a review. Avian Pathol 29: 175–194
63. van den Berg TP, Gonze M, Meulemans G (1991) Acute infectious bursal disease in poultry: Isolation and characterisation of a highly virulent strain. Avian Pathol 20: 133–143
64. van den Berg TP, Gonze M, Morales D, Meulemans G (1996) Acute infectious bursal disease in poultry: Immunological and molecular basis of antigenicity of a highly virulent strain. Avian Pathol 25: 751–768
65. 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
66. Weber S, Fichtner D, Mettenleiter TC, Mundt E (2001) Expression of VP5 of infectious pancreatic necrosis virus strain VR299 is initiated at the second in-frame start codon. J Gen Virol 82: 805–812
67. Winterfield RW, Thacker HL (1978) Immune response and pathogenicity of different strains of infectious bursal disease virus applied as vaccines. Avian Dis 22: 721–731
68. Yamaguchi T, Ogawa M, Inoshima Y, Miyoshi M, Fukushi H, Hirai K (1996) Identification of sequence changes responsible for the attenuation of highly virulent infectious bursal disease virus. Virology 223: 219–223
69. Yamaguchi T, Ogawa M, Miyoshi M, Inoshima Y, Fukushi H, Hirai K (1997) Sequence and phylogenetic analyses of highly virulent infectious bursal disease virus. Arch Virol 142: 1441–1458

70. Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103–119
71. Yao K, Goodwin MA, Vakharia VN (1998) Generation of a mutant infectious bursal disease virus that does not cause bursal lesions. J Virol 72: 2647–2657
72. Yehuda H, Pitcovski J, Michael A, Gutter B, Goldway M (1999) Viral protein 1 sequence analysis of three infectious bursal disease virus strains: a very virulent virus, its attenuated form, and an attenuated vaccine. Avian Dis 43: 55–64
73. Zierenberg K, Nieper H, van den Berg TP, Ezeokoli CD, Voss M, Müller 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
74. Zierenberg K, Raue R, Müller 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

Author's address: Dr. Matthew F. Rudd, CSIRO Livestock Industries, Australian Animal Health Laboratory, Private Bag 24, Geelong, Victoria 3220, Australia; e-mail: matthew.rudd@csiro.au