Biological and Molecular Characterisation and Crystallisation of Infectious Bursal Disease Virus and Its Major Capsid Protein

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The bravest are surely those who have the clearest purpose.....and go out to meet it.
Thucydides

Abstract

Infectious bursal disease virus (IBDV) is prevalent in most of the poultry producing areas worldwide and causes severe economic losses resulting not only from clinical disease and mortality, but also from the immunosuppressive effect in subclinically infected flocks. The research on IBDV in this thesis is divided into two main parts. In the first part, studies were carried out on the IBDV inadvertently introduced into New Zealand in 1993. Prior to that date, the country had been free from the virus. IBDV was successfully isolated from seven flocks of subclinically infected and/or seronegative chickens in SPF embryonating eggs, adapted to cell culture and identified by EM, immunocytochemistry and RT-PCR test. To evaluate the efficacy of the serological method used in the screening programme of the IBDV eradication scheme, a study was undertaken to compare three diagnostic methods. The study demonstrated that serological testing is not a reliable method for the detection of IBDV infection in New Zealand broiler flocks because antibodies may not have developed to detectable levels by the time of slaughter. Histological examination of affected bursae allowed the demonstration of IBD-like lesions, but these needed to be differentiated from those caused by other agents. The immunocytochemistry test was able to detect early IBDV infection and provided a rapid, definitive diagnosis.

Using the immunocytochemistry test to perform a longitudinal study of IBDV infection in a broiler and a layer farm, results showed the birds were infected as early as 6 to 7 days of age. The prevalence of IBDV infection was estimated to be 55% in the broiler flock. The results showed that the serological test had a sensitivity of 28.57% and a specificity of 73.68% for detecting the New Zealand IBDV strain infection. This indicated that there should be further evaluation of the use of serological testing as the sole method for the detection of IBDV infected farms in the current control scheme.

An *in-vivo* pathogenicity study using IBDV derived from a bursal tissue homogenate and carried out in SPF chickens demonstrated the low virulence of the virus present in

New Zealand. Molecular analysis of the hypervariable region of the VP2 gene of two IBDV isolates obtained in 1997 and 1998 showed they are more closely related to attenuated strains than other strains. In all three phylogenetic analyses, using neighbour joining, parsimony and split decomposition, the NZ isolates are closely related to attenuated strain PBG98 and Cu1 but split away from Australia 002-73, variant E, classical and very virulent strains. Both results support the hypothesis that an attenuated strain of IBDV was inadvertently introduced into the New Zealand poultry population in 1993.

In the second part of this thesis, studies on the structure of the IBDV virion and its major capsid protein were initiated by X-ray crystallography. The purification of IBDV for crystallisation and crystallisation trials are described. Several viral crystals were produced from the trials but only weak diffraction was obtained from these crystals.

With the aim of studying the structure of the major capsid protein of IBDV (VP2) and investigating the major antigenic site on this capsid protein, the *vp2* gene was cloned and expressed, the protein purified, and preliminary crystallisation trials performed. Recombinant VP2 was successfully expressed from a baculovirus expression system. The purification of the recombinant VP2 was also completed in the study and preliminary crystallisation screens determined several conditions favouring the production of crystals.

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Abbreviations

aa	amino acid
A	adenine
AGDP	agar gel immunodiffusion precipitaion
ATP	adenosine-5'-triphosphate
ATV	Antibiotic / trypsin / versene
bp	base pair
BLAST	basic local alignment search tool
BSA	bovine serum albumin
C	cytosine
CAA	*Chicken anaemia virus*
CAM	chorio-allantoic membrane
CEF	chicken embryo fibroblast
cDNA	complimentary DNA
CPE	cytopathic effect
CsCl	cesium chloride
dNTP	deoxynucleoside-5'-triphosphate
dH_2O	distilled water
DMSO	dimethyl sulphoxide
DNA	deoxyribose nucleic acid
ds	double-strand
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
EID_{50}	mean embryo infective dose 50%
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
ES-MS	electrospray mass spectrometry
FBS	feotal bovine serum
FMDV	*Foot-and-Mouth Disease virus*
G	guanine
GM	growth medium
H & E	haematoxylin and eosin
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
HPLC	high-performance liquid chromatography
IBDV	*Infectious bursal disease virus*
IBV	*Infectious bronchitis virus*

IF	immunofluorescence
IgG	immunoglobulin G
IgM	immunoglobulin M
IMAC	immobillised metal ion affinity chromatography
IP	immunoperoxidase
IPNV	*Infectious pancreatic necrosis virus*
IPTG	isopropylthio-ß-D-galactoside
kbp / kb	kilobase pair
kDa	kilo daltons
LB	luria broth
MAb / MCA	monoclonal antibody
MEM + n	minimal essential medium + non-essential amino acids
MM	maintenance medium
MOI	multiplicity of infection
MW	molecular weight
MWCO	molecular weight cut-off
NZ	New Zealand
ORF	open reading frame(s)
PAGE	polyacrylamide gel electrophoresis
PAUP	phylogenetic analysis using parsimony
PBS	phosphate buffered saline, pH 7.0
PCR	polymerase chain reaction
PEG	polyethylene glycol
p.i.	post-infection
PMSF	phenylmethylsulfonyl fluoride
PSK	penicillin / streptomycin / kanamycin
PVDF	polyvinylidene difluoride
RE	restriction endonuclease
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcriptase-PCR
SDS	sodium dodecyl sulphate
SFM	serum-free medium
SNT	serum neutralisation test
SPF	specific pathogen free
T	thymine

TAE	tris / actate / EDTA
TBE	tris / borate / EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl)-aminomethane
UV	ultraviolet
Vero	African green monkey cells
VN	virus neutralisation
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Abbreviations for Amino Acids

Amino Acid	Three Letter Symbol	One Letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Asparagine or Aspartic Acid	Asx	B
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glutamine or Glutamic Acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Related Publications

The results presented in chapters three and five have been published in peer-reviewed journals.

Y. F. Chai, J. Meers and N. H. Christensen (1999) Evaluation of serological, histological and immunocytochemical methods for the detection of infectious bursal disease virus infection in broiler flocks in New Zealand. New Zealand Veterinary Journal, 47:175-179.

Y. F. Chai, N. H. Christensen, C. R. Wilks and J. Meers (2001) Characterisation of New Zealand isolates of infectious bursal disease virus. Archives of Virology, 146:1-10.

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

Infectious bursal disease virus (IBDV) is a member of the family *Birnaviridae*, genus *Avibirnavirus* (van Regenmortel *et al.*, 2000). Other related viruses in this family with IBDV are *Infectious pancreatic necrosis virus* (IPNV) of fish (*Aquabirnavirus*) and *Drosophila X virus* of fruit fly (*Entomobirnavirus*) (Dobos *et al.*, 1979). The earliest report of IBDV affecting chickens was from Cosgrove in 1962 (Cosgrove, 1962) but, since the most prominent clinical sign was extreme kidney damage in infected chickens, the disease was first called "avian nephrosis". The disease was also named "Gumboro disease" since the first outbreak was observed in the area of Gumboro, Delaware, USA (Cosgrove, 1962). Later studies by (Winterfield & Hitchner, 1962; Winterfield *et al.*, 1962) succeeded in isolating the aetiological agent of the disease in embryonating eggs and referred to it as "infectious bursal agent" to recognise it as the cause of bursal lesions in the chickens. Finally in 1970 Hitchner proposed the term "infectious bursal disease" (IBD) as the appropriate name for the disease, which was characterised by causing specific lesions in the cloacal bursa in chickens (Hitchner, 1970).

Two IBDV serotypes (1 and 2) have been identified (McFerran *et al.*, 1980b), with subtypes based on antigenic variation, documented within each serotype (Saif, 1984). Both serotype 1 and 2 IBDV naturally infect chickens and turkeys, but only serotype 1 IBDV is pathogenic to chickens, causing clinical disease in the birds (Jackwood & Saif, 1983; Jackwood *et al.*, 1984). Antibody to serotype 1 and/or 2 has been found also in turkeys (Barnes *et al.*, 1982; Chin *et al.*, 1984; Jackwood *et al.*, 1982), ducks (McFerran *et al.*, 1980b), guinea fowl (Nawathe *et al.*, 1978), pheasants (Louzis *et al.*, 1979), migrating birds (Wilcox *et al.*, 1983) and penguin (Gardner *et al.*, 1997), but no clinical disease in species other than chickens has been reported.

IBDV infection is highly prevalent in most of the poultry population of the world. IBDV has a very important economic impact on the poultry industry worldwide by causing production loss from clinical disease as well as from subclinical disease as a

result of immunosuppression. Immunosuppression in IBDV infected chickens has been well documentated by numerous studies. Secondary infection following IBDV infection may result in inclusion body hepatitis (Fadly *et al.*, 1976), gangrenous dermatitis (Rosenberger *et al.*, 1975) and coccidiosis (Anderson *et al.*, 1977). Other studies have also shown interaction between IBDV infection and *Chicken anaemia virus* (Yuasa *et al.*, 1980) or *Marek's disease virus* (Cho, 1970; Sharma, 1984) infection in the flock. Vaccination failures have also been attributed to prior IBDV infection (Allan *et al.*, 1972; Faragher *et al.*, 1974; Hirai *et al.*, 1974).

Chickens are most susceptible to IBDV during the first week of life, and up to 12 weeks of age. With a short incubation period of 2 to 3 days, the clinical signs may be seen from 2 to 5 weeks of age. Earliest signs include diarrhoea, anorexia, depression, ruffled feathers, prostration and sudden death. Gross lesions observed at postmortem examination include an oedematous bursa, which may be haemorrhagic and progress to atrophy. The spleen may be enlarged with necrotic foci and there may be enlarged kidneys filled with urates. Haemorrhages in the thigh and pectoral muscles also are observed in some cases. The severity of clinical signs and the mortality in an infected flock is dependent on the virulence of the virus strain. Mortality varies from 20 to more than 50% being reported following infection with the very virulent strain of the virus (Chettle *et al.*, 1989; van den Berg *et al.*, 1991). Antigenically distinct virus strains emerged in the USA in the early eighties (Rosenberger & Cloud, 1986) and these so called "variant strains" of serotype 1 IBDV caused clinical signs in infected chickens even in those with a high concentration of maternal antibody. They caused severe immunosuppression despite the presence of antibody to serotype 1 IBDV.

A detailed review of the antigenic variation and diversity of serotype 1 IBDV is presented in section 1.9 in this chapter. Other information on IBDV such as the virion, genomic properties, viral structural proteins, pathogenesis and epidemiology will also be reviewed in the following sections.

1.2 VIRION PROPERTIES

IBDV virions are about 60 nm in diameter, single shelled, non-enveloped icosahedrons with symmetrical shape (Dobos *et al.*, 1979). A total of 260 trimeric subunits form the viral capsid, which is composed of 32 capsomers with a triangulation number T = 13 icosahedral lattice (Ozel & Gelderblom, 1985). Virion Mr is about 55 x 10^6, sedimentation rate of 435S in water (S_{20w}) and 460S in sucrose gradients (Dobos *et al.*, 1979; Todd & McNulty, 1979). The buoyant density of the mature virion in caesium chloride gradient is 1.33 g/mL (Becht *et al.*, 1988; Nick *et al.*, 1976), ranging from 1.29 to 1.34 g/mL due to incomplete/immature viral particles.

The virus is very stable at pH 3 to 9 and is resistant to treatment with ether and chloroform (Benton *et al.*, 1967a). It is resistant to heat to 60 °C for 1 hour but not at 70 °C for 30 min (Landgraf *et al.*, 1967). It is also resistant to 0.5% phenol, 0.125% thiomersol and 1% SDS at 20 °C, pH 7.5 for 30 min. Exposure to 0.5% formalin for 6 hours reduces its infectivity (Benton, Cover, & Rosenberger, 1967a).

1.2.1 Viral Genome and Viral Proteins

IBDV like other viruses in the *Birnaviridae* family has two segments of double-stranded RNA; a larger segment (A) of about 3254 base-pairs and a smaller segment (B) of about 2800 base-pairs(MacDonald, 1980; Muller *et al.*, 1979a). The synthesis of the viral structural proteins VP2, VP3 and VP4 in IBDV, other members of the *Birnaviridae* family and in *Reoviruses* (also double stranded RNA viruses), involves the synthesis and subsequent cleavage of precursor polyprotein(s) (Spall *et al.*, 1997). The genome segment A contains two open reading frames (Fernandez-Arias *et al.*, 1998) encoding a 110 kDa precursor protein and a 17 kDa protein – VP5 (Azad *et al.*, 1986; Hudson *et al.*, 1986; Kibenge & Dhama, 1997; Muller & Becht, 1982). The two ORFs are partially overlapping in the segment A genome (Mundt *et al.*, 1995; Mundt & Muller, 1995). Segment B encodes a 94 kDa polypeptide – VP1 (or VPg) (Bruenn, 1991; Kibenge *et al.*, 1996; Muller & Nitschke, 1987a; Muller & Nitschke, 1987b; Spies *et al.*, 1987). VP1 is found in virions both in a free and genome-linked form. Both genome segments

A and B contain a 5' genome-linked protein (Carvelt *et al.*, 1991) and no polyA tracts at the 3' ends of the genome (Muller & Nitschke, 1987b).

The 110 kDa precursor protein contains the pre-VP2 (47-48 kDa), VP4 (28-31 kDa) and VP3 (33-34 kDa) arranged from 5' to 3' end as NH2-pVP2-VP4-VP3-COOH. The VP4 has been shown to be the viral protease responsible for cleaving the polyprotein into three separate segments of pre-VP2 (or VPX in some publications), VP4 and VP3 (Azad *et al.*, 1987; Hudson *et al.*, 1986; Spies *et al.*, 1987). This is an autocatalytic reaction and does not involve host cellular proteases (Kibenge *et al.*, 1997). The exact cleavage sites on the polyprotein are not known. The VP4 is a virus-encoded proteinase responsible for cleaving itself out of the precusor (Azad *et al.*, 1987; Hudson *et al.*, 1986; Jagadish *et al.*, 1988; Nagy *et al.*, 1987). The result of the VP4 cleavage forms pre-VP2 and VP3 structural proteins for the virion capsid. Some predictions are that the dibasic residues (RR) are the sites for proteolytic cleavage of VP4 which acts as a serine protease (Azad *et al.*, 1987; Hudson *et al.*, 1986). The latest study by Sanchez and Rodriguez demonstrated that the specificity of the cleavage is dictated by the conserved AA dipeptide (Sanchez & Rodriguez, 1999). In contrast to IBDV, the exact cleavage sites between VP2 and VP4 in the polyprotein of the IPNV has been shown to be the Ser/Thr-X-Ala-Ser/Ala-Gly motif (Petit *et al.*, 2000). The pre-VP2 protein is later cleaved into VP2 protein (40-45 kDa) (Kibenge *et al.*, 1997). The maturation of pre-VP2 to VP2 is believed to involve the cleavage of pre-VP2 near the carboxy end (Azad *et al.*, 1987) and does not involve cellular proteases (Kibenge *et al.*, 1997). The cleavage of pre-VP2 into VP2 has proved to be incomplete as both pre-VP2 and VP2 could be detected in purified virus preparations (Dobos *et al.*, 1979). The post-translational modification of the pre-VP2 to VP2 has been suggested to occur during virus maturation and assembly as reported in *Reoviruses* and *Rotaviruses* (Cohen *et al.*, 1978; Krystal *et al.*, 1976; Muller & Becht, 1982). The regulation which underlies the transcription of the mRNA for the individual polypeptides from the two RNA segments of the genome is still not clearly identified.

VP2 and VP3 are the major structural proteins of the virus. VP2 constitutes 51% of the total capsid protein and studies have shown that it is the main host-protective antigen of IBDV; the VP2 contains the major antigenic epitope for the induction of neutralising antibodies (Fahey *et al.*, 1989; Oppling *et al.*, 1991; Reddy & Silim, 1991; Snyder *et al.*,

1988a) and the epitope is highly conformation-dependent (Heine *et al.*, 1991). The other major structural protein VP3, has a group-specific region (Becht *et al.*, 1988) and a minor neutralising site (Jagadish *et al.,* 1995). The positively charged C-terminus of VP3 might interact with the genomic dsRNA molecules and encapsidation of the RNA genome within the capsid (Hudson *et al.*, 1986; Lombardo *et al.*, 1999).

VP1 encoded by segment B is the putative virion-associated RNA-dependent RNA polymerase (Bruenn, 1991; Kibenge *et al.,* 1996). VP1 has been found as a "free" protein and is covalently linked to both ends of the dsRNA genome segment A and B in purified virions (Kibenge & Dhama, 1997; Magyar *et al.*, 1998). Lombardo's research also demonstrated that VP1 is incorporated into newly formed virus-like particles by formation of VP3-VP1 complexes (Lombardo *et al.,* 1999). Guanylyl transferase and methyl transferase activities also have been shown to be associated with the VP1 in IPNV (Dobos, 1993).

The function of the VP5 is not known, however recent research showed VP5 is not essential for viral replication in cell culture and in vivo (Mundt *et al.,* 1997). But VP5 might play an important role in viral pathogenesis since mutant VP5-defective virus had lost the ability to cause bursal lesions (Yao. *et al.*, 1998).

Schematic diagram of the IBDV genomic structure and organization is shown in Figure 1-1.

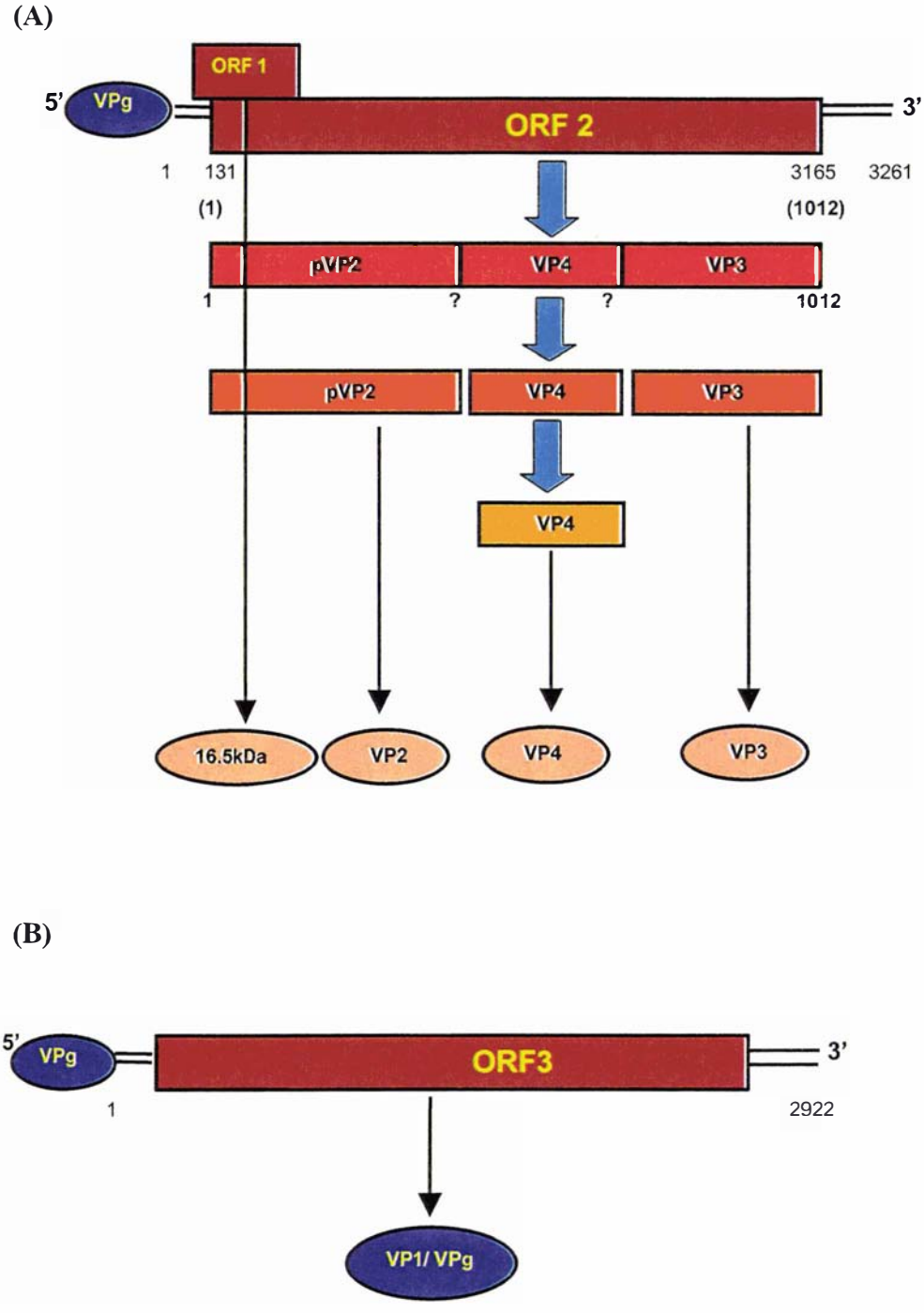


Figure 1.1: Schematic diagram of the genome organisation of Infectious Bursal Disease virus (IBDV) (Cu-1 strain). (A) Genome segment A, VP4 is the virus-encoded protease and the exact cleavage sites have not been determined. (B) Genome segment B, encoded for the viral RNA-dependent RNA polymerase, VP1 as the free-form and VPg as the genome-linked form. (Figure copied and modified from Virus Taxonomy, seventh report of the international committee on taxonomy of viruses, 2000)

1.2.2 Viral Replication

IBDV has been adapted to replicate and produce cytopathic effect (CPE) in primary cell cultures such as chicken bursal lymphoid cells, chicken embryo kidney cells and chicken embryo fibroblast cells (Lukert & Davis, 1974) as well as some mammalian continuous cell cultures such as RK-13, Vero, BMG-70 and Ma-104 (Jackwood *et al.*, 1987; Kibenge *et al.*, 1988; Lukert *et al.*, 1975; Petek *et al.*, 1973; Rinaldi *et al.*, 1974). In primary cell cultures of chicken origin, the viral replication cycle takes 10 to 16 hours compared with more than 48 hours in mammalian cells (Jackwood *et al.*, 1987; Kibenge *et al.,* 1988; Lukert & Davis, 1974; Nick *et al.*, 1976). There is also a difference in the latent period of infection between the two cell cultures, being 4 to 6 hours in chicken fibroblast cells (Lukert & Davis, 1974; Nick *et al.,* 1976) and 12 to 16 hours in Vero cells (Kibenge *et al.*, 1988). Muller and Betch have shown that synthesis of IBDV-specific polypeptides could be detected in chicken bursal lymphoid cell cultures as early as 90 min after infection, with release into culture medium 6 hours after infection (Muller, 1986).

The target organ for the pathogenic serotype 1 strains is the bursa of Fabricius, as demonstrated by experiments in which bursectomized chicken are not susceptible to lethal infection with IBDV (Kaufer & Weiss, 1980). Chickens infected soon after hatching or at an early age have marked depletion of B-cells whereas infection of chickens older than 12 weeks with IBDV dose not result in clinical signs (Becht, 1980), and a high concentration of antigen can be detected only in the B-cells of the bursa but not in thymus and spleen (Kaufer & Weiss, 1980; Nick *et al.*, 1976). *In vitro* infection studies also showed that IBDV replicates in proliferating B-cells (Muller, 1986) but not in very immature lymphoblast cells (Nakai & Hirai, 1981). Apathogenic IBDV serotype 2 does not replicate in lymphoid cells but can be propagated in chicken embryo fibroblast cells (Becht *et al.*, 1988; Nick *et al.*, 1976). In susceptibility tests of chicken lymphoid cells to both the pathogenic serotype 1 and nonpathogenic serotype 2 IBDV, Nieper and Muller (Nieper & Muller, 1996) demonstrated that the restriction of IBDV replication in lymphoid cells is not determined by the presence of specific receptor sites but may be due to the presence of different receptors: CEF had receptors common to both serotypes and specific ones for each serotype. Receptor sites common to both serotypes were also present on lymphoid cells, however, additional serotype-specific

sites were only demonstrated for apathogenic serotype 2 strains (Nieper & Muller, 1996).

Although the mechanism of synthesis of ds RNA during the replication of IBDV has not been clearly determined, other studies on IPNV showed that, after viral attachment to cellular receptors and penetration into target cells, viral RNA transcription is initiated without uncoating or degradation of the capsid. The precusor molecules gain access to the viral polymerase-template complex by passing through the capsid (Spies *et al.*,1987). Dobos et al (Dobos, 1993) demonstrated that virion-associated VP1 protein catalyses a guanylylation reaction which serves to prime viral RNA synthesis. In vitro single-stranded (ss) RNA synthesis studies have shown the virus RNA is transcribed by a semi-conservative strand displacement mechanism. There is no information on minus strand RNA synthesis, but the two mRNAs can be detected in infected cells by 3-4 hours post-infection and are synthesised in the same relative proportions throughout the replicative cycle (Dobos, 1995). The initiation of IPNV viral RNA synthesis is suggested to involve either two VP1 molecules, one serving as a primer and the other as polymerase for chain elongation or just a single VP1 molecule in both functions (Dobos, 1995).

Complete sequence information of both segment A and B including the 5' and 3' terminal non-coding sequences have been reported (Kibenge *et al*, 1996; Mundt & Muller, 1995). The results identified some regulatory sequences involved in the IBDV RNA synthesis on the viral 5'-terminal non-coding region, a 32 base-pairs of genomic segment A that contains a major promoter element for IBDV genome replication and expression (Mundt & Muller, 1995; Nagarajan & Kibenge, 1997). The 3'-terminal of both segments contains a conserved pentamer GCGGU (Kibenge *et al.*, 1996). Several palindromic sequences have been identified as well as direct and inverted repeats of both segments, which could function as a binding site for chicken 18S rRNA (Mundt *et al.*, 1995).

1.3 HOST SUSCEPTIBILITY AND EPIZOOTIOLOGY

IBDV is widely distributed in all poultry flocks world-wide, with the exception of New Zealand, which reported freedom from IBDV infection from 1973 to 1992 (Howell &

Stanislawek, 1992; Jones, 1986; With, 1985). However, serological evidence of IBDV infection was detected in commercial broiler chickens in 1993 in New Zealand (Thompson, 1994).

IBDV serotype 1 mainly infects chickens, including commercial and backyard fowls, and causes disease only in chickens. Turkeys and ducks have been reported to be seropositive against serotype 1 and the virus has been isolated from these species, but no clinical diseases has been observed in them (Jackwood *et al.,* 1982; McFerran *et al.,* 1980b; Yamada *et al.*, 1982). However, some researchers found that experimental infection of 3- to 6-week-old turkeys produced microscopic lesions in the bursa (Giambrone *et al.,* 1978). IBDV serotype 2 infects turkeys and is widely distributed in all major turkey producing countries. It was shown to be non-pathogenic to day-old poults (Barnes *et al.*, 1982; Chin *et al.*,1984; Jackwood *et al.*, 1982). Chickens and ducks can be infected with serotype 2 IBDV, with seroconversion detected by serum-neutralization test. Neither gross nor microscopic lesions were observed in chickens or ducks, following natural or experimental infection with serotype 2 IBDV (Jackwood & Saif, 1983; McFerran *et al.*, 1980b).

All breeds of chickens were susceptible to IBDV serotype 1 infection. However, a difference in genetic susceptibility has been reported, with the White Leghorn egg-laying breed having the highest mortality rate when compared to other light breed commercial birds (Bumstead *et al.,* 1993; Lukert *et al.*, 1991). Genetic resistance is now known to influence several outcomes of IBDV infection. It was shown that the genetic line influenced the severity of IBDV-induced histological lesions in the thymus (Sharma & Lee, 1983), as well as the mortality and the severity of histological lesions in the bursa (Bumstead *et al.*,1993). Disease resistance research on the major histo-compatibility complex (MHC) genes showed IBDV mortality and thymus atrophy were influenced by the MHC B haplotype of the chickens. Meat-type chicken lines are more resistant to IBDV than layer-type lines (Fadly & Bacon, 1992; Nielsen *et al.*, 1998).

Efforts to identify the natural reservoir and other susceptible hosts of IBDV have produced evidence that free range and backyard chickens may serve as a reservoir of IBDV (McFerran *et al.*, 1980a). Commercially reared quail and pheasant were reported to be susceptible to IBDV infection with resultant mortalities (Louzis *et al.*, 1979), but

some researchers failed to demonstrate disease in these species following experimental infection (Weisman & Hitchner, 1978a). Antibodies against IBDV have been demonstrated in a wide range of wild and migratory birds including quail (Weisman & Hitchner, 1978a), pigeons (Vindevogel, 1979), village weavers (*Ploceus cucullatus*), pied cordon bleus (*Uraeginthus bengalus*) (Nawathe *et al.*, 1978), magpie geese (*Anseranus semipalmata*), shearwaters (*Puffineus carneipes*), sooty terns (*Sterna fuscata*), common noddy (*Anous stolidus*), silver gulls (*Larus novaehollandiae*), and black ducks (*Anas supercilicosa*) (Wilcox *et al.*,1983). There was one report of IBDV antibodies in wild Antarctic penguins (Gardner *et al.*,1997).

1.4 EPIDEMIOLOGY OF VERY VIRULENT AND VARIANT STRAINS OF INFECTIOUS BURSAL DISEASE VIRUS

Since IBDV was reported by Cosgrove in 1962, the disease was well controlled until 1983 by effective immunisation programmes using live and inactivated vaccines in broilers, layers and parent flocks. The emergence of variant strains in poultry flocks in the United States in 1983 in the Delmarva area is not clearly understood, but it is suggested that the antigenic variation was due to natural genetic drift of an RNA-polymerase dependent RNA virus and selective immune pressure in the field. The first reported case of vaccination failure associated with the emergence of pathogenic variation outside North America was from Belgium in 1987 (van den Berg *et al.*, 1991). In contrast to the North American situation, the high mortality in the infected flock was caused by a new strain which did not appear to differ antigenically from the classic strains, but appeared to be more virulent (Berg *et al.*, 1996; Oppling *et al.*, 1991; van den Berg & Meulemans, 1991a). The new strain caused lesions typical of IBDV, was antigenically similar to the classical European strains, but was now able to infect birds in the face of maternal antibody levels that were protective against classical strains. Despite vaccination, the new strains also caused up to 25% mortality in broilers and 60-70% in layers (Chettle *et al.*,1989; Oppling *et al.*, 1991). These so called "very virulent' (vv) IBDV strains and their association with similar disease outbreaks have subsequently been reported in several parts of Europe, South Africa and Asia (Brown *et al.*, 1994; Cao *et al.*, 1998; Eterradossi *et al.*, 1992; Lin *et al.*, 1993; Pitcovski *et al.*,

1998; Zierenberg *et al.*, 2000). The vvIBDV strains not only caused high mortality in young chickens but also in older birds. Most affected birds had high maternal antibody levels or were vaccinated with classical strains. Up to 60 to 100% mortality was observed in SPF birds infected with vvIBDV strains. To date, there have been no reports of vvIBDV strains in North America, Australia or New Zealand.

Nevertheless, the reason for the emergence of variant and virulent strains is not clearly understood. Possible contributing factors could be the high density of poultry in a limited geographical area such as the Delmarva peninsula where the variant strains emerged in 1983, intensive integrated production of poultry and subsequently intensive vaccination for control of diseases. All these selective pressures in the field may have contributed to the antigenic and genetic changes of IBDV that occurred from the 1980's until the present day (Rosales *et al.*, 1989; van den Berg & Meulemans, 1991a).

1.5 PATHOGENESIS

1.5.1 Route of Infection and Transmission

IBDV infection occurs through the oral route, upper respiratory tract and conjunctiva, and contagious virus is excreted in the droppings of infected chickens 24 hours after infection. Virus shedding continues for at least 14 to 16 days (Vindevogel *et al.*, 1976; Winterfield *et al.*, 1972). Studies showed the virus is highly persistent in the environment and could survive in poultry house litter for up to 60 days (Benton *et al.*, 1967b; Petek *et al.*, 1973; Vindevogel *et al.*, 1976). Indirect transmission of virus occurs via contaminated feed, litter or workers, and through airborne transmission of virus associated with feathers and dust (Benton *et al.*, 1967b). No evidence of egg or vertical transmission was found in experimental or field observations (Lukert *et al.*, 1991). However, possible reservoir hosts including lesser mealworms (Snedeker *et al.*, 1967), *Aedes vexans* mosquitoes (Howie & Thorsen, 1981) and rats (Okoye & Uche, 1986) have been described. These species have been demonstrated to be either positive for IBDV antibodies or for virus, by virus isolation.

1.5.2 Replication *in-vivo* and Organs Affected

After natural infection through the upper respiratory tract, the virus could be detected in gut-associated lymphoid cells and macrophages within 4 to 5 hours post-infection (p.i.) (Fadly & Nazerian, 1983; Ley *et al.*, 1983). After primary replication of the virus in these sites, the virus spreads to different organs in the bird, most probably via the portal veins and main blood stream. The virus was demonstrated in Kupffer cells of the liver and tubular epithelial cells of the kidney in the early infection stage (Muller *et al.*, 1979b). Antigen detection studies by various researchers demonstrated that viral antigen could be detected in the bursa as early as 11 hours p.i (Ley *et al.*, 1983; Tanimura *et al.*, 1995). The early replication of the virus in the bursa was localised mainly in the medullary-cortical boundary or in the interfollicular connective tissue. Two hours after entry to the bursa (13 hours p.i.), the virus replicated in the lymphoid cells of medulla and cortex of the organ. By 16 hours p.i., virus was present in the epithelial and subepithelial cells of the follicles (Muller *et al.*, 1979b). After massive replication of the virus in the bursa, a secondary viraemia and replication in other organs including thymus, spleen, kidney and caecal tonsils occurs 2 to 8 days p.i. (Fadly & Nazerian, 1983; Kaufer & Weiss, 1980).

The target organ of IBDV is the Bursa of Fabricius and the target cells for the virus are the actively dividing, surface immunoglobulin M-bearing B-lymphocytes in the bursa (Hirai & Calnek, 1979; Muller & Becht, 1982; Nakai & Hirai, 1981). These cells are lysed after infection and reduced in number. The depression in B-lymphocytes is age dependent, with a greater depletion in birds infected at 1 day-old than at 3 weeks of age (Sivanandan & Maheswaran, 1980). Virus-triggered apoptosis in immature B- and T-cells is responsible for part of the immunosuppression in birds (Ojeda *et al.*, 1997; Tanimura & Sharma, 1998; Tham & Moon, 1996; Vasconcelos & Lam, 1994). The adverse effect of IBDV on cell-mediated immunity and T-cells has also been studied using a variety of research methods (Cloud *et al.*, 1992a; Cloud *et al.*, 1992b; Sharma *et al.*, 1913-1986; Sharma & Lee, 1983; Vervelde & Davison, 1997). It is suggested that mechanisms such as the activation of suppressor cells and the impairment of helper T-cells cause suppression of cellular immunity in IBDV-infected birds.

1.5.3 Clinical Signs of IBD

The clinical signs of IBDV infection have been well described in numerous poultry diseases text books (Baxendale *et al.*; Calnek *et al.*, ed; McFerran *et al.*, 1993). Since IBDV has become endemic in countries around the world and extensive vaccination programmes have been instituted in commercial poultry flocks, the typical clinical signs reported by Cosgrove and others during the early 1960s are not commonly found now (Cosgrove, 1962; Hitchner, 1963; Landgraf, Vielitz, & Kirsch, 1967; Parkhurst, 1964). In summary, the incubation period of IBDV is very short (2 to 4 days). In birds without any maternal antibody or active immunisation against IBDV, the clinical signs included pecking their own vents, soiled or urate-stained vent feathers, whitish or watery diarrhoea, anorexia, ruffled feathers, trembling, prostration and death. Morbidity is usually 100% and deaths are first observed at the third day p.i., peak at 5 to 7 days and then decline. Overall mortality following infection with classical virulent strains is usually between 20 and 30 % (Calnek *et al.*, 1993; McFerran *et al.*, 1993).

As mentioned earlier, since most commercial poultry now have moderate to high levels of maternal antibody, most birds do not become infected at as early an age as before. However, immunosuppression may result if there is a breach in the vaccination programme (such as a late booster of the second vaccination, causing decline in protective antibody), giving a window of opportunity for residual field virus to infect the flock at a later age. Thus, infection with IBDV at a later age will produce a range of clinical signs (Faragher *et al.*, 1974; Rosenberger & Gelb, 1978), depending on the nature of secondary or opportunist pathogens present in the field (see section 1.6).

For the serotype 1 variant strains that emerged in USA, the antigenically-shifted virus could escape neutralisation by either maternal or immunisation induced antibody in chicks and infect them in the first week after hatching. The variant strains cause immunosuppression and induce a variety of secondary field infections. Thus, the characteristic clinical signs, as described above, are not observed. The initial finding of variant strain infection was an increase in respiratory disease caused by agents such as *Newcastle disease virus*, *Infectious laryngotracheitis virus* and *Infectious bronchitis virus* (Rosenberger & Cloud, 1986). The clinical manifestations found in birds infected with these variant strains could also result from other secondary bacterial or protozoal infections.

On the other hand, the vvIBDV strains produce similar clinical signs to the classical virulent strains. However, vvIBDV can break through high maternal or active immunisation-induced antibody protection in birds, resulting in more acute clinical signs and higher mortality in comparison to classical strains. Mortalities up to 25% in broiler and 60% in layer pullets within a short period have been reported (Chettle *et al.*, 1989; Nunoya *et al.*, 1992; van den Berg & Meulemans, 1991a).

1.5.4 Gross Pathology of IBD

Examination of sick or dead IBDV-infected birds generally reveals dehydration, and ecchymotic haemorrhages in the thigh and pectoral muscle may be found (Lukert *et al.*, 1991). As the target organ of the virus is the bursa, the major gross lesions appear in this organ. There is a sequential change in the gross bursal lesions after infection; at 2 to 3 days p.i., the bursa is double its normal weight and size, due to oedema, and viscous transudate may surround the serosal surface. On cross-section of the swollen bursa, it may be hyperaemic and show prominent longitudinal striations. Pinpoint to patchy haemorrhages may be present on the mucosal surface (Helmboldt & Garner, 1964). By 5 days p.i., the bursa decreases in size to normal size and continues to atrophy to about one-third of its original size by 8 days (Lukert *et al.*, 1991).

The spleen may be enlarged in some cases and show grey focal lesions on the serosal surface (Morales & Boclair, 1993). A parameter used commonly to determine the virulence of strains of IBDV uses the bursal to body weight (B/BW) ratio and the spleen to body weight (S/BW) ratio in infected birds (Lucio & Hitchner, 1979; Mazariegos *et al.*, 1990; Morales & Boclair, 1993; van den Berg & Meulemans, 1991a). However, these ratios vary between different breeds of birds, so a control group comparison is required.

Occasionally, kidney enlargement and urate deposition in the renal tubules may be found, but that is a nonspecific lesion that results from water deprivation during the terminal illness of the birds (Lukert *et al.*, 1991).

The gross lesions in birds infected with variant strains of IBDV are different from those of classical strains. Infection with variant strains caused rapid and profound atrophy of the bursa without the sequence of inflammatory changes(Rosenberger & Cloud, 1985).

1.5.5 Histopathology of IBD

The predominant histopathological lesions of IBDV infection are found in B-lymphocyte structures such as the bursa of Fabricius, spleen, thymus, caecal tonsils, gut-associated lymphoid tissue (GALT), Harderian gland, head-associated lymphoid tissue (HALT) and conjunctiva-associated lymphoid tissue (Fix & Arp, 1991). The lesions in the bursa have been extensively reported (Cheville, 1967; Helmboldt & Garner, 1964; Henry *et al.*, 1980; Kaufer, 1976). The histopathological changes in the bursa progress in parallel with the gross changes. As early as 6 hours after infection, the virus can be detected in the bursal lymphocytes, and within the first day of infection, necrosis of lymphocytes in the medulla can be seen. There is also infiltration of heterophils into the medulla. By the third day, the medulla has a marked accumulation of heterophils. Later, the inflammatory reaction is so severe that only necrotic follicles surrounded by heterophils are seen. The inflammation also affects the subserosal and interfollicular connective tissue. From the fourth day after infection, the inflammatory reaction declines, leaving cystic cavities in the medulla of the follicles, filled with necrotic cellular elements, heterophils, plasma cells and phagocytes. At the same time, fibrosis occurs in the interfollicular connective tissue, and proliferation of the epithelial layer of the bursa produces a glandular structure of columnar epithelial cells, occasionally containing globules of mucin. Finally the cystic cavities fill with fibroblastic interfollicular stroma.

There is hyperplasia of reticuloendothelial cells in the spleen, which causes enlargement of the spleen. Lymphoid necrosis can be found in the germinal centres of the spleen by the third day of infection. The thymus and caecal tonsils may have mild heterophilic infiltration and necrosis of lymphoid cells. In the spleen, thymus and caecal tonsils regeneration of lymphocytes occurs after the sequences of inflammation and necrosis, and the repopulation of lymphocytes is usually completed in one week (Helmboldt & Garner, 1964; Okoye & Uzoukwu, 1990).

Studies on the Harderian gland showed there is decline in the number of plasma cells in the gland in infected birds. This decline in number is transient, and its normal population is recovered after 2 weeks (Dohms *et al.*, 1981; Survashe *et al.*, 1979).

In contrast to infection with classical strains described above, the histological lesions in birds infected with variant strains of IBDV lack an acute inflammatory reaction in the bursa. There is more severe follicular lymphoid necrosis occurring within 3 days of infection, and atrophy of plicae and bursal epithlium is observed (Sharma *et al.*, 1989).

1.6 DIAGNOSIS OF IBDV INFECTION

The prominent gross and histopathological lesions of the disease are suggestive of IBDV infection. However, the diagnosis of subclinical IBDV is complicated due to the intensive management of commercial poultry, different vaccination programmes employed and interference by maternal antibody. Also, the emergence of variant strains, which caused mainly immunosuppression in birds, result in pathological lesions of secondary infections. Laboratory diagnosis is thus needed for differential diagnosis. A description of the methods used for the confirmation of IBDV infection follows:

Virus Isolation

Organs for virus isolation are collected from acutely infected birds with clinical signs. Most studies harvest the affected bursa for isolation of IBDV, but during the viraemic stage, spleen and faeces can also successfully be used for virus isolation (Lukert *et al.*, 1989). Hitchner (Hitchner, 1970) reported that the CAM of 9- to 11-day-old embryos was the most sensitive route for isolation of IBDV. Classical serotype I strains of IBDV killed the embryo within 3-5 days, producing vascular congestion and subcutaneous haemorrhages in the embryo. Variant strains of IBDV do not cause embryo mortality, but stunting of the embryo with splenomegaly and liver necrosis occur (Rosenberger & Cloud, 1985). McFerran et al (McFerran *et al.*, 1980b) reported seven chicken isolates of IBDV that failed to grow in cell culture, but were successfully propagated in embryonating eggs.

Many strains of IBDV have been adapted to cell cultures of chicken embryo origin and produced cytopathic effects in these cultures (Petek *et al.*,1973; Rinaldi *et al.*, 1974).

Other cell cultures apart from those of chicken origin can also be used for propagation of IBDV. These include turkey and duck embryo cells (McNulty *et al.*, 1979), mammalian cell lines such as rabbit kidneys (RK-13) (Rinaldi *et al.*, 1974), monkey kidneys (Vero-cell) (Kibenge *et al.*, 1975) and baby grivet monkey kidney cells (BGM-70) (Jackwood *et al.*, 1987). The different susceptibility of the cell-lines to propagate the virus has been discussed in few studies (Jackwood *et al.*, 1987; Kibenge *et al.*, 1988; Lukert *et al.*, 1975). Some field strains of IBDV, especially the virulent strains, can not be readily adapted to cell culture, but only replicate in embryonating eggs (Lee & Lukert, 1986; McFerran *et al.*, 1980b).

Antigen Detection

IBDV isolated in embryonated eggs and cell culture can be identified by immunofluorescence (Snyder *et al.*, 1984) or electron microscopy (McFerran *et al.*, 1971). IBDV antigen could also be detected in sections of formalin-fixed and paraffin embedded bursal tissues by indirect immunofluorescence (IF) and immunoperoxidase (IP) staining (Chai *et al.*, 1999; Cho *et al.*, 1987; Cruz-Coy *et al.*, 1993; Jonsson & Engstrom, 1986; Tanimura *et al.*, 1995). Antigen also could be detected in bursal tissues from acutely infected birds using agar gel precipitation test (AGPT) (Ide, 1975), serum neutralisation test (Weisman & Hitchner, 1978b), immunorheophoresis (Raj *et al.*, 1998), latex agglutination test (Nachimuthu *et al.*, 1995), and IF, IP or electron microscopic examination on direct smear of bursal tissue (Allan *et al.*, 1984; Ide, 1975). Snyder et al (Snyder *et al.*, 1988a) developed a selective panel of monoclonal antibodies for use in antigen-capture Enzyme-Linked Immunosorbent Assay (ELISA) for detecting and differentiating IBDV isolates from clinical bursal samples.

Viral Nucleic Acid Detection

Molecular detection techniques such as nucleic acid probe, in-situ hybridisation (Hathcock & Giambrone, 1992; Jackwood *et al.*, 1990) and in-situ PCR (Liu *et al.*, 2000) were developed recently for detecting viral RNA in cells or histological sections. Amplification of viral RNA by reverse transcriptase-polymerase chain reaction (RT-PCR) to detect IBDV gene sequences in clinical samples also has been successfully demonstrated to be a rapid and specific assay (Davis & Boyle, 1990; Jackwood & Nielsen, 1997; Lee *et al.*, 1992; Qian & Kibenge, 1994; Tham *et al.*, 1995; Wu *et al.*, 1992).

Antibody Detection

The most commonly used serological test for detection of antibody against IBDV in commercial poultry flocks is the ELISA. The commercialised ELISA is rapid, quantifiable and enables the handling of large numbers of samples. It is used to monitor the efficiency of IBDV vaccination programmes and the antibody profile of the chicks and breeder birds. With the addition of automated assays and computerised processing, the ELISA became a powerful tool for monitoring the IBDV antibody status in poultry flocks (Briggs *et al.*, 1986; Marquardt *et al.,* 1980). The advantages of ELISA compared with other antibody detection methods have been extensively discussed in numerous publications (Box *et al.*, 1988; Kreider *et al.*, 1991; Snyder *et al.*, 1986; Thayer *et al.,* 1987). Overall, the ELISA provides a relatively specific and sensitive test for detection of antibody against IBDV. However, all commercial ELISA tests have been developed for the purpose of providing information on the immunity level of the flock rather than the infection status of individual birds. The test results only provide a mean flock IBD antibody titre in comparison to standard sera provided in the test kit. The interpretation of results is based on cut-off values established by the manufacturer just for a guide line, and each laboratory or farm should establish its own criterion for protective immunity based on historical antibody responses to specific vaccines or protocols.

The agar gel diffusion precipitin test (AGDP) was the original qualitative method for detection of IBDV antibody and the serum virus neutralisation test (SNT) was the original quantitative method for determining antibody titres against IBDV (Chin *et al.*, 1984; Cullen & Wyeth, 1975; Wyeth & Chettle, 1988). The AGDP test could also be used as a quantitative method as described by Cullen and Wyeth (Cullen & Wyeth, 1975). A modification of the AGDP, Immunoelectrophoresis, provides a result in 45 minutes instead of 5 to 7 days for the AGDP alone (Berg, 1982). Overall, the AGDP test is a highly specific test for serotype 1 IBDV (although it does not differentiate antigenic difference within serotype 1), but has low sensitivity as demonstrated in various studies (Box *et al.*, 1988; van den Berg & Meulemans, 1991b; Wyeth & Chettle, 1982).

In SNT, the neutralisation titre is the reciprocal of the highest dilution of the tested serum which inhibits cytopathic effect. SNT performed in a microtiter system using chicken embryo fibroblast cells was first described by Rosenberger (1989). The SNT is

highly sensitive and specific and is able to detect the different serotypes of IBDV, so it is still a useful tool for differentiating antibodies to antigenic subtypes within serotype 1 IBDV (Chin *et al.*, 1984; Jackwood & Saif, 1983; Skeeles *et al.*, 1979). Some strains of IBDV that are not adapted to cell culture can not be used in microtiter system, and inoculation of SPF eggs has to be performed for the neutralisation test.

1.7 PREVENTION AND CONTROL

The fact that IBDV has been endemic throughout the world since the 1960s (except in New Zealand), the resistance of the virus in the environment, the impossibility of preventing exposure of commercial flocks to the virus, and the continuous rearing cycle without decontamination process, all make the eradication of IBDV from commercial poultry almost impossible. Since there is no therapeutic agent for the treatment of birds after infection, the control of IBDV infection in chickens mainly depends on immunisation of the birds. The principles governing the choice and use of IBDV vaccines in commercial chickens have been well described and summarised by several authors (Lasher & Shane, 1994; Thornton, 1977). The development and availability of IBDV vaccines are also well summarised in different publications (Calnek *et al.*, 1994; Thornton & Pattison, 1975). Basically, the control of IBDV depends upon the production of active or passive immunity in chicks by vaccination either of the chicks with live virus vaccines or hens with live or inactivated or both types of vaccines. In practice, parent birds are primed with attenuated live vaccine and a booster given with inactivated (mostly oil adjuvanted) vaccines to produce uniformly high levels of circulating antibody in the hens, which later are transferred in the egg yolk to the chicks. The transferred maternal antibody protects the chicks during the early weeks of age.

This approach was found to be satisfactory for the whole growing period of broiler chickens when challenged with classical strains of IBDV. However, when the virulent strains of IBDV emerged and were found to break through passive protection, a live vaccination of chicks became necessary to induce active immunity to protect the birds. However, the choice and timing of administration of live vaccine to chicks is not straight forward. The interference by maternal antibody of live vaccine, the uneven

decline of maternal antibody in individual birds, and the residual virulence of different live vaccines make the vaccination schedule complicated. Highly attenuated live vaccines do not produce disease, bursal lesions or immunosuppression, but do not result in sufficient immunity to protect chicks against virulent strains of IBDV. Also, they are subject to interference by maternal antibody. These attenuated vaccines may work well if administration is delayed until maternal antibody has waned. However, that may allow the virulent field strains to infect the naïve birds. A second approach is to immunise the chicks that have maternal antibody using more virulent live vaccine strains. These vaccines are able to break through moderate levels of maternal antibody and induce active immunity in chicks. A large number of vaccine strains with different virulences are available. They are classified based on their ability to break through maternal antibody and their residual pathogenicity to SPF birds. Details of these vaccines and their application have been summarised by different authors (Calnek *et al.*, 1994; Thornton & Pattison, 1975).

Beside conventional vaccines, numerous researchers have aimed to develop alternative methods such as recombinant viral vaccines expressing IBDV capsid protein. These include VP2 in fowlpox virus (Bayliss *et al.*, 1991; Boyle & Heine, 1993), turkey herpesvirus (Darteil *et al.*, 1995) or fowl adenovirus (Sheppard *et al.*, 1998). Expression of IBDV immunogenic proteins in eukaryotic or prokaryotic systems and their use in inactivated subunit vaccines is also in an experimental stage (Dybing & Jackwood, 1997; Dybing & Jackwood, 1998; Fahey *et al.*, 1991; Macreadie *et al.*, 1990; Pitcovski *et al.*, 1996; Vakharia *et al.*, 1994; Vakharia *et al.*, 1993). All of these products of recombinant technology still need to be tested for efficacy and safety. The progress is promising and this approach may replace live vaccination for control of IBDV in the future.

In summary, a good immunisation programme includes close monitoring of the antibody profile of breeders and day-old chicks, selection of the correct vaccine candidate, the right vaccination programme and timing, understanding the field challenge strain(s) and lastly, constant evaluation and adjustment of the vaccination programme.

1.8 IMMUNOSUPPRESSION AND INTERACTION WITH OTHER PATHOGENS

1.8.1 Immunosuppression Studies of IBDV

IBDV infected chickens develop bursal atrophy as a result of the necrosis of lymphoid cells in the bursa of Fabricius and the inflammatory response to viral replication in B-lymphocytes (Cheville, 1967). Replication of IBDV in B-lymphocytes has also been demonstrated in an *in vitro* cell culture system (Hirai & Calnek, 1979; Hirai *et al.*, 1981). Various studies have shown only B-lymphocytes but not T-lymphocytes are susceptible to IBDV (Hirai & Calnek, 1979; Muller, 1986). Chicks bursectomized after hatching showed no clinical signs of the disease when infected with a virulent IBDV strain, compared to intact chicks which succumbed (Hiraga *et al.*, 1994). The severity of clinical signs in infected birds also depends on the age at which infection occurs. Birds infected at less than 3 weeks of age do not exhibit clinical signs or mortality but have marked bursal atrophy and are immunosuppressed. Birds infected between 3 to 6 weeks of age are most susceptible to IBDV infection and subsequently develop lesions in the bursa with necrosis of lymphocytes, a severe inflammatory reaction and high mortality. IBD is less common in birds older than 12 weeks of age (Okoye & Uzoukwu, 1981). The differences in age susceptibility, as shown by many studies, are due to the fact that IBDV has a predilection for proliferating B-lymphocytes. Because the bursa of Fabricius is a specific lymphoid organ in birds and is the reservoir of precusor B-lymphocytes during early life, the bursa of Fabricius is the target organ affected by the virus (see below).

The immunosuppressive effect of IBDV in infected birds has been extensively studied by many researchers. The very first report was from Faragher et al (Faragher *et al.*, 1974). They reported that chicks infected at 1-day-old had a suppressed antibody response to Newcastle disease vaccination. The suppression was greater when chicks were infected at 1-day-old than at 7 days or later of age (Faragher *et al.*, 1974). Many studies with IBDV-infected birds also showed that the birds were more susceptible to secondary infections such as gangrenous dermatitis (Rosenberger *et al.*, 1975), salmonellosis (Wyeth, 1975), colibacillosis (Wyeth, 1975), inclusion body hepatitis (Fadly *et al.*, 1976) and other viral infections. Also suppression of vaccination responses to Marek's disease (Cho, 1970; Sharma, 1984), infectious laryngotracheitis

(Rosenberger & Gelb, 1978), infectious bronchitis (Pejkovski *et al.*, 1979), chicken anaemia agent (Yuasa *et al.*, 1980) and coccidiosis (Anderson *et al.*, 1977; Giambrone *et al.*, 1977), have been reported.

Depression of Humoral Immunity

Most studies agree that immunosuppression following IBDV infection is due to necrosis and depletion of susceptible, proliferating, precursor B-lymphocytes in the bursa (Hirai & Calnek, 1979; Hirai *et al.*, 1981; Lin *et al.*, 1994). Suppression of humoral immunity is directly due to the reduction of circulating B-cells as a result of the lysis of the B-cells and actively dividing precusors in the bursa (Hirai & Calnek, 1979; Rodenberg *et al.*, 1994). It has been shown that the virus selectively targets immunglobulin M-bearing B-cells (Hirai & Calnek, 1979) and intra-bursal B-cell precursors (Rodenberg *et al.*, 1994), but not mature circulating B-cells and B-cells with IgG receptors (Hirai & Calnek, 1979). These findings correlate with the observation that infection during the early posthatching age is more immunodepressive than infection at later ages when more mature and differentiated circulating B-cells are present in the birds. Ivanyi and Morris found that the effect of viral damage to the IgM- producing B-cells was to cause monomeric IgM to fail to polymerise (Ivanyi, 1975; Ivanyi & Morris, 1976) and Hirai (Hirai *et al.*, 1979) demonstrated IBDV-infected birds have severe deficiency of IgG. Recent studies have shown that peripheral blood lymphocytes infected with IBDV *in vitro* have typical features of apoptosis, suggesting that IBDV-induced immunosuppression may be caused, at least in part, by the activation of programmed cell death in lymphocytes (Tanimura & Sharma, 1998; Vasconcelos & Lam, 1994). Transfection of a variety of mammalian cell-lines with an expression vector containing the VP2 or VP5 coding region demonstrated that the expression of the VP2 and VP5 protein could lead to the induction of apoptosis (Fernandez-Arias *et al.*, 1997; Tanimura & Sharma, 1998; Vasconcelos & Lam, 1995). Apoptotic cells also could be detected in the bursa and thymus of SPF chickens infected with IBDV *in vivo* (Tanimura & Sharma, 1998).

Depression of Cellular Immunity

It has been demonstrated via *in vitro* mitogen-induced proliferation studies, that impairment of T-cells in IBDV-infected chickens was not due to lack of functional T-cells but to the presence of macrophage-like suppressor cells, which were activated

during early stages of IBDV infection (Sharma & Lee, 1983). Kim et al suggested that IBDV modulates macrophages and cytokine expression by macrophages *in vitro* and so inhibits T-cell mitogenic responses of spleen cells (Kim *et al.*, 1998). An increase in the number of circulating, large immature lymphocytes incapable of mitogen-induced blastogenesis also has been detected in IBDV-infected chickens (Confer & MacWilliams, 1982).

There are also reports of lysis of lymphocytes in the peripheral lymphoid tissues during IBDV infection, which may compromise local immunity in the birds (Dohms & Jaeger, 1988; Dohms *et al.*, 1988; Vervelde & Davison, 1997). Studies on the effect of IBDV infection on the gland of Harder, which plays a vital role in local immunity of the upper respiratory tract, showed evidence of a significant reduction in the number of plasma cells in the gland (Dohms *et al.*, 1988) and in local primary antibody production (Dohms & Jaeger, 1988). Similar lymphoid necrosis also has been found in the thymus, spleen and caecal tonsils (Cheville, 1967; Fadly & Nazerian, 1983; Helmboldt & Garner, 1964; Ley *et al.*, 1983; Tanimura & Sharma, 1997; Vervelde & Davison, 1997).

1.8.2 Interaction of IBDV with Other Pathogens

The first evidence of immunosuppressive effects of IBDV infection was observed in day-old chicks which failed to respond to Newcastle disease vaccination (Allan *et al.*, 1972). Further studies supported this observation (Faragher *et al.*, 1974; Hirai *et al.*, 1974). It was demonstrated that IBDV infection may play a part in the aetiology of other clinical diseases such as adenoviral inclusion body hepatitis (Rosenberger *et al.*, 1975), chicken anaemia disease (Yuasa *et al.*, 1980), Marek's disease (Cho, 1970; Sharma, 1984), colibacillosis (Wyeth, 1975), clostridial gangrenous dermatitis (Rosenberger & Gelb, 1978) and malabsorption syndrome (Springer *et al.*, 1983). The following is a summary of the interaction of IBDV with various major poultry diseases that have been reported:

Interaction of IBDV with Respiratory Diseases in Poultry

As mentioned previously, IBDV can compromise the vaccination responses to live Newcastle disease virus vaccine and thus the chickens were more susceptible to Newcastle disease virus in the field (Faragher *et al.*, 1974). An experimental challenge study showed that birds receiving the lentogenic Newcastle disease vaccine without

IBDV infection had a lower mortality rate than birds infected with IBDV (Allan *et al.*, 1972). Giambrone (Giambrone, 1979) showed a significant reduction in the serological response to vaccination and an increase in susceptibility to challenge with *Newcastle disease virus* at 4 weeks of age in chicks infected with IBDV at either 1 day or 3 weeks of age. Similarly, Rosenberger and Gelb (Rosenberger & Gelb, 1978) demonstrated that IBDV-infected chicks had a decreased ability to respond to *Newcastle disease* and *Laryngotracheitis virus* vaccinations at 2 weeks of age. In a study of *Infectious bronchitis virus* (IBV), co-infection with IBDV caused more severe respiratory disease compared to birds infected with IBV alone (Pejkovski *et al.*, 1979).

The first immune barrier of poultry against upper respiratory disease agents such as IBV is the local mucosal immunity provided by the Harderian gland, the head associated lymphoid tissue (HALT) and the conjunctival-associated lymphoid tissue (Fix & Arp, 1991) (Davelaar & Kouwenhoven, 1977; Montgomery & Maslin, 1991). IBDV can damage both HALT and CALT, in addition to the lymphocytic depletion in the bursa, by depressing plasma cell activity in these local mucosal sites (Davelaar & Kouwenhoven, 1977; Montgomery & Maslin, 1991). The defective local mucosal immunity resulting from IBDV infection increases the susceptibility to IBV, *Pneumovirus* and *E. coli* (Dohms *et al.*, 1988; Nakamura *et al.*, 1990; Pejkovski *et al.*, 1979).

Interaction of IBDV with Marek's Disease

An increased incidence of Marek's disease in IBDV-infected flocks was observed in some areas. Cho et al (Cho, 1970) found that when IBDV was inoculated into birds before they were exposured to acute *Marek's disease virus*, there was a higher incidence of gross nerve involvement and more severe lesions than in those exposed to *Marek's disease virus* alone. Chicks inoculated at hatch with pathogenic IBDV and the *Turkey herpesvirus* (Bublot *et al.*, 1996) vaccine were poorly protected against challenge at 1 week with virulent *Marek's disease virus* (Sharma, 1984).

Interaction of IBDV with Chicken Anaemia Agent

The first report of an interaction between IBDV and *Chicken anaemia agent* (CAA) was by Yuasa (Yuasa *et al.*, 1980). He found that CAA alone could not produce clinical

signs in 2-week-old SPF chicks, but chicks infected with IBDV at 1-day-old were susceptible to CAA challenge at 2 weeks old. Rosenberger and Cloud (Rosenberger & Cloud, 1989) obtained a similar result, demonstrating that IBDV increases the susceptibility of birds to CAA and the severity of the clinical signs. CAA was later shown to suppress T-cell immunity in thymus, spleen and bone marrow (Cloud *et al.*, 1992a; Cloud *et al.*, 1992b). This adverse effect of CAA on the immune system could be further enhanced by co-infection with IBDV.

Interaction of IBDV with Other Viruses

Many outbreaks of adenoviral inclusion body hepatitis in broilers have been shown to be associated with prior infection of the birds with IBDV, and bursal atrophy was found in these birds (Rosenberger *et al.*, 1975). Simultaneous infection of chickens with IBDV and another immunosuppressive virus in poultry, *Reovirus*, produced severe bursal and intestinal lesions (Moradian *et al.*, 1990; Springer *et al.*, 1983). Narita et al (Narita *et al.*, 1991) have reported clinical signs of *Picornavirus* infection in SPF chickens, which caused nephritis in the birds only when they were co-infected with IBDV

Interaction of IBDV with Bacteria, Fungus and Protozoal

Many bacteria, fungi and protozoa are opportunist pathogens in chickens. Immunosuppression due to IBDV infection usually enhances the effects of infection with these agents, thus making the diagnosis of the actual cause(s) much more difficult in the field. Lucio and Hitchner (Lucio & Hitchner, 1980) found that infection with IBDV suppressed the immune response to *Salmonella pullorum* antigen in chicks of 4 weeks old or younger. Similarly, Wyeth (Wyeth, 1975) found chicks infected with IBDV at 3 weeks of age were significantly more susceptible to infection with *E.coli*. IBDV-infected, 7-day-old chicks that were experimentally infected with a highly virulent strain of *E.coli,* had a marked increase in mortality up to 90% compared with only 40% mortality in chicks infected with *E.coli* alone (Nakamura *et al.*, 1990). Clostridial gangrenous dermatitis was found to be associated with IBDV infection and was prevalent in poultry flocks during the early 1970s (O'Brien, 1976). IBDV infection also reduces the protection level of live coccidia (*Eimeria tenella*) vaccination, giving higher *Eimeria tenella* lesion scores following challenge (Anderson *et al.*, 1977; Giambrone *et al.*, 1977). Rare clinical signs and economic losses due to

Cryptosporidiosis and Aspergillosis were reported to be associated with IBDV infection (Levy *et al.*, 1988; Okoye *et al.*, 1991).

1.9 ANTIGENIC AND GENETIC VARIATION OF IBDV

McNulty et al first isolated IBDV from a natural infection of turkeys in Northern Ireland (McNulty *et al.*, 1979). Later, distinct serotypes from fowl, turkeys and ducks were identified and grouped into two serotypes (serotype 1 and 2) using neutralisation tests (McFerran *et al.*, 1980b). Serotype 1 and 2, although distinct by virus-neutralisation test, could not be distinguished by fluorescent antibody tests or polyclonal antibody ELISA (Snyder *et al.*, 1992). Both serotypes 1 and 2 could be isolated from chickens and turkeys and both chickens and turkeys seroconverted to each serotype when infected (Ismail *et al.*, 1988; Jackwood & Saif, 1983; Jackwood *et al.*, 1984). Cross protection studies showed that immunisation against serotype 2 does not protect against serotype 1 IBDV challenge (Saif, & Moorhead, 1988; Jackwood *et al.*, 1985). Serotype 2 IBDV does not cause any clinical disease in chickens or turkeys and serotype 1 is pathogenic in chickens but not in turkeys (Saif, & Moorhead, 1988; Jackwood *et al.*, 1985).

The earliest report of antigenic variation within the serotype 1 IBDV was from McFerran et al (McFerran *et al.*,1980b). He demonstrated several field isolates showed only a 30% cross reaction with the vaccine strain used in an area by using cross-neutralisation tests. It was hypothesised that this might explain the apparent failure of the vaccine to protect the birds. A few years later, Saif (Saif & Swayne, 1998) reported an isolate of serotype 1, designated Md strain, which was obtained from 7-day-old broilers with bursal lesions and low mortality, despite the presence of high levels of maternal antibody to IBDV. Virus-neutralisation tests indicated the Md strain was antigenically distinct from several vaccine and field strains of IBDV. Later in 1985, Rosenberger and Cloud isolated four serotype 1 viruses that also differed antigenically from standard IBDV strains (Rosenberger & Cloud, 1985). Since 1985, more IBDV isolates were shown to be antigenically different from classical strains in the poultry industry in the United States. Researchers recognised that antigenic drift was occurring in field strains and the clinical signs observed were due to the emergence of so called

"variant" strains (to differentiate them from the "classic" IBDV strains isolated before 1985). The new variant strains of IBDV serotype I were isolated from vaccinated flocks that had high levels of maternal antibodies to IBDV. They caused rapid bursal atrophy without an inflammatory reaction in the bursa, no significant clinical signs and were highly immunosuppressive. Sustained economic losses due to the emergence of these variant strains prompted more studies into the antigenicity of variant strains of the virus.

1.9.1 Various Antigenic and Genetic Diversity Studies of IBDV

Numerous methods have been applied to classify and differentiate the emerging variant strains from the classical serotype 1 IBDV. Knowledge about emerging strains and advance warning about antigenic shift in the field could improve diagnostic and immunisation programmes in the affected flocks to prevent further economic loss. Below is a summary of various studies on the antigenic and genetic diversity of the virus.

Virus-Neutralisation Test

Cross-protection studies have shown that immunisation with classic IBDV strains only offered a low degree of protection against the variant strains, however, vaccination with variant strains of IBDV serotype 1 protected the birds against variant strains as well as classical serotype I strains (Ismail & Saif, 1990; Ismail *et al.*, 1990; Kibenge & Dhillon, 1987; Rosenberger & Cloud, 1986). The variant strains were able to escape neutralising antibody induced by classic IBDV strains (Ismail & Saif, 1991; Rosenberger & Cloud, 1986). Using virus-neutralisation (VN) test, studies to determine the relatedness of the isolates from the variant strains with several vaccine and field strains of IBDV isolated before 1985, revealed six antigenic subtypes of IBDV serotype 1 (Jackwood & Saif, 1987). The VN test distinguished the variant strains in the study into one subtype, distinct from the four subtypes of classic serotype 1 and one group of serotype 2 IBDV.

Neutralising Monoclonal Antibody Tests

Snyder et al (Marel *et al.*, 1990; Snyder *et al.*, 1988a; Snyder *et al.*, 1988b) reported using a battery of monoclonal antibodies (MCAs) against strains of IBDV to analyse the antigenic differences among classic and variant serotype I IBDV strains. The panel of MCAs was used in antigen-capture enzyme immunoassays (Jiang, *et al.*, 1996) to examine the antigenicity of wild-type IBDV isolates directly from diseased bursal

tissues. Using two neutralising MCAs, the neutralisation sites expressed on the VP2 viral protein were found to differ between the classic and variant strains. The MCAs designated as R63 and B69 neutralised IBDV to a high titre in VN tests; R63 MCA neutralised nearly equally six strains of serotype 1 and one strain of serotype 2, however, B69 MCA showed almost complete absence of any neutralisation against variant strains in the study. The deletion or alteration of one of the two neutralisation sites found on the classic serotype 1 strains demonstrated the occurrence of antigenic shift in new field isolates in USA (Sellers *et al.*, 1999; Snyder *et al.*,1988b). A further study in Australia using MCAs in additive/competitive ELISAs also supported the hypothesis that there are at least two non-overlapping neutralising epitopes on VP2 viral protein (Fahey *et al.*, 1991).

RT-PCR and Restriction Fragment Length Polymorphism Analysis

Restriction endonuclease (RE) digestion of genomic RNA or cDNA and analysis of restriction fragment length polymorphisms (Kwon. *et al.*, 1999) for differentiating subtypes of a virus is a widely accepted research method. Various restriction enzymes were tested for their ability to digest the RT/PCR product amplified from genomes of different IBDV strains (Dybing & Jackwood, 1996; Dybing & Jackwood, 1998; Hassan *et al.*, 1996; Jackwood *et al.*, 1997; Jackwood & Sommer, 1998; Sellers *et al.*, 1999; Ture *et al.*, 1998). Initial results indicated that the three variant strains in the study could be differentiated from classic IBDV strains (Jackwood & Jackwood, 1994). In a later study, two restriction enzymes, *BstNI* and *Mbol,* were found useful in the RFLP assay on a RT-PCR-amplified fraction of the VP2 gene. With this RFLP assay, thirteen vaccine viruses and five IBDV field strains, which were previously characterised antigenically as serotype 1 were placed into five molecular groups. Two groups contained viruses described as being classic strains, two groups contained viruses described as being variant strains and the fifth group contained both classic and variant strains. Some field isolates in the study were distinguished from each other and did not fit into any of the five molecular groups (Jackwood & Sommer, 1997). A new molecular group in the RFLP assay designated as group 6 was later reported. Within the molecular group 6, there was a subset of viruses that had a *SspI* RE site that was suspected to be a virulence marker (Jackwood & Sommer, 1999).

Although RFLP assay has been used successfully to classify numerous viruses into distinctive genetic subtypes (Brake & Studdert, 1985; 1991; Hamelin *et al.*, 1986; Maeda *et al.*, 1995) and particularly to discriminate between virulent and avirulent strains, this is not the case with IBDV. This may be due to the fact that the cleavage sites with the enzymes used do not identify critical mutation points in the viral protein. Also it may be that there are other viral proteins beside the VP2 protein responsible for the antigenicity and pathogenicity of IBDV (Yehuda *et al.*, 1999).

RT-PCR and Comparison of Hypervariable Region

Nucleotide sequencing of genomic RNA or cDNA and phylogenetic analysis are commonly used techniques for classifying subtypes of viruses within the same family and are accepted as tools for viral taxonomy. Numerous studies have compared the hypervariable region of the VP2 genome, which is one of the major antigenic regions of IBDV, to identify the molecular difference between variant and classic serotype 1 IBDV strains (Cao *et al.*, 1998; Dormitorio *et al.*, 1997; Eterradossi *et al.*, 1998; Lin *et al.*, 1993; Pitcovski *et al.*, 1998). It is hypothesised that the variable domain, with at least three independent epitopes within the domain, is responsible for the induction of neutralising antibodies. A change in the amino acid residues in the variable domain may lead the virus to escape neutralisation by MCAs (Fahey *et al.*, 1991; Sellers *et al.*,1999; Synder *et al.*, 1994). A more detailed review on the molecular variation of IBDV, especially the VP2 protein, is included in Chapter 5, section 5.1.

1.9.2 IBDV Classification and Terminology

The classification and differentiation among classical, very virulent (vv) and variant strains of serotype 1 IBDV is not clearly defined. Most researchers at present can only differentiate virulence of IBDV by the *in vivo* challenge test on SPF birds or by the mortality caused in the field. The definition of the serotype 1 IBDV strains described below will be used in this thesis to avoid confusion with the different terminology used in different publications:

Attenuated or low virulent IBDV strains: Defined as serotype 1 attenuated IBDV strains, which do not cause mortality in SPF or conventional chickens. However, some attenuated strains still cause histological bursal lesions, which vary from mild to

moderate depending on the attenuation of the particular strain by commercial biological production. No clinical signs are observed in birds infected with these strains. Examples of this classification are PBG98 (UK) and Cu1 (Germany).

Classical virulent strains: Describes all IBDV serotype 1 strains isolated before the early 80's that caused up to 50% mortality in SPF birds but only 1-2% in commercial chickens. Classical attenuated IBDV vaccine strains provide good protection against these IBDV strains. Examples of this classification are STC (USA) and UK 52/70.

Very virulent or hypervirulent strains: Describes all IBDV serotype 1 strains isolated from the 80's until now, which cause up to 100% mortality in SPF birds, 20 to 25% in broilers and 50 to 60% in layers. Classical attenuated IBDV vaccine strains only provide partial protection against these IBDV strains. Examples of IBDV strains in this classification are Holland DV86, UK661 and Japan 90-11 strains.

Variant strains: Describes all new IBDV serotype 1 strains found in the USA that cause less than 5% mortality in SPF or conventional chickens and which are able to escape neutralisation by antibodies induced by vaccination with classical serotype 1 vaccines. Although there is no increase in mortality of the birds, severe immunosuppression and rapid bursal atrophy without signs of clinical disease is characteristic of infection by these strains. Examples of IBDV strains in this classification are Variant A, E/DEL and GLS strains.

1.10 AIM AND SCOPE OF THE THESIS

The first part of the research in this thesis was to develop a sensitive and accurate detection method for the field investigation and early detection of IBDV infection in poultry flocks in New Zealand. Using this detection method, a longitudinal study of IBDV infection in the field was carried out to investigate the time of infection, the gross and histological lesions in infected birds, and the economic impact of the infection, and to compare different detection methods.

The second part of the thesis focuses on the characterisation of the IBDV isolates in New Zealand by *in vivo* challenge in SPF birds and by molecular and phylogenetic analysis to determine the possible origin of the virus in this country.

The third major part of the thesis attempted to investigate the immunogenic site of IBDV by X-ray crystallography, which has not been reported to date. With the preliminary setting-up of the IBD viral crystals and recombinant VP2 capsid protein crystals, collection of x-ray diffraction data and computation analysis, the three-dimensional structure of the capsid structural protein of the virus and its subunit major capsid protein -VP2 could be revealed. The aim was to disclose the structure of the epitope within the capsid protein responsible for the antigenicity of the virus. A review of the literature specific to this study is included in Part II Introduction chapter.

CHAPTER 2: ISOLATION OF IBDV AND DETECTION OF IBDV INFECTION IN CHICKENS IN NEW ZEALAND

2.1 INTRODUCTION

Since the first description of IBD in the 1960s (Cosgrove, 1962), the virus has been shown to be highly prevalent among poultry flocks world wide (Lukert *et al.*, 1991). However, IBDV had not been isolated from New Zealand chickens nor had there been any clinical disease outbreaks recorded. A number of serological surveys for IBDV were carried out between 1980 and 1992 in New Zealand poultry flocks. Serum samples were tested against IBDV using agar gel immunodiffusion test (AGDP), and all samples from 1975 to 1981 (560 samples tested) (Howell *et al.*, 1982), 1984 (1793 samples tested) (With, 1985), 1985(1784 samples tested) (Jones, 1986) and 1991 (1057 samples tested) (Howell & Stanislawek, 1992) were negative. This indicated that New Zealand poultry were free of IBDV infection and IBD was classified as a notifiable disease in the country.

The presence of IBDV in New Zealand poultry was suspected in November 1993 when acute bursal inflammation in meat chickens was observed during processing (Thompson, 1994). A serological survey was carried out by the poultry industry in the following month, within at least one age group on each of 344 farms (80% of major meat and egg production farms in New Zealand). A total of 22 farms (5%) were found to be positive and 14 (3%) had suspicious reactions (Anonymous, 1996). The virus was successfully isolated and confirmed to be IBDV at the Central Animal Health Laboratory, Wallaceville (Motha, 1996). Serotyping and pathogenicity tests were conducted by the Central Veterinary Laboratory, Adlestone, UK and the IBDV isolates from New Zealand were characterised as apathogenic strains of IBDV serotype 1. However they caused bursal damage and immunosuppression in SPF chickens equal to that caused by the UK reference strain 52/70 (Motha, 1996).

Other studies on IBDV infection in New Zealand suggested that the virus was less contagious (Christensen, 1999) and genetically different from overseas strains (Tham *et al.*, 1995). A field trial was carried out (Christensen, 1999) in which susceptible 2-week-old chickens were placed in contact with serologically positive 50-week-old layers on a farm. Serological tests (AGDP) on sera from the susceptible birds at 4, 5, 6 and 7 weeks of age were negative. A reverse transcription-polymerase chain reaction amplification assay (Tham *et al.*, 1995) and comparison of the deduced amino acid sequence with overseas IBDV strains suggested that the New Zealand IBDV isolates were genetically different from overseas strains in the analytical study.

Although no compulsory eradication programme was implemented by the poultry meat and egg industries after the positive finding in 1993, all infected farms were advised on the restriction of movement of live birds for sale other than for slaughter, and cleaning and disinfecting procedures were recommended. Routine serological testing of birds at slaughter for IBDV antibody was carried out from 1993 to the present time. By 1996, 10 farms remained serologically positive out of 847 farms tested. In 1997, 22 farms tested positive; 8 farms were positive in 1998 and 1 farm remained positive in 1999 (Ryan *et al.*, 2000).

This chapter describes studies on farms that remained serologically positive in 1997 and 1998. IBDV was isolated for further study. Several methods including histological examination, immunocytochemical detection of viral antigens, serological tests, electron microscopy and PCR test were used to detect the presence of the IBD virus.

2.2 MATERIALS AND METHODS

2.2.1 Collection of Samples

Farms that had tested positive following serological testing by the Poultry Veterinary Services laboratory (Auckland) were selected for this study. The farms were located in an isolated poultry farming area near Foxton in the Manawatu area. Five to ten birds of the same age were randomly selected from each shed from both the broiler and layer flocks for virus isolation and other studies reported in this chapter, as shown in Table 2.1.

2.2.2 Blood Samples

Blood was collected from each bird via wing vein venipuncture using a 2.5 mL syringe. Blood was transferred to a clean test tube and allowed to clot for 1 hour at room temperature, then centrifuged at 100 x g for 10 minutes and the serum removed and labelled according to the bird's identity number.

2.2.3 Tissues and Organs

After blood collection, all birds were killed by stunning and neck dislocation. The whole bursa and spleen, and part of the liver were sterilely collected from the bird. The body weight, bursal and spleen weights were determined with an electronic weighing scale. The fresh bursa, spleen and liver samples were cut into two equal halves; half was fixed in 10% v/v neutral formalin solution for histological examination and the other half kept in a sterile container, and stored at –70 °C until processed for virus isolation.

2.2.4 Serology

ELISA Test for IBDV Antibody

A commercially available, direct enzyme immunoassay kit (IDEXX, USA) was used for the detection of antibody to IBDV. The preparation of sera and test procedures were performed according to the manufacturer's instructions. The wells of the microtitre plate were coated with IBDV viral antigen. Test serum (100 µL of 1:500 dilution in sample diluent) was added in duplicate and incubated for 30 minutes at room temperature. After washing away the unbound material from the well with deionized water five times, 100 µL of a goat anti-chicken horseradish peroxidase conjugate was added. Unbound conjugate was washed as above and enzyme substrate (3,3',5,5'-tetramethylbenzidine, TMB) added. After adding 100 µL of stop solution to each well, the plate was read in an ELISA plate reader (340 ATC, SLT Lab Instrument) at absorbance value of 650 nm. Positive and negative control sera were included in duplicate on each plate and for the assay to be considered valid, the difference between the positive control mean (PCx) and the negative control mean (NCx) had to be greater

than 0.075, and NCx had to be less than or equal to 0.150. The relative concentration of antibody in the tested sera was determined by calculating the sample to positive ratio (S/P) as below:

S/P Ratio = (tested sample mean – NCx) / (PCx – NCx)

Sera with a S/P ratio of less than or equal to 0.2 were considered negative, and those with a S/P ratio greater than 0.2 considered positive. Endpoint titres of all sera were calculated according to the manufacturer's instruction using the equation below:

Log_{10} titre = 1.09 (Log_{10} S/P) + 3.36

Agar Gel Immunodiffusion/Precipitation Test (AGDP) for IBDV Antibody

To prepare the agar gel test plates for the AGDP test, 6 to 7 mL of molten test agar containing 0.9% agarose (Type I: low EEO, SIGMA) and 8.5% NaCl was poured into 60 mm diameter petri dishes, and the agar set at room temperature in a laminar flow hood. A hexagonal pattern of wells consisting of 1 central and 6 peripheral wells (5 mm in diameter) was cut in each agar plate and the agar removed from each well by aspiration. The test procedure was performed as follows:

1. The central well was filled with 50 μL of prepared IBDV antigen as described in 2.2.7.
2. One of the peripheral wells was filled with positive control antiserum against IBDV (kindly supplied by Dr. W. Stanislawek, MAF, Wallaceville).
3. The remaining peripheral wells were filled with 50 μL test sera.
4. The agar plate was covered and incubated for 48 hours at room temperature in a moist chamber.
5. The test results were read at 6, 24 and 48 hours, using a strong light source from the rear with a black background.
6. The result of the tested sera was interpreted as positive when a clear precipitin line formed a "line of identity" with that of the positive control antiserum with the antigen in the central well. A negative result of the tested sera was read when no lines of precipitin were observed. The test was repeated when no clear precipitin

line formed at the positive control well or when a suspicious reaction at the tested serum well was observed

2.2.5 Macroscopic and Histological Examination

All birds were examined for clinical signs of illness prior to euthanasia. At necropsy, the birds were examined for gross abnormal lesions such as bursal hypertrophy or atrophy, inflammation or haemorrhage on the bursal mucosa.

Bursa, spleen and liver tissues were fixed in 10% v/v neutral buffered formalin, routinely processed and embedded in paraffin. Sections were cut and stained with haematoxylin and eosin (H&E) and examined microscopically for histological changes.

2.2.6 Virus Isolation

Primary Propagation in SPF Eggs

SPF eggs

All SPF eggs were supplied from MAF Qual Laboratories, Wallaceville, New Zealand. The parent flocks were tested seronegative to 12 serotypes of *Avian adenovirus, Avian aenemia virus*, *Marek's disease virus*, Avian l*eukosis virus*, *Avian influenza virus*, *Newcastle disease virus*, *Avian encephalomyelitis virus, Infectious laryngotracheitis virus, Infectious bursal disease virus,* A*vian reovirus*, *Reticuloendotheliosis virus* as well as *Mycoplasma gallisepticum*, *Mycoplasma synoviae* and *Salmonella pullorum* and *Salmonella gallinarum*.

Inoculum preparation

All frozen bursal tissues from birds of the same flock and same age were pooled together and homogenized with a Tenbroek grinder in Tryptose Phosphate broth (TPB) (Appendix D) with antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) to make a 20% w/v tissue homogenate. The tissue homogenates were frozen and thawed

three times before centrifugation at 1,500 x g for 20 minutes at 4 °C. The supernatant was then aseptically removed into sterile vial for egg inoculation.

Virus inoculation and harvest

A set of six 11-day-old embryonating SPF eggs was used for inoculation of the tissue homogenate as prepared above. Each egg was inoculated with 0.3 mL of inoculum via the chorio-allantoic membrane route. The details of the procedure were described elsewhere (Senne *et al.*, 1989). All inoculated eggs were candled once a day to check for viability. Any embryo that died within the first 24 hours after inoculation was regarded as a non-specific death. All inoculated eggs were further observed for 7 days. All embryo deaths beyond 24 hours and within the observation period were collected and any live embryo was killed by refrigeration for 4-5 hours after 7 days. Bursa, spleen and liver from each embryo were harvested and examined for lesions. If no IBDV-related lesions were found, the harvested embryo tissues were homogenised as above and used as the inoculum for a second passage in SPF eggs. Each initial inoculum was passaged one to three times in SPF eggs before passage into a continuous cell line.

Primary Cells and Cell Lines

Cell culture

Primary chicken embryo fibroblast cells (CEF) and continuous Vero cell lines were used for propagation of the IBDV isolates from virus propagated in SPF eggs. Primary CEF were prepared from 9- to 11-day-old SPF chicken embryos as described below and Vero cells were available from the cell culture collection in our laboratory, with cell passage number between 120 and 150. Standard laboratory procedures were applied for the propagation and maintenance of cell cultures, and for isolation and propagation of viruses (Freshney *et al.*, 1994; Lennette *et al.*, 1988).

Media

The growth medium (GM) consisted of minimal essential medium with non-essential amino acids (MEM+n, Sigma) supplemented with 10% v/v foetal bovine serum (FBS) and 1% v/v antibiotic solution-PSK (Appendix D). Maintenance medium (MM) was the

same as GM, except that it contained only 2% v/v FBS. For cultivation of primary CEF cells, the GM was supplemented with 10% TPB.

Preparation of primary chickens embryo fibroblast (CEF)cell culture

Nine- to 11-day-old embryonating eggs were used for the preparation of primary CEF cell culture and procedures were followed as described by Purchase and Schat (1989):

1. The egg shell surface was disinfected with tincture of iodine. Using sterile technique, the shell was opened and the embryo removed with forceps.
2. The embryo was placed on petri dish and the head, limbs and viscera were removed.
3. The body was transferred to beaker containing phosphate buffered saline (PBS) and minced with sterile scissors. The minced tissues were washed with PBS 3 to 4 times to remove red blood cells.
4. The minced tissues were poured into trypsinisation flask containing magnetic stirring bar. About 50 mL pre-warmed (37 °C) trypsin solution (0.25%) was added to flask and put on a stir plate at slow speed for 10 – 15 minutes.
5. The trypsinised suspension was poured through two layer of wire mesh into a beaker. The supernatant was transferred into a large centrifuge tube containing 5% FBS (v/v) and placed on ice for 5 minutes.
6. The supernatant was centrifugated for 10 minutes at about 350 x g. The supernatant containing the trypsin solution was discarded.
7. One hundred mL of GM was added for each mL of packed cells. The cell pellet was resuspended by gently repeated pipetting.
8. The number of viable cells and density were determined by hemocytometer using trypan blue dye exclusion method (see section 7.2.1).
9. The cells were seeded at 1 to 2 x 10^6 cells/mL for stationary cultures and 4 x 10^6 for roller bottles.

Subculturing of Vero cell culture

The Vero cells were routinely subcultured twice weekly. The monolayers were washed twice with warm PBS, pH 7.0 and cells were detached from the flask with antibiotic-trypsin-versene solution (ATV) (Appendix D), incubated at 37 °C for 5 to 10 minutes. Detached cells were resuspended in an appropriate volume of GM. A split ratio of 1:3 to 1:5 was used for subculturing the cells and reseeding into new flasks. GM was added

to make a cell count of 1-5 x 10^5 cells per mL. Cell cultures were maintained at 37 °C in a 5% CO_2 incubator.

Cryopreservation and recovery of cells

For freezing of the Vero cell line, cells were multiplied in large flasks (Nunc, 180 cm^2), harvested as above and resuspended in 10 mL of GM, counted and pelleted by centrifugation at 300 x g for 10 minutes. The cell pellet was resuspended in freezing medium containing 20% v/v FBS in GM and 10% v/v dimethyl sulphoxide (DMSO, Sigma) at 2-5 x 10^7 cells per mL. Aliquots, 1 mL each, were delivered into 1.7 mL cryo-vials (Nunc) and cooled in a freezing container (Nalgene) at –70 °C overnight, then transferred to liquid nitrogen for long term storage. To resuscitate frozen cells, the cells were thawed and transferred to a tissue culture flask. An appropriate volume of warm GM was added and the cells were incubated at 37 °C in a 5% CO_2 incubator.

Virus Inoculation and Subculturing

Virus stock prepared from the infected embryo tissue homogenate was used to inoculate cell cultures. Once the primary CEF cells or Vero cells had grown to more than 80% confluency, the GM was discarded and the cell monolayer washed twice with warm PBS. One mL of the embryo tissue homogenate was inoculated onto the cell culture, adsorbed in the incubator for 1 hour and the virus inoculum then discarded before adding MM. Incubation continued as previously described.

The inoculated cell cultures were observed for cytopathic effect (CPE) at least once daily for 7 to 9 days. After typical CPE appeared, the cell cultures were freeze-thawed three times and 1 mL of the virus suspension was transferred to a new, 80% confluent monolayer. The samples were considered negative if no CPE was observed after six passages. Electron microscopy, immunocytochemistry staining of fixed cells and PCR were used for confirmation of the presence of IBDV.

For multiplication of the IBDV in cell culture, 10 to 20 mL of the positive virus suspension was inoculated onto Vero cells in a large tissue culture flask (Nunc, 175 cm^2) or large roller bottle (1750 cm^2), and incubated until more than 80% CPE appeared. The CPE positive cell cultures were harvested, frozen and thawed three times, the cells and medium collected, and the cell debris pelleted by centrifugation at

500 x g for 15 minutes. The supernatant was collected and stored at –70 °C for further propagation and other tests.

2.2.7 Electron Microscopy Examination

The pooled bursal and spleen tissue homogenate and infected cell cultures were examined by electron microscopy for the presence of IBDV or other viruses. Tissue homogenates prepared as described in section 2.2.6 were clarified at 500 x g for 15 minutes. The supernatant was filtered through a 0.2 μm syringe filter, then ultracentrifuged and prepared as for the cell culture sample as follows.

Infected cell cultures showing more than 80% CPE were frozen and thawed three times and clarified by centrifugation at 500 x g for 10 minutes. The supernatant was ultracentrifuged at 100,000 x g for 2 hours over a 1 ml cushion of 25% w/v sucrose. The pellets were resuspended in 100 μL of distilled H_2O (dH_2O) overnight at 4 °C. The soaked pellets were dissolved by repeated pipetting up and down through a narrow pipette tip. The viral suspensions were negatively stained on formvar-coated 400 mesh copper grids. First the grids were wetted by placing coating side down onto a 50 μL drop of 1% bovine serum albumin for 10 seconds. Then they were placed on a 20 μL drop of virus sample on the coating side for 40 seconds, washed on a drop of dH_2O for 10 seconds and lastly stained with 4% phosphotungstic acid, pH 7.0 for 40 seconds. Between each transfer, excess fluid was blotted away on a filter paper and the grid was thoroughly dried before placing in a grid holder. The stained grids were examined in a Philips 201C transmission electron microscope.

2.2.8 Immunocytochemistry Staining of Fixed Infected Cells

Cells for immunocytochemistry staining were grown in eight-well chamber slides (Tissue Tek, Nunc). When cells reached more than 80% confluence, they were inoculated with 100 μL of virus suspension. After 3 to 5 days incubation at 37 °C in 5% CO_2 incubator, the cells were fixed with 20% acetone, and stained with the immunoperoxidase staining procedure described in chapter 3. The stained slides were sealed with coverslips and examined under a light microscope for any positive staining.

2.2.9 Polymerase Chain Reaction

Preparation of Samples for PCR

The IBDV isolates from the infected farm that were successfully propagated in the Vero cell cultures described in section 2.2.4 were used as antigen preparation and samples for PCR. When 80% CPE were observed in the inoculated Vero cell culture (except for flock E where no CPE was observed after six passages), the infected cells were disrupted by freezing and thawing three times, and the cell lysate and medium was clarified by centrifugation at 500 x g for 10 minutes. The supernatants were ultracentrifuged at 100,000 x g for 2 hours over a 1 mL cushion of 25% w/v sucrose. The pellets were resuspended in 100 μL of dH_2O overnight at 4 °C. The soaked pellets were dissolved by repeated pipetting up and down through a narrow pipette tip, and used for RNA extraction. For flock E, the inoculated cell cultures were harvested at the same time post inoculation as those cultures that showed CPE.

RNA Extraction from Samples

A 250 μL sample of the concentrated virus suspension prepared as above was used for extraction of the viral RNA for detection and confirmation of virus propagation in the cell line. TRIZOL LS reagent (Gibco BRL) was used for isolation of total RNA from the virus stock suspension according to the manufacturer's instructions. Briefly, 0.75 mL of TRIZOL LS reagent was added to the 250 μL sample in an eppendrof tube. After mixing and incubating for 5 minutes at 15 to 30 °C, 0.2 mL of chloroform was added per 0.75 mL of reagent used. The tube was capped and shaken vigorously by hand for 15 seconds and incubated at 15 to 30 °C for 15 minutes. The tube was centrifuged at 12,000 x g for 15 minutes at 4 °C. Following centrifugation, the colourless upper aqueous phase was carefully removed and transferred to a clean tube. Five hundred μL of isoprophyl alcohol was added and the tube incubated at 15 to 30 °C for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4 °C. The supernatant was removed and the RNA pellet washed once with 1 mL of 75% ethanol and centrifuged at 7,500 x g for 5 minutes at 4 °C. The ethanol supernatant was removed and the RNA pellet briefly air-dried. Fifty μL of DEPC-treated dH_2O was added to dissolve the RNA pellet. The concentration of RNA was determined by a GeneQuant RNA/DNA Calculator (Pharmarcia Biotech).

RT- PCR Reaction

Based on the published nucleotide sequence of the IBDV genome segment A of the STC strain (Kibenge *et al.*, 1990), a pair of oligonucleotide primers was designed to amplify a 555 bp fragment of the VP2 gene containing the hypervariable region at bases 662 to 1157. The primers were as follows:

Primer Forward 5'-TGAGACTTGGTGACCCCATAC-3'
Primer Reverse 5'- CGACGGATCCTGTTGCCACTC-3'

A one step RT-PCR was performed using Titan One Tube RT-PCR system kit (Roche) and a Perkin Elmer 9600 thermocycler (Perkin-Elmer Cetus, Norwalk, CT, USA), with a total reaction mix volume of 50 μL, containing 1 μL of sample RNA, 1 μL of enzyme mix (Taq and Pwo DNA polymerase and AMV reverse transcriptase), 10 μL of 5 x RT-PCR buffer, 2 μL of 25 mM $MgCl_2$, 2.5 μL of DTT-solution, 2 μL of 5 mM dNTP, 2 μL of each primer (10 μM) and 27.5 μL of dH_2O.

The RT reactions were carried out at 50 °C for 30 min. The PCR reactions consisted of an initial denaturation for 2 min at 94 °C, followed by 10 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and elongation at 68 °C for 45 sec, followed by 25 cycles with the same denaturation and annealing conditions but with 5 sec added to the elongation step in each successive cycle and a final elongation at 68 °C for 7 min.

Analysis of Amplified cDNA

Ten μL of the RT-PCR product was subjected to electrophoresis through a 1.5% ethidium bromide stained agarose gel (Gibco, BRL) in TBE buffer (Appendix D) at 100 volts for 40 to 60 minutes. The gels were visualised under UV light and photographed using Polaroid black and white photographic film (Polaroid 667). All samples were run alongside in the gel with a molecular size marker (*fX174* RF DNA/*Hae III* fragments, Gibco BRL) as reference.

As no positive control sample was available for reference in the RT-PCR at the time of the test, samples were considered positive if products of correct size (555bp) were visible on the gel. Confirmation of the product was performed by sequencing the product and aligning the nucleotide sequence with reported IBDV sequences in the EMBL data bank using the BLAST programme (Altschul *et al.*, 1990).

Sequencing

The cDNA was purified from the agarose gels with QIAquick Gel Extraction kit (QIAGEN, Germany) and sequenced on an ABI Prism 377 DNA sequencer using Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer).

2.3 RESULTS

2.3.1 Farm History and Collection of Samples

During the visit and selection of farms available in the area, birds were randomly sampled from farms according to the flock housing at the time of the visit. Five to ten birds were taken from different flocks at various ages as shown in Table 2.1. Some additional birds were sent to our laboratory by the farm owner for testing for IBDV infection. No significant clinical signs were observed in the IBDV infected farms. Thus, random selection of the birds was used to pick up 5 to 10 chickens from the flock at each visit. Birds' production parameters such as feed consumption, feed conversion rate (FCR), daily weight gain and mortality, and egg production were satisfactory according to the standard of the breed.

2.3.2 Serological Diagnosis

The results of serological tests of the 62 blood samples from broiler and layer birds are shown in Table 2.1. Sixteen blood samples were positive in ELISA test and in AGDP. If considered at flock level, 4 of the investigated flocks were positive in both serology tests and 5 flocks were negative in the tests.

2.3.3 Pathology

Necropsy of the birds showed that some birds had mild atrophy of the bursa, the bursa sizes and weight of the birds were variable and unevenly distributed within the same age. Occasionally, haemorrhage of the bursal mucosa could be found as seen in Figure 2.1(B).

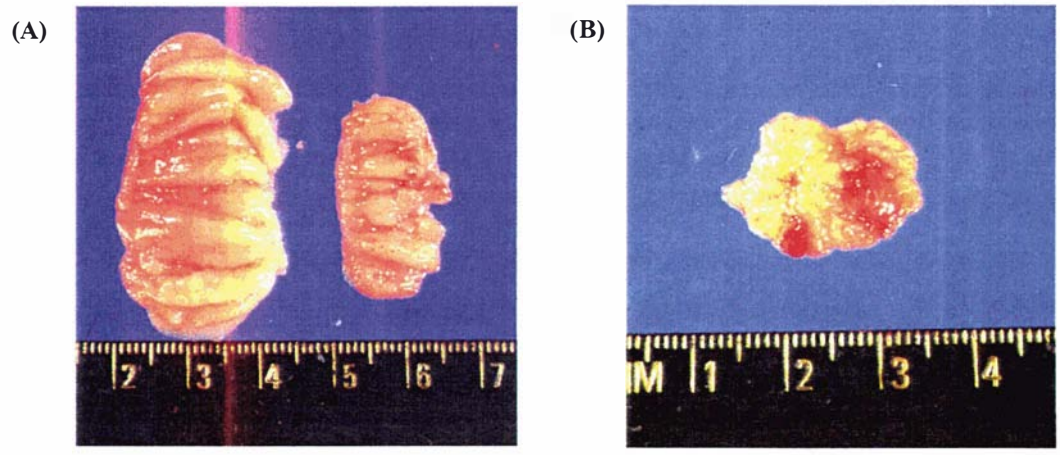


Figure 2.1: (A) Atrophied bursa from a bird in flock B (right), a normal bursa from a bird of the same age is shown on the left for comparison. (B) Bursa from a bird in flock D, several haemorrhages are evident on the bursal mucosa.

Histological examination of tissue sections showed there was some depletion and necrosis of lymphocytes in the bursa of chickens at various ages. The lesions varied from mild lymphocyte depletion from the follicles to severe lesions with pyknotic cells, infiltration of heterophils, hyperplasia of reticuloendothelial cells and fibrosis of follicles in some birds (see Figure 2.2).

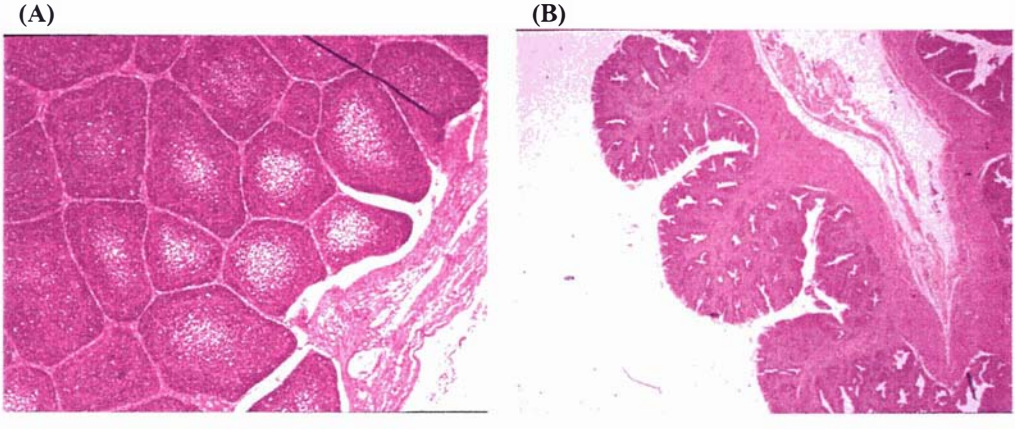


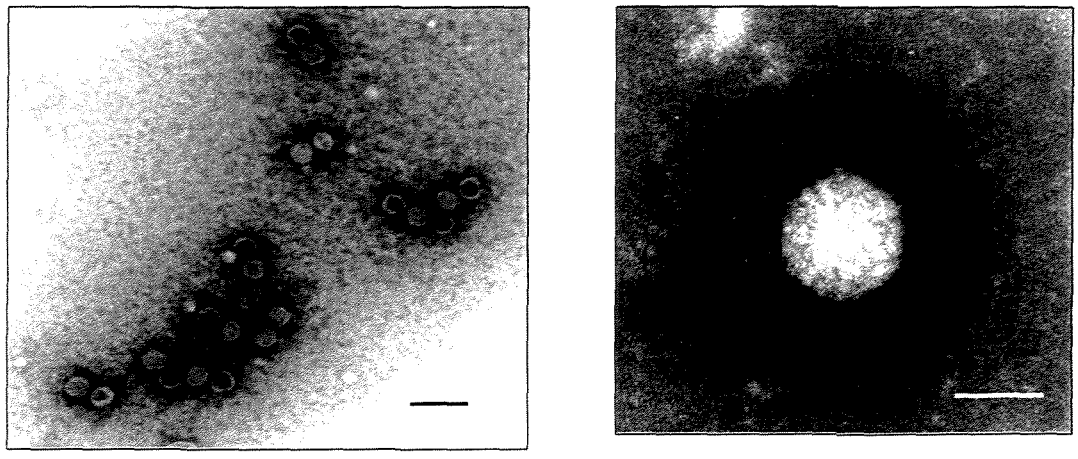
Figure 2.2: (A) Section of bursa from a bird in flock B showing loss of lymphocytes in the medulla of the follicles, H&E, x 40. (B) Section of bursa from a bird in flock D. There is follicular fibrosis and total depletion of lymphocytes from the bursal follicles, H&E, x 10.

2.3.4 Virus Isolation

The results of virus isolation from different flocks of birds are presented in Table 2.1. Viruses were isolated from pooled samples from 8 of the 9 flocks. They caused lesions in chick embryos in the first to second passage and CPE in cell culture after the third to fourth passage. Samples from only one flock (Flock E) failed to yield any virus after three passages in SPF eggs. Further examination of the viruses by EM revealed that seven isolates morphologically resembled IBDV and one isolate was a reovirus-like virus (see Figure 2.3 and 2.4). The CPE of this isolate was quite different to that of the other seven isolates. The early CPE showed formation of syncytial cells rather than rounded and detached cells as seen in the other seven isolates. RT-PCR confirmed that seven EM-positive isolates were IBDV. The reovirus-like isolate was negative on RT-PCR.

2.3.5 Electron Microscopy Examination

No virus was observed from the bursal tissue homogenate preparations under EM but was detected by EM examination of the viruses isolated and propagated in cell-culture, and prepared as described in section 2.2.7. The negatively stained EM of the virus isolates shown in Figure 2.3 (a) and (b) have the morphology of naked icosahedral virus with approximate diameter of 60 to 65 nm.



(a) (b)

Figure 2.3: Negatively stained IBDV particles. The virus was isolated (from flock H) in chick embryos and passaged in Vero cells. (a) x 12,000, bar represents 100 nm. (b) x 100,000, bar represents 50 nm.

Reovirus-like particles were found in one of the isolates that showed a different type of CPE. The negatively stained EM of the virus particle showed an outer coat with spikes and diameter of approximately 75 nm (Figure 2.4).

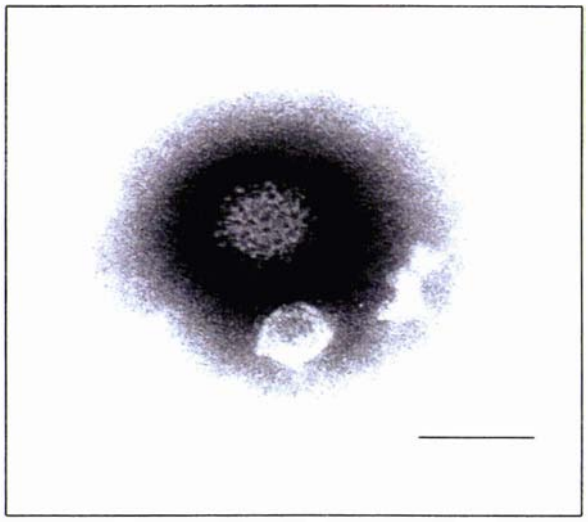


Figure 2.4: Negatively stained reovirus-like particle. The virus was isolated (from flock H) in chick embryos and passaged in Vero cells. x 50, 000, bar represents 100 nm.

2.3.6 Immunocytochemistry Staining of Fixed Infected Cells

The cultures of cells containing the seven isolates that were positive by EM and RT-PCR were positive in immunocytochemistry staining. IBDV antigens in the cytoplasm of the cells were detected by the staining as a reddish-brown precipitate as shown in Figure 2.5 (A). The cell culture that had a different CPE and in which a reovirus-like virus was identified gave a negative result. All negative control cells showed negative results.

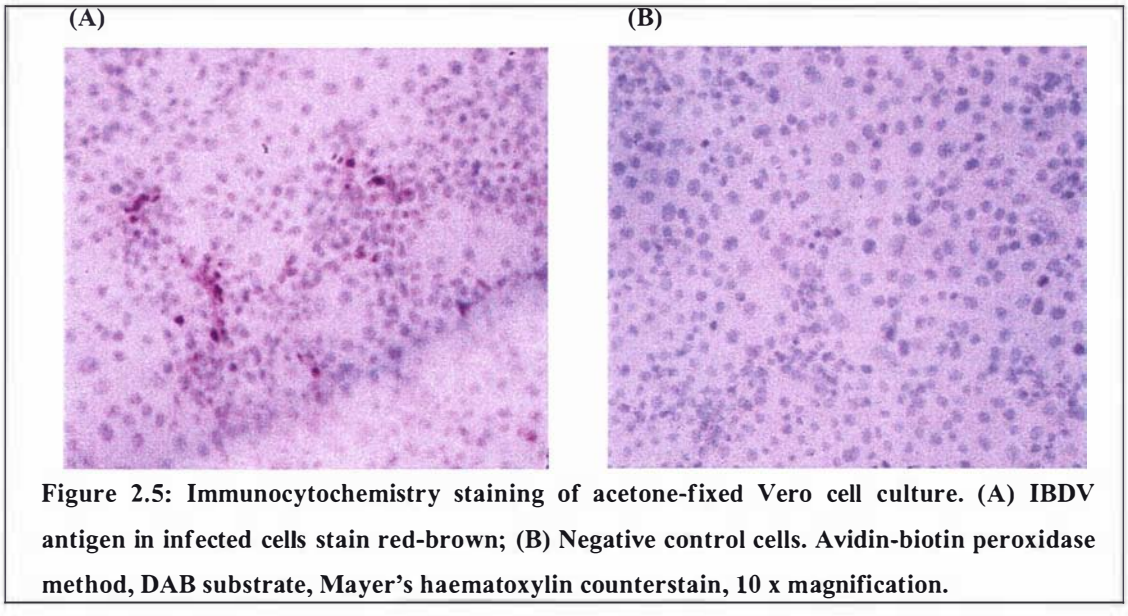


Figure 2.5: Immunocytochemistry staining of acetone-fixed Vero cell culture. (A) IBDV antigen in infected cells stain red-brown; (B) Negative control cells. Avidin-biotin peroxidase method, DAB substrate, Mayer's haematoxylin counterstain, 10 x magnification.

2.3.7 RT-PCR Detection of Isolates

As summarised in Table 2.1, viral RNA of seven isolates from seven different flocks was successfully amplified in RT-PCR and a product of predicted size (555bp) was demonstrated as shown in Figure 2.6.

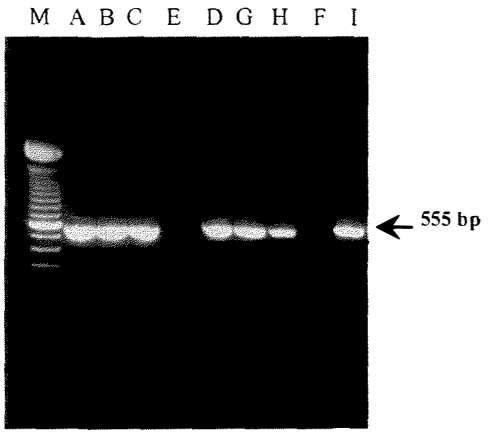


Figure 2.6:. Amplification products of RT-PCR performed on Vero cell culture supernatants and lysates. Lane M: 100 bp DNA ladder (Gibco BRL); Lane 2 to 10: Flock A to H as indicated. Expected product is 555bp.

2.3.8 Sequencing of cDNA and Sequence Comparison

All amplified cDNA products of 555 bp were sequenced and edited with Sequencer 3.1 Programme (Gene Codes Corporation). Comparison of the 555bp sequences from the seven isolates showed a high degree of identity (98 to 100%) among the isolates. The consensus sequences were analysed with the BLAST programme, which showed the amplified product had high identity scores (92 to 98%) with published IBDV sequences, confirming that the isolates were IBDV. Further analysis of this sequence data is reported in Chapter 5.

Table 2.1: Summary of results in serological, virus isolation, EM and RT-PCR tests.

Flock ID.	Type of Bird	Age (days)	ELISA Test[a]	AGPT[a]	Virus Isolation	EM Examination[b]	RT-PCR[b]
A	Broiler	7	0/5	0/5	Positive	IBDV	Positive
B	Broiler	20	0/5	0/5	Positive	IBDV	Positive
C	Broiler	34	0/5	0/5	Positive	IBDV	Positive
D	Broiler	42	10/10	10/10	Positive	IBDV	Positive
E	Layer	47	0/5	0/5	Negative	Negative	Negative
F	Broiler	11	2/10	2/10	Positive	Reovirus-like	Negative
G	Broiler	14	1/10	1/10	Positive	IBDV	Positive
H	Broiler	18	3/11	3/11	Positive	IBDV	Positive
I	Layer	13	0/5	0/5	Positive	IBDV	Positive

a: Number positive / number tested.

b: Test was performed on cell-culture adapted isolate.

2.4 DISCUSSION

2.4.1 IBDV Isolation and Detection

From the random sampling of birds from various farms in the study area, IBDV was isolated from seven of the nine flocks. However, both the ELISA and AGPT serological tests revealed only four flocks were seropositive for IBDV infection. Interestingly, broiler birds of older age (flock D) showed 100% seroconversion to the IBDV infection, but broiler birds of younger age showed a lower percentage of seroconversion (flocks G and H). The seropositive result in Flock F is likely due to the transfer of maternal antibodies and it appeared that no actual IBDV field infection occurred in the birds. This is supported by the negative results obtained by virus isolation. Flock G and H had a low percentage of seropositive birds. The seropositive results in these flocks may be due to maternal antibody or seroconversion to field challenge. Various studies have shown maternal antibody declines to non-detectable concentrations by 2 to 3 weeks of age (Ide *et al.*, 1978; Lucio & Hitchner, 1979; Muskett *et al.*, 1979). It was not determined which seropositive birds were positive in the virus isolation test because pooled samples were used. However IBDV infection was shown to have occurred in these flocks.

Four flocks (A, B C and I) were seronegative but were positive by virus isolation. As discussed in section 1.5.6, AGDP proved to be relatively insensitive for detection of low concentration of IBDV antibody (van den Berg & Meulemans, 1991) and ELISA required at least 15 – 20 individual bird samples to establish a mean flock titre to IBDV. The ELISA used in this study is not suitable for evaluating the IBDV antibody titre in single birds and was not designed to detect IBDV infection in individual birds (Kreider *et al.*, 1991; Thayer *et al.*, 1987). If the ELISA test has to be used for detecting IBDV infection in flocks or on farms (rather than individual bird), the sensitivity and specificity of the test should be determined. Based on the known or estimated IBDV antibody prevalence in the flocks, a suitable sample size could then be calculated to detect disease prevalence in the population with a predetermined, statistically valid, level of confidence. A detailed discussion and comparison of serology screening using ELISA and other tests is presented in chapter 4, section 4.4.3.

Confirmation of IBDV infection in the birds was successfully demonstrated by virus isolation, confirmed by EM, immunocytochemistry and RT-PCR detection methods. In contrast to other reports, EM and RT-PCR methods in this study could not be successfully applied directly to samples collected from infected birds. It was necessary to multiply the virus in SPF embryonating eggs and later to adapt to cell culture. Efforts to perform RT-PCR amplification on clinical samples such as bursal tissues were not successful. The RT-PCR amplifications were either inhibited or non-specific amplification occurred (data not shown). Various other studies have reported amplification of cDNA of IBDV by RT-PCR from clinical samples (Berg *et al.*, 1996; Chen *et al.*, 1998; Jackwood *et al.*, 1996; Lin *et al.*, 1993), and have demonstrated the virus by electron microscopy from infected bursa (Harkness *et al.*, 1975). It is probable that the failure in RT-PCR amplification and EM detection from tissue samples in this study was due to the low virus concentration in the collected samples. The virus was of low virulence in our subclinically infected birds compared to other studies, which were performed on highly pathogenic strains and severely affected birds. Initial propagation in SPF eggs by CAM inoculation is a sensitive route for isolation of IBDV (Hitchner, 1970) and was necessary to raise the concentration of virus for detection by PCR or EM examination.

Early confirmation of viral growth in SPF eggs should be carried out because the embryonic lesions caused by IBDV inoculation in SPF eggs are not specific. Immunocytochemical staining of the histological sections of affected embryonic liver, spleen and bursa can provide specific identification of IBDV antigen in these tissues but was not evaluated in this present study.

Adaptation of isolates, which have been propagated in SPF eggs, to Vero cell culture was done by 3 to 5 blind passages. The low passage number required for adaptation in Vero cells is consistent with these IBDV isolates being of low virulence. Numerous studies on adaptation of IBDV field strains into continuous cell line have found a common trend that virulent IBDV strains are difficult to grow in continuous cell lines of non-chicken origin (Jackwood *et al.*, 1987; Kibenge *et al.*, 1988; McFerran *et al.*, 1980). Some virulent strains may need to be grown in chicken origin cell lines before they can be propagated in continuous mammalian cell-lines (Lee & Lukert, 1986).

However, this observation is not sufficient to determine the pathogenicity of the NZ IBDV isolates. Further characterisation of the pathogenicity of these isolates is discussed in Chapter 5. The CPE caused by IBDV is not specific. The infected cells appear rounded, with granulation around the nuclei and with cells detached from the substrate (Jackwood *et al.*, 1987; Kibenge & McKenna, 1992). Some other poultry viruses exhibit CPE in Vero cells, such as the reovirus-like virus that was isolated from Flock F in this study. Further confirmation such as by EM, RT-PCR and immunocytochemistry staining are required to confirm the diagnosis.

2.4.2 Detection of Infection with Other Viruses

As mentioned earlier, a reovirus-like virus was the only virus isolated other than IBDV from the studied flocks. *Avian reovirus* is a common infection in chickens of all ages and types and has been associated with viral arthritis, malabsorption or enteritis. Usually chickens are protected from clinical signs by vaccinating breeder birds. No live *Reovirus* vaccine was used in these farms, so the reovirus-like virus that was isolated was most probably a natural infection and no related clinical signs were observed in the studied flocks.

Although *Chicken anaemia virus* (CAA) was not isolated in this study, serological tests with an ELISA against CAA showed some of the birds in some flocks were seropositive (tests done by Dr. W. Stanislawek, MAF, Wallaceville). Two flocks of birds at age 7 and 42 days were positive to CAA in this study, so the possibility that CAA had an effect on immune competence in birds infected with IBDV or vice versa can not be ignored. The adverse interaction between IBDV and CAA has been well documented by many researchers(Cloud *et al.*, 1992a; Cloud *et al.*, 1992b; Otaki *et al.*, 1989; Rosenberger & Cloud, 1989; Yuasa *et al.*, 1980), and there is evidence that prior infection with IBDV could cause immunosuppression in the birds and enhance the pathogenicity of CAA and increase its prevalence in the flock.

Other common viruses such as *Infectious bronchitis virus* (IBV), *Marek's disease virus*, *Avian adenovirus*, which could be found in NZ poultry flocks (Howell *et al.*, 1982), were not detected in our study. The cell culture conditions were not optimal for growth of other viruses. For example, primary isolation of IBV usually requires chicken-origin tracheal organ culture; mammalian cell lines do not the favour the growth of IBV.

Marek's disease virus only grows in primary cultures of chicken kidney cells (Calnek *et al.*, 1991). Secondly, since we were aiming to isolate IBDV in this study, the specimens collected were bursa, spleen and liver, which are the primary target organs of IBDV replication. Some other poultry viruses have different target organs for isolation, therefore they would have been excluded from our study simply due to the samples collected.

2.4.3 Significance of Subclinical IBDV Infection

In this study, we isolated and detected IBDV in seven flocks of birds. However, no clinical signs of IBDV infection were observed or reported during the study period. The weekly mortality rate and feed conversion rate in both broiler and layer flocks were within the normal range. The prevalence of IBDV in these flocks could not be determined from this study because of the small number of samples collected from each flock. The age of infected birds ranged from 7-day-old to near slaughter age in broiler birds, so it was worth considering the potential effect of IBDV infection on the occurrence of other diseases during the production cycle. Discussion with the flock owners concerning the history of previous flocks revealed that outbreaks of airsacculitis and colisepticaemia, and uneven slaughter weights had occurred. The immunosuppressive effect of subclinical IBDV infection and its significance in economic loss have been discussed by various authors (Allan *et al.*, 1972; Hirai *et al.*, 1974; Rosenberger & Gelb, 1978). Most studies have shown that the significant economic losses due to immunosuppressive effects were mainly non-responsiveness to live attenuated respiratory vaccines (Newcastle disease and Infectious bronchitis) and subsequent outbreaks of respiratory diseases (Rosenberger & Gelb, 1978). Fortunately, most major avian respiratory diseases are not common in New Zealand poultry flocks; in particular the country is free from *Newcastle disease virus*. It is therefore likely that the immunosuppressive effect of subclinical IBDV infection in NZ poultry flocks would be different from the impact reported overseas.

Economic loss from subclinical IBDV infection could not be demonstrated in this study, but in the report of Christensen (Christensen, 1999), it was predicted that the potential cost of losses due to an accidental introduction of clinical IBDV infection into NZ would to be about US$10 million per year. A survey in Ireland reported 14%

reduction in financial profit from broiler flocks with subclinical IBDV infection compared with unaffected flocks (McIlroy *et al.*, 1989). In that survey, no significant difference was found in mortality of the affected flocks compared with non-exposed flocks. The major reduction in profit was attributed to the reduction of slaughter weight and feed conversion efficiency in serologically positive birds.

The New Zealand poultry industry decided to embark on a programme of eradication of subclinical IBD from NZ poultry flocks after the initial discovery of the virus in 1993. The additional cost to the NZ poultry industry to eradicate the virus from the country and to be able to declare IBDV freedom, through implementation of an IBDV serological monitoring system, decontamination of infected farms and preventive measures, was estimated conservatively at NZ$8 million (Dr. Clifton King, MAF, pers comm).

2.5 SUMMARY

IBDV was successfully isolated from seven flocks of chickens. No clinical signs of IBDV infection were observed in the flocks. Subclinical IBDV infection was widely distributed in broiler chickens of all ages and one layer flock was exposed to the virus during an early age. IBDV was isolated, in SPF embryonating eggs, from birds with no serological evidence of infection, adapted to cell culture and detected by EM, immunocytochemistry and RT-PCR test.

CHAPTER 3: EVALUATION OF IMMUNOCYTOCHEMISTRY, SEROLOGY AND HISTOLOGICAL DIAGNOSTIC METHODS FOR THE DETECTION OF INFECTIOUS BURSAL DISEASE VIRUS INFECTION IN BROILER FLOCKS IN NEW ZEALAND

3.1 INTRODUCTION

The diagnosis of IBDV infection can be made using a variety of methods (Lukert *et al.*, 1991). If the disease is acute and severe, the diagnosis can often be made on clinical signs and gross pathology alone. Microscopic examination of bursal tissues may reveal characteristic lesions primarily in the lymphoid organs – cloacal bursa, spleen, thymus, harderian gland and caecal tonsil (Jackwood & Saif, 1987; Lukert *et al.*, 1991). Serological assays such as AGPT, ELISA and virus neutralisation test are used for the detection of IBDV antibodies (Box *et al.*, 1988; Chin *et al.*, 1984; Cullen & Wyeth, 1975; Kreider *et al.*, 1991; Thayer *et al.*, 1987). Confirmation and identification of the agent can be achieved by isolation and propagation of IBDV in SPF embryonating eggs and cell culture (Hitchner, 1970). Various cell lines have proved to be successful for growing certain strains of IBDV (Kibenge *et al.*, 1988; Lukert *et al.*, 1975). Several blind passages may be required until CPE is observed, but reports show some IBDV field strains failed to grow in primary or continuous cells (Lee & Lukert, 1986; McFerran *et al.*, 1980). Direct examination by electron microscopy has proved to be useful for early detection in some cases (McFerran *et al.*, 1978). Identification of the virus by direct immunofluorescent staining or immunoenzyme staining of the affected organs has been reported in various papers and these methods have been valuable for early detection and identification of IBDV (Chai *et al.*, 1999; Cho *et al.*, 1987; Cruz-Coy *et al.*, 1993a; Tanimura *et al.*, 1995).

With the method of macroscopic and histologic examination of the affected organs, differential diagnosis must be made between infection with other common immunosuppressive agents such as *Chicken anaemia virus* (Yuasa *et al.*, 1980), *Marek's disease* (Cho, 1970), *Infectious bronchitis* (Pejkovski *et al.*, 1979) and *Avian adenovirus* (Rosenberger *et al.*, 1975). Additionally, the emergence of variant strains of serotype 1 IBDV, which are characterised by sub-clinical infection but which lead to immunosuppression with other consequences of infection are increasingly recognised (Rosenberger & Cloud, 1985). Therefore adjunct methods to distinguish IBDV infection must be applied.

Serological tests for detection of IBDV antibodies have been used extensively in the poultry industry for monitoring the disease and vaccination status of flocks. Commercially available ELISA kits have been designed for several important diseases in chickens and are user friendly and have acceptable sensitivity and specificity. However, with IBDV infection it has been shown that antibodies are not detectable in the AGP test until 7 to 10 days after infection (Cho *et al.*, 1987; Ley *et al.*, 1983; Takase *et al.*, 1993; Tanimura *et al.*, 1995). Some ELISA tests may achieve higher sensitivity and specificity but the cut-off value for interpretation (i.e. positive / negative discrimination) used in all ELISA kits are designed for average flock seroconversion evaluation. Therefore these ELISAs are not suitable as tools to demonstrate freedom from infection in a eradication programme. Overall, serological tests are not suitable for early detection and identification of IBDV infection in chickens, especially when there is infection with IBDV strains of low pathogenicity and there is a low prevalence level of positive birds. Negative results of the serological tests under these conditions have low predictive value and create problems in deciding whether or not the flock is infected.

The most frequently used technique for confirmation of diagnosis is virus isolation in cell culture (Kibenge *et al.*, 1988; Lukert *et al.*, 1975), but this technique is not sufficiently sensitive for IBDV since some strains of IBDV do not grow in cell culture and the CPE is not easily differentiated from that of other viruses. Also, it may require several days or even weeks before the cultures can confidently be classed as negative. There are some diagnostic methods that could offer detection and identification of the

causative agent as early as a few hours post-infection. These include direct immunohistochemistry to demonstrate the viral antigens by immunofluorescence or immunoenzyme staining of frozen or fixed tissue sections (Cho *et al.*, 1987; Tanimura *et al.*, 1995). Both of the methods are rapid and specific, but the frozen section technique has poor preservation of histological detail and interpretation of the presence of virus in infected tissues is rather subjective. Immunohistochemical staining of formalin-fixed tissues provides the advantages of ability to detect viral antigens in parallel with histological examination, accuracy of identification of the presence and location of viral antigen, and the ability to perform retrospective study on histological specimens.

The avidin-biotin-peroxidase complex (ABC) method has proven to be a sensitive and specific immunohistochemical staining technique that can be performed on frozen and formalin-fixed tissues (Hsu *et al.*, 1981a; Hsu *et al.*, 1981b). In this chapter, development of an ABC immunocytochemical staining method on formalin-fixed bursal tissues of chickens to detected IBDV antigens is described. The ABC method was compared with the serological methods, ELISA and AGPT, for detection of early infection of IBDV in suspected broiler farms. The need for parallel testing using these methods in the IBDV eradication programme was demonstrated.

3.2 MATERIALS AND METHODS

3.2.1 Sample Collection

Retrospective Study

Bursal tissue samples had been submitted in 1997, before the commencement of this study to the Poultry Laboratory, Department of Veterinary Pathology and Public Health, Massey University, from two broiler farms (Farm A and B) suspected to be infected with IBDV. Serum samples from birds on these farms had given negative results when tested in AGDP. Bursal tissues from 5 birds from a 45-day-old flock on farm A (flock A1) and from 3 birds from a 49-day-old flock on farm B (flock B1) had been collected, fixed in formalin and embedded in paraffin blocks. All paraffin-embedded bursal tissues from these birds were recalled for routine histopathological sectioning, staining with

Haematoxylin and Eosin, and examined microscopically. Unstained sections also were cut for immunocytochemical staining as described in 3.2.4.

Field Investigation

In 1998, Farms A and B were revisited and serum and tissue samples collected from various ages of birds as shown in Table 3-1. One single age of 47-day-old birds was sampled from Farm A (flock A2), but several successive samplings were done on Farm B from birds in the same flock ranging in age from 7 to 42 days (flock B2).

3.2.2 Serology Test

All serum samples from Farms A and B submitted in 1997 were tested for IBDV antibody with AGP test as described in section 2.2.4. All serum samples collected in 1998 were tested with a commercially available ELISA kit and an AGDP test for IBDV antibodies as mentioned in section 2.2.4.

3.2.3 Histopathology

All bursal tissue samples were fixed in 10% neutral buffered formalin, dehydrated in graded ethanol, cleared with xylene and infiltrated and embedded in paraffin. Embedded tissues were sectioned and stained with Haematoxylin and Eosin. Sections were examined and scored for IBD lesions using a scale of 0 – 4 as described by Cruz-Coy et al (Cruz-Coy *et al.*, 1993a). Briefly, scoring was as follows:

0 = no lesions

1 = a few necrotic lymphoid cells

2 = numerous necrotic lymphoid cells

3 = numerous necrotic lymphoid follicles and an increase in inflammation and connective tissue between follicles

4 = lymphoid follicles atrophied and cystic

3.2.4 Immnunocytochemical Staining on Formalin-Fixed Tissues

A monoclonal antibody (Mab) against the IBDV variant strain A, Mab G11 (Cruz-Coy *et al.*, 1993b) was used in the avidin-biotin-peroxidase complex (ABC) method (Wang *et al.*, 1995) as follows:

1. Formalin-fixed, paraffin-embedded tissues were sectioned at 5 μm thickness.
2. The cut sections were deparaffinised and hydrated through xylene and graded ethanol of 100%, 95%, 80% and 70% at room temperature (24 °C).
3. Hydrated sections were rinsed in distilled water and reacted with 3% hydrogen peroxidase in methanol for 15 minutes.
4. Sections were washed with phosphate buffered saline (PBS), pH 7.4 for 20 minutes.
5. Sections were incubated with 1% trypsin solution in 0.5% $CaCl_2$ for 30 minutes at 37 °C.
6. Sections were washed as in step 4 and blocked with 1% normal horse serum for 20 minutes.
7. Sections were blotted and incubated with 1:10 dilution of Mab G11 for 30 minutes in a humidified chamber at room temperature.
8. Step 4 was repeated and slides incubated with 1:200 dilution of anti-mouse biotinylated antibody (Vector Laboratories, USA) for 30 minutes.
9. Step 4 was repeated and slides were incubated with Vectastain ABC reagent (Vector Laboratories) for 30 minutes.
10. Step 4 was repeated and slides were incubated for 15 – 30 minutes in Diaminobenzidine tetrahydrochloride solution.
11. Slides were washed in tap water for 5 minutes.
12. Slides were counterstained with Mayer's stain solution, dehydrated through graded alcohols and xylene, and mounted.

All prepared sections were examined and scored for intensity of staining using a scale from 0 to 3 as described by Cruz-Coy et al (Cruz-Coy *et al.*,1993a). Briefly, scoring was as follows:

0 = no staining

1 = few lightly stained positive cells

2 = widely scattered positive stained cells

3 = numerous darkly stained cells

Samples to be used for Negative Control were collected from specific pathogen free (SPF) birds from the Central Animal Health Laboratory flock, Wallaceville, MAF, New Zealand. The Positive Control slides used in the immunocytochemistry staining procedure were the samples collected from a positive flock which was confirmed by RT-PCR test described in Chapter 2.

3.3 RESULTS

3.3.1 Serology

As shown in Table 3.1, all sera tested by AGDP from Farm A in 1997 were negative for IBDV antibodies, while sera from birds at Farm B in 1997 were positive. No ELISA test was done on these sera samples. Serum samples were tested from the same farms in 1998. From Farm B, no antibodies to IBDV were detected from birds aged 7 days to 34 days in either the AGDP or ELISA tests. However, when the birds were grown to 42-day-old, all birds had seroconverted to IBDV and were positive in both ELISA and AGDP tests. No IBDV antibodies were detected in sera of birds from Farm A in 1998 or of the SPF birds.

3.3.2 Histopathology

The bursal tissues from the broiler birds submitted in 1997 showed varying degrees of histopathological lesions, with scores ranging from 2 to 4 (Table 3.1). Typically, there was depletion and necrosis of lymphocytes, numerous pyknotic cells, infiltration of heterophils, hyperplasia of reticuloendothelial cells between the cortex and medulla, and an increase in the interfollicular connective tissue (Figure 3.1, Figure 3.2); The bursa of birds from Farm B collected in 1998 had a range of histology lesion scores from 0 to 4, with the lowest scores occurring in the youngest birds. There were no histological lesions observed in the bursa of birds from Farm A in 1998 or in SPF birds.

3.3.3 Immunocytochemical Staining

Tissues of birds from both farms (A & B) in the retrospective studies in 1997 showed a range of positive immunoperoxidase staining reaction, with scores from 1 to 3 (Table 3.1). There was generally an even distribution of dark-brown stained lymphoid cells within the bursal follicles (Figure 3.3, Figure 3.4). The most intensely stained cells were found around and within the histopathological lesions as described in 3.3.2. Samples from 1998 of Farm B were scored from 0 to 3. The 7-day-old birds had no positive stained cells, while all 20- to 42-day-old birds at Farm B had a moderate to large number of stained cells. The sections of birds from Farm A in 1998 and the SPF birds showed no positive staining cells (Table 3.1).

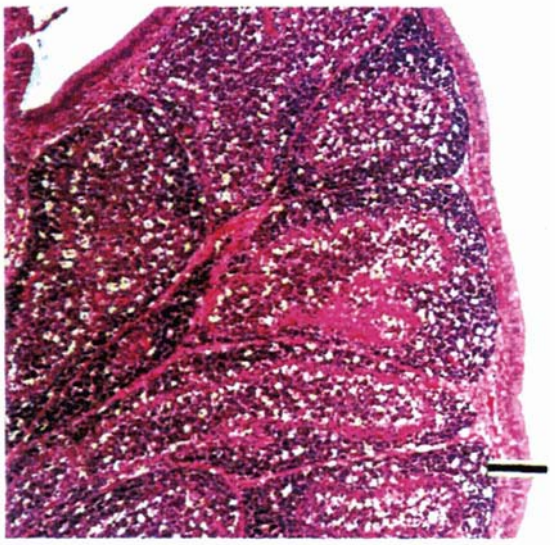


Figure 3.1: Section of bursa from bird A/11 which had a histological score of 2 showing depletion of lymphocytes in the medulla of bursal follicles, with prominent reticular endothelium between the cortex and medulla. H&E. Bar = 100 μm.

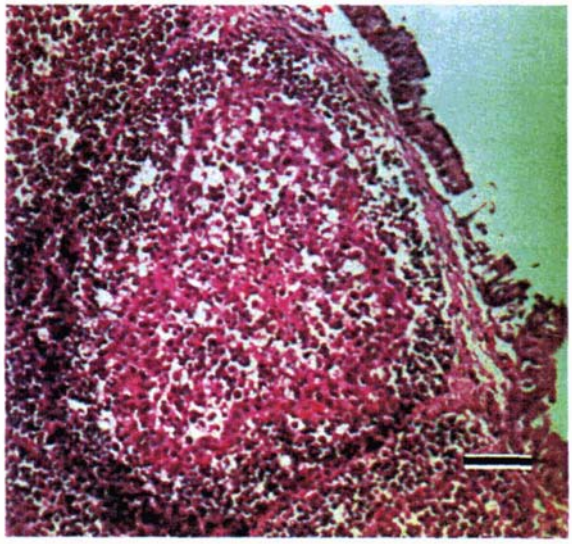


Figure 3.2: Section of bursa from bird B/28 which had a histological score of 3, showing extensive necrosis of lymphocytes and marked pyknotic cellular debris within the medulla of a follicle and hyperplastic reticuloendothelial cells. H&E. Bar = 50 μm.

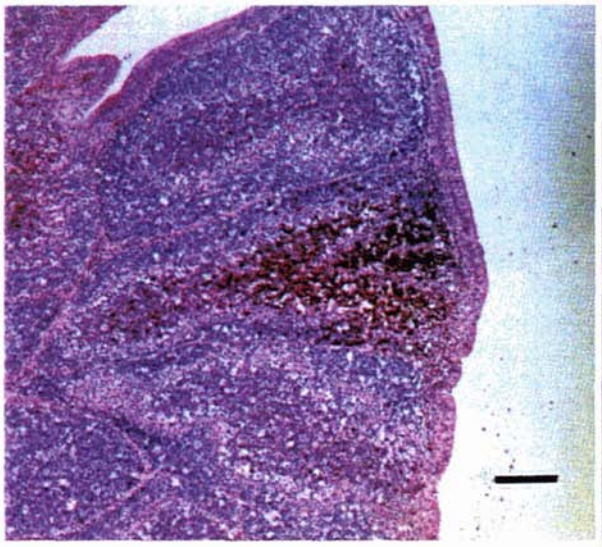


Figure 3.3: IBDV antigen in lymphoid cells in the bursal follicles shown in Figure 3.1 with an immunoperoxidase score of 2. Avidin-biotin peroxidase method, DAB substrate, Mayer's heamatoxylin counterstain. Bar = 100 μm.

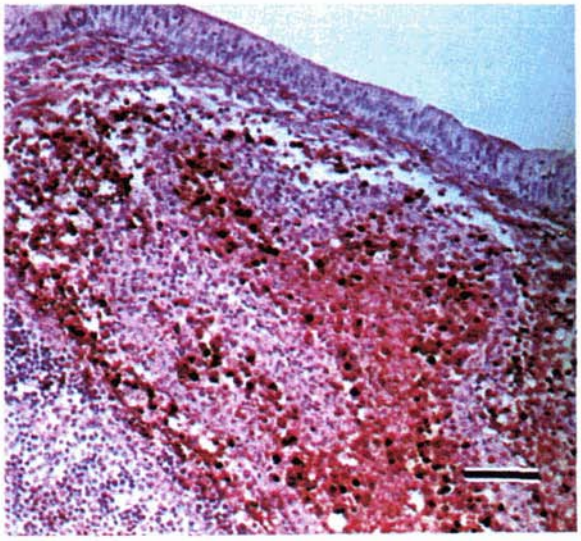


Figure 3.4: Higher magnification of bursal follicle from Figure 3.3. showing IBDV antigen in lymphocytes in the cortex and medulla. Avidin-biotin peroxidase method, DAB substrate, Mayer's heamatoxylin counterstain. Bar = 50 μm.

Table 3.1: Flock details, age, histological lesion score, immunoperoxidase staining score and serological results of chickens in the study.

Farm/ Flock No. (year)	Bird	Type, age of bird (days)	Histological lesion Score[a]	Immuno-peroxidase Score[b]	Serology[c] ELISA	AGP
A1	A/11	Broiler, 45	2	2	n.t.	-
(1997)	A/12	Broiler, 45	3	1	n.t.	-
	A/13	Broiler, 45	3	1	n.t.	-
	A/14	Broiler, 45	3	3	n.t.	-
	A/15	Broiler, 45	3	1	n.t.	-
B1	B/11	Broiler, 49	4	2	n.t.	+
(1997)	B/12	Broiler, 49	4	2	n.t.	+
	B/13	Broiler, 49	4	2	n.t.	+
A2	A/21	Broiler, 47	0	0	-	-
(1998)	A/22	Broiler, 47	0	0	-	-
	A/23	Broiler, 47	0	0	-	-
	A/24	Broiler, 47	0	0	-	-
B2	B/21	Broiler, 7	0	0	-	-
(1998)	B/22	Broiler, 7	1	0	-	-
	B/23	Broiler, 7	3	0	-	-
	B/24	Broiler, 20	4	3	-	-
	B/25	Broiler, 20	4	2	-	-
	B/26	Broiler, 34	4	2	-	-
	B/27	Broiler, 34	4	3	-	-
	B/28	Broiler, 42	3	3	+	+
	B/29	Broiler, 42	3	2	+	+
	B/210	Broiler, 42	3	3	+	+
SPF[d]	S104	SPF, 63	0	0	-	-
(1998)	S105	SPF, 63	0	0	-	-

a Histological scores from 0 to 4 (Cruz-Coy et al, 1993a). 0 = no lesions, 1 = a few necrotic lymphoid cells, 2 = numerous necrotic lymphoid cells, 3 = numerous necrotic lymphoid follicles and an increase in inflammation and connective tissue between follicles, 4 = atrophied and cystic lymphoid follicles.

b Immunoperoxidase scores from 0 to 3 (Cruz-Coy et al, 1993a). 0 = no staining, 1 = few lightly stained positive cells, 2 = widely scattered positive cells, 3 = numerous darkly stained cells.

c Detection of IBDV antibody, using IDEXX ELISA test kit and agar gel precipitation (AGP) test : - = negative, + = positive, n.t. = not tested.

d Specific-pathogen-free.

3.4 DISCUSSION

3.4.1 Retrospective Study

Immunocytochemical testing of samples from the suspected IBDV infected Farm A and serologically positive Farm B in 1997 confirmed that IBDV infection was present in the farms. Although histopathological lesions present in all the bursae of affected birds was suggestive of IBDV infection, the serological evidence was not consistent and did not support the diagnosis of IBD in Farm A, which was negative in the AGDP test. But positive AGDP results were obtained in Farm B. This highlights some of the problems associated with the diagnosis of acute, mild IBDV infection by histological and serological methods. The histological lesions of IBD are variable and depend on the strain of virus (Cheville, 1967; Helmboldt & Garner, 1964; Henry *et al.*, 1980; Kaufer, 1976). Highly pathogenic strains of IBDV cause characteristic bursal lesions, with almost total destruction of the bursa of Fabricius, often with severe haemorrhage (Cheville, 1967; Ley, Yamamoto, & Bickford, 1983). However, infection with mild to moderately pathogenic strains can result in lesions that may be difficult to differentiate from those caused by other agents (Jackwood & Saif, 1987; Lukert *et al.*, 1991). In older birds, the appearance of physiological atrophy of the bursa that occurs naturally with maturation of chickens could be confused with the bursal atrophy and lymphoid depletion caused by less pathogenic strains of IBDV (Glick, 1991; Glick, 1994).

The serological monitoring of birds and farms for IBDV infection by collecting blood samples at the processing plant has proved to be inadequate if used solely as the basis of the test and removal programme. As shown in the serological test results of Farm A in 1997, infected flocks could slip through the detection net. The failure of the birds to seroconvert by 45-days of age in Farm A may be due to infection late in the life of the birds so that they were sampled prior to the development of detectable antibody. Also a poor immune response to this mild strain of IBDV may have occurred or there may have been co-infection with other immunosuppressive agents. It has been demonstrated that in chickens infected with IBDV, antibodies are not detectable in the AGDP test until 7 – 10 days after inoculation (Cho *et al.*, 1987; Ley *et al.*, 1983; Takase *et al.*, 1993; Tanimura *et al.*, 1995). Also, the low sensitivity of the AGDP test must be taken

into consideration despite its high specificity (Box *et al.*, 1988; van den Berg & Meulemans, 1991; Wyeth & Chettle, 1982).

3.4.2 Field Investigation

The absence of any histopathological, serological or immunocytochemistry evidence of IBDV infection in the birds on Farm A in 1998 indicates that the flock had not been exposed to the virus despite the positive results in a flock on this farm the previous year in 1997. According to the farm history, a strict biosecurity programme had been adopted since the infection was identified in 1997. The farm had been completely depopulated and sheds cleaned thoroughly before re-population. The farm was also converted into an all-in-all-out single-age rearing system. This suggests that the eradication programme instituted on Farm A was effective and demonstrates that strict biosecurity measures can be successful in eradicating IBDV. However, since only a small number of birds were tested from this farm, it would be necessary to either test a much larger number at slaughter and/or to repeat testing of subsequent flocks to be certain that the farm was now free of infection. In any case testing of subsequent flocks would be advisable to check for any re-introduction of the virus onto the farm.

However, the results of the birds from Farm B were positive from 20 days of age onwards as demonstrated by immunocytochemistry, but were still negative for IBDV antibodies at 34 days of age. Seroconversion was not detected until the following time of sampling, at 42 days of age. Similar to the results of the retrospective study, the results of the Farm B study in 1998 demonstrated the problem of relying on serological testing in IBDV control programmes. The false negative serological results in IBD infected birds between 20 and 30 days of age on Farm B would allow infected birds to slip through any serological screening conducted by the farm at that time. As biosecurity measures applied to non-infected flocks are less stringent than those applied to infected flocks, false negative results may lead to serious risk of dissemination of virus through the industry.

The birds on Farm B in 1998 were seropositive at 42 days of age, which is before the common slaughter age of broiler chickens in New Zealand (45-day-old). Thus, this

flock would have been detected by serological testing at slaughter. However, if infection of the flock had occurred one week later, it is possible that the birds would not have seroconverted by 45 days of age, and would not have been detected at slaughter. Although it appears that infections of this flock occurred sometime between 7 and 20 days of age, the exact day is unknown. The route by which the virus entered this flock is also unknown. Therefore, it is clearly possible that other flocks on this farm or on other farms could become infected with IBDV at an age that would not allow time for seroconversion to occur before slaughter. This is presumably the situation that occurred in flock A1. Control programmes based on serological testing of birds at slaughter would not detect such flocks. A similar finding had been reported by Christensen (Christensen, 1999).

3.4.3 Comparison of the Three Diagnostic Methods

The immunocytochemistry test used in this study offered advantages over the other diagnostic methods. It was sensitive and specific, and infection could be detected by this method prior to positive serological results. Other authors have also found that immunocytochemistry was able to detect IBDV and other viral infections before either histopathology or serology (Cho *et al.*, 1987; Tanimura *et al.*, 1995). Additionally, viral antigen could be detected in formalin-fixed, paraffin-embedded tissue, allowing the convenience of a single sample submission and the ability to perform retrospective studies on stored paraffin blocks. The monoclonal antibody used in this study is able to detect both subtypes of IBDV, including the variant strains of serotype 1 (Cruz-Coy *et al.*, 1993b). Additionally, the IP staining method required only 4 hours to complete, after histological processing, enabling a rapid diagnosis to be made. This study clearly demonstrated the advantage of using immunocytochemistry for rapid and accurate diagnosis of IBDV infection in New Zealand.

3.5 SUMMARY

The evaluation of the three diagnostic methods for detecting IBDV infection in commercial poultry flocks in this study has demonstrated that serological testing is not a reliable method for the detection of IBDV infection in New Zealand broiler flocks because the antibodies may not have developed to detectable levels by the time of slaughter. Histological examination of bursae allowed the demonstration of IBD-like

lesions, but these need to be differentiated from those caused by other agents. The immunocytochemistry test was able to detect early IBDV infection. It provided a rapid, definitive diagnosis and may be useful in control programmes. It could be used in an eradication programme as a second test to confirm the classification of flocks that are presumed free of infection on the basis of a serological test at slaugther.

CHAPTER 4: LONGITUDINAL CASE STUDY OF A BROILER AND A LAYER FARM INFECTED WITH INFECTIOUS BURSAL DISEASE VIRUS

4.1 INTRODUCTION

By December 1993, one month after the discovery of the presence of IBDV in New Zealand poultry flocks (Thompson, 1994), serological surveys on most commercial layer, broiler and breeder flocks had confirmed IBDV infection on 32 layer and pullet rearing farms and 2 broiler farms. All parent breeding farms and small egg producer farms remained free of infection. As the IBDV isolate was confirmed to be a mild strain and given the large number of farms believed to be infected, the poultry meat and egg industries decided not to adopt an eradication programme for IBD, but to assist individual farmer, whose flocks were infected, to institute practical decontamination and control measures (Ryan *et al.*, 2000).

In the disease survey programme, all broiler farms were required to submit blood samples from the birds at slaughter (40 to 45 days of age) for IBDV antibody testing at the designated laboratory. Blood samples were collected from layer birds at different ages during the rearing period. However, an outbreak of IBD in the southern North Island in March 1997 showed that solely relying on serological evidence of infection as a basis for the eradication programme was inadequate (Christensen, 1999). We demonstrated (Chapter 3) that detectable seroconversion in infected birds could be delayed as late as 3 weeks (at 42 days old) after the first detection of viral antigen when birds were 20 days old.

Furthermore, the decision in 1995 to allow the vaccination of breeder flocks with inactivated IBDV vaccine confused the whole picture of serological monitoring and created the possibility of late infection in flocks due to presence of maternal antibodies against IBDV in day-old chicks. Vaccination of parent flocks achieved protection of

progeny from IBDV infection for the first two weeks of age. However, without secondary vaccination of the progeny with live vaccine and following an uneven decline of maternal antibodies, a proportion of the birds became susceptible to IBDV infection from environmental sources, as has been demonstrated in many studies (Berg & Meulemans, 1991; Eidson *et al.*, 1980; Naqi *et al.*, 1983; Wyeth, 1980; Wyeth & Cullen, 1978a).

In this chapter, a longitudinal case study of an IBDV-infected broiler flock and a layer flock is described. Serological testing for antibodies, histological examination for lesions and immunocytochemistry for detection of viral antigens were performed at various times to determine the age at which birds became infected.

4.2 MATERIALS AND METHODS

4.2.1 Study Design

A layer and a broiler farm were selected for longitudinal study. The broiler flock comprised 10,000 birds, housed in one of three sheds on a multiple age broiler-rearing farm. The farm had been tested positive serologically since 1997 and confirmed to be IBDV infected as described in the study in Chapter 3. The layer farm had 15,000 birds reared in layer cages from day 0 of age. The layer birds in the same house from the previous rearing period had tested positive against IBDV infection by ELISA serological tests.

Ten live birds reared in the broiler farm were selected at random from one of the sheds at 0, 4, 7, 11, 14, 18, 21, 28, 35 and 42 days of age and 5 live birds were sampled from the laying farm at 0, 6, 13, 20, 27, 34 and 41 days of age. Each bird was labelled with an ID number so individual test results could be related to each bird.

4.2.2 Farm Recording

All parameters recorded on the farms, such as feed consumption, feed conversion rate (FCR), weight gain, mortality rate and vaccination/medication treatment schedule were collected to monitor the performance of the birds and to detect any abnormality in the flocks.

4.2.3 Macroscopic and Histological Examination

All birds were examined for clinical signs of illness and necropsied for abnormal lesions such as bursal hypertrophy or atrophy and inflammation or haemorrhage on the bursal mucosa. Bursa and spleen weight of each bird were recorded and the means bursal to body weight (B/BW) ratio and spleen to body weight (S/BW) ratio were determined according to Hassan and Saif (Hassan & Saif, 1996), for indication of any significant change in size of the bursa or spleen following the calculation:

B/BW ratio = (Bursal weight, gram/ Body weight, gram) x 1000
S/BW ratio = (Spleen weight, gram/ Body weight, gram) x 1000

The means of the B/BW and S/BW ratios were calculated for each age group, and box plots were used to present the distribution of the bursal and spleen weight of the flock over the observation period (NCSS 2000 statistic programme).

Bursa, spleen and liver tissues fixed in 10% v/v neutral formalin were processed by routine histopathological sectioning, stained with Haematoxylin and Eosin and examined microscopically for histological changes. Histological scores, as described in section 3.2.3, were given for each bird. Mean histological lesion scores were calculated for each age group.

4.2.4 Serology Test

A commercially available indirect enzyme immunoassay kit (FlockChek IBD, IDEXX, USA) was used for the detection of antibody to IBDV in the collected sera. The preparation of sera, the test procedure and the calculation of antibody titre were performed as described in section 2.2.4. The interpretation of results was according to the manufacturer's instructions; sera with antibody titres greater than 396 should be considered as positive against IBDV.

4.2.5 Immunocytochemistry Staining on Formalin-Fixed Tissues

All tissue sections prepared for histological examination were also cut for immunocytochemical staining for IBDV antigen. Immunocytochemistry was performed and immunoperoxidase staining scores were given for each section, as described in section 3.2.4.

4.2.6 Disease Prevalence and Comparison of Diagnostic Tests

Using the data from Table 4.1, we determined the IBDV infection prevalence in the flock and evaluated the sensitivity, specificity, predictive value of the ELISA test in comparison to the immunocytochemistry test in this study (Win Episcope 2.0), and determined the sample sizes of both tests based on the efficacy and disease prevalence in this study (Survey Toolbox).

4.3 RESULTS

4.3.1 Clinical and Necropsy Findings

Broiler Flock

No significant daily mortality rate was observed in the broiler flock during the study period from day 0 to day 42 (slaughter age). Weekly weight gain was consistent with the breed standard. Average slaughter weight at 42 days of age was 2.1 kg.

Necropsy of the broiler birds did not reveal any significant gross lesions at any age. The bursal and spleen weights of birds at various ages are presented in Figure 4.1 and the mean ratios of B/BW and S/BW are presented in Table 4.1. The bursal and spleen weight of the broiler birds showed a stead y increase as the birds grew older but a greater variation in distribution of the bursal weight was observed from day 21 onward. As a result of the significant deviation of the bursal and spleen weight value in each age, there were no statistically significant differences of mean B/BW and S/BW ratios between the different age groups within the broiler birds.

Layer Flock

No significant mortality rate was observed in the layer flock during the study period; the average weekly mortality rate from day 0 to day 41 (0.1%) was below the expected mortality rate. However, following the period of study, the egg production of the flock from week 20 to 75 was just below the expected standard and there was a slight increase in the average weekly mortality rate from 35 to 75 weeks of age, with 1 to 2% above the expected mortality rate (data not shown).

Postmortem examination of layer birds did not reveal any significant lesion. The bursal and spleen weight are shown in Figure 4.1 and the mean B/BW and S/BW ratios are presented in Table 4.2. Both the bursal and spleen weight increased over the observation period. There were no significant differences between age groups in the B/BW and S/BW ratios of the layer birds from day 13 to 41 of age.

4.3.2 Serological Test

The ELISA antibody titres to IBDV of the broiler and layer flocks are shown in Figure 4.2. The number of seropositive birds and the mean ELISA titres at various ages are presented in Table 4.1 and 4.2.

The mean antibody titre profile of the broiler birds showed a concave curve from day 0 to day 42 as shown in Figure 4.2. The day-old birds had significant antibody against IBDV and this decreased as the flock grew older. The mean antibody titre fell below the cut-off value (titre = 396) of the ELISA test from days 7 to 35, but increased above the cut-off value at day 42 of age.

In the layer flock, all sera had values in the IBDV ELISA below the cut-off value from 1- to 27-day-old except for one bird that had a titre above the cut-off value at days 34 and another bird at day 41.

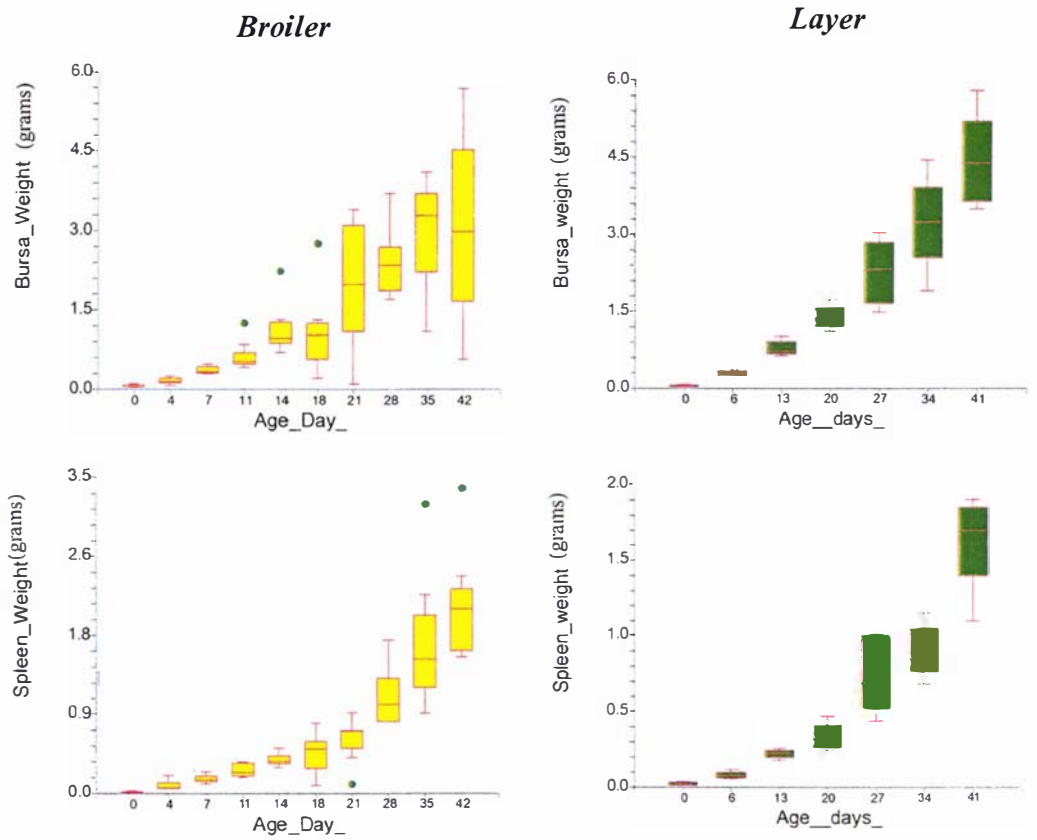


Figure 4.1: Box-plots showing the bursal (upper) and spleen (lower) weight of the broiler (left) and layer (right) flocks over the observation period. The rectangular box represented 50% distribution of the data within the indicated age group and 25% deviation on each side of the box.

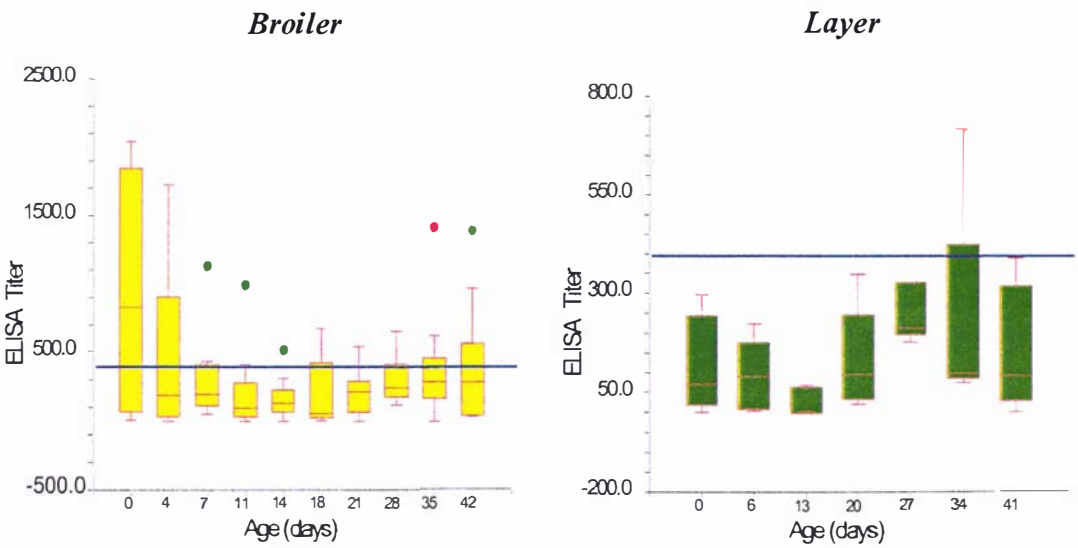


Figure 4.2: Box-plot showing IBDV ELISA titre of the broiler and layer flocks (the blue line indicates the cut-off titre value of 396).

4.3.3 Histopathology

The mean histological lesion scores of the broiler and layer flocks are summarised in Tables 4.1 and 4.2 respectively. No histological lesions suggestive of IBDV infection were observed in broiler birds at day 0 and 4, but lesions were detected from day 7 onward. Mean lesion scores increased from day 7 to day 21, then decreased slightly until the end of the observation period. The mean lesion score over the entire study period was 2.

No significant lesions were detected in the layer flock at day 0, but were detected from day 6 onwards. The mean lesion score was below 1.0 on day 6, 13, 27 and 33, but was above 1.0 on days 20 and 41.

4.3.4 Immunocytochemistry Staining on Formalin-Fixed Tissues

The number of birds with positively stained sections of bursal tissue and the mean immunoperoxidase staining scores of the broiler and layer flocks are summarised in Tables 4.1 and 4.2 respectively. No staining was found in broiler birds at 0 and 4 days of age, but at day 7, 8 of 10 birds were IP positive and the mean IP score was 1.8. The number of positive birds and the mean IP scores varied over the remaining period, with the highest proportion of positive birds (10/10) occurring at days 11, 14 and 42 and the highest mean IP score of 2.2 occurring at day 11 and 14.

No staining was found in layer birds at day 0. At day 6, the bursa from 2 of 5 birds were IP positive, with a mean IP score for the group of 0.6. The mean IP score and the number of birds with positively staining bursal sections varied over the remainder of the study period (Table 4.2).

Table 4.1: Summary of B/BW, S/BW ratio, ELISA, IP and Histological scoring of broiler flock.

Age (days)	Mean B/BW ratio[a]	Mean S/BW ratio[b]	#Positive/#Tested (Mean ELISA Titre[c])	#Positive/#Tested (Mean IP Score[d])	Mean Histo. Score[d]
0	1.64+/-0.41	0.37+/-0.15	7/10 (953.46+/-813)	0/10	0
4	1.94+/-0.47	1.15+/-0.88	5/10 (506.34+/-592)	0/10	0
7	2.29+/-0.32	1.04+/-0.29	3/10 (16.19+/-316)	8/10 (1.8+/-1.20)	0.9+/-1.10
11	2.18+/-0.72	0.89+/-0.17	2/10 (211.01+/-297)	10/10 (2.2+/-0.78)	1.5+/-0.70
14	2.69+/-0.84	0.91+/-0.009	1/10 (167.77+/-149)	10/10 (2.2+/-0.91)	1.7+/-0.40
18	2.19+/-0.69	1.01+/-0.28	3/10 (223.20+/-243)	6/10 (1.3 +/-1.27)	1.7+/-1.19
21	2.66+/-1.02	0.92+/-0.14	1/10 (211.48+/-157)	4/10 (0.5+/-0.70)	2.3+/-0.94
28	2.25+/-0.41	0.99+/-0.22	3/10 (300.60+/-167)	1/10 (0.1+/-0.31)	2.2+/-1.03
35	1.98+/-0.52	1.07+/-0.30	3/10 (391.83+/-397)	6/10 (0.8+/-0.90)	1.4+/-1.26
42	1.48+/-0.76	0.98+/-0.22	4/10 (403.49+/-445)	10/10 (1.7+/-0.80)	1.6+/-0.69

a Bursal/body weight ratio (x 1000) : mean +/- standard deviation.

b Spleen/body weight ratio (x 1000) : mean +/- standard deviation.

c IDEXX ELISA test kit, titer > 396 considered positive.

d See Material and Method for scoring method.

Table 4-2: Summary of B/BW, S/BW ratio, ELISA, IP and histological scoring of layer flock.

Age (days)	Mean B/BW ratio[a]	Mean S/BW ratio[b]	#Positive/#Tested (Mean ELISA Titre[c])	#Positive/#Tested (Mean IP Score[d])	Mean Histo. Score[d]
0	1.46+/-0.31	0.65+/-0.21	0/5 (119.83+/-121)	0/5	0
6	3.88+/-0.67	1.04+/-0.31	0/5 (92.75+/-90)	2/5 (0.6+/-0.89)	0.8+/-0.44
13	5.41+/-1.04	1.54+/-0.19	0/5 (25.60+/-35)	4/5 (1.6+/-1.14)	0.4+/-0.54
20	6.41+/-1.16	1.49+/-0.29	0/5 (130.98+/-130)	5/5 (1.4+/-0.54)	1.6+/-0.54
27	5.97+/-0.89	1.94+/-0.43	0/5 (252.91+/-70)	4/5 (0.8+/-0.44)	0.8+/-.044
34	6.95+/-1.76	1.92+/-0.33	1/5 (224.26+/-276)	3/5 (0.8+/-0.80)	0.8+/-0.44
41	6.79+/-0.66	2.54+/-0.42	1/5 (151.86+/-158)	5/5 (1.4+/-0.54)	1.4+/-0.54

a Bursal/body weight ratio (x 1000) : mean +/- standard deviation.

b Spleen/body weight ratio (x 1000) : mean +/- standard deviation.

c IDEXX ELISA test kit, titer > 396 considered positive.

d See Material and Method for scoring method.

4.4 DISCUSSION

4.4.1 Broiler Flock

Throughout the whole observation period, there were no clinical signs of IBDV or other illness in the broiler birds. Although no significant lesions were found at necropsy of the birds and the bursal weight increased over the observation period, greater variation in the bursal weight was observed from day 21 onward. The variation of bursal weight, which occurred in the later age may be due to physiological differences of the bursal development as it was shown in the mean B/BW ratio that no significant difference was found over the observation period. In general, the bursa grows proportionally to the body weight until sexual maturity, the body weight is about 400 times more than the bursal weight during the first three weeks and attains a plateau after which there is rapid atrophy (Glick, 1994; Petersen, 1992). The mean B/BW and S/BW ratios of the broiler flock in this study indicated no significant gross abnormality although the following tests clearly show that these birds were infected with IBDV. This indicates that gross examination is not sufficient for the diagnosis of the mild IBDV infection in New Zealand poultry flocks.

The serological profile of the broiler flock using the ELISA test demonstrated the flock had a high concentration of transferred maternal antibody against IBDV at one day of age. Infection at day-old and seroconversion at the time of testing is not a feasible explanation for this finding. As shown in Figure 4.2, the maternal antibody had an uneven distribution among the day-old chicks. This is mainly due to the fact that vaccination of the breeder flock at that time was with a single dose of inactivated IBDV vaccine only. Without priming with a live IBDV vaccine (which was not available in NZ) and a second booster vaccination with inactivated vaccine, the immune responses in the breeder flock and transfer of maternal antibody to the progeny would be variable and not persistent (Cullen & Wyeth; Eidson *et al.*, 1980; Naqi *et al.*, 1983; Wyeth & Cullen, 1978a; Wyeth & Cullen, 1978b; Wyeth & Cullen, 1979). More than 50% (7/10) of the sampled birds were serologically positive at day-old, but the number of positive birds and the mean antibody titre decreased through day 7 to 21. Various studies have shown that maternal antibody in chicken falls below detectable levels by 15 to 25 days of age (Lucio & Hitchner, 1980; Reddy & Koteeswaran, 1999; Tsukamoto *et al.*, 1995; Wood *et al.*, 1981). Interestingly, at the later sampling times of 35 and 42 days, the

number of serologically positive birds and the mean antibody titre increased, indicating the birds had experienced an IBDV field challenge on the farm. By the day of slaughter (day 42), about 40% of the birds were detected antibody positive although the mean titre was low.

Compared to the serological detection of IBDV infection by ELISA, more birds were found positive by the immunocytochemical detection of IBDV antigen in the bursa. Some birds had a negative result in the ELISA test but a positive result in the immunocytochemistry test. By detecting IBDV antigen, the results indicated that field IBDV infection started as early as day 7 in the flock. More than 50% of the sampled birds were infected by IBDV at day 7 and 100% of the birds were positive at days 11, 14 and 42. Another benefit of the immuocytochemistry test is that it is not affected by the transfer of maternal antibody to the progeny and viral antigen may be detected in the affected organ as early as 6 to 12 hours post-infection (Tanimura *et al.*, 1995). This is far earlier than seroconversion or development of detectable histological lesions.

Overall, from the study in this broiler flock, the results demonstrated the birds started to get infected with IBDV as early as 7 day after placement as day-old chicks into the shed. Maternal antibody was present in the chickens but the concentration of this in individual birds would have varied and, during the first week, it would have declined so that the flock became susceptible to residual IBDV in the shed. Infection of the more susceptible birds would have lead to shedding of virus from the infected birds, so that more birds were infected during the growing period. This scenario is consistent with the results of the ELISA test with more seropositive birds, higher antibody titres and birds becoming positive by immunocytochemistry. Due to the low pathogenicity of the IBDV strain in the field, no direct clinical signs nor increased mortality appeared in the infected flocks. It is possible that transient immunosuppression due to the IBDV infection may have induced secondary opportunistic infection, but this was not observed during the period of study.

4.4.2 Layer Flock

No clinical signs were observed or lesions detected at necropsy in the layer birds from day 0 to 42 (6 weeks). There was a slight increase in the average weekly mortality rate from 35 to 75 weeks of age, with 1 to 2% above the expected mortality rate. Although

IBDV infection was confirmed as early as when the chickens were 6 days-old in this flock, the slightly poorer performance during the egg-laying period could not be definitely confirmed to be a consequence of IBDV infection in this study. The bursal and spleen weight of the layer birds steadily increased as the birds grow older. No significant differences were detected in the mean B/BW and S/BW ratios in the various age groups from day 13 to 41. As a general indication, the body weight is about 200 times greater than the bursal weight in Leghorn chickens before sexual maturity (Petersen, 1992).

Unlike the broiler flock, the layer birds were negative for antibodies to IBDV from day 0 to the end of the observation period, except for one bird at each of days 34 and 41. Although the layer birds were serologically negative from day 0 to day 27, the immunocytochemical detection of IBDV antigen revealed the layer birds were infected from day 6. The different management of layer birds compared to broiler birds may explain the lower frequency of seroconversion in the layer birds. The layer birds were kept in rearing cages from day old so they had less contact with other birds and floor litter. Stricter biosecurity management may also have limited the extent of the field IBDV challenge to the layer birds. The mean IP scores of the positive layer birds were lower than in the broiler birds, indicating less IBDV antigen in the bursae and suggesting that the virus did not replicate as extensively. More limited viral replication in the layer birds would be consistent with lower antibody response compared to the broiler birds. It may also be that genetic differences between these two breeds results in the different size of the antibody responses to IBDV as has been reported by others(Cheng & Lamont, 1987; Cheng & Lamont, 1988; Ismail *et al.*, 1984; Reddy *et al.*, 1997; Solano *et al.*, 1986; Stewart-Brown & Grieve, 1992).

The study in the layer flock demonstrated the layer chicks were infected with IBDV as early as 6 days of age. Although the cage rearing and stricter biosecurity management in the layer house comparing to the broiler flock, the layer birds still became infected with IBDV, which was most probably residual in the shed. This indicated the decontamination procedure employed in the layer shed during the empty period was not sufficient to eradicate the residual virus from the farm. During the growing period, more birds were infected by IBDV as shown in the immunocytochemical tests and mild

histological lesions appeared in the bursa. The significance of economic loss due to the subclinical IBDV infection in the layer flock could not be demonstrated in this study.

4.4.3 Disease Prevalence and Evaluation of Diagnostic Tests

Overall prevalence of IBDV infection of the broiler flock from days 0 to 42, estimated from the immunocytochemical test, (assuming 100% sensitivity and specificity to detect the virus infection) was 55%, with 95% confidence interval of 45.24% to 64.75%. With 55% prevalence in 10,000 birds in the shed, the minimum sample size using immunocytochemical test to be 95% confidence to detect at least one positive bird is 4 (Win Episcope 2.0). If no positive birds are detected, the probability that the population is infected at prevalence of 55% is 0.0410. This indicated the sampling size (n=10) used in this study using immunocytochemical test was sufficient to detect the IBDV infection in the flock.

To evaluate the performance of the ELISA antibody test, only the test results of birds older than 21 days were used for the calculation because any positive results in birds younger than 3 weeks old may be derived from residual maternal antibody. If the immunocytochemical test is regarded as the "gold standard" for the diagnosis of IBDV infection in the flock, by comparing the two test methods, the sensitivity and specificity of the ELISA test can be estimated. The calculation shows the sensitivity of the ELISA is 28.57% (9.25% to 47.89%) and the specificity is 73.68% (53.88% to 93.48%). With a prevalence of 0.525 from day 21 to 42, the predictive value for positive test result is 0.54 (0.25 – 0.83) and the predictive value for negative test result is 0.48 (0.30 – 0.66). Considering the low sensitivity and specificity of the ELISA, the sample size on each sampling occasion in this study (n=10) is not sufficient to be 95% confident of detecting at least one positive birds and proving the population to be free from IBDV infection. However, there was at least one positive bird detected by ELISA on each of the sampling times from days 21 to 42. The probability of observing one positive in a sample of 10 animals from the flock with disease prevalence of 55% using the ELISA is 0.19. This indicates that either the prevalence of infection is higher than the value estimated above or the sensitivity of the ELISA is better than the calculated values shown above.

The manufacturer of the ELISA kit claims that the specificity and the sensitivity of the test are both over 95% and that the test results are highly correlated to the virus-neutralisation test. However, the test efficacy was determined using in vivo challenge with an overseas pathogenic IBDV strain, and pathogenic strain usually induce a high antibody titre 7 to 14 days post-exposure. On top of the above, the comparison of the two tests in this statistical calculation was different to the manufacturer's as we are comparing an antibody detection method (ELISA) with an antigen detection method (immunocytochemistry). As such, a trial to compare the agreement of the ELISA and VN tests should be carried out with the New Zealand IBDV strain to determine the performance of the ELISA test used in the control programme.

4.5 SUMMARY

In this study, the presence of IBDV was demonstrated in both the broiler and layer farms. The broiler and layer birds were infected as early as 6 to 7 days of age. Detection of IBDV antigen in affected bursal tissues by immunocytochemistry could be achieved in the early infection stage before either serology or histopathology, and was not interfered by the presence of maternal antibody.

The subclinical IBDV infection within the two flocks did not appear to cause any significant economic losses. However, damage to the bursae of a number of birds was demonstrated. It is possible that such damage, even if only transient, could have immunosuppressive consequences. These consequences were not further evaluated in this study.

To minimise false negative or positive results in the eradication screening programme, parallel or negative-herd re-testing strategies should be considered if the ELISA test continues to be used as the screening method for detection of the remaining IBDV-infected farms. One possible approach would be to test a sample of flock for the presence of viral antigen in the bursa by immunocytochemistry.

CHAPTER 5: CHARACTERISATION OF NEW ZEALAND ISOLATES OF IBDV

5.1 INTRODUCTION

5.1.1 Molecular Variation in Antigenicity and Pathogenicity

The emergence of variant strains of IBDV in the USA in 1985 (Rosenberger & Cloud, 1985) and later the discovery of vvIBDV in different parts of Europe and Asia (Lin *et al.*, 1993; van den Berg *et al.*, 1991), stimulated research into molecular characterisation of the virus in the hope that it would help in identifying the genomic differences responsible for the differences in antigenicity and virulence. In early studies that compared pathogenic serotype 1 strains and nonpathogenic serotype 2 strains, the identity of nucleotide and amino acid sequences in segment A (83-84% and 90% respectively) were lower than those of segment B (89% and 93-98% respectively). In segment A, the major sequence variation occurred in the region of amino acid residues 206 to 350 (numbering from the sequence of segment A of a serotype 1 strain, strain 52/70 of IBDV) (Bayliss *et al.*, 1990), sometimes referred to as the *AccI – SpeI* fragment or the hypervariable region. This hypervariable region of 145 – 146 amino acid residues, was shown in many studies to encode a conformational epitope recognised by various monoclonal antibodies (Bayliss *et al.*, 1990; Kibenge *et al.*, 1990) and to correspond to serotype-specific epitope(s) in the VP2 structural protein. This region has been classified further into two symmetrically spaced hydrophilic regions, located between amino acid residues 212 – 224 and 314 – 324 (Schnitzler *et al.*, 1993).

The VP2 coding region is responsible for inducing virus neutralising antibodies (Fahey *et al.*, 1989). The first hydrophilic region was predicted to be responsible for stabilising a conformational epitope and the second hydrophilic region for recognition by VN monoclonal antibodies (Heine *et al.*, 1991). Sequencing of the VP2 gene of numerous IBDV strains from different geographic regions has been reported (Brown *et al.*, 1994; Eterradossi *et al.*, 1999; Eterradossi *et al.*, 1998; Lin *et al.*, 1993; van den Berg & Meulemans, 1991a; Yamaguchi *et al.*, 1996b). Many researchers have focused on the

VP2 hypervariable region and have found that this variable domain may represent the molecular basis of the antigenic variation of the virus but is not solely responsible for the variation in pathogenicity (Jackwood & Jackwood, 1994; Mahardika *et al.*; Schnitzler *et al.*, 1993).

Figure 5.1 shows the alignment of the deduced amino acid residues within the hypervariable domain of VP2 (Nagarajan & Kibenge, 1997). Using this alignment, I have summarised the findings to date on the characterisation of regions within the VP2 hypervariable region that may be responsible for variation in pathogenicity and antigenicity. (All amino acid residues numbered following the sequence of segment A of serotype 2, strain OH of IBDV, as shown in Figure 5.1).

1st hydrophilic region (amino acid residues 212 to 224): In this region, the only significant finding was a difference of 4 to 5 amino acids between the pathogenic serotype 1 and nonpathogenic serotype 2 strains. Various publications reported a change in the amino acid residue at position 222 in some vvIBDV strains (from P to A) and in some variant strains such as variant A, E/Del and GLS (P to Q or T) (Heine *et al.*, 1991; Lana *et al.*, 1992). The possibility that this particular amino acid residue is responsible for the virulence of the virus was also suggested. Eterradossi et al (Eterradossi *et al.*, 1999; Eterradossi *et al.*, 1998; Eterradossi *et al.*, 1997) found that failure to react with Mab in virus neutralisation tests or AC-ELISA of vvIBDV was associated with changes in the Proline-Glycine pair at positions 222-223. The amino acid residue at position 222 was also predicted by Brown et al (Brown *et al.*, 1994) and Yamaguchi et al (Yamaguchi *et al.*, 1996b) to be one of the three unique amino acid residues that may contribute to the virulence difference between vvIBDV and classical serotype 1 strains. However, the variation in this region is not as high as in the other regions described below, so it was speculated that it is only responsible for stabilising the conformational epitope of the VP2 protein (Heine *et al.*, 1991).

2nd hydrophilic region (amino acid residues 315 to 326): Numerous studies have shown that amino acid substitutions in this region are present in variant strains and these changes enable the variant strains to escape from neutralisation by antibodies induced by classical serotype 1 strains. Both variant A and E/Del strains have an amino acid change at position 319 from G to D. Variant GLS and DS326 have a substitution at

position 322 from A to E (Heine *et al.*, 1991). In the studies by Snyder et al (Snyder *et al.*, 1988a; Snyder *et al.*, 1988b; Snyder *et al.*, 1992) using a select panel of monoclonal antibodies to screen different strains of IBDV in an AC-ELISA, there were two distinctive MAbs B69 and R63, which were found to be able to target two different epitopes on VP2 and successfully differentiate variant and classical strains. The variant strains did not react with the B69 MAb but reacted with all classical strains except PBG98. The R63 MAb reacted with all serotype 1 strains except variant GLS and DS326 strains. These findings further supported the hypothesis that amino acid substitutions at positions 319 and 322 were responsible for the VN MAb binding sites, and that mutations in the second hydrophilic regions of the VP2 gene contributed to antigenic variation of the virus.

Heptapeptide region (amino acid residues 327 to 333): This region is adjacent to the second hydrophilic region and contains a conserved, serine-rich heptapeptide motif S-W-S-A-S-G-S in various vvIBDV strains and variant strains (Eterradossi *et al.*, 1992; Lin *et al.*, 1993; van den Berg & Meulemans, 1991a). One common finding was that any substitution in this motif appears in less virulent strains. The nonpathogenic serotype 2 OH strain has three substitutions to other amino acid residues in the region (position 329, 330 and 333). Classical strains of low virulence, e.g. PBG98 and Cu-1 have fewer serine residues than those of high virulence (Heine *et al.*, 1991; Vakharia *et al.*, 1994), and attenuated vaccine strains also have substitution(s) in this region (Yamaguchi *et al.*, 1996a; Yamaguchi *et al.*, 1996b).

Hydrophobic region between amino acid residues 225 and 314: This region is located between the two hydrophilic regions, and some researchers predicted that the virulence marker of IBDV may be located in this region. For example, the nonpathogenic serotype 2 strain, OH, has an extra insertion of a serine residue at position 249 as shown in Figure 5.1. There is a large number of differences between serotype 1 and serotype 2 strains in a cluster of amino acids from residues 249 to 263. Early work by Bayliss et al (Bayliss *et al.*, 1990) demonstrated that the virulent strain 52/70 and its attenuated vaccine strain PBG98 had five amino acid changes in the hydrophobic region. Yamaguchi et al (Yamaguchi *et al.*, 1996a) compared a highly virulent strain of IBDV and its cell culture attenuated strain, and their results revealed only five amino acid

differences between the two strains: four in the VP2 hypervariable domain and one in the VP3 protein, The amino acid substitutions at position 280 (from D to N) and 285 (from A to T) in the hydrophobic region of VP2 were common substitutions found in other cell culture-adapted strains (Lim BoonLeong. *et al.*, 1999; Mundt, 1999; Proffitt *et al.*, 1999; Yamaguchi *et al.*, 1996a). The substitution of these two amino acids may contribute to the alteration of the structure of VP2 (Yamaguchi *et al.*, 1996a). Brown *et al* (1994) found three unique amino acid residues were conserved in European highly virulent strains, two of which were located in this region (position 257 and 295).

5.1.2 *In-Vivo* Pathogenicity Study in SPF Birds

Although the application of molecular virology to viral poultry diseases has made considerable progress in recent years, the specific antigenic and pathogenic determinants within the variable region of VP2 are still not clearly identified. Investigation of nucleotide changes in other parts of the genome of IBDV such as VP1, VP3 and VP4 may reveal that these areas also contribute to variation of the virus (Brown *et al.*, 1994; Jagadish & Azad, 1991; Yamaguchi *et al.*, 1997; Yehuda *et al.*, 1999). To date, the only reliable criteria for determination of the pathogenicity of an IBDV strain still depend on the *in-vivo* challenge test in SPF birds or inoculation into embryonating eggs. The classification of IBDV strains based on their virulence in SPF birds by challenge test was described in section 1.10.

In this chapter, a partial genetic characterisation of NZ isolates of IBDV is presented and pathogenicity of one isolate is determined using an in-vivo challenge test in SPF birds.

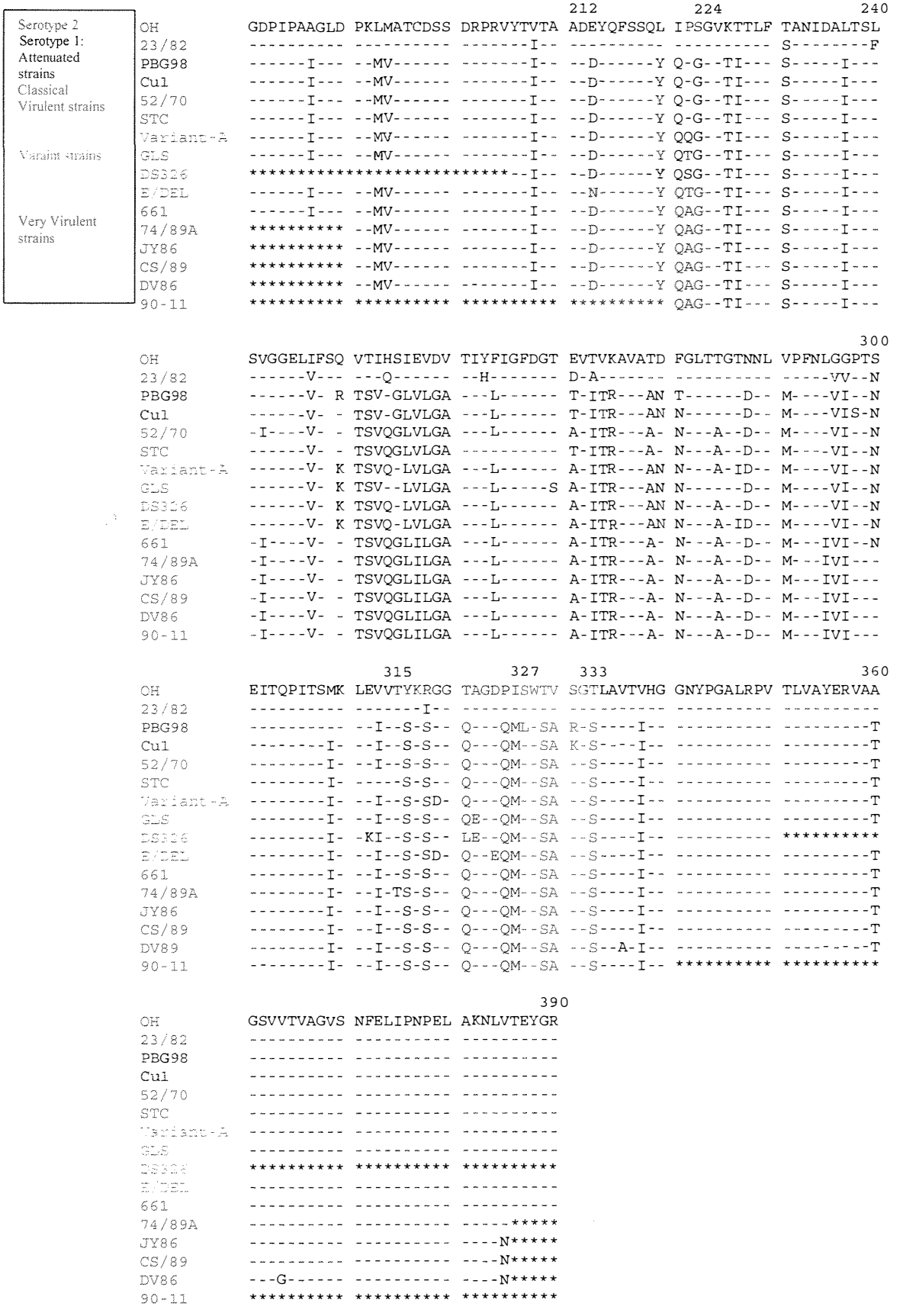


Figure 5.1: Deduced amino acid sequences of VP2, from amino acid position 181-390, numbering from the sequence of segment A of serotype 2, strain OH of IBDV (Nagarajan and Kibenge, 1995). Hyphens denote sequences identical to IBDV strain OH, gaps represent deletion (marked by □), and * represents unavailable sequence. The two hydrophilic regions are in blue letters and the heptapeptide region is in red letters.

5.2 MATERIALS AND METHODS

5.2.1 Genetic Characterisation of IBDV

IBDV Isolates

The IBDV isolates were originally from the bursae collected in 1997 and 1998 from serology positive farms as described in section 2.2.6. Two Vero cell-culture adapted strains designated as NZ2103/97 and NZ1105/98, which were passaged less than five times in cell-culture, were used for genetic characterisation.

RNA Extraction from IBDV Isolate

IBD viral RNA was extracted from the infected cell culture as described in section 2.2.9.

Reverse-Transcriptase-Poymerase Chain Reaction (RT-PCR)

The RT-PCR reaction was carried out as described in section 2.2.9.

cDNA Nucleotides Sequencing

The cDNA PCR products were loaded and run on an agarose gel as described in section 2.2.9. The cDNA was purified from the agarose gels with QIAquick Gel Extraction kit (QIAGEN, Germany) and sequenced on an ABI Prism 377 DNA sequencer using Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer).

To ensure accuracy of the sequence data, each isolate was subjected to three separate RT-PCR reactions and both strands of the cDNA of the three RT-PCR products from each isolate were sequenced with the designed forward and reverse primers (total of 12 cDNA sequences from each isolate).

Nucleotides, Amino Acid alignment and Analysis

Both strands of the cDNA sequences of both isolates were aligned and edited using Sequencer 3.1 program (Gene Codes Corporation). The consensus nucleotide sequence and deduced amino acid sequence of both isolates were analysed using Wisconsin

Package 8.1 and compared to corresponding published sequences of the following IBDV strains:

Two classical IBDV strains: STC and UK-70 (EMBL accession numbers D00499 and D00869)

One very virulent strain: DV86 (Z25482)

Two attenuated strains: Cu1 and PBG98 (X16107 and D00868)

One variant strain: Var E (D10065)

One Australian strain: A002-73 (M64738)

Phylogenetic Analysis

Amino acid sequences of the NZ isolates and the overseas published IBDV strains were aligned using Clustal W version 1.5. All aligned deduced amino acid sequences were used for tree building. A phylogenetic network was first constructed using SplitTree programme (Hudson, 1998), which implements the method of Split Decomposition (Bandelt & Dress, 1992). Neighbour joining and parsimony (PAUP 3.1.1) were also used to build the IBDV phylogeny from the 9 taxon cases.

5.2.2 *In-Vivo* Pathogenicity Study

IBDV Challenge Strain

Bursae collected in 1997 from IBDV antibody-positive birds were used to prepare the inoculum for the challenge test. The bursal tissues were homogenised and inoculated into 11-day-old embryonated SPF eggs via the chorioallantoic membrane route as described in section 2.2.6. Inoculated eggs were incubated at 38.5 °C for 3 to 5 days, and tissues from infected embryos with gross lesions were collected and homogenised. The tissue homogenates from SPF eggs were stored at –70 °C until use.

SPF Eggs and Birds

All White Leghorn SPF eggs were purchased from SPAFA, Australia. Upon arrival at the laboratory, the SPF eggs were incubated in an egg incubator at 37.5 °C and relative humidity of 60-65%. The eggs were manually turned three times per day until day 18 of incubation. At day 18, all incubated eggs were candled to remove infertile and dead-in-

shell eggs, and fertile eggs were set onto hatching trays in the incubator until the chicks hatched.

Hatched chicks were kept in a positive pressure isolator, and autoclaved water and feed were provided *ad libitum*. A water-soluble vitamins supplement (Stressol, Technik NZ) was added in drinking water from day 1 until the end of the trial. The temperature was maintained at 32.5 °C from day 0 to 14 and at 28 – 30 °C after day 14. Sixteen hours lighting per day was provided.

Virus Titration

Eleven-day-old embryonated SPF eggs were used for the titration of the virus stock (tissue homogenates from the infected SPF eggs). The eggs were candled to check for the viability of the embryo before use in the titration. The tissue homogenate was diluted in tryptose phosphate broth (20% w/v) with antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) in 10-fold serial dilution series from 10^{-1} to 10^{-6}. Two hundred μL of each dilution was inoculated into each of eight 11-day-old embryonated SPF eggs via the chorioallantoic membrane route. Eight eggs were used as the negative control and were each inoculated with 200 μL of tryptose phosphate broth containing antibiotics only. The inoculated eggs were incubated in the egg incubator horizontally for 7 days and candled daily. Any embryo deaths within the first 24 hours after inoculation were considered to be due to nonspecific causes such as trauma and were discarded from the experiment. All embryos that died between 24 hours p.i. and 7 days p.i. were examined for gross lesions due to IBDV infection, such as cutaneous congestion and petechial haemorrhages, focal necrosis and ecchymotic haemorrhages in the liver, heart and kidney (Lukert *et al.*, 1991). At the end of the incubation period, all remaining viable embryos were opened and examined for gross lesions. Embryos with gross lesions typical of IBDV were considered as positive, any embryos without gross lesions were counted as negative. The titre of the virus stock was calculated by the method of Reed and Muench (Reed & Muench, 1938) and expressed as EID_{50}/mL (mean embryo infectious dose per mL).

Challenge of SPF Birds

Twenty-five 3-week-old SPF chickens were used in the challenge trial and a group of 10 birds of the same age served as an unchallenged control. The challenge group birds were kept in a negative pressure isolator and the negative control group was kept in an animal facility (Small Animal Breeding Unit, Massey University). Each bird was identified by a numbered leg band. Blood was collected from all birds before the challenge. The challenge group birds were each given an intraocular inoculation of $10^{2.5}$ EID_{50} dose of the prepared infected embryo homogenate. All birds were observed daily, and any birds that showed clinical signs were euthanased and necropsied. All remaining birds were euthanased at 10 days post-inoculation.

Post-Challenge Examination and Data Collection

The following parameters were collected pre- and post-inoculation of the birds:

Serological Test

About 0.5 mL of blood was collected from all birds in the challenge test (including the negative control birds) via the wing's brachial vein before inoculation and 10 days post-inoculation. The blood was allowed to clot at 4 °C for 1 hour and the sera were separated from the blood clot by centrifugation at 2000 x g for 20 minutes, collected and labelled accordingly. All sera were stored at –70 °C until further testing.

All serological tests were performed at the end of the challenge test. All sera were tested for the presence of IBDV antibody using an antibody ELISA test kit (IDEXX laboratories, USA) according to the manufacturer's instructions as described in section 2.2.4.

Gross and Histpathological Examination

Following euthanasia, all birds were examined for gross lesions and samples of bursa, liver, spleen and any tissues were collected and fixed in 10% neutral formalin for histopathological examination. Histopathological lesion scores were given to each bursal section as described in section 3.2.3, but slightly modified as follows:

0 = No lesions

1 = Mild lymphoid necrosis in isolated follicles

2 = Moderate generalised lymphoid depletion or isolated follicles with severe depletion

3 = Over 50% of follicles with severe lymphocyte depletion

4 = severe lymphocyte depletion and follicles either atrophic, cystic or fibroplastic.

Bursa to Body Weight and Spleen to Body Weight Ratios

The individual body, bursa and spleen weights of each of the challenge and negative control birds were measured at necropsy. The mean bursa to body weight ratio (B/BW) and spleen to body weight ratio (S/BW) of the two groups were calculated as described in section 4.2.3 and analysed for statistical significance.

Statistical Analysis

The differences between the mean B/BW and S/BW ratio of the two groups were statistically analysed by Student's *t*-test in NCSS 2000 programme. The median histopathological lesion scores of the two groups were determined and analysed by Mann-Whitney test.

5.3 RESULTS

5.3.1 Genetic Characterisation of New Zealand's IBDV Isolates

RT-PCR and cDNA Sequences

Three separate RT-PCRs of both isolates resulted in amplification products of the predicted size of 555 bp, indicating no deletion or insertion of nucleotide(s) occurred in the amplification. Alignment and comparison of the 555-bp sense and nonsense cDNA sequences of each isolate showed a high degree of homogeneity in this region. A final consensus nucleotide sequence of each isolate was obtained from the alignment of the 12 cDNA sequences. Deduced amino acid sequences were translated from the consensus nucleotide sequences.

The nucleotide and deduced amino acid sequences of the two isolates were submitted to GenBank with the accession numbers AF281311 (NZ2103/97) and AF281312 (NZ1105/98) as shown in Figure 5.2 and 5.3.

Consensus Nucleotide and Deduced Amino Acid Sequences Alignment

The consensus nucleotide sequences of each of the two isolates were aligned with other reported strains as shown in Figure 5.2. The nucleotide sequences of the two isolates from NZ had 99.5% identity with each other, 96 - 97% identity with the attenuated strains (Cu1 and PBG98), 94% identity with the classical strains (STC and UK52/70), 92% identity with the very virulent strain DV86, 93% identity with Variant E and 88-89% identity with the Australian 002-73 strain.

```
                662                                                       721
NZ2103/97        TTGACCCAAAAATGGTAGCCACATGTGACAGTAGTGACAGGCCCAGAGTCTACACCATAA
NZ1105/98        ....T.....................T.............................
Cul              ............................C.............................
PBG98            ............................C.............................
STC              ............................C.............................
UK52/70          ................T.....C.....C.............................
DV86             *****.............A...........C.............................
Var E            ..................A...........C.............................
A002-73          .C..........................................................

                722                                                       781
NZ2103/97        CTGCAGCCGATGATTACCAATTCTCATCGCAGTATCAACCAGGTGGGGTAACAATCACAC
NZ1105/98        ............................................................
Cul              ..........................A.....C...........................
PBG98            ..........................A.....C...........................
STC              ..........................A.....C...........................
UK52/70          ..........................A.....C...........................
DV86             ..........................A.....C...G.......................
Var E            ..........A...............A.....C...A.......................
A002-73          ....C..A..................A................A..G..G.........

                782                                                       841
NZ2103/97        TGTTCTCAGCCAACATCGATGCCATCACAAGCCTCAGCGTTGGGGGAGAGCTCGTGTTTC
NZ1105/98        ............................................................
Cul              ..............T.............................................
PBG98            ..............T.............................................
STC              ..............T.....T.......................................
UK52/70          ..............T.....T.............A.......................
DV86             .........T..T.....................A.C........A.............
Var E            ..............T............T.........................CA
A002-73          ..............T........T..C.A......T.....A.............C.

                842                                                       901
NZ2103/97        GAACAAGCGTCCAAGGCCTTGTACTGGGCGCCACCATCCACCTCATAGGCTTTGATGGGG
NZ1105/98        ............................................................
Cul              A...........C.....................T...................A
PBG98            ............C.....................T...................A
STC              A.............T...................T..T.T.............A
UK52/70          A.....................................T....T.............A
DV86             A.................A.......T..T......T....T.............A
Var E            A............A.....................T....T.............A
A002-73          A...................G..AAA.........TT....GG............A

                902                                                       961
NZ2103/97        CAACGGTAATCACCAGGGCTGTGGCCGCAAACAATGGGCTGACGACCGGCACCGACAACC
NZ1105/98        ............................................................
Cul              ........................T...................................
PBG98            ............................C...............................
STC              .T.........................G.............G.............T.
UK52/70          .TG.............A...........G.T...........G.............T.
DV86             .TG.............A...........G..........A...G.......T.......
Var E            .TG.............A.........................G......T......T.
A002-73          .C.......C......A...........GG............G..............
```

```
              962                                                        1021
NZ2103/97      TTATGCCATTCAATCTTGTGATTCCAACAAACGAGATAACCCAGCCAATCACATCCATCA
NZ1105/98      ............................................................
Cul            ..........................T.................................
PBG98          ............................................................
STC            ................................C...........................
UK52/70        ......................C.........C..T.........................
DV86           ................A.............C.G...........................
Var E          ..............................C..T.........................
A002-73        .C...........C..............C.GT..............G.T........T.

              1022                                                       1081
NZ2103/97      AACTGGAGATAGTGACCTCCAGAAGTGGTGGTCAGGCAGGGGATCAGATGTCATGGTTGG
NZ1105/98      ............................................................
Cul            ......................A.....................................C..
PBG98          ......................A.............................T.....C..
STC            ........G............A.........C.........................C..
UK52/70        ......................A.....................................C..
DV86           ......................A................G....................CA.
Var E          ........................A.....A..............A.............C..
A002-73        ..............A........A.......A.........T..A...........C.......

              1082                                                       1141
NZ2103/97      CAAGAGGGAGCCTAGCAGTGACGATCCATGGTGGCAACTATCCAGGGGCCCTCCGTCCCG
NZ1105/98      ............................................................
Cul            ...A........................................................
PBG98          ............................................................
STC            ....T......................C.................................
UK52/70        ....T........................................................
DV86           ....T..............C.........C...............................
Var E          ....T.........................................A..............
A002-73        ....T....A............A..T........A.....C.....T........C....

              1142          1157
NZ2103/97      TCACGCTAGTGGCCTA
NZ1105/98      ................
Cul            ................
PBG98          ................
STC            ....A.....A.....
UK52/70        ....A.....A.....
DV86           ....A.....A.....
Var E          ....A...........
A002-73        ....A.....A.....
```

Figure 5.2: Nucleotide sequences alignment of the hypervariable region (position 662-1157) of the VP2 gene. A dot indicates positions where the sequence is identical to the New Zealand isolate NZ2103/97 in the upper line. * indicates sequence not available. See section 5.2.1 for EMBL accession numbers.

The deduced amino acid sequences were also aligned and compared with the seven overseas strains as shown in Figure 5.3. The two NZ IBDV isolates showed 100% similarity with each other, 95% similarity with the two attenuated strains, 93% with classical strains, 91% with both the Variant E and Australian 002-73 strains, and only 90% with the very virulent strain DV86.

```
           190                  212          224                      249
NZ2103/97  DPKMVATCDSSDRPRVYTITAADDYQFSSQYQPGGVTITLFSANIDAITSLSVGGELVFR
NZ1105/98  ...........................................................
PBG98      ...........................................................
Cu1        ..........................................................Q
STC        ..........................................................Q
UK52-70    ..........................................................I.....Q
DV86       *.........................A.................I.....Q
Var E      ....................N........T.....................K
A002-73    ..........................................................N.....Q

           250                                                        309
NZ2103/97  TSVQGLVLGATIHLIGFDGATVITRAVAANNGLTTGTDNLMPFNLVIPTNEITQPITSIK
NZ1105/98  ...........................................................
PBG98      ...H.....Y.....T.....T.....
Cu1        ...H.....Y.....T.....S.....
STC        .....YF....T.....D....A.....
UK52-70    .....Y.....TA.....D....A.....
DV86       .....I....Y.....TA.....D....A.....I....S.....
Var E      ....S.....Y.....TA.....A.I.....
A002-73    .....N...Y.V....T..T.....G....A.....S.....V....

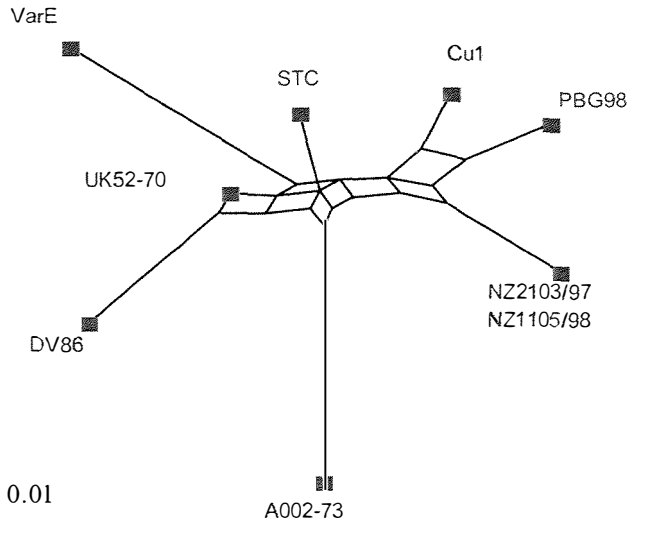
           310  315      324   330                  353
NZ2103/97  LEIVTSRSGGQAGDQMSWLARGSLAVTIHGGNYPGALRPVTLVA
NZ1105/98  ..........................................
PBG98      .....K.....L.S.....
Cu1        .....K.....S.K.....
STC        ..V...K.....S.S.....
UK52-70    .....K.....S.S.....
DV86       .....K.....S.S....A.....
Var E      .....K.D....E....S.S.....
A002-73    .....K.....S.N.....
```

Figure 5.3: Deduced amino acid sequences alignment of the VP2 variable region (from position 190 to 353) (Nagarajan and Kibenge, 1995). A dot indicates positions where the sequence is identical to the New Zealand isolate NZ2103/97. The two hydrophilic regions are underlined and the heptapeptide region is in bold italic letters. * indicates sequence not available.

Phylogenetic Analysis

The strict consensus of the best branch and bound parsimony trees favours a close relationship between PBG 98 and Cu1 (Figure 5.6). In contrast, neighbour joining on amino acid sequences places PBG98 as most closely related to the two NZ isolates (Figure 5.5). Phylogenetic analysis of the deduced amino acid sequences by splits decomposition showed the NZ IBDV isolates to be genetically closest to the attenuated PBG98 and Cu1 strains, and to be distant from the classical, very virulent, variant and the Australian strain (Figure 5.4). The splitgraph shows that PBG98 is almost equally close to Cu1 and the NZ isolates. Unlike other tree building methods, split decomposition makes no assumption that the data are treelike, representing it as such only when the data are truly treelike(Bandelt & Dress, 1992; Hudson, 1998). As shown in Figure 5.4, the splitgraph boxes indicate the presence of some contradictory signals in the data that do not fit onto a bifurcating tree (as shown by parsimony and neighbour joining methods).

Overall, in the Neighbour Joining, Parsimony and splitdecomposition analyses the NZ isolates, PBG98 and Cu1 are grouped together and split away from the other strains.



Fit=87.2 ntax=9 nchar=164 const=132 non parsi=152 – dsplits_hamming

Figure 5.4: Splitgraph showing the phylogenetic relationship between isolates of IBDV from the deduced amino acid sequences. The boxiness between the NZ isolates, Cu1 and PBG98 suggests there are patterns in the sequences indicating a close relationship between PBG98 and NZ isolates, and between PBG98 and Cu1. Unlike the other tree building methods, split decomposition makes no assumption that the data are treelike, representing it as such only when the data are truly treelike.

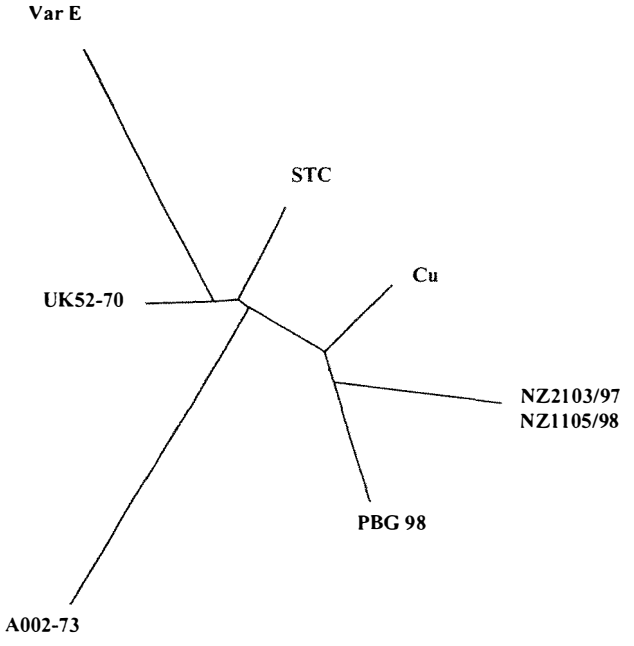


Figure 5.5: Neighbour Joining tree showing the phylogenetic relationship of the NZ IBDV isolates with overseas IBDV strains. It placed the NZ IBDV isolates as most closely related to UK attenuated strain PBG98.

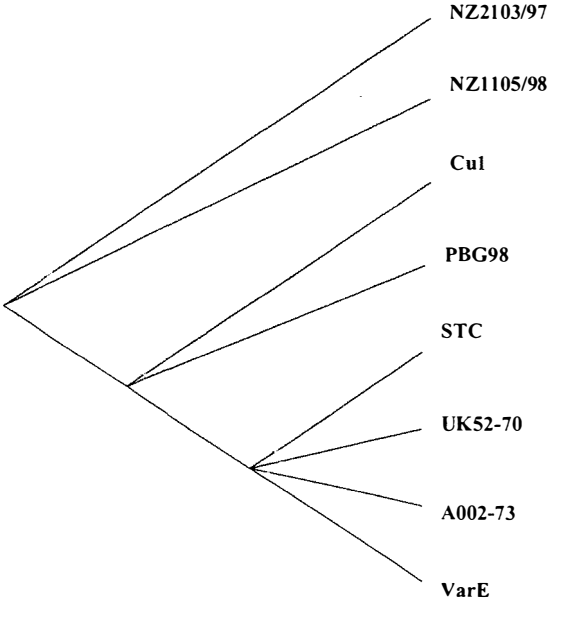


Figure 5.6: Branch and Bound parsimony tree showing the phylogenetic relationship of the 8 taxa. Unlike the split decomposition method, the strict consensus of the best branch and bound parsimony trees favours a closer relationship between PBG98 and Cu1 strains, making the NZ IBDV isolates split away from PBG98 and Cu1.

5.3.2 In-Vivo Pathogenicity Study

Virus Titration

The titre of the bursal homogenate was $5x10^{3.75}$ EID_{50} per mL.

Serology

All of the birds were seronegative to IBDV before inoculation. Five birds in the challenge group were antibody positive at 10 days post-inoculation and the remaining 20 birds in this group were antibody negative, as shown in Table 5.1. All of the birds in the negative control group were antibody negative at the end of the observation period.

Clinical Signs and Pathology

None of the IBDV-inoculated birds showed any clinical signs during the 10-day observation period, and no significant reduction of feed or water intake was observed. No significant gross lesions were found during necropsy examination. The histological lesion scores of the inoculated birds ranged from 0 (5 birds) to 4 (1 bird), the remaining 19 birds having scores of 2, giving a median score value of 2. All the birds in the negative control group had lesion scores of 0.

B/BW and S/BW ratio

As shown in Table 5.1, there was a significant difference ($p<0.05$) between the bursal to body weight ratio of inoculated and control groups. The spleen to body weight ratio showed no significant difference between the two groups.

Table 5.1: Serological results, bursa and spleen to body weight ratios, and histological lesion scores of IBDV-inoculated and uninoculated groups of birds.

Group (number of birds)	Serology[a] pre-inoculation	Serology[a] 10 days post-inoculation	Mean bursal/body weight ratio[b]	Mean spleen/body weight ratio[c]	Median histological lesion scores[d]
IBDV-inoculated birds (25)	0/25	5/25	4.656+/-1.176	2.012+/-0.845	2
Control birds (10)	0/10	0/10	5.713+/-1.468	1.858+/-0.556	0
			P=0.028[e]	P=0.584[e]	P < 0.001[f]

[a] Number of IBDV antibody positive birds/total birds tested.

[b] Bursal/body weight ratio (x 1000) : mean +/- standard deviation.

[c] Spleen/body weight ratio (x 1000) : mean +/- standard deviation.

[d] Histological lesion scores : see section 5.2.2 of scoring method.

[e] Statistical analysis by Student's t-test, p<0.05 is significantly different.

[f] Statistical analysis by Mann-Whitney test, p<0.05 is significant different.

5.4 DISCUSSION

5.4.1 In-Vivo Pathogenicity Study

Clinical Signs and Mortality

No clinical signs or mortality were observed in the IBDV inoculated SPF birds during the observation period. To determine the pathogenicity of the NZ isolate by comparing the results of this study with those of similar challenge tests overseas, we found there is no generally accepted challenge dose of IBDV for pathogenicity testing of isolates. In this study, a challenge dose of $10^{2.5}EID_{50}$ per bird was used. This dose is slightly higher than that used by others with the challenge test on the standard American pathogenic classical (STC) strain of IBDV ($10^{2}EID_{50}$) (Hassan & Saif, 1996), so it is reasonable to use those results for comparison with ours. Challenge of 2-week-old SPF chickens with the STC strain at a dose of $10^{2}EID_{50}$ did not cause any mortality but histological lesions were found. The STC strain was clearly classified as classic serotype 1 IBDV with mild pathogenicity in SPF birds. Another challenge study used a serotype 1 classical virulent IBDV strain (Edgar strain) with $10^{3}EID_{50}$ per bird. This strain produced severe clinical signs and mortality (>20%) as early as 48 hours post-inoculation (Rosales *et al.*, 1989).

Zierenberg *et al* (2000) used a high challenge dose of $10^{4.38}ELD_{50}$/mL in SPF birds with a vvIBDV strain and produced more than 60% mortality. Higher challenge doses (up to $10^{4.5}EID_{50}$) have been used to test the protective effect of vaccines (Nakamura *et al.*, 1994) but not usually for pathogenicity testing in SPF birds. Other pathogenicity studies have used a challenge dose of 10^4 to 10^6 $TCID_{50}$ per bird (for tissue culture adapted IBDV strains) or $100LD_{50}$ for virulent IBDV strain titrated in SPF birds (Cao *et al.*, 1998; Hassan & Saif, 1996; van den Berg & Meulemans, 1991a; van den Berg & Meulemans, 1991b). These pathogenicity tests can not be directly compared with the present study. However, the absence of mortality after a sufficient challenge dose in this study suggests the NZ isolate should be grouped with the apathogenic strains of IBDV.

Gross and Histopathological Lesions

However, despite the lack of clinical signs, the bursal to body weight ratio did indicate that there was some degree of bursal atrophy in the infected birds. This ratio is widely used as an indication of bursal damage and immunosuppression following IBDV infection (Hassan *et al.*, 1996; Hassan & Saif, 1996; Sharma *et al.*, 1989; van den Berg *et al.*, 1991). No evidence of spleen enlargement was found in this study. Although the median histological lesion score of infected birds was only 2, moderate to severe lymphoid depletion was observed in the bursa of some birds. Similar histological lesions and lesion scores are generally found in infection with mild IBDV strains (Hassan *et al.*, 1996) or following vaccination with pathogenic live IBDV vaccine strains (so called "intermediate" to "hot" strains) (Thornton, 1976; Thornton, 1977; Thornton & Pattison, 1975). Neither of these types of virus completely compromise cellular immunity but cause partial and transient lymphoid depletion in the bursa. The depleted lymphoid follicles are later regenerated by the remaining lymphoid stem cells (Mazariegos *et al.*, 1990). However, chickens in the stage of transient lymphoid depletion have impaired immune responses and may be susceptible to secondary infection. Although this outcome was not investigated in this study, Motha (Motha, 1996) found evidence of the immunosuppressive effect of NZ isolates on SPF birds when he showed that SPF birds inoculated with a NZ strain of IBDV had depressed responses to *Brucella abortus* antigen. These findings may explain the poor growth in broiler flocks when the infection was first discovered in NZ in 1993 and in a later outbreak in 1997 (Christensen, 1995; Christensen, 1999). However, in this study it was not determined whether the lymphoid depletion in the bursa caused by the challenge

virus was transient or permanent. But further work could be done by using several separate challenge groups for longer observation periods.

Serological Test

Only 5 of 25 birds in the challenge group had seroconverted to IBDV by the end of the observation at 10 days p.i. The lack of seroconversion at 10 days p.i. in most of the inoculated birds is consistent with our findings from a previous study (Chai *et al.*, 1999). In that study, we showed that in a field situation, seroconversion to IBDV could be delayed up to 20 days post-infection. In the similar challenge test with STC strain in SPF birds, VN antibody was detectable as early as 5 days p.i. (Hassan *et al.*, 1996). The lymphoid depletion in the bursa found in the histopathological examination may partially contribute to this delay in seroconversion.

5.4.2 Genetic Characterisation of New Zealand's IBDV Isolates

The hypervariable region of the VP2 gene of IBDV lies between the *AccI* and *SpeI* sites at residues 206 and 350 respectively. Mutations within this region have been shown to cause antigenic changes that allow escape from neutralising antibodies and to result in changes in virulence (Bayliss *et al.*, 1990; Vakharia *et al.*, 1994). The sequence of the NZ isolates in this region was characteristic of an attenuated IBDV strain, and phylogenetic analysis confirmed the close relationship between the isolates in this study and attenuated strains.

There are two hydrophilic peaks within the hypervariable region of VP2, the first from residues 212 to 224 and the second from 314 to 324. Substitution of proline for any other amino acid at position 222, within the first hydrophilic region, induces changes in both virulence and antigenicity, as seen in vvIBDV strains (Brown *et al.*, 1994; Cao *et al.*, 1998; Eterradossi *et al.*, 1998; Lin *et al.*, 1993; Pitcovski *et al.*, 1998) and variant strains (Dormitorio *et al.*, 1997; Ture *et al.*, 1993) respectively. Other substitutions in the first hydrophilic region also contribute to antigenic drift of classical serotype 1 strains, leading to vaccination failures (Heine *et al.*, 1991; Schnitzler *et al.*, 1993). The two NZ isolates in this study showed the same sequence in this region as the classical and attenuated serotype 1 strains. The NZ isolates had a lysine (K) to arginine (R) substitution at residue 316, within the second hydrophilic region. A tissue-culture

attenuated IBDV strain (GT101) from Australia was reported to have the same substitution (Proffitt *et al.*, 1999), but it is not yet clear whether this site serves as a marker for attenuation.

The serine-rich heptapeptide, SWSASGS, lying next to the second hydrophilic region has been shown to be highly conserved in classical, vvIBDV and variant strains, but mutation within this region is commonly found in attenuated strains (Chen *et al.*, 1998). The NZ isolates in this study had two substitutions within this heptapeptide. The serine (S) to arginine (R) substitution that was present at position 330 is commonly found in attenuated strains (Berg *et al.*, 1996; Brown *et al.*, 1998; Eterradossi *et al.*, 1998; Pitcovski *et al.*, 1998). The NZ isolates and the Australian isolate A002-73 had an additional substitution, at position 328, of serine (S) to leucine (L). This substitution has been described in other Australian isolates (Proffitt *et al.*, 1999). However, other substitutions which are characteristic of Australian isolates, at positions 258, 264, 272, 279, 305 and 332, were not found in the NZ isolates in this study.

A number of other amino acid substitutions between the two hydrophilic regions have been shown to correlate with the virulence of the isolate (Berg *et al.*, 1996; Brown *et al.*, 1998; Cao *et al.*, 1998; Yamaguchi *et al.*, 1996a; Yamaguchi *et al.*, 1996b). At two positions within this region, the NZ isolates had residues consistent with those of attenuated viruses. At position 279, the NZ isolates, the attenuated strains PBG98 and Cu1, and the Australian strain A002-73 had an aspartic acid (D) to asparagine (N) change. The NZ isolates and the attenuated strains had an alanine (A) to threonine (T) substitution at position 284.

The method of split decomposition was used to analyse the evolution of the amino acid sequences of the NZ isolates and other overseas strains. Neighbour joining and parsimony methods were also used to analyse the same data. In all three analyses, using neighbour joining, parsimony and split decomposition, the NZ isolates are closely related to attenuated strain PBG98 and Cu1 but split away from Australia 002-73, variant E, classical and very virulent strains. Only the hypervariable region between the *AccI* and *SpeI* sites at residues 206 and 350 (145 amino acid residues) was used in the phylogenetic analysis, the greatest amount of amino acid sequence variation in VP2 among various strains of IBDV occurs in this region. Numerous reports found

phylogenetic analysis of this region useful for predicting the relationship of different IBDV strains and enabled epidemiological studies of emerging new strains (Brown *et al.*, 1994; Cao *et al.*, 1998; Chen *et al.*, 1998; Dormitorio *et al.*, 1997; Sellers *et al.*, 1999; Zierenberg *et al.*, 2000). The cluster pattern derived from this region has a high degree of similarity with those constructed from the complete sequence of VP2 or the VP2-4-3 polyprotein (Brown *et al.*, 1997; Yamaguchi *et al.*, 1997).

The two NZ isolates were obtained from chickens on the same farm, sampled in two consecutive years. There were only two nucleotide differences in the region sequenced between these two isolates, suggesting that little genetic drift had occurred during the 1-year time period. Passage of field isolates of virus in tissue culture has the potential to select for subpopulations or for mutations adapted to the *in vitro* conditions. However, in this study the two isolates used for genomic characterisation had been passaged less than five times. Also, since isolates from two different years were shown to have almost identical sequences, it is unlikely that significant mutation had occurred during *in vitro* propagation. Further, the *in vivo* pathogenicity tests, which used infected SPF embryo homogenate would be expected to have revealed any more virulent subpopulation should that have existed in the original field material.

Based on the finding that the infected SPF birds showed no clinical signs, the NZ isolates could be classified as apathogenic strains. However, the bursal atrophy index (B/BW ratio) and the histological lesion scores indicate that the NZ isolate was capable of causing lymphoid depletion and possibly immunosuppression and should thus be classified as a mild strain of IBDV. Although the genetic markers responsible for antigenicity and pathogenicity of IBDV have not been fully determined, the NZ isolates contained many of the substitutions reportedly associated with attenuated or mild strains. Phylogenetic analysis confirmed the close relationship between the isolates in this study and attenuated strains of IBDV. Although the source and the route by which IBDV entered NZ remain unknown, this study supports the hypothesis that in 1993, an attenuated vaccine strain of the virus was inadvertently introduced into the NZ poultry population (O'Neil, 1995). The control programme which began in 1994 by the poultry industry has effectively reduced the seropositive farms and, in 1999, only one layer farm was tested seropositive (Ryan *et al.*, 2000). However, constant field monitoring of any possible remaining virus should be carried out, not only in the commercial sector,

but also in the non-commercial sector (such as backyard chickens) and wild birds. Before the virus is totally eradicated from the country, it is conceivable that the high mutation rate of the RNA-polymerase dependent RNA virus in the field could lead to a new strain with different antigenic structure and virulence.

5.5 SUMMARY

Isolates of IBDV were obtained from chickens on the IBDV-positive farms described in Chapter 3 in New Zealand in 1997 and 1998. An *in-vivo* pathogenicity study carried out in SPF chickens demonstrated the low virulence of one of the virus isolates. The nucleotide sequences of the hypervariable region of the VP2 gene of two isolates were determined and compared with published sequences of strains from other countries. The deduced amino acid sequence of the two New Zealand IBDV isolates showed 100% identity with each other, suggesting that little genetic drift had occurred. Phylogenetic analysis showed that the New Zealand isolates were more closely related to two attenuated IBDV strains (Cu1 and PBG98) than to classical (STC and 52/70), very virulent (DV86), variant (variant E) or Australian strains. The results support the hypothesis that an attenuated strain of the virus was inadvertently introduced into the NZ poultry population in 1993.

PART II LITERATURE REVIEW OF VIRUS AND CAPSID PROTEIN STRUCTURAL STUDY

II.1 INTRODUCTION

To date, although various "Hot Spots" within the VP2 gene have been identified in the different sequences of the IBDV genome that have been published, the true pattern of the antigenic and virulence markers has still not been unequivocally identified. The pathotyping of a particular IBDV isolate is based on both the mortality rate and observation of lesions from *in-vivo* challenge tests in SPF or commercial birds or from the inoculation of embryonating eggs with the virus isolate (Lukert *et al.*, 1991) The precise location of the amino acid residues in the IBDV capsid protein that are predicted to be responsible for virulence factors, their possible interactions with both host receptor and antibodies, and the way that sequence differences cause functional changes in these interactions is not known. One of the most effective ways to explore the effects of changes in amino acid sequences within the IBDV structure, especially the major capsid protein VP2, is to determine its three-dimensional structure to high resolution using X-ray crystallography. Such a structure is necessary to understand the precise nature of the interaction between the virus and host, and how sequence changes in the viral epitopes are involved in antibody-protein interactions.

Structural studies of *Human rhinovirus* (HRV) and *Poliovirus* at high resolution together with later studies of the virus-antibody complexes revealed the presence of a ligand-binding canyon in viral protein 1 (VP1) (Hogle *et al.*, 1985; Rossmann *et al.*, 1985). A number of antiviral compounds have been reported to bind in this pocket and inhibit the replication of the virus by either inhibiting disassembly, disrupting binding to the host cell receptor or preventing the delivery of viral nucleic acid to host cell (Pevear *et al.*, 1989). Thus, structural studies of virus particles not only aid the understanding of native viral function, but also are a valuable tool in the design of antiviral compounds that target the immunogenic domain(s).

II.2 VIRUS STRUCTURAL STUDY

The first observation that a germ-free fluid extracted from diseased plants could be used to transmit the same disease to healthy leaves was made in 1892 by the Russian botanist D. Iwanowski, working with *Tobacco mosaic virus* (TMV). The Latin word "virus" (poison) was first used by Martinus Beijerink in 1898 to describe the filtered disease plant fluid. Loeffler and Frosch (1898) experimented with material from animals infected with Foot-and-Mouth Disease (FMD), and confirmed that an infectious agent that could pass through filters, which prevent the passage of bacteria, was capable of transmitting the disease in cattle. Many more viruses were characterised by the end of the nineteenth century using both biophysical and biochemical methods. Direct visualisation of viruses, however was not possible until the development of the electron microscope between 1932 and 1936. The rapid development of high resolution electron microscopy plus the introduction of various specimen preparation techniques, especially negative staining (Brenner and Horne, 1959), revolutionised the study of virus structure. As the technology continued to improve, more information about the detailed architecture of virus particles was revealed. Eventually improvements in electron microscopy, together with the availability of X-ray diffraction techniques, resulted in the determination of virus structures at medium to high resolution, so that the individual proteins making up the capsid could be observed in molecular detail.

II.2.1 Electron Microscope (EM)

With modern, high resolution electron microscopes, low structural details of virus particles between 20 and 300 nm can be visualised. These images show the relative orientation of the proteins making up the capsid and their relationship to one another, and may reveal broad structural features such as "canyons" and other surface features (Crowther *et al.*, 1970). However, detailed molecular structure is not seen. The necessity of using metal stains to prepare specimens for visualisation contributes to the lack of detail visible in the early structures determines using this technique. Nevertheless, the overall shape and symmetrical arrangement of the protein components of viruses (icosahedral, helical or non-symmetrical shape), which laid the foundation for understanding viral architecture and the geometrical arrangement of the capsid protein, is readily seen.

II.2.2 Cryo-Electron Microscopy (cryo-EM)

Cryo-EM is the technique of visualising virus particles by observing unstained specimens in a frozen hydrated state using high resolution EM, followed by image reconstruction. Since the virus specimens are preserved in a flash-frozen aqueous environment, the image obtained represents a more "natural" structure and more details. Advances in image processing techniques resulted in both internal and external features of the virus being revealed. Because of the icosahedral symmetry of the capsid of many viruses, three-dimensional models could be constructed from the two-dimensional EM images using Fourier transformation techniques. The resolution of the virus structures obtained using these methods ranged between 10 and 30 Å, allowing the visualisation of far more structural detail than had previously been possible. Cryo-EM requires only a small amount of virus specimens to provide unique structural information within a short time. Since the first report of the reconstruction of an icosahedral virus structure (Crowther *et al.*,1970), more than a hundred virus structures have been determined using cryo-EM. Most of them have not been analysed at higher resolution by X-ray crystallography. A detailed review on cryo-EM can be found in Baker et al (Baker *et al.*, 1999).

II.2.3 Atomic Force Microscopy (AFM)

Atomic force microscopy is a technique for defining a sample's surface by raster-scanning a small tip back and forth over the surface of the sample. The tip is on the end of a cantilever, which is deflected when the tip encounters a feature on the sample surface. This deflection is sensed with an optical lever: a laser beam reflecting off the end of the cantilever onto a segmented photodiode, which magnifies small cantilever deflections into a large change in the relative intensity of the laser light onto two segments of the photodiode. In this way, the AFM constructs a topographical map of the sample surface. Although AFM reveals only surface topology, the combination of data from both AFM and cryo-EM proved to be a powerful tool in increasing knowledge about the detail of virus structure (Muller *et al.*, 1997; Ohnesorge *et al.*, 1997).

II.2.4 X-Ray Crystallography

Despite the developments in electron and atomic force microscopy, X-ray crystallography remains the only method for visualising protein and virus structure at high resolution (2 to 3 Å). Spherical viruses, being uniform molecular assemblies,

crystallise in much the same way as proteins, so that the techniques of X-ray crystallography can be used to determine their structure. Although the structures of simple spherical plant viruses have been studied since the early 1950s using some of the techniques described above, it was only in the 1970s that the tertiary structures of such molecules began to be investigated using X-ray diffraction. This was a consequence of advances in technology, in particular, the availability of intense synchrotron X-radiation and supercomputers to analyse the massive amount of data collected in such structure determinations. The first X-ray structure of a spherical virus determined at high resolution was that of *Tomato bushy stunt virus* (TBSV) (Harrison & Jack, 1975). Since then, numerous plant, insect and mammalian viruses have been studied at moderate to high resolution using X-ray crystallography. These are listed in Table II-1.

A number of structures of plant spherical viruses have been studied at low to moderate resolution. These include TBSV (Harrison & Jack, 1975; Jack & Harrison, 1975; Ziegler *et al.*, 1974), *Southern bean mosaic virus* (SBMV) (Johnson *et al.,* 1976; Johnson *et al.*, 1974) and TMV (Jones & Liljas, 1984). Mammalian viruses, which are larger and more complex, make data collection and phase determination at high resolution technically challenging. It is also difficult to obtain a large quantity of highly purified and stable virus for structural studies in contrast to plant viruses, which are relatively easy to isolate in gram quantities. Finally, it has proved difficult to produce crystals of mammalian viruses that diffract well. The crystal structure of *Poliovirus* was the first animal virus structure determined by X-ray crystallography (Finch & Klug, 1959). Such structures initially took many years to complete, however, by utilising noncrystallographic symmetry (symmetry that pertains to a virion, but is not propagated throughout the crystals) (Rossmann & Blow, 1962; Rossmann & Blow, 1963) has greatly simplified determining the structure of viruses and has increased the precision of the analysis compared to earlier methods(Arnold *et al.*, 1984).

The X-ray structure of the first viral pathogen in veterinary medicine was that of the *Foot-and-Mouth Disease virus* (FMDV), which was determined by David Stuart and Graham Fox in 1987(Fox *et al.*, 1987). This structure, together with the structures of *Rhinovirus* (causing common cold in humans) (Kim *et al.*, 1989; Verdaguer *et al.*, 1999), and *Poliovirus* (causing poliomyelitis in humans) (Hogle, 1982; Hogle *et al.*, 1985), all of which belong to the family *Picornaviridae*, are the only mammalian

viruses which have been studied extensively at high resolution by X-ray crystallography. Sequence comparison of each of the four structural proteins that comprise the capsid (VP1, VP2, VP3 and VP4) from these three viruses, showed there was little homology among.their sequences. Despite this, all the capsid proteins of these three viruses exhibit striking structural similarities (Acharya *et al.*, 1989; Rossmann *et al.*, 1985). Intriguingly, these structural similarities extend to the coat proteins of some of the spherical plant viruses such as TBSV and SBMV (Akimoto *et al.*, 1975; Harrison & Jack, 1975; Jack & Harrison, 1975; Johnson *et al.*, 1976; Johnson *et al.*, 1974; Ziegler *et al.*, 1974), indicating that *Picornaviruses* and the spherical plant viruses may have diverged from a common ancestor.

So far, X-ray crystallography is the most powerful biophysical technique for studying high resolution structures of macromolecular assemblies. However, the major drawback in X-ray crystallography analysis of viral structure is the necessity of purifying enough virus for crystallisation and obtaining diffraction quality crystals.

Table II.1: Summary of viruses that have been studied by X-ray crystallography (data extracted from VIPER, http://mmtsb.scripps.edu/viper.html).

Virus group/name	Virus family/group	T number	Reference
Animal and Insect Viruses			
Black beetle virus	Nodaviridae	3	Sehnke et al. J. of Crystal Growth (1998) 90,222-230.
Bluetongue virus	Reoviridae	13	Grimes et al. Virology (1995) 210,217-220.
Bovine enterovirus	Picornaviridae	P3	Smyth et al. J. Mol. Biol. (1993) 231,930-932.
Canine parvovirus (CPV) Empty	Parvoviridae	1	Wu et al. Acta Cryst. (1993) D49,572-579.
Feline panleukopenia virus	Parvoviridae	1	Agbandje et al. Proteins:Struc. Func. Gen. (1993) 16,155-171.
Flock house virus	Nodaviridae	3	Fisher et al. Acta. Cryst. (1992). B48,515-520.
Foot-and-Mouth Disease virus	Picornaviridae	P3	Fox et al. J. Mol. Biol. (1987) 196,591-597.
Hepatitis B virus	Hepadnaviridae	4	Zlotnick et al. Acta Cryst. (1999) D55, 717-720.
Human rhinovirus (HRV) 1A	Picornaviridae	P3	Kim et al. J. Mol. Biol. (1989) 210,91-111.
HRV 2	Picornaviridae	P3	Verdaguer et al. Acta Cryst. (1999) D55,1459-1461.
HRV 3	Picornaviridae	P3	Zhao et al. Structure (1996) 4, 1205-1220.
HRV 14	Picornaviridae	P3	Erickson et al. Proc.natl. Acad. Sci. USA(1983)80,931-934.
HRV 16	Picornaviridae	P3	Oliveira et al. Structure (1993) 1, 51-68.
Mengo encephalomyocarditis virus	Picornaviridae	P3	Luo et al., Science (1987) 235,182-191.
Murine minute virus	Parvoviridae	1	Llamas-Saiz et al. Acta Cryst. (1997) D53,93-102.
Murine polyomavirus	Papovavirus	7	Stehle and Harrison, Stucture (1996) 4,183-194.
Poliovirus Empty	Picornaviridae	P3	Basavappa et al. Prot. Sci. (1994) 3,1651-1669.
Simian virus 40	Papovavirus	7	Lattman et al. Science (1980) 208,1048-1050.
Theiler MEV BeAn	Picornaviridae	P3	Luo et al. Proc., Natl. Acad. Sci.USA (1992) 89,2409-2413.
Theiler murine encephalo-myelitis virus	Picornaviridae	P3	Grant et al. Proc.Natl. Acad.Sci. USA (1992) 89,2061-2065.
Plant Viruses			
Alfalfa mosaic virus	Bromoviridae	1	Yusibov et al., J.Gen. Virol. (1996) 77,567-573.
Bean pod mottle virus	Comoviridae	P3	Sehnke et al. J. of Crystal Growth (1998) 90,222-230.
Carnation mottle virus	Tombusviridae	3	Morgunova et al. FEBS Letters (1994) 338,267-271.
Cowpea chlorotic mottle virus	Bromoviridae	3	Speir et al., Virology (1993) 193,234-241.
Cowpea mosaic virus	Comovirus	P3	Lin et al. Virology (1999) 265,
Coxsackievirus B3	Picornaviridae	P3	Muckelbauer et al. Acta Cryst.(1995) D51,871-887.
Cricket paralysis virus	Picornaviridae	P3	Tate et al. Nature Struc. Biol. (1999) 6,756-774.
Cucumber mosaic virus	Bromoviridae	3	Smith et al. J. Virol (2000) 74, 7578-7586.
Densovirus	Parvoviridae	1	PDB entry
Echovirus 1	Picornaviridae	1	PDB entry
Nodamura virus	Nodaviridae	3	PDB entry
Nudaurelia capensis virus	Tetraviridae	4	Cavarelliet al. Acta Cryst. (1991) B47,23-29.
Physalis mottle virus	Tymovirus	3	Krishna et al., J., Mol. Biol. (1999) 289,919-934.
Red clover mottle virus	Comoviridae	P3	Lin et al. Virol. (2000) 74,493-504.
Rice yellow mottle virus	Sobemovirus	3	Qu et al., (2000) *in press*
Satellite panicum mosaic virus	Tombusviridae	3	Day et al. J. Mol. Biol. (1994) 238,849-851.
Satellite tobacco mosaic virus	Tobamovirus	1	PDB entry
Satellite tobacco necrosis virus	Tombusviridae	1	Liljas et al., J. Mol. Biol.(1982) 159,93-108.
Sesbania mosaic virus	Sobemovirus	3	Subramanya et al. J. Mol. Biol. (1993) 229,20-25.
Southern bean mosaic virus	Sobemovirus	3	Johnson et al.j. Ultrarruc. Res. (1994) 46,441-451.
Tobacco necrosis virus	Necovirus	3	Fukuyama et al. J. Mol.Biol. (1987) 196,961-962.
Tobbacco ringspot virus	Nepovirus	P3	PDB entry
Tomato bushy stunt virus	Tombusviridae	3	Harrison and Jack, J. Mol. Biol.(1975) 97,173-191.
Turnip crinkle virus	Carmovirus	3	Hogle et al. J. Mol. Biol. (1986) 191,625-638.
Turnip yellow mosaic virus	Tymovirus	3	Canady et al. (1995) Proteins: Struc. Func. Gen. 21,78-81.
Others			
Bacteriophage FR	Leviviridae	3	Bundule and Pumpens J. Mol. Biol. (1993) 232,1005-1006.
Bacteriophage G4	Microviridae	1	McKenna et al. J. Mol. Biol. (1996) 256,736-750.
Bacteriophage GA	Leviviridae	3	Tars et al. J. Mol. Biol. (1997) 271,759-773.
Bacteriophage HK97	Leviviridae	3	Wikoff et al., Acta Cryst. (2000) D55,763-771.
Bacteriophage MS2	Leviviridae	3	Valegard et al. J. Mol. Biol. (1986) 190,587-591.
Bacteriophage PP7	Leviviridae	3	reference
Bacteriophage Qb	Leviviridae	3	Valegard et al. Acta Crust. (1994) D50,105-109.
Bacteriophage x174	Microviridae	1	Willingmann et al. J. Mol. Biol. (1990) 212,345-350.

II.3 VIRUS QUATERNARY STRUCTURE AND SYMMETRY

The outer shell of the virus referred to as the capsid, forms a protective shell around the nucleic acid, either single-stranded or double-stranded RNA or DNA. Together they are referred to as the virus particle or virion. The capsid is built up by one or a few kinds of protein subunits (sometimes referred to as capsomers) encoded by the viral genome. In more complex viruses, the capsid may be surrounded by an envelope constructed of a lipid bilayer and with inserted glycoproteins. Crick and Watson (Crick & Watson, 1956) proposed that since the small size of viral nucleic acid limits the number of proteins that can be encoded by its genome, some form of regular or symmetrical packing of repeats of a limited number of protein molecules was required to construct the viral shell. The quaternary structures of virus capsids depend primarily upon protein-protein interactions resulting in the formation of either a helical rod or an icosahedral capsids.

For helical viruses, such as TMV, the coat protein subunits associate to form a helical tube. In this structure, the protein subunits are placed around the circumference of a circle to form discs that are then arranged into a helical structure that encloses its nucleic acid. The rods formed are normally 15 to 19 nm wide with a length between 300 and 500 nm depending on the size of the viral genome. The helix structure is characterised by its amplitude and pitch. TMV is one of the well studied helical nucleocapsid viruses. A review of such structures can be found in Bloomer et al (Bloomer, 1978).

In 1956, Crick and Watson (Crick & Watson, 1956) proposed that the protein coat structures of most simple spherical viruses consisted of regular polyhedra identically arranged on the surface of a sphere. For each of these to have an identical environment, icosahedral symmetry is required utilising a minimum of 60 subunits to completely cover the surface of the sphere. An icosahedron is composed of 20 facets, each an equilateral triangle, and has twelve vertices, as shown in Figure II-1. This shows the symmetrical elements of such an arrangement: six 5-fold axes pass through the vertices; ten 3-fold axes extending through each face; fifteen 2-fold axes through each edge as shown in Figure II-1. As more EM images and X-ray structures of spherical viruses were reported, it became obvious that all spherical viruses display an icosahedral arrangement of capsid proteins, but with the number of subunits per virus often being larger than 60. Later in 1962, Caspar and King (Caspar & Klug, 1962) proposed the

concept of quasi-symmetry as the basis for the structural characterisation of isometric viruses. They suggested that all surface lattices are identified by a T (triangulation) number that is equal to the number of quasi-equivalent units in an icosahedral asymmetric unit. An icosahedron has 20 equilateral triangular facets, therefore 20T structure units. By subdividing the triangular faces of an icosahedron into integral numbers of equally sized equilateral triangles, an icosadeltahedron results. There are 60T subunits in the whole capsid. The value of T must be restricted, such that $T = (h^2 + hk + k^2)f^2$ where h, k and f are integers, if the quasi-symmetry is to be retained. Although the quasi-equivalent environments usually contain identical objects in the proposal, it was later found that some viruses could have an alternative arrangement of their icosahedral grouping in terms of structure units and capsomers. For such structures, the subunits do not need to be identical to form the predicted lattice. As shown in Figure II-2, this arrangement was described as "pseudo-symmetric" with a P rather than a T number. Within each of these subunits there may be a single protein (T=1 lattice), three copies of a single protein (T=3 lattice), or three different protein domains each with a unique primary structure but similar tertiary structure (P=3) as shown in Figure II-2.

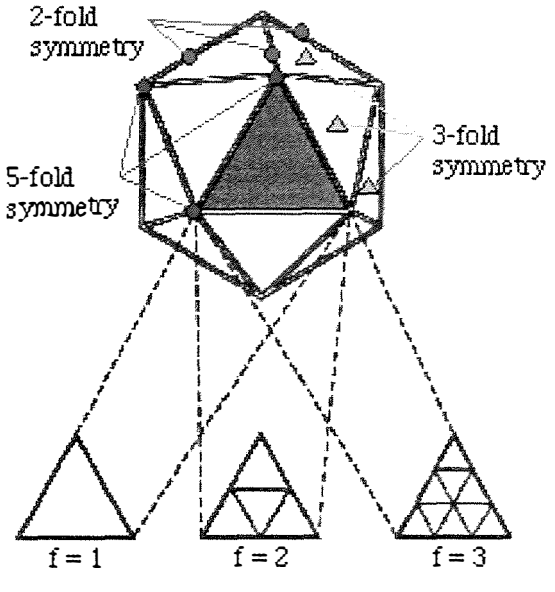


Figure II.1: Diagram of a regular icosahedron showing the 2-fold, 3-fold and 5-fold rotational symmetry axes. (The Figure is copied from a web site located at:http://www.tulane.edu)

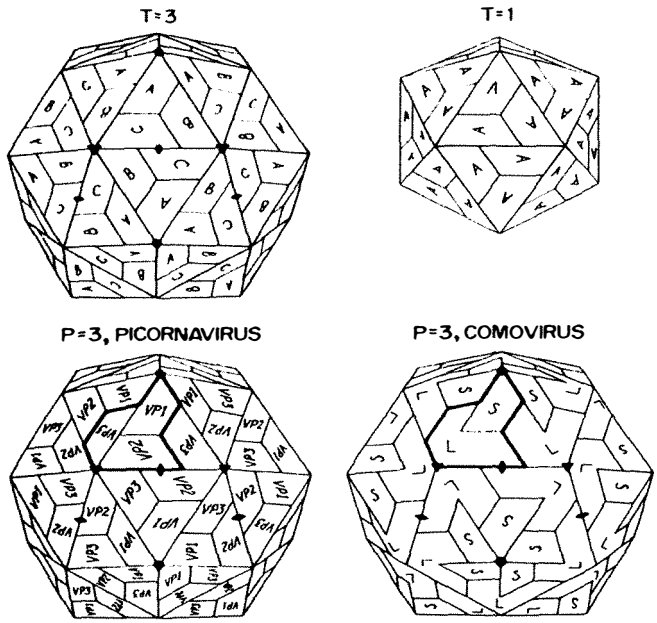


Figure II.2 Examples of the virus capsid protein in T unit. The T=1 shell contains 60 subunits related by icosahedral symmetry. The asymmetric unit of the T=3 capsid is the central triangle containing subunit A, B and C, the subunits labelled A, and C have the same amino acid sequence but are in slightly different environment. The P=3 picornavirus shell, technically a T=1 particle, is closely related to the T=3 shell, being formed by 180 ß-barrel domains. The three subunits VP1, 2 and 3 are distinct proteins. The comovirus formed by only two protein types (L and S), the large protein (L) is composed of two ß-barrel domain covelently linked together, the small protein (S) is a single ß-barrel domain. (Figure copied from Schnke et al (1988)).

II.4 CAPSID PROTEIN TERTIARY STRUCTURE

In most of the viral capsid proteins reported, the most common folding motif comprised two back-to-back, four-stranded β–sheets that follow the "jelly roll topology" proposed by Richardson in 1981 (Richardson, 1981)(Figure II-3). This was demonstrated for the capsid proteins of the family *Picornaviridae* (*Rhinovirus*, *Poliovirus*, *Mengo virus* and FMDV), where the VP1, VP2 and VP3 capsid proteins all display the eight-stranded, antiparallel β-barrel motif (Acharya *et al.*, 1989). A comparison of these structures and the role of the β-barrel domain have been reviewed by Rossmann and Johnson (1989).

A different folding motif in the coat protein of other viruses has been reported recently. The outer capsid of *Bluetongue virus* (family *Reoviridae*) has been shown to contain proteins that have a helical inner domain and a β-sandwich outer domain (Grimes *et al.*, 1995). The protein core of *Hepatitis B virus*, whose structure was determined using both cryo-EM and X-ray diffraction, was shown to be entirely α-helical (Bottcher *et al.*, 1997).

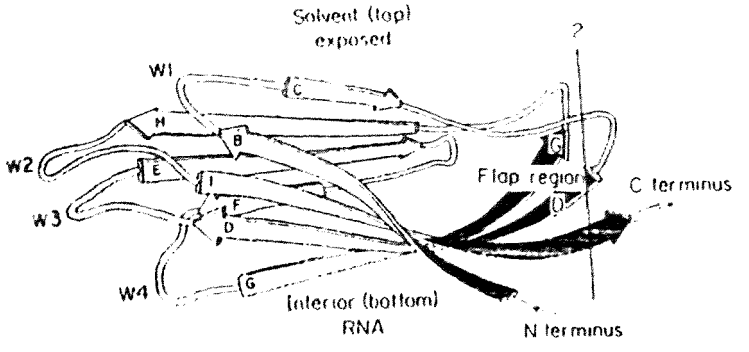


Figure II.3: The jellyroll fold consisting of 8 β-strands (B through I) forming 2 antiparallel sheets. The wedge shape is apparent with its sharp end formed by loops W1 to W4 connecting strands B and C, I and H, D and E, and F and G respectively. The fold is oriented relative to the indicated 2-fold axis for structures with a triangulation number of 3. (Figure copied from Chelvanayagam et al, 1992)

II.5 VIRAL CAPSID FUNCTIONS

Crystallographic studies of virus structures have not only elucidated the molecular anatomy of the viral capsid/coat protein(s), but have also revealed the functions of the capsid proteins involved in viral assembly, in the packaging of viral RNA/DNA, details of the infection process, how the virus binds to host receptors, how mutation in sequence lead to the virus escaping host immunity and how they function as primers for viral genomic replication (Rossmann & Johnson, 1989; Sehnke *et al.*, 1988). Knowledge of the specific interactions between the different subunits reveals the biological and biochemical functions of the virus to the environment or host. A brief review of the functions of the viral capsid proteins follows:

II.5.1 Protection of Nucleic Acid Genome

Undoubtedly, assembly of the protein subunits (capsomers) into a protective capsid to protect the viral genetic material from physical, chemical or enzymatic damage from the external or host environment is the major function of the viral capsid. This process is not yet fully understood. It has been shown that when purified TMV RNA and coat protein were mixed together, helical virus particles formed that were identical to the native form of the virus, indicating that the virus assembly is one of minimum free energy (Fraenkel-Conrat & Williams, 1955). The strong protein-protein interactions between capsomer subunits maintains the structure of the virion. These interactions include hydrophobic, hydrogen bonds and salt bridges (Jurnal, 1984).

II.5.2 Protein-Nucleic Acid Interactions

The specificity of the interaction between the capsid protein and the viral nucleic acid is essential for the correct packaging of the virus genome in an environment that contains an enormous amount of host cellular nucleic acid. Various studies have identified the specific sequences in the viral genome that specifically bind to its coat protein aggregates (Casjens, 1985; Wiley, 1985; Wiley, 1984). Although some studies have shown that this packaging of the viral genome of some simple viruses is nonspecific, for most viruses it has been shown to be a process that is controlled by the preformed capsid. In this process, the DNA/RNA is inserted into the capsid via a transient packaging complex formed between the nucleic acid material and a docking protein (Wiley, 1985; Rossmann & Johnson, 1989).

II.5.3 Receptor Recognition

Most viruses initiate infection and entry into host cells by attaching to receptors on the host cell membrane. The receptor attachment site of most viruses has to remain conserved in order for viruses to bind to the same cellular receptors. In contrast the viral capsid is able to undergo rapid mutation in order to escape the specific neutralising antibodies produced against it by the immune system. Structural studies on viruses from the family *Picornaviridae*, particularly the HRV showed the presence of major canyons or pits on the outer surface of the capsid. They were found to form receptor-binding sites through which the virus is targeted to specific cells (Luo *et al.*, 1987; Smith & Baker, 1999; Smith *et al.*, 1993b). A variety of data indicate that these canyons are the sites of receptor attachment in *Rhinoviruses* (Luo *et al.*,1987). While such depressions

would hinder the binding of antibodies to the virus, they may provide specific binding sites for receptors on cells. In this way, the virus is able to conserve its ability to bind to specific receptors, while still rapidly changing its more exposed surfaces to avoid host immune surveillance (Luo *et al.*, 1988). The discovery of these "canyons" was instrumental in the development of some antiviral compounds against human rhinovirus and was a direct contribution from the structural information provided by the X-ray structure of the capsid protein (Smith & Baker, 1999).

In spite of the close relationship of FMDV to HRV, the former does not seem to have any major canyons on its surface. This may be related to the size of its capsid proteins, VP1, 2 and 3, which are smaller than those of HRV, resulting in a generally thinner protein shell. In FMDV, the cores of each of these proteins are placed at the same radius and in similar orientations to those of the other *Picornaviruses*. VP1 shows the most significant rearrangement. For VP2 and VP3, the axis of the β-barrel is approximately in the plane of the capsid whereas for VP1 it is canted up from the plane towards the 5-fold axis (Acharya *et al.*, 1989). Structural studies of the individual capsid proteins of FMDV successfully identified the cell attachment site, the calcium-binding site and the major epitope of the virus (Acharya *et al.*, 1989; Logan *et al.*, 1993). In 1993, Logan et al reported the identification of the major immunogenic site on FMDV. They demonstrated that the attachment of FMDV to its cellular receptor involves a long, highly antigenic loop containing the conserved sequence, Arg-Gly-Asp, a motif known to be a recognition element in many integrin-dependent cell adhesion processes. Examination of the three-dimensional structure of FMDV at 2.9 Å resolution revealed that the major neutralising region of the virus comprises residues 133-158 and 141-160 of VP1. These residues are part of a prominent loop in the protein structure (Acharya *et al.*, 1989). Other studies showed that peptides incorporating these sequences promote an immediate immune response with the production of high levels of neutralising antibody (Bittle *et al.*, 1982). Thus, knowledge of the structures of the FMDV itself and its capsid proteins, was critical for the identification of the most immunogenic portion of the capsid, and may lead to the development of improved and novel potent peptide vaccines and the design of anti-viral drugs.

Another X-ray crystallography study on the capsid protein of *Bluetongue virus*, explored the structure of VP7. Analysis of this structure resulted in the identification of

a possible cellular attachment site, which contained a conserved Arg-Gly-Asp tripeptide (Grimes *et al.*, 1995). Three dimensional structure of *Canine parvovirus* showed that there are two major depressions on the viral surface that are reminiscent of the "canyon" found in *Rhino-* and other *Picornaviruses* (Rossmann *et al.*, 1985). All of this emerging knowledge of viral receptor sites in different viruses expands our understanding of the roles played by each particular coat protein in initiating viral infection, and may subsequently lead to prevention methods for virus infection rather than treatment after infection has occurred. The recently approval specific influenza neuraminidase inhibitor drug, oseltamivir phosphate, is a good example of application of structural information on the active site of the viral enzyme to the design of a drug which inhibits its action, thereby inhibiting the release of virus particles from infected cells (Kim *et al.*, 1997).

II.5.4 Virus-Antibody Interaction

Antibodies to viruses act by binding to the virus surface. However, only certain surface areas are useful targets for neutralisation. The neutralising antibodies can either cross-link with several virus particles or interfere with viral functions such as cell attachment, membrane penetration and uncoating. Attempts to study the virus-antibody interaction at a molecular level by X-ray crystallography were not initially successful. The virus-antibody complexes formed immune precipitates rather than crystallising. Thus far, crystallographers have been able to study only structures of the Fab fragments of antibodies complexed with specific capsid proteins (Chen *et al.*, 1998; Chiu & Smith, 1994). The structure of a complex between rhinovirus and its Fab antibody fragment has been revealed using a combination of a cryo-EM reconstructed map of the complex and the X-ray structures of each separated structure of the Fab or rhinovirus by X-ray diffraction (Rossmann *et al.*, 1985; Smith *et al.*, 1993a; Smith *et al.*, 1993b). Analyses of the structure confirmed that the antibody bound in a bivalent manner across the icosahedral two fold axes by flexing at the Fab elbow (Smith *et al.*, 1993b).

II.5.5 Other Functions of Viral Capsid Protein

After the packaging of viral genome is completed, the capsid undergoes a controlled, limited proteolysis referred as "capsid maturation". This process often involved proteolytic cleavage of protein components and transforms the viral particle from an unstable pro-virion to a mature stable virion (Curry *et al.*, 1997; von Schwedler *et al.*, 1998), and enables the virus to deliver its nucleic acid to a suitable host cell for

replication. The processing of capsid also involves the binding of metal ions (Hadfield *et al.*, 1997; Zhao *et al.*, 1997), formation of disulfide bonds (Li *et al.*, 1998; Sapp *et al.*, 1998) and other covalent links (Hendrix & Duda, 1998), and phosphorylation (Lewis *et al.*, 1998).

II.6 SUBUNIT CAPSID PROTEIN STUDY

Many viruses, especially the enveloped viruses, are difficult to study at moderate or high resolution as their complex, nonsymmetric structure prevents crystallisation. Structure determination of the individual subunit capsid proteins has proved a valuable tool in understanding the structure of these groups of viruses and their interaction between each subunit capsid protein. For example, the hexon unit of *Adenovirus*, a virus without envelope but with spikes that inhibit its crystallisation, was the first animal viral subunit protein to be crystallized (Pereira *et al.*, 1968). The haemagglutinin and neuraminidase protein structures of *Influenza virus*, an enveloped virus, have each been determined at high resolution by first removing them from the membrane by proteolytic cleavage, then crystallizing the isolated proteins (Colman *et al.*, 1983; Varghese *et al.*, 1983; Wiley & Skehel, 1987). The structures of subunit capsid proteins have been shown to be immunologically and morphologically similar to those structural components of the intact virions. The above-mentioned two viruses (*Adenovirus and Influenza virus*) are the most extensively studied subunit proteins at high resolution. These studies have lead to significant new insights into the architectural principles involved in the design and assembly of viral particles and interaction with their hosts. Further review of the two protein structure can be found in various publications (Athappilly *et al.*, 1994; Burnett, 1985; Wiley & Skehel, 1987).

Advances in molecular biology have resulted in the identification of the coding region of many viral subunit proteins and allowed expression of recombinant capsid proteins in various systems. This has made the structural determination of individual viral capsid proteins more accessible. Extensive structural studies on *Adenovirus* proteins by expressing the recombinant hexon, penton and fiber proteins provided information that proved essential to understanding the function of this complex virus (Fender *et al.*, 1997; Schoehn *et al.*, 1996). The three separated protein shells of the complex icosahedral *Rotavirus* were expressed in a baculovirus expression system and each

protein was crystallised (Petitpas *et al.*, 1998). The full length of VP7 of *Bluetongue virus* was expressed and crystallised, and the structure data showed that the conformation of the protein was the same as that in the intact virus (Basak *et al.*, 1992; Grimes *et al.*, 1995). Structure studies of each viral subunit domain have since been reported using various recombinant proteins expressed in different expression vectors (Belyaev *et al.*, 1994; Petitpas *et al.*, 1998; Saad *et al.*, 1999; Yusibov *et al.*, 1996). This approach to understanding virus structure by studying its parts individually will accelerate the rate of our understanding of viruses, and their interaction with host cells.

II.7 THE AIMS AND SCOPE OF PART II

In the part 2 of this thesis, crystallisation trials were carried out on IBDV and recombinant VP2 capsid protein. The purification experiment carried out on the IBDV is reported in Chapter 6. A preliminary crystallisation screen was carried out on the purified IBDV, and preliminary X-ray diffraction trials performed on suitable virus crystals. Biochemical characterisation of the native IBDV capsid protein-VP2 was necessary before undertaking the cloning and expression of recombinant VP2 as reported in Chapter 7. N- and C-terminal sequencing, mass-spectrometry and gel electrophoresis were used to determine the actual cleavage site of the VP2 protein and any post-translation modifications that were present on the native proteins.

Cloning and expression of the major capsid protein of IBDV, VP2, using a Baculovirus Expression system (Bac-to Bac baculovirus expression system, Lifetech) is described in Chapter 7. With the expressed and purified recombinant VP2 capsid protein, preliminary crystallisation screens were carried out on the purified recombinant VP2 protein to investigate the crystallisation condition(s) for the recombinant VP2 and obtain suitable crystals for X-ray diffraction trial.

The long term goals of this project were to determine the three-dimensional structure of IBDV and/or the VP2 capsid protein using X-ray crystallography to gain better understanding of how mutations in the amino acid sequences of VP2 are responsible for the changes in virulence and phenotype.

CHAPTER 6: IBDV PURIFICATION AND CRYSTALLISATION

6.1 INTRODUCTION

The earliest attempt to study the structure of IBDV was to classify the virus in terms of the established taxonomic groups based on the structural properties of the virus as revealed by negative-staining electron microscopy. In the early 1960s, IBDV was tentatively classified as a member of the family *Reoviridae* based on the morphology using electron microscopy of ultrathin sections of infected tissue from chickens (Cheville, 1967; Lunger & Maddux, 1972). Early low resolution electron microscopy studies showed that IBDV appeared to be a nonenveloped icosahedron with a diameter that ranged from 55 to 65 nm (Almeida & Morris, 1973; Harkness *et al.*, 1975; Nick *et al.*, 1976). Hirai and Shimakura (Hirai & Shimakura, 1974; Hirai *et al.*, 1974) first reported the ultrastructure of IBDV using the negative-staining technique and electron microscopy. The details of the morphology suggested that the virus measured about 55 nm in diameter and had no envelope. It showed the expected icosahedral symmetry, with the capsid constructed from a single layer of 32 capsomers arranged in 5:3:2 symmetry, to give T = 3. Later, Hirai et al (Hirai *et al.*, 1979) re-examined the IBDV structure using high-resolution electron microscopy and rotational enhancement of the image. They found that the virus particle had a diameter of 62 to 63 nm, that the triangulation number appeared to be 3 and that there were apparently 32 morphological units of the capsid. Further examination of the capsid symmetry of the three members in the tentatively named Birnavirus group of bisegmented double-stranded RNA viruses, (including IBDV) was carried out using high resolution negative staining and shadowing EM techniques followed by rotational enhancement analysis. This showed that IBDV consists of single-shelled particles that have capsid diameters between 61 – 65 nm. These are clearly distinguishable from the double-shelled *Reoviruses* which are between 80 – 85 nm in diameter (Ozel & Gelderblom, 1985). The capsid symmetries of IBDV and *Reovirus* are skew, with a triangulation number T = 13.

Analysis of unstained, frozen, hydrated virus particles using electron microscopy combined with image processing resulted in a three-dimensional map of IBDV being constructed at 20 Å resolution (Bottcher *et al.*, 1997). The map showed that the capsid of IBDV belongs to a T = 13 lattice, with trimeric clustering of the subunits. The overall thickness of the capsid is about 9 nm, and the relative arrangement of the trimers produces a honeycomb pattern on a roughly spherical surface with an outer radius between 31 and 33 nm. As they have inner radii between 26 and 30 nm, the trimers have triangular or Y-shaped profiles and pack closely to form a nearly continuous shell. The outer surface contains 260 trimers with some capsid proteins protruding from an almost continuous shell, whereas the inner surface of the capsid has only 200 trimers. A possible interpretation of the features seen in this three-dimensional map was that the outer surface of the capsid was formed almost entirely by VP2. The Y-shaped features seen on the inside of the capsid could be formed by part of VP3, with the basic region of the protein extending inwards to interact with the packaged RNA. The material forming the rim around each of the fivefold axes on the inner surface of the capsid might then correspond to VP4.

Details of the structure of IBDV at atomic level have, until recently, been obtained primarily by electron microscopy at about 20 Å resolution. To obtain structural information at higher resolution, an X-ray diffraction study of either the intact virus, or the individual coat proteins is required. A requirement for such study is suitable virus crystals or crystals of the coat proteins. The purification of IBDV, and crystallisation trials undertaken to grow diffraction quality crystals of IBDV are reported in this chapter. The cloning, expression, biochemical characterisation and crystallisation trials on the coat protein VP2 are described in Chapter 7.

6.2 MATERIALS AND METHODS

6.2.1 Virus Sample

The IBD virus used in the study were supplied from SELECT MERIAL LABORATORIES, Georgia, USA (MAF import permit # 1999007791). The attenuated vaccine virus strain was cultured in primary chicken embryo fibroblast cells in roller

flasks, in 199 medium supplemented with 5% foetal bovine serum. Other additives in the medium included minimum amino acids, protein lyophilisation stabilizer and antibiotics. Multiplied viruses were grown to a titre of 8.0 Log 10 $TCID_{50}$/mL, then collected in bulk (approximately 800 mL per bottle). A total of 20 litres of virus suspension was supplied. The virus was kept at –70 °C during storage and transportation.

6.2.2 Virus Purification

Different methods to purify the IBD virus were tested and a best method further developed to obtain the highest recovery and purity of the virus particles was used for crystallisation trials. Final yields of concentrated virus were examined by electron microscopy as described in chapter 1 and analysed by SDS-gel electrophoresis as described below. Only those preparations that were free from contamination by other proteins were used in crystallisation trials.

(a) Ammonium Sulfate Precipitation Method

After removal of cellular debris from the virus culture suspension by centrifugation at 10,000 x g for 30 minutes at 4 °C, solid ammonium sulfate was added slowly to the virus supernatant with stirring at 4 °C to 85% saturation. When all solid ammonium sulfate had completely dissolved, the suspension was equilibrated for 2 hours or overnight at 4 °C. The virus was pelleted using centrifugation (10,000 x g for 30 minutes at 4 °C), and the supernatant discarded. The pellet was resuspended in TBS buffer (0.01M Tris-HCl, 0.1M NaCl, pH 7.6) to give a volume of about 1% of the initial virus suspension. Remaining insoluble materials were removed by centrifugation at 10,000 x g for 5 minutes. The resuspended precipitate was then dialysed against TBS buffer to remove residual ammonium sulfate. Virus particles were concentrated by ultracentrifugation at 120,000 x g on top of a 25% sucrose cushion for 2 hours at 4 °C. After discarding the supernatant, 100 µL of TBS buffer was added to the pellet and left to stand at 4 °C overnight. All dissolved pellets were resuspended gently by aspirating through a narrow pipette tip a few times and pooled. The pooled suspension was recentrifuged at low speed of 5,000 x g for 15 minutes to remove any insoluble material before further purification by ultracentrifugation in a CsCl isopycnic gradient.

(b) Polyethylene Glycol Precipitation Method

Solid PEG 6000 was slowly added to the virus suspension to a concentration of 7% (w/v) followed by solid NaCl to a concentration of 0.5 M. The resulting solution was centrifuged at 10,000 x g for 30 minutes to pellet the virus. The supernatant was removed by decanting and the pellet redissolved in TBS buffer of a volume of about 1% of the initial virus suspension. The pooled suspension was recentrifuged at low speed of 5,000 x g for 15 minutes to remove any insoluble material and then subjected to ultracentrifugation using a CsCl isopycnic gradient as described below.

(c) Direct Ultracentrifugation Method

After removal of cellular debris from the virus culture medium, the clear virus supernatant was carefully layered onto the top of a 30% sucrose cushion and subjected to ultracentrifugation at 120,000 x g for 2 hours at 4 °C. The supernatant was carefully decanted from the pellet, and the residual fluid drained from the tube by inversion. One hundred μL of TBS buffer was added to each tube and the pellet allowed to soften overnight at 4 °C. The virus pellet was then resuspended by pipetting as previously described, all pellet suspensions were pooled, then subjected to centrifugation at 5,000 x g to remove insoluble material. This was followed by ultracentrifugation on a CsCl isopycnic gradient as described below.

(d) Cesium Chloride Isopycnic Gradient Purification

A linear CsCl gradient (25 to 50%) was prepared in an ultracentrifugation tube (SW40, Beckman) by mixing 6 mL of 25% (w/v) CsCl with 6 mL of 50% (w/v) CsCl solution using a gradient mixer. The outlet tube from the mixer was placed at the bottom of the centrifuge tube so that the linear gradient density increased from the top to the bottom of the tube. One and a half (1.5) mL of the virus suspension prepared from the three methods described above was then carefully layered on top of the CsCl linear gradient. The tubes were balanced with virus suspension making sure that a space between 2 – 3 mm was left at the top of the tube. The linear gradient was ultracentrifuged at 30,000 rpm for 18 hours at 10 °C.

At the end of the centrifugation run, the virus band was detected by holding the tube under a narrow beam of light against a dark background, where it appeared as a bluish

opalescent narrow band in the middle of the gradient. A whitish band could be seen above and/or below the virus band depending on which preparation was used. A 24G needle with a 5 mL syringe was used to side puncture the tube slightly below the virus band but avoiding the whittish band, and the virus band was withdrawn carefully.

Virus bands from different runs were pooled together and CsCl was removed by ultrafiltration using several changes of 0.1M HEPES (pH 7.0). The sample was then concentrated using the same ultrafiltration units (Centricon-500, Amicon) following the manufacturer's instructions.

6.2.3 Assessment of Virus Concentration and Purity

The virus concentration was determined by measuring the absorbance at 260 nm. The final virus samples were desalted by ultrafiltration and the buffer exchanged three times using a Centricon-500 kDa MWCO (molecular weight cut-off) as described above. The concentrate was then reconstituted into a 2 mL volume with 0.1M HEPES buffer and 200 μL was used to measure the concentration. The virus concentration was estimated using the assumption that an absorbance of 5.0 at 260 nm indicates the presence of 1 mg per mL of purified virus (Bottcher *et al.*, 1997; Smith *et al.*, 1969).

The purity of the final yield virus was assessed using SDS-PAGE as described below and examination by electron microscopy as described in section 2.2.7.

6.2.4 Polyacrylamide Gel Electrophoresis (PAGE)

The purified IBDV major viral structural protein VP2 (40 – 45kDa), VP3 (32 – 34kDa), VP4 (27 – 30 kDa) and minor protein VP1 (90 – 95 kDa), VP5 (16 – 17 kDa) were separated and detected by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis using the Bio-Rad Mini Protean II Dual Slab Cell system and the method described by Laemmli *et al* (1970). The 12% separating and 4% stacking gels (80 x 100 x 0.75 mm) were made as described in Bio-Rad Mini Protean II instruction manual. Five μL of the concentrated virus sample was mixed with 1 μL of 10 x concentrated SDS loading sample buffer, and heated at 100 °C in a boiling water bath

for 5 minutes before being loaded into the well of a mini-gel (8 x 10 cm). A constant voltage of 200 volts was applied across the electrodes until the dye front reached the bottom of the resolving gel. The gels were removed, fixed and stained in a solution of 0.1% (w/v) Coomassie Brilliant Blue R250 (in 40% methanol and 10% acetic acid in water) for 30 minutes. The background was removed by destaining the gel with destaining solution (40% methanol and 10% acetic acid in water).

Molecular weights of proteins were estimated by comparison with known standards. Low molecular weight protein markers (Bio-Rad) were routinely used in all gels. Stained gels were vacuum dried using a Bio-Rad gel drier by sandwiching the gels between two sheets of cellulose acetate or placing onto Whatman filter paper after soaking in a solution of 2% glycerol in distilled water for 1 hour.

6.2.5 Electroblotting of Viral Capsid Protein from Acrylamide Gels

For analysis of the native IBDV capsid proteins, the proteins separated by SDS-PAGE were transferred onto a PVDF membrane (Applied Biosystems) using electroblotting (Mini Trans-Blot, Bio-Rad). In order to minimise N-terminal blocking, the gels were left to polymerise overnight (Walker & Gasstra, 1987). Samples were loaded onto a SDS polyacrylamide gel in a symmetrical pattern, so that once the gel was run it could be cut in half to give two mirror images. 1 mM sodium thioglycolate was added to the cathode buffer, to prevent the formation of free radicals that can induce acetylation at the N-terminus of proteins (Walker & Gasstra, 1987).

After electrophoresis, the gel was cut in half and one half stained with Coomassie Blue R250 in order to detect the protein bands. The other half was soaked in a transfer buffer (10 mM CAPS, 20% methanol, pH 11) for 15 minutes to prepare the gel for electroblotting. The gel piece was placed next to a sheet of PVDF membrane that had been wetted in methanol, equilibrated in transfer buffer, and assembled into a sandwich between sheets of Whatman No. 3 filter paper presoaked in transfer buffer according to the manufacturer's instructions. Proteins were transferred from the gel (anode) to the PVDF membrane (cathode) in a Bio-Rad electroblotter at a constant current of 100 mA for 90 minutes. When electroblotting was complete, the membrane was removed and

soaked in water for 10 minutes. The blotted gel was stained by Coomassie Blue to assess the degree of transfer in comparison with the reference gel.

6.2.6 Detection of Viral Capsid Protein by Protein Blotting and Immunodetection.

Detection of PVDF membrane-bound proteins was carried out using a BM chemiluminescence blotting system (Boehringer Mannheim). Detection is based on the oxidation of diacylhydrazides such as luminol in the presence of hydrogen peroxide (H_2O_2) by peroxidases (POD) such as horseradish peroxidase (HRP) attached to secondary antibodies (anti-mouse or anti-rabbit). An activated intermediate reaction product is formed, which decays to the ground state by emitting light. This system uses 4-iodophenol to aid the transmission of oxygen radicals formed by the peroxidase to luminol, thus enhancing light emission.

The electroblotted membrane was first blocked with 1% blocking solution (w/v) for 1 hour at room temperature to avoid any non-specific binding of primary antibody. After the blocking step, the membrane was incubated for 1 hour with primary antibody diluted in 0.5% blocking solution (w/v). Mouse monoclonal antibody R63 directed against the VP2 epitope (Snyder *et al.*, 1988) was used for detection of the VP2 protein only, while rabbit anti-IBDV polyclonal antibody (kindly supply by Dr. Jagoda Ignatovic, CISRO, Australia) was used to detect all of the IBDV capsid proteins (VP1 – VP5). Dilutions used for the monoclonal antibody R63 and polyclonal antibody were 1:4,000 and 1:2,000 respectively. After the incubation was complete, the membrane was washed twice in 50 mM Tris-HCl, 150 mM NaCl, 0.0125% Tween 20, pH7.5 and twice with 0.5% blocking solution, each wash lasting 10 minutes. Anti-mouse Ig-POD (BM) or anti-rabbit Ig-POD (Sigma) were used as the secondary detection reagent depending on the primary antibody used. Both secondary antibodies were diluted in 0.5 % blocking solution at a ratio of 1:5000 and 1: 20,000 respectively. The membrane was left to react with the secondary antibody for 30 minutes at room temperature. It was then washed four times with large volumes of washing buffer for 15 minutes each time.

For chemiluminescent detection, the membrane was incubated with the detection reagent (a mixture of substrate solution A and starting solution B in a ratio of 100:1, at

room temperature) for 60 seconds. Excess detection reagent was drained from the membrane which was then placed between two transparent films, making sure that no air bubbles were trapped. This sandwich was then placed in a file cassette, with the protein side up, covered with a sheet of X-ray film (Kodak X-Omat AR) and exposed for 60 seconds. The exposure was repeated using a new film and different exposure times, depending on the signal intensity obtained on the first film. The X-ray films were developed by an automatic X-ray film processor (100 Plus, All-Pro Imaging).

6.2.7 N-Terminal Sequencing of Viral Capsid Protein

Once the IBDV capsid proteins had been electroblotted onto the PVDF membrane, the membrane was stained briefly in Coomassie Blue to visualise the protein bands. The membrane was rinsed in Milli-Q water for 5 minutes and thoroughly air-dried before the desired bands were excised, and stored in Eppendorf tubes at –20 °C for N-terminal sequencing. N-terminal sequencing was carried out by the Protein Sequencing Service, Institute of Molecular BioSciences (IMBS), Massey University on an automated Applied Biosystems model 476A protein sequencer.

6.2.8 Electrospray Ionisation Mass Spectrometry (ES-MS)

To determine the actual molecular weight of the IBDV capsid proteins, capsid proteins of the purified IBDV were separated using SDS-PAGE as described in section 6.2.5. The SDS-PAGE gel was negatively stained using a Zinc-stain (Bio-Rad) according to the manufacturer's instructions. Each IBDV capsid protein band was carefully excised with a clean scalpel and stored in an Eppendorf tube. Three of the bands were pooled together for electro-elution.

Protein was eluted from the gel band using electroelution or sonication, and analysed using electrospray MS-Mass Spectrometry to determine the molecular weight of each capsid protein. Mass spectrometry was done on a Sciex API 300 instrument by MasSpec Services, IMBS, Massey University.

6.2.9 Detection of Deglycosylated Substrate with Digoxigenin (DIG) Glycan/Protein Double Labelling Kit

In order to determine if native VP2 capsid protein of the IBDV is a glycosylated protein, the digoxigenin glycan/ protein double labelling kit (Roche) was used. This kit can be used to detect glycosylated and deglycosylated proteins on both polyacrylamide gels and blots. The native VP2 protein was separated from the purified IBDV by SDS-PAGE and then immobilised onto a PVDF membrane for glycan/protein detection. The kit has a detection limit of about 50 ng for proteins and can detect glycoprotein at a minimum concentration of 10 ng. The principle of detection is based on the sugar and protein fractions of a glycoprotein each being labeled with a different hapten, which is then detected with the aid of a corresponding antibody coupled to different reporter enzymes. The sugar side chains are labelled by specific oxidation of the sugar hydroxyl groups to aldehydes with periodate followed by covalent coupling of the DIG-hapten to the aldehyde groups created. The DIG-hydrazide is specifically detected by reaction with anti-digoxigenin-POD antibody conjugate that contains peroxidase (POD) as a reporter molecule. The substrate for the peroxidase is TETON (4-triethylenetrioxy-1-naphtnol), a blue coloured precipitation is obtained from the reaction, indicating the presence of sugar side chains on the electroblotted protein. Similarly, free amino groups on the non-glycosylated or deglycosylated proteins are labelled by fluorescein (FLUOS) and are specifically detected by anti-fluorescein-AP antibody conjugate. The anti-fluorescein-AP conjugate contains alkaline phosphatase that uses 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazollum chloride (INT) and X-phosphate as substrates to give a reddish-brown precipitation indicating the presence of non-glycosylated or deglycosylated protein.

6.2.10 Virus Crystallisation Trials

Conditions reported for all successful virus crystallisations to date use either ammonium sulfate or polyethylene glycol (PEG) as precipitants (Ducruix & Giege, 1992) (see Table II-1). A set of experiments was designed using ammonium sulphate and PEG 8000 to find suitable conditions for crystallising IBDV. These are listed in Tables 6.1 to 6.4. The preliminary screening was then further modified according to observations made over a period of time. All crystallisation experiments were performed using the hanging drop method (Ducruix & Giege, 1992) at room temperature in 24 well Linbro

plates (Hampton, USA). One to 2 μL drops of concentrated virus solution were mixed with equal volumes of crystallisation solution from each well. Crystal growth was observed weekly after the initial set-up.

Any crystals that grew to a suitable size were mounted in siliconised glass capillaries of 0.5 mm diameter, for diffraction trials as described below.

6.2.11 Crystal Diffraction Trials

All crystals mounted were tested for X-ray diffraction on a Rigaku R-Axis II C image plate detector using a Rigaku RU-200B rotating anode source and an AXCO capillary optic to select monochromatic radiation. The program DENZO was used to index and integrate any images obtained.

Table 6.1: IBDV crystallisation screen # 1.

Condition	Precipitant	Buffer and pH	Salt
1	1.0% PEG 8K + 0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 6.0	1 mM Mg Acetate
2	1.0% PEG 8k + 0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 6.5	1 mM Mg Acetate
3	1.0% PEG 8k + 0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.0	1 mM Mg Acetate
4	1.0% PEG 8k + 0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.5	1 mM Mg Acetate
5	1.5% PEG 8k + 0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 6.0	1 mM Mg Acetate
6	1.5% PEG 8k + 0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 6.5	1 mM Mg Acetate
7	1.5% PEG 8k + 0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.0	1 mM Mg Acetate
8	1.5% PEG 8k + 0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.5	1 mM Mg Acetate
9	2.0% PEG 8k + 0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 6.0	1 mM Mg Acetate
10	2.0% PEG 8k + 0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 6.5	1 mM Mg Acetate
11	2.0% PEG 8k + 0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.0	1 mM Mg Acetate
12	2.0% PEG 8k + 0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.5	1 mM Mg Acetate
13	0.2 M Ammonium Sulfate	50 mM K_2HPO_4, pH 6.0	1 mM Mg Acetate
14	0.2 M Ammonium Sulfate	50 mM K_2HPO_4, pH 6.5	1 mM Mg Acetate
15	0.2 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.0	1 mM Mg Acetate
16	0.2 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.5	1 mM Mg Acetate
17	0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 6.0	1 mM Mg Acetate
18	0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 6.5	1 mM Mg Acetate
19	0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.0	1 mM Mg Acetate
20	0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.5	1 mM Mg Acetate
21	0.6 M Ammonium Sulfate	50 mM K_2HPO_4, pH 6.0	1 mM Mg Acetate
22	0.6 M Ammonium Sulfate	50 mM K_2HPO_4, pH 6.5	1 mM Mg Acetate
23	0.6 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.0	1 mM Mg Acetate
24	0.6 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.5	1 mM Mg Acetate

Table 6.2: IBDV crystallisation screen # 2.

Condition	Precipitant	Buffer and pH	Salt
1	0.2 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.1	1 mM Mg Acetate
2	0.2 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.3	1 mM Mg Acetate
3	0.2 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.5	1 mM Mg Acetate
4	0.2 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.7	1 mM Mg Acetate
5	0.2 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.9	1 mM Mg Acetate
6	0.2 M Ammonium Sulfate	50 mM K_2HPO_4, pH 8.1	1 mM Mg Acetate
7	0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.1	1 mM Mg Acetate
8	0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.3	1 mM Mg Acetate
9	0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.5	1 mM Mg Acetate
10	0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.7	1 mM Mg Acetate
11	0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.9	1 mM Mg Acetate
12	0.4 M Ammonium Sulfate	50 mM K_2HPO_4, pH 8.1	1 mM Mg Acetate
13	0.6 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.1	1 mM Mg Acetate
14	0.6 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.3	1 mM Mg Acetate
15	0.6 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.5	1 mM Mg Acetate
16	0.6 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.7	1 mM Mg Acetate
17	0.6 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.9	1 mM Mg Acetate
18	0.6 M Ammonium Sulfate	50 mM K_2HPO_4, pH 8.1	1 mM Mg Acetate
19	0.8 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.1	1 mM Mg Acetate
20	0.8 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.3	1 mM Mg Acetate
21	0.8 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.5	1 mM Mg Acetate
22	0.8 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.7	1 mM Mg Acetate
23	0.8 M Ammonium Sulfate	50 mM K_2HPO_4, pH 7.9	1 mM Mg Acetate
24	0.8 M Ammonium Sulfate	50 mM K_2HPO_4, pH 8.1	1 mM Mg Acetate

Table 6.3: IBDV crystallisation screen # 3.

Condition	Precipitant	Buffer and pH	Salt
1	1.0% PEG 8000	0.1 M HEPES, pH 7.0	None
2	1.0% PEG 8000	0.1 M HEPES, pH 7.5	None
3	1.0% PEG 8000	0.1 M K_2HPO_4, pH 7.0	None
4	1.0% PEG 8000	0.1 M K_2HPO_4, pH 7.5	None
5	1.5% PEG 8000	0.1 M HEPES, pH 7.0	None
6	1.5% PEG 8000	0.1 M HEPES, pH 7.5	None
7	1.5% PEG 8000	0.1 M K_2HPO_4, pH 7.0	None
8	1.5% PEG 8000	0.1 M K_2HPO_4, pH 7.5	None
9	2.0% PEG 8000	0.1 M HEPES, pH 7.0	None
10	2.0% PEG 8000	0.1 M HEPES, pH 7.5	None
11	2.0% PEG 8000	0.1 M K_2HPO_4, pH 7.0	None
12	2.0% PEG 8000	0.1 M K_2HPO_4, pH 7.5	None
13	2.5% PEG 8000	0.1 M HEPES, pH 7.0	None
14	2.5% PEG 8000	0.1 M HEPES, pH 7.5	None
15	2.5% PEG 8000	0.1 M K_2HPO_4, pH 7.0	None
16	2.5% PEG 8000	0.1 M K_2HPO_4, pH 7.5	None
17	3.0% PEG 8000	0.1 M HEPES, pH 7.0	None
18	3.0% PEG 8000	0.1 M HEPES, pH 7.5	None
19	3.0% PEG 8000	0.1 M K_2HPO_4, pH 7.0	None
20	3.0% PEG 8000	0.1 M K_2HPO_4, pH 7.5	None
21	3.5% PEG 8000	0.1 M HEPES, pH 7.0	None
22	3.5% PEG 8000	0.1 M HEPES, pH 7.5	None
23	3.5% PEG 8000	0.1 M K_2HPO_4, pH 7.0	None
24	3.5% PEG 8000	0.1 M K_2HPO_4, pH 7.5	None

Table 6.4: IBDV crystallisation screen # 4.

Condition	Precipitant	Buffer and pH	Salt
1	0.2 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.1	1 mM Mg Acetate
2	0.2 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.3	1 mM Mg Acetate
3	0.2 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.5	1 mM Mg Acetate
4	0.2 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.7	1 mM Mg Acetate
5	0.2 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.9	1 mM Mg Acetate
6	0.2 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 8.1	1 mM Mg Acetate
7	0.4 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.1	1 mM Mg Acetate
8	0.4 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.3	1 mM Mg Acetate
9	0.4 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.5	1 mM Mg Acetate
10	0.4 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.7	1 mM Mg Acetate
11	0.4 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.9	1 mM Mg Acetate
12	0.4 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 8.1	1 mM Mg Acetate
13	0.6 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.1	1 mM Mg Acetate
14	0.6 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.3	1 mM Mg Acetate
15	0.6 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.5	1 mM Mg Acetate
16	0.6 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.7	1 mM Mg Acetate
17	0.6 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.9	1 mM Mg Acetate
18	0.6 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 8.1	1 mM Mg Acetate
19	0.8 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.1	1 mM Mg Acetate
20	0.8 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.3	1 mM Mg Acetate
21	0.8 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.5	1 mM Mg Acetate
22	0.8 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.7	1 mM Mg Acetate
23	0.8 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 7.9	1 mM Mg Acetate
24	0.8 M Ammonium Sulfate	0.1 M K_2HPO_4, pH 8.1	1 mM Mg Acetate

6.3 RESULTS AND DISCUSSION

6.3.1 Virus Purification and SDS-PAGE Analysis

Ammonium sulfate and/or PEG 6000 precipitation of the IBDV from the virus culture media did not significantly increase its purity. SGS-PAGE analysis of both the pellet and the supernatant showed that, although the pellet was enriched in virus, it still contained proteins from the virus culture media such as foetal bovine serum albumin (Figure 6.1) that made the subsequent purification steps difficult. It was almost impossible to separate those proteins from the virus particles.

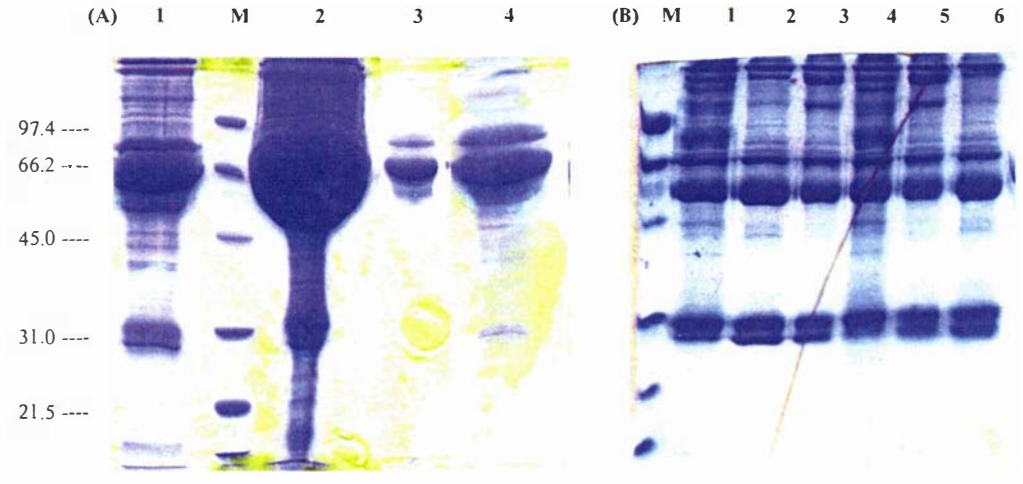


Figure 6.1: SDS-PAGE analysis of the (A) Ammonium sulfate (A/S) precipitation of IBDV. M: Low range molecular weight standards; Lane 1: Cell free IBDV virus culture; Lane 2: 85% A/S precipitated pellet; Lane 3: 85% A/S precipitation supernatant; Lane 4: 5% foetal bovine serum sample (FBS).

(B) PEG 6000 precipitaion of IBDV. M: Low range molecular weight standards; Lane 1 - 3: 7%, 10% and 20% PEG precipitated pellet; Lane 4 – 6: PEG precipitation supernatant from lane 1 to 3.

Direct ultracentrifugation (100,000 x g, 2 hours) of the culture media over a 30% sucrose cushion proved to be a successful method for separating the virus particles from the vast majority of the protein contaminants in the virus culture media as shown in Figure 6.2. These contaminants were concentrated in the whitish band that could be seen on top of the virus band. The remaining contaminating proteins were separated from the virus particles using ultracentrifugation with a linear CsCl isopycnic gradient as shown in

Figure 6.2, lane 1 and 2. Occasionally, a second CsCl isopycnic gradient was needed when the virus bands became contaminated with the protein bands (whitish in colour) during collection.

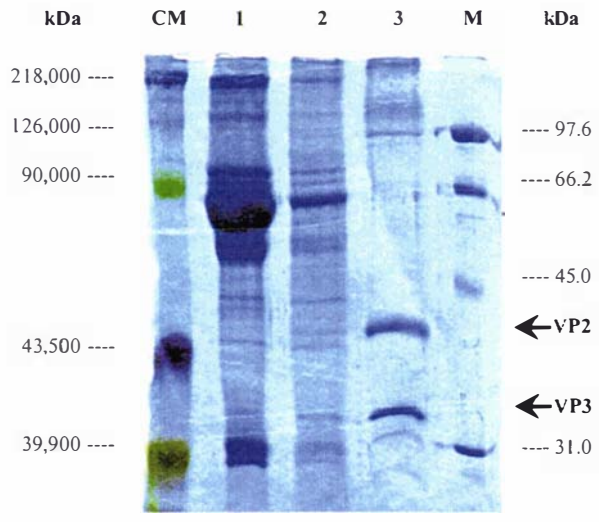


Figure 6.2: SDS-PAGE analysis of the direct centrifugation method. CM: Kaleidoscope prestained standards (Bio-Rad); M: Low range molecular weight standards; Lane 1: Whittish band on top of the sucrose cushion; Lane 2: Supernatant above the sucrose cushion; Lane 3: Redissolved virus pellet.

6.3.2 Purified Virus Concentration and EM Examination

All purified IBDV were concentrated to a final concentration of about 10 mg per mL by ultrafiltration and equilibrated in 0.1M HEPES, pH 7.0 using a Centricon-500 MWCO.

The purified virus suspensions were examined using EM to estimate both purity and uniformity as showed in Figure 6.3.

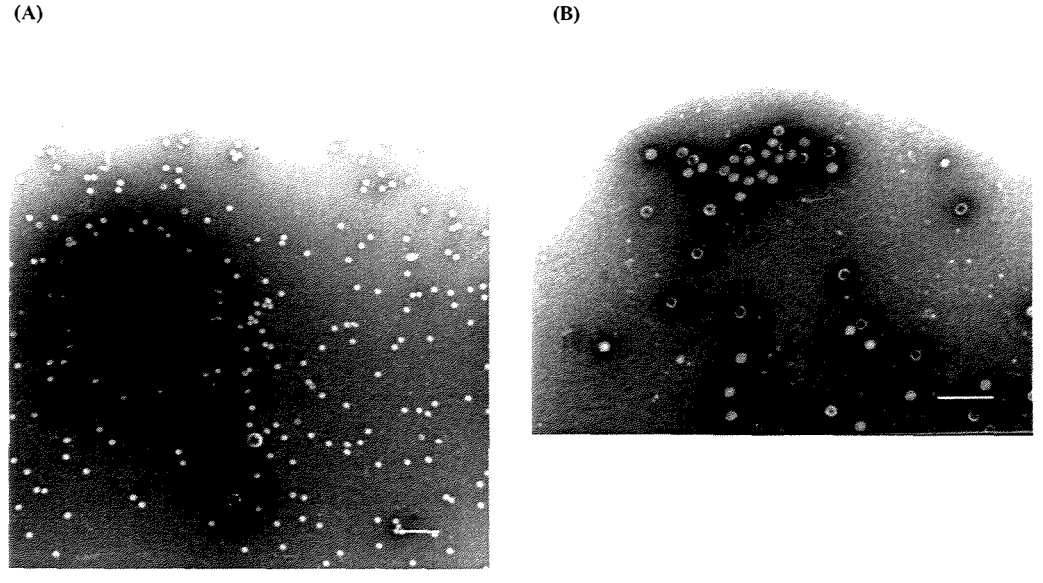


Figure 6.3: EM examination of the purified IBDV from direct ultracentrifugation method. (A) Low magnification of 15, 300 x, bar = 500 nm; (B) Higher magnification of the virus at 31, 800 x, bar = 500 nm.

6.3.3 Detection of Viral Capsid Protein by Protein Blotting and Immunodetection.

Figure 6.4 shows an westernblot of the native IBDV capsid proteins using the mouse monoclonal antibody R63 directed against the VP2 and rabbit anti-IBDV polyclonal antibody for detecting all of the IBDV capsid proteins (VP1 – VP5). The mouse monoclonal antibody R63 bound to a protein band of about 45 kDa MW (VP2) and the rabbit polyclonal antibody bound to protein bands of about 45 (VP2) and 35 kDa (VP3) MW. The rabbit polyclonal antibody failed to detect the other capsid proteins VP1, VP4 and VP5 probably because of the low concentration of these capsid proteins in the virus.

Interestingly, the band identified as VP2 that was detected using the rabbit polyclonal antibody was less intense than the VP2 band detected by the mouse monoclonal antibody. This is probably because the rabbit anti-IBDV polyclonal antibody was produced from an Australian IBDV serotype 1 strain, and the virus used in this study is a classical serotype 1 strain from the USA. Molecular and phylogenetic analyses have demonstrated that the Australian IBDV strains are more diverse than the classical serotype 1 strains found in other parts of the world (Proffitt *et al.*, 1999).

It has been found that most sequence differences observed between different strains of virus occur in VP2 (see chapter 5 introduction), while VP3 is highly conserved. This would explain why the rabbit polyclonal antibody binds less strongly to the VP2 from the strain used in this study.

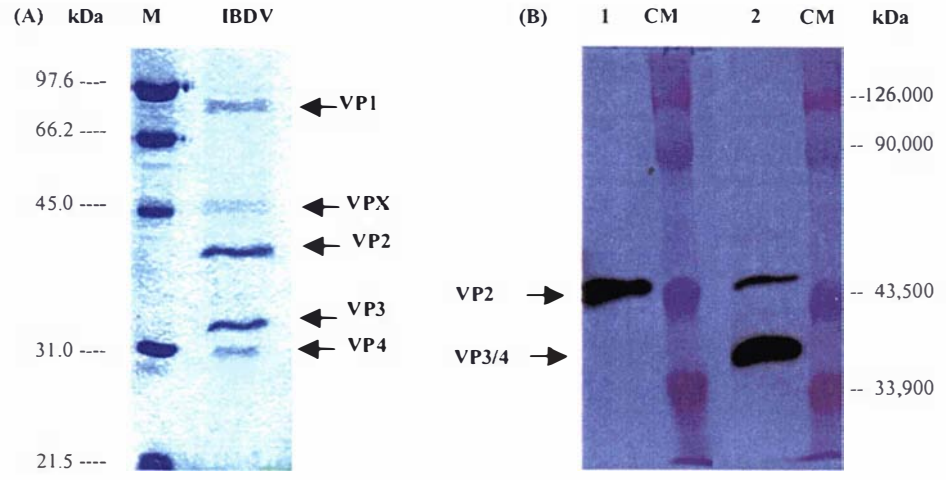


Figure 6.4: (A) SDS-PAGE analysis of purified IBDV showing capsid proteins VP1, 2, 3, 4 and VPX (precusor VP2), M: Marker; IBDV: Purified IBDV ;(B) Western immunodetection of IBDV capsid proteins, Lane 1: Immunodetection with mouse monoclonal antibody R63; lane 2: Immunodetection with rabbit anti-IBDV polyclonal antibody; CM: Kaleidoscope prestained standards.

6.3.4 N-Terminal Sequencing and Electrospray Ionisation Mass Spectrometry (ES-MS) of Viral Capsid Protein

N-terminal sequencing and an accurate molecular weight determination of the viral capsid proteins were necessary to determine the actual cleavage sites of the IBDV polyprotein NH_2-pVP2-VP4-VP3-COOH into the individual proteins VP2, VP4 and VP3. Theoretically, by identifying the N-terminal amino acid residues of each protein, and measuring an accurate mass for each, the C-terminal amino acid residue of each capsid protein should be able to be identified using sequence analysis. The results from the Edman sequencing of the native VP2 capsid protein confirmed that the N-terminus had the sequence **T-N-L-Q** as previously reported by other researchers (Bayliss *et al.*, 1990; Kibenge *et al.*, 1990) . The N-termini of both VP3 and VP4 appeared to be blocked. This may have been a result of gel electrophresis and blotting or they may

naturally be acetylated as has been shown with the capsid proteins of some other viruses (Yusibov *et al.*, 1996).

It was found unfortunately that the individual capsid proteins could not be eluted from the SDS gel in high enough concentrations for ES-MS. All methods that were tried to elute the individual proteins from the gel, and remove SDS from the protein were unsuccessful. Due to the limited amount of purified IBDV available for the tests, the experiment was not carried out further. However, this should be possible once appropriate conditions have been determined and, in the future, may be a good way to determine the actual cleavage sites of the polyprotein.

6.3.5 Detection of Deglycosylated Substrate with Digoxigenin (DIG) Glycan/Protein Double Labelling Kit

Figure 6.5 shows the result of protein/glycan detection by the DIG kit. The band identified as VP2 using monoclonal antibody (45 kDa in lane 3) has a reddish-brown colour indicating the protein is non-glycosylated. There is also a weak reddish-brown precipitation colour band at about 35 kDa in lane 4 just below the VP2 band, that has tentatively been identified as the VP3. The weak brown colour also suggests that VP3 is not glycosylated. Similar results have been found for other capsid proteins of IPNV, another birnavirus, confirming that IPNV structural proteins are not glycosylated (Perez *et al.*, 1996).

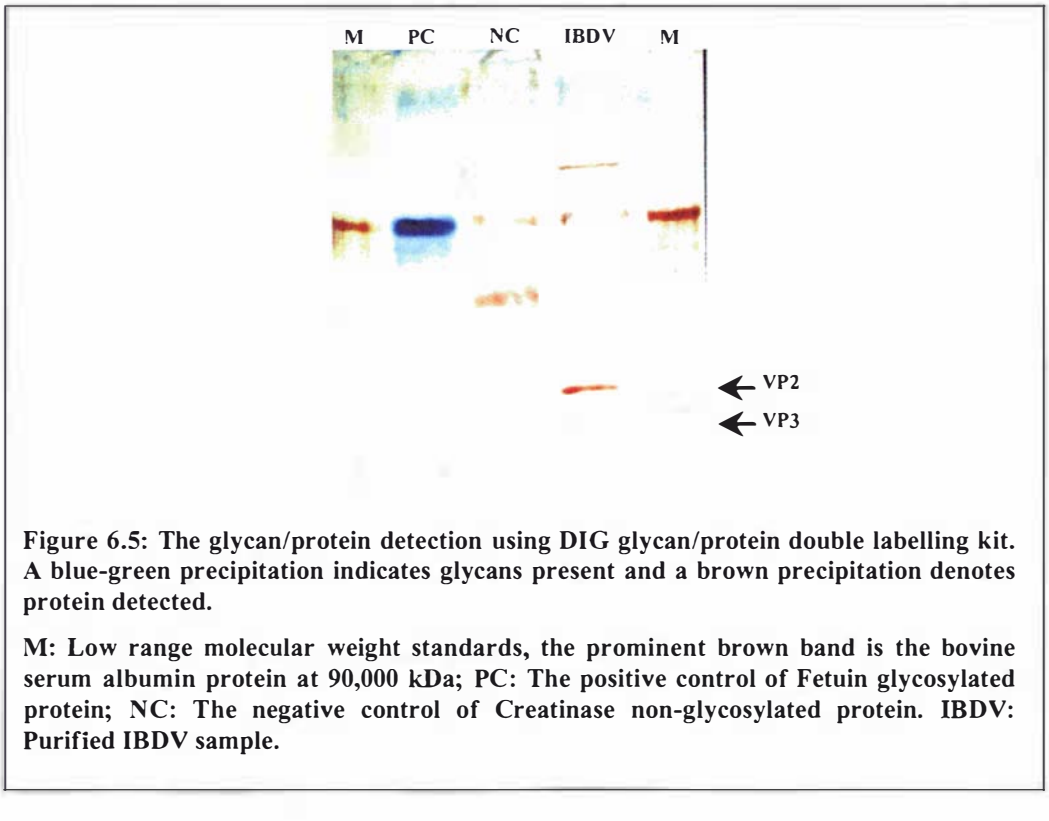


Figure 6.5: The glycan/protein detection using DIG glycan/protein double labelling kit. A blue-green precipitation indicates glycans present and a brown precipitation denotes protein detected.

M: Low range molecular weight standards, the prominent brown band is the bovine serum albumin protein at 90,000 kDa; PC: The positive control of Fetuin glycosylated protein; NC: The negative control of Creatinase non-glycosylated protein. IBDV: Purified IBDV sample.

6.3.6 Virus Crystallisation and DiffractionTrials

In the first screen using PEG 8000 and ammonium sulfate as precipitants (Table 6.1), crystals of IBDV were obtained between 0.2 and 0.4 M ammonium sulfate, 0.05 M K_2HPO_4, pH 7.0 and 7.5 and 1 mM Mg Acetate (conditions 15, 16 and 20 in Table 6.1). Single rhombohedral shaped crystals appeared after 5 weeks as shown in Figure 6.6 (a) to (c). The crystals measured 0.08 to 0.1 mm in the longest dimension. Multiple small icosahedral shaped crystals were grown from 0.6 M ammonium sulfate (Figure 6.6 (d) to (f)). No crystals grew using PEG 8000 as precipitant. One crystal was successfully mounted in a siliconised glass capillary for X-ray diffraction trial, but failed to diffract. The other two crystals were damaged during mounting.

A second crystallisation trial (Table 6.2) was carried out to optimise the crystallisation conditions identified from the first screen. Three single rod-like crystals were formed using 0.2 to 0.4 M ammonium sulfate as precipitant, 0.05 M K_2HPO_4, between pH 7.5 and 8.1 with 1 mM Mg Acetate (condition # 3, 5, 6 and 10) (Figure 6.6 (g) and (h)). These crystals had dimensions averaging 0.15 x 0.05 x 0.05 mm. All three single crystals were mounted for X-ray diffraction trials but again failed to diffract.

Only one irregular shaped crystal formed at 3.0% PEG 8000 in 0.1 M HEPES, pH 7.0, but did not grow large enough to test. Such conditions have been reported to be successful for the crystallisation of other viruses(Arnold *et al.*, 1984; Fisher *et al.*, 1992; Luo *et al.*, 1988; Luo *et al.*, 1987).

The fourth, fifth, sixth, seventh and eighth crystallisation screens were carried out based on the conditions shown in Table 6.4. Single crystals grew between 0.4 to 0.6 M ammonium sulfate (0.1 M K_2HPO_4, pH 7.1 to 7.9 and 1 mM Mg Acetate). They were the same shape (rhombohedral) as those that had been previously obtained and measured approximately 0.1 mm in the longest dimension. Three crystals were mounted for X-ray diffraction trials. These crystals diffracted, but only very weakly and could not be indexed.

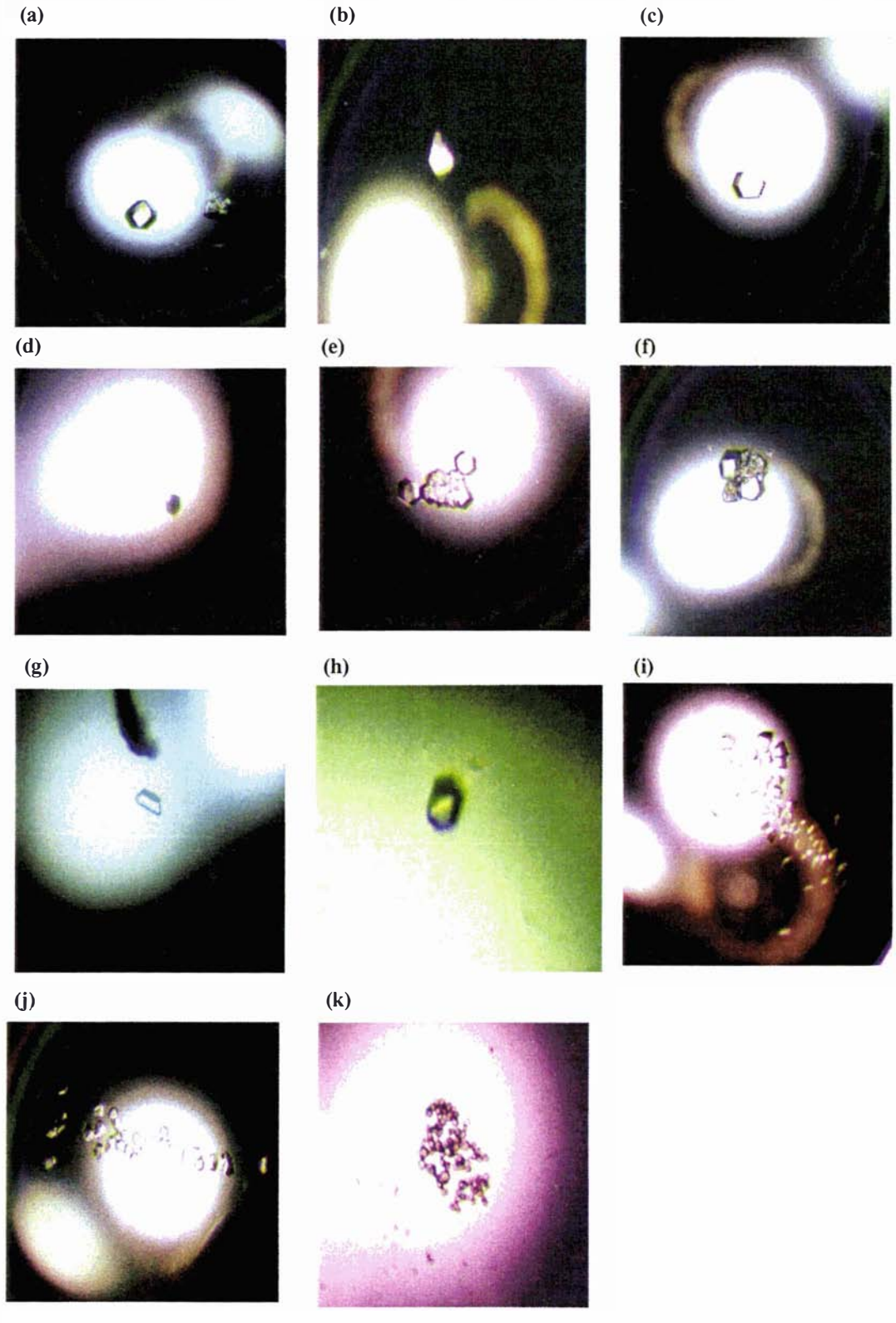


Figure 6.6: IBDV crystals grown using the conditions described in section 6.3.6. (a) to (c): single rhombohedral crystals; (d) to (f): single or multiple icosahedra; (g) and (h): single long rods; (i) to (k): multiple irregularly shaped crystals.

6.4 CONCLUSION

Although the preliminary efforts to crystallise IBD virions resulted in the growth of crystals, all crystals failed to diffract. Ammonium sulfate appeared to be the best precipitant, with the optimal pH being between 7.0 and 8.0. Buffer type had little effect on the growth of crystal. None of the crystals grew larger than 0.1 mm in the longest direction.

The weak diffraction data obtained from the crystals is probably due to the poor ordering of the molecules. However, the fragile nature of virus crystals is well known and mounting them in capillaries can cause severe damage. It is therefore possible that inexperience in handling these crystals may have contributed to their failure to diffract. Most virus crystals have extremely large unit cells. It is therefore necessary to use strong radiation such as synchrotron X-radiation for data collection. Nevertheless, a weak, low resolution diffraction patterns should have been observed with the rotating anode source if the crystals had been ordered.

The crystallisation conditions for IBDV need to be further optimised based on the preliminary conditions determined from this experiment. Different precipitants or additives need to be screened in order to improve crystal size and diffraction quality.

The IBDV purification experiments in this chapter resulted in a reliable, relatively short protocol for purifying high quality virus. This will certainly benefit any future structural investigations. Unfortunately, due to the limited availability of high titre virus (this preparation used 20 L of virus culture) and the small size and fragility of the crystals, the trials could not be continued. However, structural studies on IBDV are a novel approach in poultry virology research, and a molecular model of this virus should answer the question of why different strains of virus react differently to antibodies. This will potentially contribute enormously to the design of new drugs, new vaccines and also to the fundamental understanding of antigenicity.

CHAPTER 7: IBDV CAPSID PROTEIN EXPRESSION, PURIFICATION AND CRYSTALLISATION

7.1 INTRODUCTION

Various studies have determined that the major capsid protein, VP2, is the main antigenic protein of IBDV with at least two virus-neutralising epitopes being located in the protein (Crisman *et al.*, 1993; Fahey *et al.*, 1991; Hudson *et al.*, 1986; Reddy & Silim, 1991; Reddy *et al.*, 1992; Snyder *et al.*, 1988; Whetzel & Jackwood, 1995). The *vp2* gene has been cloned and expressed in many different expression systems to produce protein that has been used for diagnostic studies of IBDV (Dybing & Jackwood, 1997; Snyder *et al.*, 1988), identification of the neutralisation epitope(s) (Jagadish *et al.*, 1991; Jagadish *et al.*, 1990), and for the development of a recombinant capsid protein vaccine (Dybing & Jackwood, 1998; Fernandez-Arias *et al.*, 1998; Hu *et al.*, 1999; Kibenge *et al.*, 1999; Pitcovski *et al.*, 1996; Snyder & Vakharia, 1993; Vakharia *et al.*, 1993; Wang *et al.*, 1994).

The *vp2* gene has been cloned into various vectors, both alone and as part of the whole segment A gene expressing the polyprotein VPX-VP4-VP3. The early work done by Jagadish et al to express the polyprotein in *E.coli* and yeast (Jagadish *et al.*, 1991; Jagadish *et al.*, 1990) showed that recombinant VP2 was immunogenic and that it was recognised by various monoclonal antibodies against *vp2* epitopes. Vaccination of hens with the recombinant protein expressed in *Saccharomyces cerevisiae,* conferred passive protection to progeny against IBDV (Macreadie *et al.*, 1990). Intriguingly, when hens were vaccinated with *vp2* expressed in *E. coli*, their progeny were not passively protected against IBDV infection and no virus-neutralising antibodies could be identified in serum of the hens (Azad *et al.*, 1991). Further efforts to develop a vaccine for IBDV involved expressing the *vp2* gene in fowlpox virus. Vaccination of chickens with this recombinant fowlpox virus resulted in variable levels of active protection (Bayliss *et al.*, 1991; Heine & Boyle, 1993; Heine *et al.*, 1994). When the *vp2* gene was cloned into *Turkey herpesvirus,* vaccination of chickens with the recombinant virus

resulted in a significant level of protection. However, for both of these recombinant vaccines, the level of protection was lower than that provided by an oil adjuvanted inactivated whole IBDV vaccine (Heine *et al.*, 1994).

The *vp2* gene was subsequently expressed using baculovirus and vaccinia virus expression systems (Dybing & Jackwood, 1997; Dybing & Jackwood, 1998; Fernandez-Arias *et al.*, 1998; Hu *et al.*, 1999; Kibenge *et al.*, 1999; Pitcovski *et al.*, 1996; Snyder *et al.*, 1994; Vakharia *et al.*, 1993). Eukaryotic expression systems have several advantages compared to prokaryotic expression systems. They reliably produce recombinant protein that is antigenically and functionally similar to the native protein, and any post-translational protein modifications of the protein will be more likely to mimic those of the native protein (O'Reilly *et al.*, 1994). Vakharia's group was the first to report the production of IBDV structural proteins in the baculovirus system. Inoculation of susceptible chickens with these recombinant proteins was shown to induce virus-neutralising antibodies in the chickens, conferring up to a 79% protection against IBDV challenge (Vakharia *et al.*, 1993). A chimeric *vp2* gene constructed from the cDNA of both variant and classical strains of IBDV, was expressed in baculovirus. When chickens were vaccinated with this recombinant chimeric subunit vaccine, it markedly improved active cross-protection against both classic and variant IBDV challenges (Snyder *et al.*, 1994). Other laboratories have also reported the expression of VP2, VP2/VP4 and VP2/VP4/VP3 in baculovirus systems and used the recombinant protein as a subunit vaccine, reporting variable levels of active or passive protection (Dybing & Jackwood, 1997; Dybing & Jackwood, 1998; Pitcovski *et al.*,1996).

In this chapter, the expression of the capsid protein VP2 in a baculovirus expression system (Bac-to-Bac Baculovirus Expression System, Lifetech), its purification, partial characterisation and crystallisation are discussed.

7.2 MATERIALS AND METHODS

7.2.1 Insect Cells (Sf9 cells) Culture Procedure

The *Spodoptera frugiperda* (Sf9) insect cells were supplied from Gibco, BRL in a 3 mL cryogenic vial containing 5.0 x 10^6 viable cells/ mL (Cat. no.11496). The cells are adapted for serum-free growth in Sf-900 II SFM (Gibco, BRL), a serum-free medium (SFM) optimised for the growth of Sf9 insect cells. The Sf9 insect cell line is a clonal isolate derived from the parental *Spodoptera frugiperda* cell line IPLB-Sf-21-AE. The Sf9 cultures were incubated at 28 °C ± 0.5 °C in a non-CO_2, ambient air equilibrated incubator. Cell cultures were grown in shaker flask culture as described below.

Recovery of Cryopreserved Sf9 Culture

The cryopreserved Sf9 cells were shipped on dry ice. For recovery and propagation of the cell line, the following procedures were followed:

1. Cryovials were rapidly thawed by placing them in a 37 °C water bath. The entire contents of the cryovial were transferred into a 125 mL Erlenmeyer flask containing 25 mL of pre-warmed Sf-900 II SFM and incubated at 28 °C ± 0.5 °C in a non-humidified, ambient air regulated incubator on an orbital shaker platform rotating at 135-150 rpm. Oxygenation/aeration was accomplished through sterilised, loose capped non-absorbant cotton wool.
2. The culture was maintained at a cell density of 5 x 10^5 to 2 x 10^6 viable cells/mL during the two subcultures or passages following recovery from cryopreservation.
3. After the two recovery passages, the cell density was allowed to reach 2 to 4 x 10^6 viable cells/mL before the cells were subcultured into another fresh Sf-900 SFM at a density 3 to 5 x 10^5 viable cells/mL.
4. The culture was expanded through continued sub-culturing to provide a sufficient number of Sf9 cells (at a low passage seed) for cryopreservation following the cryopreservation protocol outlined below. A fresh culture of cryopreserved Sf9 cells was recovered every 3 months or 30 passages to maintain a viable culture as described from step 1 to 3 in the above.

Sf9 Insect Cells Culture Density and Viability Determination

Cell density and viability of Sf9 cells were determined by counting the cells using a haemocytometer and the trypan blue exclusion method as described in the laboratory manual (Burleson *et al.*, 1992). The cell density and viability were calculated as follows:

% viable cells = 100% x (viable cell count / total cell count)
Total cells/mL = (total cell count / 5) x (1 / dilution) x 10^4
Viable cells/mL = (viable cell count / 5) x (1 / dilution) x 10^4

Sf9 Suspension Cell Culture Maintenance and Subculturing

The Sf9 culture was subcultured when the cell density reached its mid-log phase of growth (2 to 4 x 10^6 viable cells/mL). The cells were passaged into 25, 50 or 100 mL of SFM in 125 or 250 mL Erlenmeyer flasks as follows:

1. A new flask containing the desired volume of Sf-900 SFM medium was inoculated with 3 to 5 x 10^5 viable cells/mL.
2. The culture was incubated as a suspension using an orbital shaker (135 to 150 rpm) in a 28 °C ± 0.5 °C non-humidified, ambient air regulated incubator.
3. The culture was incubated until the cell count reached 2 to 4 x 10^6 viable cells/mL (approximately 2 to 4 days post seeding), then subcultured again as above.
4. Once every three weeks, the cell suspension from shaker cultures was gently centrifuged (100 x g) for 5 minutes and the cell pellet resuspended in fresh Sf-900 SFM in order to reduce the accumulation of cell debris and metabolic by-products.

Monolayer Culture of Sf9 Insect Cells

Sf9 monolayer cultures were grown in 6-well plates in order to carry out the recombinant baculovirus plaque assay and a small-scale recombinant protein expression trial.

1. Mid-log phase Sf9 cells (5 x 10^5 cells/mL) were resuspended in pre-warmed fresh Sf-900 SFM.
2. 2 mL of the prepared cell suspension was dispensed into each well under sterile conditions.

3. Cells were allowed to settle to the bottom of plate and incubated in a 28 °C ± 0.5 °C non-humidified, ambient air regulated incubator for 1 hour.
4. The monolayer was observed under an inverted microscope in order to confirm cell attachment and 50% confluence.

Cryopreservation of Serum-Free Sf9 Insect Cell Cultures

The initial recovered cryopreserved Sf9 cell culture was expanded to a sufficient number of flasks in order to cryopreserve low passage master stock for further experiments. All cells were cryopreserved as followed:

1. Sf9 cells grown in 250 mL shaker flasks and with viability > 90%, were harvested in mid-log exponential phase.
2. The viable cell count was determined using trypan blue dye exclusion as described above and the volume of cryopreservation medium (7.5% DMSO in fresh Sf-900 SFM) required to yield a final cell density of 0.5 to 1.0 x 10^7 viable cells/mL calculated.
3. The calculated volume of cryopreservation medium was equilibrated at 4 °C.
4. Cells were pelleted by centrifugation at 500 x g for 5 to 10 minutes. The supernatant was aseptically decanted and the cell pellet resuspended in the chilled cryopreservation medium.
5. Aliquots of this suspension were then dispensed into 4.5 mL cryovials (Nunc).
6. The cell suspension was gently mixed in the cryovials to achieve a homogenous cell suspension, and placed in a controlled-rate freezing apparatus (Nalgene Cryo 1°C) in –75 °C freezer. The freezing rate in this apparatus decreases at a rate of 1 °C per minute to –75 °C.
7. Following completion of cryopreservation, the frozen ampoules were immediately transferred to liquid nitrogen (-125 to –200 °C) for storage.
8. Viability and recovery of cryopreserved cells were checked 24 hours after storage of vials in liquid nitrogen by following the thawing and recovery of one frozen ampoule as procedure outlined above.

7.2.2 RT-PCR Amplification of *vp2* Gene

The VP2 capsid protein gene was amplified for cloning by RT-PCR using a forward primer, 5' ATGACAAACCTGCAAGATCAAAC 3', flanking the VP2 protein N-

terminus, and a reverse primer 5' TTACCTTATGGCCCGGATTATGTCTT 3' covering the proposed C-terminal cleavage site for VP2 (between the dibasic amino acids RR at positions 452 and 453) (Kibenge *et al.*, 1990). A stop codon (TAA) was inserted at the 3' end using the downstream primer as shown in Figure 7.1. RNA was extracted from the purified IBD virus (Chapter 6) using Trizol LS (Lifetech) following the manufacturer's instructions.

cDNA for *vp2* was synthesised using the isolated RNA as a template for RT-PCR using the Titan One-Step RT-PCR kit (Roche). The reaction mixture contained 2 μL of primer (10μM), 10 μL of 5x buffer, 3 μL $MgCl_2$, 2.5 μL DTT, 1 μL of enzyme mix (Taq and Pwo DNA polymerase and AMV reverse transcriptase), 0.25 μL RNAse inhibitor, 25.25 μL of DEPC-treated dH_2O and 2 μL of the RNA template. The RT-PCR cycle was started by synthesis of the cDNA at 50 °C for 30 minutes, then amplification by 35 cycles of annealing at 60 °C for 30 seconds followed by elongation at 68 °C for 2 minutes, with a final elongation cycle of 68 °C for 7 minutes.

The RT-PCR product was loaded onto a 1.5% agarose gel and electrophoresed at 100V for 40 minutes. The gel contained 5% ethidium bromide for visualisation. The RT-PCR reaction resulted in the production of amplification products of the predicted size of 1359 bp.

The *vp2* gene was extracted and purified from the gel using a QIAquick Gel Extraction kit (QIAGEN, Germany), then sequenced on an ABI Prism 377 DNA sequencer using a Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) and the primer pair described above. Nucleotide and the deduced amino acids sequences are shown in Appendix A.

7.2.3 Cloning of *vp2* Gene Into the pFASTBAC Donor Plasmids

The first step in cloning the *vp2* gene using the BAC-to-BAC Baculovirus Expression system is shown in Figure 7.1. In order to clone the *vp2* cDNA gene amplified in the RT-PCR step described above, it was necessary to use blunt–end ligation to place the

vp2 gene in the pFASTBAC vector (Rapid DNA Ligation kit, Roche). Competent *E.Coli* cells (LE DH5α) were used for the initial cloning.

Klenow and Polynucleotide Kinase Treatment of Insert Gene

The *vp2* cDNA gene was treated with Klenow enzyme to produce the blunt ends. A reaction mixture containing 3.9 μL of 10x Klenow buffer, 0.312 μL 0.5mM dNTPs, 3.9 μL of 1mg/mL BSA, 0.75 μL of Klenow (5U/μL) and 0.138 μL of dH_2O was prepared. After thorough mixing, 3 μL of the reaction mixture was added to 10 μL of *vp2* cDNA (25 ng/μL). The tube was incubated at room temperature for 15 minutes before being heated to75 °C for 10 minutes to inactivate the enzyme.

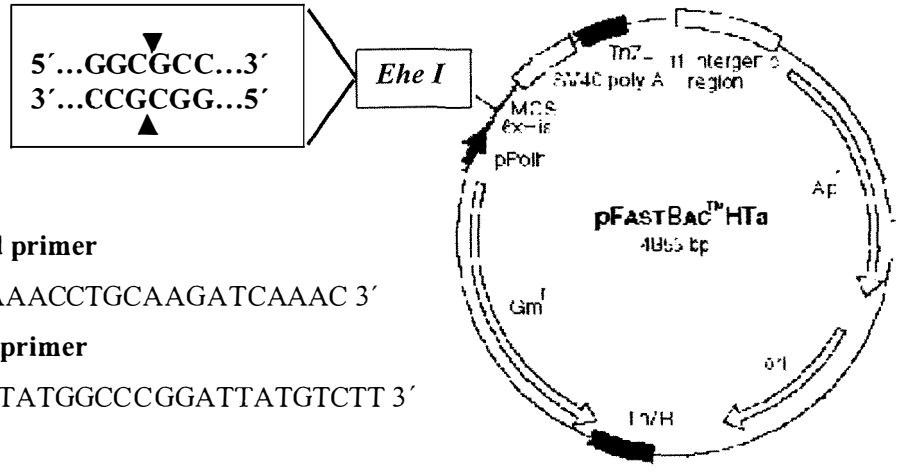
The 5' hydroxyl terminal of the *vp2* cDNA was phosphorylated using T4 polynucleotide kinase. One μL of a solution made up of 0.6 μL of 5x buffer, 0.42 μL of 100mM ATP, 0.75 μL of T4 polynucleotide kinase, and 1.23 μL of dH_2O, was added to the reaction tube and incubated at 37 °C for 60 minutes. The enzyme was then inactivated by incubation at 65 °C for 20 minutes. The prepared *vp2* cDNA was stored at –20 °C.

Digestion, Alkaline Phosphatase Treatment and Purification of Donor Plasmid

The pFASTBAC HTa donor plasmid map and restriction sites are shown in Figure 7.1. The *Ehe I* restriction site of the pFASTBAC HTa donor plasmid was used to clone the *vp2* gene. Two μL of the *Ehe I* restriction enzyme (4U/μL) was added to a tube containing 8 μL of the prepared *vp2* cDNA from the above followed by, 2 μL of 10x NE buffer 2 (Appendix D) and 10 μL of dH_2O. The digestion mixture was incubated at 37 °C overnight.

In order to prevent self-ligation of the plasmid after the blunt-end digestion, the 5′ phosphate groups were removed from the DNA insert using alkaline phosphatase. Ten (10) μL of the alkaline phosphatase mixture (prepared by mixing 1 μL of thermosensitive alkaline phosphatse (TsAP, Lifetech), 2 μL of 25mM $MgCl_2$, 1 μL of 10x buffer and 6 μL of dH_2O) was added to the digestion tube and incubated at 65 °C for 15 minutes. The digested and dephosphorylated donor plasmid was analysed by gel electrophoresis (0.8% agarose gel, 80 V for 1 hour). The linearised plasmid of 4855bp was visualised under UV and the agarose band excised for purification using a

QIAquick Gel extraction kit (QIAGEN,Germany). The concentration of the linearised plasmid was determined using a GeneQuant RNA/DNA calculator (Pharmacia Biotech).



***vp2* forward primer**

5′ ATGACAAACCTGCAAGATCAAAC 3′

***vp2* reverse primer**

5′ TTACCTTATGGCCCGGATTATGTCTT 3′
stop
codon

Figure 7.1: Map of pFASTBAC HTa donor plasmid showing the *Ehe I* restriction site for cloning and the primer set used for the amplification of the *vp2* insert gene in RT-PCR.

Ligation of *vp2* Gene Insert Into pFASTBAC HTa Donor Plasmid

The prepared *vp2* insert was ligated into the donor plasmid vector using the Blunt End High Efficiency Ligation kit (Roche). Three μL of the prepared insert, *vp2* gene and 1 μL of the linearised vector were added to a sterile Eppendorf tube containing 1 μL of 5x buffer, 5 μL of 2x ligation buffer and 0.5 μL of T4 ligase enzyme. The tube was incubated at room temperature overnight to produce the pFBHTaVP2 plasmid.

The pFBHTaVP2 plasmid was transformed into LE DH 5α competent cells using heat shock according to the manufacturer's instructions. The ligation reaction mixture was used in the transformation procedure without any further purification.

For transformation, 2.5 μL of ligation reaction was added to an Eppendorf tube and put on ice. Then, 50 μL of competent LE DH 5α cells was added to the tube and the mixture incubated on ice for 30 minutes. The cells were heat-shocked by incubation at 42°C for 1 minute then recovered on ice for 2 minutes before being diluted with 450 μL of SOC broth (Appendix D), and incubated at 37 °C with shaking at approximately 180

rpm for 1 hour. Following incubation, the entire contents of the tube were spread onto an agar plate (LB, 100 mg/mL ampicillin) and incubated inverted, overnight at 37 °C.

Colony Screening

Transformed colonies were screened for positive clones using PCR with the primers directed at sequences on either side of the *EheI* RE site of pFBHTaVP2. A correctly transformed plasmid containing the *vp2* insert should produce a PCR product that is 2093 bp in size.

In order to determine whether the gene had been inserted in the correct orientation, the plasmid was subjected to another PCR reaction using a forward primer directed to the sequence flanking the vector and a reverse primer directed to the 3′ end of the *vp2* insert gene. Amplification of the correctly incorporated gene should give a PCR product of 1479 bp as illustrated in Figure 7.2.

Positive colonies were transferred into 5 mL of LB broth containing 100 μg/mL ampicillin and incubated overnight at 37 °C with constant shaking (180 rpm). Plasmid DNA was extracted from the overnight culture broth using the Mini-Prep Extraction kit (Roche). The concentration of the extracted plasmid DNA was determined using a GeneQuant RNA/DNA calculator (Pharmacia) and the orientation of the insert verified by PCR as described above. The recombinant plasmid DNA was also sequenced using the plasmid specific primer set to verify the 5' and 3' sequences of the VP2 insert. Positive cloned plasmid DNA in both the PCR reactions and with the correct 5' and 3' sequences was used to transpose DH10BAC.

Uncut pFASTBAC HTa plasmid DNA without insert was used in the PCR screening as negative control.

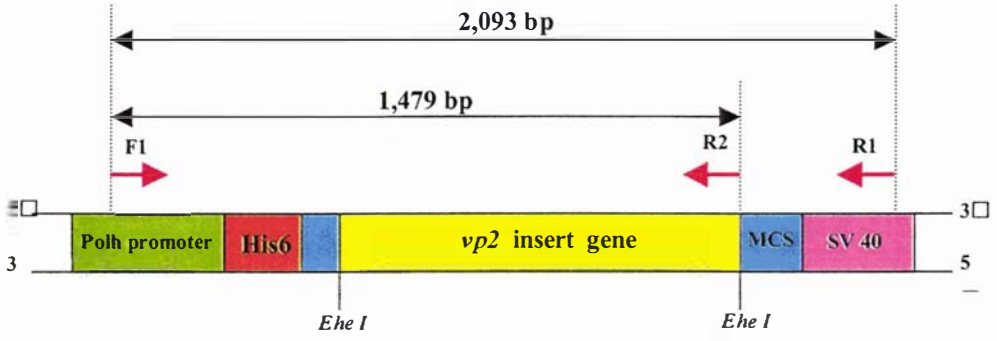


Figure 7.2: Schematic diagram showing predicted PCR products using various primer pairs on the successfully and correctly ligated plasmid pFBHTaVP2.

F1: Forward primer directed at the sequences on the vector.
R1: Reverse primer directed at the sequences on the vector.
R2: Reverse primer directed at the sequences of the 3'end of the *vp2* insert gene.

7.2.4 Transposition of Recombinant Donor Plasmid Into DH10 BAC Competent Cells

The second step in the Bac-to-Bac system is to produce recombinant bacmids using a baculovirus shuttle vector (bacmid) propagated in *E.coli*. The bacmid contains the low-copy-number mini-F replicon, a kanamycin resistance marker, and a segment of DNA encoding the *lacZ*α peptide from a pUC-based cloning vector. The bacmid propagates in *E.coli* DH10BAC as a large plasmid that confers resistance to kanamycin and can complement a *lacZ* deletion present on the chromosome to form colonies that are blue (*Lac*+) in the presence of a chromogenic substrate such as Blue-gal or X-gal and the inducer IPTG.

Based on site-specific transposition of an expression cassette in the bacmid, recombinant bacmids are constructed by transposing a mini-Tn7 element from a pFASTBAC donor plasmid to the mini-*att*TN7 attachment site on the bacmid when the Tn7 transposition functions are provided *in trans* by a helper plasmid (pMON7124). The helper plasmid confers resistance to tetracycline and encodes the transposase. Insertions of the mini-Tn7 into the mini-*att*Tn7 attachment site on the bacmid disrupts expression of the *lacZ*α peptide, so colonies containing the recombinant bacmid are

white in a background of blue colonies that harbour the unaltered bacmid. Recombinant bacmid can therefore be easily identified and picked for culturing.

Five μL of the diluted pFBHTaVP2 donor plasmid were used to transform 100 μL of DH10BAC competent cells as directed in the manufacturer's instructions. For the transposition, 5 μL (approximately 1 ng) of recombinant donor plasmid was added to an Eppendorf tube and put on ice. One hundred μL of DH10BAC competent cells was added to the tube and gently mixed. The cells were incubated on ice for 30 minutes before being heat shocked by incubation at 42 °C for 1 minute, followed by recovery on ice for 2 minutes. Nine hundred μL of SOC medium (Appendix D) was added to the tube and the mixture incubated at 37 °C with medium agitation (225 rpm) for 4 hours. After incubation, the cells were serially diluted with SOC medium to give 10^{-1}, 10^{-2} and 10^{-3} fold dilutions. One hundred μL of each dilution was spread on Luria Agar plates containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 100 μg/mL X-gal and 40 μg/mL IPTG. The plates were inverted and incubated at 37 °C for 24 to 48 hours.

Plates were stored at 4 °C overnight to enhance the colour differences. White colonies (approximately 20) were selected from the plate and re-cultured on a fresh plate in order to verify the phenotype. After incubation at 37 °C overnight, a single white colony was picked, inoculated into a 5 mL LB broth containing the antibiotics (50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline) and incubated overnight at 37 °C with constant shaking (225 rpm). The 5 mL overnight culture was used for the isolation of recombinant Bacmid DNA.

7.2.5 Extraction and Screening of Recombinant Bacmid

The CONCERT High Purity Plasmid Isolation System (Lifetech) was used to isolate the recombinant Bacmid DNA from the 5 mL overnight LB broth culture and the yield of recombinant bacmid DNA determined using a GeneQuant RNA/DNA calculator (Pharmacia) as before.

Recombinant bacmid DNA was analysed using PCR and gel electrophoresis in order to verify that *vp2* gene had been correctly transposed from the pFBHTaVP2 donor plasmid

to the bacmid DNA. The pUC/M13 amplification primers that are directed at sequences on either side of the mini-*att*Tn7 site within the *lacZα*-complementation region of the bacmid were used for PCR analysis. If the *vp2* gene was correctly transposed, the PCR product produced by the pUC/M13 primers should be 3786 bp (2430 bp + 1356 bp) in size. Additionally, in order to verify that the *vp2* gene had been inserted into the bacmid in the correct orientation, the pUC/M13 forward primer and the VP2 gene–specific reverse primer were used in a PCR reaction. In this case, the product should be 3006 bp as shown in Figure 7.3.

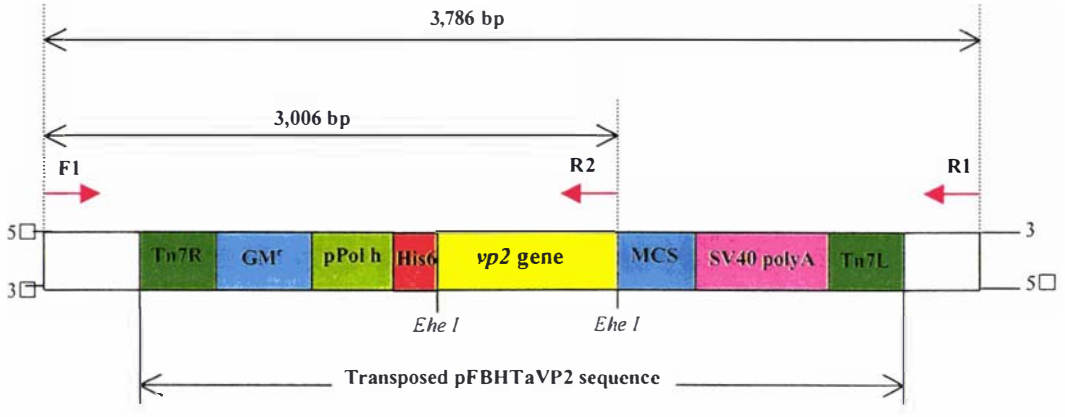


Figure 7.3: Schematic diagram showing predicted PCR products using the pUC/M13 forward (F1) and pUC/M13 reverse (R1) directed on either side of the mini-attTn7 of the bacmid in the verification of transposition. For gene-specific amplification, the M13/pUC forward and the reverse primer (R2) directed at the 3'end of the *vp2* insert gene were used.

Five μL of the isolated recombinant bacmid DNA was also analysed by agarose gel electrophoresis. Five μL of the recombinant bacmid DNA was mixed with 2 μL of loading dye, loaded onto 0.5% agarose gel made with TAE buffer and subjected to a constant voltage of 23 V for 12 hour. The presence of the recombinant bacmid DNA should be indicated by a band that migrates more slowly than the 23.1 kb fragment of the *λDNA/HindIII* fragment.

7.2.6 Transfection of Sf9 Cells with Recombinant Bacmid DNA

Transfection of the recombinant bacmid DNA into Sf9 cells to generate recombinant baculovirus carrying the *vp2* gene for the expression of His-tagged VP2 protein was carried out as below:

1. Cells from a 3- to 4-day-old suspension culture in mid-log phase with a viability of >97% were added to 2 mL Sf-900 II SFM containing penicillin/streptomycin at 0.5 times the final concentration (50 units/mL penicillin, 50 μg/mL streptomycin), in a 6 well plate to give 9 x 10^5 cells per well.
2. The cells were allowed to attach to the surface for at least 1 hour at 27 °C.
3. The following solutions were prepared in 12 x 75-mm sterile tubes:
   Solution A: Five μL of the recombinant bacmid DNA was added to 100 μL Sf-900 II SFM without antibiotics.
   Solution B: Ten μL of CELLFECTIN reagent (Lifetech) was added to 100 μL Sf-900 SFM without antibiotics.
4. Solution A was added to solution B, mixed gently, and incubated for 45 minutes at room temperature to form lipid-DNA complexes.
5. Cells seeded from step 1 were washed once with 2 mL of Sf-900 SFM without antibiotics.
6. At the end of the incubation, 0.8 mL of Sf-900 SFM was added to the tube containing the lipid-DNA complexes. After gently mixing, the wash medium was removed from cells by aspiration, and the cells overlaid with the diluted lipid-DNA complexes.
7. Cells were incubated for 5 hours at 27 °C.
8. The transfection mixture was removed and 2 mL of Sf-900 II SFM containing antibiotics was added to the cells, which were then incubated at 27 °C for 72 hours.
9. At the end of the 72 hours, the recombinant baculovirus was ready for harvesting as described below.

7.2.7 Harvest and Titration of Recombinant Baculovirus

The recombinant baculovirus was transferred from the well into a sterile tube and clarified by centrifugation for 5 minutes at 500 x g. The virus-containing supernatant

was transferred to a fresh tube and titrated using the Viral Plaque Assay described below:

Viral Plaque Titration Assay

1. Twelve wells (2 x 6-well plates) were seeded with 2 mL of Sf9 cells at 5 x 10^5 cells/mL per well. The plates were covered and incubated at room temperature for 1 hour to allow the cells to attach to the plates.
2. A bottle containing sterile 4% low-melting point (LMP) agarose gel was equilibrated at 70 °C. An empty 100 mL bottle and a bottle of 1.3 x Sf-900 II insect medium were equilibrated at 40 °C.
3. After one hour's incubation at room temperature, the plates were inspected using an inverted microscope to confirm that cell attachment had occurred with the formation of a monolayer of 50% confluence.
4. An eight-log serial dilution of the harvested recombinant baculovirus was prepared by sequentially diluting 0.5 mL of the previous dilution in 4.5 mL of Sf-900 II SFM, resulting in 5 mL each of a 10^{-1} to 10^{-8} fold dilution of the original stock.
5. The plates from step 1, containing 2 x 6 wells with 50% confluent monolayers of cells were labelled in duplicate to indicate 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} dilution. The supernatant was sequentially removed from each well and immediately replaced with 1 mL of the appropriate virus dilution (in the duplicate wells). The plates were incubated for 1 hour at room temperature.
6. Near the end of the virus incubation, a plaquing overlay was prepared by mixing 10 mL of the liquified 4% LMP agarose with 30 mL of the pre-warmed 1.3x Sf-900 II insect medium in the pre-warmed 100 mL bottle. The plaquing overlay mixture was returned to the 40 °C water bath until use.
7. After one hour, the virus inoculum was sequentially removed (from high to low dilution) from the wells and replaced with 2 mL of the plaquing overlay. The gel was allowed to harden for 10 to 20 minutes before the plates were placed in a 27 °C humidified incubator for 5 days.

8. After the 5th day of incubation, each well was stained with Neutral Red in order to identify the plaques. Freshly prepared 0.1% (w/v) neutral red stain solution in sterile PBS (0.5 mL) was added to each well and the plates incubated for 1 hour.
9. The staining solution was gently removed from each well by aspiration and the plaques in each well were counted under the microscope. The plaques appear as clear circles with diameters of approximately 1 to 2 mm against the stained red/russet red background.
10. In order to determine the titre (pfu/mL) of the recombinant baculovirus, the plaques in wells containing 10 to 50 plaques per well were counted. The titre was calculated using the following formula:

    Pfu/mL = 1/dilution factor x number of plaques x 1/(mL of inoculum/plate)

7.2.8 Determination of Optimal MOI and Incubation Time for Recombinant VP2 Expression

Once the titre of the recombinant baculovirus stock had been determined, the virus stock was stored at 4 °C and protected from light. One mL of the virus stock was stored at –70 °C for long-term storage. To determine the optimal expression of the recombinant VP2 protein, the infection parameters were optimised using different Multiplicity of Infection (MOI) and incubation times.

1. Three 25 mL Sf9 cell cultures were grown to a cell density of 1 x 10^6 to 2 x 10^6 cells/mL.
2. Each flask was infected with the recombinant baculovirus stock to obtain a MOI value of 1, 5 and 10 using the following formula:

    $$\text{Inoculum required (mL)} = \frac{\text{desired MOI x Total number of cells}}{\text{Titre of viral inoculum (pfu/mL)}}$$

3. The infected flasks were incubated at 28 °C ± 0.5 °C with constant shaking (140 rpm).
4. Two mL of cell suspension was collected from each flask at 48, 72, 96 and 120 hours post-infection, and the cells pelleted by centrifugation (5 minutes, 500 x g). The supernatant was discarded and the cell pellet from each MOI and incubation interval stored at –70 °C until the test was completed.

5. The cell pellets were thawed and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.5, 5 mM 2-mercaptoethanol, 100 mM KCL, 1 mM PMSF, 1% Nonidet P-40 at 4 °C) (5 volumes of lysis buffer per gram of cells).
6. The cells were ruptured by sonication (three times for 10 seconds each). Cell debris was removed by centrifugation at 10,000 x g for 10 minutes, and the supernatant carefully transferred to a new tube.
7. Ten µL of the supernatant from each tube was mixed with 2 µL of 10 x loading buffer, boiled for 5 minutes and analysed by SDS-PAGE gel as described in section 6.2.4.
8. Proteins from the SDS-PAGE gel were electroblotted onto a PVDF membrane as described in section 6.2.5. Recombinant VP2 protein was probed with anti-IBDV monoclonal antibody R63 and polyclonal antibody using standard techniques as described in section 6.2.6.

7.2.9 Amplification of Recombinant Baculovirus Working Stock

Once expression of recombinant VP2 protein using the baculovirus system had been confirmed, the transfected virus stock was amplified to make a larger working stock for further expression trials. Four flasks containing 25 mL suspensions of Sf9 cell cultures were inoculated with the transfected virus stock at MOI of 0.01, 0.05, 0.1 and 0.2 according to the formula described in section 7.2.8. Virus from each MOI was harvested 5 days (120 hour) post-infection and the titre determined for each MOI dose following the protocol described in section 7.2.6. The MOI dose that gave the highest progeny recombinant virus titre was used for further experiments. Working stock viruses were stored at 4 °C.

An overview of the generation of recombinant baculovirus and gene expression of recombinant VP2 protein with the Bac-to-Bac Expression system is shown in Figure 7.4.

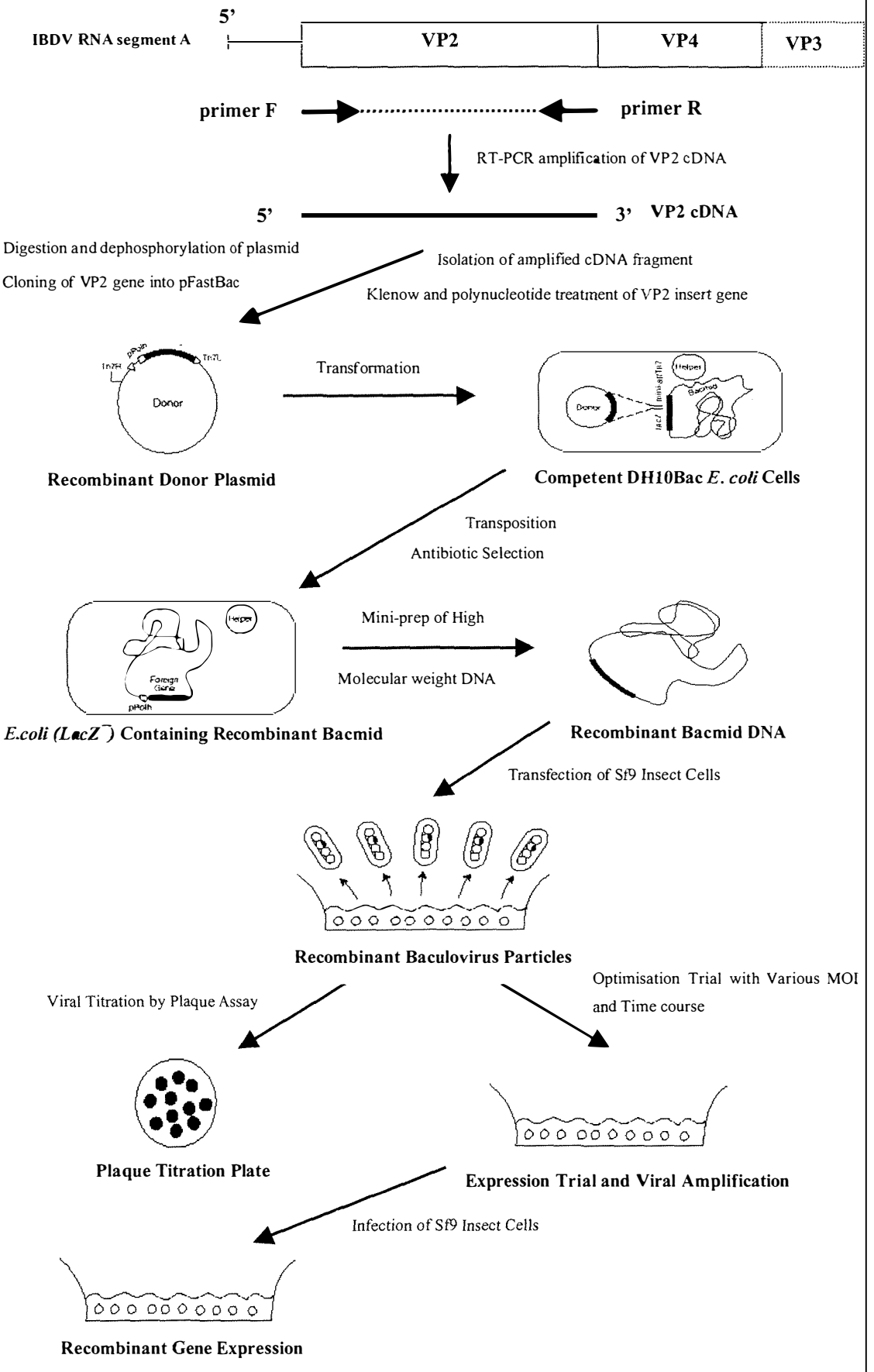


Figure 7.4: Overview of generation of recombinant baculovirus and rVP2 expression with BAC-TO-BAC Expression System.

(Figure adapted from Lifetech Bac-To-Bac Baculovirus Expression System manual, Lot No. MF1PO1-0699)

7.2.10 Expression and Purification of Recombinant VP2 protein Using Immobilised Metal Affinity Chromatography (IMAC)

After determining the optimal expression parameters for both MOI and incubation time, a large scale of 100 mL suspension culture was prepared for recombinant VP2 protein production and preliminary purification trials. A 100 mL suspension culture of Sf9 cells, cultured at a density of 2 x 10^6 cells/mL, was inoculated with the pre-determined optimal MOI and incubated at 28 °C with constant shaking (140 rpm). At the end of incubation (72 hours), culture media was centrifuged for 10 minutes at 1,500 x g. The resulting cell pellet was processed following the procedure below, or stored at –80 °C until needed.

1. The thawed or fresh cell pellet was resuspended in 5 times volume of loading buffer (20 mM Tris-HCl, 500 mM NaCl, pH 8.0). One tablet of Complete™ EDTA-free protease inhibitor (Roche) was added to 50 mL of cell suspension according to the manufacturer's instructions.
2. Cells were lysed using a French Pressure Cell (AMINCO, USA). Cellular debris was removed by centrifugation at 10,000 x g for 30 minutes. The supernatant was transferred to a new tube and centrifuged once more to ensure complete removal of any remaining particulate matter.
3. As the recombinant VP2 protein carries a 6xHistidine-Tag at its N-terminus end, immobilised metal affinity chromatography was used to simplify purification. A metal chelating Sepharose Fast Flow resin (Pharmacia Biotech) was used, following the procedure recommended in the manufacturer's instruction manual. The cell free supernatant was filtered through a 0.2 μm filter before loading onto a Ni-NTA column (16 mm x 20 cm, XK16, Pharmacia) that had been equilibrated in 10 x column volume of loading buffer (20 mM Tris-HCl, 500 mM NaCl, pH 8.0), at a flow rate of 0.5 mL/min, and was eluted with a step-wise increase in the concentration of imidazole (0 – 0.5 M) in the loading buffer, at a flow rate of 0.5 mL/min, for 10 x column volume each.
4. Two mL of each elution fraction (0 – 0.5 M imidazole in loading buffer) was concentrated by ultrafiltration to 100 μL (Centricon-10, Amicon) and 10 μL of the concentrated fraction was added to 2 μL of 10 x SDS-loading buffer and loaded

onto a 12% SDS-polyacrylamide gel as described in section 6.2.4 in order to determine the protein composition of each elution fraction.

7.2.11 Purification of Recombinant VP2 Protein by Chromatographic Methods

Ion Exchange Chromatography (IEX) – UNO-Q Column

Recombinant VP2 protein was eluted at 5 – 10 mM imidazole from the Ni-NTA column. However, SDS-PAGE showed that it needed further purification. Ion Exchange Chromatography (IEX) separates proteins on the basis of the net charge on their surface at a given pH. The surface distribution of charge, as well as the overall net charge on a protein dictates its unique behaviour in an ion exchange environment. Ion exchange resins bear functional groups that have either positive or negative charges over particular pH ranges. These charges are balanced by counter ions such as Cl- for anion exchange and Na+ for cation exchange. The net charge on the protein is the same as those of the counter ions and thus the protein displaces the counter ions to bind to the ligand. Proteins carrying no charge or the same net charge as the column pass through the column in the mobile phase.

The calculated pI value of the recombinant VP2 protein was 5.36 (ProtParam Tool, ExPASy tools; http:// www. Expasy.ch/tools/protparam.html). At pH > 6.0, the protein should have an overall negative charge, and therefore is likely bind to an anion exchange column. The pooled samples from the IMAC elution fractions were extensively dialysed against ten times the sample volume of loading buffer (10 mM Bis-Tris, pH 6.5), until the conductivity and pH was identical to that of the loading buffer. A pre-packed Uno-Q1 column (Bio-Rad) was connected to an FPLC (Waters, model 650) and equilibrated with 10 column volumes of loading buffer at 4 °C. The sample was loaded at 0.5 mL per minute, then washed with loading buffer until the absorbance at 280 nm (A_{280}) was stable at a value less than 0.02 absorbance units. The bound protein was eluted by applying a gradient of 0.0 to 1.0 M NaCl in the loading buffer over 6.5 hours at a flow rate of 0.5 mL per minute. Fractions of 2.5 mL (5 minutes per tube) were collected and analysed by SDS-PAGE.

7.2.12 Size Exclusion Chromatography (SEC) with Superdex-75

SEC was used as a final purification step. Pores within the beads making up the resin act like a molecular sieve, so that large proteins are completely excluded from the pores and pass through in the void volume (V_0). Smaller proteins on the other hand, are able to penetrate the pores to different extents, depending on their size. Consequently, these proteins are eluted between the void volume (V_0) and the total column volume (V_t) in the order of decreasing molecular weight.

Fractions containing recombinant VP2 from the IEX were pooled and concentrated to 10% their original volume by ultrafiltration in a stirred cell concentrator (Amicon 8400 with an Amicon YM-10 membrane). Both the concentrator and membrane had been pre-rinsed with 0.1% Thesit detergent (Calbiochem) then rinsed again with distilled water to reduce protein loss via surface absorption. A pre-packed super fine Superdex-75 HR10/30 column (10 mm x 31 cm, Pharmacia) was used for this step. The concentrated protein solution (250 µL) was loaded onto the column using a rheodyne injection valve, and isocratically eluted in 10 mM HEPES buffer (pH 7.5) at a rate of 0.2 mL per minute. One (1) mL fractions were collected and analysed by SDS-PAGE. The purest fractions were pooled and the protein concentration was determined using either the Bradford assay or the A_{280} UV absorbance method described in section 7.2.13. The protein was concentrated by ultrafiltration in a stirred cell as described above or in a Centricon-10 (Amicon) to a final concentration of 10 to 15 mg/mL, stored at 4 °C and used for crystallisation screening trials.

An overview of the purification scheme for the recombinant VP2 protein is presented in Figure 7.5.

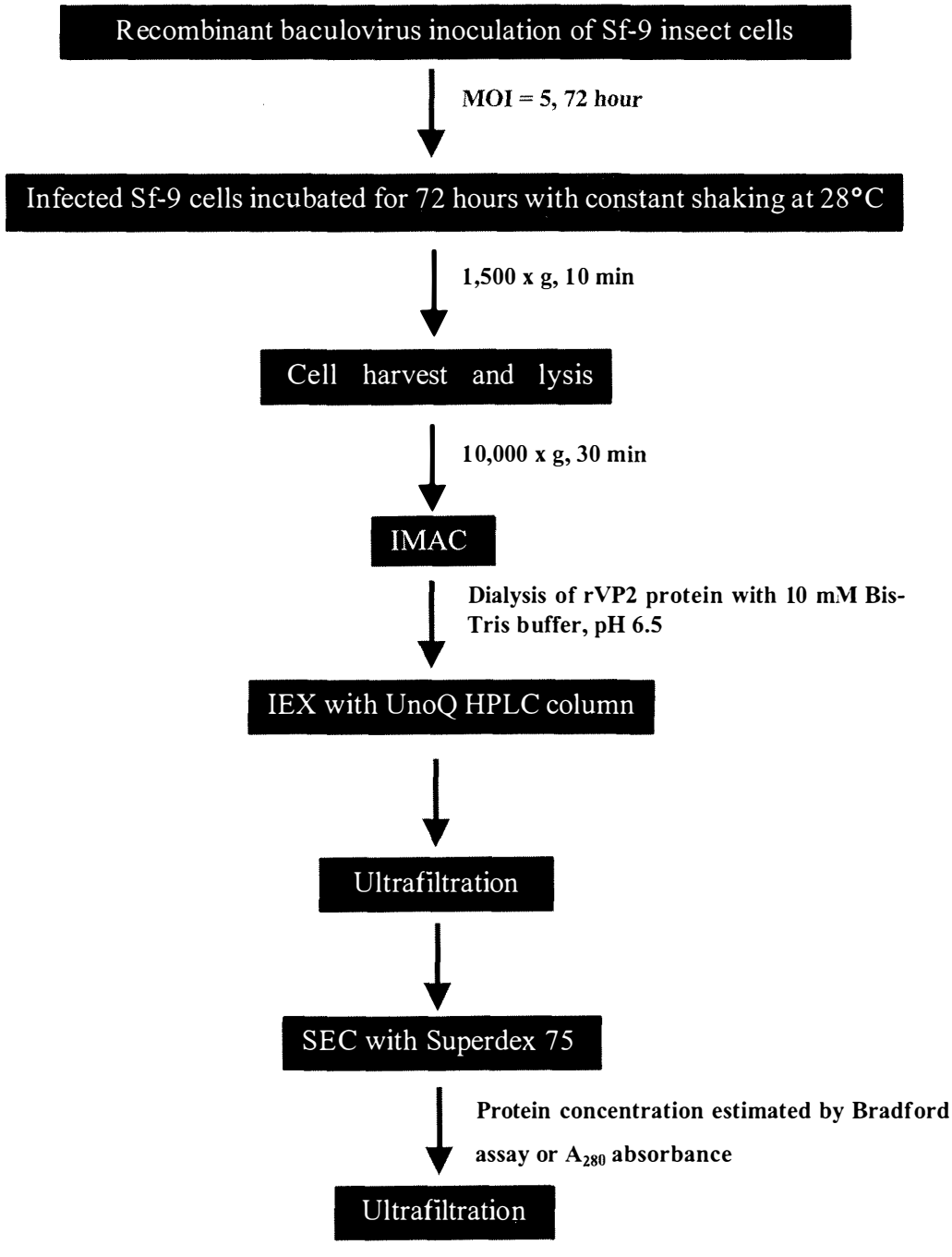


Figure 7.5: A summary of the purification scheme of recombinant VP2 protein.

7.2.13 Protein Quantification

Bradford Method

The dye-binding method using Coomassie Blue G-250 was performed as described by Bradford (Bradford, 1976). The Bradford reagent containing 100 mg of Coomassie Blue G-250 disssolved in 50 mL 95% ethanol and 100 mL concentrated phosphoric acid, was made up to 200 mL with Milli-Q water and filtered before use. Protein standards of bovine serum albumin (BSA), at concentration of 100, 50, 25 and 5 μg /100 μL were prepared in the same buffer solution as the protein sample to be measured. The Bradford dye concentrate was diluted 5x with Milli-Q water and 5 mL of the diluted dye was added to100 μL of each of the standards and the samples, and the colour allowed to develop for at least 5 minutes, but not longer than 30 minutes and their absorbance read at a wavelength of 595 nm. A standard curve was prepared using a Varian Cary-1 UV-Visible spectrophotometer and the protein concentration of the unknown determined using the standard curve.

A280 UV Absorbance Method

The calculated extinction coefficient of the rVP2 protein at 280 nm is 0.795 (ExPASy Tools, http://www.Expasy.ch/tools/protparam.html). Thus the rVP2 protein concentration could be calculated according to the equation below:

$$\text{rVP2 concentration (mg/mL)} = (A_{280} / 0.795) \times \text{dilution factor}$$

7.2.14 Biochemical Analyses of the Recombinant VP2 Fusion Protein

In order to determine an accurate molecular weight and to confirm that the rVP2 protein had the correct sequence and was folded, the purified protein was analysed using Electrospray Mass Spectrometry, N-terminal sequencing, circular dichroism spectroscopy, electron microscopy, immunoblotting and immunodiffusion techniques.

7.2.15 Immunodiffusion Test of rVP2

One way to determine whether the recombinant protein is correctly folded is to investigate its interaction with monoclonal and/or polyclonal antibodies. It has been

suggested that only proteins with native folds will give a positive response to antibodies raised against wild type protein(Chiu & Smith, 1994; Smith & Chase, 1992). To determine whether the active immunogenic site(s) of the expressed rVP2 were identical to those of the wild type, double diffusion immuno-precipitation tests were performed against two mouse anti-VP2 monoclonal antibodies, R63 and B69, and a rabbit anti-IBDV polyclonal antibody, using a technique similar to that used in the AGDP test described in section 2.2.4. In this test, the central well was filled with each individual antibody and the outer wells were filled with the partially purified or purified rVP2 at different concentrations. The IBDV antigen prepared as described in section 2.2.7 was used as a positive control. Cell lysate of non-infected Sf9 cells was used as a negative control.

7.2.16 Circular Dichroism Spectroscopy

Circular dichroism is a spectroscopic method that is often used to predict the presence of α or β structure in mature proteins. β sheets and α helices give characteristic maxima in the far UV region of the spectrum in contrast to random coil. A folded protein would therefore be expected to show a spectrum that contained α and β structure or both.

The purified rVP2 at a concentration of 0.15 mg/mL (in 20 mM phosphate buffer, pH 7.2) was scanned over the far-UV region (185 – 250 nm) using a Jasco Model J-720 spectropolarimeter (Jasco, Hachioji City, Tokyo, Japan) in a temperature-controlled room at 20 ± 0.5 °C. The spectra of the protein solutions were measured in 0.5 mm cells. Solutions were scanned at 20 nm/min using a 2 s time constant, a 0.2 nm step resolution, a 1 nm bandwidth, and a sensitivity of 10 m° (millidegrees). An average of five scans was recorded. The baseline spectrum (with buffer) was subtracted from each spectrum and the resultant rotation (m°) values were converted to the molar CD extinction coefficients.

7.2.17 Multiangle Laser Light Scattering (MALLS) Photospectrometry

To investigate the homogeneity of rVP2, the purified protein was subjected to SEC using a Superose 6HR 10/30 column (Pharmacia) that has an optimal separation range between 5,000 and 5 x 10^6 Daltons, with an exclusion limit of approximately 4 x 10^7.

Because of the large sized exclusion range, both the extent of aggregation and the distribution of aggregate size can be determined. The chromatography system consisted of a superose 6HR 10/30 column, a UV absorbance detector (GBC Scientific Equipment Ltd, Victoria, Australia) operating at 280 nm, a SAWN-DSP MALLS photometer (Wyatt Technology, Santa Barbara, CA) fitted with a helium-neon laser (λ=632.8 nm) and a K-5 flow cell, and a DRI detector.

The SEC column was attached to a GBC HPLC system (GBC Scientific Equipment Ltd). The purified rVP2 was filtered through a 0.22 μm filter and 50 μL of the rVP2 was injected onto the SEC column using a syringe pump. A solution containing 20 mM imidazole and 50 mM NaCl at pH 7.0 was used as the elution buffer at a flow rate of 0.4 mL/min.

7.2.18 Cleavage of Fusion Protein with rTEV Protease

The His-tag on the N-terminus of the expressed VP2 protein was removed using a recombinant TEV (rTEV) protease (Gibco, BRL). This protease recognizes a consensus cleavage site sequence that spans seven amino acids, Glu-Asn-Leu-Tyr-Phe-Gln-Gly, at the C-terminal end of the poly-His tag. Cleavage occurs between Gln and Gly, resulting in an extra Gly(G) at the N-terminus of the VP2 protein compared to the native sequence. Following digestion, rTEV protease was removed from the rVP2 protein via its poly-His tag by passing the reaction mixture back down the Ni-NTA affinity column.

To determine the optimal parameters for cleavage of the recombinant VP2 protein, the following experiment was carried out:

1. The following components were added to a microcentrifuge tube:

Recombinant VP2 protein	: 100 μg
20X rTEV buffer	: 50 μL
0.1 M DTT	: 10 μL
rTEV protease	: 10 μL (100 units)
Distilled water	: to 1000 μL

2. The tube was incubated at 25 °C with constant shaking, and 20 μL of reaction mix was removed at 24, 48, 72, 96 and 120 hours.

3. Ten µL of 10 x SDS-loading buffer was added to each sample which was stored at –20 °C until the experiment was completed
4. Samples were heated at 100 °C for 5 minutes before being analysed by SDS-PAGE as described in section 6.2.4.

7.2.19 Removal of rTEV Protease and Cleaved Poly-His Tag

The rTEV protease contains a histidine tag at its amino terminus for easy removal by batch affinity chromatography:

1. Ni-NTA resin was added to the cleavage reaction mixture (1 mL of resin binds 4 to 8 mg of protein).
2. The tube was incubated for 1 hour at 4 °C. The resin was pelleted down by centrifugation at 5,000 x g for 15 minutes. The supernatant containing the cleaved rVP2 protein was carefully removed by aspiration. Both the digested poly-His tag from the fusion protein and the rTEV protease remained attached to the pelleted resin.

7.2.20 Recombinant VP2 Protein Crystallisation Trials

Purified recombinant VP2 protein was screened for crystallisation conditions using the sparse matrix screen of Jancarik and Kim (Jancarik & Kim, 1991). The screen conditions are shown in Appendix C. The 98 crystal screen formulations were supplied by Hampton Research, USA.

The protein concentration used in all screens was approximately 10 mg/mL in 10 mM HEPES, pH 7.0. Hanging drops consisting of 1 µL of protein plus 1 µL of well solution were pipetted onto siliconized 22 mm diameter circular cover slides. Crystallisation trials were performed in duplicate with one set of plates (98 conditions) being set down at 4 °C while the second set was incubated at room temperature. The plates were examined under a stereo microscope immediately after they were set up to record all drop conditions, then re-examined weekly.

7.2.21 Crystal Diffraction Trials

Any resulting crystals were either mounted in capillaries or frozen at 113 K, and subjected to X-ray analysis using Rigaku RU-200B rotating anode source, with a device for selecting monochromatic radiation (AXCO capillary optic). Data was collected on a Rigaku R-Axis II C image plate detector. The program DENZO was used to index and integrate the images.

7.3 RESULTS

7.3.1 RT-PCR Amplification of *vp2* Gene

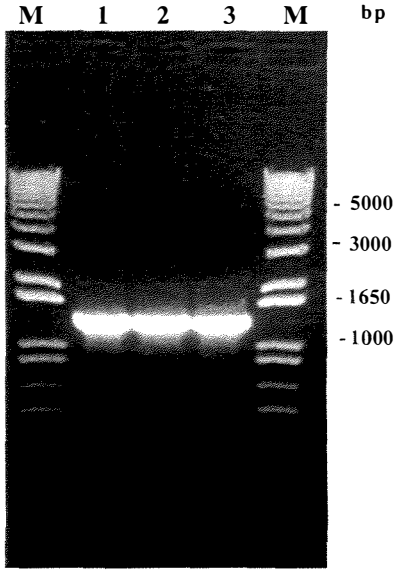


Figure 7.6: RT-PCR amplication of the VP2 insert gene.from purified IBDV M: 1 kb plus DNA ladder (Gibco BRL); Lane 1, 2, 3: Amplified RT-PCR product of IBDV cDNA.

The RT-PCR resulted in the amplification of a product with the predicted size of 1359 bp (Figure 7.6). The sequence obtained for this PCR product confirmed it contained the complete nucleotide sequence of the *vp2* gene (Appendix A), with the correct 5' and 3' termini. The stop codon (TAA) was inserted correctly at the 3' end.

The deduced amino acid sequence of the amplified *vp2* gene is shown in Appendix A. It has a calculated molecular weight of 48.45 kDa.

7.3.2 Cloning of *vp2* Gene Into pFASTBAC Donor Plasmid

After the gene fragment isolated above was inserted into the linearised pFASTBAC plasmid, and transformed into competent *E.coli* cells, the cells were plated out to screen for the correct directional insert. From a total of 50 colonies, 20 were selected for PCR analysis as described in section 7.2.3.

Amplification of the correctly oriented recombinant plasmid was expected to result in a PCR product of 1479 bp (Figure 7.7). Colonies in lanes 4, 8, 10, 11, 16 and 18 showed amplification products of this size indicating that they contained the *vp2* gene in the correct orientation in the plasmid.

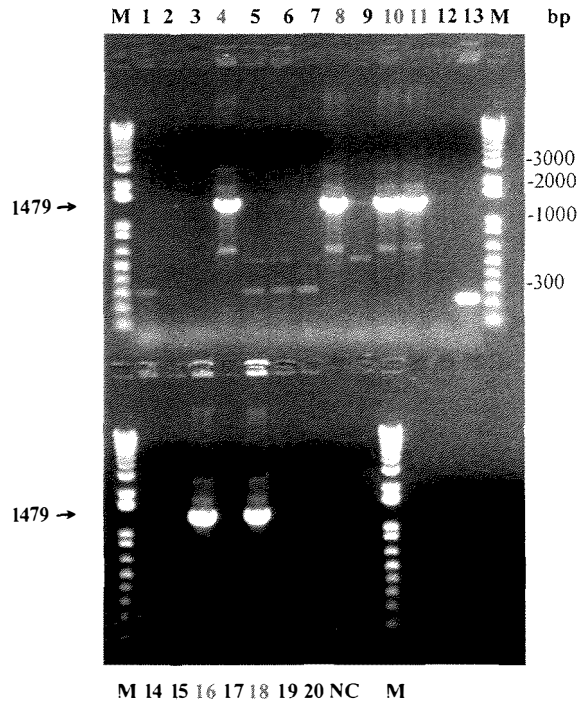


Figure 7.7: Results from colony screening of the donor plasmid pFASTBAC HTa for correct insertion of *vp2* gene. M: 1 kb plus molecular ladder (Gibco BRL); lanes 1-20: colonies 1 to 20 from transformation (lane number in red for positive colonies); NC: negative control, plasmid without insert gene.

7.3.3 Transposition of the pFBHTaVP2 into DH10BAC Competent Cells

The results from this experiment showed that a dilution of the 10^{-3} gave the optimum number of white colonies. It was found that storage of the plates at 4 °C overnight facilitated the differentiation between blue and white colonies.

About 35 white colonies were selected from the plate (approximately 100 colonies) and bacmid DNA prepared for screening, using PCR with the pUC/M13 amplification primers directed to sequences on either side of the mini-*att*Tn7 site within the *lacZ*α-complementation region. The results are shown in Figure 7.8. Colonies in lanes 2, 4, 14, 15 and 31 gave a PCR product of 3786 bp, the size expected if the recombinant plasmid contained the *vp2* insert.

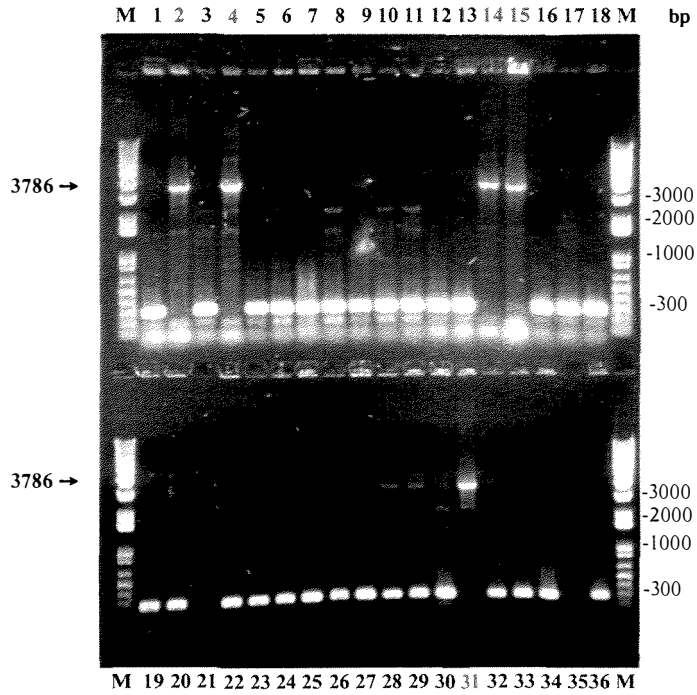


Figure 7.8: Amplification products from PCR amplification of bacmid DNA using pUC/M13 primers. M: 1 kb plus molecular ladder (Gibco BRL); lanes 1-36: colonies 1 to 36 from transposition. Expected product size of bacmid transposed with pFASTBAC HTa + VP2 gene insert is approximately 3786 bp (indicated in red) and nontransposed bacmid alone is about 300bp.

Five of the verified colonies were re-streaked on a fresh plate to verify the phenotype and incubated overnight at 37 °C. By using the pUC/M13 forward primer and the *vp2* gene–specific reverse primer, it was possible to verify that the vp2 gene was correctly oriented in the bacmid. Results are shown in Figure 7.9, where it can be seen that all five selected colonies contained the correctly oriented *vp2* gene.

A single white colony was picked from this second plate and used to inoculate a 5 mL LB broth containing antibiotics (50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline). The culture was grown at 37 °C with constant shaking at 225 rpm overnight, and used to isolate recombinant Bacmid DNA.

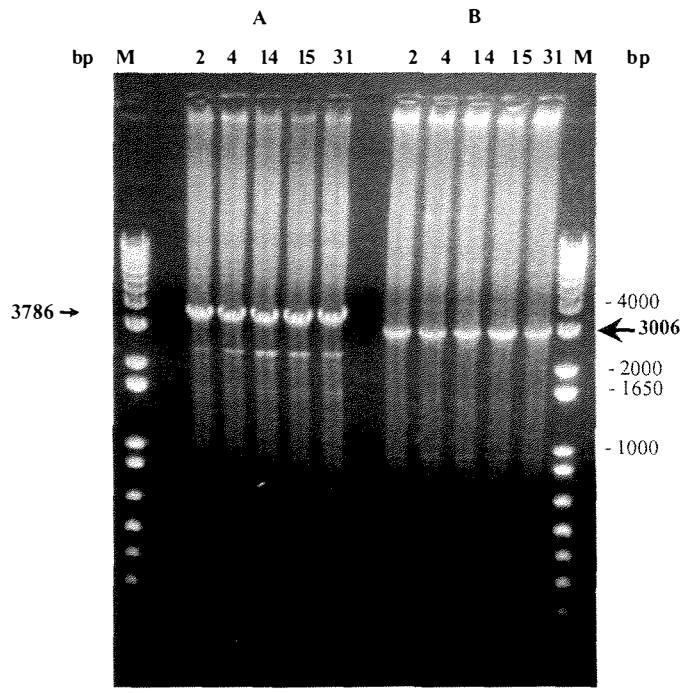


Figure 7.9: Amplification products of the PCR reaction carried out using recombinant bacmid from colonies 2, 4, 14, 15 and 31 as template, (A) with pUC/M13 primers, expected product size 3786 bp; (B) with pUC/M13 forward and *vp2* insert gene reverse primers, expected product size 3006 bp. M: 1 kb plus molecular ladder (Gibco BRL).

Agarose gel electrophoresis of the isolated recombinant bacmid DNA from the mini-prep also verified the presence of bacmid DNA as shown in Figure 7.10, lanes 2 to 6 where a band migrating slightly slower than the 23.1 kb fragment of the *λDNA/HindIII* fragment marker is visible. The concentration of the isolated bacmid DNA was between 60 to 80 ng/μL as determined by a GeneQuant RNA/DNA calculator (Pharmacia). Colony # 4 had the highest concentration and strongest band intensity in the gel and was therefore selected to transfect the insect cells to generate recombinant baculovirus.

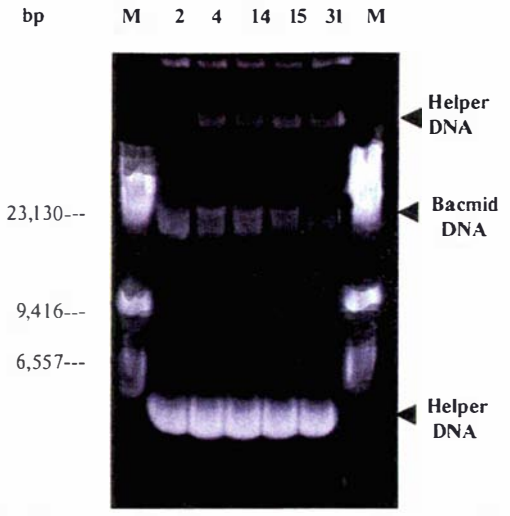


Figure 7.10: Agarose gel electrophoresis of mini-prep bacmid DNA. M: DNA/Hind III ladder (Gibco BRL); Lane 2 – 6: Mini-prep of recombinant bacmid from colonies 2, 4, 14, 15 and 31.

7.3.4 Transfection of Sf9 Cells with Recombinant Bacmid DNA, Harvest and Titration of Recombinant Baculovirus

Recombinant baculovirus containing the *vp2* gene were harvested from the transfection wells 72 hours post-incubation. Plaque titration of the initial transfected recombinant baculovirus yielded a viral titre of 1 x 10^7 PFU/mL. Figure 7.11 shows a plaque titration well stained with 0.1% neutral red stain.

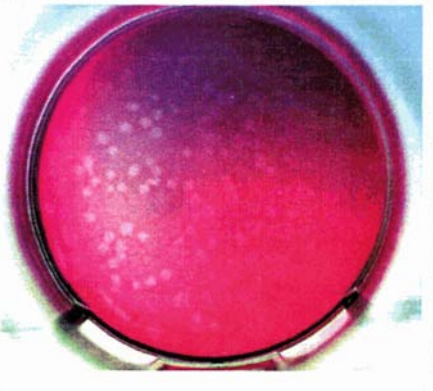


Figure 7.11: A plaque titration well showing clear plaques in the overlaid gel against the russet red background.

7.3.5 Determination of Optimal MOI and Incubation Time for Recombinant VP2 Expression

The results of the experiments carried out to optimise the expression of recombinant VP2 (rVP2) proteins are shown in Figure 7.12. Recombinant VP2 protein was found to

be optimally expressed at an MOI of between 5 and 10, with an optimal incubation time between 48 and 72 hours. There was a decrease in the production of rVP2 if incubation continued for more than 72 hours, but there was no difference between MOI values of 5 and 10. Subsequent rVP2 expression trials were carried out using an MOI of 5 and an incubation time of 72 hours post-infection.

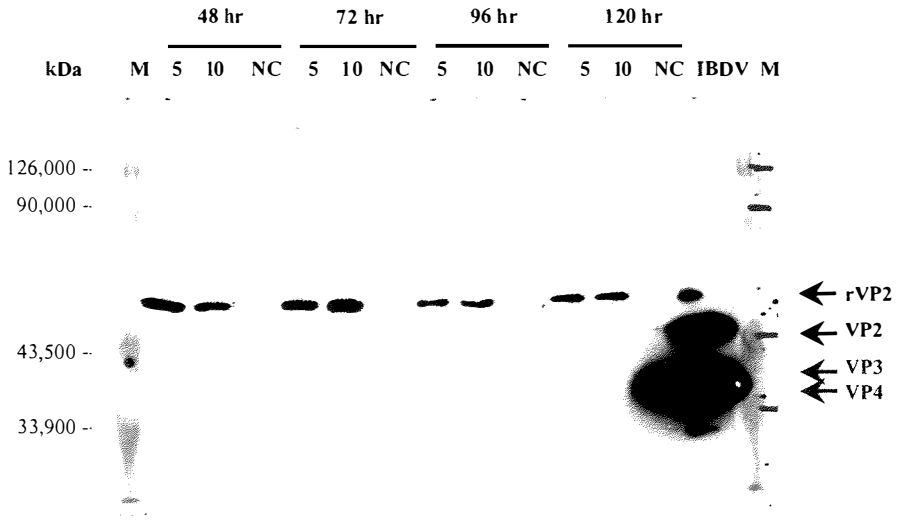


Figure 7.12: Western immunodetection of rVP2 from cultures of different MOI and incubation times using rabbit anti-IBDV polyclonal antibody. Samples were prepared from cells with MOIs of 5 and 10, that had been incubated for 48, 72, 96 and 120 hours

M:Kaleidoscope prestained standards; NC: lysed uninfected insect cells as negative controls; IBDV: purified IBDV virus as positive control sample.

7.3.6 Amplification of Recombinant Baculovirus Working Stock

The initial recombinant baculovirus stock (viral passage # P0) was used to amplify a larger scale working stock (viral passage #P1 and P2). An optimisation experiment was carried out using MOIs of 0.01, 0.05, 0.1 and 0.2. Once it had been harvested, the virus culture was titrated as described in section.7.2.7. Cultures with a MOI between 0.1 and 0.2 gave the highest amplification 120 hours post-infection. Titration of the subpassaged recombinant baculovirus working stock yielded a titre of approximately 1-2 x 10^8 PFU/mL.

7.3.7 Purification of rVP2 Protein Using Immobilised Metal Affinity Chromatography (IMAC)

The first step of the purification of the expressed rVP2 utilised the hexa-histidine tag at the N-terminus of rVP2 that has an affinity for metals such as Ni^{2+} or Zn^{2+}. The crude cell lysate was passed down a pre-equilibrated Ni^{2+}-chelating Sepharose Fast Flow resin (Pharmacia) packed in a 16 mm x 20 cm column (XK 16/20, Pharmacia). Unbound proteins were removed by extensive washing with loading buffer. The bound proteins were eluted using a stepwise imidazole gradient (5, 10, 20, 50, 100, 200 and 500 mM) as described in section 7.2.10. A sample from each elution fraction was concentrated and analysed by SDS-PAGE as shown in Figure 7.13. The majority of the rVP2 protein (45 kDa in molecular weight) eluted between 5 and 10 mM imidazole, with several other proteins. A weak band, 45 kDa in size was eluted between 20 to 200 mM imidazole. Fractions eluted between 5 and 10 mM imidazole and between 20 to 200 mM imidazole were pooled separately for the next purification step. No protein was eluted in the stripping buffer step (lane 11), indicating all proteins had been removed by the stepwise gradient.

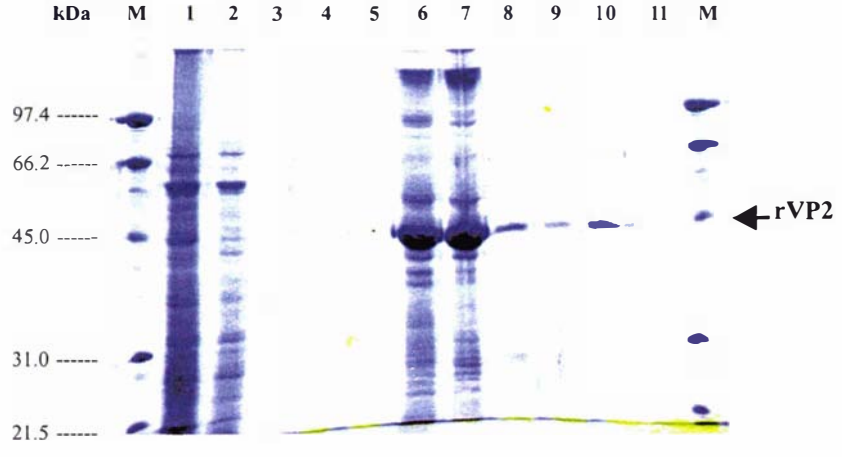


Figure 7.13: SDS-PAGE analysis of fractions from Ni^{2+} charged IMAC column purification.

M: SDS low molecular weight standards (Bio-Rad).
Lane 1: Cell lysis supernatant.
Lane 2: Unbound material (flow through).
Lane 3-5: washing step with 0.5, 0.75 and 0.5 M NaCl in loading buffer.
Lane 6-10: Elution fractions with 5, 10, 20, 50 and 200 mM imidazole in buffer.
Lane 11: Elution fraction of 0.1 M EDTA in stripping buffer.

All fractions were concentrated 20 fold and 10 μL of concentrate was loaded per well except lane 1.

7.3.8 Anion Exchange Chromatography (IEX) – UNO-Q Column

Figure 7.14 shows 5 well separated peaks were eluted with increasing NaCl concentration. SDS-PAGE analysis confirmed that the second peak contained the rVP2 protein. However, SDS-PAGE (Figure 7.15) indicated that peak 2 also contained many other proteins both higher and lower in molecular weight. Analysis of these bands by western-immunodetection showed that they all interacted with the VP2 monoclonal antibody. It could only be assumed that bands of higher molecular weight were aggregates of rVP2, while those of lower MW represented cleaved protein. Several attempts were made to disrupt these aggregates using salt, different buffers and detergents, but none of these were successful. The pooled fractions from peak 2 were used in the next purification step.

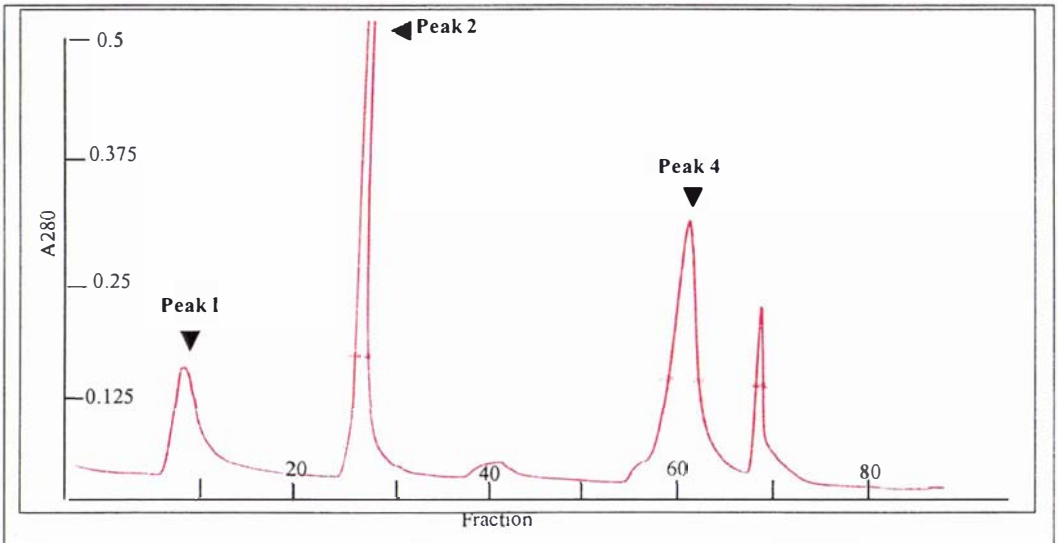


Figure 7.14: Chromatogram of the elution from UnoQ column of the rVP2 protein. SDS-PAGE analysis of peaks 1, 2 and 4 are shown in Figure7.15.

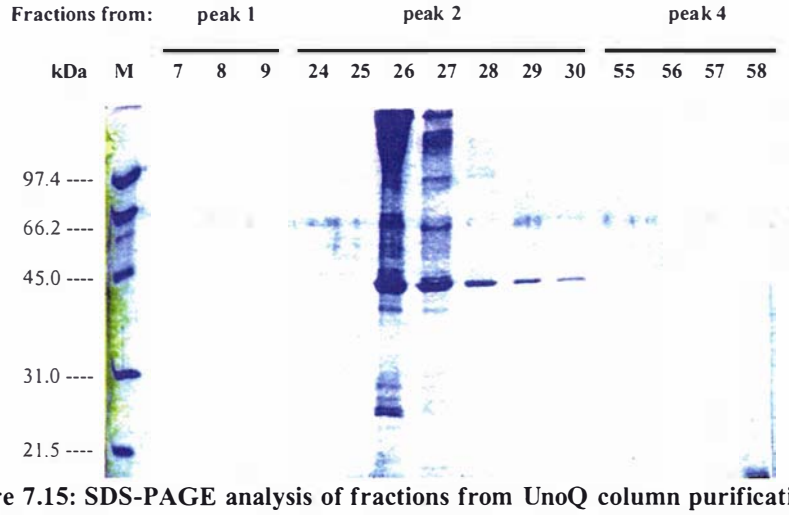


Figure 7.15: SDS-PAGE analysis of fractions from UnoQ column purification. Fractions are numbered according to the chromatogram shown in Figure 7.14.

All fractions were concentrated 20 fold and 10 µL of concentrate was loaded per well.

7.3.9 Size Exclusion Chromatography (SEC) with Superdex-75 (HR10/30)

When the protein was subjected to size exclusion chromatography, it was apparent that rVP2 protein eluted in the void volume of the column as shown in Figure 7.16. Analysis of peak 1 by SDS-PAGE showed that it contained rVP2, and that many of the contaminating bands had been removed. However, because the protein eluted in the void volume, its molecular weight was likely to be greater than 100,000 kDa, indicating that the protein was probably forming aggregates of a size out of the optimal separation range of this column. As a result of these findings, the protein was subjected to SEC on a Superdex 200 HR10/30, which has a separation range of 10,000 –600,000 MW. However, the protein still eluted in the void volume (data not shown).

In spite of this, SDS-PAGE analysis showed that the rVP2 eluted in the void volume fractions from each column was free from contamination (Figure 7.16). The purest fractions were pooled and the protein concentration was measured using either the Bradford or A280 UV absorbance methods as described in section 7.2.13.

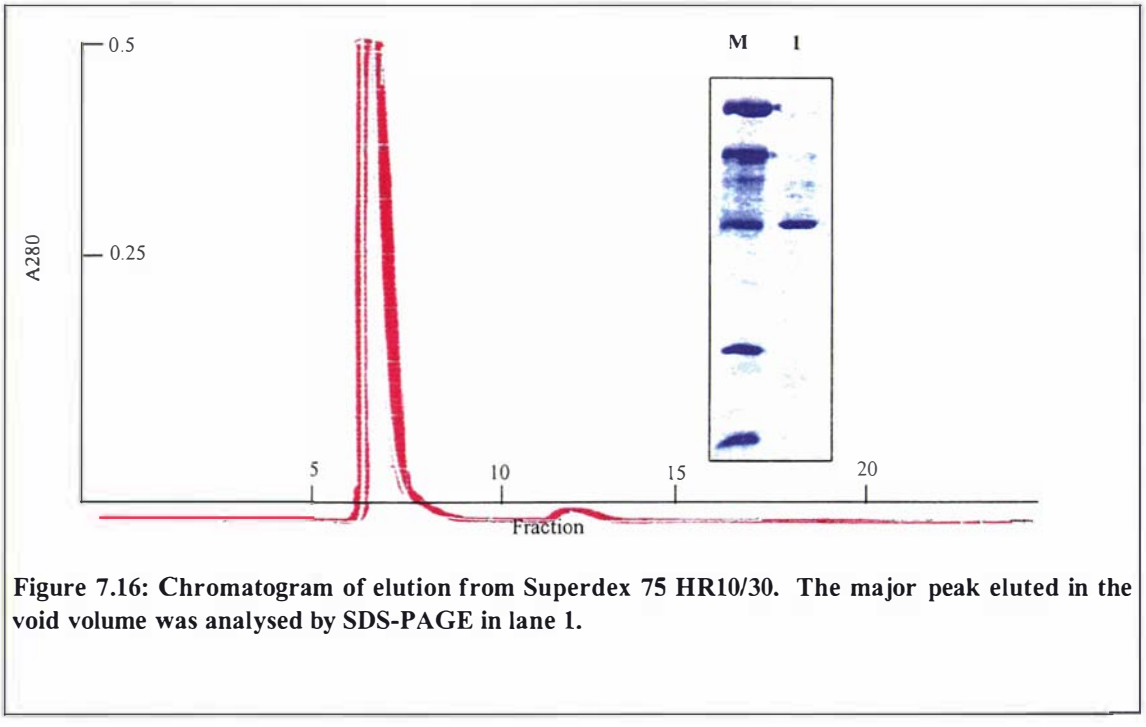


Figure 7.16: Chromatogram of elution from Superdex 75 HR10/30. The major peak eluted in the void volume was analysed by SDS-PAGE in lane 1.

7.3.10 Cleavage of Fusion Protein with rTEV Protease and Removal of rTEV Protease

RTEV protease was used to cleave the N-terminal linked poly-His tag from the purified rVP2 protein. The time course digestion of rVP2 by rTEV is shown in Figure 7.17. The SDS gel showed that only about 50% of the poly-His tag was cleaved from the rVP2. Increasing both the length of digestion and the rTEV protease concentration did not improve the efficiency of the digestion (data not shown).

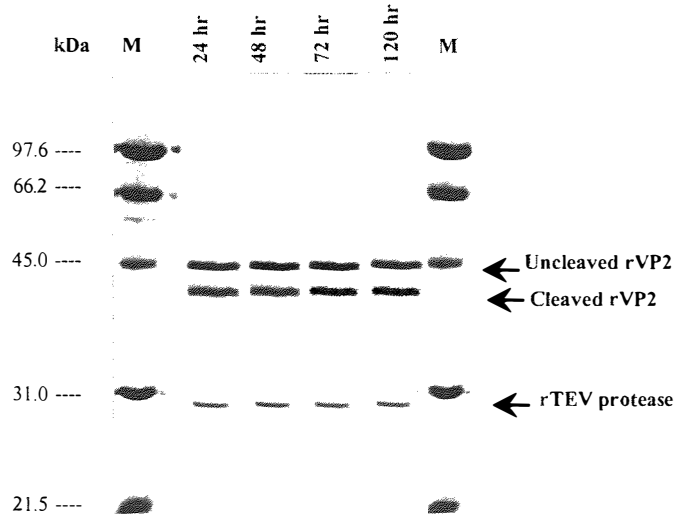


Figure 7.17: Cleavage of rVP2 protein with rTEV protease, incubated at 25°C for 24, 48, 72 and 120 hours as indicated in each lane.

Attempts to separate cleaved rVP2 from the uncleaved rVP2 following the cleavage reaction were not successful. It is possible that the property of the protein to aggregate makes it impossible to separate them by IMAC or other chromatographic procedures. Before attempting to solve this problem, it was decided to characterise the fusion protein, as reports in the literature suggested that this tag would have little effect on the structure and function of the capsid protein (Hu *et al.*, 1999; Johnson & Chiu, 2000; Liljas, 1999; Phelps *et al.*, 2000; Wang. *et al.*, 2000).

7.3.11 Analysis of Expressed Recombinant VP2 (rVP2) Protein

Western Immunodetection of rVP2

A western blot of the rVP2 using the mouse monoclonal antibody R63 and the rabbit anti-IBDV polyclonal antibody is shown in Figure 7.18. The monoclonal antibody R63 was able to detect both the rVP2 protein and the native viral VP2 protein, indicating that the native and the recombinant proteins have similar epitopes recognised by the monoclonal antibody. The polyclonal antibody also recognised the rVP2 protein as well as the native viral VP2.

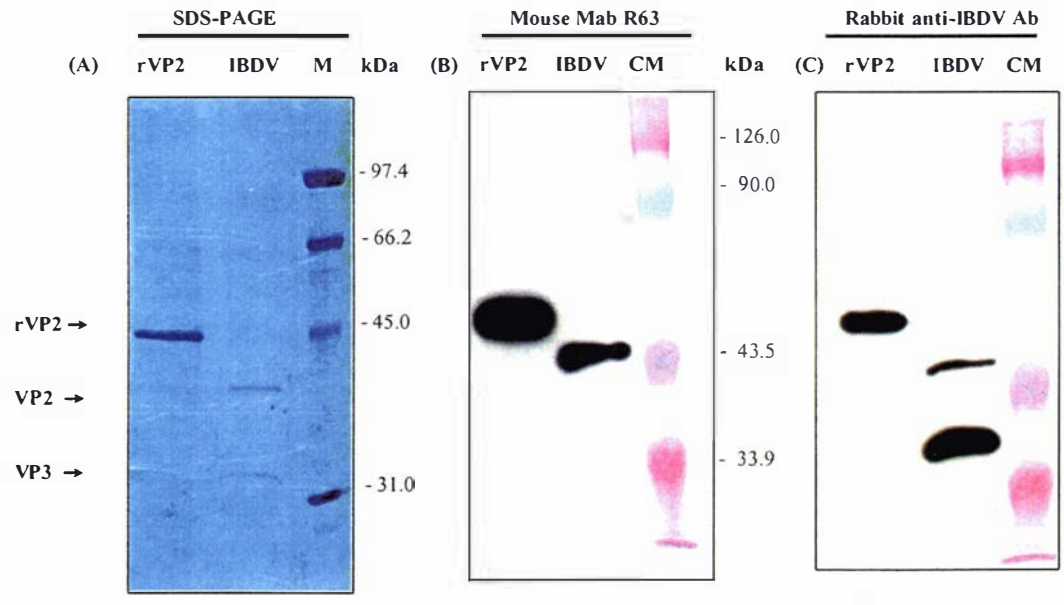


Figure 7.18: SDS-PAGE and Western immunoblotting of recombinant VP2 protein and native IBDV proteins. (A) SDS-PAGE analysis; (B) western immunodetection with mouse monoclonal antibody R63; (C) western immunodetection with rabbit anti-IBDV polyclonal antibody. M: SDS-PAGE low range weight standards (Bio-Rad); CM: Kaleidoscope prestained standards (Bio-Rad); rVP2: purified recombinant VP2 protein; IBDV: purified IBDV virion.

Immunodiffusion Test of rVP2

As shown in Figure 7.19, the rVP2 reacted with both the monoclonal and polyclonal antibodies forming a strong line of precipitation in the diffusion test. The precipitation lines of the rVP2 were identical to those for the purified IBDV indicating that the rVP2 had similar immunogenic site(s) to the native IBDV protein.

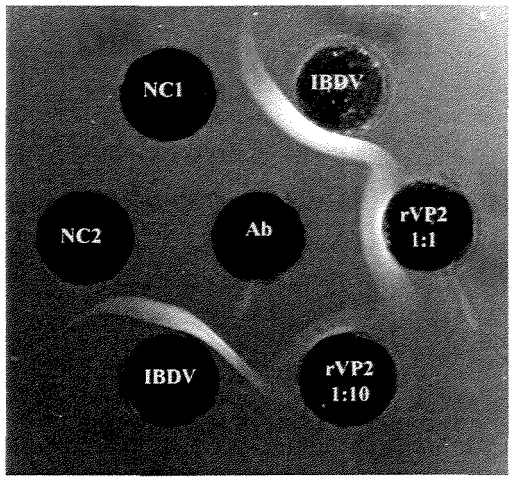


Figure 7.19: Immunodiffusion test of rVP2 against mouse monclonal antibody R63. The central well was filled with mouse monoclonal antibody (or polyclonal antibody in other test). The outer wells were filled with: IBDV (purified IBDV as positive control); NC1 (lysed uninfected insect cells as negative control); NC2 (culture supernatant from uninfected insect cells as a negative control); rVP2 (purified rVP2 protein in 1:1 and 1:10 dilution) in the indicated wells.

N-terminal Protein Sequencing

Attempts to sequence the N-terminus of the expressed rVP2 were not successful. It was assumed that the N-terminus was blocked – a reasonable assumption since the N-terminal residue is serine (see Appendix A), which is commonly acetylated during the blotting process (Walker & Gasstra, 1987).

Mass Spectrometry

Figure 7.20 shows the electrospray mass spectrum of rVP2 (with the poly-His tag). The molecular weight was found to be 51387.0 Da. The calculated molecular weight of the

protein based on the amino acid sequences is 51344.0 Da (Expasy Tools, http://www.expasy.ch/tools/protparam.html). This correlates to a difference in molecular weight of 43 Da. The difference in molecular weight can be explained by the modification of the N-terminal serine by an acetyl group and this would be consistent with the inability of the protein to be sequenced using Edman methods.

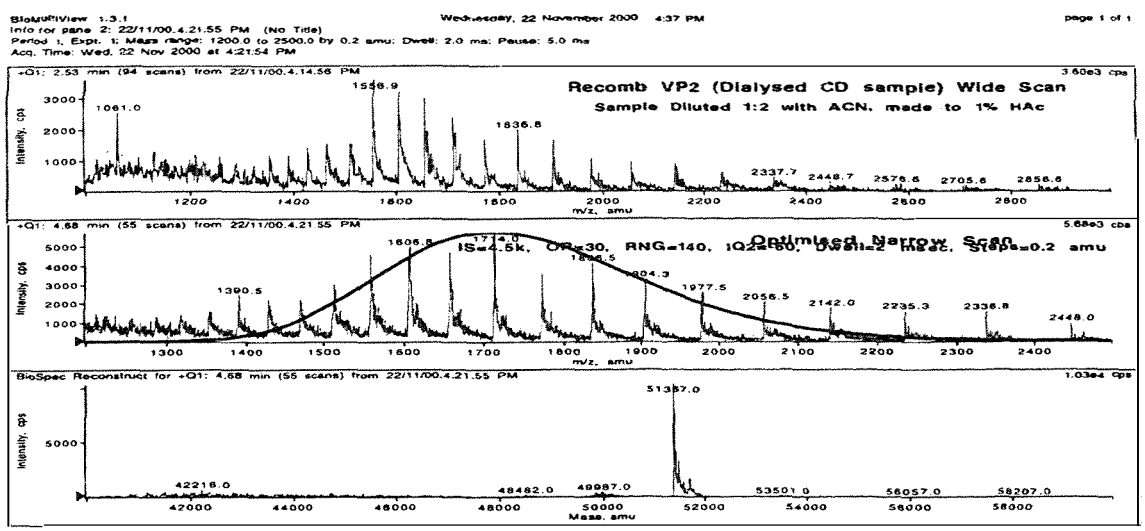


Figure 7.20: Results of the ES-Mass spectrometry of the purified rVP2 protein.

Circular Dichroism Spectroscopy

The far-UV CD spectrum of rVP2 is shown in Figure 7.21. The strong positive spectra centred between 195 and 197 nm, and the negative spectra centred near 218 nm suggested a high proportion of ß pleated sheet, which would be expected if rVP2 has a structure similar to other viral coat proteins (Pysh, 1966). Unfortunately, no native VP2 was available to carry out a comparison. Nevertheless, this indicates that the protein did have ordered secondary structure, and was therefore likely to be

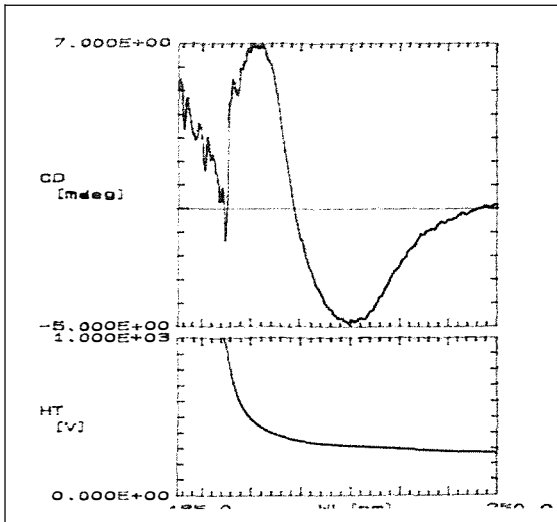


Figure 7.21: Result of the circular dichroism spectroscopy.

folded correctly.

Concentration and Aggregation of rVP2 Protein

For cleavage, it was necessary to concentrate the rVP2 to approximately 5 times the original concentration using ultrafiltration. Figure 7.22 shows that when this was done, a high M.W. band appeared in the SDS-gel. The higher the concentration, the more intense these higher bands became. It is possible that the high M.W. band is the result of strong hydrophobic interactions between rVP2 molecules, that increase with increasing concentration. Such interactions are very strong and result in very stable aggregates. This hypothesis was confirmed using western blotting that showed the high molecular weight bands were recognised by monoclonal antibodies specific for VP2 (data not shown). Analysis of the protein using SEC and MALLS confirmed that the protein did exist as very large aggregates with a molecular weight > 100,000. Obviously, in SDS-PAGE, some of the monomers are disupted and appear as a 45 kDa band. Further confirmation of aggregation was obtained from electron microscopy that showed rVP2 is able to form virus-like particles (VLP). Each spherical shaped VLP may consist of between 300 and 500 kDa of protein as has been reported by Hu *et al* (Hu *et al.*, 1999). They found that VLP 20 nm in diameter was formed by aggregates of recombinant VP2. When two capsid proteins, VP2 and VP3 were jointly expressed, VLPs of approximately 60 nm spontaneously assembled (Fernandez-Arias *et al.*, 1998; Kibenge *et al.*, 1999).

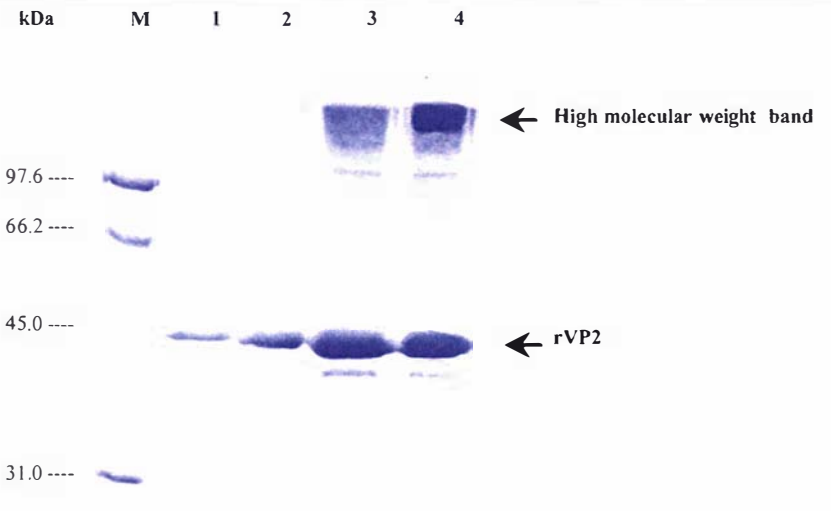


Figure 7.22: Concentration of rVP2 protein resulted in aggregation of the protein. M: Low ranged molecular weight standards (Bio-Rad); Lane 1: Diluted rVP2 protein; Lane 2 - 4: 5 x, 10 x and 40 x concentrated rVP2 protein.

Electron Microscopy Examination of rVP2 Protein

Evidence of the formation of high molecular weight aggregates of rVP2 from SDS-PAGE analysis coupled with reports of the self-assembly of recombinant capsid proteins of IBDV and other viruses (Fernandez-Arias *et al.*, 1998; Hu *et al.*, 1999; Kibenge *et al.*, 1999; Petitpas *et al.*, 1998; Yusibov *et al.*, 1996) prompted examination of rVP2 protein under the electron microscope (EM). The protein was coated and negatively stained on a copper grid as described in section 2.2.7. Two different types of structure were observed under the EM as shown in Figure 7.23. Figure 7.23 (A) shows a population of spherical particles that are either single or part of a clump. In Figure 7.23 (B) tubes having variable diameters and lengths are visible against a background of spherical particles. The surface details of these tubular structures suggest that the smaller particles aggregate to form them as the concentration of the protein is increased.

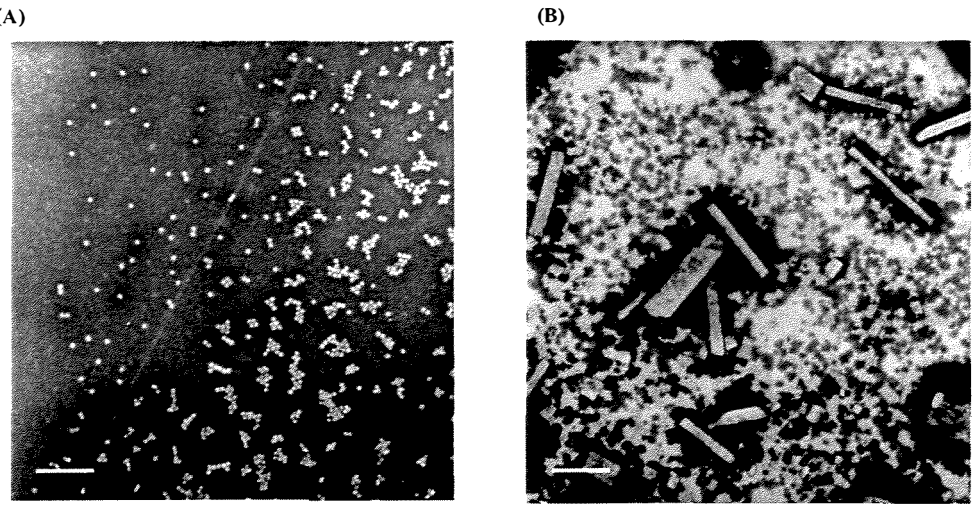


Figure 7.23: Characterisation of the self-assembly of rVP2 by negative-staining EM. (A) Spherical forms of the rVP2, approximately 12 to 13 nm diameter. (B) Association of the rVP2 into tubular structures of various diameter. The background shows the high density of the spherical form of rVP2. Bar = 100 nm.

7.3.12 Multiangle Laser Light Scattering Photospectrometry

The SEC profile for the rVP2 and the responses from the DRI, UV and MALLS 90° LS detectors were analysed. Only one major peak eluted from the SEC column, and a plot of weight-average (M. Wt.) as a function of elution time is shown in Figure 7.24. This clearly shows that there is a single species in the elution peak. The weight-average (M.Wt.) and the average rms Radius (Rg) of the protein were measured as 2.5 x 10^6 g/mol and 12.8 nm respectively. Overall, the data presented here suggest that rVP2 exists as aggregates of monomer that assemble into a spherical shape with an average diameter of 12.8 nm. These assemblies are smaller size than the native virus (60 - 70 nm), but they appear to exist as a fairly homogenous population.

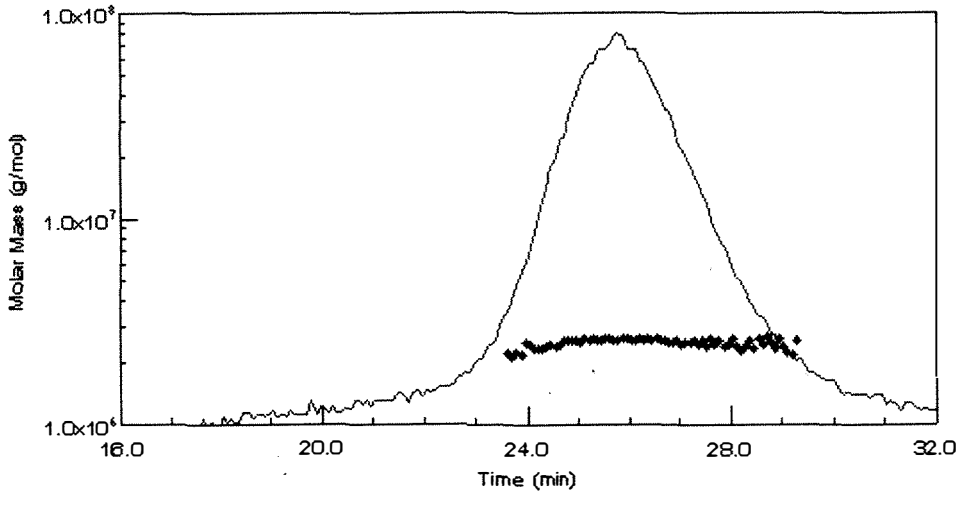


Figure 7.24: SEC-DRI elution profile (—) of the predominant elution peak of the rVP2 proteins. The elution profile is overlaid with the calculated molar mass (◆).

Low Temperature Effect on rVP2 Aggregation

Interestingly, when the concentrated rVP2 was stored at 4 °C for longer than 24 hours, SDS-PAGE showed that the high molecular weight bands disappeared, leaving a single band of 45 kDa as shown in Figure 7.25. This is further evidence that the aggregates form because of strong hydrophobic interactions between monomers. Hydrophobic interactions are non-covalent interactions that have a large entropy component, and hence are affected by temperature. Unlike other non-covalent interactions such as H-bonds and charge-charge interactions, they are weakest at low temperatures and become stronger as the temperature increases.

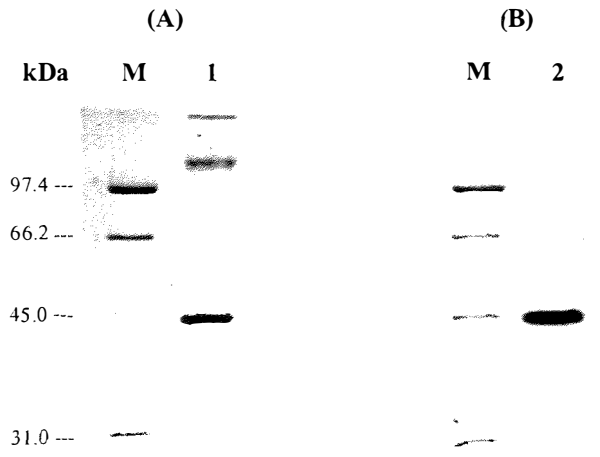


Figure 7.25: SDS-PAGE of purified and concentrated rVP2, (A) after concentration; (B) after concentration and stored at 4 °C >24 hours. M: SDS-PAGE low range weight standards (Bio-Rad).

7.3.13 Overall Purification of rVP2 Protein

The yields from the purification of rVP2 protein are shown in Table 7.1. Approximately 6.0 mg of purified rVP2 protein could be obtained from one litre of culture.

Table 7.1: Summary of the purification of recombinant VP2 protein from 500 mL of culture.

Purification Step	Volume (mL)	Protein Conc. (mg/mL) [a]	Total Protein (mg)	Yield (%) [b]
Cell pellet lysis	30	4.01	120.3	100
Cell lysis pellet	10	4.68	46.8	38.9
Cell free supernatant	50	1.47	73.5	61.1
IMAC non-bound fraction	80	0.34	27.2	22.6
IMAC fraction (20 mM IMD) [c]	170	0.10	17.0	14.1
IMAC fraction (200 mM IMD) [c]	100	0.02	2.0	1.60
UnoQ pooled fraction	10	0.70	7.0	5.80
Superdex 75 pooled fraction	30	0.10	3.0	2.50
Concentrated fraction	0.3	10.0	3.0	2.50

a: Protein concentration determined by the Bradford assay.

b: Yield was claculated by dividing the total protein from the cell pellet lysis step x 100%.

c: IMD - imidazole concentration in the elution buffer.

7.3.14 Recombinant VP2 Protein Crystallisation Trials

Crystals grew overnight in a well containing 12% PEG 20,000 and 0.1 M MES, pH 6.5 at 4 °C (condition #22, screen 2). Crystals grew as spherulites as shown in Figure 7.26. Experiments to optimise these conditions using 10 – 16% PEG 20,000 and 0.1 M MES pH 5.5 to 8.0 at 4 °C have been done but thus far, have produced no crystals.

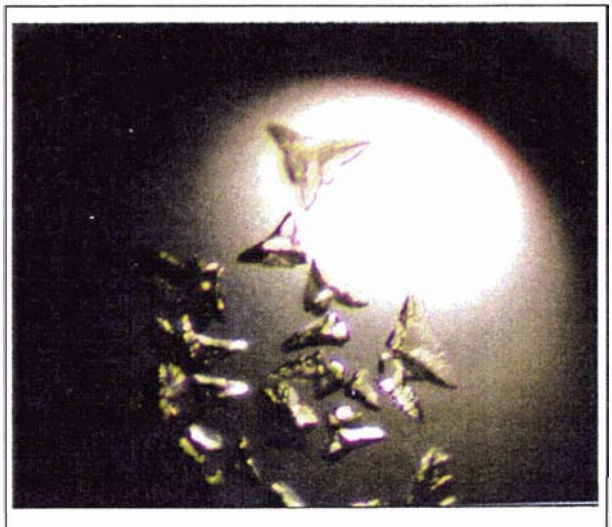


Figure 7.26: Crystals grown in 12% PEG 20,000, 0.1 M MES, pH 6.5 at 4°C.

Results from the initial trials indicated that other conditions from screens 1 and 2 favour crystal growth. These used 30% v/v 2-methyl-2, 4-pentanediol (MPD) as precipitant, buffers ranging from pH 4 to

9 (0.1M citric acid, sodium acetate, MES, HEPES, tris-HCl and bicine) and ammonium sulfate as the additive (0 – 0.4 M). These conditions have also failed to produce crystals to date.

Crystals were obtained after 4 weeks in rods and cubes shape at room temperature from 30% MPD, 0.5 M ammonium sulfate, in 0.1 M HEPES, pH 7.5. These had dimensions of 0.02 x 0.5 mm (rods) and 0.2 x 0.2 x 0.2 mm (cubes).

7.3.15 Crystal Diffraction Trials

The crystals obtained from the preliminary screen were used for an X-ray diffraction trial. Due to the small number of crystals, higher concentrations of PEG 20,000 and glycerol (15% PEG 20,000 and 20% glycerol) were trialed as cryo-protectants. The crystals were taken from the drop with a cryoloop and quickly dipped into the cryo-protectant before being flash frozen in a stream of liquid nitrogen at 113 K.

Three crystals were mounted for diffraction and small oscillation photographs taken. These showed that low order weak diffraction indicating the crystals were disordered. However, the data were consistent with the crystals being formed of protein.

7.4 DISCUSSION

7.4.1 Cloning and Expression of rVP2 Protein

The culturing and maintenance of the Sf9 insect cells was simple but was time and labour consuming. Because positive selection by antibiotic resistance is not used, strict sterile regimens have to be followed during the subpassaging and handling processes. Optimal cell density and good viability are important for successful subculture of the cells to another healthy passage. Subculture of cells from cultures of too high density or cultures of low viability always resulted in a weak cell line, which would either not grow to log-phase or took a much longer time to reach optimal density. It was found that it was better to start a new cell line from the cryopreservation stock when problems arose during the subculturing.

High fidelity polymerases (Expand High Fidelity enzyme blend consisting of Taq and Pwo DNA polymerase) were used in the amplification of the *vp2* insert. The possibility of error being introduced during PCR was deemed to be lower than using Taq polymerase alone. The sequences of the inserted gene were determined by sequencing the cDNA products as shown in Appendix A. Six sequences were obtained from the sense and nonsense cDNA strands of the RT-PCR products, with a final consensus sequence being obtained from the alignment of these six nucleotide sequences. Thus the nucleotide sequence should be a true representation of the native VP2 protein. Unfortunately, the sequences of the IBDV strain used in this study had not been published, so verification of these results was impossible. In order to avoid any frameshift during the expression of rVP2, it was confirmed that there were no insertions or deletions in the cDNA product prepared for cloning into the donor plasmid.

In the initial transfection procedure, recombinant baculovirus was produced in a titre similar to the titre recommended in the manufacturer's manual (2×10^7 to 4×10^7 PFU/mL). However a 2-log amplification of the working P1 and P2 virus stocks could not be attained. Even when incubation time was extended, only a 1-log amplification of the titre could be obtained. Using different MOIs (0.01 to 0.1) did not increase the titre. The presence of "defective interfering particles" in the initial recombinant baculovirus stock could explain these results (O'Reilly *et al.*, 1994). These defective particles have extensive mutations in their genome and are helper-dependent. The replication has been shown to interfere with the replication of the recombinant virus, resulting in a substantial reduction of the infectious virus yield. Plaque selection from the initial virus stock to start a new working stock may remove defective virus strains, but screening for optimal protein expression from the new stock would have to be done in order to get the required expression from the selected plaque. The titre of the working stock in this study was 1 to 3×10^8 PFU/mL, and as such was sufficient for optimal expression of rVP2. In order to avoid the accumulation of defective virus in the working stock, the number of passages of the working stock in cell culture was kept to a minimum, by preparing a large low-passage stock (only up to #P3 in this study). Secondly, inoculation was done at low MOI when preparing the working stock. Future subculturing of the working stock to higher passage number should include plaque purification of the recombinant virus to eliminate any defective virus in order to ensure optimal titre and expression.

The expression of the recombinant baculovirus varies between cell-lines, MOI and incubation time. The final yield of the expressed rVP2 in this study was 5 to 6 mg per litre of Sf9 cell lysate. There was certainly some expressed rVP2 protein in the cell culture medium due to cell lysis and loss during the purification procedure. Wang *et al* (Wang *et al.*, 1994) reported production of recombinant VP2 in a baculovirus expression system with a final yield of 120 mg/L protein. Although they used a similar cell-line (Sf9 cells) for the expression, they used different culture medium and a different vector (Invitrogen, CA). Other reports of the production of recombinant VP2 in different baculovirus expression system have a yields ranging from 50 to 70 mg/L (Pitcovski, *et al.*, 1996; Wang *et al.*, 1994). For example, Dybing and Jackwood (1997) used the High Five® insect cell line (BTI-TN-5B1-4, Invitrogen) and increased the yield of recombinant protein five fold compared to what they obtained in Sf9 cells. Since correct processing of the expressed protein was the main reason for using baculovirus, and since the quantity of the expressed rVP2 protein obtained was sufficient for this study, further experiments with different baculovirus expression and culture systems were not carried out. This is something that should possibly be done in the future.

The major advantage of the baculovirus expression system (Bac-to-Bac®) used in this study is the ability to generate the recombinant baculovirus by site-specific transposition in *E.coli,* enabling early selection of the recombinant bacmid that contains the baculovirus shuttle vector and the *vp2* insert gene. This is in contrast to the conventional homologous recombination methods used to generate recombinant baculovirus that uses linearised parental baculovirus DNA, and requires multiple rounds of plaque purification to isolate the recombinant from nonrecombinant virus. The time normally required for this process is from 4 to 6 weeks. The Bac-to-Bac system reduced this time to only 7 to 10 days. During the initial isolation of recombinant bacmid and transfection to generate recombinant baculovirus for expression trials, one of the recombinant baculovirus failed to express the *vp2* gene. Interestingly, substantial virus titre was obtained from this "defective" isolate. Possible reasons for the failure of expression from this particular recombinant virus are not understood. The bacmid is constructed by insertion of approximately 12.5 kb of heterologous DNA into the baculovirus genome, which may make it unstable resulting in inhibition of expression but not the replication of the genome (Luckow *et al.*, 1993). This is not an uncommon

phenomenon as it has been found by other researchers using this system (Dr. Vernon Ward, Department of Microbiology, Otago University, personal commumication). It is therefore important that several different bacmid isolates are selected and used for transfection in order to generate a few different recombinant baculoviruses that can be screened for protein expression. This should save the time and effort necessary to repeat the transposition step to generate new bacmids.

Although plaque purification of the initial recombinant baculovirus stock was not recommended by the manufacturer, it may be desirable to do this in order to prevent the defective baculovirus getting into the stock used for large-scale of subculturing. Another disadvantage with this system was the low expression level of protein obtained when the *vp2* gene was inserted as a DNA cassette into the baculovirus gennome by transposition, compared to the levels obtained when the recombinant baculovirus was generated using homologous recombination in insect cells followed by purification of the plaques by the plaque assay (Dybing & Jackwood, 1997; Pitcovski *et al.*, 1996; Wang *et al.*, 1994). If higher levels of protein expression are required, the conventional homologous recombination method could be chosen.

7.4.2 Purification of rVP2 Protein

A poly-His tag was incorporated at the N-terminus of the expressed rVP2 protein to facilitate purification using IMAC. The majority of rVP2 eluted between 5 and 10 mM imidazole showing that the binding of rVP2 to the IMAC support was weak. It has been demonstrated that the addition of as few as three accessible histidines to the protein surface can increase the binding affinity of the protein to IMAC by as much as 1000 fold (Johnson *et al.*, 1996). The reason for the weak binding affinity of the 6x histidine affinity tag on rVP2 in this study is not known. One possible explanation may be the possibility of steric hindrance of the His-tag due to the way rVP2 folds, or the interaction observed between rVP2 molecules may mask the tag. Analysis of the eluted rVP2 showed that it co-eluted with many other proteins. An additional washing step with a higher NaCl concentration of 0.75 M before the elution with imidazole helped to reduce the non-specific binding of most of the contaminating proteins.

Another unexpected result from the IMAC purification was the rVP2 protein did not run on SDS-polyacrylamide gels according to its calculated molecular weight (51.34 kDa). Instead, it ran at a molecular weight of about 45 kDa. The 45 kDa band was confirmed to be rVP2 by western blotting and *immunodetection with* monoclonal and polyclonal antibodies (Figure 7.19). Anomalies in molecular weight determination of proteins in SDS-PAGE is not uncommon. Under SDS-PAGE conditions, all reduced polypeptides bind the same amount of SDS on a weight basis and should be separated on the basis of their size and charge density. However, post-translational modifications and sequence peculiarities can result in proteins running anomalously on SDS-polyacrylamide gels (Shi & Jackowski, 1998).

The second purification step using anion exchange at pH 6.5 (10mM Bis-Tris buffer) successfully separated the rVP2 protein from most of the contaminating proteins. The second narrow peak was well resolved and contained mainly rVP2 as shown by SDS-PAGE. However there were several other high molecular weight and low molecular bands in this elution fraction. Addition of higher concentrations of SDS in the loading buffer reduced the intensity of the high molecular weight band and increased the intensity of the 45 kDa band. This indicated that increasing the concentration of SDS broke up the large aggregates to give a larger proportion of monomers. Interestingly, the high M.W. band did not appear in the SDS-polyacrylamide gels when the rVP2 protein was diluted and in lower ionic strength buffer (Figure 7.22)

7.4.3 Analysis of Expressed rVP2 Protein

EM Examination

The aggregation of the rVP2 protein into spherical or tubular forms was not expected even though the formation of virus-like-particles (VLP) had been reported for the production of the VP2-4-3 polyprotein of IBDV in baculovirus by various authors (Fernandez-Arias *et al.*, 1998; Hu *et al.*, 1999; Kibenge *et al.*,1999) and for other recombinant viral capsid proteins (Petitpas *et al.*, 1998; Yusibov *et al.*, 1996). The mechanism by which virus-like-particles are formed from subunit capsid proteins is not yet clearly understood. It is generally believed that the subunit proteins interact in such a way as to maintain minimum free energy and this results in the formation of these

particles. However, there are several reports that suggest the binding of viral nucleic acid to the N- or C-terminus of the capsid protein is the trigger that activates the polymerisation of the capsid proteins (Baer *et al.*, 1994; Jaspars, 1985; Van Der Kuyl *et al.*, 1991). Yusibov *et al* (1996) reported that the production of recombinant coat protein from *Alfalfa mosaic virus* with an N-terminal extension, resulted in a homogenous population of spherical virus particles in contrast to the assembly of bacilliform particles by the native coat proteins. The environment of the capsid proteins has also been shown to affect the self-assembly of the proteins into either tubular or native structures (Petitpas *et al.*, 1998). On the other hand, Lamb *et al* (Lamb *et al.*, 1996) demonstrated that the native coat protein of *Potato leafroll virus* does not spontaneously form particles. Interestingly, VLPs were formed when the recombinant protein contained a 6 x histidine-tag on the N-terminus. It has also been reported that the inclusion of a histidine-tag on the C-terminus of IBDV VP2, can promote VLP formation (Hu *et al.*, 1999).

It has been shown that the expression of single capsid protein (VP2) alone, results in formation of smaller VLPs of 20 nm in diameter, compared to the particles of 60 nm formed from three capsid proteins (Hu *et al.*, 1999). In studies on herpesvirus assembly, it was found that by expressing two of the six herpesvirus procapsid genes, an icosahedral particle of smaller size was formed (Chandran *et al.*, 1999; Saad *et al.*, 1999). This indicates that the assembly of virus particles often does not require all the gene products found in native virus. The formation of tubular shapes or string-like structures has also been reported in the purification of the IBD virion by a few authors (Harkness *et al.*, 1975; Hirai *et al.*, 1979; Hirai & Shimakura, 1974; Ozel & Gelderblom, 1985). It has been suggested that the local pH and ionic concentration affects the state of hydration of the virion and disassembly of the capsid subunit to form tubular shape (Harkness *et al.*, 1975).

Although the recombinant rVP2 produced in this study could assemble into a VLP, analyses with SEC MALLS showed that the population of protein particles was quite homogenous and, therefore theoretically could be crystallised in the same way that the native virus can be crystallised. The capsid protein structures of other viruses have been solved from crystals of VLP using X-ray crystallography (Petitpas *et al.*, 1998; Yusibov *et al.*, 1996). The results from the Multiangle Laser Light Scattering photospectrometry

proved that the rVP2 consists of a homogenous population made up of spherical assemblies approximately 12 nm in diameter.

Mass-Spectrometry

The molecular weight of the rVP2 protein determined by Electrospray-Mass Spectrometry was higher (51387.0 Da) than the calculated molecular weight (51344.0 Da) of the deduced amino acid sequence. The difference in the molecular weight may be due to the acetylation of the first serine residue (+43 Da) at the N-terminus of the rVP2. The fact that the protein appeared to be N-terminally blocked when sequenced using Edman degradation supports the likelihood that the N-terminal serine is acetylated.

Circular Dichroism Spectroscopy

The result from the circular dichroism spectroscopy is interesting. The result from the CD spectroscopy suggested the rVP2 is made up predominantly of β-sheet. Structural studies of various viral capsid proteins have shown that most viral capsid proteins display β-barrel like architecture with a "jellyroll" architecture, consisting of eight antiparallel β-strands (Chelvanayagam *et al.*, 1992). The results confirm that rVP2 contains secondary structure characteristic of other viral coat proteins. Whether it resembles that of the native protein or not, cannot be determined because of the lack of a spectrum from the native protein for comparison. However, the fact that both the monoclonal and polyclonal antibodies recognise the recombinant protein strongly suggest that the protein is in a native-like conformation.

Immunodetection

The western immunodetection and agar gel diffusion tests show that rVP2 carried immunogenic site(s) similar to the native VP2 capsid protein. The monoclonal antibodies R63 and B69 used in the tests recognise separated distinctive neutralisation sites (Snyder *et al.*, 1988; Snyder *et al.*, 1992), which are presented in the rVP2 protein.

This would indicate that rVP2 was properly processed and folded in the baculovirus system.

Immunodiffusion Test

The immunodiffusion test of the rVP2 protein against anti-VP2 monoclonal antibodies and anti-IBDV polyclonal antibodies also showed that the recombinant protein carried the immunogenic site(s) similar to those found on the native VP2 protein. To further analyse the immunogenic character of rVP2, the protein could be injected into a live animal and the immune response evaluated by testing the antibodies elicited by the protein in western blots and by immunodiffusion with native proteins and in functional tests such as virus neutralisation.

7.4.4 Preliminary Crystallisation and Diffraction Trial of rVP2

So far, two of the conditions from the preliminary screen have yielded crystals. One grew overnight, and was found to diffract poorly. The fragility of these crystals together with the weak patterns, indicated strongly that the crystals were protein. Poor diffraction of the crystals is a result of disorder of the crystal lattice, and may be because the crystals grew too quickly (within 48 hours after setting-up). Secondly, there were not enough crystals to allow optimisation of the cryoprotectant used. The use of a suitable cryoprotectant is essential if good resolution and low mosaic spread of data is to be obtained. The second took longer to grow, and has not yet been tested to see if they diffract. Further work will be necessary to obtain better-ordered crystals and, when these are obtained, it will be necessary to identify a suitable cryoprotectant.

The precipitation and solubility information obtained from the preliminary screens indicated other conditions that may produce crystals. Although these conditions were tried, no crystals have yet grown. Trials are continuing.

7.5 SUMMARY

In summary, the expression of recombinant VP2 capsid protein in the chosen baculovirus expression system has been successful. The affinity purification procedure using the N-terminal linked His-tag protein did not result in a single step purification, as it was found that ion-exchange and gel filtration steps were needed to obtain homogenous protein. After these additional procedures however, enough pure rVP2 protein was obtained to carry out the preliminary crystallisation screen. For future work, production of recombinant capsid protein in a different baculovirus expression system, with different cloning strategies could be tried. For example, expressing the protein without the His-tag, or placing the His-tag on the C-terminus instead of the N-terminus, may result in expression of larger amounts of the protein. It may also result in a reduction in the self-assembly of the protein observed in this study.

The preliminary crystallisation screen in this study has resulted in the identification of some conditions that may lead to the growth of suitable crystals for X-ray diffraction studies. More protein will be required to optimise these conditions in order to produce quality crystals necessary for structure determination. These results provide a solid foundation for continuation of the structure determination of VP2.

CHAPTER 8: GENERAL DISCUSSION

Since the inadvertent introduction of a mild IBDV strain into New Zealand in 1993, the poultry industry has undertaken a nationwide serological survey of all poultry farms and implemented control programmes on seropositive farms. The screening and control programmes have achieved satisfactory results as shown by the decrease in seropositive farms from 1993 to the present time (Ryan *et al.*, 2000). However, there is evidence that serological testing that depends solely on the ELISA test method is not sufficient to detect those farms that remain positive as the disease prevalence has decreased year by year. Additionally, although no clinical signs of IBD were observed in the field on these seropositive farms, bursal lesions were consistently observed in slaughtered broilers, and substantial economic losses may be caused by subclinical infection (Christensen, 1999). An initial study, reported in the first part of this thesis, evaluated the serological test used in the control programme and described the development of an alternative or complementary test method (immunocytochemistry test) with higher sensitivity and specificity for the diagnosis of IBDV infection in New Zealand. This test method allowed determination of the prevalence of IBDV infection and the age of the infection in the flock. The efficiency of the ELISA test could also be estimated so that the new test could be used as a parallel test in the eradication scheme.

Based on overseas experience, the commercially available ELISA test is highly sensitive and specific. It was used effectively in the early stages of the control programme to identify infected flocks. However it became apparent that birds infected with the NZ strain of IBDV had a weak and delayed antibody response. Further, as the number of infected properties decreased, the predictive value of the test also decreased. In the final stages of the eradication programme alternative tests with high sensitivity and specificity will be required to detect the few remaining infected flocks. Immunocytochemical detection of IBDV antigens in bursal tissue may form the basis of such a test but further research is required to determine how to use this test in the most effective and efficient way.

In the initial random sample of IBDV-infected farms (chapter 2), it was obvious that the subclinical IBDV infection was widely distributed in flocks and the ELISA test had failed to detect many IBDV-infected birds, especially those in the early infection stage. In contrast, virus isolation in SPF eggs and cell culture was able to confirm the presence of the virus, which could then be detected either by EM or a RT-PCR test. However, these isolation techniques require weeks to positively identify the virus from clinical samples and are not feasible for large scale screening such as is necessary in an eradication scheme. An immunocytochemistry test was therefore developed, which offered some advantages over other diagnostic methods (chapter 3). The test is highly sensitive and specific (100% sensitivity and specificity compared to ELISA test) and is able to detect IBDV infection earlier, before either histopathology or serology. It also makes it possible to carry out large scale of testing in parallel to the histopathological examination.

By using the immunocytochemistry test in a longitudinal study of an infected farm (chapter 4), the earliest age at which IBDV infection occurred in the flock could be determined. Comparing the results obtained from ELISA and immunocytochemistry, the prevalence of IBDV infection in the farm was estimated to be about 52.5% and demonstrated the performance of the ELISA test used in the serology detection. The gold standard used in our analysis (antigen detection by immunocytochemistry) may not offer 100% sensitivity and specificity, but it is still a reliable method for evaluating the ELISA test. However, a proper control experiment should be carried out to determine the accuracy of the ELISA test, such as using experimental inoculation of NZ IBDV isolates into birds, and comparing the ELISA and immunocytochemistry test results with an independent, valid criterion such as virus-neutralisation test, which could be used to define the "true" infection status. The manufacturer and the ELISA used in this study is the same kit used by the NZ poultry industry in their screening programme. It is claimed that the kit has a very high specificity (99.9%) and sensitivity in comparison with the virus-neutralisation test. The performance results of the kit provided were determined using an overseas pathogenic strain of IBDV for challenging the tested birds. However, *in vivo* challenge tests in SPF birds with the New Zealand IBDV strain in the pathogenicity study (Chapter 5) demonstrated only 5 out of 25 birds had detectable seroconversion after 10 days p.i. with this ELISA test kit. With these results, the efficiency of the ELISA used in the eradication scheme has to be verified carefully.

Although it would be possible to increase the sensitivity of detection of IBDV-infected flocks by increasing the sample size, an alternative is to use complementary tests such as immunocytochemistry on selected birds from flocks that test negative by ELISA. The convenience of the ELISA test offered a great advantage in the earlier control programme, but the performance of this serological test had not been evaluated with birds infected with the New Zealand IBDV isolates. In the preliminary comparison of the performance of two tests, the results demonstrated that the sensitivity of the test is much lower than the manufacturer's claims. This may be due to the different antigens coated in the ELISA plates compared to the New Zealand IBDV strains, or the poor and delayed seroconversion of birds infected with the New Zealand IBDV strains as shown in our and other studies (Chai *et al.*, 1999; Christensen, 1999; Motha, 1996). Further evaluation of the serological test with local IBDV strains and approaches may be needed.

Understanding the genomic characteristics and pathogenicity of the New Zealand IBDV isolates as reported in chapter 5 will help in the design of more efficient control programmes for the industry, to enable the country to regain its freedom from IBDV. The results from this study also pointed out the possible origin of the IBDV that was introduced into New Zealand in 1993. The *in-vivo* pathogenicity study in challenged birds showed the New Zealand IBDV isolate only has mild pathogenicity in SPF birds and the genetic study and analysis of the New Zealand IBDV strains grouped them with the attenuated IBDV strains. These results were consistent with other studies, which determined the low pathogenicity and infectivity characteristics of the virus (Motha, 1996; Tham *et al.*, 1995). The epidemiological investigation of the initial IBDV-infected farms in 1993 by MAF also demonstrated that the temporal clustering of positive farms was produced by one hatchery in September 1993 (O'Neil, 1995). These findings, support the hypothesis that an attenuated IBDV strain was introduced to the layer pullets produced from the hatchery and subsequently spread to broiler birds. The possible origin of the virus may be from contaminated vaccine used in the hatchery (Ryan *et al.*, 2000) or some other source at that focal point. In order to remain free from IBDV in the face of the highly pathogenic IBDV emergence in the nearby Pacific region and the frequent international trading among countries, it will be essential to avoid any accidental introduction of IBDV in the future. This will require high biosecurity standards and regulation of imports. Since importation of live virus vaccine for disease prevention, such as live Marek's disease vaccine, is necessary for the poultry

industry in New Zealand, methods of identifying unwanted contaminating virus in the imported vaccines should be re-evaluated. More sensitive and refined method such as PCR, RT-PCR or DNA probe technology could be used in the testing of imported live vaccines to detect any possible minor contamination. Additionally and possibly more importantly it will be necessary to ensure that the methods used to manufacture the vaccines overseas and the quality assurance measures in place are adequate to prevent contamination and to ensure that only the specified product is imported.

IBDV infection in commercial poultry farms outside New Zealand continues to remain an important economic threat to the worldwide industry because of its impact on the performance and growth of the birds. The high mutation rate of this RNA-polymerase dependent RNA virus has lead to vaccination failures since the late 1980's and has further impacted on the industry in terms of performance and cost of poultry production. Many studies have tried to determine which part(s) of the virus structure are involved in the antigenic and pathogenic mutations as discussed in chapters 1 and 5. To date, although several epitopes have been identified, it has not been possible to unequivocally identify all the sites that allow the virus to escape immune surveillance (chapter 6 and 7). A structural approach to understanding how changes in sequence affect the interaction of the virus immunogenic sites was initiated, and is described in part II of this thesis. This involved development of methods for structural determination of capsid proteins using X-ray crystallography. The structures of the capsid proteins of various animal and human disease viruses have been reported, as well as virus-antibody, capsid-Fab, and peptide-Fab structures (Chen *et al.*, 1998; Smith & Baker, 1999). Elucidation of the role of epitope structure in antibody-virus interactions had lead to the development of improved and new drugs in the fight against viral infections. The most exciting example is the recent development and marketing of an antiviral drug against the human influenza virus, which was based on detailed knowledge of the virus-receptor interaction obtained from X-ray diffraction studies, identification the three dimensional structures of proteins involved in the virus-host receptor mechanism. (Kim *et al.,* 1997; Olson *et al.*, 1993; Smith *et al.*, 1993).

As yet, there has been no X-ray structural data reported for IBDV. To start the pilot experiment on IBDV with this technique, many tasks had to be achieved in the initial step as described in the two chapters. Obviously this is a huge task, one that cannot be

completed in this course of study. However, significant progress has been made towards achieving this goal. IBDV has been purified to homogeneity, and preliminary crystallisation trials yielded a number of crystals. However, none of which diffracted X-rays. The reasons for this need to be investigated, as because virus crystals are notoriously fragile, it could simply be due to the way they were handled during mounting. Regardless, other crystallisation conditions need to be investigated to obtain diffracting crystals to continue this study. The information generated in this study could serve as the basis for further virus crystallisation trials on IBDV in order to solve the viral structure.

The expression of one of the IBDV capsid proteins, VP2 (rVP2) in a baculovirus system, its purification and characterisation and the results of preliminary crystallisation trials on the purified protein are reported in Chapter 7. In order to investigate possible processing problems, folding and aggregation properties were investigated using CD, SEC, MALLS, and epitope recognition with both monoclonal and polyclonal antibodies. Results from these tests confirmed that the capsid protein was most likely to be folded correctly. Crystals were obtained from two different precipitants, but due to time restraints, could not be further investigated. Valuable experience in handling recombinant viral capsid protein was obtained in this part of the project. It was found that the recombinant capsid protein tended to self-assemble at reasonably low concentration (5 mg/mL), which made obtaining the concentrated homogenous concentration needed for crystallisation difficult. Improvements that could be made to the expression of capsid protein in the baculovirus system were discussed in chapter 7. Likewise, results from crystallisation trials indicate that the optimisation of the conditions where crystals were obtained will produce crystals suitable for data collection. The accuracy of expression of the recombinant protein indicates that this will be a useful approach to producing sufficient protein for X-ray crystallography, which will provide information on structure that will be analogous to that of the native protein.

The molecular structure of both IBDV and the VP2 capsid is thus far from completed. The structure determination of IBDV using X-ray crystallography will be a massive task, requiring hundreds of diffraction quality crystals. The results presented in this study form the basis for a large study aimed at understanding the structural basis of epitope efficiency. The final goal is to solve the virus structure and extend the study to

virus-Fab and capsid-Fab complexes in order to understand the complexities of virus-antibody interactions and the basis of immune defence in host cells. The success so far was totally dependent on collaboration between The Centre for Structural Biology in the Institute of Molecular Biosciences and Veterinary Virology Unit in the Institute of Veterinary, Animal and Biomedical Sciences at Massey University. Eventual solution of the three dimensional structure of this virus at this University will depend on continuing collaboration between these two laboratories.

In conclusion, results from the attempts to determine the three dimensional structure of both IBDV and the VP2 capsid protein of this virus presented in the second part of this thesis are encouraging. It is hoped that this work will be continued and will lead to the successful structure determination of both the virus and its capsid protein in the near future. Further work using site-directed mutagenesis could then rationally explore the effect of sequence changes in the interactions between VP2 and the immunogenicity characteristics of the virus. This would not only provide explanations for the practical problem of vaccine failures but perhaps enable prediction of the nature of future failures.

Recent technological advancements in other techniques such as cryo-EM and atomic force microscopy may improve and accelerate viral structural studies in the near future, These new techniques could make up for some of the drawbacks of the X-ray crystallography method. The most recent reports of structure determination of large macromolecular assemblies using cryo-EM have broken the 10 Å resolution barrier and determined the helical structure of the hepatitis B core protein (Bottcher *et al.*, 1997; Conway et *al.*, 1997) showing a promising aspect of combination of lower resolution techniques such as cryo-EM with high resolution technique such as X-ray crystallography will be optimised to enable detailed structure determination of large viral structures in a rapid and easier way.

APPENDICES

APPENDIX A: SEQUENCES OF THE *vp2* INSERT GENE (The nucleotide bases are shown in black and the deduced amino acid sequences are shown in red colour. The stop codon are shown in bold italic and underlined)

```
   1 ATG ACA AAC CTG CAA GAT CAA ACC CAA CAG ATT GTT CCG TTC ATA CGG AGC
     > M   T   N   L   Q   D   Q   T   Q   Q   I   V   P   F   I   R   S
  52 CTT CTG ATG CCA ACA ACC GGA CCG GCG TCC ATT CCG GAC GAC ACC CTG GAG
     > L   L   M   P   T   T   G   P   A   S   I   P   D   D   T   L   E
 103 AAG CAC ACT CTC AGG TCA GAG ACC TCG ACC TAC AAT TTG ACT GTG GGG GAC
     > K   H   T   L   R   S   E   T   S   T   Y   N   L   T   V   G   D
 154 ACA GGG TCA GGG CTA ATT GTC TTT TTC CCT GGA TTC CCT GGC TCA ATT GTG
     > T   G   S   G   L   I   V   F   F   P   G   F   P   G   S   I   V
 205 GGT GCT CAC TAC ACA CTG CAG GGC AAT GGG AAC TAC AAG TTC GAT CAG ATG
     > G   A   H   Y   T   L   Q   G   N   G   N   Y   K   F   D   Q   M
 256 CTC CTG ACT GCC CAG AAC CTA CCG GCC AGT TAC AAC TAC TGC AGG CTA GTG
     > L   L   T   A   Q   N   L   P   A   S   Y   N   Y   C   R   L   V
 307 AGT CGG AGT CTC ACA GTG AGG TCA AGC ACA CTT CCT GGT GGC GTT TAT GCA
     > S   R   S   L   T   V   R   S   S   T   L   P   G   G   V   Y   A
 358 CTA AAC GGC ACC ATA AAC GCC GTG ACC TTC CAA GGA AGC CTG AGT GAA CTG
     > L   N   G   T   I   N   A   V   T   F   Q   G   S   L   S   E   L
 409 ACA GAT GTT AGC TAC AAT GGG TTG ATG TCT GCA ACA GCC AAC ATC AAC GAC
     > T   D   V   S   Y   N   G   L   M   S   A   T   A   N   I   N   D
 460 AAA ATT GGG AAC GTC CTA GTA GGG GAA GGG GTC ACC GTC CTC AGC TTA CCC
     > K   I   G   N   V   L   V   G   E   G   V   T   V   L   S   L   P
 511 ACA TCA TAT GAT CTT GGG TAT GTG AGG CTT GGT GAC CCC ATT CCC GCA ATA
     > T   S   Y   D   L   G   Y   V   R   L   G   D   P   I   P   A   I
 562 GGG CTT GAC CCA AAA ATG GTA GCC ACA TGT GAC AGC AGT GAC AGG CCC AGA
     > G   L   D   P   K   M   V   A   T   C   D   S   S   D   R   P   R
 613 GTC TAC ACC ATA ACT GCA GCC GAT GAT TAC CAA TTC TCA TCA CAG TAC CAA
     > V   Y   T   I   T   A   A   D   D   Y   Q   F   S   S   Q   Y   Q
 664 CCA GGT GGG GTA ACA ATC ACA CTG TTC TCA GCC AAC ATT GAT GCC ATC ACA
     > P   G   G   V   T   I   T   L   F   S   A   N   I   D   A   I   T
 715 AGC CTC AGC GTT GGG GGA GAG CTC GTG TTT CAA ACA AGC GTC CAC GGC CTT
     > S   L   S   V   G   G   E   L   V   F   Q   T   S   V   H   G   L
 766 GTA CTG GGC GCC ACC ATC TAC CTC ATA GGC TTT GAT GGG ACA ACG GTA ATC
     > V   L   G   A   T   I   Y   L   I   G   F   D   G   T   T   V   I
 817 ACC AGG GCT GTG GCC GCA AAC AAT GGG CTG ACG ACC GGC ACC GAC AAC CTT
     > T   R   A   V   A   A   N   N   G   L   T   T   G   T   D   N   L
 868 ATG CCA TTC AAT CTT GTG ATT CCA ACA AAC GAG ATA ACC CAG CCA ATC ACA
     > M   P   F   N   L   V   I   P   T   N   E   I   T   Q   P   I   T
 919 TCC ATC AAA CTG GAG ATA GTG ACC TCC AAA AGT GGT GGT CAG GCA GGG GAT
     > S   I   K   L   E   I   V   T   S   K   S   G   G   Q   A   G   D
 970 CAG ATG TCA TGG TCG GCA AGA GGG AGC CTA GCA GTG ACG ATC CAT GGT GGC
     > Q   M   S   W   S   A   R   G   S   L   A   V   T   I   H   G   G
1021 AAC TAT CCA GGG GCC CTC CGT CCC GTC ACG CTA GTG GCC TAC GAA AGA GTG
     > N   Y   P   G   A   L   R   P   V   T   L   V   A   Y   E   R   V
1072 GCA ACA GGA TCC GTC GTC ACG GTC GCT GGG GTG AGC AAC TTC GAG CTG ATC
     > A   T   G   S   V   V   T   V   A   G   V   S   N   F   E   L   I
1123 CCA AAT CCT GAA CTA GCA AAG AAC CTG GTT ACA GAA TAC GGC CGA TTT GAC
     > P   N   P   E   L   A   K   N   L   V   T   E   Y   G   R   F   D
1174 CCA GGA GCC ATG AAC TAC ACA AAA TTG ATA CTG AGT GAG AGG GAC CGT CTT
     > P   G   A   M   N   Y   T   K   L   I   L   S   E   R   D   R   L
1225 GGC ATC AAG ACC GTC TGG CCA ACA AGG GAG TAC ACT GAC TTT CGT GAA TAC
     > G   I   K   T   V   W   P   T   R   E   Y   T   D   F   R   E   Y
1276 TTC ATG GAG GTG GCC GAC CTC AAC TCT CCC CTG AAG ATT GCA GGA GCA TTC
     > F   M   E   V   A   D   L   N   S   P   L   K   I   A   G   A   F
1327 GGC TTC AAA GAC ATA ATC CGG GCC ATA AGG TAA
     > G   F   K   D   I   I   R   A   I   R stop
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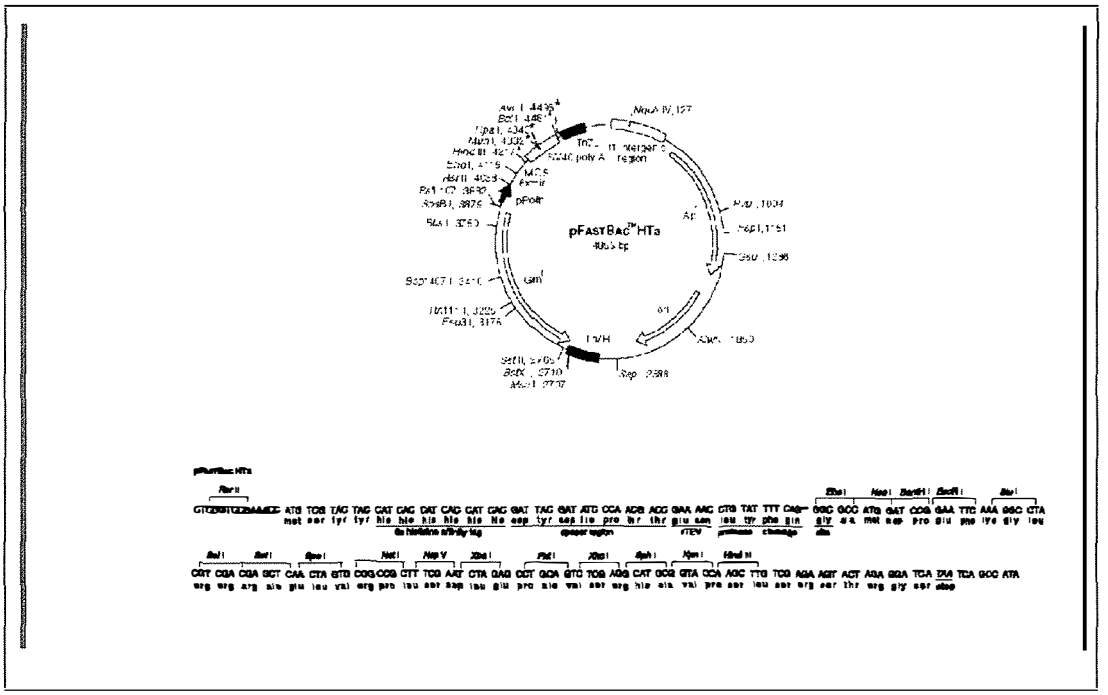
APPENDIX B: MAPS AND RESTRICTION ENDONUCLEASE SITES FOR pFASTBAC HTa VECTOR.



APPENDIX C: CRYSTAL SCREEN FORMULATION # 1

Tube	Salt	Tube	Buffer	Tube	Precipitant
1	0.02 M Calcium Chloride dihydrate	1	0.1 M Sodium Acetate trihydrate pH 4.6	1	30% v/v 2-Methyl-2, 4-pentanediol
2	None	2	None	2	0.4 M Potassium Sodium Tartrate tetrahydrate
3	None	3	None	3	0.4 M mono-Ammonium dihydrogen Phosphate
4	None	4	0.1 M Tris Hydrochloride pH 8.5	4	2.0 M Ammonium Sulfate
5	0.2 M tri-Sodium Citrate dihydrate	5	0.1 M HEPES-Na pH 7.5	5	30% v/v 2-Methyl-2, 4-pentanediol
6	0.2 M Magnesium Chloride hexahydrate	6	0.1 M Tris Hydrochloride pH 8.5	6	30% w/v Polyethylene Glycol 4000
7	None	7	0.1 M Sodium Cacodylate pH 6.5	7	1.4 M Sodium Acetate trihydrate
8	0.2 M tri-Sodium Citrate dihydrate	8	0.1 M Sodium Cacodylate pH 6.5	8	30% v/v iso-Propanol
9	0.2 M Ammonium Acetate	9	0.1 M tri-Sodium Citrate dihydrate pH 5.6	9	30% w/v Polyethylene Glycol 4000
10	0.2 M Ammonium Acetate	10	0.1 M Sodium Acetate trihydrate pH 4.6	10	30% w/v Polyethylene Glycol 4000
11	None	11	0.1 M tri-Sodium Citrate dihydrate pH 5.6	11	1.0 M mono-Ammonium dihydrogen Phosphate
12	0.2 M Magnesium Chloride hexahydrate	12	0.1 M HEPES-Na pH 7.5	12	30% v/v iso-Propanol
13	0.2 M tri-Sodium Citrate dihydrate	13	0.1 M Tris Hydrochloride pH 8.5	13	30% v/v Polyethylene Glycol 400
14	0.2 M Calcium Chloride dihydrate	14	0.1 M HEPES-Na pH 7.5	14	28% v/v Polyethylene Glycol 400
15	0.2 M Ammonium Sulfate	15	0.1 M Sodium Cacodylate pH 6.5	15	30% w/v Polyethylene Glycol 8000
16	None	16	0.1 M HEPES-Na pH 7.5	16	1.5 M Lithium Sulfate monohydrate
17	0.2 M Lithium Sulfate monohydrate	17	0.1 M Tris Hydrochloride pH 8.5	17	30% Polyethylene Glycol 4000
18	0.2 M Magnesium Acetate tetrahydrate	18	0.1 M Sodium Cacodylate pH 6.5	18	20% Polyethylene Glycol 8000
19	0.2 M Ammonium Acetate	19	0.1 M Tris Hydrochloride pH 8.5	19	30% v/v iso-Propanol
20	0.2 M Ammonium Sulfate	20	0.1 M Sodium Acetate trihydrate pH 4.6	20	25% w/v Polyethylene Glycol 4000
21	0.2 M Magnesium Acetate tetrahydrate	21	0.1 M Sodium Cacodylate pH 6.5	21	30% v/v 2-Methyl-2, 4-pentanediol
22	0.2 M Sodium Acetate trihydrate	22	0.1 M Tris Hydrochloride pH 8.5	22	30% w/v Polyethylene Glycol 4000
23	0.2 M Magnesium Chloride hexahydrate	23	0.1 M HEPES-Na pH 7.5	23	30% v/v Polyethylene Glycol 400
24	0.2 M Calcium Chloride dihydrate	24	0.1 M Sodium Acetate trihydrate pH 4.6	24	20% v/v iso-Propanol
25	None	25	0.1 M Imidazole pH 6.5	25	1.0 M Sodium Acetate trihydrate
26	0.2 M Ammonium Acetate	26	0.1 M tri-Sodium Citrate dihydrate pH 5.6	26	30% v/v 2-Methyl-2, 4-pentanediol
27	0.2 M tri-Sodium Citrate dihydrate	27	0.1 M HEPES-Na pH 7.5	27	20% v/v iso-Propanol
28	0.2 M Sodium Acetate trihydrate	28	0.1 M Sodium Cacodylate pH 6.5	28	30% w/v Polyethylene Glycol 8000
29	None	29	0.1 M HEPES-Na pH 7.5	29	0.8 M Potassium Sodium Tartrate tetrahydrate
30	0.2 M Ammonium Sulfate	30	None	30	30% w/v Polyethylene Glycol 8000
31	0.2 M Ammonium Sulfate	31	None	31	30% w/v Polyethylene Glycol 4000
32	None	32	None	32	2.0 M Ammonium Sulfate
33	None	33	None	33	4.0 M Sodium Formate
34	None	34	0.1 M Sodium Acetate trihydrate pH 4.6	34	2.0 M Sodium Formate
35	None	35	0.1 M HEPES-Na pH 7.5	35	0.8 M mono-Sodium dihydrogen phosphate 0.8 M mono-Potassium dihydrogen phosphate
36	None	36	0.1 M Tris Hydrochloride pH 8.5	36	8% w/v Polyethylene Glycol 8000
37	None	37	0.1 M Sodium Acetate trihydrate pH 4.6	37	8% w/v Polyethylene Glycol 4000
38	None	38	0.1 M HEPES-Na pH 7.5	38	1.4 M tri-Sodium Citrate dihydrate
39	None	39	0.1 M HEPES-Na pH 7.5	39	2% v/v Polyethylene Glycol 400, 2.0 M Ammonium Sulfate
40	None	40	0.1 M tri-Sodium Citrate dihydrate pH 5.6	40	20% v/v iso-Propanol, 20% w/v Polyethylene Glycol 4000
41	None	41	0.1 M HEPES-Na pH 7.5	41	10% v/v iso-Propanol, 20% w/v Polyethylene Glycol 4000
42	0.05 M mono-Potassium dihydrogen Phosphate	42	None	42	20% w/v Polyethylene Glycol 8000
43	None	43	None	43	30% w/v Polyethylene Glycol 1500
44	None	44	None	44	0.2 M Magnesium Formate
45	0.2 M Zinc Acetate dihydrate	45	0.1 M Sodium Cacodylate pH 6.5	45	18% w/v Polyethylene Glycol 8000
46	0.2 M Calcium Acetate hydrate	46	0.1 M Sodium Cacodylate pH 6.5	46	18% w/v Polyethylene Glycol 8000
47	None	47	0.1 M Sodium Acetate trihydrate pH 4.6	47	2.0 M Ammonium Sulfate
48	None	48	0.1 M Tris Hydrochloride pH 8.5	48	2.0 M mono-Ammonium dihydrogen Phosphate
49	1.0 M Lithium Sulfate monohydrate	49	None	49	2% w/v Polyethylene Glycol 8000
50	0.5 M Lithium Sulfate monohydrate	50	None	50	15% w/v Polyethylene Glycol 8000

APPENDIX C: CRYSTAL SCREEN FORMULATION # 2

Tube	Salt	Tube	Buffer	Tube	Precipitant
1	2.0 M Sodium cloride	1	None	1	10% w/v PEG 6000
2	0.01 M Hexadecyltrimethylammonium Bromide	2	None	2	0.5 M Sodium Chloride, 0.01 M Magnesium Chloride hexahydrate
3	None	3	None	3	25% v/v Ethylene Glycol
4	None	4	None	4	35% v/v Dioxane
5	2.0 M Ammonium Sulfate	5	None	5	5% v/v iso-Propanol
6	None	6	None	6	1.0 M Imidazole pH 7.0
7	None	7	None	7	10% w/v Polyethylene Glycol 1000 10% w/v Polyethylene Glycol 8000
8	1.5 M Sodium Chloride	8	None	8	10% v/v Ethanol
9	None	9	0.1 M Sodium Acetate trihydrate pH 4.6	9	2.0 M Sodium Chloride
10	0.2 M Sodium Chloride	10	0.1 M Sodium Acetate trihydrate pH 4.6	10	30% v/v MPD
11	0.01 M Cobaltous Chloride hexahydrate	11	0.1 M Sodium Acetate trihydrate pH 4.6	11	1.0 M 1,6 Hexanediol
12	0.1 M Cadmium Chloride dihydrate	12	0.1 M Sodium Acetate trihydrate pH 4.6	12	30% v/v Polyethylene Glycol 400
13	0.2 M Ammonium Sulfate	13	0.1 M Sodium Acetate trihydrate pH 4.6	13	30% w/v Polyethylene Glycol Monomethyl Ether 2000
14	0.2 M Potassium Sodium Tartrate tetrahydrate	14	0.1 M tri-Sodium Citrate dihydrate pH 5.6	14	2.0 M Ammonium Sulfate
15	0.5 M Ammonium Sulfate	15	0.1 M tri-Sodium Citrate dihydrate pH 5.6	15	1.0 M Lithium Sulfate monohydrate
16	0.5 M Sodium Chloride	16	0.1 M tri-Sodium Citrate dihydrate pH 5.6	16	4% w/v Ethylene Imine Polymer
17	None	17	0.1 M tri-Sodium Citrate dihydrate pH 5.6	17	35% v/v tert-Butanol
18	0.01 M Ferric Chloride hexahydrate	18	0.1 M tri-Sodium Citrate dihydrate pH 5.6	18	10% v/v Jeffamine M-600
19	None	19	0.1 M tri-Sodium Citrate dihydrate pH 5.6	19	2.5 M 1,6 Hexanediol
20	None	20	0.1 M MES pH 6.5	20	1.6 M Magnesium Sulfate heptahydrate
21	0.1 M Sodium dihydrogen phosphate mono 0.1 M mono-Potassium dihydrogen Phosphate	21	0.1 M MES pH 6.5	21	2.0 M Sodium Chloride
22	None	22	0.1 M MES pH 6.5	22	12% w/v Polyethylene Glycol 20,000
23	1.6 M Ammonium Sulfate	23	0.1 M MES pH 6.5	23	10% v/v Dioxane
24	0.05 M Cesium Chloride	24	0.1 M MES pH 6.5	24	30% v/v Jeffamine M-600
25	0.01 M Cobaltous Chloride hexahydrate	25	0.1 M MES pH 6.5	25	1.8 M Ammonium Sulfate
26	0.2 M Ammonium Sulfate	26	0.1 M MES pH 6.5	26	30% w/v Polyethylene Glycol Monomethyl Ether 5000
27	0.01 M Zinc Sulfate heptahydrate	27	0.1 M MES pH 6.5	27	25% v/v Polyethylene Glycol Monomethyl Ether 550
28	None	28	None	28	1.6 M tri-Sodium Citrate dihydrate pH6.5
29	0.5 M Ammonium Sulfate	29	0.1 M HEPES pH 7.5	29	30% v/v MPD
30	None	30	0.1 M HEPES pH 7.5	30	10% w/v Polyethylene Glycol 6000, 5% v/v MPD
31	None	31	0.1 M HEPES pH 7.5	31	20% v/v Jeffamine M-600
32	0.1 M Sodium Chloride	32	0.1 M HEPES pH 7.5	32	1.6 M Ammonium Sulfate
33	None	33	0.1 M HEPES pH 7.5	33	2.0 M Ammonium Formate
34	0.05 M Cadmium Sulfate hydrate	34	0.1 M HEPES pH 7.5	34	1.0 M Sodium acetate
35	None	35	0.1 M HEPES pH 7.5	35	70% v/v MPD
36	None	36	0.1 M HEPES pH 7.5	36	4.3 M Sodium Chloride
37	None	37	0.1 M HEPES pH 7.5	37	10% w/v Polyethylene Glycol 8000, 8% v/v Ethylene Glycol
38	None	38	0.1 M HEPES pH 7.5	38	20% w/v Polyethylene Glycol 10,000
39	0.2 M Magnesium Chloride hexahydrate	39	0.1 M TRIS pH 8.5	39	3.4 M 1,6 Hexanediol
40	None	40	0.1 M TRIS pH 8.5	40	25% v/v tert-Butanol
41	0.01 M Nickel(II) Chloride hexahydrate	41	0.1 M TRIS pH 8.5	41	1.0 M Lithium Sulfate monohydrate
42	1.5 M Ammonium Sulfate	42	0.1 M TRIS pH 8.5	42	12% v/v Glycerol anhydrous
43	0.2 M mono Ammonium dihydrogen Phosphate	43	0.1 M TRIS pH 8.5	43	50% v/v MPD
44	None	44	0.1 M TRIS pH 8.5	44	20% v/v Ethanol
45	0.01 M Nickel(II) Chloride hexahydrate	45	0.1 M TRIS pH 8.5	45	20% w/v Polyethylene Glycol Monomethyl Ether 2000
46	0.1 M Sodium Chloride	46	0.1 M Bicine pH 9.0	46	30% w/v Polyethylene Glycol Monomethyl Ether 550
47	None	47	0.1 M Bicine pH 9.0	47	2.0 M Magnesium Chloride hexahydrate
48	2% v/v Dioxane	48	0.1 M Bicine pH 9.0	48	10% w/v Polyethylene Glycol 20,000

APPENDIX D: BUFFERS AND SOLUTIONS

ANTIBIOTIC / TRYPSIN / VERSENE (ATV)

Trypsin Difco, 1:250	0.5 g
Versene (EDTA) tetrasodium salt	0.2 g
NaCl	8.0 g
KCl	0.4 g
Dextrose	1.0 g
$NaHCO_3$	0.58 g
Penicillin	2×10^5 units
Streptomycin	0.1 g
Phenol red	0.02 g

Adjust to pH 7.2, filter through 0.2 μm filter, dispense into 10 mL aliquots and store at –20 °C.

CAESIUM CHLORIDE STOCKS (50%, w/v)

Caesium chloride	50 g
TBS	up to 100 mL

Mix and stir to dissolve. Dilute as needed to make other concentrations.

DEPC-TREATED H_2O

Add diethylpyrocarbonate (DEPC) to a final volume 0.01% (v/v). Stir at 37 °C for an hour. Leave at 37 °C overnight, followed by autoclaving.

EAGLE MINIMUM ESSENTIAL MEDIA + NON-ESSENTIAL AMINO ACIDS, WITH L- GLUTAMINE (MEM+N)

MEM + n (Sigma)	9.7 g
$NaHCO_3$	2.2 g
Distilled H_2O	up to 1000 mL

Sterillise by filtration through 0.2 μm.

GEL LOADING BUFFER

1 X TBE

50% glycerol

0.005% Bromophenol Blue

0.025% Xylene Cyanol

PENICILLIN STREPTOMYCIN KANAMYCIN (PSK)

Streptomycin	1.0 g
Kanamycin	1.0 g
Penicillin	1 x 10^6 units

Make up to 100 mL with PBS, filter through 0.2 μm filter, dispense into 20 mL aliquots and store at –20 °C until required.

PHOSPHATE BUFFERED SALINE, pH 7.0 (PBS)

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g

Make up to 1000 mL with H_2O, adjust to pH 7.0 and sterilise by autoclaving.

PHOSPHOTUNGSTIC ACID STAIN, 4%

Potassium phosphaotungstate	4 g
Distilled H_2O	up to 100 mL

Adjust pH 7.2 with KOH and filter through 0.2 μm filter.

SDS STOCK SOLUTION

10% SDS in H_2O filtered through 0.2 μm filter.

SDS-PAGE LOADING BUFFER (10X)

dH_2O	4.0 mL
0.5 M Tris-HCl, pH 6.8	1.0 mL
Glycerol	0.8 mL
10% (w/v) SDS	1.6 mL
DTT	0.77 g
0.05% (w/v) bromophenol blue	0.2 mL

SDS-PAGE ELETROPHORESIS TANK BUFFER, pH 8.3 (5X)

Tris base	15 g
Glycine	72 g
SDS	5 g
dH_2O	up to 1000 mL

Dilution 60 mL 5x stock with 240 mL dH_2O for one electrophoretic run.

SOC MEDIUM

Tryptone (Gibco BRL)	2 g
Yeast extract (Gibco BRL)	0.55 g
1 M NaCl	1 mL
1 M KCl	1 mL

Stir to dissolve, autoclave, and store at RT. Immediately before use, add 2 M $MgCl_2$ (1 mL) and 2 M glucose (1 mL), which had been filtered through 0.2 μm filter.

60% SUCROSE (w/w)

Sucrose	60 g
TSB	40 g

Mix and stir until dissolved. Do not heat. Store at 4 °C.

30% SUCROSE (w/w)

60% sucrose	30 mL
TBS	30 mL

TAE ELECTROPHORESIS BUFFER STOCK SOLUTION (50X)

Trizma base	242 g
EDTA, sodium salt	18.6 g
Distilled H_2O	up to 1000 mL

Dissolve in approximately 800 mL H_2O. Adjust to pH 8.0 with glacial acetic acid (57 mL/L). Make up to final volume of 1000 mL. Store at RT.

TB MEDIUM

Tryptone (Gibco BRL)	12 g
Yeast extract (Gibco BRL)	24 g
Glycerol	4 mL
Distilled H_2O	up to 900 mL

Stir to dissolve, autoclave and cool to RT. Add 100 mL of Phosphate Buffer and mix well.

TBE ELECTROPHRESIS BUFFER STOCK SOLUTION (10X)

Trizma base	1.21 g
Boric acid	55.0 g
EDTA, sodium salt	9.3 g
Distilled H_2O	up to 1000 mL

TRIS BUFFERED SALINE (TBS)

NaCl	5.844 g
Trizma base	1.211 g
Distilled H_2O	up to 1000 mL

Dissolve in approximately 800 mL H_2O. Adjust to pH 7.6 with conc. HCl. Make up to final volume of 1000 mL. Filter through 0.2 μm and store at RT.

TRYPTOSE PHOSPHATE BROTH (TPB)

Tryptose (Gibco BRL)	20.0 g
Dextrose	2.0 g
NaCl	5.0 g
Na_2HPO_4 (anhydrous)	2.5 g
Distilled H_2O	up to 1000 mL

Adjust pH 7.3, dispensed into 200 mL aliquots and sterilised by autoclaving.

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Erratum

Page number / line number	Alterations to text
xviii	Additional abbreviation: DRI dairy research institute MALLS multiangle laser light scattering photospectrometry SEC size exclusion chromatography
213 / 4	(***Journal of Structural Biology***) not (*Structural Biology*)
226 / 8	Insert journal title: ***Avian Diseases***
232 / 26	***(Biophysical Journal)*** not (*Biophysic Journal*)
235 / 14	***(American Journal of Hygiene)*** not (*American Journal of Hygene*)
242 / 7	Delete repeated reference