Infectious bursal disease virus receptor identification with anti-peptide antibodies

by

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PREFACE

The experimental work described in this dissertation was carried out in the School of Molecular and Cellular Biosciences, Department of Biochemistry, University of KwaZulu-Natal, Pietermaritzburg, from March 2002 to December 2003 under the supervision of Professor Theresa H. T. Coetzer.

These studies represent original work by the author and have not been submitted in any other form to another university. Where use has been made of the work of others, it has been duly acknowledged in the text.

Habtom Haileselassie Habte
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ABSTRACT

Infectious bursal disease virus (IBDV) has a tropism for the lymphoid tissue of poultry and infects actively dividing and differentiating B-lymphocytes in the bursa of Fabricius. This results in a high mortality rate and severe immunosuppression. These immunodepressed chickens are highly susceptible to secondary infections and have a reduced capacity to respond to vaccination. The principal method to control IBDV is through extensive vaccination using either attenuated live or inactivated IBDV vaccines. However, in recent years due to the emergence of new virulent strains, risk of reversion to pathogenicity, cost considerations and intervention by maternal antibodies, the effectiveness of these vaccines in the veterinary field is being reduced. An alternative approach to prevent infection is by interfering with the binding of IBDV to its receptor protein on the surface of bursal cells. Hence this study was undertaken on the characterisation of a possible IBDV receptor on bursal membranes.

Infectious bursal disease virus was isolated from infected bursal tissue using CsCl density gradient centrifugation and visualised with Tris-Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transmission electron microscopy. Following purification of double stranded RNA from infected bursal tissue and commercially available live IBDV vaccines, a polymerase chain reaction (PCR)-based diagnostic assay based on sequences from the highly conserved viral protein (VP2) region was performed. The presence of the virus was demonstrated by the amplification of a 150 bp band in 2% agarose and 15% nondenaturing PAGE gels. The correctness of this product was confirmed by restriction digestion with a specific restriction endonuclease (*Bam*HI) that resulted in the predicted digestion fragments of 93 and 57 bp.

Following preparation of bursal membrane proteins from uninfected bursal tissue, using sucrose density gradient centrifugation, isolation of IBDV receptor protein was carried out by immobilising IBDV on a Sepharose 4B chromatography matrix. After affinity purification, two prominent protein bands around 40 kDa were visualised using a silver stained Tris-Tricine SDS-PAGE gel. Previous work in this laboratory identified two possible IBDV receptor proteins on bursal membranes of 32 and 40 kDa. Antibodies against peptide sequences derived from the 32 kDa receptor protein were raised in rabbits in the present study. These anti-IBDV

receptor peptide antibodies recognised the affinity purified native 40 kDa IBDV receptor proteins in an enzyme-linked immunosorbent assay (ELISA). However, due to the possible epitope denaturation by the reducing treatment buffer prior to Tris-Tricine SDS-PAGE such as SDS and 2-mercapthethanol or detergent (Na-deoxycholate) used during the affinity purification of the IBDV receptor protein, the anti-IBDV receptor peptide antibody did not recognise the receptor protein on a western blot.

An inhibition assay was performed in an ELISA format by coating the 40 kDa IBDV receptor protein to see if the anti-IBDV receptor peptide antibody could inhibit IBDV binding to the receptor. The result showed that the anti-IBDV receptor peptide antibody effectively inhibited the binding of IBDV to the receptor. This result could pave the way for reducing IBDV infection by interfering at the viral attachment stage prior to crossing the bursal cell membrane barrier.

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ABBREVIATIONS

ω	angular velocity
3D-PSSM	three-dimensional position-specific scoring matrix
A	adenine
A_{405}	absorbance at 405 nm
AC-ELISA	antigen-capture enzyme-linked immunosorbent assay
AEBSF	4-(2-aminoethyl)benzenesulfonylfluoride
Ala	alanine
AMV	avian myeloblastosis virus
Anti-Id	antiidiotype antibodies
Arg	arginine
Asn	asparagines
Asp	aspartic acid
ATBS	2,2'-Azinobis[3-ethyl-2,3-dihydrobenzthiazole-6-sulphonate]
ATP	adenosine tri-phosphate
*Bam*HI	*Bacillus amyloliquefaciens* H
BDB	bis-diazotized benzidine
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
C	cytosine
cDNA	complementary deoxyribonucleic acid
CEF	chicken embryo fibroblast
CsCl	cesium chloride
C-terminal/terminus	carboxy terminal/terminus
dATP	deoxy adenosine tri-phosphate
DCI	dichloroisocoumarin
dCTP	deoxy cytosine tri-phosphate
ddH_2O	distilled, deionised water
DEPC	diethylpyrocarbonate
DESs	sodium oligooxyethylene dodecyl ether sulfates

DFP	diisopropylphosphofluoride
dGTP	deoxy guanidine tri-phosphate
dist.H_2O	distilled water
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxy nucleotide tri-phosphate
ds	double stranded
dT	oligo
DTT	dithiothreitol
dTTP	deoxy thymidine tri-phosphate
E-64	[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-amido(4-guanidino)butane]
EGTA	ethylene glycol-bis (β-aminoethylether)-N, N, N', N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EtBr	ethidium bromide
Fab	hypervariable antigen binding
FAE	follicular-associated epithelium
FCA	freund's complete adjuvant
FIA	freund's incomplete adjuvant
FITC	fluorescent isothiocyanate
FMDV	foot and mouth disease virus
g	relative centrifugal force
G	guanine
HA	influenza virus
HEPES	4-(2-hydroxyethyl)-1-piperazin-ethan-sulfonaure
His	histidine
HIV	human immunodeficiency virus
HRPO	horse radish peroxidase

HRSV	human respiratory syncytial virus
HSV	herpes simplex virus
HV	high virulent
IBD	infectious bursal disease
IBDV	infectious bursal disease virus
IBDVR	IBDV receptor peptide sequence
IgG	immunoglobulin g
IgM	immunoglobulin M
IgY	egg yolk immunoglobulin
IPNV	infectious pancreatic necrosis virus
kb	kilo-base
kDa	kilo-Dalton
KLH	keyhole limpet hemocyanin
Lys	lysine
M	medulla
MAb	monoclonal antibody
MBS	m-maleimidobenzoilacid-N-hydroxysuccinimide ester
MEC	molecular exclusion chromatography
MHC II	major histocompatibility complex class II
M_r	molecular mass
mRNA	messenger RNA
N	Avogadro's number
Na_2-EDTA	ethylenediaminetetra-acetic acid disodium salt
N-terminal/terminus	amino terminal/terminus
OD	optical density
ORF	open reading frames
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
Phe	phenylalanine
PMSF	phenylmethylsulfonylfluoride

Pro	proline
PTA	phosphotungstic acid
r	radius
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolution per minute
RSA	rabbit serum albumin
RSV	respiratory syncytial virus
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SCOP	structural classification of proteins
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide –gel electrophoresis
SF	simian para influenza virus
SH	sulfohydril
SIgM	surface immunoglobulin M
SPF	specific pathogen free
ss	single strand
T	thymine
TAE	tris-acetate buffer
TBE	tris, boric acid and EDTA
TBS	tris buffered saline
TEM	transmission electron microscopy
TEMED	N,N,N,'N'-tetramethylethylenediamine
TEN	tris, EDTA, sodium chloride
Thr	threonine
Tm	meltimg temperature
Trp	tryptophan
Tth	polymerase
Tyr	tyrosine
UV	ultraviolet

V	volume
VAP	viral attachment protein
VOPBA	virus overlay protein blot assay
VP	viral protein
VSV	vesicular stomatitis virus
VV	very virulent
pg	density gradient
pm	buoyant density

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Infectious bursal disease virus (IBDV) was first discovered in chickens by Cosgrove in 1962 in the Gumboro area of the Southern Delaware, USA (McIlroy *et al.*, 1992; Yamaguchi *et al.*, 1997). Because of its origin, further outbreaks were subsequently known as 'Gumboro disease'. The virus infects most prominently the bursa of Fabricius, thus the current name of the disease. This tropism for the bursa was confirmed by electron microscopy analysis, which detected large numbers of virions in the bursa of infected birds (Murphy *et al.*, 1999). Based on its structure/morphology, IBDV was temporarily classified as a picornavirus, adenovirus or orbivirus, until the essential structural features of the cloned virus were determined that showed significant homology with infectious pancreatic necrosis virus (IPNV) (Becht, 1994). Like IPNV and DrosophilaX virus, IBDV belongs to the family of *Birnaviridae* (Lejal *et al.*, 2000).

Infectious bursal disease (IBD) is a highly transmittable disease of young chickens, typically in the age between two and eight weeks, with mortality rates up to 80% (Mundt and Muller, 1995). The virus replicates in the developing bursa of Fabricius, thymus, spleen and coecal tonsils of young birds and causes severe B-cell depletion by inducing apoptosis, resulting in severely immunodepressed chickens. For that reason chickens are highly susceptible to secondary infections and have a reduced capacity to respond to vaccination (Boot *et al.*, 2000b). The acute form is characterised by sudden onset, short course and extensive destruction of lymphocytes particularly in the bursa of Fabricius and also in other lymphoid tissue (Jordan, 1990).

Economically IBDV is a very important viral disease throughout the world, which results in mortality and heavy financial losses to the poultry industry (Baxendale, 1981; Pitcovski *et al.*, 1996). Direct economic losses can be as a result of high mortality rates due to an acute course

of the disease. The indirect impact of the disease is due to the virus-induced immunosuppression and/or potential interactions between IBDV and other viruses, bacteria or parasites. These indirect losses induce growth retardation and condemnation of carcasses at the slaughterhouse. Furthermore, the increased use of antibiotics against the secondary infections constitutes a growing public health concern (van den Berg *et al.*, 2000).

The first option for IBDV control is vaccination using either attenuated live or inactivated IBDV vaccines (Amakye-Anim *et al.*, 2000). Using a series of live attenuated and killed commercial vaccines the disease was successfully controlled until the late 1980's (Brown and Skinner, 1996). However, as a consequence of the appearance of new virulent variants resulting in heavy outbreaks of the disease worldwide, the effectiveness of these vaccines has decreased (Muller *et al.*, 1992; Pitcovski *et al.*, 1999). Since 1987 a growing number of acute cases of IBDV with high mortality have been reported in many European countries (Bumstead *et al.*, 1993), Asia (Mandeville *et al.*, 2000) and Africa (Brown and Skinner, 1996). The latest emergence of the highly virulent strains of IBDV in Europe and the United Kingdom made IBDV a major problem in the poultry industry and the need for the development of a new vaccine has become critical (Bayliss *et al.*, 1991).

1.2 Geographical distribution of infectious bursal disease virus

Three years after the first case of IBD was diagnosed in the Gumboro area of Delaware, USA, the virus was detected in other regions of the USA, and very soon appeared in the United Kingdom and Western Europe (Lasher and Davis, 1997; Sainsbury, 2000). From 1962 to 1974, the disease was identified in the Middle East, Southern and Western Africa, India, the Far East and Australia (van den Berg *et al.*, 2000).

Outbreaks of "very virulent" IBDV strains with high mortality rates have been gradually increasing (Fernandez-Arias *et al.*, 1997). In 1987 a pathogenic variant of IBDV appeared in the Benelux countries and spread rapidly through Europe including the United Kingdom (Bayliss *et al.*, 1991), Germany (Yamaguchi *et al.*, 1996a) and France (Eterradossi *et al.*, 1992). The term "very virulent" (VV) was used for the IBDV strain as it caused high flock mortalities up to 70% for pullets, 25% for broilers and 100% for specific pathogen free birds

(SPF), (Lim *et al.*, 1999). Based on pathological data, VV IBDV appears to have reached South Africa by 1989 and Poland by 1991 (Brown and Skinner, 1996). Since the late eighties, outbreaks of newly evolved viral strains have caused significant economic losses to the poultry industry in China (Lim *et al.*, 1999) and Japan (Nunoya *et al.*, 1992; Lin *et al.*, 1993). Unreliable reports indicate that the pathogenic variant may also be present in the Middle East, Asia and in Central Africa but have not been identified in Northern, Central or South America and Australia (Mandeville *et al.*, 2000).

1.3 Taxonomy and classification of IBDV

Infectious bursal disease virus belongs to the family *Birnaviridae*, which gets its name from their bi-segmented RNA (Porterfield, 1989). This family is made up of three genera: *Avibirnavirus*, *Aquabirnavirus* and *Entomobirnavirus*. The genus *Aquabirnavirus* includes IPNV of salmonid fish and viruses of oysters and crabs. Viruses that infect only insects belong to the genus *Entomobirnavirus* (Murphy *et al.*, 1999) while the virus infecting chickens, belongs to the genus *Avibirnavirus* (Brown *et al.*, 1994).

Infectious bursal disease virus is nonenveloped, icosahedral virus made up of 132 morphological subunits with a diameter of 60 to 70 nm (Spies *et al.*, 1987; Granzow *et al.*, 1997; Schröder *et al.*, 2000). The single shell capsid has a thickness of *ca* 9 nm, with 780 subunits clustered as 260 outer trimers, arranged with a triangulation number of T=13. This arrangement of IBDV was confirmed by cryoelectron microscopy and image processing analysis (Caston *et al.*, 2001). At the outer radii between 31 and 33 nm, the comparative arrangements of trimers produce a honeycomb like structure on the surface of the virus. At inner radii between 26 and 30 nm, the trimers have a triangular or Y-shaped pattern and arrange more closely to form a continuous shell. The subunits adjacent to the five-fold axes lie at a larger radius from the core of the particle than those perform around the three- or two-fold axes, giving the capsid a markedly nonspherical shape (Böttcher *et al.*, 1997).

There are currently two different serotypes of IBDV, namely serotype I and II. Serotype I, which is a wild type, isolate is pathogenic for chickens, while serotype II isolates, which are mainly from turkeys are considered as non-pathogenic for chickens (Boot *et al.*, 2000b). Viral

strains may also be classified according to their virulence i.e. mortality and bursal lesion. Serotype I strains can be divided into four groups: classical virulent strains, attenuated strains, antigenic variant strains and very virulent strains. Classical virulent strains can cause bursal inflammation and severe lymphoid necrosis, which results in immunodeficiency and moderate mortality rates (20 to 30% in SPF chickens). Antigenic variant strains have the ability to escape cross neutralisation by antisera against classical strains. Unlike classical strains, variant strains are characterised by severe atrophy of the bursa without showing inflammation. Attenuated strains, which are made by adapting the classical and variant strains to chicken embryo fibroblast (CEF) cells or other cell lines through serial passage, do not cause disease in chickens and some of them are therefore being used as live vaccines (Lim *et al.*, 1999).

1.4 Stability of the virus

Infectious bursal disease virus is reasonably resistant to environmental factors such as extreme pH, temperature, a wide range of chemical treatments, photodynamic and UV radiation (Lasher and Davis, 1997). The study by Alexander and Chettle (1998) confirmed that IBDV is extremely resistant to temperatures up to 80°C for 1 min. In an early study Cho and Edgar (1969) showed that after the virus was exposed to 60°C for 90 min, it retained nearly 100% of its infectivity in live birds. In a prior study infectivity of the virus was analysed following exposure of the virus at 56°C for 5 h and no difference in infectivity was found in comparison to the control (Benton *et al.*, 1967). It is difficult to achieve consistent denaturation of IBDV either by heat denaturation or heat in the presence of dimethylsulfoxide. Consequently for the purposes of translation and reverse transcription, the genetic material has to be heated in the presence of methyl mercuric hydroxide. It was found that the melting temperature of the dsRNA in the presence of 150 mM NaCl is 95.5°C (Davis and Boyle, 1990).

Despite its resistance to temperature, infectivity can be reduced by treatment of the virus with 1% formalin and 1% phenol or cresol for 1 h (Becht, 1994). Although infectivity is not affected at pH 2, the virus is totally inactivated in sodium hydroxide at pH > 12. It is also sensitive to iodinated and chlorinated derivatives, to formaldehyde and glutaraldehyde (van den Berg *et al.*, 2000). However, it is resistant to organic solvents and can remain active in the environment for at least 4 months (Jordan, 1990).

1.5 Modes of transmission and progression of the disease

Mode of transmission is horizontal via the faecal-oral route (Sharma *et al.*, 2000). The virus is extremely resistant; it can continue to live in feed and in pens for over seven weeks and four months respectively. Since usual cleaning and sterilisation measures often do not lead to elimination of the virus from contaminated premises, indirect transmission through water, contaminated feed, dust, litter, clothing or mechanical spread via insects may reintroduce the virus. Vertical transmission probably occurs via the egg. However, there is no evidence that older birds act as carriers or that the virus is egg transmitted. Meal worms and litter mites have been found infected with the virus eight weeks after an outbreak of disease in chickens on the premises and could be important in the transmission of infection. Due to the stability of the virus outside the host, fomites are also of significance; wild birds and vermin perhaps act as mechanical carriers. Some reports also stated that the virus could directly spread through air or imported by migratory bird species (Jordan, 1990; Ogawa *et al.*, 1998a).

A kinetic study using immunofluorescence showed that four hours after oral inoculation the virus is found in the lymphoid tissues related with the digestive tract, where the first cycle of viral replication occurs. The virus then enters the circulatory system via the hepatic portal vein. A phase of primary viraema proceed, during which the virus reaches the bursa, 11 hours after infection, and major secondary replication cycle occurs (van den Berg *et al.*, 2000).

1.6 Host range

Infectious bursal disease virus affects chickens from different avian species (Sanchez and Rodriguez, 1999). The virus has been shown to occur wherever poultry are kept intensively. Although there was no strong evidence that the virus affected birds other than fowl, turkeys have been found to be susceptible even if ten years earlier they were not (Baxendale, 1981). The serotypes determine the host range of the virus. Only chickens (*Gallus gallus*) develop IBD following infection by serotype I viruses. The pekin duck (*Cairina moschata*) and the turkey (*Meleagris gallopavo*) may be asymptomatic carriers of serotype I and II respectively. Anti-IBDV antibodies have been identified in common pheasants (*Phastanus colchicus*), guinea fowl (*Numida meleogris*), and ostriches (*Struthio camelus*), which also appear to carry

serotype II viruses. Neutralisation and precipitating antibodies have also confirmed the presence of the virus in various species of wild duck, goose, tern, puffin, crow and penguin (van den Berg *et al.*, 2000). The virus has also been isolated from mealworms (Andrewes *et al.* 1978), mites, mosquitoes (Porterfield, 1989), and ducks (Murphy *et al.*, 1995).

1.7 Economic impact of IBDV

Since the emergence of the VV strains in the late 1980's, IBDV has become a pathogen of considerable economic interest to the poultry industry worldwide (Yehuda *et al.*, 1999). Due to its severe immunosuppressive effect in young chickens, IBDV causes mortality and heavy financial losses to the poultry industry. Until 1987 most of the IBDV infections were sub-clinical, with insignificant financial losses due to impaired growth and acquired immunodeficiency. However, the emergence of a highly virulent strain of serotype I IBDV (HV-IBDV) in 1987 in the Netherlands and Belgium caused high mortality not only in young chickens but also in older birds (Yamaguchi *et al.*, 1997). The best way to control the virus is by vaccination using live attenuated or inactivated vaccines. However, despite the implementation of bio-security measures and the intensive use of vaccines, the virus still causes substantial economic losses (Hassan and Saif, 1996).

1.8 Bursa of Fabricius

The bursa was discovered by Hieronymus Fabricius in 1621. Its function was not clear until Bruce Glick (1956) revealed that it plays an important role in the production of antibody (Pllard, 1967). This was confirmed using chickens with surgically removed bursa, which failed to produce antibodies following immunisation with the *Salmonella typhimurium* O antigen. The bursa is a sac-like lymphatic organ situated dorsal to the cloaca in the caudal body cavity (Fig. 1.1) (Nieminen *et al.*, 2001).

The small subset of B-cell which survive the selection during apoptosis and emigrating to the peripheral lymphoid organ has been shown to express high levels of B-L (a chicken major histocompatibility complex (MHC) class II molecule) and Chl12, a 38-40 kDa alloantigen also

expressed by a range of thymocytes, peripheral T-cells and haematopoietic precursors (Lampisuo *et al.*, 1998).

1.9 Clinical signs of IBD

Newly introduced IBDV can increase morbidity to approximately 100% and mortality may be up to 90%. The disease is most severe in young chickens especially at age three to six weeks, when the bursa of Fabricius reaches its maximal stage of development. However, due to the presence of maternal antibodies chicks between the ages of 1 to 14 days are less sensitive. Although they produce antibodies to the virus, birds older than six weeks hardly show signs of disease (Murphy *et al.*, 1999). Two or three days after infection birds usually show clinical signs such as anorexia, huddling, lack of co-ordination, severe depression, prostration and white or watery diarrhea (Andrewes *et al.*, 1978; Sainsbury, 2000). Ruffling of feathers with a messy flock appearance are also clinical signs of IBDV infection. Normally infection is followed by a sharp increase in temperature within two days and a high death rate two or three days later followed by a rapid decline so that the course of the disease is about seven or eight days. In less acute forms no symptoms are observed. However, due to their severe immunosuppressive effect, these sub-clinical infections are economically important (Cummings *et al.*, 1986; Jordan, 1990).

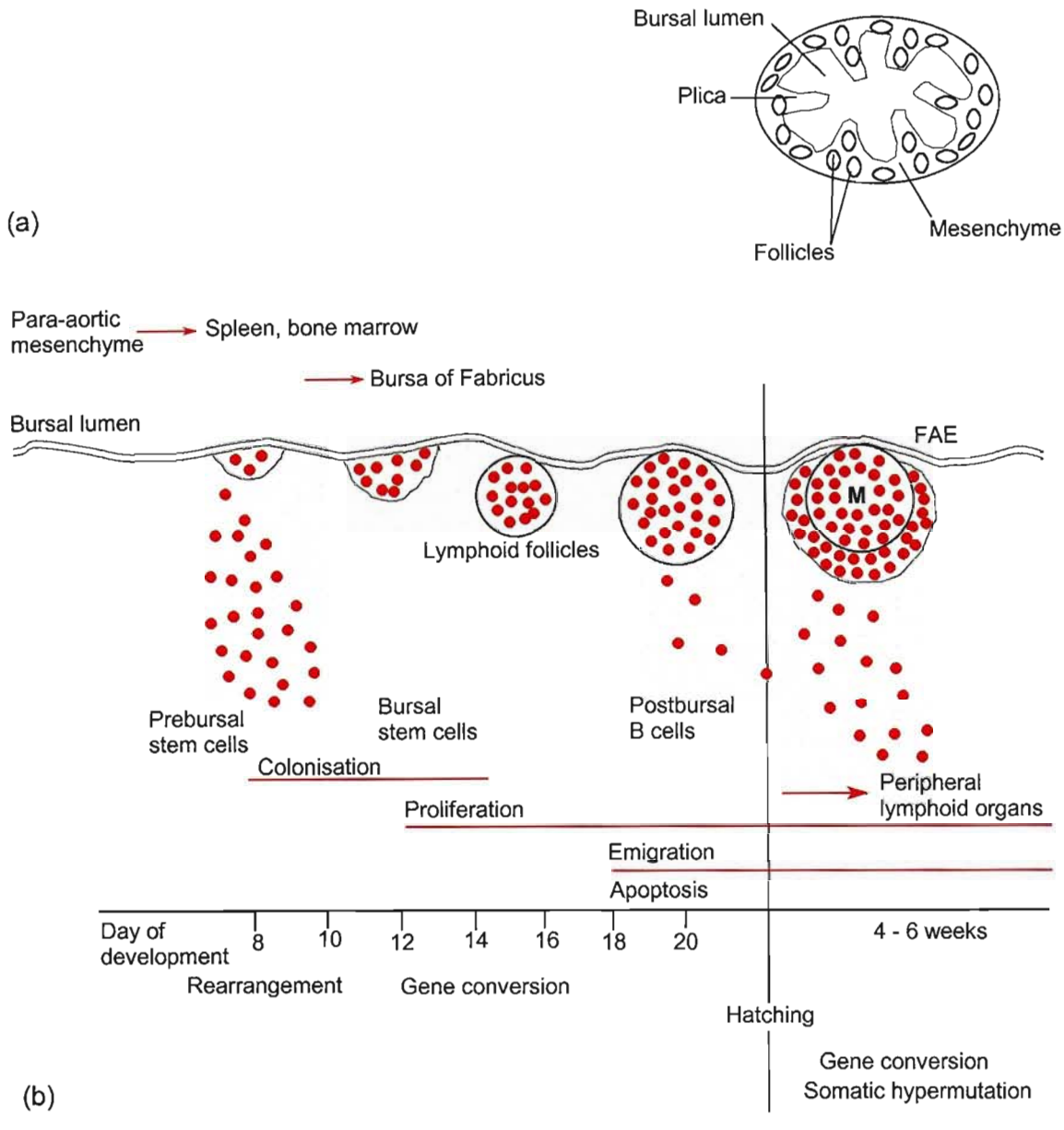


Figure 1.1 Anatomy and structure of the avian bursa of Fabricius. (a) Cross-section of the bursa of Fabricius. The bursa with a highly folded surface surrounding the central lumen is connected to the intestine by the bursal duct. The lymphoid follicles are positioned within the longitudinal plicae. In total, the bursa contains around 10 000 follicles. The follicular-associated epithelium (FAE), is situated inbetween the bursal medulla and lumen. (b) Avian B-cell development. The para-aortic mesenchyme is the origin for the progenitor cells seeding the bursal primordium. Before bursal colonisation, the perbursal 'stem cells' are committed to the B-cell linage. Inside the bursal follicles the surface immunoglobulin-expressing bursal stem cells start to divide and diversify their immunoglobulin V genes. The proliferating bursal B cells are densely packed in the cortical (C) part of the bursal follicles and the loosely packed lymphocytes and stromal cells are mainly contained in the medulla (M). Although following hatching, the diversified B-cells start to emigrate from the bursa, more than 95% of bursal cells die by apoptosis. By gene conversion and somatic hypermutation, postbursal B cells can further modify their immunoglobulin gene specificity within the peripheral lymphoid organs. However, the peripheral lymphoid organs cannot replace the important function of the bursa as a mutant-generating organ (after Nieminen *et al.*, 2001).

1.10 Pathogenesis of IBDV

Infectious bursal disease virus causes more extensive lymphoid cell necrosis in the bursa although lymphoid organs such as thymus, spleen, coecal tonsil and Harderian glands may show various degrees of lymphoid cell necrosis (Alamsyan *et al.*, 1993). Depending on their degree of pathogenicity IBDV strains induce lesions in the coecal tonsils, thymus, spleen and bone marrow. For that reason VV IBDV strains cause more severe thymic atrophy, greater lymphoid cell depletion in the coecal tonsil and spleen, and greater hematopoietic cell depletion in bone marrow than attenuated strains (Tanimura *et al.*, 1995).

After two days of infection the bursa becomes congested and oedematous. This becomes noticeable four days later. The acute form is characterised by severe inflammation of the mucosa and yellow transudate on the serosal surface. This is followed by an increase in size of the bursa (it becomes twice the normal size), and gradual fading of lymphocytes, proliferating reticulendothelia cells and intrafollicular oedema with central necrosis (Porterfield, 1989). Pathological examinations revealed bursal and thymus necrosis, pancytopenic bone marrow, followed by acute hepatitis, aplastic anaemia and functional abnormality of the liver (Nakamura *et al.*, 1992). Death is characterised by bursal atrophy and gray, enlarged kidneys, with accumulation of urates due to dehydration and immune complexes in the glomeruli (Murphy *et al.*, 1999).

Infectious bursal disease virus infection is characterised by morphological and biochemical features of apoptosis in chicken embryo fibroblasts and chicken peripheral blood lymphocytes (Tanimura and Sharma, 1998). Apoptosis is an individual and active type of cell death that is accompanied by nuclear fragmentation and cellular breakdown into apoptotic vesicles. There is no release of cellular components into the interstices and no inflammation surrounding the dead cells, which are characteristics of necrosis. Although pathological stimuli, such as viral infections, can also be the triggering factors, this type of cellular self-destruction is usually initiated by physiological stimuli such as maturation of lymphoid cells, metamorphosis, organogenesis, and during hormone or growth factor removal-induced involution (Vasconcelos and Lam, 1994; Vasconcelos and Lam, 1995; Roulston *et al.*, 1999). Despite the

above understanding the mechanism by which these events are triggered, regulated and coordinated is poorly understood (Hara *et al.*, 1996).

The initial stage of apoptosis is characterised by cell shrinkage and disconnection from the adjacent cells. The reduction of the mitochondrial transmembrane potential, and activation of the caspase proteolytic signalling cascades are also indications of an early event in the apoptotic pathway. Subsequently the apoptotic cells undergo extensive membrane blebbing, and chromatin condensing (Tham and Moon, 1996). Membrane blebbing is associated with an unfolding of the lipid bilayer leaflets and exposure of phosphatidylserine residues. The chromatin condensation induces nuclear fragmentation by activating an endogenous endonuclease that cleaves genomic DNA at internucleosomal sites. Such DNA degradation has become a unique characteristic of apoptosis. In the final stages of apoptosis, the cell fragments, which form apoptotic bodies, are eliminated by macrophages to prevent the extra-cellular leakage of cellular constituents and the development of an inflammatory response (Tham and Moon, 1996; Compton and Wickliffe, 1999).

Developing B-cell populations in the bursa of Fabricius have been reported to be highly susceptible to programmed cell death (Neiman *et al.*, 1991). Since the replication of IBDV specifically takes place in developing B-lymphoid cells in the bursa of Fabricius, the replicating IBDV induces apoptosis, which leads to a rapid reduction in the number of B- cells (Boot *et al.*, 2000b). Although the mechanism of IBDV-induced apoptosis remains unknown, two viral proteins (VP2 and VP5) have been suspected to play a major role in the induction of apoptosis (Jungmann *et al.*, 2001).

1.11 Virus receptor site

The viral receptor is the structure on the surface of a cell membrane to which a virus binds prior to crossing the membrane barrier and enter the cell. The presence or absence of a viral receptor determines whether a specific cell can be infected by a specific virus (Lentz, 1990). There are also membrane structures on cell surfaces to which a virus binds, but they do not serve as receptors for viral entry into the cell. However, viral binding to such sites could have

other biologically important consequences, such as cellular metabolism induced by surface structures or the generation of an immune response against the virus. The identity of a receptor is usually determined by measuring the biological effect of receptor-virus binding in a biologically relevant manner (Tradieu *et al.*, 1982). Three major criteria, derived from models of ligand-receptor interactions, need to be satisfied by a potential receptor protein, i.e. saturability, specificity, and competition as elaborated on below.

Saturability. During interaction of viral particles with receptor sites only a limited number of sites will be available for viral binding. Therefore viral binding can continue until no further binding sites are available (saturation point). This can be confirmed experimentally by determining viral binding as a function of increasing viral concentration (Tradieu *et al.*, 1982).

Specificity. Specificity can only be confirmed according to "biologic parameters". That is viruses can only bind to host cells with specific receptors for the virus or to cells where viral binding induces some other biologically measurable response. Although most viruses bind to cells in a biologically relevant manner or with specificity, a certain proportion of viral binding is non-specific and unrelated to specific viral receptors. The nonspecific component contained in any binding curve is usually not saturable and therefore it is a linear function, which can be measured, and subsequently subtracted from total binding to reveal "specific" binding (Tradieu *et al.*, 1982).

Competition. It should be possible to inhibit specific viral binding competitively using a second ligand, which has the affinity to bind to the same receptor. In neuropharmacology, for example, specific binding of acetylcholine to muscarinicacetylcholine receptor can be blocked by atropine. Such types of specific blockers are usually unavailable in viral systems and blocking can only be accomplished by the binding of radio-labeled virus with unlabeled virus or if possible, the viral attachment protein (Tradieu *et al.*, 1982; Lentz, 1990).

Due to the fact that virus particles can adhere to many substances, including inert materials, identification of virus receptors has proved to be very difficult, especially to differentiate

between non-specific and biologically relevant binding. Non-specific binding of viruses to cell surfaces can be mediated through electrostatic interactions. During internalisation of solutes and small particles via fluid-phase or constitutive endocytosis, there is a possibility that some non-specific viruses might be internalised through this non-specific mechanism. Another major problem, which makes receptor identification very difficult, is availability of specific virus receptors in low numbers on the cell surface. There is also a possibility that some viruses can use more than one type of receptor (Lentz, 1990).

Until the mid 1980s the only virus receptor that was clearly identified was sialic acid for the myxoviruses and paramyxoviruses. However, many more virus receptors have since been identified due to advances in technology, and the list is rapidly growing (Haywood, 1994). For example, sialyloligosaccharides are receptors for influenza virus and phosphatidylserine and phosphatidylinositol are receptors for vesicular stomatitis virus (VSV). The CD4 molecule is a receptor for human immunodeficiency virus type one (HIV- 1) (Borrow and Oldstone, 1992), the C3d receptor, CR2, for Epstein-Barr virus (Roberts *et al.*, 1996), the acetylcholine receptor for rabies virus (Gastka *et al.*, 1996) and foot and mouth disease virus (FMDV) uses RGD-dependent integrins as receptors (Jackson *et al.*, 2000).

A flow cytometric virus-binding assay showed that most of the cells, which bound IBDV, were surface immunoglobulin M (SIgM)-positive (Ogawa *et al.*, 1998b). Treatment of the cells with proteases and N-glycosylation inhibitors appeared to affect the binding of IBDV to the LSCC-BK3 cells. This finding revealed that the IBDV host range is mainly controlled by the presence of a virus receptor composed of N-glycosylated proteins (Ogawa *et al.*, 1998b). Results of Nieper and Muller (1996) showed that IBDV serotypes I and II specifically bound to proteins of 40 kDa and 46 kDa exposed on the surface of lymphoid cells. This result was proved by competition experiments, which indicated that these proteins might represent the common receptor sites of IBDV.

1.11.1 Identification of receptor

A useful screening procedure is the virus overlay protein blot assay (VOPBA) (Haywood *et al.*, 1994). In this technique membrane proteins are separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Membrane strips are incubated with the virus and viruses bound to the receptor are detected with anti-virus antibody. Visualisation of bound antibodies is done with radio or enzyme labelled secondary antibodies (Dalziel *et al.*, 1991). Prior to SDS-PAGE analysis, membrane proteins should be carefully purified to remove associated components such as lipids by using detergent. For carbohydrate receptor identification, glycolipids can be separated by thin-layer chromatography, followed by the virus overlay test for binding (Haywood *et al.*, 1994).

1.11.2 Biochemical and immunological approches

Direct purification and biochemical characterisation has been the most frequently used techniques for identification of various icosahedral viral receptors on host cell surfaces. Most of the purification methods couple viral attachment proteins to an affinity matrix to isolate specific cell surface receptors. This purification strategy is a powerful technique for the isolation and purification of specific viral receptors. However, poor affinity for monomeric virus-receptor complexes in comparison to the multivalent binding which functions *in vivo* is the major limitation of this approach (Bass and Greenberg, 1991).

Two immunological approaches, i.e. anti-cell surface monoclonal antibodies and antiidiotype antibodies have been used in the study of viral receptors. In both approaches, antibodies are raised against cell surface epitopes, which are essential for viral binding. Such reagents are generally selected based on their ability to block viral attachment and infection. However, the use of these reagents to identify biologically essential receptors can cause production of antibodies directed against the actual receptor. Antiidiotype antibodies (anti-Id), are based on the concept that the hypervariable antigen binding sites of antibodies may function as antigens. Hence an antibody (an anti-Id) against the binding site of an antibody against an antibody,

against an antigen may be structurally the same as the original antigen (Bass and Greenberg, 1991). If the original antigen is the viral receptor protein, the anti-Id may attach to the anti-receptor antibody (Fig. 1.2).

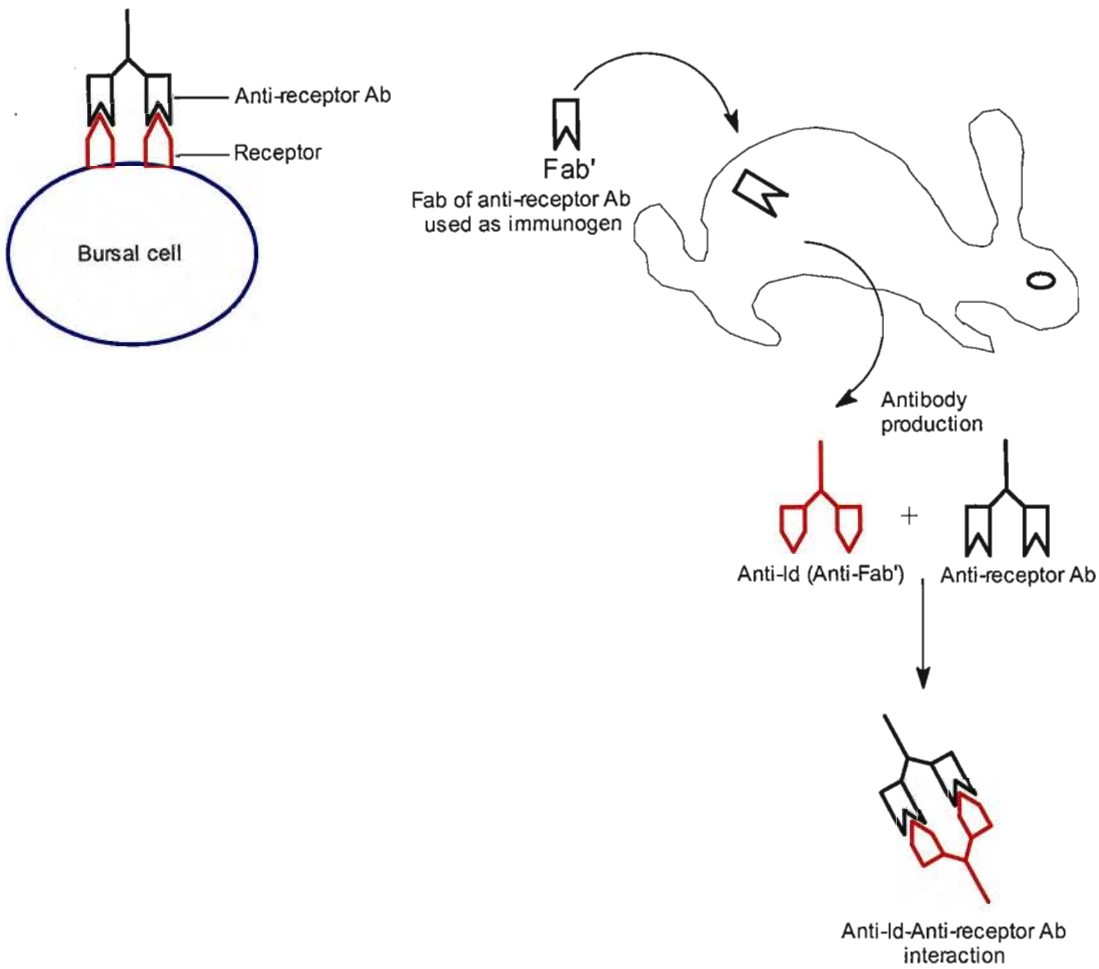


Figure 1.2 Schematic representations of anti-Id production in rabbits. Production of antibody against the Fab (hypervariable antigen binding) region of the anti-receptor antibody resulted in anti-Id. The Fab portion of the anti-Id is structurally equivalent to the original antigen, i.e. the receptor protein.

1.12 Viral attachment and penetration

The attachment of the virus particle to the host cell membrane is the initial stage in the viral infectious life cycle. Attachment is mediated by the interaction of a viral surface protein (VAP) with a cell surface molecule; such molecules could be proteins, carbohydrates or lipids,

which serve as a virus receptor (Lentz, 1990; Freed and Martin, 1995). Attachment is followed by a series of events including internalisation, uncoating, replication, and assembly that finally results in viral replication (Tradieu *et al.*, 1982).

Binding of the virus to the receptor on a host cell triggers the stage of crossing the host membrane barrier, allowing viruses to enter the host cell by one of two pathways. These are either direct fusion with the plasma membrane or by adsorptive or receptor mediated endocytosis (Schneider-Schaulies, 2000). In the adsorptive endocytosis pathway the receptor-virus complex is internalised in clathrin-coated pits and transported to endosomes. Following triggering of the acidic interior of the endosomes, a conformational change occurs in the viral fusion protein. Hydrophobic sequences thus exposed induce fusion of the viral membrane or virus particles with the endosome membrane, allowing the viral genome to enter the host cytoplasm. Before fusion can occur, some viruses require proteolytic cleavage of a fusion protein or haemagglutination protein by a host protease to expose a new hydrophobic N-terminal region. Similarly, direct fusion of virions with the plasma membrane releases the genome into the cytoplasm (Fig. 1.3). Many viruses appeared to enter by both mechanisms, while some viruses may preferentially utilise one of the two pathways (Lentz, 1990).

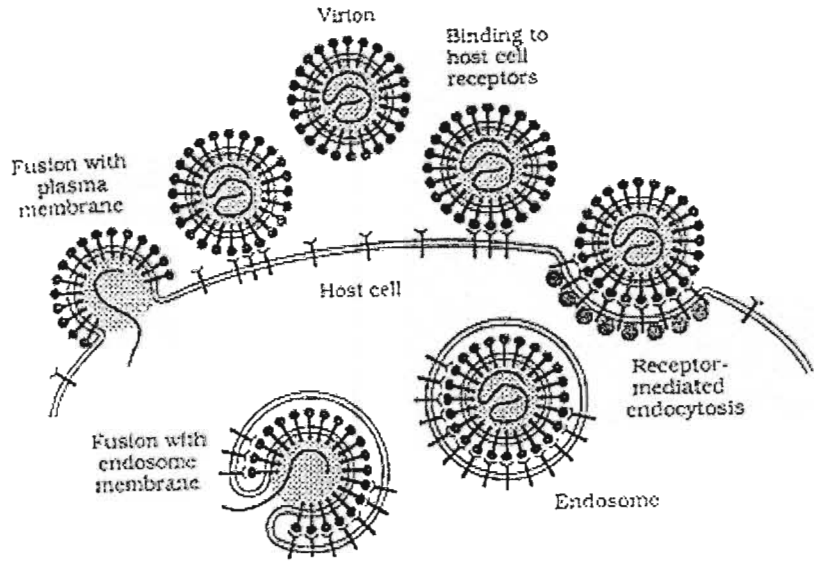


Figure 1.3 Entry of enveloped viruses into cells. The virus particle bears VAPs embedded in its plasma membrane, which interact with the virus receptor, hence attaching the virion to the cell surface. The virus can then cross the cell membrane barrier through one of two pathways. The membrane of the virion may fuse directly with the plasma membrane releasing the genome into the cytoplasm. Alternatively, the virus particle is internalised by adsorptive or receptor mediated endocytosis and transported to an endosome. The acidic pH triggers fusion of the viral membrane with the endosome membrane, thereby releasing the genome (Lentz, 1990).

Surface glycoproteins (G and F) of the human respiratory syncytial virus (HRSV), as with other members of the family, play an important role in the initial stages of the infectious cycle. The G and F glycoproteins mediate binding of the virus to an as yet unidentified cell surface receptor and triggers fusion of the viral and cellular membranes respectively, to allow transportation of the virus ribonucleoprotein into the host cell cytoplasm. In addition, the F protein promotes fusion of infected cell membranes with those of neighboring cells resulting in formation of characteristic syncytia (Garcia-Beato and Melero, 2000). Similar F protein structures were found in the influenza virus HA, simian parainfluenza virus 5 F, human immunodeficiency virus (HIV) gp41, mouse moloney leukemia virus envelope transmembrane protein, and Ebola virus gp2 protein. While structurally different, the tick-borne encephalitis virus E protein is also functionally analogous (Schneider-Schaulies, 2000). Measles virus appears to use CD 46 in the process of cell recognition and attachment. Although it lacks haemagglutinin and neuraminidase activities, respiratory syncytial virus (RSV) has a type two integral membrane glycoprotein that is believed to mediate attachment. In consequence of

their high carbohydrate content, G proteins are significantly important in pneumovirus attachment (Gorman *et al.*, 2001).

VP2 and VP3, the major viral proteins of IBDV, are responsible for viral attachment and penetration into the bursal cells. This was shown by the inhibition of virus attachment to the susceptible cells using anti-VP3 virus neutralising monoclonal antibody. The amino acid residue at position 805 was believed to play an important role in receptor-mediated virus cell attachment, although the recognition site for the monoclonal antibody was not clearly defined. Two specific amino acid changes in the VP2 region of VV IBDV at positions 279 (Asp to Asn) and 284 (Ala to Thr) resulted in tissue culture adaptation and attenuation of the virus. It was supposed that the two amino acid residues were important for virulence of IBDV. Since these amino acid substitutions were responsible for attenuation of the virus it was speculated that the amino acid residues might play a significant role in virulence of IBDV as viral attachment protein (Yamaguchi *et al.*, 1996b). Subsequent work by van Loon *et al.* (2002) showed that amino acid residues at positions 279 and 284, in the variable region of VP2 were responsible for infection of tissue culture. IBDV binding or membrane penetration activates intracellular signals in chicken embryo fibroblasts. Report by Repp *et al.* (1998) showed that the K^+ current changes induced by IBDV were not due to virus replication, but were as the result of IBDV attachment and penetration. This phenomenon may delay the apoptotic process in CEFs after IBDV infection

1.13 IBDV genome and protein composition

Infectious bursal disease virus has a dsRNA genome that is divided over two segments, the A- and B-segment. These segments encode five viral proteins namely VP1, VP2, VP3, VP4 and VP5 (Tacken *et al.*, 2000). The VP2 is the most abundant, making up 51% followed by VP3 (40%), VP4 (6%), and VP1 (3%) (Muller and Becht, 1982; Kibenge *et al.*, 1988). Segment A (3.2 kb) is made up of two open reading frames (ORFs), namely a long ORF of 3039 bp and a short ORF of 438 bp, which overlaps with the 5' end of the long ORF (Fernandez-Arias *et al.*, 1997). The smaller ORF, designated ORFA-2, encodes the non-structural viral protein, VP5 consisting of *ca* 145 amino acid residues (17 kDa) (Tacken *et al.*, 2000). This protein is only

detected in cytoplasms of IBDV infected cells and not in the virion. It is highly basic, cysteine rich and conserved among all serotype I IBDV strains (Mundt *et al.*, 1997; Yao *et al.*, 1998). Although it is important in virus release and dissemination (Lombardo, *et al.*, 2000), its function is not clearly understood (Barlic-Maganja *et al.*, 2002).

The 110 kDa precursor polyprotein (NH_2-VP2-VP4-VP3-COOH), encoded by the larger ORF of segment A is autoproteolytically cleaved into three proteins: VPX (*ca* 48 kDa), VP3 (32 kDa) and VP4 (28 kDa) (Dybing and Jackwood, 1998; Schröder *et al.*, 2001). The VPX (also known as PVP2) is further proteolytically processed to VP2 (41 kDa) by cleavage near its C-terminus (Lejal, *et al.*, 2000). VP4, the viral protease, is responsible for the inter-domain proteolytic autoprocessing of the VP2-VP4-VP3 polyprotein (Birghan *et al.*, 2000). The dibasic residues Arg 452 - Arg 543 and Lys 722 - Arg 723 are suspected VP2-VP4 and VP4-VP3 cleavage sites respectively (Yamaguchi *et al.*, 1997).

The proteinacious capsid of the virus is made up of the two major structural proteins VP2 and VP3 (Da Costa *et al.*, 2002). VP2 possesses the conformational dependent epitopes in its hypervariable region, which are capable of inducing virus-neutralising antibodies, suggesting that it is at least partly exposed on the outer surface of the capsid. The second major structural protein, VP3, contains a very basic carboxy terminus that may interact with the packaged RNA and is therefore expected to be on the inner surface of the capsid (Tacken *et al.*, 2000). VP3 forms a complex with VP1; this complex may play an important role in the morphogenesis of IBDV (Brandt *et al.*, 2001).

The B-segment (2.9 kb) contains one large ORF and encodes the RNA dependent RNA polymerase, the 91 kDa VP1. This protein is believed to be linked to the 5'-ends of the genomic RNA segments. Although the exact mechanism of replication and transcription of IBDV is not clearly understood, VP1 is reported to be involved in IBDV replication and transcription (Boot *et al.*, 2000b).

1.14 Immunology of IBDV

The 32 kDa structural protein (VP3) has been revealed as the major immunogen of IBDV (Azad *et al.*, 1986). This was confirmed after VP3 electro-eluted from SDS-PAGE gels and injected into chickens, produced antibodies that neutralised the virus and provided passive protection (Azad *et al.*, 1987). This was further confirmed after chickens, immunised with recombinant VP3, expressed as a β-galactosidase fusion protein in *Escherichia coli,* produced low levels of neutralising antibodies, which gave passive protection. However, similar preparations of VP2 did not induce neutralising antibodies (Bayliss *et al.*, 1991).

Western blotting analysis using IBDV antibodies also showed that VP3 was recognised as a major immunogen (Fahey *et al.*, 1989). However, these experiments were undertaken in the presence of SDS, which is a powerful denaturating agent (Fahey *et al.*, 1991). This detergent may destroy important immunogenic sites on the other structural polypeptides of IBDV (Fahey *et al.*, 1985a; 1985b). This was proved by the finding that neutralising monoclonal antibodies raised against virus particles recognized VP2 under non-denaturing conditions (Jagadish *et al.*, 1988; Bayliss *et al.*, 1991).

VP2, the major host-protective protein of IBDV, is highly conformationally dependent with at least two to three closely linked antigenic positions responsible for inducing the production of virus neutralising antibodies and serotype specificity (Schnitzler *et al.*, 1993; Liu *et al.*, 1994). Sequencing studies showed that changes in any region of VP2 result in antigenic or pathogenic variants of the virus. This was proved by a change from Pro to Thr at position 22 in variants that caused the virus to lose an epitope and escape neutralisation by maternal antibody (Dormitorio *et al.*, 1997). VP2 is made up of 454 amino acid residues that form the external surface of the virion. Expression studies have shown that amino acid positions between 206 and 305 of VP2 represents a region called "VP2 variable domain" which is a major conformational and neutralising antigenic domain. Most of the amino acid residue changes between IBDV strains take place in this region (Eterradossi *et al.*, 1998). The region consists of hydrophobic residues, flanked by two hydrophilic peaks A and B, which include residues 210 to 225 and residues 312 to 324, respectively (Crisman *et al.*, 1993). In conclusion, a small number of changes in the amino acid sequence within the viral proteins, particularly in the

variable domain of VP2 can cause extreme changes in the ability of the host immune system to give protection against virulent viruses (Lana *et al.*, 1992).

1.15 Diagnosis of IBDV

In the acute form of the disease diagnosis can be made based on the clinical symptoms of the disease and post-mortem examination of the pathogenic lesions (van den Berg *et al.*, 2000). However, in the less severe form of the disease, which does not show any clinical symptoms, further diagnostic tests are required. This includes haemorrhagic syndrome tests for muscular and other types of haemorrages; avitaminosis A for caseous plugs in the bursa; and visceral gout, fatty liver and kidney syndrome and water deprivation for swollen kidneys and excess renal urates is required (Jordan, 1990).

The best technique to diagnose IBDV is direct visualisation using electron microscopy. However, sensitivity, cost and time are among the limitations of electron microscopy as a diagnostic tool. It requires skilled manpower using very expensive equipment (Gupta and Yamatoto, 2000). A relatively cheap diagnostic method, i.e. agar gel precipitation, can be used to demonstrate IBDV-specific antigens in bursal samples (Becht, 1994). Antibody against the virus and a sample suspected to have viral antigen are placed in opposing wells cut in an agarose gel. Following diffusion a visible precipitation line will be formed if antigen reactive with the antibody is present in the sample (Murphy *et al.*, 1999). However, it is not a very sensitive technique and time consuming, taking 48 h for results to be obtained (van den Berg *et al.*, 2000).

An agglutination test, using latex beads coated with antiviral antibody is the simplest immunoassay and results can be visualised within one min. However, lack of sensitivity and specificity as well false positive results are the main limitations of this method (Murphy *et al.*, 1999). Qualitative detection and quantitative estimation of IBDV can also be made using rocket immunoelectrophoresis. This method can also be used to quantify IBDV specific antigen in commercial killed vaccines (Raj *et al.*, 2000).

Identification of IBDV can also be done using an immunofluorescent staining technique, which is a simple, highly specific and quick technique. This method requires a specific labeled antibody to locate and identify the antigen of interest inside the cell or tissue. The identification could be either by direct or indirect immunofluorescence. The direct method requires fluorescent isothiocyanate (FITC) labelled specific antibody against the virus to be identified, while the indirect method uses unlabelled primary antibody and labelled secondary antibody. The direct technique is more specific but less sensitive than the indirect method (Burleson *et al.*, 1992).

Identification of IBDV in the bursal follicles of infected chickens can also be carried out using horseradish peroxidase coupled antibody specific to the antigen (van den Berg *et al.*, 2000). Binding is visualised using a substrate forming a coloured insoluble precipitate and light microscopy. The superiority of this method over immunofluorescence is that preparations are permanent and requires relatively inexpensive equipment. Another powerful diagnostic method is radioimmunoassay, but although highly sensitive, the cost of the equipment and the health hazard of working with radioisotopes make the technique impractical in small laboratories (Murphy *et al.*, 1999).

Infectious bursal disease virus diagnosis by isolation of the virus from inoculated eggs and/or cell cultures and the demonstration of bursal lesions is a common practice in veterinary laboratories. The disadvantage of this technique is that not all pathogenic strains of IBDV grow readily in eggs or cell cultures on primary isolation. Although these stains can be isolated by experimental infection of SPF chickens, this method has evident drawbacks for routine diagnosis (Allan *et al.*, 1984). Although it is not convenient for routine diagnostic purposes, the virus neutralisation assay in cell culture and embryonated chicken eggs can be used as a diagnostic tool to identify antigenic subtypes of IBDV (Jackwood and Jackwood, 1997).

The antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) is very sensitive, specific, simple to perform, and a relatively cheap diagnostic method. The presence of IBDV in infected bursal tissue and from experimentally infected SPF chickens can be analysed using

monoclonal AC-ELISA. Despite its simplicity and low cost, it is slightly less sensitive than cell culture or embryonated egg titration (Hassan *et al.*, 1996b). Detection using serological diagnosis by specific immunoglobulin M (IgM) against the virus is also a commonly used method. However, a false positive result due to cross-reactivity is a major limitation of this method (Mosquera *et al.*, 2002).

Although all these diagnostic methods are very important in IBDV diagnosis, they all experience various limitations such as being time-consuming, expensive, labour-intensive, nonspecific or insensitive. Therefore to control the disease, a specific and sensitive diagnostic method is required (Wu *et al.*, 1992). Many of the above limitation can be resolved using polymerase chain reaction (PCR), which is an excellent tool for the direct detection of viruses in clinical samples (Mosquera, *et al.*, 2002). Reverse transcription polymerase chain reaction (RT-PCR), which is a very sensitive and specific diagnostic tool for viruses, has been used successfully to amplify the IBDV VP2 region with oligonucleotide primers selected from the highly conserved VP2 gene. The detection sensitivity of IBDV in bursal tissue is higher when nested primers are included in the PCR amplification (Barlic-Maganja *et al.*, 2002).

1.16 IBDV control

Although hygienic safety measures including cleaning, disinfection and depopulation of infected chickens are extensively used to eradicate infection on premises, the virus is extremely persistent and it is unlikely to be completely eliminated (Mandeville *et al.*, 2000; Sainsbury, 2000). However, these precautions are probably helpful in reducing the degree of infection and should be practiced wherever possible (Jordan, 1990). The principal means for IBDV control is vaccination (Snyder, 1990; Snyder *et al.*, 1992). This could be through a passive route where young birds are protected by the passive immunity transmitted from the hen to the egg or an active route through vaccination using live vaccines (van den Berg and Meulemans, 1991).

1.16.1 Passive protection

Passive protection can be achieved against IBDV for the first few weeks after hatching (the critical period when young chickens are most susceptible to the immunodepressive effects of the disease), via maternal antibodies (Macreadie *et al.*, 1990). Maternal antibody levels in the fertilised egg can be effectively increased by injecting breeder hens (previously sensitised to live IBDV) with inactivated vaccine to ensure high levels of maternal antibody in the fertilised eggs and hence the newly hatched chickens to reduce problems caused by immunosuppression (Azad *et al.*, 1985; Nakamura *et al.*, 1992; Darteil *et al.*, 1995). However, the level of passive immunity is inconsistent and unpredictable (Kibenge *et al.*, 1988).

1.16.2 Active protection

Vaccination with a live vaccine during the first three weeks of life is the best way to control IBDV (Kibenge *et al.*, 1988). The use of modified live or inactivated vaccines prepared from classical serotype I strains is very successful (Fahey *et al.*, 1991; Heine *et al.*, 1991; Hassan *et al.*, 1996a). However, the interference of maternally derived antibody is the main problem and only vaccines of intermediate virulence were shown to be efficient in vaccination of the offspring (van den Berg *et al.*, 1996). Secondly, due to its cost and the difficulty to propagate the virus in the bursa of Fabricius of SPF chickens, the currently used inactivated vaccine is not convenient for practical purpose (Azad *et al.*, 1986). Live IBDV vaccines can be applied either *in ovo* or at hatching and in the field via booster vaccinations (Fussell, 1998).

The best way to control IBDV effectively is through extensive vaccination; hence there is a continuous need to develop efficacious vaccines for commercial poultry production. The currently available vaccines are tissue culture, egg embryo or bursa of Fabricius propagated (Hassan and Saif, 1996). Adaptation of wild-type IBDV correlates with attenuation of the virus. Adapted IBDV can infect non-B-lymphoid chicken cells, probably resulting in a reduced viral load in the B-lymphoid cells in the bursa of infected chickens (Boot *et al.*, 2000a)

Commercially used IBDV vaccines are selected based on their ability to induce strong, long lasting antibody responses (Kim *et al.*, 2000; Juul-Madsen *et al.*, 2002). An immune complex vaccine has been also used. This vaccine is made by mixing a certain amount of specific antibodies obtained from the serum of hyperimmunised chickens with live IBDV (Jeurissen *et al.*, 1998). Additionally it was found that infectivity of IBDV could be reduced under higher-pressure conditions. This pressure inactivated IBDV with its original immunogenic properties can induce long lasting high titre of virus neutralising antibody (Tian *et al.*, 2000).

Generally, vaccination managed to limit the occurrence and diversity of IBD in chickens (Lana *et al.*, 1992). However, the effectiveness of conventional vaccines used in the field, including attenuated or inactivated vaccines, is gradually reducing due to the emergence of new virulent or variant strains of IBDV (Ho *et al.*, 1999). Improper administration of the vaccine, antigenic differences among viruses and insufficient potency of live-attenuated vaccine virus may also affect the ability to control IBDV infections through vaccination (Jackwood and Sommer, 1998). Interference from maternal antibodies, the date of vaccination of the offspring, improper storage, non-observance of recommended doses, non-observance of the expiry date, deficiency in vaccination techniques, may also contribute to the failure of live-virus vaccinations (van den Berg *et al.*, 2000).

1.16.3 Subunit vaccine

Subunit vaccines with the advantage of using only part of the infectious microorganisms to induce a protective immune response are vaccines of choice. Since subunit vaccines cannot replicate inside the host, there is no risk of pathogenicity (Roitt, 1997). The composition of a subunit vaccine can be clearly defined, which is a major advantage in terms of safety and minimisation of side effects. Production of subunit vaccines is based on peptides, proteins or polysaccharides that appeared to contain protective epitopes (Hansson *et al.*, 2000). Many of the cell surface carbohydrates of pathogenic microorganisms are important antigenic determinants for vaccine development (Mundt and Muller, 1995). In prior studies subunit vaccines for IBDV were produced using VP2, the surface protein of IBDV. Reports by

Macreadie *et al.* (1990) and Fahey *et al.* (1991) showed that neutralising and protective antibodies were produced in chickens using VP2 isolated from the virus.

The advantage of using a subunit vaccine is no risk of pathogenicity, large-scale production, various delivery systems, and a possibility of further modification (Table 1.1). However, subunit vaccines require multiple doses and the use of adjuvants (Hansson *et al.*, 2000).

Table 1.1 A comparison of some properties of different vaccine types (Hansson *et al.*, 2000).

Vaccine type	Advantages	Disadvantages
Live vaccines (attenuated)	- One or few doses normally required - Long-lasting protection - Both humoral and cellular responses	- Controlled attenuation normally required - Risk of reversion to pathogenicity - Certain risk of transmission - Poorly defined composition
Killed vaccines	-No risk of reversion to pathogenicity - No risk of transmission	- Multiple doses typically required - Poorly defined composition - Antigen must be produced by cultivation of a pathogen - Mainly humoral responses - Adjuvants normally needed
Subunit vaccine (non-recombinant)	- Defined composition - Various delivery systems available	- Multiple doses typically required - Adjuvants needed
Subunit vaccine (recombinant)	-No risk of pathogenicity since the pathogen organism is not present - Defined composition - Various delivery system available - Simplified large-scale production - Further engineering possible	- Multiple doses typically required - Adjuvants needed

Advances in immunology and protein engineering have allowed the design and production of recombinant subunit vaccines. The epitopes recognised by neutralising antibodies are usually found on the surface of the pathogenic microorganisms. In the case of IBDV the major neutralising epitopes that determine the immunogenic properties of the virus are found in a specific region of VP2 on the surface of IBDV (Tian *et al.*, 2000). Isolation of the genes encoding such epitopes and their expression in heterologous hosts form the basis of recombinant-subunit-vaccine development (Hansson *et al.*, 2000). The method is in use in veterinary medicine and involves the use of virus as vector to carry the genes for the protective antigens of other viruses. Recently a complete, long-lasting recombinant vaccine against IBDV was developed by single vaccination with an avian herpesvirus vector expressing VP2 antigens. The herpesvirus of turkey expressing larger amounts of VP2 antigen conferred complete protection against a lethal IBDV challenge, which is expected to persist for the lifetime of the chicken (Tsukamoto *et al.*, 2002).

The concept of this vaccine involves insertion of the VP2 gene of IBDV into the genome of the vector. Following injection the virus can multiply and present the VP2 gene to the immune system of the chicken and the chicken will mount both a humoral and a cell-mediated immune response to it (Murphy *et al.*, 1999; Tsukamoto *et al.*, 2002). The advantage of using single proteins displaying immunodominant epitopes as vaccine is to induce protective immunity without having side effects and immune reactions caused by other parts of the pathogenic organism. It is a safe and cost effective production systems can be used. Recombinant strategies further offer the possibility of delivering protein subunits with the help of live delivery systems, bacterial or viral, or even as antigen-encoding genes, so-called nucleic acid vaccines (Table 2) (Hansson *et al.*, 2000).

However, variable immune response, inefficient delivery, risk of integration into host chromosomes, risk of reversion when using attenuated pathogenesis as a carrier and cumbersome large-scale production are the main disadvantages of recombinant subunit vaccines (Hansson *et al.*, 2000).

Table 1.2 Recombinant subunit vaccines and examples of their advantages and drawbacks (Hasson et al., 2000).

Recombinant vaccine	Advantages	Disadvantages
Protein immunogens	-No risk of pathogenicity since the pathogenic organism is not present -Efficient production system available	- Multiple doses required - Adjuvants needed
Live delivery system	-May induce both humoral and cellular response -Adjuvants normally not needed	- Risk of reversion when using attenuated pathogens as carriers
Bacterial	-Surface display of antigens possible -Mucosal administration possible	
Viral	- Efficient induction of cellular responses	
Nucleic acid vaccine	- No risk of pathogenicity - Simple production -May induce both humoral and cellular responses	-Variable immune responses -Inefficient transfection
DNA	- Dedicated delivery systems exist	- Inefficient delivery -Risk of integration into host chromosomes must be considered -Moderate and variable immune responses
RNA	- No risk of integration in to host chromosomes -No need to inter the nucleus for translation -In vivo amplification systems available	-Cumbersome large-scale production -Unstable

1.16.4 Control based on interfering with virus-receptor interaction

Unlike CD4, the receptor for HIV-1, which is a component of the molecular complex that facilitates the interaction of the T-cell receptor with MHC class II molecules (Levesque *et al.*, 2003; Staudinger *et al.*, 2003), the biological role of IBDV receptor protein on the surface of bursal cells is not yet known. A safe and effective vaccine against viruses should elicit humoral responses that are effective against entire strains of the virus (Fouts *et al.*, 2002). Some reports showed that generation of cross-reactive neutralising antibodies is possible using complexes of virus surface proteins and receptors. Fouts *et al.* (2002) and Moulard *et al.* (2002) showed that covalently crosslinked complexes of soluble human CD4 and HIV-1 envelope glycoproteins (gp 120 or gp 140) generated antibodies that neutralised a wide range of primary HIV-1 isolates. Their result suggests that these complexes induce long lasting neutralising humoral responses that, in part, may be directed against novel epitopes found on the HIV-1 envelope. Production of such antibodies could be possible using covalently crosslinked complexes of soluble IBDV receptor protein and IBDV attachment protein (VP2). This complex commences a series of conformational changes that are the foundation of the fusion machinery leading to viral penetration (Moulard *et al.*, 2002).

Reduction of Hantaan virus infection was obtained by blocking the binding of Hantaan virus to the 30 kDa receptor protein on the surface of Vero-E6 cells using the anti-30 kDa receptor antibody (Kim *et al.*, 2002). In previous studies the same method was used to block Hepatitis B virus binding to human hepatocytes (Petit *et al.*, 1992). Thus blocking of IBDV binding to the IBDV receptor protein using anti-IBDV receptor antibody could be applied to reduce IBDV infection.

In conclusion, these approaches are essential for the development of molecular models for virus-host interaction. Furthermore, such models can allow the rational design of agents, which might be effective in preventing virus interaction by blocking the attachment stage of the infections cycle (Lentz, 1990). Virus-receptor interactions could be blocked with either anti-receptor antibodies or with soluble receptors. Additionally, other chemicals or

pharmacologies could be developed that specifically block virus attachment either at the level of the cell receptor or the viral attachment protein (Fig. 1.4).

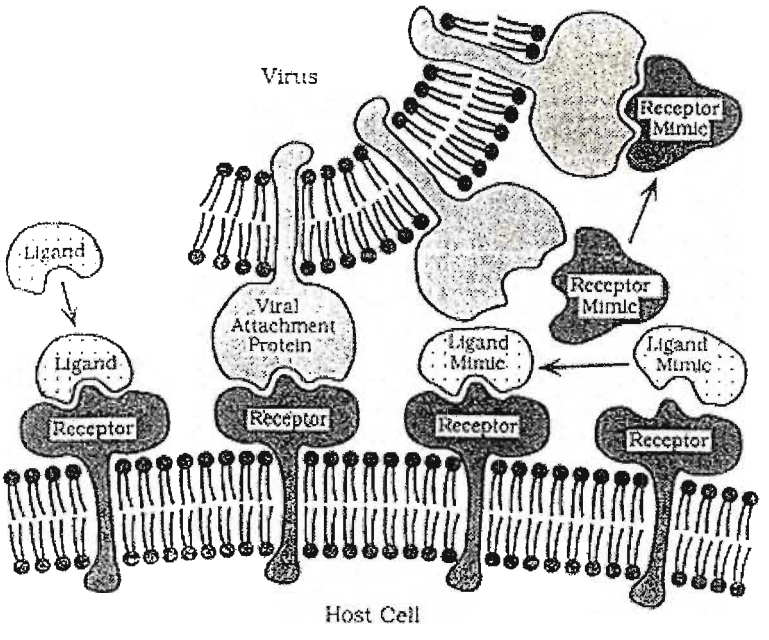


Figure 1.4 Interaction of virus with a host cell receptor and agents interfering with the attachment step. Virus binding to the receptor is mediated by the interaction of a VAP (forming the envelope or capsid of the virus) with receptors on the cell surface. The receptor is a structural molecule on the cell surface and may function as a receptor for a physiological ligand such as a hormone, neurotransmitter, growth factor or immunoglobulin. The ability of the virus to bind to the receptor is conferred by a portion of the VAP that mimics the normal ligand structurally or conformationally. Following binding of the virus to the receptor, internalisation of the virus may proceed either by receptor-mediated endocytosis or by direct fusion with the plasma membrane (see Fig. 1.3). Binding of virus to the receptor can be blocked using two groups of substance at the attachment step, i.e. ligand or receptor mimics. Ligand mimics resemble the ligand and will bind to the receptor. Such ligand mimics include antibodies against the binding site of the receptor, anti-idiotypic antibodies against anti-ligand antibodies, natural ligands of the receptor, and designed or engineered ligand mimics such as drugs or synthetic peptides. Receptor mimics resemble the binding domain of the receptor and will bind to the VAP. Receptor mimics include antibodies against the binding domain of the ligand, anti idiotypic antibodies against anti-receptor antibodies, soluble forms of the receptor itself, and designed receptor mimics such as synthetic peptides representing the binding domain of the receptor (Lentz, 1990).

1.17 Objectives of this study

The main objectives of this study were to evaluate a PCR-based diagnostic assay for IBDV based on sequences from VP2 and confirmation of the identity of an IBDV receptor on the

surface of bursal cells. To this aid anti-putative IBDV receptor peptide antibodies were raised for possible blocking of viral binding to the receptor. This would confirm that the putative receptor previously identified in this laboratory is a specific receptor for IBDV.

For the diagnostic purposes, IBDV was isolated from IBDV infected bursal tissue using cesium chloride (CsCl) density gradient centrifugation and the identity of the virus was confirmed by Tris-Tricine SDS-PAGE and transmission electron microscopy. Double stranded RNA was isolated from the virus for the PCR-based diagnostic studies. Primers were designed from the highly conserved VP2 genome and synthesised for the amplification of VP2 protein by RT-PCR. The RT-PCR assay was evaluated using commercially available live IBDV vaccines and the resulting PCR product were subjected to restriction digestion with a specific restriction endonuclease (*Bam*HI) to confirm the correctness of the amplified PCR product (Chapter 3).

For the identification of IBDV receptor protein, membrane proteins were isolated from uninfected bursal tissue. IBDV was attached to Sepharose 4B chromatography matrix for affinity purification of the viral receptor protein and purity was assessed on Tris-Tricine SDS-PAGE (Chapter 4). Anti-IBDV receptor peptide antibodies were raised against the 32 kDa IBDV receptor protein sequences obtained in this laboratory by Edwards (2000) in rabbits. Inhibition assays was performed in an ELISA format to see if the anti-IBDV receptor peptide antibodies could inhibit the binding of IBDV to the receptor, thus confirming the identity of the IBDV receptor (Chapter 5).

CHAPTER 2

MATERIALS AND METHODS

This chapter describes general methods used during the course of this research project. Methods pertaining only to particular sections of the study are described in the relevant chapter. For ease of reference all the materials used in this study are listed here with their suppliers.

2.1 Materials

General chemicals used in this study were purchased from, BDH, Fluka or Roche, and were of the highest purity available. Coomassie brilliant blue G-250, cesium chloride, lithium chloride, 5, 5'-dithio-bis (2-nitrobenzoic acid) (Ellman's reagent), pepstatin A, rabbit serum albumin, m-maleimidobenzoilacid-N-hydroxysuccinimide ester (MBS), agarase I, agarose, deoxycholic acid (sodium salt), Sepharose 4B, 4-chloro-1-naphthol, L-cysteine, Sephadex G-10 and Freund's adjuvants were from Sigma. DNA molecular weight marker II, 1,4-dithiothreitol (DTT), bovine serum albumin, ovalbumin, titan one tube RT-PCR kit, 2,2'-Azinobis[3-ethyl-2,3-dihydrobenzthiazole-6-sulphonate] (ATBS), proteinase K, 4-(2-hydroxyethyl)-1-piperazin-ethan-sulfonaure (Hepes), DNA molecular weight marker XIII and phenylmethylsulfonylfluoride (PMSF) were from Roche. Ethylenediaminetetra-acetic acid disodium salt (Na_2-EDTA), *N, N, N,' N*'-tetramethylethylenediamine (TEMED) and *N, N*'-methylenebisacrylamide were from BDH. Dialysis tubing was from PIERCE. *N, N*-dimethylformamide (DMF), 2-mercaptoethanol and dimethylsulfoxide (DMSO) were from Fluka. Low molecular weight marker, nitrocellulose (Hybond^{TM}- C extra), 0.45Micron and Sephadex G-25 were from Amersham Pharmacia Biotech. 8-hydroxy-chinolin was from Merck. Goat anti-rabbit antibody (HRPO linked) was from Jackson Immuno-Research laboratory. ELISA plates (Nunc-Immuno^{TM}, MaxiSorp^{TM}) were from Nunc^{TM}. Low melting point agarose was from Whitehead Scientific. Deoxy nucleotide tri-phosphates (dNTPs) stock solution was from Takapo. Thin walled PCR reaction tubes (0.2ml) were from QSP (Petaluma, USA). Restriction enzymes were from Promega (Madison, USA). Commercial vaccines nobils® gumboro D78 and nobils® gumboro 228E were from Intervet (South Africa). Tad

Gumboro vac forte 1000ds and tad Gumboro vac 1000ds (cloned) were from Lohmann Animal Health Germany.

2.2 Protein quantification

The Bradford dye-binding protein assay (Bradford, 1976) is a frequently used method to quantify proteins in biochemical and clinical laboratories. It is a sensitive assay with a capacity to detect less than 5 μg of protein and very easy to use requiring only one reagent. Furthermore it is rapid, requiring a short time for colour development. An additional advantage is its relative freedom from interference by common laboratory reagents with the exception of relatively high concentrations (1%) of detergents and basic buffers (Bradford, 1976).

The dye consists of Serva blue or Coomassie brilliant blue G-250 dissolved in ethanol and phosphoric acid. The assay is based on the binding of the dye to basic (Arg, His, Lys) or aromatic (Phe, Tyr, Trp) amino acid residues. This binding causes a shift in the absorbance maximum of the dye from the cationic red form at 465 nm to the anionic blue form at 596 nm (Compton and Jones, 1985). The limitation of this dye-protein binding assay is variability of colour developments with different protein samples, hence variability in absorbance (Congdon *et al.*, 1993). Read and Northcote (1981) modified the Bradford method to decrease the level of protein-protein variability in dye binding and to increase the sensitivity of the assay, by replacing the Coomassie brilliant blue G-250 with Serva blue G dye, and by increasing the concentration of the dye in the assay solution or decreasing the concentration of phosphoric acid.

2.2.1 Materials

Dye Reagent. Coomassie brilliant blue G-250 (50 mg) was dissolved in 88% phosphoric acid (50 ml) and 99.5% ethanol (23.5 ml). The solution was made up to 500 ml with dist.H_2O, stirred for 30 min on a magnetic stirrer, filtered through Whatman No.1 filter paper and stored in a brown bottle. The solution could be stored for up to 6 months, although visual checks for

precipitation were made before use. If precipitation was visible, the reagent was filtered and re-calibrated before use.

Standard protein solution. Ovalbumin (1 mg), was dissolved in 1 ml of dist.H_2O and the solution was diluted to 100 μg/ml in dist.H_2O.

2.2.2 Method

Protein standard (0-50 μl of the 100 μg/ml solution, i.e. 1-5 μg) was diluted to a final volume of 50 μl with dist.H_2O in 1.5 ml polyethylene microfuge tubes. Dye reagent (950 μl) was added, mixed in by inversion and allowed to stand for 2 min for colour development. The absorbance was read at 595 nm against an appropriate blank (950 μl of dye reagent + 50 μl of dist.H_2O) in 1 ml plastic micro-cuvettes. Mean values of four replicates were used to construct a standard curve. The concentration of the samples of unknown concentration was calculated from an equation generated by linear regression analysis of the standard curve data using Microsoft Excel.

2.3 Concentration of protein samples

Dilute affinity purified IBDV receptor protein samples (Section 4.4.2.2) were concentrated prior to Tris-Tricine SDS-PAGE, analysis. A simple, and inexpensive concentrating technique, employing dialysis against polyethylene glycol (PEG) (M_r 20 000), was found to be sufficiently gentle to keep proteins in their native state and was the method of choice for the concentration of protein samples. Dialysis against the soluble high molecular weight PEG, which is too large to pass into the dialysis membrane, was only used when the presence of sucrose, which diffuses into the dialysis bag (M_r 12 000 cut-off), was not desirable. The principle of concentration by these polymers is based on the movement of water along a concentration gradient out of the dialysis bag to dissolve the dry polymer, leaving a concentrated sample solution inside the dialysis bag. In cases where more concentrated sample was required prior to Tris-Tricine SDS-PAGE analysis, a rapid, small-scale method, based on the precipitation of SDS-protein complexes by KCl, was used and resulted in an overall 50-fold concentration of samples. However, the SDS-KCl precipitation method was found to be

unsuitable for non-reducing SDS-PAGE analysis, possibly as a result of the very high salt concentration in the samples.

2.3.1 Dialysis against PEG 20 000

The protein sample to be concentrated was placed in a dialysis bag (M_r 12 000 cut-off). Following sealing, it was placed in a plastic tray, and covered with (PEG 20 000) at 4°C for a limited time (5 to 10-fold concentration was usually achieved in 2-4 h), the bag was briefly rinsed in dist.H_2O and the sample was squeezed out.

2.3.2 SDS/KCl Precipitation

2.3.2.1 Materials

5% (w/v) SDS. SDS (0.5 g) was dissolved in 10 ml of dist.H_2O.

3 M KCl. KCl (2.24 g) was dissolved in 10 ml of dist.H_2O.

2.3.2.2 Method

5% SDS (10 μl) was added to the sample (100 μl) in a 1.5 ml polyethylene microfuge tube. The solution was mixed by inverting the tube followed by the addition of 3 M KCl (10 μl). The mixture was again inverted and centrifuged (12 000 *g*, 2 min, RT). The supernatant was discarded and the precipitate was dissolved in reducing treatment buffer (20 μl, solution G, Section 2.4.1).

2.4 SDS-PAGE (Laemmli and Tricine) systems

SDS-PAGE (both Laemmli and Tris-Tricine) was used to evaluate the identity of purified IBDV bursal membrane proteins and the affinity purified IBDV receptor protein. When proteins are treated with the anionic detergent, SDS, the secondary, tertiary and quaternary structure of the protein is denatured resulting in a negatively charged linear polypeptide chain of a constant charge/mass ratio and uniform shape. Denaturation is assisted by the presence of

2-mercaptoethanol that reduces all disulfide bonds (Smith, 1984; Boyer, 1993). The resulting negatively charged detergent-polypeptide complexes will have similar charge-to-mass ratios and hence size dependent anodal migration in an electric field. The anionic detergent binds tightly to most hydrophobic regions of the denatured protein chain in a constant ratio of about 1.4 g of SDS to 1 g of protein (Reynolds and Tanford, 1970).

The most commonly used technique is that developed by Laemmli (1970). This method uses two different gel systems, a running (lower) and stacking (upper) gel with different pore sizes, pH and ionic strength. The function of the stacking gel is to concentrate large volume samples, resulting in improved resolution of proteins.

Protein samples prepared in low conductivity buffer (0.06 M Tris-HCl buffer, pH 6.8) are loaded between the higher conductivity electrode (0.025 M Tris, 0.192 M glycine, pH 8.3) and stacking gel (0.125 M Tris-HCl buffer, pH 6.8) buffers. When the power is applied, a voltage drop develops across the sample solution, driving the samples into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is quickly formed with the highly mobile chloride ions at the front and the relatively slow glycinate following behind. A restricted high-voltage gradient created between the leading and trailing ion fronts causes the samples to form a thin zone (stack) and migrate between the chloride and glycinate phases. Because of the larger pore size in the staking gel no retardation of sample migration occurs. Once the samples reach the running gel, movement is restricted by the pore size. The glycinate ions overtake the proteins, which then move in a zone of uniform pH (pH 9.5) formed by the Tris and glycine. Samples thus separate according to their size, with the smaller molecules migrating the longest distance down the gel (Garfin, 1990).

The limitation of the Laemmli method is that it does not separate small proteins (< 20 kDa) very well, hence an alternative method with a higher resolving power for small proteins was needed. Schägger and von Jagow (1987) described an alternative SDS-PAGE method, using a Tricine buffer, which gives exceptionally good resolution for proteins under 20 kDa. The power of this technique for resolving proteins, particularly in the range of 5 to 30 kDa, is

mainly due to the addition of Tricine as the trailing ion and making the stacking limits as narrow as possible within the low molecular mass range.

Despite its higher molecular mass, Tricine migrates much faster than glycine in a stacking gel at pH values between 6.8 and 8.8, because much more Tricine is in the migrating, anionic form. As a result, the stacking limit is shifted to the low-molecular mass range. The stacking and destacking of proteins therefore could be achieved at lower acrylamide concentrations than those in the glycine systems (Schägger and Jagow, 1987).

2.4.1 Materials for Laemmli SDS-PAGE

Solution A: Monomer Solution [30% (w/v) acrylamide, 2.7% (w/v) bis-acrylamide]. Acrylamide (73 g), and bis-acrylamide (2 g) were dissolved and made up to 250 ml with dist.H_2O and stored in an amber coloured bottle at 4°C.

Solution B: 4 × Running Gel Buffer (1.5 M Tris-HCl buffer, pH 8.8). Tris (45.37 g), was dissolved in 200 ml of dist.H_2O, adjusted to pH 8.8 with HCl and made up to 250 ml.

Solution C: 4 × Stacking Gel Buffer (500 mM Tris-HCl buffer, pH 6.8). Tris (3 g), was dissolved in 40 ml dist.H_2O, adjusted with HCl to pH 6.8 and made up to 50 ml. This buffer was made up weekly, because, as a result of its poor buffering capacity at 2.1 pH units below the pKa of Tris at 4°C, pH drift led to anomalous running patterns in non-reducing SDS-PAGE.

Solutions A, B, and C were filtered through Whatman No. 1 filter paper before use.

Solution D: 10% (w/v) SDS. SDS (10 g) was dissolved in 100 ml dist.H_2O.

Solution E: Initiator [10% (w/v) ammonium persulfate]. Ammonium persulfate (0.1 g) was made up to 1 ml just before use.

Solution F: Tank buffer [250 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3]. Tris-base (15 g), and glycine (72 g) were dissolved and made up to 5 litres with dist.H_2O. Prior to use, 2.5 ml of SDS stock (solution E) was added to 250 ml.

Solution G: Reducing Treatment Buffer [125 mM Tris-HCl buffer, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]. Buffer C (2.5 ml), 10% SDS (4 ml) (solution D), glycerol (2 ml) and 2-mercaptoethanol (1 ml) were made up to 10 ml with dist.H_2O.

Solution H: Non-reducing Treatment Buffer [125 mM Tris-HCl buffer, 4% (w/v) SDS, 20% (v/v) glycerol, pH 6.8]. Buffer C (2.5 ml), 10% SDS (4 ml) (solution D) and glycerol (2 ml) were made up to 10 ml with dist.H_2O.

Molecular weight markers. The standard molecular weight markers were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Lyophilised markers were reconstituted in non-reducing treatment buffer (100 μl) and heated for 5 min for Coomassie staining or western blot analysis. For silver staining the reconstituted markers were diluted 1:50 in non-reducing treatment buffer (solution H), and stored at 4°C.

Table 2.1 Preparation of Laemmli running and stacking gels

Reagent	Volume (ml)	
	Running gel (12%)	Stacking gel (4%)
A	6.25	0.94
B	3.75	0
C	0	1.75
D	0.15	0.07
E	0.075	0.035
Dist.H_2O	4.75	4.3
TEMED	0.0075	0.015

2.4.2 Method

The Bio-Rad mini-PROTEAN II® unit was assembled as described in the manufacturer's manual. This involved cleaning a smaller and larger glass plate with ethanol for each of the two sides of the apparatus, and clamping these with two 1.5 mm polyethylene spacers separating them at the edges in the casting stand. The running gel was run into the space between the plates, to a depth of 3 cm from the top of the larger glass plate, and overlayered with dist.H_2O to allow for even polymerisation. Once the gel had set (evidenced by the appearance of the interface between the gel solution and water, usually 1 h), the water was removed with a syringe. Staking gel solution was poured on top of the running gel layer, and a comb was inserted to form the sample application wells. Once this gel had set (about 30 min) the comb was removed and the wells were rinsed with dist.H_2O. The gel assemblies were clamped to the inner cooling core.

Tank buffer, containing SDS, was poured into the upper and lower electrode compartments. Samples were prepared with an equal volume of reducing treatment buffer and boiled for 2 min. A marker dye, bromophenol blue (5 μl), which migrates with the buffer front, was added to each sample before loading onto the gels. Suitable amounts of samples (at least 1 μg of protein per band for the Coomassie blue R-250 staining procedure) were applied to the wells using a Hamilton microsyringe. The gel unit was connected to a power pack and run at 18 mA per gel until the bromophenol blue tracker dye was about 0.5 cm from the bottom of the running gel. At this point, the apparatus was disconnected from the power supply, the plates were removed, and levered apart using a plastic spacer. The gel was removed and stained (Section 2.7).

2.4.3 Materials for Tris-Tricine SDS-PAGE

Solution A: [3 M Tris-HCl buffer, 0.3% (w/v) SDS, pH 8.45]. Tris (72.7 g), was dissolved in 180 ml of dist.H_2O, SDS [6 ml of a 10% (w/v) solution] was added, adjusted to pH 8.45 with HCl, and made up to 250 ml.

Solution B: [49.5% (w/v) acrylamide, 3% (w/v) bis-acrylamide]. Acrylamide (48 g), and bis-acrylamide (3 g) were dissolved and made up to 100 ml with dist.H_2O and stored in an amber coloured bottle at 4°C.

Solution D: [10% (w/v) ammonium persulfate]. Ammonium persulfate (0.1 g) was made up to 1 ml just before use.

Anode buffer [200 mM Tris-HCl buffer pH 8.9]. Tris (24.22 g) was dissolved in approximately 950 ml of dist.H_2O, adjusted to pH 8.9 with HCl and made up to 1 litre.

Cathode buffer [100 mM Tris-HCl buffer, 100 mM Tricine, 0.1% (w/v) SDS, pH 8.25]. Tris (12.2 g), and Tricine (17.9 g) were dissolved in 950 ml of dist.H_2O, SDS [10 ml of 10% (w/v) solution] was added, adjusted to pH 8.25 with HCl, and made up to 1 litre.

2.4.4 Method

The procedure outlined in Section 2.4.2 was followed, except that the running and stacking gels were prepared as shown in Table 2.2. The cathode buffer was placed in the top chamber and the anode buffer in the bottom chamber of the vertical slab gel electrophoresis unit. Samples were combined with reducing treatment buffer and prepared as described in Section 2.4.2 before loading onto the gels. Two gels were run at 80 V until the tracking dye had entered the running gel, before the voltage was increased to 100 V. Electrophoresis continued until the tracker dye was about 0.5 cm from the bottom of the gel. The gel was removed as before and stained (Section 2.7).

Table 2.2 Preparation of Tricine-SDS-PAGE running and, stacking gels

Reagent	Volume (ml)	
	Running gel (10%)	Stacking gel (4%)
Solution A	5	1.5
Solution B	3	0.5
Dist.H_2O	7	4
Ammonium persulfate	0.05	0.03
TEMED	0.0075	0.015

2.5 Nondenaturing PAGE for DNA Separation

Due to its higher resolution power especially for small size nucleic acid fragments (Table-2.3), nondenaturing PAGE was used to evaluate the homogeneity of the purified double stranded viral RNA (dsRNA), amplified PCR products and restriction digestion fragments. These gels are run at very low voltage (1-8 V/cm) to avoid denaturation of DNA fragments by the electric current. Double stranded DNA migrates in nondenaturing polyacrylamide gels at a rate inversely proportional to the $\log_{10}$ of its size. DNA's mobility can also be affected by the base composition and sequence, therefore DNA-strands of exactly the same size can differ in their mobility by up to 10%. This is may be caused by kinks that form at specific sequences in double stranded DNA (Sambrook *et al.*, 1989).

Table 2.3 Effective range of separation of DNAs in polyacrylamide gels (Sambrook *et al.*, 1989)

Acrylamide (% [w/v])	Effective range of separation (bp)
3.5	1000-2000
5	80-500
8	60-400
12	25-150
20	6-100

2.5.1 Materials

30% Acrylamide. Acrylamide (29 g), and *N*, *N*'-methylenebisacrylamide (1 g) were dissolved in 100 ml of dist.H_2O.

1 × TBE [89 mM Tris-borate buffer, 2 mM EDTA, pH 8.0]. Tris (10.8 g), boric acid (5.5 g) and EDTA (0.93 g) were dissolved in 950 ml of dist.H_2O, adjusted to pH 8.0 with HCl and made up to one litre.

5 × TBE. Tris (54 g), boric acid (27.5 g) and EDTA (4.65 g) were dissolved in 950 ml of dist.H_2O, adjusted to pH 8.3 with HCl and made up to one litre.

10% Ammonium persulfate. Ammonium persulfate (1 g) was dissolved in 10 ml of dist.H_2O the solution was made up fresh weekly and stored at 4°C.

Staining solution [0.5 μg/ml ethidium bromide]. Ethidium bromide 10 μl of a 5 mg/ml solution was made up to 100 ml with 1 × TBE.

Table 2.4 Volume of reagents used to cast nondenaturing polyacrylamide gels of different concentrations (Sambrook *et al.*, 1989)

	Volume (ml) for Running gel %
Reagent	15%
30% Acrylamide	50
Dist.H_2O	29.4
5 × TBE	20
10% Ammonium persulfate	0.7
TEMED	0.0088

2.5.2 Method

The Bio-Rad Mini-PROTEAN II® electrophoresis apparatus was assembled as described in Section 2.4.2. After the running gel mixture (Table 2.4) was poured into the space between the glass plates, a plastic comb was inserted immediately and carefully to avoid entrapment of air bubbles and the gel allowed to set for 60 min at room temperature. Following polymerisation, the comb was removed carefully and wells were rinsed immediately with distilled water followed by 1 × TBE buffer.

After the 1 × TBE buffer was poured into the upper and lower electrode compartments, samples (PCR product combined with gel loading buffer, Section 2.6.1) were loaded into the wells using a Hamilton™ syringe and electrophoresis was performed at 30 V for 16 h. The gels were removed and stained in ethidium bromide (0.5 μg/ml ethidium bromide in 1 × TBE) for 45 min at room temperature.

2.6 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse the identity of purified IBDV dsRNA, amplified PCR products and restriction digestion fragments in this study. Despite their superiority in protein analyses, PAGE and SDS-PAGE with smaller pore sizes are not suitable

to analyse large nucleic acid fragments or intact DNA molecules. However, agarose gels are available to analyse RNA and DNA in the range of 200 to 50 000 base pairs (50 kilobases). Agar is extracted from various genera of the class *Rhodophyta* and consists of a mixture of polysaccharides. The polysaccharides are mostly alternating copolymers of 1,4 linked 3,6 anhydro-α-L galactopyranose and 1,3 linked β-D- galactopyranose (Boyer, 1993). Agaropectin, a heterogeneous mixture of smaller molecules, occur in less significant amounts. They contain negatively charged constituents, i.e. sulfate, pyruvate and glucuronate. They have poor gelling capacity and is removed from the readily gelling agarose molecules, using anion exchange chromatography (Fukui *et al.*, 1983).

Agarose gels are prepared by dissolving the required amount of agarose in warm electrophoresis buffer to give a specific % gel. Upon solidifying, the agarose gel forms a matrix, the density of which is determined by the concentration of the agarose. When an electric current is applied, nucleic acids, which are negatively charged at neutral pH, migrate toward the anode. The mobility of nucleic acids in an agarose gel is influenced both by concentration of agarose and size of the nucleic acids. Like proteins in SDS-PAGE, nucleic acids migrate in an agarose gel according to their size, with the smallest molecules migrating the greatest distance. As a result, molecular weight can be estimated based on the electrophoretic mobility of the nucleic acids in relation to that of molecular weight markers (nucleic acids or DNA fragments of known molecular weight) (Boyer, 1993). Nucleic acids are detected in agarose and polyacrylamide gels with ethidium bromide, a fluorescent dye that intercalates between stacked base pairs, extending the length of linear and nicked circular nucleic acid molecules and making them more rigid (Sambrook *et al.*, 1989).

2.6.1 Materials

EtBr stock solution (10 mg/ml EtBr). EtBr (1 mg) was dissolved in dist.H_2O (100 ml) by stirring for several hours on a magnetic stirrer. The container was wrapped in aluminium foil to prevent photo bleaching, and the solution was stored at RT. EtBr was used at a working concentration of 0.27 μg/ml.

Gel loading buffer [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 1 mM Na_2-EDTA, pH 8.0 ,50% (v/v) glycerol]. Bromophenol blue (50 mg), and xylene cyanol FF (50 mg) were dissolved in dist.H_2O (8 ml), 100 mM Na_2-EDTA (200 μl) and 99.5% (v/v) glycerol (10 ml) were added and the volume made up to 20 ml. The solution was sterilised by autoclaving and stored at RT.

50 × Tris-acetate buffer (TAE) (2 M Tris-acetate, 50 mM Na_2-EDTA, pH 8.0). Tris base (242 g), glacial acetic acid (57.1 ml) and Na_2-EDTA (18.61 g) were dissolved in dist.H_2O (500 ml), adjusted to pH 8.0 and made up to 1 litre. The stock solution was stored at RT and diluted 50-fold with dist.H_2O to yield a 1 × TAE working solution.

1 × TAE working solution. 50 × TAE buffer (20 ml) was made up to 1 litre with dist. H_2O.

2.6.2 Method

The casting tray was placed on a level surface and the open ends sealed with masking tape. The comb was positioned in the tray to provide about 1mm of space between the ends of the teeth and the surface of the plate. Agarose (0.3 g) was dissolved in 30 ml of 1 × TAE buffer in an Erlenmeyer flask, its weight was recorded and boiled in a microwave oven for 1 min. The boiled solution was weighed and the volume was readjusted by adding more TAE buffer. After addition of EtBr (0.7 μl) the gel was cooled to ± 55°C by rotating the flask under running tap water and poured rapidly and carefully into the prepared casting tray. Any air bubbles were removed and the gel was allowed to set completely at RT (30 min). The comb was carefully removed and the casting tray positioned on the centre platform of the unit. The unit was filled with approximately 250 ml of 1 × TAE buffer until a depth of about 1 mm. EtBr (5 μl) was added to the tank buffer and dispersed with gentle agitation of the pod. Gel loading buffer (3 μl) was mixed with nucleic acid samples and molecular weight markers, and samples were loaded into the wells. Following electrophoresis (100 V, unlimiting current, 1 h, RT) the gel was examined by UV light and scanned.

2.7 Protein staining methods

The permanent and growing interest in the detection of very small amounts of proteins and nucleic acids in polyacrylamide gels required staining techniques which are highly sensitive, inexpensive and easy to operate (Blum *et al.*, 1987). Different proteins exhibit different staining properties and mechanisms. Although depending on pH proteins may be stained with anionic (acidophilia) or cationic (basophilia) dyes, only their staining with anionic dyes is of practical significance. Anionic dyes are negatively charged due to the presence of sulphonate (SO_3^{2-}) groups on the dyes. At pH < 7.0, the net charge of proteins is positive, contributed by the positively charged amino groups of Lys, Arg, and His residues. Once electrostatic attraction took place between protein and dye, the staining mechanism is through hydrophobic interactions, van der Waals, ionic forces and hydrogen bond formation (Horobin, 1988; Prento, 2001; Puchtler *et al.*, 1988). The two staining methods used in this study are Coomassie blue dye and silver staining.

2.7.1 Coomassie blue R-250 staining

Due to its simplicity and reliability, Coomassie blue staining is the most widely used staining technique. However, it requires long staining/destaining times and has a detection limit of 1 μg protein/band (Choi *et al.*, 1996). In addition it causes variable background staining (Goldring and Ravaioli, 1996). In slight acid media, the dye-anion is electrostatically attached to the NH_3^+ groups of the protein, and within this primary fusion van der Waals forces hold the reactants together. The dye-protein complex is fixed, although fully reversible by dilution under appropriate conditions of pH (Fazekas De St. Groth *et al.*, 1963).

2.7.1.1 Materials

Stain stock solution [1% (w/v) Coomassie blue R-250]. Coomassie blue R-250 (1 g) was dissolved in 100 ml of dist.H_2O by magnetic stirring for 1 h at room temperature. The solution was filtered through Whatman No. 1 filter paper.

Staining solution [0.125% (w/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid]. Stain stock (62.5 ml) was mixed with methanol (250 ml) and acetic acid (50 ml), and made up to 500 ml with dist.H_2O.

Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (500 ml) was mixed with acetic acid (100 ml) and made up to 1 litre with dist.H_2O.

Destaining solution II [7% (v/v) acetic acid, 5% (v/v) methanol]. Acetic acid (70 ml) was mixed with methanol (50 ml), and made up to 1 litre with dist.H_2O.

2.7.1.2 Method

The gel was removed from the glass plates using gloves (Section 2.4.2) and placed into staining solution for 4 h. The staining solution was removed at the end of this time and, following rinsing with dist.H_2O, the gel was placed into destaining solution I, overnight, and into destaining solution II to effect complete destaining . Gels were stored in polythene zip-seal bags and kept well hydrated until photographed. They were stable in this form for long periods.

2.7.2 Silver staining

Silver staining is a common technique used to detect low concentration samples separated by SDS-PAGE. Silver staining is reported to be 50-100 times more sensitive than Coomassie blue staining and is comparable to the sensitivity of autoradiography of [^{35}S] methionine-labeled proteins. This sensitivity allows for the detection of less than 1 ng protein in a gel. This method is very rapid and relatively simple and results can be obtained within 2 h. Colour development is as a result of silver complexes formed with ionic amino acid side chains in proteins (Nielsen and Brown, 1984). Despite its sensitivity and simplicity silver staining has still a number of limitations: (1) purity of the water can affect the background; (2) different proteins can be stained differently, i.e. some proteins can be stained perfectly, others may stain only weakly or not at all (Fernandez-patron *et al.*, 1995). However, a modification of the silver staining method by Blum *et al.* (1987) prevents unspecific surface or background staining

during image development. This was achieved by the addition of low amounts of thiosulfate for the pre-treatment of fixed gels, which can chemically dissolve the formation of insoluble silver salts in the developer by complexation.

2.7.2.1 Materials

Fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.019% (v/v) formaldehyde. Methanol (100 ml), acetic acid (24 ml) and formaldehyde (100 μl 0f 37% solution) were diluted to 200 ml with dist.H_2O.

Pre-treatment solution (4 mg/ml $Na_2S_2O_3$, $5H_2O$). $Na_2S_2O_3.5H_2O$ (40 mg), was dissolved in 200 ml dist.H_2O.

Impregnation solution [0.2% (w/v) $AgNO_3$, 0.028% (v/v) formaldehyde]. $AgNO_3$ (400 mg), was dissolved in 200 ml of dist.H_2O and formaldehyde (150 μl of a 37% solution) was added.

Developing solution [0.019% (v/v) formaldehyde, 0.0004% $Na_2S_2O_3.5H_2O$, and 60 g/l Na_2CO_3]. Na_2CO_3 (12 g) was dissolved in 190 ml of dist.H_2O and $Na_2S_2O_3.5H_2O$ (4 ml of pre-treatment solution) and 37% (v/v) formaldehyde (100 μl) were added and the volume made up to 200 ml.

Stopping solution [50% (v/v) methanol, 12% (v/v) acetic acid]. Methanol (50 ml) and acetic acid (12 ml) were diluted to 100 ml with dist.H_2O.

Washing solution [50% (v/v) methanol]. Methanol (50 ml) diluted to 100 ml with dist.H_2O.

2.7.2.2 Method

All steps were carried out on an orbital shaker (50 rpm; RT) and in scrupulously cleaned glass containers to minimise background staining. The electrophoresis gel was soaked in fixing solution (100 ml, 1 hr or overnight), before incubation in washing solution (3 × 20 min), followed by soaking in pre-treatment solution (1 min). The gel was rinsed in dist.H_2O (3 × 20 s) and soaked in impregnation solution (20 min). Following rinsing in dist.H_2O (2 × 20 s) the gel was incubated in developing solution until the first protein bands become visible.

Developing solution was immediately replaced with dist.H_2O and the gel washed until colour was sufficiently developed. Development was stopped by immersing the gel in stopping solution (10 min). The gel was washed in 50% methanol and stored in polythene zip-seal bags

2.8 Isolation of Antibodies

Antibody (IgG) was isolated from serum using the neutral water-soluble high molecular mass polymer PEG 6 000, which proved to be very simple and efficient in the purification of both IgG and IgY to near homogeneity (Polson *et al.*, 1964; Polson *et al.*, 1985). PEG precipitation is performed by exclusion of water from the hydration pocket of protein molecules, which alters the dielectric constant of the surrounding medium. This may change the steric relationships of the hydrophilic groups of the protein molecules and proteins precipitate because they exceed their solubility limit (Fried and Chun, 1971; Giorgione and Epand, 1997; Goldring and Coetzer, 2003).

2.8.1 Materials

Borate buffered saline, pH 8.6. Boric acid (2.16 g), NaCl (2.19 g), NaOH (0.7 g) and 37% HCl (0.62 ml) were dissolved in 950 ml of dist.H_2O, adjusted to pH 8.6 and made up to 1 litre.

100 mM Na-phosphate buffer, 0.02% (w/v) NaN_3, pH 7.6. $NaH_2PO_4.H_2O$ (13.8 g) and NaN_3 (0.2 g) were dissolved in 950 ml of dist.H_2O, adjusted to pH 7.6 and made up to 1 litre.

2.8.2 Method

Isolation of serum from blood was carried out according to the method of Harlow and Lane (1988). Collected blood was allowed to clot for 60 min at 37°C. The clot was separated from the sides of the container (ringing), using a Pasteur pipette and placed at 4°C overnight to allow the clot to contract. The serum was removed from the clot and any remaining insoluble material was removed by centrifugation (10 000 *g*, 10 min, 4°C) and stored at -20°C. One volume of rabbit serum was mixed with two volumes of borate buffered saline. Solid PEG 6 000 was added to the diluted serum to 14% (w/v), dissolved with constant gentle stirring,

and the mixture was centrifuged (12 000 *g*, 10 min, RT). The pellet was re-dissolved in the original serum volume, using 100 mM Na-phosphate buffer pH 7.6. PEG was again added to 14% (w/v), dissolved with stirring, and the solution was centrifuged (12 000 *g*, 10 min, RT). Finally the pellet was re-dissolved in half the original serum volume using 100 mM Na-phosphate buffer, pH 7.6, containing 30% (v/v) glycerol, and stored at -20°C.

2.9 Enzyme linked immunosorbent assay (ELISA).

The ELISA technique was used to monitor the progress of anti-peptide antibody production during the immunisation period. ELISA is a frequently used technique to determine the reactivity of biological molecules (antigens) with their antibodies. Antigens or lectins are immobilised on the wells of microtiter (ELISA) plates. Since immobilisation of proteins onto plastic surfaces depends on hydrophobic interaction, protein antigens are adsorbed by hydrophobic interaction (Suzuki *et al.*, 1997).

ELISA is usually carried out in microtitre wells (96 well plates, 8 or 12 well strips). Following coating with antigen, all unoccupied sites are blocked with a non-reactive protein, such as BSA. A primary antibody specific for the antigen of interest is used to allow the antigen-antibody interaction to take place. After washing, the bound primary antibody is detected using an enzyme-linked secondary antibody, which upon addition of a substrate produces a soluble colored product that can be quantified visually or photometrically (Thorpe and Kerr, 1994; Switzer and Garrity, 1999).

2.9.1 Materials

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), $Na_2HPO_4.2H_2O$ (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in 950 ml of dist.H_2O, adjusted to pH 7.2 and made up to 1 litre.

0.5% (w/v) Bovine serum albumin-PBS (BSA-PBS). BSA (0.5 g) was dissolved in 100 ml of PBS.

0.1% (v/v) PBS-Tween. Tween 20 (1 ml) was made up to 1 litre in PBS.

0.15 M Citrate-phosphate buffer, pH 5.0. A solution of citric acid.H_2O (21.0 g/l) was titrated with a solution of $NaHPO_4.2H_2O$ (35.6 g/l) to pH 5.0.

Substrate solution [0.05% (w/v) ABTS and 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer]. ABTS (7.5 mg) and H_2O_2 (7.5 μl) were dissolved in citrate-phosphate buffer, pH 5.0 (15 ml) for one ELISA plate.

Stopping buffer (citrate-phosphate buffer-0.1% (w/v) NaN_3). NaN_3 (0.1%) was made up to 100 ml in citrate-phosphate buffer.

2.9.2 Method

The IBDV receptor peptide (Chapter 5) was coated (5 μg/ml) in PBS (150 μl per well, overnight at 4°C). Non-specific binding of antibody was prevented by blocking the wells with 0.5% BSA-PBS (200 μl, 1 h at 37°C), and the plates were washed three times with PBS-Tween. Serial two-fold dilutions of primary antibody, starting from 100 μg/ml was prepared on the plate in 0.5% BSA-PBS and incubated (100 μl, 2 h at 37°C). The plates were washed three times with PBS-Tween, and HRPO-linked secondary antibody, at a suitable dilution (1 in 20 000 in 0.5% BSA-PBS) was added to each well and incubated (120 μl, 1 h at 37°C). Following washing three times with PBS-Tween, substrate solution (150 μl) was added to each well and colour was allowed to develop in the dark against the background of the controls (usually 10-15 min) and the A_{405} of each well was measured in a Titertek ELISA plate reader.

2.10 Western blotting

Western blotting has been a powerful molecular biology technique for the identification and characterisation of proteins separated by SDS-PAGE and electrophoretically transferred to a nitrocellulose sheet for detection by antibodies (Hughes *et al.*, 1988). In this technique binding of protein-SDS complexes to the nitrocellulose membrane is enhanced by the addition of methanol into the transfer buffer. After blocking of the unoccupied binding sites on the

nitrocellulose membrane with non-fat milk, the proteins (antigens) are available to react with their corresponding primary and secondary detection antibodies. The detection system comprises a secondary antibody which has a strong affinity for the primary antibody, labelled with an enzyme such as HRPO, which catalyses a reaction leading to the formation of insoluble (precipitating) coloured product.

2.10.1 Materials

Blotting buffer. Tris (27.23 g) and glycine (64.8 g) were dissolved in 3.5 litres of dist.H_2O, and methanol (900 ml) was added. The volume was made up to 4.5 litres. Prior to use, 10% (w/v) SDS was added (4.5 ml solution Section 2.4.1).

Tris buffered saline (TBS; 20 mM Tris, 200 mM NaCl, pH 7.4). Tris (2.42 g), and NaCl (11.69 g) were dissolved in 950 ml of dist.H_2O, adjusted to pH 7.4 with HCl, and made up to 1 litre.

Blocking solution [5% (w/v) non-fat milk powder]. Low fat milk powder (5 g) was dissolved in TBS (100 ml).

1% (w/v) Ponceau S. Ponceau S (0.1 g), was dissolved in 100 ml of 1% (v/v) glacial acetic acid.

4-chloro-1-naphthol substrate solution [0.06% (w/v) 4-chloro-1-naphthol, 0.0015% (v/v) H_2O_2]. 4-chloro-1-naphthol (0.03 g), was dissolved in methanol (10 ml). Two ml of this solution was diluted to 10 ml with TBS, with the addition of 30% hydrogen peroxide (4 μl).

2.10.2 Method

Following SDS-PAGE, usually on duplicate gels, one gel was stained to show the total protein pattern, while the other was used for blotting. Nitrocellulose was cut to a suitable size. To avoid entrapment of air the nitrocellulose was carefully floated onto blotting buffer, before being totally immersed. The immersed nitrocellulose was sandwiched, with the gel lying squarely on top of it, between three pieces of Whatman No.4 filter paper and two pieces of

Scotchbrite foam, also totally immersed in blotting buffer. The sandwich was placed into a Western blotting apparatus filled with blotting buffer. The apparatus was connected to a power supply so that the nitrocellulose was on the anodal side of the gel, and blotting was effected for 16 h at 200 mA. The buffer was stirred by a magnetic stirrer throughout the process to ensure even distribution of cooling. After 16 h, the sandwich was removed and the filter paper was peeled off the gel. The gel was carefully removed, and stained to assess the efficiency of blotting.

The nitrocellulose sheet was removed from the filter paper and air dried for about 1.5 h. The nitrocellulose strip was blocked for 1 h with 5% (w/v) low fat milk powder in TBS, washed in TBS (3 × 5 min), and incubated for 2 h in primary antibody in 0.5% BSA-PBS. Following washing in TBS (2 × 5 min), it was incubated in HRPO-linked secondary antibody in 0.5% BSA-PBS for 1 h. After washing with TBS (3 × 5 min), it was immersed in substrate solution and reacted in the dark until bands were clearly evident against a lightly stained background. Finally the strip was removed from the substrate solution, and washed in dist.H_2O and dried between filter paper. This last step insured good preservation of the bands before scanning.

2.11 Reverse transcription polymerase chain reaction (RT-PCR)

A very sensitive diagnostic method called reverse transcription polymerase chain reaction (RT-PCR) has been used successfully to amplify the IBDV viral protein-2 (VP2) gene region of the virus with specific primers designed from the highly conserved region of VP2 (Barlic-Maganja *et al.*, 2002). This molecular technique is very fast, sensitive and specific that allows the detection and the analysis of very small samples (Lee *et al.*, 1992).

2.12 Polymerase chain reaction (PCR)

Although direct amplification of RNA using a single enzyme called polymerase (*Tth*) from *Thermus thermophilus* is possible, originally the procedure was based on the reverse transcription (RT), of the RNA into cDNA by a reverse transcriptase prior to amplification by *Taq* polymerase (Kawasaki, 1990) (Fig. 2.1). The reaction components are a DNA template containing the region to be amplified, two primers flanking this region, and thermostable DNA

polymerase isolated from *T. aquaticus*, called *Taq* polymerase. This technique is based on three successive cycles of denaturation, annealing and synthesis at different suitable temperatures. The primary step is denaturation of the double stranded DNA template at 90-95°C. After cooling the mixture to the annealing temperature, which is approximately 55°C, the target specific oligonucleotide primers anneal to the 5' end of the two single stranded templates. For the third step, which is the extension step, the temperature is raised to 72°C and the primer target hybridisations serve as initiation points for the synthesis of new DNA strands. The time of incubation required for each step is usually between 1-2 min. One cycle of PCR comprises this sequence of three steps. In the second cycle, the newly synthesised DNA strand serves as a template for the next cycle (Fig 2.2) (Edel, 1998). Normally, PCR is carried out for about 30-40 cycles, which is sufficient to amplify specific parts of DNA, which can be visualised in the gel after staining with ethidium bromide (Lee *et al.*, 1992).

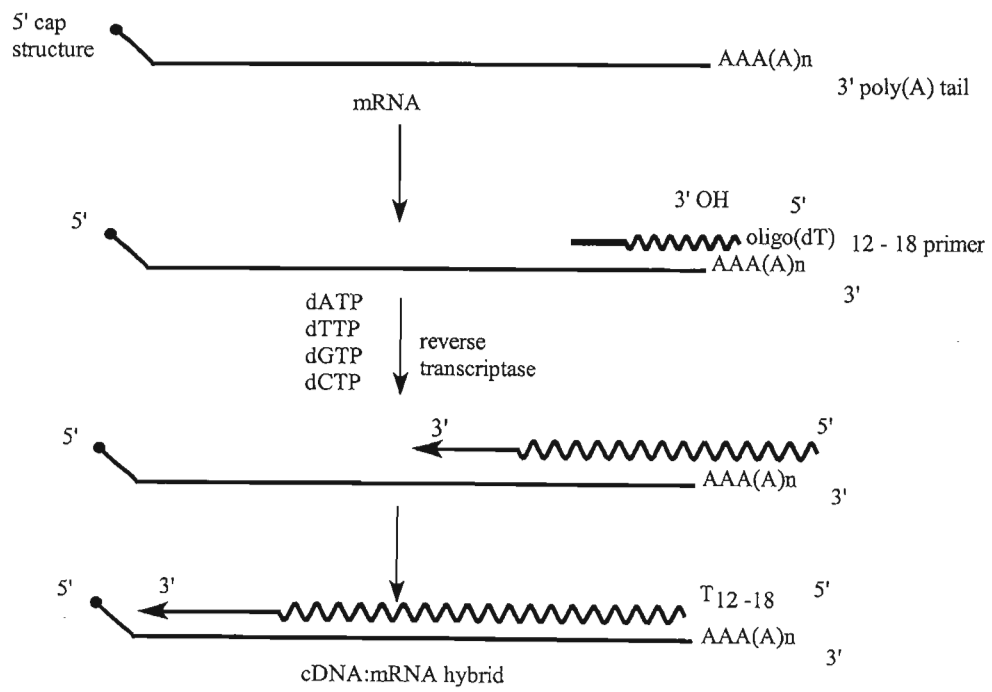


Figure 2.1 Synthesis of the first strand of cDNA using an oligo(dT) primer and reverse transcriptase (after Sambrook and Russell, 2001).

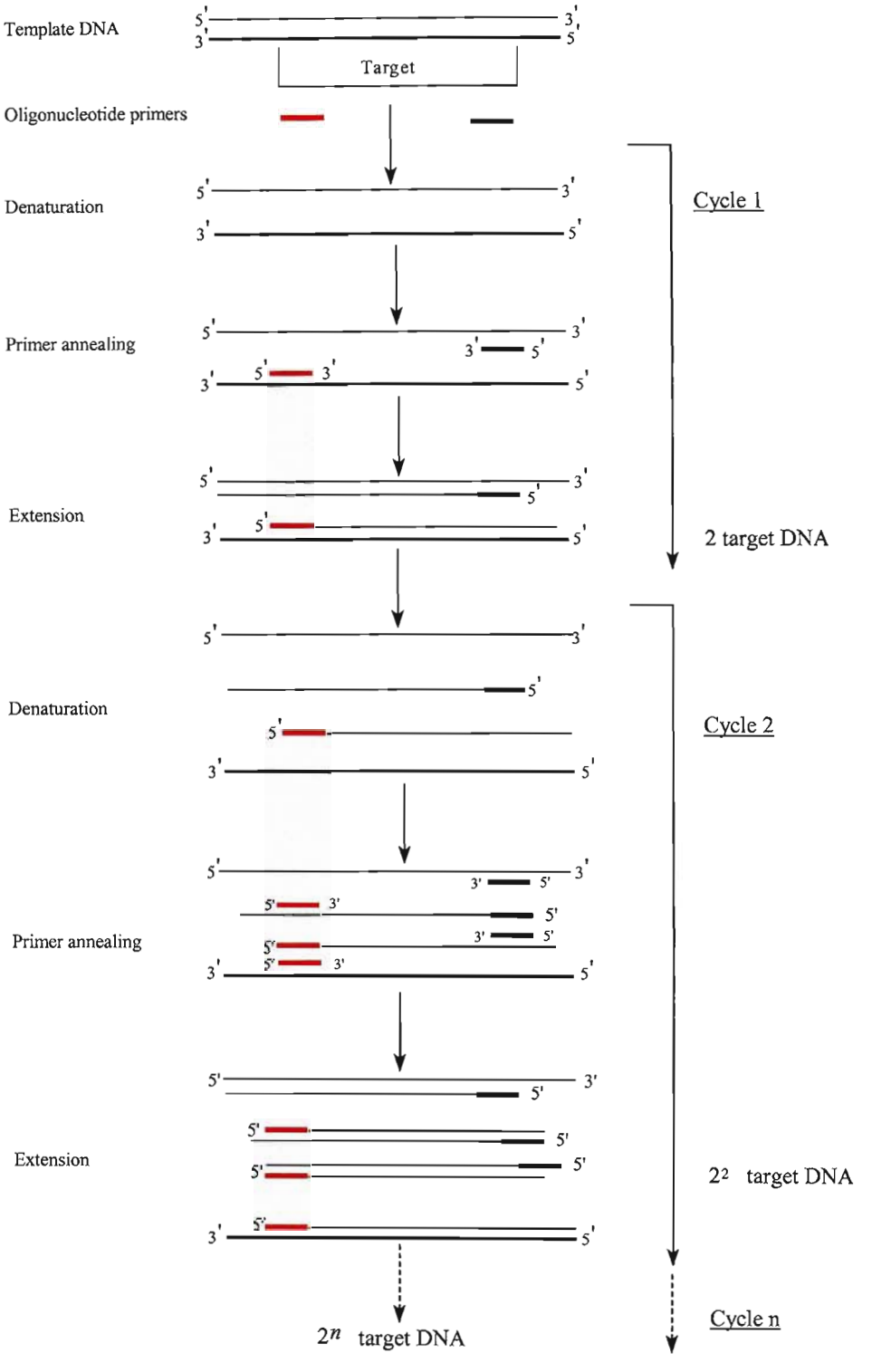


Figure 2.2 **Principle of PCR amplification** (after Edel, 1998).

2.12.1 Materials

Primer 1 (anti-sense primer) stock solution (Section 3.6.1). 720.1 OD/ml (4036.92 μM).

Primer 2 (sense primer) stock solution (Section 3.6.1). 890.5 OD/ml (4538.36 μM).

Primer 1 (anti-sense primer) working solution (30 μM). Primer stock solution (0.37 μl) was diluted in 49.63 μl of DEPC treated deionised H_2O to final volume of 50 μl.

Primer 2 (sense primer) working solution (30μM). Primer stock solution (0.33 μl) was diluted in 49.67 μl of DEPC treated deionised H_2O to final volume of 50 μl.

Enzyme mix: Expand High Fidelity enzyme mix, reverse transcripytase, AMV, in storage buffer (100 μl). From Roche.

RT-PCR reaction buffer (5 × Conc.) with 7.5 mM $MgCl_2$ and DMSO (1.0 ml). From Roche.

$MgCl_2$ solution (25 mM) (1.0 ml). From Roche.

Dithiothreitol solution (100 mM) (1.0 ml). From Roche.

dNTP stock solution [2.5 mM of each dATP, dCTP, dGTP and dTTP]. From Takapo.

DEPC treated ddH_2O [0.1% (v/v) DEPC]. Diethylpyrocarbonate (DEPC) (100 μl) was made up to 100 ml with deionised dist.H_2O, the solution incubated at 37°C for 12 h and autoclaved at 121°C for 15 min.

2.12.2 Method

One tube RT-PCR was performed according to Barlic-Maganja *et al.* (2002). RNA sample (100 ng), AMV (1 μl), sense and anti-sense primer (0.6 μl/20 pmol), 5 mM DTT (2 μl), mixed dNTP (4 μl/0.2 mM) and 5 × RT-PCR buffer with Mg^{2+} (10 μl) was made up to 50 μl with DEPC treated ddH_2O. The RT-PCR reaction was carried out with uninterrupted thermal cycling under the following conditions: 45 min at 48°C for reverse transcription and 2 min at

94°C for AMV reverse transcriptase inactivation and RNA/cDNA/primer denaturation, 40 cycles of 30 s at 94°C, 1 min at 60°C and 2 min at 68°C for PCR, and a final extension step of 7 min at 68°C. The reaction products were analysed by electrophoresis on a 2% agarose gel and stained with ethidium bromide (ethidium bromide was mixed with the gel prior to electrophoresis) (Section 2.6.2).

CHAPTER 3

DIAGNOSTIC METHODS FOR INFECTIOUS BURSAL DISEASE VIRUS (IBDV)

3.1 Introduction

The objective of this part of the study was the development of diagnostic tests for IBDV infection. Infectious bursal disease virus was isolated from IBDV infected bursal tissue using CsCl density gradient centrifugation and its identity was analysed using Tris-Tricine SDS-PAGE and electron microscopy. Double stranded IBDV RNA (dsRNA) was purified from IBDV using proteinase K digestion followed by phenol:chloroform extraction and ethanol precipitation. For further purification, the impure RNA was run on a low melting point agarose gel and double stranded RNA was subsequently extracted from the gel and recovered by agarase I enzyme. The homogeneity of the purifications was evaluated by agarose gel electrophoresis. Specific primers were designed from the highly conserved genome coding for the IBDV VP2 and RT-PCR was performed on the isolated dsRNA. Following isolation of the double stranded RNA; four commercial IBDV live vaccines were also tested for the presence of IBDV using RT-PCR. The test was proved to be positive by the amplification of a 150 bp band. The correctness of the amplified PCR product was confirmed by restriction digestion with a specific restriction endonuclease (*Bam* HI) resulting in correct digestion fragments of size 93 and 57 bps.

3.2 IBDV purification using CsCl density gradient centrifugation

Various biological studies of viruses require the presence of highly purified and concentrated virus particles, either from clinical or infected tissue culture cells. The most frequently used purification method has been Freon (tri-chloro-tri-fluoroethane) extraction and concentration of viruses by pelleting, followed by CsCl gradient centrifugation (Chen and Ramig, 1992). Purification of viral particles has been performed using cesium chloride isopycnic density gradient centrifugation, a method which can exploit the difference in G + C content of the host cellular and viral nucleic acids (Mellerick and Fraser, 1987).

The usual method used to establish a CsCl gradient is through homogenisation of the virus with the CsCl solution and centrifugation until the sedimentation-diffusion equilibrium is reached. The time required for the separation of the viruses by their buoyant density depends on the type of rotor used. When a fixed-angle rotor is used, the time ranges from 36 to 50 h, because the centrifugation time is mostly spent in collecting the virus molecules into the equilibrium bands. This time problem can be solved by the use of a steep step gradient technique i.e. when the virus is placed in the upper or lower portions of the CsCl solution, the centrifugation time required for the separation of the virus reduces to 12 h. Further reduction can be achieved, with swing out rotors; this technique can reduce the time required for the formation of virus bands in their equilibrium position to 2 h.

During the course of centrifugation using an isopicnic density gradient the force affecting viral sedimentation is

$$F = \frac{MV}{N}(\rho m - \rho g)\omega^2 r,$$

where M is the molecular weight of the viruses, V is the partial specific volume of the viruses, N is Avogadro's number, ρm is the equilibrium buoyant density of the viruses, ρg is the density of the gradient at the position of the viruses, ω is the angular velocity, and r is the distance from the centre of the rotation (Babykin and Zinchenko, 1984).

CsCl gradient centrifugation is a powerful technique for purification of viruses from infected tissue with higher yield and purity. In this project IBDV purification was performed according to the method of Fernandez-Arias *et al.* (1998) with slight modifications. Homogenisation buffer containing only Tris-HCl was used in this study instead of TEN buffer (10 mM Tris-HCl, pH 7.2; 100 mM NaCl and 1 mM EDTA). Since the objective was to purify IBDV from bursal cells there was no need of stabilising the inclusion bodies following lysis of the cell (Sambrook and Russell, 2001). A two-step density gradient centrifugation using 1.37 and 1.27 g/ml of CsCl solutions was employed as a replacement for the one-step density gradient centrifugation using 1.3 g/ml CsCl solution, as clearer bands were evident following the addition of the second step i.e. 1.27 g/ml of CsCl solution.

3.2.1 Materials

Homogenisation buffer (0.02 M Tris-HCl buffer pH 7.8). Tris (2.42 g), was dissolved in 950 ml of dist.H_2O, adjusted to pH 7.8 and made up to 1 litre.

40% (w/v) sucrose. Sucrose (60 g) was dissolved in a minimum of homogenisation buffer and made up to 150 ml with homogenisation buffer.

Dialysis buffer (0.01 M Tris-HCl buffer pH 7.8). Tris (1.21 g), was dissolved in 950 ml of dist.H_2O, adjusted to pH 7.8 and made up to 1 litre.

Cesium chloride solutions. Two solutions of cesium chloride were prepared by dissolving cesium chloride in homogenisation buffer until the density reached 1.27 g/cm^3 and 1.37 g/cm^3 respectively. The density of the solution was estimated by withdrawing 1 ml fractions of the solution and determining the mass on a balance. The density was adjusted by adding more cesium chloride or homogenisation buffer.

3.2.2 Method

An equal volume of homogenisation buffer was added to the infected bursal tissue and homogenised using a Potter S homogeniser. After removing the cell debris by low speed centrifugation (17 000 *g*, 15 min, 0°C), the supernatant was recovered, loaded on top of 2 ml sucrose cushions (40%) and centrifuged in a Beckman SW 40 rotor (86 000 *g*, 2.5 h, 2°C). After centrifugation, the pellet was thoroughly resuspended in homogenisation buffer and layered over half its volume of a density of 1.37 g/cm^3 CsCl in homogenisation buffer. The preparation was centrifuged (86 000 *g*, 6 h, 2°C). After centrifugation, a viral band was clearly visible towards the top of the gradient. This band was collected by aspiration using a Pasteur pipette, resuspended in homogenisation buffer and dialysed overnight against dialysis buffer for removal of cesium chloride. The dialysed suspension was layered again over half its volume of a density of 1.27 g/cm^3 cesium chloride and centrifuged (86 000 *g*, 6 h, 2°C). Virus

particles from the interface were resuspended in homogenisation buffer, dialysed overnight against dialysis buffer and analysed by Tris-tricine SDS-PAGE (Section 2.4.4).

3.3 Identification of IBDV using transmission electron microscopy

Electron microscopy is a useful diagnostic tool to identify infectious virus particles (Curry, 2000). IBDV can be identified using a negative staining diagnostic method, which is used to resolve the shape, size or structure of a particular microorganism using high-resolution electron microscopy. The viral suspension is fixed in a negative stain, e.g. a metal such as phosphotungstic acid (PTA) and uranyl acetate. On drying, the electron-dense metal atoms surround the specimen. The difference in intensity between the specimen and the surrounding heavy metal atoms produces the necessary contrast. The specimen appears bright, surrounded by the dark background of dried metallic stain. The electron beam passes though the low electron density of the specimen (virus), but not though the metallic background. The specimen base is revealed by the penetration of the stain into its holes and crevices. The clarity of the specimen depends on the degree to which the stain remains nebulous as it dries, as well as on the thickness of the dried negative stain envelope (Hayat, 1989).

3.3.1 Materials

Washing buffer (0.05 M cacodylate buffer, pH 7.1). Sodium cacodylate (8 g), was dissolved in 950 ml dist. H_2O, adjusted to pH 7.1 with NaOH and made up to 1 litre.

2% (w/v) uranyl acetate. Uranyl acetate (2 g) was dissolved in washing buffer (100 ml).

3.3.2 Method

Electron microscopy analysis of infectious bursal disease virus was performed according to the method of Fernandez-Arias *et al.* (1998). Purified infectious bursal disease virus particles (viral suspension obtained after centrifugation though 40% sucrose cushion, Section 3.2.2), were attached to electron microscopy (EM) copper grids covered with collodion and carbon that had previously been made hydrophilic by glow discharge. These grids were placed on

drops of the viral suspension, and incubated for 2 min. Adsorbed particles were negatively stained for 30 s with a 2% solution of uranyl acetate and viewed on the EM 120 Brotwin TEM at 80 KU.

3.4 RNA isolation

Infectious bursal disease virus has a double stranded viral RNA (Boot *et al.*, 2000a). There are several methods for the detection, isolation and characterisation of double stranded RNAs (dsRNAs) from viruses. Most of the methods developed involve enzymatic treatment for the extraction of total nucleic acids from virally infected tissues (Diaz-Ruiz and Kaper, 1978). Purification of double stranded IBDV RNA requires purification of IBDV from infected bursal tissue and dsRNA extraction from the purified viral particles by proteinase K digestion over an extended period of time. Following proteinase K digestion the resulting IBDV RNA is obtained by ethanol precipitation (Akin *et al.*, 1998). The IBDV dsRNA can be recognised according to its electrophoretic mobility in 1% agarose gels (Diaz-Ruiz and Kaper, 1978).

3.4.1 Materials

Homogenisation buffer (0.02 M Tris-HCl buffer pH 7.8). As per Section 3.2.1

40% (w/v) sucrose. As per Section 3.2.1

Diethylpyrocarbonate (DEPC) treated ddH_2O [0.1% (v/v) DEPC]. DEPC (100 μl) was made up to 100 ml with ddH_2O, incubated at 37°C for 12 h and the solution was autoclaved (121°C, 15 min).

0.5 M Tris-HCl buffer, pH 8.0. Tris (30.28 g) was dissolved in 450 ml of dist.H_2O, adjusted to pH 8.0 and made up to 500 ml. The solution was sterilised by autoclaving prior to use.

0.1 M Tris-HCl buffer, pH 8.0. Tris (6.05 g) was dissolved in 450 ml of dist.H_2O, adjusted to pH 8.0 and made up to 500 ml. The solution was sterilised by autoclaving prior to use.

Viral lysis buffer [100 mM Tris-HCl buffer, 150 mM NaCl, 12 mM EDTA, 1% (w/v) SDS, pH 7.5]. Tris base (3.03 g), and NaCl (2.19 g) were dissolved in dist.H_2O (150 ml), 100 mM EDTA (30 ml) and 10% (w/v) SDS (25 ml) were added. The pH was adjusted to 7.5 with HCl, and made up to 250 ml. The solution was sterilised by autoclaving prior to use.

Tris-saturated phenol. Equilibration of phenol was performed according to the method of Sambrook and Russell (2001). Crystallised phenol (stored at -20°C) was allowed to warm at room temperature and melted in a boiling water bath (68°C) in a fume hood. Hydroxyquinoline was added to a final concentration of 0.1% (v/v). An equal volume of 0.5 M Tris-HCl buffer, pH 8.0 was added and the mixture stirred for 15 min. After the mixture had settled, as much as possible of the aqueous (upper) phase was removed by aspiration. The same process was repeated using 0.1 M Tris-HCl buffer, pH 8.0 until the pH of the phenolic phase was > 7.8 (as measured with pH indicator paper). After the phenol was equilibrated, 0.1 volume of 0.1 M Tris-HCl, pH 8.0 was added and stored at 4°C in an amber bottle. Hydroxyquinoline is an antioxidant, a partial inhibitor of RNase, and a weaker chelator of metal ions. In addition, its yellow color provides a convenient way to identify the organic phase.

Proteinase K (5 mg/ml). Proteinase K from *Tritirachium album* (5 mg) was dissolved in sterile ddH_2O (1 ml), dispensed into aliquots of (50 µl each) and stored at -20°C. Multiple freeze-thaw cycles were avoided.

25 × agarase I buffer (0.75 M Bis-Tris buffer, 0.25 M EDTA, pH 7.1). Tris (60.55 g), EDTA (4.65 g) and glacial acetic acid (14.27 ml) were dissolved in 200 ml of dist.H_2O, adjusted to pH 7.1 and made up to 250 ml.

3 M sodium acetate buffer pH 5.2. Sodium acetate (24.61 g), was dissolved in 80 ml of dist. H_2O adjusted to pH 5.2 and made up to 100 ml.

10 mM sodium phosphate buffer, pH 7.0. $NaH_2PO_4.2H_2O$ (1.56 g) was dissolved in 950 ml of dist.H_2O adjusted to pH 7.0 and made up to 1 litre.

Acridine orange stock solution (50 mg/ml acridine orange). Acridine orange (0.5 g) was dissolved in 10 ml of 10 mM sodium phosphate buffer, pH 7.0 and stored at RT. Stock solution was diluted with dist.H_2O to give a working concentration of 30 μg/ml.

3.4.2 Method

Viral pellets isolated as described in Section 3.2.2 were resuspended in viral lysis buffer (depending on size of the pellet) for RNA isolation according to Fernandez-Arias *et al.* (1998). The resuspended pellets were digested with proteinase K (500 μg/ml) for 1 h at 37°C. The solution was extracted with half a volume of phenol and half a volume of chloroform and the aqueous phases were removed into a clean microfuge tube. A second phenol:chloroform extraction was performed on this aqueous phase. Viral RNA was precipitated by the addition of 2 volumes of cold ethanol and stored at -20°C for 2 h. Total RNA was pelleted by centrifugation (15 000 *g*, 15 min, 0°C) and resuspended in an appropriate volume of DEPC (diethyl polycarbonate) treated ddH_2O.

Crude RNA was subjected to 1% low-melting point agarose gel electrophoresis (Section 2.6.2) to separate the two genomic segments of IBDV. The large RNA segment (dsRNA) of approximately 3.2 kb was excised from the gel and recovered by agarase I. The weight of the excised band was determined (100 mg = 100 μl agarose gel) and 0.04 volumes of 25 × agarase I buffer was added before incubating at 65°C for 15 min or until the agarose gel melted. The agarose was cooled to 45°C and 0.01 μl of agarase I per 1 mg of gel was added and incubated for 1 h at 45°C. To isolate the dsRNA, 0.1 volume of 3 M sodium acetate buffer, pH 5.2 was added to the melted agarose solution and incubated on ice for 15 min. Oligosaccharides were pelleted by centrifugation (16 000 *g*, 15 min, RT) and the supernatant was placed in a suitable centrifuge tube. Three volumes of ice-cold ethanol was added to the supernatant and kept for 1 h at -20°C. The tubes were centrifuged (14 000 *g*, 25 min, 4°C) and the RNA pellet was air dried by careful aspiration before resuspended in DEPC treated ddH_2O.

Following nondenaturing gel electrophoresis (Section 2.5.2), the gel was incubated in the dark in acridine orange solution (30 μg/ml, 30 min) and destained in an enamel pan through a

number of changes of 10 mM phosphate buffer. The enamel adsorbs acridine orange and thus reduces the time needed to remove the high background fluorescence of the gel. The gel was viewed using UV light.

3.5 Design of VP2 primers

Despite the fact that primers are crucial elements of the PCR system, which can decide the failure or success of the amplification reaction, there are a few guidelines available for primer design (David, 1998). It is highly advisable to use one of the software packages available such as Basic Local Alignment Search Tool (BLAST) (www.expasy.org/swis-prot).

During primer design some of the factors listed below should be taken into account.

(a) Melting temperature (*Tm*), one of the most essential parameters, should be between 68-78°, though temperatures of 50-55° can be used, but with great risk of mispriming. In the absence of a computer program, the 2 + 4 rule can be used as an alterative quick estimation i.e. $Tm = 2(A + T) + 4(G + C)$.

(b) Primer length, although there is no rule which specifies the exact length of primers to use, the length can vary from a minimum of 18 to a maximum of 30 bases, and an average of 20-22 is commonly used.

(c) There should not be more than four of any single base in succession, particularly at the 3'-end of the primer where polymerisation is initiated.

(d) The presence of simple sequence repeats, such as CACACA or GTGTGT, in any of the primers should be avoided to prevent binding of the primer to simple repeats present at many sites in the genome.

(e) To allow higher binding and efficient polymerisation, the primers should end in a G or C "clamp" rather than with A or T (David, 1998).

3.5.1 Method

Reverse transcription polymerase chain reaction amplification of IBDV was performed using two appropriate oligonucleotide primers. The primers were selected from the highly conserved VP2 genome region based on a previously published sequence using the computer program BLAST (Wu *et al.*, 1992). The sequences of the two primers are given in Fig. 3.1. The inter primer spacing for primer 1 and primer 2 is 108 base pairs. This set of primers specified a 150-base-pair sequence located at nucleotide position 1760 to 1910 of the IBDV large RNA segment (Fig. 3.2).

Primer 1 5' - CAA CAG TGT AGT CTC TCC CG -3' (anti-sense primer)
Primer 2 5'- AGA TGT TTG CTG TCA TTG AAG G -3' (sense primer)

Figure 3.1 Oligonucleotide primer pair designed for the detection of the VP2 genome of IBDV.

3.6 Titan® one tube RT-PCR system

The currently used two-step RT-PCR system increases the risk of contamination during the addition of reverse transcriptase and *Taq* DNA polymerase at different intervals of the reaction process, which results into higher error rates and low amplification (Barlic-Maganja *et al.*, 2002). These problems can be solved using the Titan® one tube RT-PCR system that uses one optimised reaction buffer and a blend of enzymes to offer maximum performance. A single tube also lowers the risk of contamination. The objective of this method is to avoid contamination and achieve full-length transcription using a single buffer. Roche's Expand® PCR system's blend of thermostable enzymes, results in very low error rates and allows for larger amplified PCR products (up to 6 kb). The Expand® PCR system uses a proofreading polymerase and *Taq* DNA polymerase to produce the amplification product. This enzyme combination is optimised to ensure a three-fold increase in fidelity, when compared to using *Taq* polymerase alone (Roche, product information sheet, 2003). This technique enables reverse transcription of IBDV RNA into cDNA and then amplification by PCR under a single set of conditions, hence the possibility of contamination due to opening the reaction tube after

the initial reverse transcription, and the hands-on work required in the two-step technique is reduced (Barlic-Maganja *et al.*, 2002).

3.6.1 Materials

Buffers and reagents as described in Section 2.12.1

Primer 1 (anti-sense primer) 5' CAA CAG TGT AGT CTC TCC CG 3'.

Primer 2 (sense primer) 5' AGA TGT TTG CTG TCA TTG AAG G 3'.

Primers used in the present study were synthesised by the department of Molecular and Cellular Biology University of Cape town.

3.6.2 Method

As described in Section 2.12.2

>gi|58691|emb|X03993.1|BIBDVRNA Infectious bursal disease virus large RNA segment

AGTAGAGATCAGACAAACGATCGCAGCGATGACAAACCTGTCAGATCAAACCCAGCAGATTGTTCCGTTT
ATACGGAGCCTTCTGATGCCAACAACCGGACCGGCGTCCATCCCGGACGACACCCTGGAGAAGCACACTC
TCAGGTCAGAGACCTCAACCTACAATTTGACTGTGGGGGACACAGGGTCAGGGCTAATTGTCTTTTTCCC
TGGATTCCCCGGCTCAATTGTAGGTGCTCACTACACGATGCAGAGCAATGGGAACTACAAGTTCGACCAG
ATGCTCCTGACGGCTCAGAACCTACCAGCGAGCTACAACTATTGCAGGCTAGTGAGTCGGAGTCTAACAG
TAAGGTCAAGCACACTCCCTGGTGGCGTTTATGCACTAAATGGCACCATAAACGCCGTGACCTTCCAAGG
AAGCCTGAGTGAACTGACAGATGTTAGCTACAATGGCTTGATGTCCGCGACAGCCAACATCAACGACAAA
ATTGGGAATGTCTTAGTAGGGGAAGGGGTCACCGTCCTCAGCTTACCCACATCATATGACCTTGGGTATG
TGAGGCTTGGTGACCCCATTCCTGCCATAGGACTCGACCCAAAAATGGTAGCCACATGTGACAGTAGTGA
CAGGCCCAGAGTCTACACCATAACTGCCGCAGATGATTACCAATTCTCATCACAGTATCAACCAGGTGGA
GTGACGATCACACTGTTCTCAGCCAACATTGATGCCATTACCAACCTCAGTGTTGGAGGAGAGCTCGTGT
TCCAAACAAGCGTCCAAGGCCTTGTGCTAAACGCCACCATTTACCTGGTAGGCTTTGATGGGACCACGGT
AACCACCAGAGCTGTGGCCGCAGGCAATGGGCTGACGGCCGGCACCGACAACCTCATGCCATTCAACCTT
GTGATTCCAACCAGTGAGATAACCCAGCCAGTTACATCCATTAAACTGGAGATAGTAACCTCCAAAAGTG
GAGGTCAGGCTGGAGATCAGATGTCCTGGTTGGCAAGTGGGAACCTAGCAGTGACAATTCATGGTGGAAA
CTACCCAGGTGCCCTCCGCCCCGTCACACTAGTAGCCTATGAAAGAGTGGCAACAGGATCTGTTGTAACG
GTCGCTGGGGTGAGCAACTTCGAGCTGATCCCGAATCCTGAGCTAGCCAAGAACCTAGTCACAGAATATG
GCCGATTTGACCCAGGAGCCATGAACTACACAAAACTAATCCTGAGTGAGAGGGACCGTCTTGGTATCAA
GACCGTCTGGCCAACAAGGGAGTACACCGACTTTCGTGAGTACTTCATGGAGGTGGCTGACCTCAACTCC
CCCCTTAAGATTGCAGGAGCTTTTGGCTTCAAAGACATAATCAGGGCCATAAGAAGGATAGCTGTGCCGG
TGGTTTCTACATTGTTCCCACCAGCCGCTCCCCTAGCCCATGCAATTGGGGAAGGTGTAGACTACCTGCT
GGGCGATGAGGCCCAGGCCGCTTCAGGAACTGCTCGAGCCGCGTCAGGAAAAGCAAGAGCTGCCTCAGGC
CGCATAAGGCAGCTAACTCTCGCAGCTGACAAGGGGTACGAGGTAGTTGCGAATCTATTCCAGGTGCCCC
AGAATCCCGTAGTTGACGGAATTCTTGCCTCACCTGGAGTGCTCCGCGGTGCACACAACCTCGACTGCGT
GCTAAGGGAGGGTGCAACGTTATTCCCCGTTGTCATCACGACCGTGGAAGACGCCATGACACCCAAAGCA
CTGAACAGCAAGATGTTTGCTGTCATTGAAGGCGTGCGAGAAGACCTCCAACCTCCATCTCAAAGAGGAT
CCTTCATACGAACTCTCTCCGGCCACAGGGTCTATGGCTATGCTCCAGATGGGGTGCTCCCTCTGGAGAC
CGGGAGAGACTACACTGTTGTCCCAATAGACGATGTATGGGACGACAGCATCATGCTGTCTAAAGACCCC
ATACCTCCCATTGTGGGGAACAGTGGCAATCTAGCCATAGCCTACATGGATGTATTCAGACCCAAGGTTC
CCATCCATGTAGCTATGACAGGAGCCCTCAATGCCTACGGCGAAGTTGAGAAAGTAAGCTTCAGAAGCAC
CAAACTCGCCACTGCACACCGACTTGGTCTCAAGTTGGCTGGGCCTGGTGCATTTGACATAAACACCGGG
CCCAACTGGGCGACGTTCATCAAACGTTTCCCCCACAATCCACGCGACTGGGACAGGCTCCCCTACCTTA
ACCTCCCATATCTCCCTCCCAGTGCAGGACGCCAATACCATCTTGCCATGGCCGCCTCAGAGTTCAAAGA
GACCCCCGAACTCGAAAGTGCCGTCAGAGCCATGGAGGCGGCAGCCGACGTGGACCCACTGTTCCAATCC
GCACTCAGTGTGTTCATGTGGCTAGAAGAAAACGGGATTGTGACCGACATGGCCAACTTTGCACTCAGCG
ACCCAAACGCCCATCGGATGCGCAACTTTCTTGCAAACGCACCACAAGCAGGCAGCAAGTCGCAAAGAGC
CAAGTACGGAACAGCAGGTTACGGCGTGGAGGCCAGGGGCCCCACACCAGAGGAGGCCCAGAGGGAAAAA
GACACTCGGATCTCAAAGAAGATGGAGGCCATGGGCATCTACTTTGCAACACCAGAATGGGTAGCACTCA
ATGGGCACCGAGGGCCAAGCCCTGGCCAGCTAAAGTACTGGCAGAACACACGAGAAATACCAGACCCAAA
CGAAGACTACCTAGACTATGTGCATGCAGAGAAGAGCCGGTTGGCATCAGAAGAACAGATCCTACGGGCC
GCCACCTCGATCTACGGGGCTCCAGGACAGGCAGAGCCACCCCAAGCATTCATAGATGAAGTTGCCAAAG
TCTATGAAATCAATCATGGGCGTGGCCCAAACCAAGAACAGATGAAAGATCTGCTCTTGACTGCGATGGA
GATGAAGCATCGCAATCCCAGGCGGGCTCCACCAAAGCCCAAGCCAAAACCCAATGCTCCATCACAGAGA
CCCCCTGGTCGGCTGGGCCGCTGGATCAGAACGGTCTCTGACGAGGACCTTGAGTAAGGCTCCTGGGAGT
CTCCCGACACCACCCGCGCAGGTGTGGACACCAATTCGGCCTAGTAACA

Figure 3.2 Primer selection using BLAST from the highly conserved VP2 genome of infectious bursal disease virus large RNA segment. The primer in red is the sense primer and the primer in blue is the anti-sense primer. Bases in yellow is a *Bam*HI restriction site.

Table 3.1 Summary of the reaction components for one tube RT-PCR

Component	Volume (μl)	Final concentration in the RT-PCR (μM)
Master mix 1:		
DEPC treated ddH_2O	16.67	
dATP, PCR Grade, 10 Mm	1	200
dCTP, PCR Grade, 10 mM	1	200
dGTP, PCR Grade, 10 mM	1	200
dTTP, PCR Grade, 10 mM	1	200
Down stream primer, 30 μM	0.6	0.4 (20 pmol)
Upstream stream primer, 30 μM	0.6	0.4 (20 pmol)
Template RNA (42 μg/ml)	0.63	2 ng/μl
DTT-solution (100 mM)	2.5	5000
Total volume	25	
Master mix 2:		
DEPC treated ddH_2O	14	
5 × RT-PCR buffer with Mg^{2+}	10	1500 μM $MgCl_2$
Enzyme mix AMV and Expand High fidelity PCR-system	1	
Total volume	25	

3.7 PCR product cleavage by *Bam*HI restriction endonuclease

The 645 restriction endonucleases described to date with 137 distinct specificities, are divided into three general classes based on their size and their co-factor requirements for DNA cleavage. High molecular weight multienzyme complexes are called type I endonucleases, which require magnesium, ATP and adenosylmethionine for DNA cleavage. Type II endonucleases are of lower molecular weight and consist mostly of homodimers, require divalent cations with a preference for magnesium for cleavage. Type III endonucleases require

ATP and magnesium for cleavage and adenosylmethionine as an allosteric effector. Due to the ease of data acquisition and the high specificity of DNA sequence recognition, type II endonucleases are suitable model systems for studying protein and DNA recognition, and specific DNA strand cleavage.

A restriction enzyme *Bam*HI (from *Bacillus amyloliquefaciens* H), which is a type II restriction endonuclease, recognises the duplex symmetrical sequence 5'-GGATCC-3'. In the presence of Mg^{+2}, the enzyme catalyses DNA cleavage between the guanines (GG), generating 5'-phosphoryl and 3'-hydroxyl staggered termini (Hensley *et al.*, 1990). *Bam*HI was used to determine the correctness of the amplified PCR product. Thus, as shown in Fig. 3.10, cleavage of the150 base pair PCR product should result in fragments of 93 and 57 base pairs.

3.7.1 Materials

*Bam*HI from Promega.

10 × restriction buffer from Promega.

Acetylated bovine serum albumin from Promega.

3.7.2 Method

PCR product (13 μl), 10 × restriction buffer (2 μl), acetylated BSA (0.2 μl) and restiction enzyme, *Bam*HI (2 μl) were made up to 20 μl with DEPC treated ddH_2O (Section 3.4.1). The sample was mixed in a microfuge (12 000 *g*, 5 s, RT) and incubated overnight at 37°C. Prior to analysis by nondenaturing PAGE (Section 2.5.2) and agarose gel electrophoresis (Section 2.6.2), the tube was incubated in a water bath (65°C, 10 min) to inactivate the restriction enzyme.

3.8 Results

3.8.1 IBDV purification

Infectious bursal disease virus purification was performed using CsCl density gradient centrifugation (Section 3.2.2). A band of viral particles was clearly visible towards the top of the gradient (Fig. 3.3).

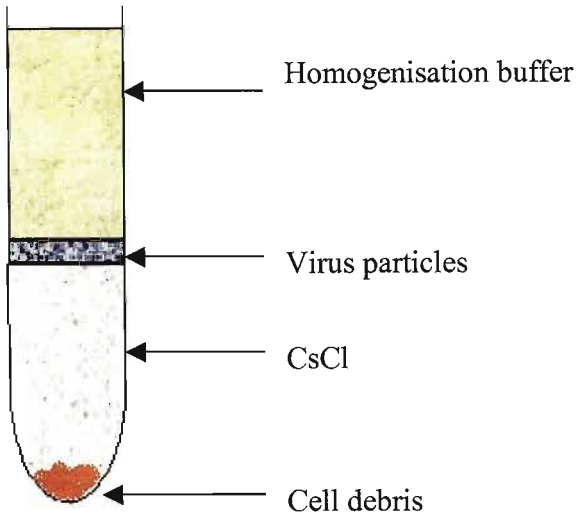


Figure 3.3 Schematic representation of IBDV purification from infected bursal tissue using CsCl density gradient centrifugation.

3.8.2 Tris-Tricine SDS-PAGE analysis of IBDV purification

To determine the success of IBDV purification using CsCl density gradient centrifugation, Tris-Tricine SDS-PAGE analysis was performed on the purified IBDV particles (Fig. 3.4). Purified infectious bursal disease virus showed five protein bands at *ca* 90, 42, 32, 28 and 17 kDa (lane 5). The results presented here illustrate the value of the second CsCl density (1.27 g/cm^3) gradient centrifugation step that results in clearly visible protein bands (lane 4 compared to lane 5).

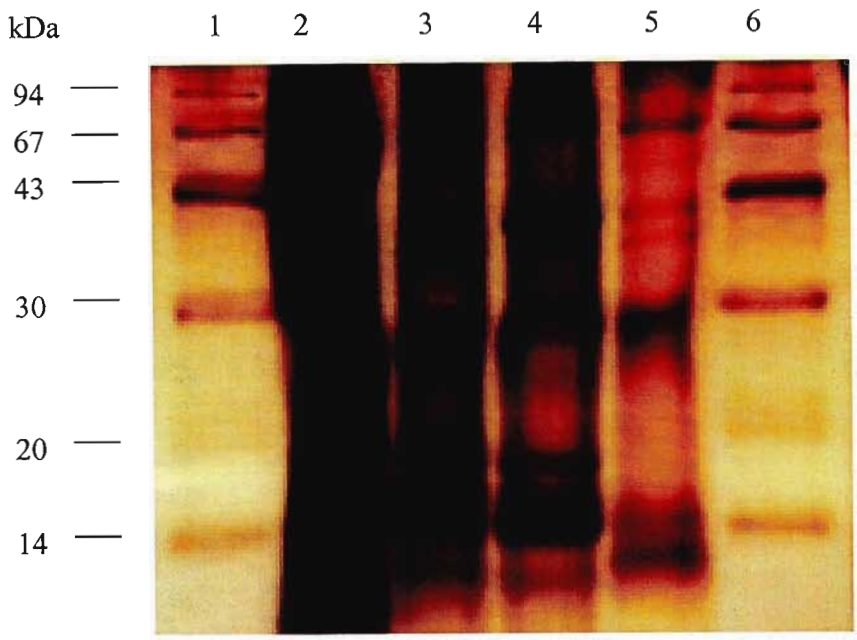


Figure 3.4 Tris-Tricine SDS-PAGE analysis of IBDV purification steps from infected bursal tissue. Samples (5 μg) were boiled for 3 min in 20 μl of reducing treatment buffer and loaded onto 10% Tris-Tricine SDS-PAGE. Samples were visualised by silver staining (Section 2.7.2.2). Lanes 1 and 6, molecular weight markers, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa); Lane 2, homogenised bursal tissue; Lane 3, pellet obtained after centrifugation in a 40% (w/v) sucrose gradient; Lane 4, viral particles from the interface after centrifugation over density gradient of 1.37 g/cm^3 cesium chloride; and Lane 5, viral particles from the interface after centrifugation over density gradient of 1.27 g/cm^3 cesium chloride.

The same gel was stained with Coomassie blue R250 (Section 2.7.1.2) but the bands were not clearly visible, probably as a result of the lower sensitivity of this staining technique.

3.8.3 IBDV detection using transmission electron microscopy

Electron microscope analysis (Section 3.3.2) was performed to determine the presence of infectious bursal disease virus in the sample purified by CsCl gradient centrifugation. The result is presented in Fig. 3.5 and shows the same structural features as those described by Böttcher *et al.* (1997); the virus is an isometric particle with a diameter of about 60 to 65 nm. It is non-enveloped and appears to be single shelled with a relatively dark body.

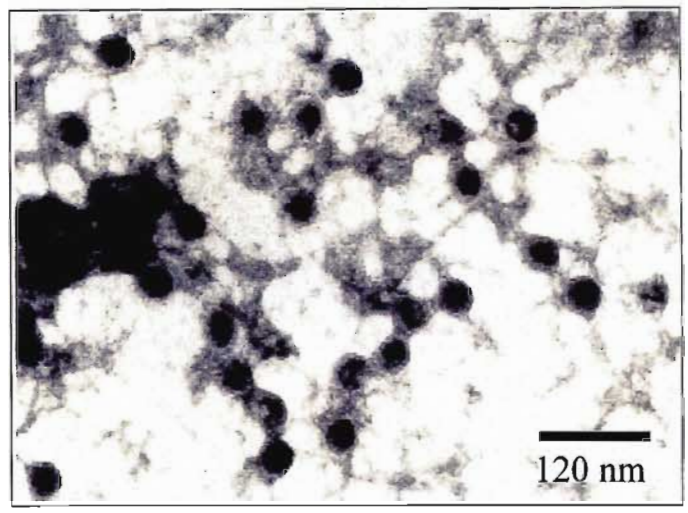


Figure 3.5 Negative staining electron microscopic analysis of infectious bursal disease virus. Bar represents 120 nm

3.8.4 RNA purification

Even though the success of IBDV purification was confirmed by Tris-Tricine SDS-PAGE and electron microscope analysis it was decided to make sure that IBDV was present in the purified sample using the size of its double stranded RNA (dsRNA) genome. Secondly for RT-PCR amplification purposes, it was necessary to purify double stranded IBDV RNA. As shown in Fig. 3.6 (lane 3) the isolated IBDV dsRNA has a size of approximately 3200 bp. There was contamination by chicken ssRNA from the bursa as shown by a band at 350 bp (Fig. 3.6, lane 2). A second purification step using the agarase I purification kit (Section 3.4.2) was thus needed to purify the double stranded viral RNA from the crude/contaminated sample (lane 3).

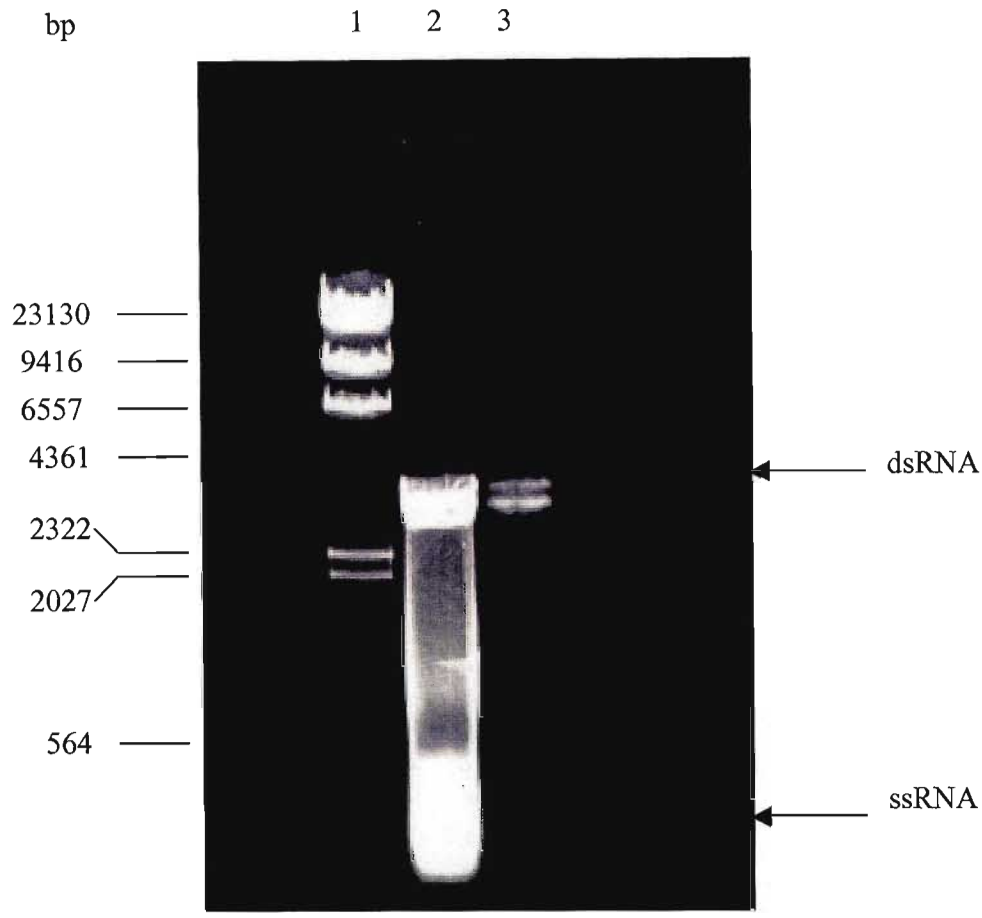


Figure 3.6 RNA purification from IBDV infected bursal tissue. Lane 1, molecular weight markers; Lane 2, crude RNA and Lane 3, purified double stranded IBDV RNA. The positions of ds and ssRNA are indicated by the arrows.

To determine whether the purified IBDV RNA is double stranded RNA, the crude RNA (Fig. 3.6, lane 2) was subjected to nondenaturing PAGE and stained with acridine orange (Section 3.4.2). The upper bands around 3200 bp appeared as bright green which is an indication of dsRNA, while the band at the bottom of the gel i.e. band around 350 bp appeared bright red which is an indication of ssRNA (result not shown).

3.8.5 Amplification of IBDV double stranded RNA using RT-PCR

Reverse transcription polymerase chain reaction amplification (Section 2.12.2) was performed using the primers detailed in Fig. 3.1. Double stranded RNA of IBDV was detected as indicated by a band at 150 bp (Fig. 3.7). Due to the small size of the amplified product (150 base pairs) it was very difficult to see the amplified PCR product in a 1% (w/v) agarose gel, but this problem was solved by increasing the agarose gel percentage to 2% (w/v) (Fig. 3.7A) and by using a 15% nondenaturing polyacrylamide gel (Fig. 3.7B) which resulted in amplified bands that could be easily observed. Due to the large number and close proximity to one another of the DNA molecular weight markers, their sizes are only listed in the figure legend.

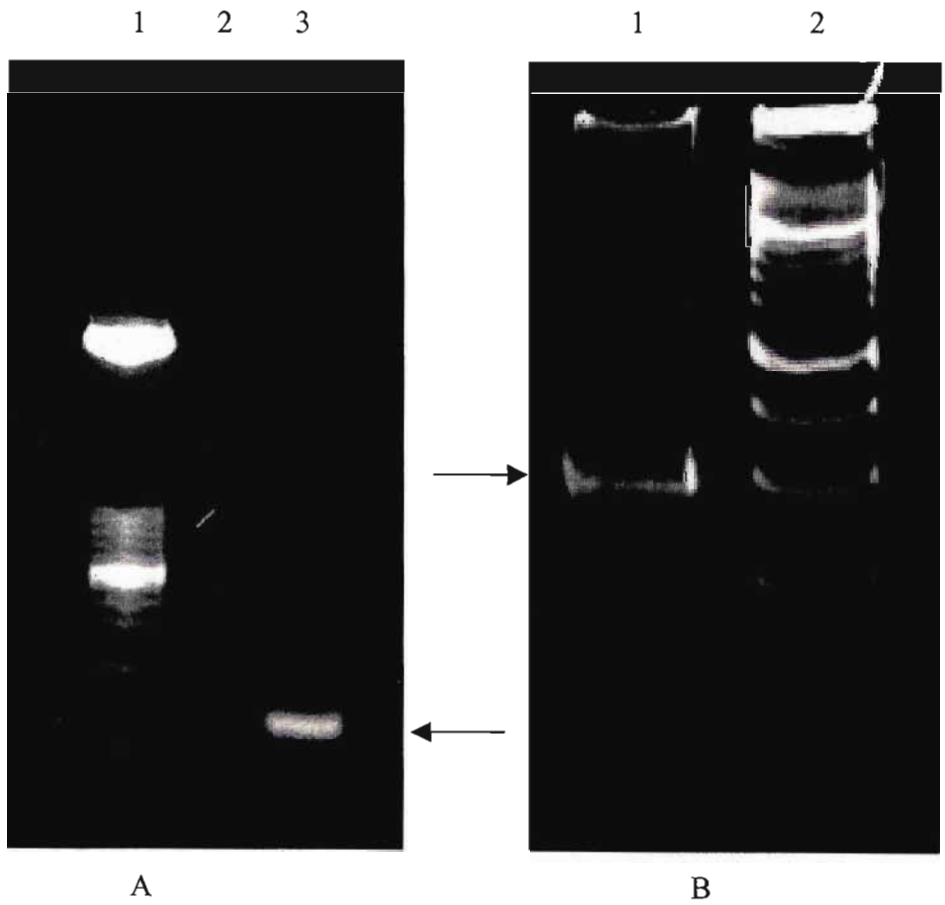


Figure 3.7 Amplification of double stranded IBDV RNA using one tube RT-PCR reaction. A) Samples were analysed on a 2% (w/v) agarose gel after 40 cycles of one tube RT-PCR and stained with ethidium bromide (Section 2.6.2). Lane 1, DNA molecular weight markers (2642 bp, 750 bp, 500 bp, 250 bp 200 bp, 150 pb, 100 bp, 50 bp); Lane 2, negative control without template; and Lane 3, double stranded IBDV RNA (100 ng). B) 15% non-denaturing PAGE gel stained with ethidium bromide (Section 2.5.2). Lane 1, double stranded IBDV RNA (100 ng); and Lane 2, DNA molecular weight markers. Arrows indicate the position of the amplified product.

3.8.6 Evaluation of commercial live IBDV vaccines using one tube RT-PCR

Due to the fact that most commercially available IBDV vaccines consist of killed or attenuated viruses, they were useful for the evaluation of the one tube RT-PCR assay for the diagnosis of IBDV. Four commercial IBDV vaccines, namely NOBILS® GUMBORO D78, NOBILS® GUMBORO 228E, TAD Gumboro Vac 100ds (cloned) and TAD Gumboro Vac forte 1000ds were used in the assay. Since the RNA is inside the virus, direct amplification using the primers detailed in Fig. 3.1 was impossible. Instead, RNA first had to be isolated according to the method in Section 3.4.2. A 150 bp band was amplified in all cases (Fig. 3.8). As a result of the small size of the amplicon, the samples were also electrophoresed on a 15% nondenaturing polyacrylamide gel (Fig. 3.8B). This resulted in improved resolution of the 150 bp amplicon.

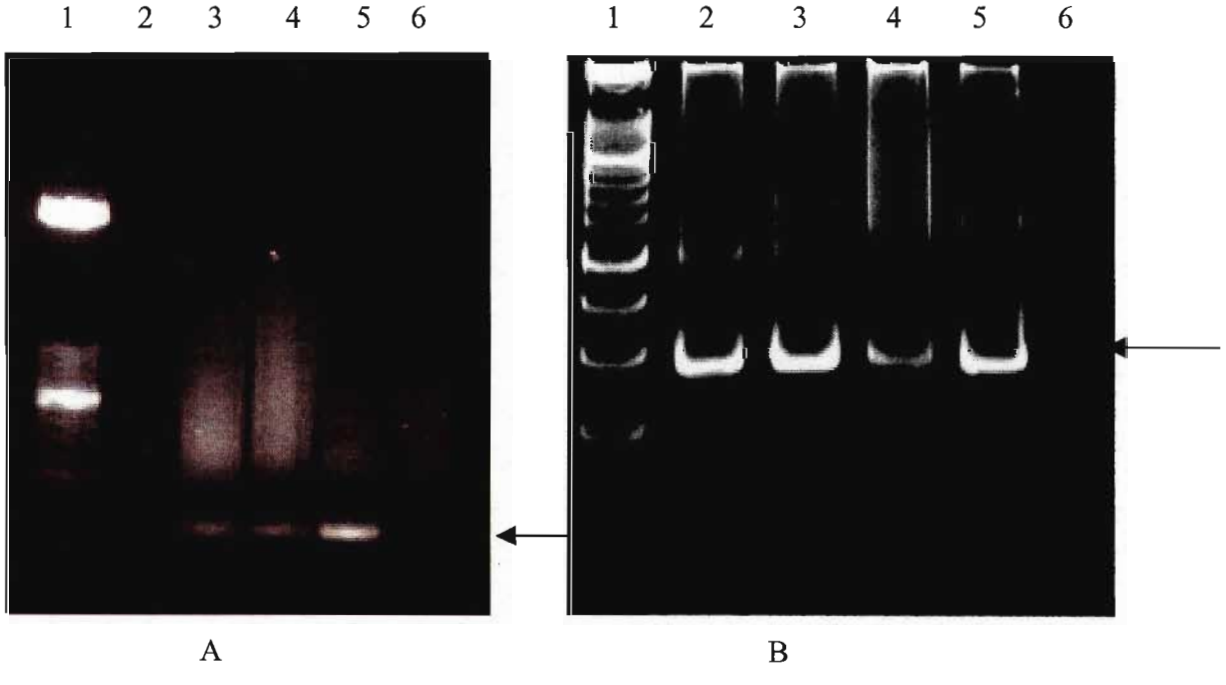


Figure 3.8 Amplification of live commercial IBDV vaccines using one tube RT-PCR. A) Samples were analysed on a 2% (w/v) agarose gel after 40 cycles of one tube RT-PCR and stained with ethidium bromide (Section 2.6.2). Lane 1, DNA molecular weight markers (2642 bp, 750 bp, 500 bp, 250 bp 200 bp, 150 pb, 100 bp, 50 bp); Lane 2, NOBILS® GUMBORO D78 (100 ng); Lane 3, NOBILS® GUMBORO 228E (100 ng); Lane 4, TAD Gumboro Vac forte 1000ds (100 ng); Lane 5, TAD Gumboro Vac 1000ds (100 ng) (cloned); and Lane 6, negative control without template. B) The same samples as in panel A, analysed on a 15% nondenaturing PAGE gel stained with ethidium bromide (Section 2.5.2).

3.8.7 *Bam*HI restriction digestion of the RT-PCR amplified IBDV product

To confirm the identity of the amplified 150 bp RT-PCR product (Section 3.8.5), restriction digestion using *Bam*HI was performed. The cleavage site of *Bam*HI in the IBDV sequence is shown in Fig. 3.9. *Bam*HI cleaved the 150 bp PCR product into two fragments of the expected size, i.e. 93 and 57 base pairs (Fig. 3.10).

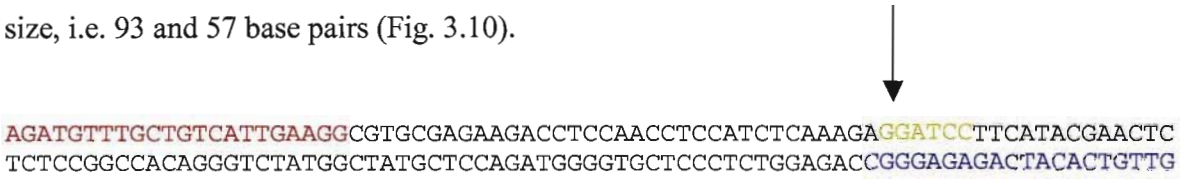


Figure 3.9 Nucleic acid sequence of the amplified 150 bp PCR product of IBDV dsRNA showing the *Bam*HI restriction site. The primer sequences are shown in red and blue. Sequences shown in yellow are duplex symmetrical sequence and the arrow indicates the *Bam*HI cleavage site.

3.9 Discussion

This chapter described diagnostic techniques for IBDV, which included IBDV purification using CsCl density gradient centrifugation, Tris-Tricine SDS-PAGE analysis of the protein profile, electron microscopic analysis of IBDV particles, dsRNA purification and RT-PCR amplification of the VP2 gene.

Despite reports that CsCl purification affects the stability of some purified viruses (Chen and Ramig, 1992), IBDV purification using CsCl density gradient centrifugation was successful giving a satisfactory yield of viral suspension. This was confirmed by Tris-Tricine SDS-PAGE analysis (Fig. 3.4), which indicated that the isolated virus was probably IBDV showing five viral proteins namely VP1, VP2, VP3, VP4 and VP5 with approximate molecular weights of 91, 42, 32, 28 and 17 kDa respectively, which agree with reported values of Schöder *et al.* (2000). This result is also similar to that reported by Boot *et al.* (2000a) showing that IBDV has dsRNA that is divided into two segments, A and B. The A-segment (3.3 kb) contains two partly overlapping open reading frames (ORFs). The first, smallest ORF encodes the non-structural viral protein 5 (VP5, 17 kDa). The second ORF encodes a polyprotein (110 kDa), which is autocatalytically cleaved into VP2 (42 kDa), VP4 (28 kDa) and VP3 (32 kDa). The

B-segment (2.9 kb) contains one large ORF, encoding the 91 kDa VP1 protein. VP2 and VP3 are the major viral proteins, with VP2 on the outer surface of the virus and it is believed to be a viral attachment protein (VAP) to the receptor on the surface of bursal cells.

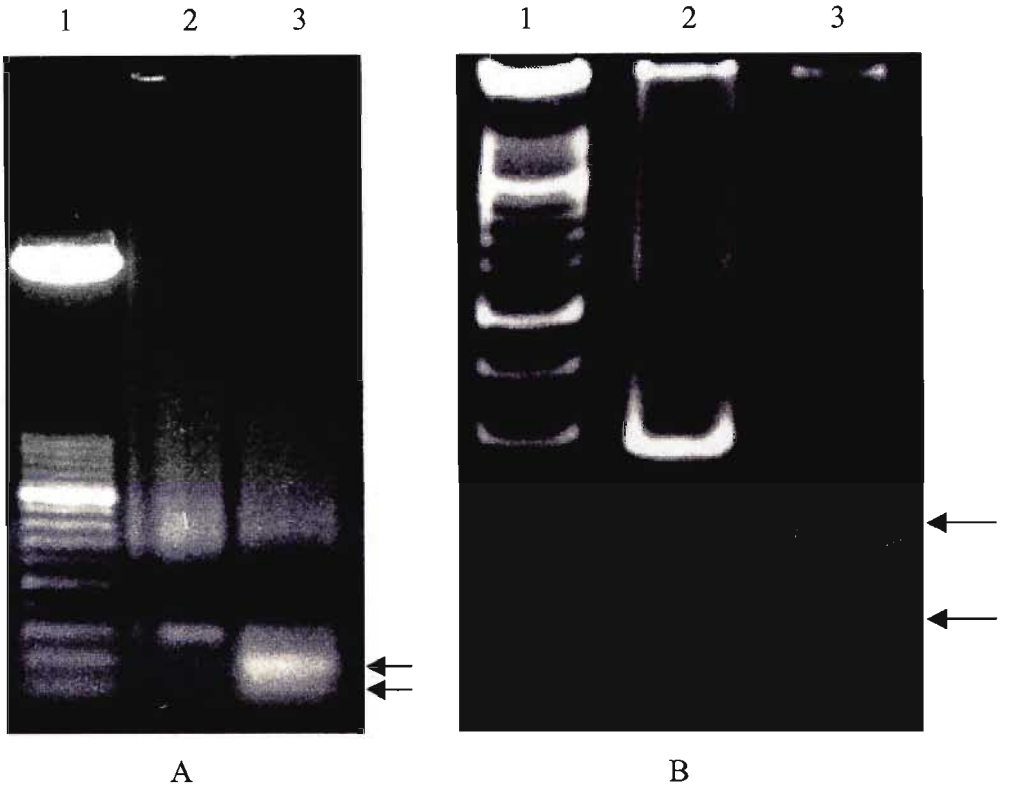


Figure 3.10 **Restriction digestion of IBDV 150 bp PCR product using *Bam*HI.** A) Restriction digestion samples were analysed on a 2% (w/v) agarose gel and stained with ethidium bromide (Section 2.6.2). Lane 1, DNA molecular weight markers (2642 bp, 750 bp, 500 bp, 250 bp, 200 bp, 150 pb, 100 bp, 50 bp); Lane 2, undigested 150 bp PCR product; and Lane 3, digested PCR product. B) The same samples as shown in panel A were analysed on a 15% nondenaturing PAGE gel stained with ethidium bromide (Section 2.5.2). The positions of the 93 and 57 cleavage products are indicated by the arrows.

Since some reports revealed the instability of some strains of IBDV on banding in CsCl gradients (Böttcher *et al.* 1997), an IBDV sample obtained with centrifugation in a 40% (w/v) sucrose cushion was used for electron microscopy examination. The isolated viral particles showed a nonenveloped isometric structure with a diameter of *ca* 60 to 65 nm, consistent with the findings of Böttcher *et al.* (1997).

The purification of viral dsRNA proved very difficult because there was contamination by chicken ssRNA during homogenisation of bursal tissue. This was shown by the presence of single and double stranded RNAs in the agarose gel (Fig. 3.6 Lane 2). Attempts to purify double stranded viral RNA using 8 M LiCl were not successful. However, this problem was solved using the agarase I purification kit (Section 3.4.2). Another problem encountered was the effect of RNase during RNA purification. RNases are ubiquitous and cannot be easily deactivated, even through autoclaving. This problem was resolved by using DEPC (diethylpyrocarbonate), which is an RNase inhibitor in every buffer used with the exception of Tris containing buffers. This is because in aqueous solution, DEPC hydrolyses rapidly to CO_2 and ethanol, with a half-life in phosphate buffer of *ca* 20 min at pH 6.0 and 10 min at pH 7.0. This hydrolyses is greatly accelerated by Tris and other amines, which themselves became consumed in the process. Therefore DECP cannot be used to treat solutions that contain Tris-buffers (Sambrook and Russell, 2001).

The purified dsRNA of IBDV had a size of *ca* 3200 bp (Fig. 3.6 lane, 3) and its double stranded nature was shown with acridine orange staining. These results match the findings of Azad *et al.* (1985). Acridine orange can either intercalate into double stranded helical nucleic acids (green fluorescence at 530 nm) or bind electrostatically to phosphate groups of single-stranded molecules (red fluorescence at 640 nm) (McMaster and Carmichael, 1977). The size of contaminating red staining chicken ssRNA (350 bp) matches with the size reported by Delany (2000).

Finally to make sure that the virus being used throughout the present study was IBDV, an additional powerful and sensitive diagnostic method was performed. The Titan® one tube RT-PCR reaction was carried out to amplify viral RNA using specific primers. The primers were

from the highly conserved VP2 genome region of IBDV and a 150 base pair amplified PCR product was evident both on 2% (w/v) agarose and 15% nondenaturing PAGE gels (Fig. 3.7). This result matches with the size reported by Wu *et al.* (1992). Titan® one tube RT-PCR amplification was also performed on commercial IBDV vaccines to test for the presence of IBDV and the result was positive (Fig. 3.8). The ability of the oligonucleotide primers to bind to sequence within the genomic RNA of IBDV to initiate amplification by PCR indicated that the oligonucleotide primers were relatively specific for IBDV (Wu *et al.*, 1992). Barlic-Maganja *et al.* (2002) and Wu *et al.* (1992) used uninfected bursae as a negative control. In addition to that Wu *et al.* (1992) included genomic nucleic acids of turkey hemorrhagic enteritis virus, infectious bronchitis virus and reovirus. Due to their tropism to chickens, genomic nucleic acids of Newcastle disease, Marek's disease and infectious bronchitis should have been included as further negative controls.

Live vaccines were used in this experiment, which contain lyophilised IBDV. The vaccine virus is grown in culture containing either allantoic fluid from eggs or tissue culture medium. During purification of viral RNA from the vaccines there might be some contamination of chicken RNA. This could result in the smeared banding patterns of the purified RNA (result not shown) and amplified RT-PCR product (Fig. 3.8)

To determine the correctness of the 150 bp amplified PCR product, it was subjected to restriction digestion with a specific restriction endonuclease (*Bam*HI). B*am*HI, in the presence of magnesium ions, cleaves between the guanine bases (GG) in the duplex symmetrical sequence (restriction site) 5'-GGATCC-3' that is present in the IBDV VP2 gene sequence (Hensley, 1990). The enzyme cleaved the PCR product to produce digestion fragments of the expected sizes of 93 and 57 bases (Fig. 3.10).

The PCR technique is an exquisitely powerful and sensitive method for diagnosis of IBDV. In the present study it was possible to detect IBDV in as little as 2 ng/μl of viral RNA from infected bursal tissue by ethidium bromide staining after 40 cycles of PCR. This technique is based on the principle that DNA is amplified exponentially by repeating cycles of heat denaturation, annealing, and primer extension. Theoretically, if PCR is amplified for 50 cycles, the original

DNA template will be amplified 1.13×10^{15} times. Reports indicated that for the detection of most viruses, the optimum sites for PCR primers should be within sequences that are unique to the viral genome in so far that they are conserved among all isolates or strains of the virus (Wu *et al.*, 1992). This report is supported by that by Barlic-Maganja *et al.* (2002), where oligonucleotides for RT-PCR were selected from the highly conserved VP2 genome region of IBDV. The forward primer corresponding to nucleotide positions 733-756 and the reverse primer corresponding to nucleotide positions 1212-1189 were selected to amplify 479 base pairs. In the present study the forward (sense) primer corresponded to nucleotide positions 1760-1782 and the reverse (anti-sense) primer corresponded to nucleotide positions 1910-1890 of the highly conserved VP2 genome region to amplify 150 base pairs.

All the above diagnostic methods confirmed that the purified virus was indeed IBDV. Chapter 4 describes the coupling of this virus to Sepharose 4B to form a matrix for affinity purification of an IBDV receptor protein from uninfected bursal membranes.

CHAPTER 4

IDENTIFICATION OF AN INFECTIOUS BURSAL DISEASE VIRUS RECEPTOR ON THE SURFACE OF BURSAL CELLS

4.1 Introduction

A wide range of cell surface molecules serves as receptors for virus binding. Such receptors range from cell specific transmembrane proteins, such as CD4 as a receptor for HIV, to the more ubiquitous cell surface-associated carbohydrate moieties e.g. sialic acid for influenza virus (Kim *et al.*, 2002). These surface molecules are mainly responsible for the host cell specificity and the site of triggering of the infection (Petit *et al.*, 1992). Viruses exploit these cell surface components to carry out their own life cycles, mostly to the disadvantage of the host. Viral host and tissue specificity is determined by the recognition of the cellular receptors by virus particles (Rossmann, 1989). The binding of the virus to the receptor is followed by a biologically significant response, usually infection of the cell (Uncapher *et al.*, 1991; Gallagher *et al.*, 1992; Varthakavi and Minocha, 1996). Especially regarding the physiology of a virus, this step is crucial because it determines the events that follow, i.e. penetration and replication (Hennache and Boulanger, 1977; McDermott *et al.*, 2000). Therefore, to understand the pathogenesis, mode of infection and the detailed mechanism of virus-cell interaction, it is very important to identify its corresponding receptor and to characterise the molecular forces involved in viral binding (Gershoni *et al.*, 1986; Wang *et al.*, 1991).

Identification of virus receptors has proven to be very difficult due to the following: (i) virus particles, in addition to binding to cells via specific cell receptors in a biologically relevant manner, also bind non-specifically followed by internalisation through fluid phase or constitutive endocytosis; (ii) the presence of low numbers of specific receptors on the surface of host cells; (iii) the possibility of multiple receptors for a single virus, as in the case of human immunodeficiency virus 1 (HIV-1) and herpes simplex virus (HSV), and (iv) involvement of intermediate molecules (Varthakavi and Minocha, 1996). Due to the fact that binding of the virus to the receptor plays a major role in determining penetration and tissue tropism, identification of the IBDV receptor on the surface of bursal cells is essential to understand the molecular

mechanism of IBDV infection and facilitate implication of pathogenesis and the design of antiviral agents that can intervence early in viral infection (Ogawa *et al.*, 1998b).

The objective of the part of the study reported in this chapter was the purification of an infectious bursal disease virus receptor identified previously in this laboratory on the surface of bursal cells (Edwards, 2000). The receptor was subsequently used for an inhibition assay to examine if inhibition of IBDV binding to the receptor was possible using anti-IBDV receptor peptide antibodies (Chapter 5). Membrane proteins were isolated from uninfected bursal tissue according to the method of Nieper and Muller (1998). IBDV was coupled to a Sepharose 4B chromatography matrix according to the method of Hennache and Boulanger (1977), for affinity purification of the viral receptor protein from uninfected bursal membranes.

4.2 Isolation of membrane proteins

The most important step for structural and functional characterisation of receptor proteins is the availability of high-level expression of cell surface receptors and preparation of sufficient membrane fractions. For this purpose membrane fractions containing the receptors of interest at a high density must be used. Such high receptor densities are only in rare cases directly available from natural sources (Pick *et al.*, 2003). It is important that prior to purification proteins should be solubilised from their tissue, biological membranes or inclusion bodies (Vuillard *et al.*, 1995). Membrane proteins include peripheral and integral membrane proteins (Thomas and McNamee, 1990). There is no clear indication yet whether the IBDV receptor on the surface of bursal cells is a peripheral or integral membrane protein. Thus the whole complement of bursal membrane proteins was isolated for the affinity purification of IBDV receptor protein.

4.2.1 Peripheral membrane proteins

Peripheral membrane proteins are surface proteins, hydrophilic in nature and bound to the surface through electrostatic interactions. They are water-soluble and can be easily purified by usual chromatographic methods. Peripheral membrane proteins should be prepared under isosmotic conditions i.e. 0.15 M NaCl. Ionic strength of the solution is very crucial, because high ionic strength may cause premature solubilisation and low ionic strength non-specific association of

peripheral membrane proteins respectively (Thomas and McNamee, 1990). However, if the samples are intended for electrophoretic evaluation or ion exchange chromatography a very low salt concentration is required (Vuillard *et al.*, 1995).

During the purification of peripheral membrane proteins the following conditions have to be considered. Gentle conditions including low-ionic-strength buffers, 0.1-1 mM EDTA, or high-ionic-strength solutions with or without up to 1 M NaCl or KCl. To avoid permanent protein denaturation, the pH should be in the range of 6-8 and isolation steps should be performed in the cold. Due to their chaotropic and detergent-like solubilising properties, anions, such as iodide and diiodosalicylate, have to be avoided (Findlay, 1990). Disruption of the membrane by detergents is always accompanied by protease activation. For example, EDTA and thiol reagents may activate proteolytic enzymes making the inclusion of protease inhibitors essential (Kaiser *et al.*, 2002).

Some of the functional inhibitors include EDTA and ethylene glycol-bis (β-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA) (0.1-5mM), which inhibit divalent cation-dependent proteases (Thomas and McNamee, 1990). Phenylmethylsulfonyl (PMSF), diisopropylphosphofluoride (DFP) and 3,4-dichloroisocoumarin (3,4-DCI) inhibits serine proteases. Due to its toxicity and short half-life in aqueous solutions at 25°C of only 35 or 110 min at pH 8.0 and 7.0 respectively, PMSF should be prepared just before use and handled with care (Findlay, 1990). However, this problem can be solved by the use of 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF), like PMSF is an inhibitor of serine protease but more stable at physiological pH than PMSF (Markwardt *et al.*, 1974). Pepstatin A and [*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-amido(4-guanidino)butane] (E-64) are added to inhibit aspartic and cysteine proteases respectively (Salvesen and Nagase, 1989). Dithiothreitol (DTT, 0.1-1 mM) is often added to prevent oxidation. Since most of these compounds have the potential to covalently modify and/or alter the activity of membrane proteins, caution is advised when using them (Thomas and McNamee, 1990).

Many peripheral membrane proteins are not recovered by the above gentle conditions and they require harsher treatments, which result in irreversible protein denaturation. Such treatments include 6 M guanidine hydrochloride, 8 M urea, 1 mM p-chloromercuribenzoate, dilute acids, pH

2.0-3.0 and dilute alkali, pH 9.5-11. However, because of its precipitant behaviour, which could interfere with membrane recovery, alkali treatment is favoured ahead of acidic conditions (Findlay, 1990; Josic and Zeilinger, 1996).

4.2.2 Integral membrane proteins and their isolation using detergents

Integral membrane proteins, however, are proteins having both hydrophilic and hydrophobic regions, with hydrophilic regions exposed to the aqueous environment and hydrophobic regions embedded in between the lipid matrix. (Thomas and McNamee, 1990). To analyse the biological activity of integral membrane proteins, proteins should first be removed from their associated lipid in the plasma membrane. These methods depend heavily on the use of detergents and the nature of the detergent has a significant influence on the success of the purification (Diaz *et al.*, 1992; Hammond and Zarenda, 1996).

Lipids can be extracted from the matrix using organic solvents, but this can cause protein denaturation. Chaotropic agents, urea, guanidine and enzymatic digestions have also been applied, but these methods are not efficient enough for membrane proteins, which are more strongly bound to the lipid matrix. For such aggregate molecules the use of detergents such as synthetic detergents, bile salts and saponins is suitable (Helenius and Simons, 1975). Even in the presence of detergents membrane proteins have a tendency to form aggregates, which affects the efficiency of the purification method. Therefore choice of non-denaturing and effective solubilising detergent is essential to determine the minimum ionic strength, which is suitable for the protein of interest to be in consistently aqueous state and the maximum ionic strength, which will not affect protein aggregation (Von Jagow *et al.*, 1994).

Detergents are relatively small molecules possessing both hydrophilic and hydrophobic portions, which are soluble in an aqueous environment through their hydrophilic portion. If they are present in excess amounts, molecules associate through their hydrophobic portions into thermodynamically stable aggregates (called micelles), which are in equilibrium with the free monomers. Membrane solubility can be achieved by addition of detergents; when added to membranes they swiftly separate into membrane bilayers until a point is reached whereby the

membrane is no longer stable and begins to split up. If adequate amounts of detergent are added the membrane is ultimately solubilised into its lipid and protein components (Findlay, 1990).

However, different detergent properties need to be considered when selecting a detergent for a particular application, i.e. (a) purity, stability, and characterisability; (b) solubility in aqueous media; (c) removability; (d) minimum interference with other assays (e.g., protein and binding assay); (e) cost; (f) optical transparency; (g) non-ionic or zwitterionic character (meaning that the protein will carry the same charge as the native protein and that the pH at which the detergent can be used is not restricted); and absence of phase separation (Shepherd and Holzenburg, 1995).

To facilitate events such as solubilisation, penetration of enzymes and other reagents, chromatography, aliquoting, and accurate concentration determinations, membrane protein purification should regularly take place in detergents (Stutzenberger, 1992; Staudinger and Bandres, 2000; Kraft *et al.*, 2001). However, removal of detergents from purified membranes is a critical step for the restoration of membrane biological activity (Vuillard *et al.*, 1995; Hannam *et al.*, 1998).

4.3 Preparations of bursal membranes

Due to its simplicity and rapidity the method of Nieper and Muller (1998), was used for the preparation of plasma membrane from bursal tissue. The bursa of Fabricius was excised from an area dorsal to the cloaca in the caudal body cavity (Fig. 4.1) (Nieminen *et al.*, 2001). The bursal cells were placed in a hypotonic medium to allow the cells to swell and the surface membrane to rise off the underlying cytoplasm, leaving a clear area under the surface membrane. The cells are then broken in a dounce homogeniser with a tightly fitting pestle, which acts by building up and suddenly releasing pressure (Kaiser *et al.*, 2002). A bag with a large hole through which the nucleus and cytoplasm were ejected remains. Membranes are then isolated by differential centrifugation on gradients of sucrose (Warren, 1974).

4.3.1 Materials

1 M $MgCl_2.6H_2O$. $MgCl_2.6H_2O$ (2.03 g) was dissolved in 10 ml of dist.H_2O.

Washing buffer [25 mM HEPES, 154 mM NaCl, 0.5 mM $MgCl_2$, pH 7.4]. HEPES (0.9 g), NaCl (1.35 g), and 75 μl of a 1 M $MgCl_2$ solution were dissolved in 130 ml of dist.H_2O, adjusted to pH 7.4 with NaOH and made up to 150 ml.

Homogenisation buffer [25 mM HEPES, 30 mM NaCl, 0.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1 μM pepstatin A, pH 8.0). HEPES (0.6 g), NaCl (0.175 g) and 50 μl of a 1 M $MgCl_2$ solution were dissolved in 80 ml of dist.H_2O, adjust to pH 8.0 with NaOH and made up to 100 ml. Before use, DTT (15 mg), PMSF (17 mg in 100 μl of DMSO) and pepstatin A (65 μg, 13 μl of a 5 mg/ml solution in DMSO) were added.

Restoration buffer [25 mM HEPES, 600 mM NaCl, 0.5 mM $MgCl_2$, pH 8.0]. HEPES (0.9 g), NaCl (5.25 g), and 75 μl of a 1 M $MgCl_2$ solution were dissolved in 130 ml of dist.H_2O, adjusted to pH 8.0 with NaOH and made up to 150 ml.

25% (w/v) sucrose solution. Sucrose (12.5 g) was dissolved in homogenisation buffer and made up to 50 ml with homogenisation buffer.

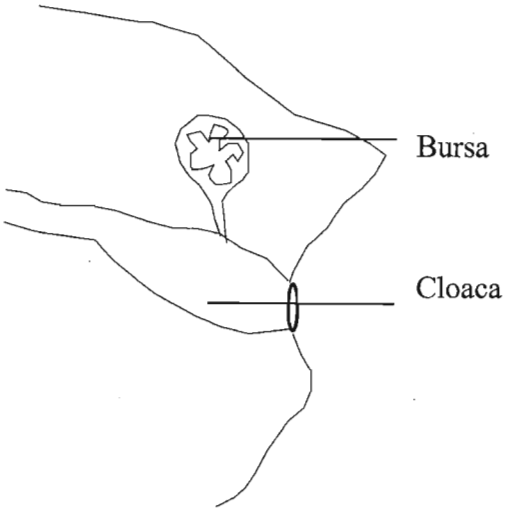


Figure 4.1 The location of the bursa of Fabricius in chickens (after Clark, 1991).

4.3.2 Method

The bursa (Fig. 4.1) was excised with scissors and washed three times in washing buffer. Bursal material (10 ml) was resuspended in a 3-fold (w/v) excess of homogenisation buffer and kept on ice for 10 min. Thereafter, the cells were disrupted by 30-40 strokes in a 'tight fitting' Dounce homogeniser. Immediately after homogenisation, restoration buffer was added in order to achieve a final NaCl concentration of 154 mM. EDTA was added to a final concentration of 1 mM. The homogenate was centrifuged (5000 *g*, 10 min, 4°C) and two volumes of the clear supernatant was layered over half a volume of a 25% sucrose cushion. After centrifugation (100 000 *g*, 1 h, 4°C) (SW40 rotor, Beckman), plasma membranes forming a white band at the interface were collected, diluted with washing buffer and sedimented by centrifugation (100 000 *g*, 30 min 4°C). The membrane pellet was resuspended in washing buffer and aliquots of the membranes were stored frozen at -20°C.

4.4 Methods for IBDV Receptor Isolation

Recently a number of viral receptors have been isolated for different infectious viruses using an array of methods. Virus receptors can be identified using anti-receptor antibodies and anti-idiotypic antibodies. Anti-idiotype antibodies are made against neutralising anti-receptor antibodies. The anti-idiotype antibodies that bind to cell membrane proteins and inhibit either virus binding and/or infection of cells usually bear the internal image of the original ligand and thus are useful for the identification and isolation of the corresponding cell surface receptors (Xue and Minocha, 1993).

The virus overlay protein-blotting assay is a more recent affinity based biochemical approach for identification of receptors (Varthakavi and Minocha, 1996). Following separation of cells or tissue by SDS-PAGE, proteins are electro-blotted to a nitrocellulose membrane and probed with virus. Membrane proteins having higher affinity for the virus are detected as bands on the nitrocellulose. The limitation of this technique is denaturation of membrane proteins, which can occur during electrophoresis. As a result the virus may not recognise the denatured receptors (Bass and Greenburg, 1991). According to the work by Edwards (2000), bursal membrane proteins were electro-blotted onto nitrocellulose and incubated with IBDV. Following incubation

with anti-IBDV antibody, the bands were detected using enzyme linked-secondary antibody (rabbit anti-chicken IgY alkaline phosphatase conjugate). The result was not absolutely accurate due to the interactions between the enzyme linked secondary antibody and the chicken bursal membrane proteins i.e. protein bands at *ca* 40 and 80 kDa were also detected in the control where no primary antibody and no virus were included in the incubations.

In the present study affinity chromatography was performed to isolate the IBDV receptor protein in uninfected bursal tissue membrane preparations. Infectious bursal disease virus was used as a ligand and attached to an activated Sepharose 4B chromatography matrix assuming that a bursal membrane protein that acts as a receptor would bind to the virus (IBDV) (Bass and Greenburg, 1991).

4.4.1 IBDV affinity column preparation

Cyanogen bromide activation of cellulose and agarose derivatives is the most commonly used method for protein immobilisation. Any polymeric matrix with cleavable vicinal hydroxyl groups can be used in this method. Agarose is a linear polymer consisting of D-galactose and 3,6-anhydro-D-galactose (Boyer, 1993). Sepharose is a trademark for beaded agarose prepared by Pharmacia. During the activation process cyanogen bromide reacts with agarose beads to form a cyclic reactive imidocarbonate, which is susceptible to nucleophilic attack by amino groups present in the ligand protein to form *N*-substituted imidocarbonates as demonstrated in Fig. 4.2. The cyclic imidocarbonate may be converted to a cyclic carbonate in aqueous solutions yielding *N*-substituted carbamates as the end product (Wong, 1991).

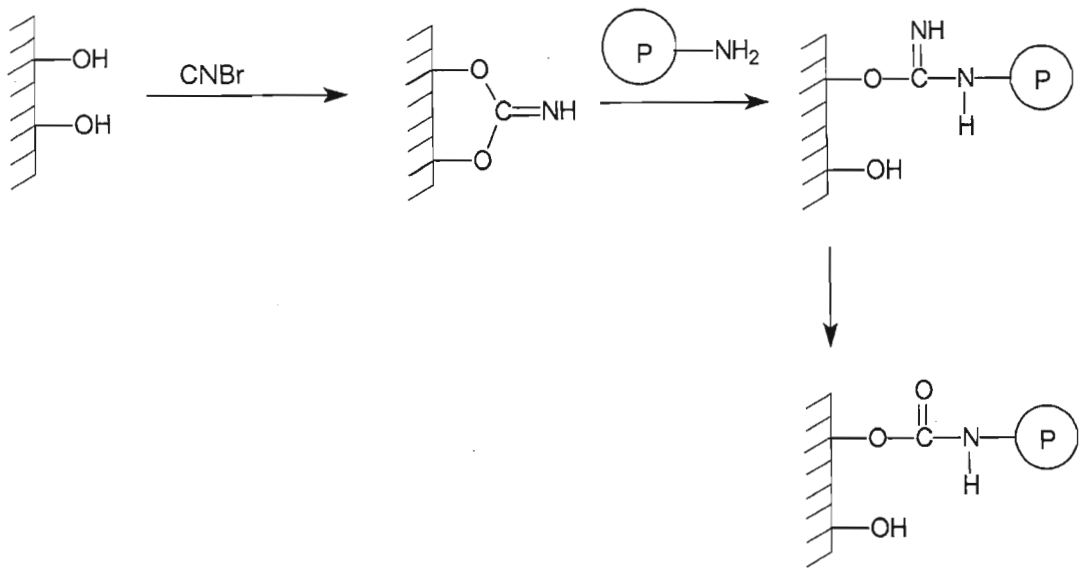


Figure 4.2 Immobilisation of proteins on cyanogen bromide activated vicinal diols on beaded agarose (after Wong, 1991). See text for details

Column preparation was performed according to the method of Hennache and Boulanger (1977) used for the coupling of adenovirus to a CNBr-activated Sepharose 4B matrix. These authors used a high salt concentration (600 mM NaCl) to elute adenovirus receptor protein from the column.

4.4.1.1 Materials

2 M sodium carbonate. Na_2CO_3 (21.19 g) was dissolved in dist.H_2O and made up to 100 ml.

Wash buffer A [200 mM $NaHCO_3$ buffer, pH 9.6]. $NaHCO_3$ (8.4 g) was dissolved in 450 ml of dist.H_2O, adjusted to pH 9.6 with NaOH, and made up to 500 ml.

Wash buffer B [200 mM $NaHCO_3$ buffer, pH 9.2]. $NaHCO_3$ (0.84 g) was dissolved in 180 ml of dist.H_2O, adjusted to pH 9.2 with NaOH, and made up to 200 ml.

Wash buffer C [100 mM sodium acetate buffer, 500 mM NaCl, pH 4.0]. Acetic acid (5.72 ml) and NaCl (14.61 g) were dissolved in 450 ml of dist.H_2O, adjusted to pH 4.0 with NaOH and made up to 500 ml.

Coupling buffer [100 mM $NaHCO_3$ buffer, 500 mM NaCl, pH 8.3). $NaHCO_3$ (4.2 g), and NaCl (14.61 g) were dissolved in 450 ml of dist.H_2O, adjusted to pH 8.3 with NaOH, and made up to 500 ml.

Blocking agent [1 M ethanolamine-HCl buffer, pH 8.0]. Ethanolamine (6.06 ml), was added to 80 ml of dist.H_2O, adjusted to pH 8.0 with HCl and made up to 100 ml.

Chromatography buffer [10 mM Tris-HCl buffer, 50 mM NaCl, 0.1% (w/v) Na-deoxycholate, 0.02% (w/v) NaN_3, pH 8.0]. Tris (1.21 g), NaCl (2.92 g), Na-deoxycholate (1 g) and NaN_3 (0.2 g) were dissolved in 950 ml of dist.H_2O, adjusted to pH 8.0 with HCl and made up to 1 litre.

4.4.1.2 Method

Sepharose 4B gel (5 ml) was washed with 150 ml dist.H_2O in a Büchner funnel, on Whatman No.1 filter paper, and transferred to a small beaker. After the gel settled the supernatant was removed. Dist.H_2O (5 ml) and 2 M Na_2CO_3 (10 ml) were added to the gel and the mixture placed on ice, in a fume hood, and mixed gently until the temperature decreased to 4°C. The rate of stirring was increased and 1 M CNBr solution in acetone was added as rapidly as possible.

The gel mixture was mixed for 75 min, transferred to a Büchner funnel and washed with 100 ml (dist.H_2O), 100 ml (wash buffer A) and 100 ml (wash buffer B). During washing, the gel was stirred gently with a glass rod to prevent it from caking during filtration. The activated gel was washed with excess coupling buffer, transferred to a glass bottle and allowed to settle. Excess coupling buffer was removed by aspiration, and approximately 10 mg of IBDV in coupling buffer was added to the suspension and mixed end-over-end for 14 h at 4°C.

The gel was allowed to settle and the excess coupling buffer, containing any unbound IBDV, was removed. Blocking agent (10 ml) was added to the remaining gel and the slurry was mixed end-over-end (2 h, RT). The gel was washed on a Büchner funnel with coupling buffer and wash buffer C, to ensure that no free ligand remained ionically linked to the gel. The gel was finally washed and suspended in chromatography buffer, packed into a column (6 cm x 0.5 cm) and washed with 10 column volumes of chromatography buffer.

4.4.2 Affinity purification of IBDV receptor proteins

4.4.2.1 Materials

Chromatography buffer. As per Section 4.4.1.1

Dissolving buffer [10 mM Tris-HCl, 50 mM NaCl, 0.5% (w/v) Na-deoxycholate, 0.02% (w/v) NaN_3, pH 8.0]. Tris (0.30 g), NaCl (0.73 g), Na-deoxycholate (1.25 g) and NaN_3 (0.05 g) were dissolved in 200 ml of dist.H_2O, adjusted to pH 8.0 with HCl and made up to 250 ml.

Chromatography buffers [10 mM Tris-HCl, 150/600 mM NaCl, 0.1% (w/v) Na-deoxycholate, 0.02% (w/v) NaN_3, pH 8.0]. Tris (0.30 g), NaCl (2.19/8.77 g), Na-deoxycholate (0.25 g) and NaN_3 (0.05 g) were dissolved in 200 ml of dist.H_2O, adjusted to pH 8.0 with HCl and made up to 250 ml.

Dialysis buffer [10 mM Tris-HCl, 50 mM NaCl, 0.02% (w/v) NaN_3, pH 8.0]. Tris (1.21 g), NaCl (2.92 g) and NaN_3 (0.2 g) were dissolved in 950 ml of dist.H_2O, adjusted to pH 8.0 with HCl and made up to 1 litre.

4.4.2.2 Method

Bursal membrane preparations (Section 4.3.2) (10 mg) were solubilised by mixing with 10 ml of dissolving buffer by stirring for 30 min at 4°C in order to prevent the aggregation and precipitation of proteins (Thomas and McNamee 1990). The total volume of solubilised membranes were passed through the column once and cycled through the column overnight at 4°C (flow rate 10 ml/h). Following elution of unbound proteins with chromatography buffer, loosely and tightly bound proteins were eluted with the same buffer containing 150 and 600 mM NaCl respectively. Elution of protein was monitored by the absorbance measuring the absorbance at 280 nm of 1 ml samples. The peak corresponding to the fractions eluted with 600 mM NaCl was pooled and dialysed overnight against dialysis buffer and concentrated using PEG 20 000 (Section 2.3.1). The concentrated fraction was further concentrated with SDS-KCl precipitation (Section 2.3.2.2) prior to analysis by reducing Tris-Tricine SDS-PAGE (Section 2.4.4).

4.5 Results

4.5.1 Tris-Tricine SDS-PAGE analysis of bursal membrane purification

Bursal membrane proteins were isolated from uninfected bursal tissue according to the method of Nieper and Muller (1998) using homogenisation and sucrose density gradient centrifugation. Following isolation the membrane proteins were analysed by Tris-Tricine SDS-PAGE. This showed that the bursal membrane contains a number of receptor proteins with molecular weights in the range between 94 and 14 kDa (Fig. 4.3). In order to determine which of the membrane proteins acts as a receptor for infectious bursal disease virus, affinity purification was done on an IBDV-Sepharose 4B column.

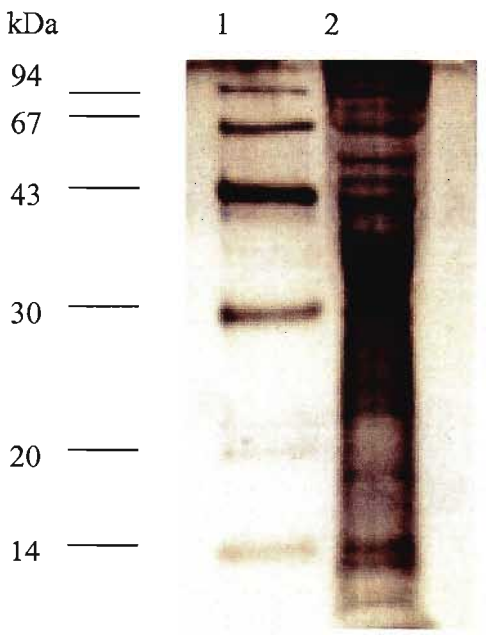


Figure 4.3 Tris-Tricine SDS-PAGE evaluation of bursal membrane protein isolation. Sample (5 μg) was boiled for 90 s in 20 μl of reducing treatment buffer and separated on 10% Tris-Tricine SDS-PAGE gel. Proteins were visualised by silver staining (Section 2.7.2.2). Lane 1, molecular weight markers, as in Fig. 3.3 and Lane 2, isolated membrane proteins from non-infected bursal tissue.

4.5.2 IBDV receptor protein isolation using IBDV affinity chromatography

Infectious bursal disease virus was coupled to a Sepharose 4B chromatography matrix for affinity purification of IBDV receptor proteins. Membrane preparations isolated from virus free bursal tissue were circulated overnight through the affinity column at 4°C. It was assumed that proteins in the mixture not having affinity to the virus would pass through the column, while proteins that have specific affinity for the virus (i.e. receptors) would bind to the immobilised virus and be retained on the column (Robyt and White, 1987). Following removal of impurities and non-specifically attached membrane proteins using 50 mM followed by 150 mM NaCl in the chromatography buffer, receptor proteins were eluted from the column using the same buffer of high salt concentration (600 mM NaCl) (Fig. 4.4). The 150 mM NaCl-containing chromatography buffer was used to remove non-specifically bound receptor proteins, since a certain proportion of viral binding is non-specific and unrelated to specific viral receptors (Tradieu *et al.*, 1982).

The elution profile showed that three peaks of membrane proteins were eluted. The first peak represents unbound membrane proteins, eluted using 50 mM NaCl chromatography buffer. The second peak represents loosely bound membrane proteins, possibly proteins that adhere non-specifically to the virus and washed from the column using chromatography buffer containing 150 mM NaCl. The last peak represents specific IBDV receptor protein eluted using chromatography buffer of a high salt concentration of 600 mM NaCl as shown by Tris-Tricine SDS-PAGE (Section 4.5.3).

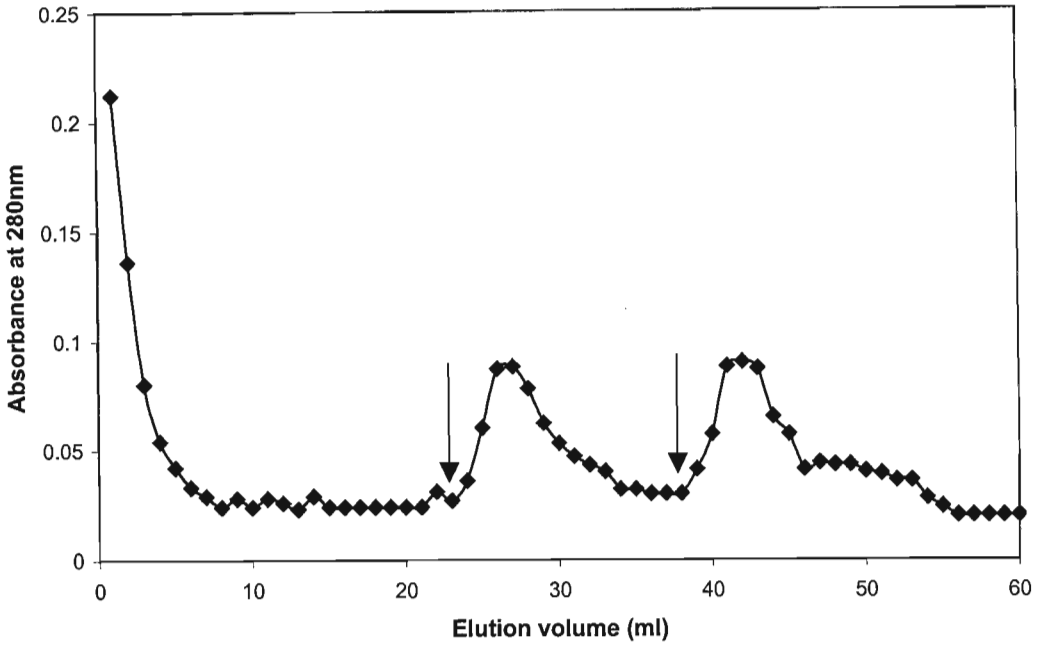


Figure 4.4 Affinity purification of IBDV receptor protein on an IBDV affinity column. Solubilised bursal membranes were circulated overnight through the column (3 ml bed volume, equilibrated in chromatography buffer) at 4°C with a flow rate of 10 ml/h. Following elution of unbound proteins with chromatography buffer, loosely and tightly bound proteins were eluted by 150 mM and 600 mM of NaCl in the same buffer. Arrows indicate points during the elution where the NaCl concentration was increased.

4.5.3 Reducing Tris-Tricine SDS-PAGE analysis of viral receptor protein

To determine whether IBDV has a single or multi receptor sites on the surface of bursal cells, the peak corresponding to the fraction eluted with 600 mM NaCl from the IBDV affinity column (Fig. 4.4) was analysed by reducing Tris-Tricine SDS-PAGE (Fig. 4.5). Since the collected fraction was very dilute successive concentrating methods were performed using PEG 20 000 (Section 2.3.1) and SDS-KCl precipitation (Section 2.3.2.2) to attain a reasonable concentration prior to Tris-Tricine SDS-PAGE analysis. In this fraction a doublet with a molecular weight of 40 kDa was visible (Fig. 4.5, lane 2).

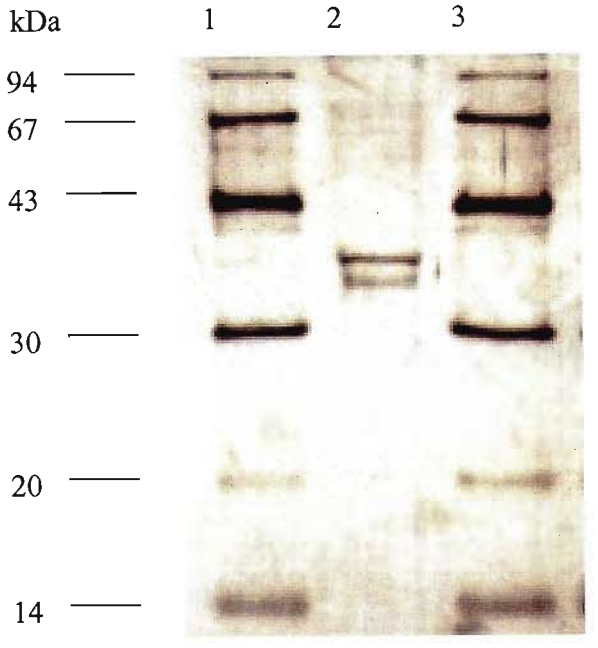


Figure 4.5 Reducing Tris-Tricine SDS-PAGE analysis of IBDV receptor protein. Sample (5 μg) was boiled for 90 s in 20 μl of reducing treatment buffer and separated on 10% Tris-Tricine SDS-PAGE gel. Proteins were visualised by silver staining (Section 2.7.2.2). Lanes 1 and 3, molecular weight markers as in Fig. 3.3 and Lane 2, purified receptor protein (the peak corresponding to the fraction eluted with 600 mM NaCl from the IBDV affinity column).

4.6 Discussion

In this chapter, an effort was made to isolate an IBDV receptor protein from the surface of chicken bursal cells. Bursal membranes were isolated, solubilised and a receptor protein purified on an IBDV-Sepharose 4B affinity column. The purified receptor proteins were subsequently analysed using reducing Tris-Tricine SDS-PAGE.

Preparation of bursal membrane protein was performed according to the method of Nieper and Muller (1998). Since solubilisation of membrane proteins using detergents activates the release of harmful proteases, inhibitors such as PMSF, had to be added prior to solubilisation to effect inhibition of serine proteases. To stabilise the nuclear membrane during incubation in hypotonic homogenisation buffer, $MgCl_2$ was used at a relatively high concentration to aggregate the lymphoid cells. A dounce homogeniser was used to homogenise bursal cells to avoid the liberation of peripheral membrane proteins that could occur when a more vigorous lysis method

such as agitation with glass beads is used (Kaiser *et al.*, 2002). Restoration buffer containing 25 mM HEPES, 600 mM NaCl and 0.5 mM $MgCl_2$, pH 8.0 (Section 4.3.1) was included to retain innate biological activities in the plasma membranes by maintaining isotonic condition of the homogenate. The presence of divalent cations after restoration can lead to aggregations of plasma membranes with the nucleus and may also activate metalloproteases (Nieper and Muller, 1998). EDTA was employed to chelate divalent cations. The isolated bursal membrane preparation obtained in the present study (Fig. 4.3) showed a similar protein banding pattern to that reported by Nieper and Muller (1998).

The biochemical technique that was applied in this section for the identification of IBDV receptor protein was a useful and specific technique. Hennache and Boulanger (1977) used a similar method for the purification of adenovirus receptor proteins using an adenovirus-Sepharose 4B affinity column. This affinity matrix has been shown to retain the biological activity of the affinity-purified products and it is relatively easy to use. In addition this approach may allow for the actual isolation and purification of receptors involved in viral attachment (Bass and Greenberg, 1991). However, the amino groups generated on the Sepharose by CNBr activation could result to lack of specificity in ligand attachment. Due to the fact that virus particles can non-specifically adhere to many substances, identification of virus receptors has proved to be extremely difficult (Varthakavi and Minocha, 1996). But this problem was improved by the three-step increment of NaCl concentration from 50 to 150 and then to 600 mM (Hennache and Boulanger, 1977). Non-specifically bound receptor proteins were eluted from the column using elution buffer with 150 mM NaCl (Fig. 4.4). Due to the possibility of a high affinity of the virus for its receptor, high salt concentration (600 mM NaCl) was expected to disrupt the interaction between the virus and the receptor. The application of this technique in the purification of monovalent virus-receptor complex is not as effective when compared to multivalent virus-receptor complexes (Bass and Greenberg, 1991). However, this method could be helpful for the isolation of specific receptor proteins of other infectious viruses.

The IBDV receptor isolated in the present study from the surface of bursal cells showed a molecular weight of 40 kDa. The 40 kDa receptor protein appeared to be fragmented showing another band below it. This could be due to the effect of denaturation by mercaptoethanol and

high temperature during sample treatment with the reducing treatment buffer prior to reducing Tris-Tricine SDS-PAGE analysis. A duplicate sample should be run in non-reducing Tris-Tricine SDS-PAGE to see if that was the reason for having a doublet. However, due to scarcity of material non-reducing Tris-Tricine SDS-PAGE was not carried out.

Previous work in this laboratory identified two possible receptor proteins on the surface of bursal cells of 32 and 40 kDa (Edwards, 2000). Nieper and Muller (1996) reported that IBDV bound to proteins with molecular masses of 40 and 46 kDa expressed on chicken embryo fibroblast cells and chicken lymphocytes by using a virus overlay protein blotting assay (VOPBA). Based on the above three independent results, the 40 kDa receptor protein seems to be the major IBDV receptor protein on the surface of bursal cells. Unlike the present study where only the doublet was observed a number of other minor bands are evident on the gels besides the major bands at 40 and 46 kDa (Nieper and Muller 1996) and at 40 and 32 kDa (Edwards 2000). Although the bursal material used in the present study was excised from young and adult chickens, no observable difference was evident in the banding pattern of the isolated receptor protein.

IBDV seems to have a receptor protein on the surface of bursal cells with a molecular weight of 40 kDa. In contrast, many other viruses exhibit possible multiple receptors e.g. HIV and herpes simplex virus. HIV uses CD4 as its main attachment receptor but also interacts with glycolipid galactosyl ceramide to mediate infection and herpes simplex virus binds to mannose-6-phosphate R and heparan sulfate (Varthakavi and Minocha, 1996; Schneider-Schaulies, 2000; Kim *et al.*, 2002). This result could encourage further inhibition studies because it would be easier to interfere with the binding of one receptor compared to inhibiting multi-receptors. The above statement could be further elaborated in such a way that if inhibition is for example using anti-receptor peptide antibodies it will require isolation, sequencing and production of anti-receptor peptide antibody for each receptor. This is expensive and using many antibodies at a time could lead to complications.

In Chapter 5, production of anti-IBDV receptor peptide antibodies in rabbits will be presented. Furthermore, an inhibition assay of IBDV binding to the receptor using anti-IBDV receptor peptide antibody will be examined in an ELISA format.

CHAPTER 5

INHIBITION OF INFECTIOUS BURSAL DISEASE VIRUS BINDING USING ANTI-RECEPTOR PEPTIDE ANTIBODIES

5.1 Introduction

Generally, antibodies have been raised against proteins purified from their natural sources or bacterially expressed fusion proteins (Danbolt *et al.*, 1998). The use of synthetic peptides has several advantages over this type of approach. It is relatively easy to prepare and allow the rapid translation of sequence information into a reagent that targets a specific protein domain (Field *et al.*, 1998). The amino acid properties of the peptide sequence must be considered in order to determine whether the synthetic peptide will have the appropriate epitope(s) that will result in immunogenicity capable of producing antibodies, which will interact with the native protein. Such properties include: hydrophilicity, areas of high segmental mobility, hydropathicity, surface accessibility and high concentration of proline residues which form part of the B turns (corners) known to form parts of epitopes (Thorpe, 1994).

It is generally recommended that in order to raise anti-peptide antibodies against small peptides it is essential to enhance their immunogencitiy by coupling them to carrier proteins (Van Regenmortel *et al.*, 1988). Moreover, when short peptide sequences are used as the immobilised antigen in solid-phase immunoassays, the use of peptide-carrier conjugates is required since peptides of 6 to 15 residues usually do not bind efficiently to plastic surfaces (Suzuki *et al.*, 1997). It is worthwhile to test if an conjugated peptide binds to the plate. The selection of carrier molecule is normally based on criteria such as availability of reactive sites, size, solubility, immunogenicity, commercial availability and cost. The commonly used carrier molecules are bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) (Verdoliva *et al.*, 1995). Currently different coupling methods are available to prepare peptide-carrier conjugates. The most frequently used coupling agents are glutaraldehyde, bis-diazotized benzidine (BDB), carbodiimides and m-maleimidobenzoyl-N-hydroxy-succinimide ester (MBS) (Briand *et al.*, 1985).

The immune response can be improved by the use of adjuvants, e.g. Freund's adjuvant can enhance the immune response by causing a 'depot' effect, i.e. allowing a slow release of antigen (Westwood and Hay, 2001). Based on the amount of serum required, source and availability of the antigen, antibodies can be raised in different experimental animals (Thorpe, 1994).

The objective of the study reported in this Chapter was the preparation of anti-peptide antibodies against a sequence in the IBDV receptor protein and block viral binding to the receptor using the anti-receptor peptide antibodies as additional proof that the receptor isolated is that for IBDV. The epitope prediction program, Predict7 (Cármenes *et al.*, 1989), was used to analyse the potential immunogenicity of the peptides from the 32 kDa receptor protein previously identified as a possible IBDV receptor protein in this laboratory (Edwards, 2000). Two peptides were synthesised, conjugated to a carrier protein, rabbit serum albumin, using MBS and immunised subcutaneously into rabbits. IgG was isolated from rabbit serum and the production of anti-peptide antibodies was monitored using ELISA with immobilised peptide. IgG was affinity purified on column prepared by immobilising the antigenic peptides. The affinity-purified antibodies were used for ELISA, western blot and assays for inhibition of viral binding.

5.2 Anti-peptide antibodies

Antibodies are the main tool for the identification, localisation, and purification of biologically interesting molecules. Furthermore, they are useful to define the structure and function of proteins, to localise the antigenic determinants contributing to idiotypic systems, and to obtain sequence specific ligands useful for the development of affinity purification strategies. Synthetic peptides are used as candidate vaccines for various infectious diseases including HIV, foot-and mouth disease, influenza, hepatitis B and cholera (Posnett *et al.*, 1988; Van Regenmortel *et al.*, 1988; Verdoliva *et al.*, 1995).

Polyclonal antibodies raised against a multivalent antigen bind to different epitopes on the antigen with a varied range of affinities. In contrast in monoclonal antibody (mAb) production, the B-cell producing a single one of these antibodies is selected and proliferated in large quantity. This results in relatively large amounts of antibody with consistent quality and characteristics (Thorpe,

1994). Monoclonal antibodies are highly specific but have the drawback that they may be non-representative of the antibody population as a whole (Westwood and Hay, 2001). Production of site-specific antibodies using synthetic peptides for various biochemical and functional studies has become a well-accepted method (Dupont *et al.*, 2003). Peptides, which represent proteins with a limited number of antigenic determinants, are very convenient for the production of anti-native protein antibodies. This implied that the peptides have a similar conformation as the sequence in the native protein (Gerritse *et al.*, 1990).

In comparison to conventional polyclonal antibody production methods the amount of useful antibody produced by synthetic peptides is relatively small, because of the restricted number of epitopes in the short peptide sequence. Secondly, due to the small size of the epitope, anti-peptide antibodies also rarely cross-react with other proteins. The other limitation is that recognition depends on the region of the protein where the peptides are from i.e. if the chosen peptide is buried within the protein, the resulting antibody may not recognise the native protein (Field *et al.*, 1998).

For anti-peptide antibody production appropriate epitopes can be designed from the protein of interest based on their hydrophilicity, areas of high segmental mobility, hydropathicity and surface accessibility. These criteria are described in detail in Section 5.3.

5.3 Designing of peptides for anti-peptide antibody production

The only structural information accessible for most of the proteins identified to date is their amino acid sequence; usually deduced from the nucleotide sequence of the corresponding gene. The need to raise specific anti-peptide antibodies and current attempts to develop synthetic peptide vaccines have emphasised the usefulness of predicting the location of continuous epitopes in proteins. Thus the accumulated information of antigenic structure of a few well-characterised proteins has served as empirical rules for predicting the position of continuous epitopes in proteins from certain characteristics of their primary structure (Van Regenmortel *et al.*, 1988).

One of the most commonly used methods for predicting the location of epitopes involves identifying regions in the polypeptide sequence that possess a high proportion of hydrophilic

residues. It is known that hydrophobic amino acids are buried inside the native structure of globular proteins, whereas hydrophilic proteins correspond to residues, which are exposed to the soluble environment at the surface of the molecule where they can interact with water. The relationship between hydrophilicity and antigenicity reproduce the general relationship between surface accessibility and antigenicity. As antibodies bind to the surface of proteins, the sequences that constitute epitopes will have a surface orientation (Van Regenmortel, 1986).

There is a correlation between mobility and surface exposure, since regions of proteins, which are buried inside the molecule tend to be less flexible than those found on the surface of the molecule. When anti-antibodies were produced against peptide fragments of the protein myohaemerythrin that represent the mobile and non-mobile regions of the molecule, the correlation between antigenicity and atomic mobility was empirical. Anti-peptide antibodies against the highly mobile residues reacted strongly with the protein whereas antibodies raised against the less mobile regions did not (Stern, 1991). Peptide sequences with a higher degree of structural variety are also more preferred as immunogens, because they may contain different structures that are unique to different genetic backgrounds (Richman and Reese, 1988).

Predict7 a computer program for epitope prediction (Cármenes *et al.*, 1989) was performed on the two amino acid sequences of the 32 kDa viral receptor protein identified and partially sequenced in this laboratory (Edwards, 2000) to see if they are immunogenic. This program analyse protein structural features using seven different algorithms. They include N-glycosylation sites, hydrophilicity, hydropathy, flexibility, surface probability, antigenicity, and secondary structure analysis. Peptide sequences with higher values of hydrophilicity, flexibility, surface probability and antigenicity are considered to be good immunogens. In this study sequences in red, replacing the residue X with H (Fig. 5.1) were used to raise antibodies in rabbits.

KXIEDG

MKLKNTLGFAIGSIIAATSFGATAQGQGAVEGELFYKKQYNDSVKHIEDG

YNPGASIGYF

FNPGARIGYFLTDDLSLNLSYDKTNHTRSNDGTGSQKIGGDTSSLTAQYH

FGQAGVDSLRPYVEGGFGHQSRGNVKADGHSGRDQSTLAIAGAGVKYYFT

NNVYARAGVEADYALDNGKWDYSALVGLGVNFGGNAGAAAPAPTPAPAPE

NLADFMAQYPATN

PTPEPEPVAQVVRVELDVKFDFDKSVVKPNSYGDVKNLADFMAQYPATNV

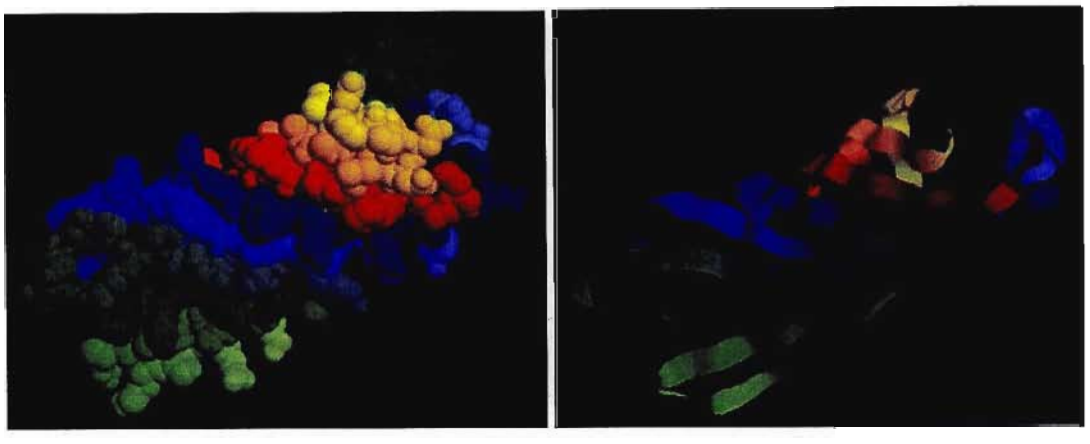
EVAGHTSIGPDAYNQKLSQRRADRVKQVLVKDGVAPSRITAVGYGESRPV

ADNATEAGRAVNRRVEASVEAQAQ

Figure 5.1 Amino acid sequence of the root adhesin from *Pseudomonas fluorescens* and internal peptide sequence of the 32 kDa chicken bursal membrane protein. Black sequence is that of the root adhesin. Sequence in red is that obtained from digestion of the 32 kDa bursal membrane protein. X, represents residues without information and the underlined sequences represent mismatches. Sequences in red are those used to raise antibodies in rabbits (Edwards, 2000).

According to Edwards (2000) the N-terminal amino acid sequence of the 32 kDa bursal receptor protein showed homology with the amino acid residues of a root adhesin from *Pseudomonas fluorescens*. In the present study a computer search using the program BLAST 2.0, from the Swiss Prot website (http://www.expasy.ch) was done to determine if any new sequences were added to the data bank since 2000 that could be aligned with the IBDV receptor sequences elucidated by Edwards (2000). Homology was still only found with the above adhesin. A computer search for structural homology was also carried out using the three-dimensional position-specific scoring matrix (3D-PSSM) software package (http://www.sbg.bio.ic.ac.uk/servers/3dpssm/). The method employs structural alignments of homologous proteins of similar three-dimensional structure in the structural classification of proteins (SCOP) database to obtain a structural similarity of residues (Kelley *et al.*, 2000). Structural homology of 95% was obtained between *Escherichia coli* outer membrane protein A transmembrane domain and *Pseudomonas fluorescens* adhesin (Fig. 5.2). Using this program a sequence identity of only 18% was obtained with the

outer membrane protein A transmembrane domain of *Escherichia coli*, which is too low for sequence alignment.



A B

Figure 5.2 The 3D structure of the outer membrane protein A transmembrane domain from *Escherichia coli* that shows structural homology with the 32 kDa IBDV receptor protein. Panel (A) shows space fill and (B) ribbon models. The individual antiparallel beta strands forming a transmembrane beta barrel are shown in separate colours.

5.4 Choice of an appropriate carrier

For antibody production by B-cells, antigens need to stimulate both B-cell and helper T-cell responses. To accomplish these criteria antigens must possess the following epitopes, (1) an epitope that can bind to the surface antibody of a virgin B-cell (this epitope will indicate the specificity of the antibody produced), and (2) on degradation must generate fragments that can induce a response from helper T-cell by stimulating binding to both class II MHC molecule and a T-cell receptor. The best way for the antigens to accomplish the above two requirements is coupling the peptides/antigens to carrier molecules (Field *et al.*, 1998). The commonly used criteria for successful carrier proteins for coupling of antigenic peptides are as follows, the potential for immunogenicity, the presence of suitable functional groups for conjugation with peptides, solubility properties even after derivatization although this is not an absolute

requirement, as precipitated molecules can be highly immunogenic, and lack of toxicity in *vivo* (Hermanson, 1996).

Because the majority of the antibody production is usually against the carrier, antibodies against the peptide of interest constitute a very small percentage. To avoid these problems antigenic peptides should be conjugated to self-proteins (Posnett *et al.*, 1988). Since the aim of this project was to produce anti-peptide antibodies against an IBDV receptor protein for studying receptor-ligand interaction, and because the one peptide appeared not to be particularly immunogenic, it was considered important to keep the antibody response to the carrier molecule to a minimum. For these reasons peptides were coupled to rabbit serum albumin (RSA) prior to antibody production in rabbits.

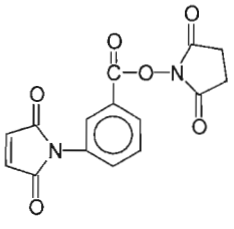
5.5 Conjugation of the IBDV receptor peptide to a carrier protein

A most important consideration during the design of a peptide for anti-peptide antibody production is the method to be used for coupling of the peptide to a carrier protein. Most coupling methods require a free amino, sulfhydryl, phenolic, or carboxylate group (Harlow and Lane, 1988). Sulfhydryl groups are found on cysteine side chains, phenolic groups on tyrosines, and carboxylate groups on aspartate, glutamate and the carboxy-terminus. Free amino groups are found on lysine side chains or on the amino-terminus. Coupling methods should be used that link the peptide to the carrier via either the carboxy- or amino terminal residue. If antibody should be prepared against the amino-terminal residues of the peptide, coupling should be performed through the carboxy-terminus of the peptide and *vice versa*. The simplest way to ensure exclusive coupling through either N-or C-terminal residue is to add an extra cysteine residue on either terminus of the peptide for MBS coupling (Harlow and Lane, 1988).

In this project all peptides were extended with an extra cysteine residue at the C-terminus to facilitate chemical coupling with MBS to RSA. MBS is a heterobifunctional cross-linking reagent (Table 5.1), which is used for the directional coupling of synthetic peptides containing a free SH group to carrier proteins in a two-step procedure (Fig. 5.3). It contains one maleimide group (which is SH-group reactive), and one *N*-hydroxysuccinimide group (which is amino reactive). The first step involves activation of the carrier that involves the reaction of MBS with the amino

groups of the carrier. Free unreacted MBS is removed before activated carrier (which now has a number of maleimide groups incorporated) is reacted in the second step with free SH groups in the peptide sample. For this purpose the peptide sample is reduced with DTT, 2-mercapthoethanol, or sodium borohydride immediately prior to coupling (Danbolt *et al.*, 1998). The reduced peptide is separated from unreacted reducing agent using molecular exclusion chromatography as described in the next section.

Table 5.1 Properties of the MBS coupling reagent (Thorpe, 1994)

Cross-linker	Structure	Reaction	Comment
M-maleimidobenzoil acid N-hydroxy succinimide (MBS) ester		Couples to SH groups on a peptide or protein	Results in a specific conjugate if a peptide has a single cysteine residue

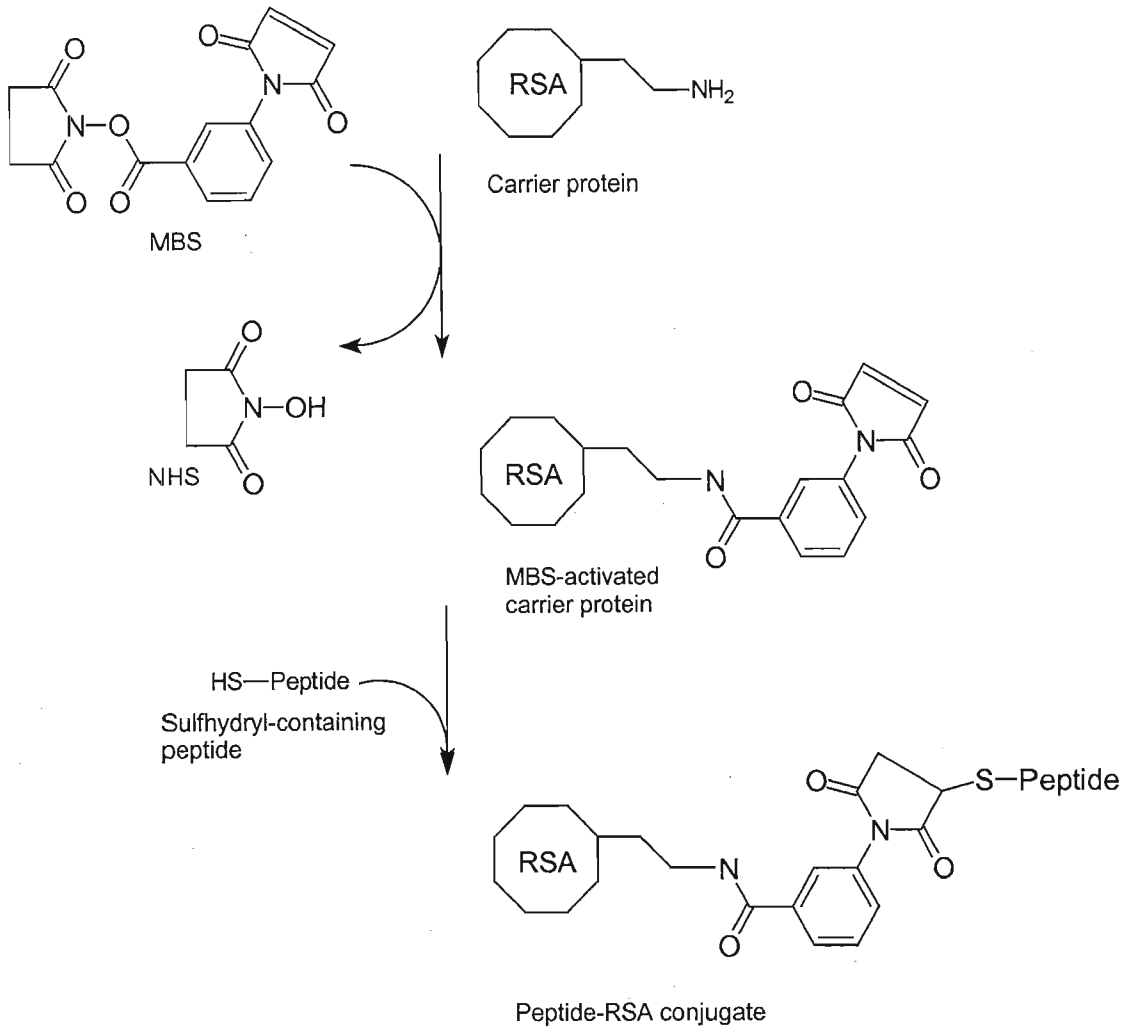


Figure 5.3 Preparation of peptide-carrier conjugate using MBS. The MBS malemeide group reacts with the primary amine group (NH_2) on the carrier molecules (RSA) to form a stable amide bond (MBS-activated carrier molecule). The MBS-activated carrier molecule targets sulfhydryl-containing peptides to form the peptide-carrier-conjugate (after Hermanson, 1996).

5.6 Molecular exclusion chromatography

Molecular exclusion chromatography was used to separate activated carrier from MBS and reduced peptides from DTT during peptide-carrier protein conjugation (Section 5.6.4) and peptide affinity column preparation (Section 5.9.3). The separation of macromolecules on cross-linked dextrans according to their size has been a common method to purify biological materials from a mixture. Separation is caused by the geometry-dependent partition of the macromolecules

between a continuous phase and the interior pores of the gel (Whitaker, 1950; Porath and Flodin, 1959; Brooks *et al.*, 2000). Following equilibration and packing of the spongelike, porous matrix/gel into a column, sample consisting of different sized molecules is applied to the column, followed by the equilibration buffer. Molecules too large to enter the small pores of the matrix move through the space between the pores and elute from the column before small molecules, which are able to enter the pores. The latter molecules diffuse in and out of the pores, which delays their movement and elution from the column (Reiland, 1971).

5.6.1 Materials

MEC buffer [100 mM $NaH_2PO_4.2H_2O$, 0.02% (w/v) NaN_3, pH 7.0]. $NaH_2PO_4.2H_2O$ (15.6 g) and NaN_3 (0.2 g) were dissolved in 950 ml of dist.H_2O, adjusted to pH 7.0 with NaOH, and made up to 1 litter.

5.6.2 Method

From the calculations made to obtain a packed bed volume of 10 ml Sephadex G-10 (4 g) or 15.5 ml Sephadex G-25 (5.5 g) dry gel were placed in separate beakers containing appropriate volumes of MEC buffer. The gel was mixed gently with a glass rod before incubating at RT overnight to allow the gel to swell. Following swelling, half the volume of the supernatant was decanted. The gel suspension was transferred into a vacuum flask containing appropriate volumes of degassed buffer respectively. Degassing was performed using a vacuum pump with occasional swirling of the flask. After the gel materials had settled, approximately 90 % of the supernatant were removed by suction in order to remove fine particles. The slurries of degassed gels were transferred into appropriate columns 1 × 12 cm for Sephadex G-10 and 1 × 23 cm for Sephadex G-25. Each column was equilibrated with at least 20 column volumes of MEC buffer before use.

5.6.3 Materials for conjugation

MEC buffer [100 mM $NaPO_4$ buffer, 0.02% (w/v) NaN_3, pH 7]. As described in Section 5.6.1

Reducing buffer [100 mM Tris-HCl buffer, 1 mM Na_2EDTA, 0.02% (w/v) NaN_3, pH 8]. Tris (1.21 g), Na_2EDTA (0.037 g) and NaN_3 (0.02 g) were dissolved in 80 ml of dist.H_2O, adjusted to pH 8 with HCl and made up to 100 ml.

Ellman's reagent buffer [100 mM Tris-HCl, 10 mM Na_2EDTA, 0.1% (w/v) SDS, pH 8]. Tris (1.21 g), Na_2EDTA (0.37 g) and SDS (1 g) were dissolved in 80 ml of dist.H_2O, adjusted to pH 8 with HCl and made up to 100 ml.

DTT (10 mM). 7.71 mg of DTT was dissolved in 5 ml of Reducing buffer just before use

Ellman's reagent (10 mM). 10 mg was dissolved in 2.5 ml of Ellman's reagent buffer just before use.

MBS (3.2 mM). MBS (1 mg) was dissolved in dimethylformamide (DMF, 200 μl). DMF must be amine free and dry to prevent undesired side reaction.

5.6.4 Method

Carrier protein (2.14 μmol, equivalent to 3.65 mg RSA) was dissolved in phosphate buffer (1664 μl Section 2.8.1). MBS (336 μl, 4.28 μmol, giving a 1:40 molar ratio of carrier: MBS) was added and stirred slowly and acylation of the carrier allowed to proceed at RT for 30 min. The activated carrier was separated from free MBS by molecular exclusion chromatography (MEC) on a Sephadex G-25 column (1 × 20 cm) pre-equilibrated in MEC buffer at a flow rate of 10 ml/h. The absorbance at 280 nm of the eluate was monitored and the activated carrier collected as the first peak. The subsequent peak, representing unreacted MBS was discarded.

Peptide sequence one (IBDVR-1) (4 mg, 2.72 μmol, giving a molar ratio of peptide to activated carrier of 40:1) was dissolved in DMSO (100 μl) and reducing buffer (900 μl) added. DTT (1 ml) was added and reduction of the peptide allowed to proceed for 1.5 h at 37°C. Reduced peptide was separated from excess DTT by MEC on a Sephadex G-10 column (1 × 13 cm) pre- equilibrated in MEC buffer at a flow rate of 10 ml/h. Fractions (1 ml) were collected in microfuge tubes and 10 μl of each fraction mixed with an equal volume of Ellman's reagent to construct the elution

profile. A yellow colour was an indication of the presence of the eluted peptide, thereafter, fractions with excess DTT, indicated by an intensely yellow colour, were eluted. The reduced peptide was immediately mixed with activated carrier and incubated for 3 h at RT. The same method was used for conjugation of peptide sequence two (IBDVR-2). Carrier protein (2.56 μmol, equivalent to 4.34 mg RSA) and MBS (403 μl, 5.11 μmol, giving a 1:40 molar ratio of carrier: MBS) were used.

5.7 Production of anti-IBDV receptor peptide antibodies in rabbits

Immunogens, such as peptide-carrier conjugates are routinely mixed with an adjuvant to improve the immune response in experimental animals. Adjuvants are immunopotentiators, which activate B and T-cell responses to elicit long lasting antibody responses (ten Hagen *et al.*, 1993). They play a major role in the immune response by increasing the persistence of the antigen in the host's immune system, that is, they allow progressive release of the antigen from the immunisation site, and protect immunogens from proteolytic enzyme degradation (Maurer and Callahan, 1980). Antigenic processing by macrophages is a crucial aspect of the immune system, hence any step, which can affect macrophage activation, will stimulate the immune response (Maurer and Callahan, 1980). The presence of bacteria, especially in Freund's complete adjuvant (Table 5.2), cause inflammation at the site of injection and infiltration by macrophages. These events enhance the immune response effectively (Diano *et al.*, 1987). For these reasons addition of adjuvants during immunisation will result in a high level of antibody production (Audibert *et al.*, 1982).

Table 5.2 Composition and use of Freund's adjuvants (Thorpe, 1994)

Adjuvant	Composition and use
Freund's complete adjuvant (FCA)	Mineral oil containing heat-killed mycobacteria (*Mycobacterium tuberculosis* or *M. butyricum*). Used as an emulsion with aqueous immunogen in the first immunisation
Freund's incomplete adjuvant (FIA)	Mineral oil Used as emulsion with aqueous immunogen for booster injections

5.8 Choice of animal

The choice of animal for immunisation depends on the amount of antibody required and on the source of the antigen. In principle it is not possible to raise antibodies using self-proteins, for example producing antibodies to rabbit proteins in rabbits. Although in rare cases this can be done with intracellular domains of membrane proteins, e.g. the C-terminal domain of the excitatory amino acid carrier glutamate transporter, which is identical in rabbit, humans, and rat (Danbolt *et al.*, 1998).

Although chickens are usually the best choice for antibody production because they are cheap, easy to handle and higher amounts of antibody can be collected from egg yolk than the equivalent volume of rabbit serum (Goldring and Coetzer, 2003). The present study required the production of anti-peptide antibodies against a chicken bursal receptor protein. Rabbits were thus used and represent a suitable alternative in terms of cost, ease of handling and relatively ease in bleeding and being good responders.

Immunogen dose depends on the type of antigen and animal species used and normally ranges from 50 to 200 μg per immunisation (Verdoliva *et al.*, 1995). Although dose of antigen is not necessarily related to the amount of antibody produced, it is essential that the amount of antigen to be injected is not very small because it can lead to tolerance towards the antigen (Florence and Hau, 1994).

Two rabbits per peptide-conjugate were immunised subcutaneously with 200 μg of peptide-RSA conjugate per immunisation per animal (Coetzer *et al.*, 1991). The peptide-conjugates were administered with FCA at a 1:1 (v/v) ratio for the first injection and with FIA for booster injections, in a total volume of 1 ml per rabbit. Rabbits were immunised five times at three week-intervals (0, 3, 6, 9 and 12). Blood was collected 9 weeks after the first immunisation by bleeding of the ear vein, and screened for anti-peptide reactivity by an indirect enzyme-linked-immunosorbent assay (ELISA) (Section 2.9.2). Sera obtained before the primary immunisation served as a negative (non-immune) control for ELISA, western blot and inhibition assays.

5.9 Affinity chromatography

Affinity purification was used to purify specific anti-peptide antibodies and the IBDV receptor protein by immobilising the synthetic peptides (Section 5.9.3) or IBDV (Section 4.4.1.2) to the respective affinity matrices. In affinity chromatography a specific type of molecule (ligand) is covalently bound to a suitable chromatography matrix, typically 4% cross-linked beaded agarose (Sepharose-4B) (Fig. 5.4). The ligand has a specific affinity for one of the molecules in the mixture to be purified. A sample with a mixture of macromolecule to be purified is applied to the column. During the circulation process molecules not having affinity for the ligand directly elute from the column, while molecules with an affinity for the ligand bind reversibly with high affinity and specificity (Robyt and White, 1987).

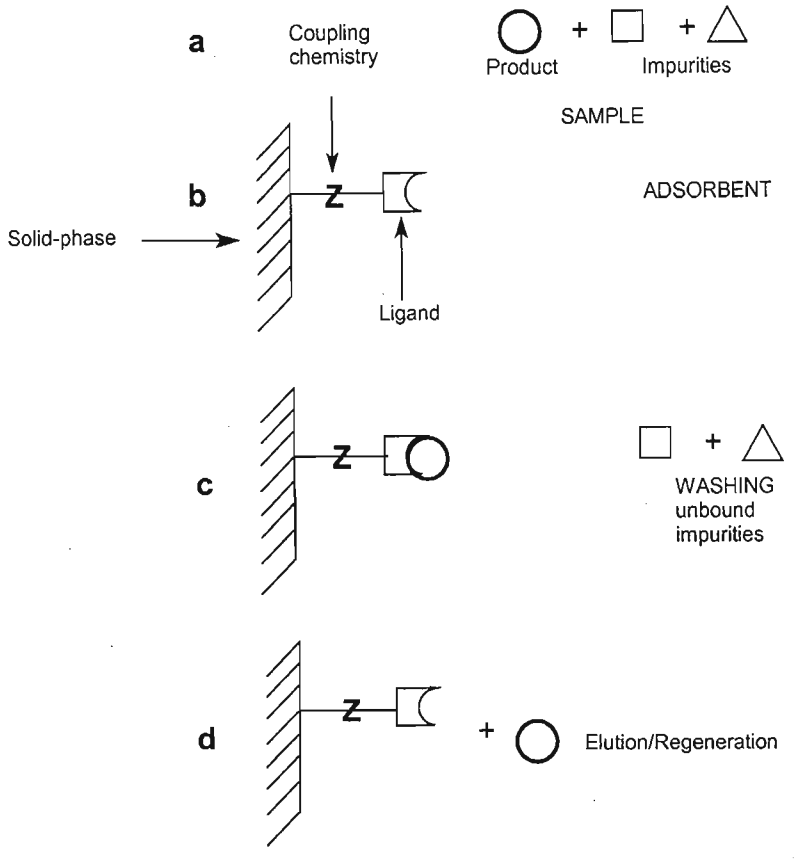


Figure 5.4 Schematic diagram illustrating the principles of affinity separation. Sample (a) comprising product and impurities is brought into contact with a solid phase (b) derivatised with a specific ligand. Product is selectively adsorbed at the expense of impurities (c) and desorbed (d) by application of an appropriate mobile phase. The solid phase is finally is regenerated for further use (after Mohan and Lyddiatt, 1997).

Following washing to remove unbound and non-specifically bound molecules, conditions are changed to elute the molecule of interest from the ligand in its natural state. Due to the fact that binding of the molecule to the ligand is as a result of electrostatic and hydrophobic interactions and van der Waals forces, samples are desorbed from the ligand by changing the pH or salt concentration (Mayers and van Oss, 1992). If elution of the molecules is not possible using the above methods, a mild denaturing agent can be used to deform the structure of the protein, hence to reduce the interaction of the molecule with the ligand. The most commonly used reagents are HCl, CNS^-, ClO^-_4, and CCl_3COO^-, but they must be used with care because they may cause permanent structural deformation (Boyer, 1993).

5.9.1 Peptide-affinity column preparation

Rabbit anti-peptide IgG preparations obtained by PEG precipitation from serum (Section 2.8.2) were purified using affinity chromatography on a column prepared by immobilising the antigenic peptides. Matrices containing iodoacetyl agents are excellent activated supports for the immobilisation of ligands containing sulfhydryl groups. The matrices will offer extremely stable thioether bonds between the ligands and matrices (Fig. 5.5). The coupling reaction with sulfhydryl ligand is very fast, taking only 15 min and can be carried out at pH 8.0-8.5 (Hermanson *et al.*, 1992). To make binding more efficient, steric hindrance can be reduced by the use of spacer arm (Pierce SulfoLinkTM data sheet).

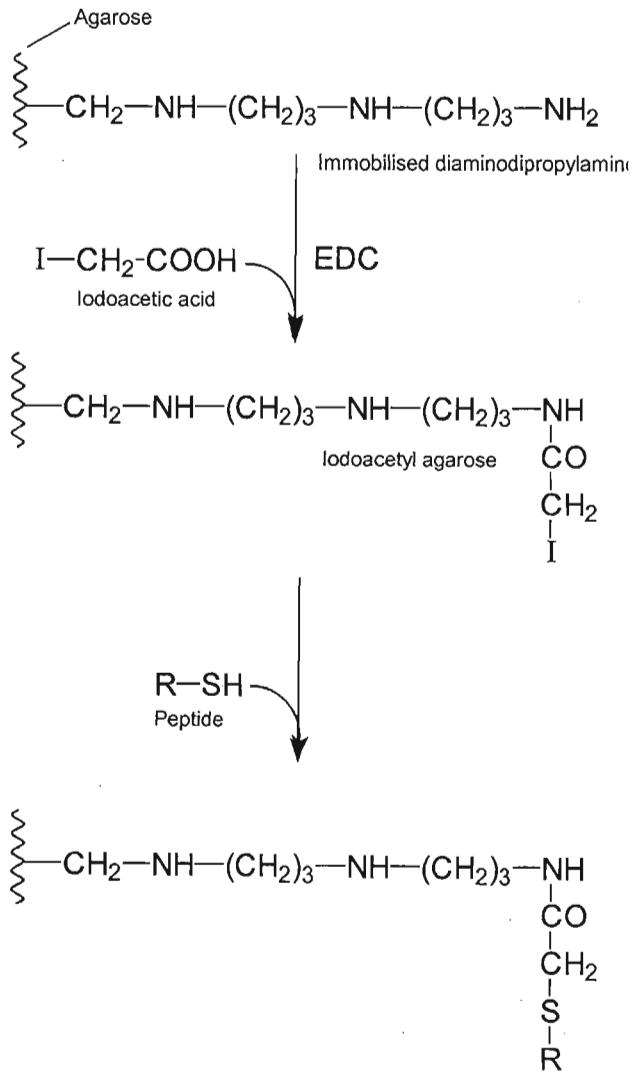


Figure 5.5 **Immobilisation of peptides to iodoacetyl-containing matrices** (after Hermanson *et al.*, 1992).

Prior to coupling, the peptides with a cysteine residue at the C-terminal were first reduced by DTT to produce sulfhydryl groups to react with iodoacetyl agarose of the SulfoLink^{TM} gel. The manufacturers, Pierce, prepare iodoacetyl agarose by the addition of iodoacetic acid to the agarose.

5.9.2 Materials

Tris buffer (50 mM Tris-HCl buffer, 5 mM EDTA pH 8.5). Tris (6.05 g) and EDTA (1.86 g) were dissolved in 950 ml of dist.H_2O adjusted to pH 8.5 with HCl and made up to 1 litre.

MEC buffer [100 mM $NaPO_4$ buffer, 0.02% (w/v) NaN_3, pH 7]. As described in Section 5.6.1

10 mM DTT. As described in Section 5.6.3

Ellman's reagent buffer [100 mM Tris-HCl, 10 mM Na_2EDTA, 0.1% SDS (w/v), pH 8]. As described in Section 5.6.3

10 mM Ellman's reagent. As described in Section 5.6.3

Blocking buffer (50 mM Cysteine, 50 mM Tris, 5 mM EDTA, pH 8.5). Cysteine (8.78 mg) was dissolved in 1 ml of Tris buffer (before use).

1 M NaCl, 0.05% (w/v) NaN_3: NaCl (58.44 g) and NaN_3 (0.5 g) were dissolved in 1litre of dist.H_2O.

0.1 M Na-phosphate buffer, 0.02% (w/v) NaN_3, pH 7.6: $NaH_2PO_4.H_2O$ (15.6 g) and NaN_3 (0.2 g) were dissolved in 950 ml of dist.H_2O, adjusted to pH 7.6 with NaOH and made up to 1 litre.

5.9.3 Method

Peptide sequence one (IBDVR-1) and IBDVR-2 (5 mg) of each were individually dissolved in 100 μl of DMSO and 400 μl Tris-HCl buffer. The peptides were reduced by adding 500 μl of 10 mM DTT to the dissolved peptide with stirring. The mixture was incubated for 1.5 h at 37°C.

The reduced peptide was separated from free DTT on a Sephadex G-10 column (1×13 cm), pre-equilibrated in MEC buffer at a flow rate of 10 ml/h. Fractions (1 ml) were collected and the elution profile was determined by the addition of Ellman's reagent (10 μl) to an equal sample volume from each fraction. The first fractions with a light yellow colour indicated the elution of the reduced peptide and an intensely yellow colour the elution of unreacted DTT peak.

The SulfoLink^{TM} gel was supplied as a 50% slurry and 2 ml of the slurry was packed into a polystyrene chromatography column. The liquid was drained from the gel until the wet cake remained. The gel was washed with 6 column volumes (6 ml) of Tris-HCl buffer. The reduced peptide was added to the gel. The gel was mixed at RT by end over end rotation for 15 min and incubated at RT for 30 min without mixing. The buffer was drained. The column was washed with three column volumes (3 ml) of Tris-HCl buffer. The non-specific binding sites on the gel were blocked by 1 ml of blocking buffer. The gel was mixed at RT by end over end rotation for 15 min and incubated at RT for 30 min without mixing. The column was washed with 16 column volumes (16 ml) of 1 M NaCl, 0.05% (w/v) NaN_3 and with 2 column volumes (2 ml) of Na-phosphate buffer. The column was stored at 4°C until use.

5.10 Modified western blot to enhance recognition by renaturation of IBDV receptor protein in the gel

Dunn (1986) reported two modifications of western blots, which enhance immunochemical recognition of proteins. The first modification is the use of carbonate blot buffer at pH 9.9, instead of the more commonly used Tris-glycine buffer at pH 8.3 during electrophoretic transfer. This alteration was reported to improve the recognition of *Escherichia coli* F1-ATPase by monoclonal antibodies. The second modification was incubation of the gel in renaturation buffer, intended to aid the renaturation of proteins, prior to the electrophoretic transfer step. According to Dunn (1986), 20% glycerol in 50 mM Tris-HCl, pH 7.4 appeared to be the most effective renaturation buffer.

5.10.1 Materials

3 mM Na_2CO_3. Na_2CO_3 (0.318 g) was dissolved and made up to 1 litre.

10 mM $NaHCO_3$. $NaHCO_3$ (0.84 g) was dissolved and made up to 1 litre.

Carbonate blot buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, 20% methanol, pH 9.9). A solution of 3 mM Na_2CO_3 (0.318 g/l) was titrated with a solution of 10 mM $NaHCO_3$ (0.84 g/l) to pH 9.9 and 20% methanol was added.

Renaturation buffer (50 mM Tris-HCl, 20% glycerol pH 7.4). Tris (6.05 g) and glycerol (200 ml) were made up to 950 ml with dist.H_2O, adjusted to pH 7.4 with HCl and made up to 1 litre.

5.10.2 Method

Following Tris-Tricine SDS-PAGE of affinity purified 40 kDa IBDV receptor protein as described in Section 2.4.4., the gel was incubated in renaturation buffer for 1 h at room temperature. At 15 min-intervals the buffer was removed and fresh buffer added. The Bio-Rad blotting equipment was assembled as described in Section 2.10.2 and the gel was blotted for 4 h at 400 mA in carbonate blot buffer. After blotting, detection of the 40 kDa IBDV receptor protein on nitrocellulose membrane was carried out using the anti-IBDVR-1 and IBDVR-2 antibodies as described in Section 2.10.2.

5.11 Inhibition assay

In the present study to determine whether the anti-IBDV receptor peptide antibodies could block IBDV binding to the receptor, an inhibition assay was performed on ELISA plate. A schematic presentation of the ELISA is given in Fig. 5.10 (the Figure is placed in Section 5.12.5 to aid interpretation of the results).

5.11.1 Materials

ELISA reagents as per Section 2.9.1

Purified 40 kDa IBDV receptor protein from Section 4.4.2.2.

IBDV from Section 3.2.2.

5.11.2 Method

The purified 40 kDa IBDV receptor protein was coated (1 μg/ml) in PBS (150 μl per well, 4°C, overnight). Non-specific binding of antibody was prevented by blocking the wells with 0.5% BSA-PBS (200 μl, 1 h, 37°C), and the plates were washed three times with PBS-Tween. Wells were incubated with serial two-fold dilutions of anti-IBDV receptor peptide antibody (panel I), IBDV (panel II), IBDV + anti-IBDV receptor peptide antibody (panel III), non-immune rabbit IgG (panel IV) and BSA-PBS (panel V) of step 2 (Fig. 5.10), starting from 100 μg/ml prepared on the plate in 0.5% BSA-PBS and incubated (100 μl, 2 h, 37°C). The plates were washed three times with PBS-Tween. The wells were incubated with serial two-fold dilutions of IBDV (panels I and IV), anti-IBDV receptor peptide antibody (panel II) and PBS (panel III and V) of step 4 (Fig. 5.10), starting from 100 μg/ml prepared on the plate in 0.5% BSA-PBS and incubated (100 μl, 2 h, 37°C). The plates were washed three times with PBS-Tween. HRPO-linked secondary antibody (goat anti-rabbit-HRPO-linked), at a suitable dilution (1 in 20 000 in 0.5% BSA-PBS) was added to each well and incubated (120 μl, 1 h, 37°C). Following washing three times with PBS-Tween, substrate solution (150 μl) was added to each well and colour was allowed to develop in the dark against the background of the controls (usually 10-15 min) and the A_{405} of each well was measured in a Titertek ELISA plate reader.

5.12 Results

5.12.1 Epitope mapping of putative IBDV receptor peptide sequences

Epitope mapping of the two peptide sequences of the 32 kDa bursal receptor protein was carried out using Predict7 to determine regions of hydrophilicity, flexibility, surface probability and antigenicity (Fig. 5.6).

Peptide sequence one (IBDVR-1) appeared to be more immunogenic than IBDVR-2. The IBDVR-1 sequence (Fig. 5.6 A) showed the highest degree of hydrophilicity, surface probability, flexibility and antigenicity towards its N-terminus, thereby suggesting that the N-terminus is more immunogenic. Conjugation to the carrier protein would thus be done via the C-terminus. The IBDVR-2 sequence (Fig. 5.6 B) appeared not to be very immunogenic overall, but slightly more hydrophilic and antigenic at the N-terminus. The peptide had almost the same values of flexibility both at its N-and C-termini. The only parameter that had higher values at the C-terminus was surface probability, but the peptide was nevertheless coupled to the carrier protein via its C-terminus. Since these peptides were immunogenic towards their N-terminal, an extra cysteine residue was added at their C-termini to facilitate coupling of peptides to carrier proteins using MBS.

IBDVR-1: Lys-His-Ile-Glu-Asp-Gly-Tyr-Asn-Pro-Gly-Ala-Ser-Ile-Gly-Tyr-Phe-Cys-NH_2

IBDVR-2: Asn-Leu-Ala-Asp-Phe-Met-Ala-Gln-Tyr-Pro-Ala-Thr-Asn-Cys-NH_2

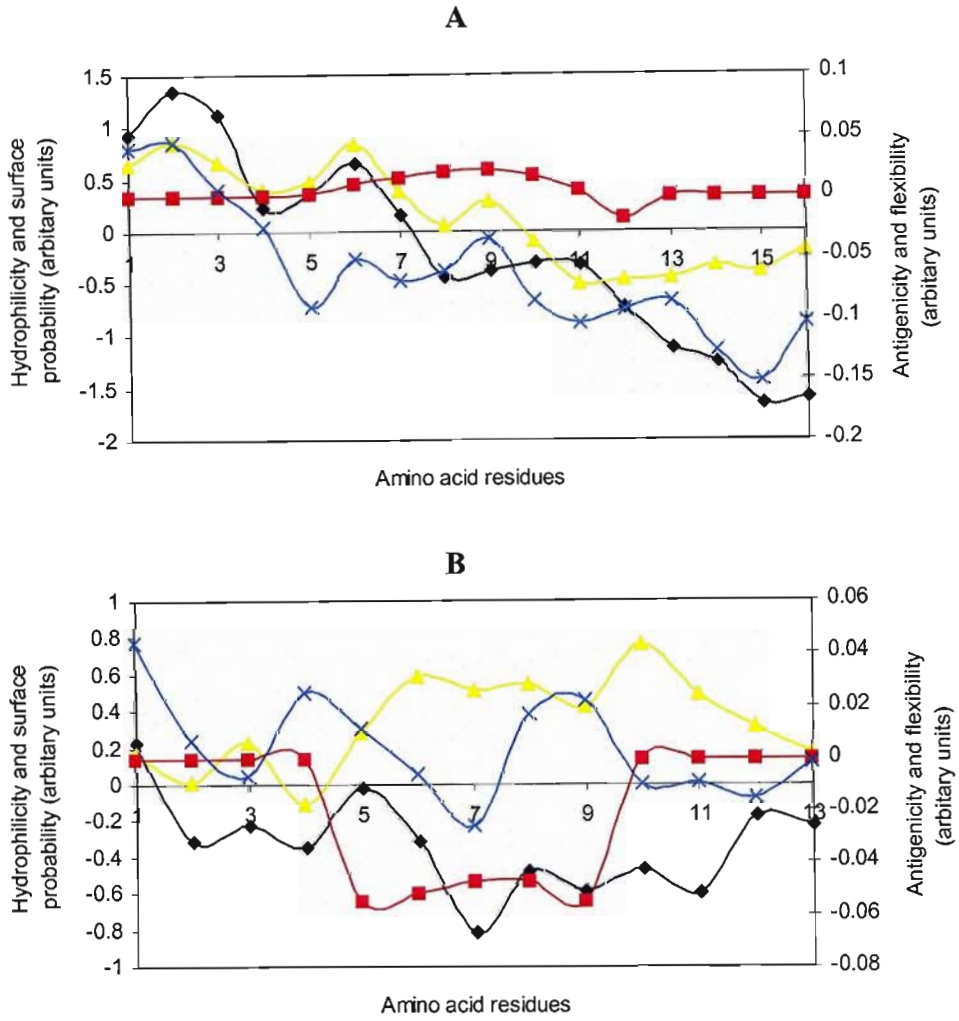


Figure 5.6 Epitope prediction plots for the 32 kDa IBDV receptor protein peptide sequences (N-terminal sequencing done by Edwards, 2000). (A) Sequence IBDVR-1, KHIEDGYNPGASIGYF and (B) Sequence IBDVR-2, NLADFMAQYPATN. The parameters plotted are: hydrophlicity (♦), surface probability (▲), antigenicity (×) and flexibility (■).

5.12.2 Evaluation of anti-IBDV receptor peptide antibody production

To assess the production of anti-peptide antibodies against the peptide sequences of the IBDV receptor protein in rabbits, ELISAs (Section 2.9.2) were used to monitor antibody production by titrating test antibodies against the free peptides coated as antigens. Although two rabbits were used for each peptide, but due to their close similarity in antibody response to the same peptide, the response of only one rabbit per peptide is shown. In the present study only antibodies from

week zero and nine were monitored because all the rabbits died before further blood samples were taken. The ELISA result showed that antibodies were produced in rabbits against IBDVR-1 (Fig. 5.7 A) and IBDVR-2 (Fig. 5.7 B) when compared to non-immune antibody production. Although IBDVR-2 appeared to be less immunogenic than IBDVR-1 when analysing using Predict7 (Fig. 5.6 B), almost the same antibody response was obtained as against IBDVR-1. Anti-IBDVR-1 antibodies appeared to have relatively higher reactivity to the peptide especially at low concentrations (6.25, 12.5 and 25 μg/ml) when compared to anti- IBDVR-2 antibodies.

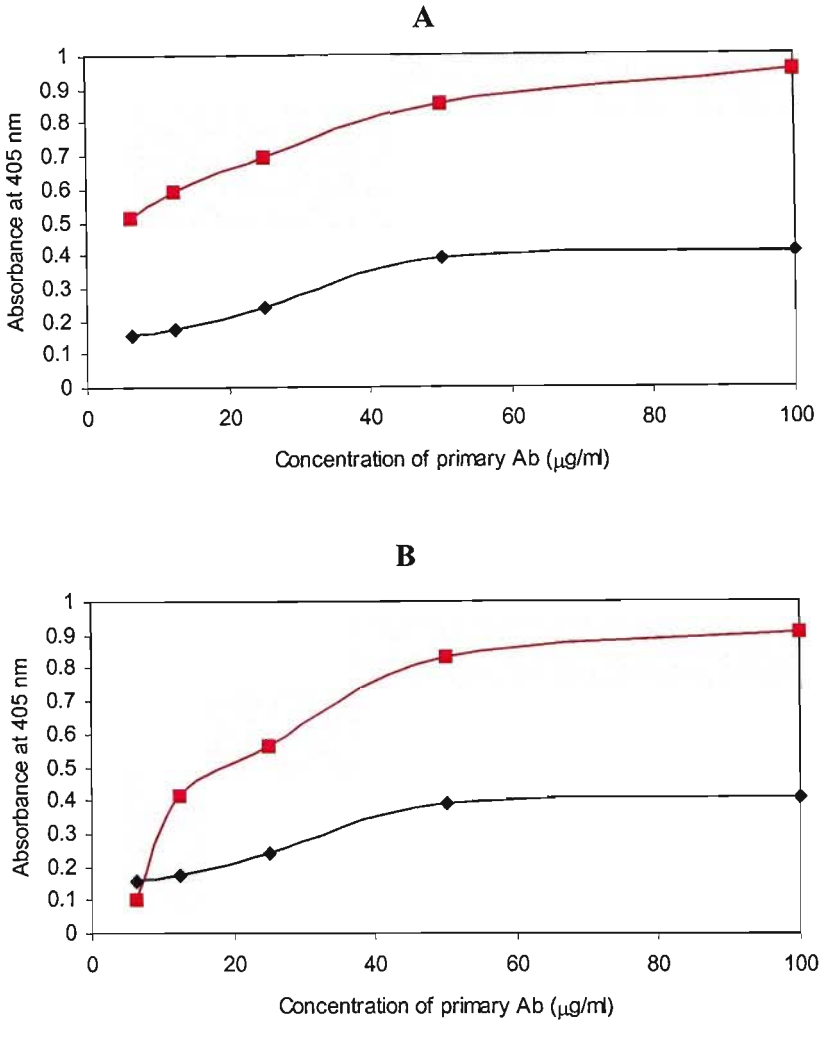


Figure 5.7 ELISAs monitoring titre of rabbit anti-IBDV receptor peptide antibodies produced against peptides corresponding to sequences in the IBDV receptor protein. Microtitre plates were coated with IBDVR-1 (panel A) or IBDVR-2 (panel B) (5 μg/ml in PBS, 4°C, overnight). Plates were incubated with serial two-fold dilutions of week zero (◆) and week 9 (■) antibodies. The concentration of the antibodies was between 100 μg/ml and 6.25 μg/ml. Antibody binding was detected using goat anti-rabbit-HRPO-linked antibodies and visualised with ABTS/H_2O_2 substrate. Absorbance values were read at 405 nm in a Titertek ELISA plate reader. Each point is the average absorbance of duplicate samples.

5.12.3 Evaluation of affinity purified anti-IBDV receptor peptide antibodies

To evaluate the reactivity of affinity purified anti-IBDV receptor peptide antibodies against their corresponding peptides, ELISAs (Section 2.9.2) were performed against the free peptides coated

as antigens. The ELISA result showed that the affinity-purified anti-IBDV receptor peptide antibodies appeared to have increased reactivity against the respective peptides (Fig. 5.8 A and B) compared to the non- affinity purified antibodies (Fig. 5.7).

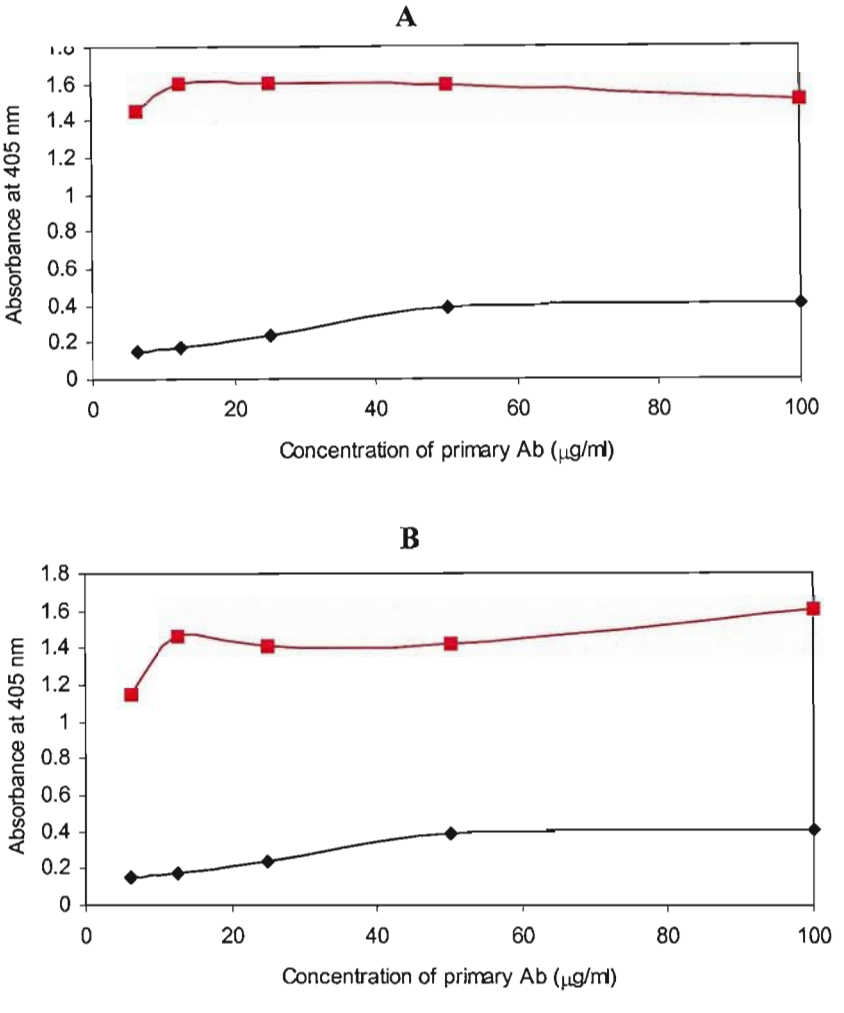


Figure 5.8 Reactivity of affinity-purified anti-IBDV receptor peptide antibodies against peptides corresponding to sequences in the IBDV receptor protein in ELISA. Microtitre plates were coated with IBDVR-1 (panel A) or IBDVR-2 (panel B) (5 μg/ml in PBS, 4°C, overnight). Plates were incubated with serial two-fold dilutions of non-immune (◆) and affinity purified anti-IBDV receptor peptide antibodies from week 9 (■). The concentration of the antibodies was between 100 μg/ml and 6.25 μg/ml. Antibody binding was detected using goat anti-rabbit-HRPO-linked antibodies and visualised with ABTS/H_2O_2 substrate. Absorbance values were read at 405 nm in a Titertek ELISA plate reader. Each point is the average absorbance of duplicate samples.

An ELISA was also carried out to determine whether the affinity purified anti-IBDV receptor peptide antibody could recognise the whole IBDV receptor protein. The affinity purified native 40 kDa IBDV receptor protein was coated on an ELISA plate. The result showed that the anti-IBDVR-1 antibodies recognised the native 40 kDa IBDV receptor protein very well (Fig. 5.9 A) but anti-IBDVR-2 antibodies showed very low recognition of the IBDV receptor protein (Fig. 5.9 B).

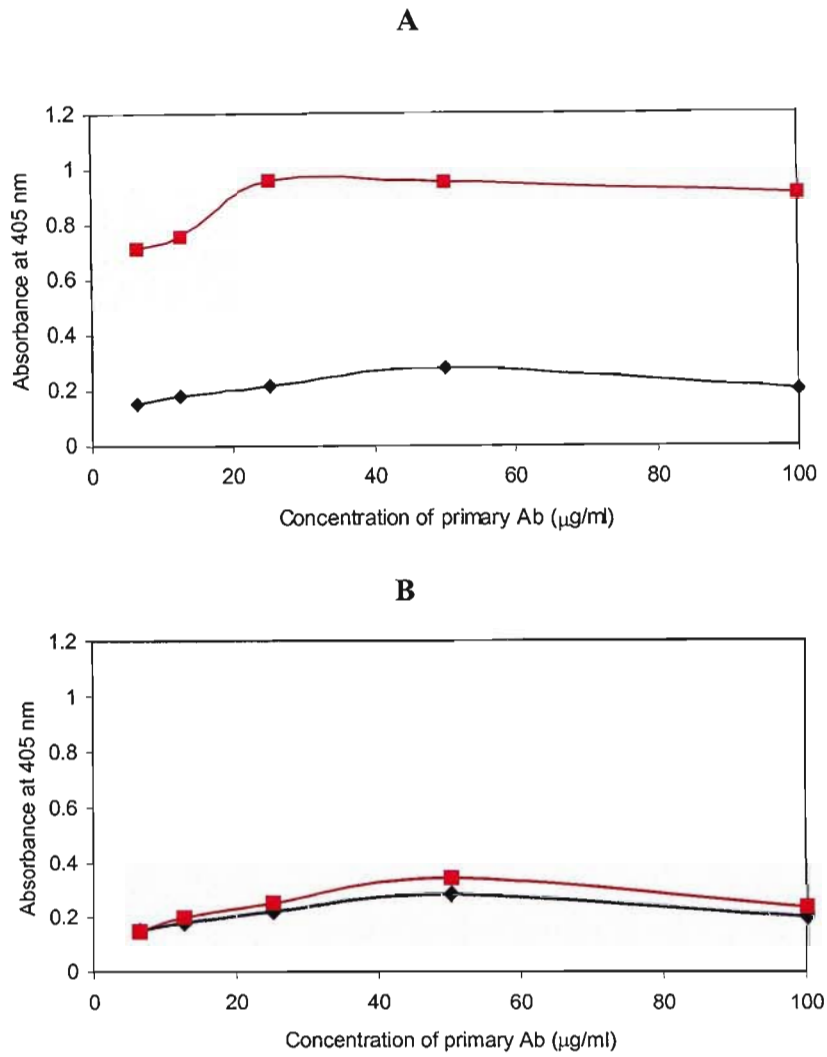


Figure 5.9 Recognition of native 40 kDa IBDV receptor protein using anti-IBDV receptor peptide antibodies in an ELISA. Microtitre plates were coated with affinity purified 40 kDa IBDV receptor protein (1 µg/ml in PBS, 4°C, overnight). Plates were incubated with serial two-fold dilutions of affinity purified anti-IBDVR-1 (panel A) or IBDVR-2 (panel B) (■) or non-immune antibodies (◆). The concentration of the antibodies was between 100 µg/ml and 6.25 µg/ml in BSA-PBS. Antibody binding was detected using goat anti-rabbit-HRPO-linked antibodies and visualised with ABTS/H_2O_2 substrate. Absorbance values were read at 405 nm in a Titertek ELISA plate reader. Each point is the average absorbance of duplicate samples.

5.12.4 Western Blotting

Western blot analysis was performed on the affinity purified IBDV receptor protein using affinity purified anti-IBDVR-1 and IBDVR-2 antibodies. No reaction was observed. The western blot was repeated using different concentrations of both the anti-IBDV receptor peptide antibodies and IBDV receptor protein. The modified western blot procedure using the modified transfer buffer and renaturation of proteins in the gel prior to electro blotting were done (Section 5.10.2), but no reaction was observed.

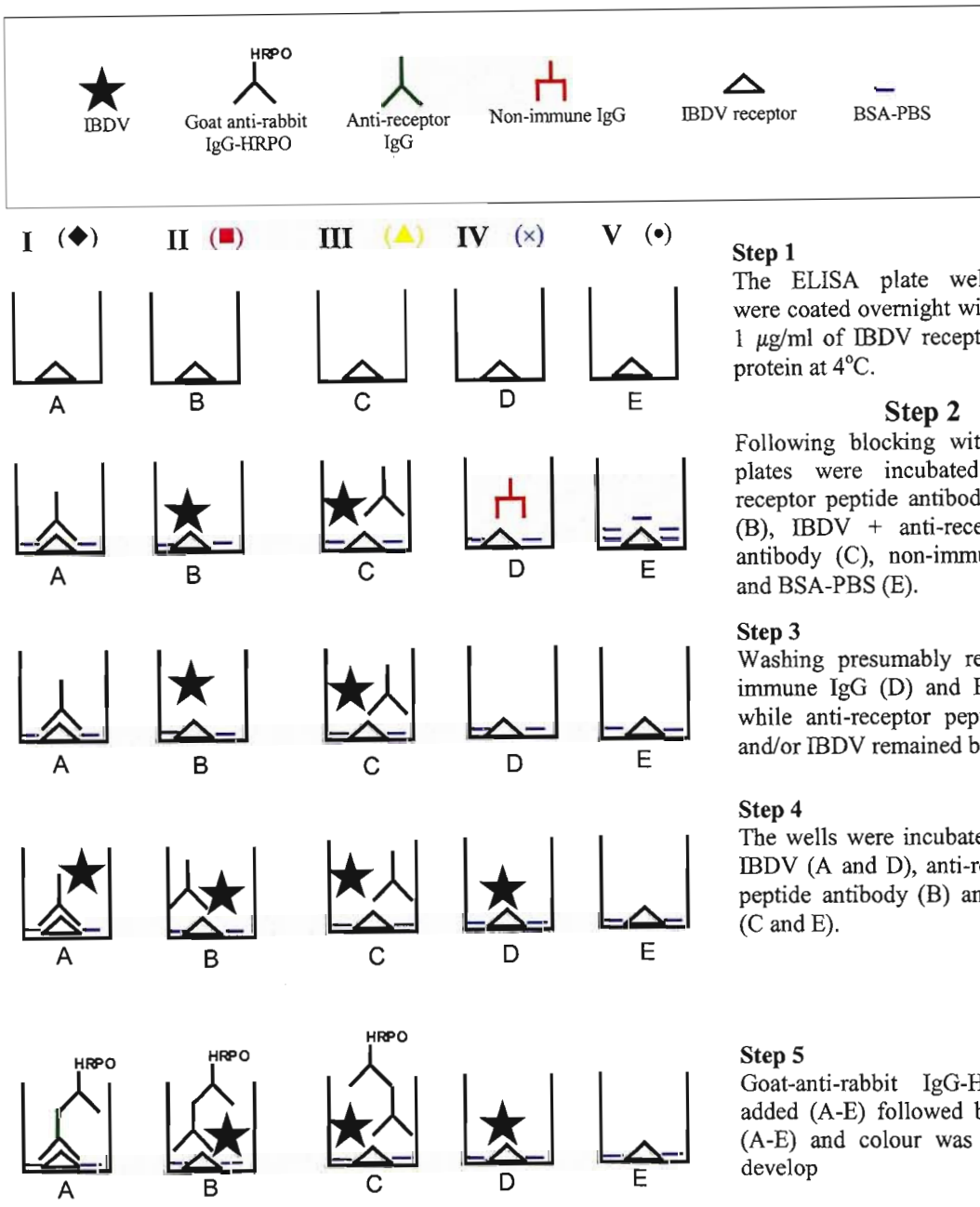
5.12.5 Blocking of IBDV binding to its receptor using anti-receptor peptide antibodies

Anti-peptide antibody against the IBDV receptor protein was used to determine whether the anti-receptor peptide antibody could block viral binding to the receptor. To assess this an inhibition assay was carried out in an ELISA format. Diagrammatic presentations of the assays are given in Fig. 5.10. An ELISA plate was coated with purified 40 kDa IBDV receptor protein (Fig. 5.10, panel I). Wells were incubated with anti-receptor peptide antibody. This was followed by incubation firstly with IBDV and secondly by goat anti-rabbit-HRPO conjugate (detection antibody). The result showed that anti-receptor peptide antibody strongly blocked the binding of IBDV to the receptor as evidenced by the high absorbance values (dark blue line, Fig. 5.11).

To ascertain whether IBDV could block anti-receptor peptide antibody binding to the receptor, IBDV receptor coated wells were incubated with IBDV (Fig. 5.10, panel II). The wells were incubated with anti-receptor peptide antibody followed by detection antibody. Lower absorbance readings (pink line, Fig. 5.11) than those described above were recorded, suggesting that IBDV did not completely block anti-peptide antibody binding to the receptor as anti-receptor antibody did (dark blue line, Fig. 5.11).

In a further assay competition between anti-receptor peptide antibody and IBDV for binding to the receptor was determined (Fig. 5.10, panel III). When compared to binding of anti-receptor peptide antibodies to receptor (dark blue line, Fig. 5.11) there was a decrease in absorbance values (yellow line, Fig. 5.11), indicating some degree of competition i.e. that some virus bound to

receptor, thereby preventing anti-receptor peptide antibody binding. This result confirmed the high affinity of the anti-receptor peptide antibodies for receptor. As a negative control wells were incubated with non-immune rabbit IgG followed by IBDV and detection antibody (Fig. 5.10, panel IV). The result (light blue line, Fig. 5.11) showed that non-immune IgG did not affect IBDV binding to the receptor and that the effects seen in the above assays were not due to non-specific binding of rabbit IgG to the receptor. A second control was also included to determine whether the HRPO-linked detection antibody recognised the IBDV receptor protein non-specifically (Fig. 5.10, panel V). No signal was observed (green line, Fig. 5.11).



Step 1
The ELISA plate wells were coated overnight with 1 μg/ml of IBDV receptor protein at 4°C.

Step 2
Following blocking with PBS-BSA, plates were incubated with anti-receptor peptide antibody (A), IBDV (B), IBDV + anti-receptor peptide antibody (C), non-immune IgG (D) and BSA-PBS (E).

Step 3
Washing presumably removed, non-immune IgG (D) and BSA-PBS (E) while anti-receptor peptide antibody and/or IBDV remained bound

Step 4
The wells were incubated with IBDV (A and D), anti-receptor peptide antibody (B) and PBS (C and E).

Step 5
Goat-anti-rabbit IgG-HRPO was added (A-E) followed by substrate (A-E) and colour was allowed to develop

Figure 5.10 Schematic representation of blocking IBDV binding to IBDV receptor using anti-receptor peptide antibodies in an ELISA. Symbols next to panel numbers refer to those used in Fig. 5.11 depicting the results of the ELISA.

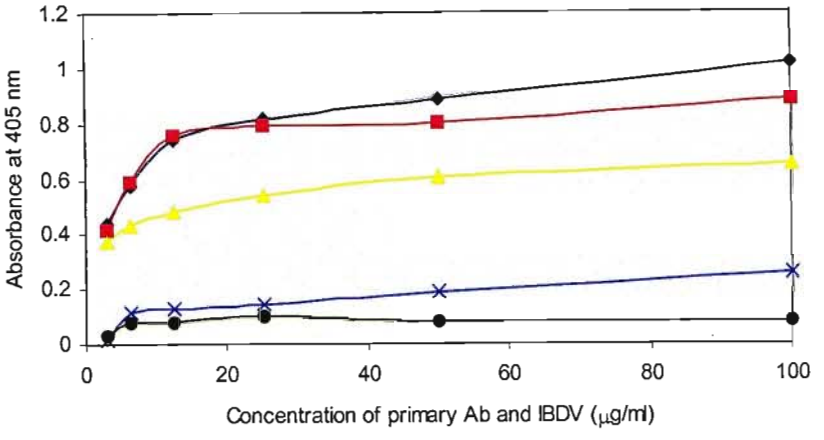


Figure 5.11 Blocking of IBDV binding to its 40 kDa receptor protein using anti-receptor peptide antibody. Affinity purified native 40 kDa IBDV receptor protein was coated onto microtitre plates (1 μg/ml in PBS, 4°C, over night). The wells were incubated with serial two-fold dilutions of anti-receptor peptide antibody (◆), IBDV (■), anti-receptor peptide antibody + IBDV (▲), non-immune IgG (×) and BSA-PBS (•). The wells were then incubated with serial two-fold dilutions of IBDV (◆), anti-receptor peptide antibody, (■), PBS (▲), IBDV (×) and PBS (•). Antibody binding was detected using goat anti-rabbit-HRPO-linked antibodies and visualised with ABTS/H_2O_2 substrate. Absorbance values were read at 405 nm in a Titertek ELISA plate reader. Each point is the average absorbance of duplicate samples.

5.13 Discussion

In this chapter evaluation of the immunogenicity of IBDV receptor peptides was done. This was followed by conjugation of the peptides to a carrier protein and production of anti-IBDV receptor peptide antibodies in rabbits. To determine the reactivity of the anti-IBDV receptor peptide antibodies before and after affinity purification, ELISAs were carried out by immobilising the antigenic peptides to microtitre plates. Western blotting was also included to determine the specificity of the antibody to the IBDV receptor protein transferred to the nitrocellulose membrane. An inhibition assay was done in an ELISA format to determine whether the anti-IBDV receptor peptide antibodies could block IBDV binding to the receptor, thereby giving further evidence that the receptor isolated in the present study is specific for IBDV.

Immunogenicity was evaluated using hydrophilicity, flexibility, antigenicity and surface probability parameters (Van Regenmortel *et al.*, 1988) of the amino acid sequences obtained in a

previous study in this laboratory (Edwards, 2000). These values were determined using a computer program for prediction of protein antigenic determinants from amino acid sequences, Predict7 (Cármenes *et al.*, 1989). All the above parameters are related to accessibility at the surface of the molecule. Since antibodies bind to the surface of proteins it is expected that epitopes would tend to consist of residues exposed at the protein surface (Schulze-Gahmen *et al.*, 1986; Van Regenmortel *et al.*, 1988).

Procedures in anti-peptide antibody production usually recommended conjugation of peptides to carrier molecules since they are assumed to be too small to be immunogenic by themselves (Richman and Reese, 1988). For this reason peptides were conjugated to RSA via MBS. The possibility of antibody production in rabbits using RSA as carrier protein was explored. According to many authors one of the criteria for a successful carrier molecule is the potential for immunogenicity (Hermanson, 1996). However, the use of self-proteins as carriers in antibody production to minimise antibody production against the carrier has been reported (Posnett *et al.*, 1988). It is thought that this directs the immune response principally toward the hapten, rather than the carrier (Hermanson, 1996). Using this strategy in the present study good antibody production against the peptides was obtained. However, tests should have been done to determine antibodies recognition of RSA in an ELISA. Although not specifically tested, no antibody production against the carrier is suspected. In a parallel study in this laboratory, using rabbit α_2-macroglobulin complexed with a protease antigen to produce anti-protease antibodies in rabbits, no antibodies were produced against α_2-macroglobulin (Laura Huson personal communication).

Coupling of peptides to carrier proteins has a marked effect on the antigenic determinants of a peptide. It appeared that conjugation of peptides to a carrier with carbodiimide results in linkage between carboxylic side chains and amine groups (Posnett *et al.*, 1988). Richman and Reese (1988) reported that conjugation of a helical peptide to KLH had altered its native structure sufficiently that the antibodies raised against this conjugated peptide were unable to recognise the corresponding protein structure. Furthermore, destruction of epitopes had also been evident in the glutaraldehyde conjugation of a tobacco mosaic virus peptide to bovine serum albumin. Conjugation by means of a terminal cysteine, is among the least intrusive coupling methods (Posnett *et al.*, 1988). Because of its ability to avoid modification of internal lysines in the

peptide, the thiol coupling method is preferable to use (Field *et al.*, 1998). Peptides were therefore conjugated to RSA by means of a C-terminal cysteine via MBS.

In the present study antibodies were produced against both peptides although IBDVR-2 appeared to be non immunogenic according to the Predict7 immunogenicity analysis. This result suggests that with careful synthesis, coupling, dosage and immunisations, most sequence might be used to induce antibodies specific for the peptide itself. Secondly, conjugation with the carrier protein may modify determinants of the peptide sequence sufficiently to enhance immunogenicity (Posnett *et al.*, 1988).

Since the peptide usually represents only a minor fraction of the total molecular weight of the peptide-carrier conjugate, the antibodies of interest may represent only a minor fraction of the total amount of antibodies produced, depending on the immunogenicity of the carrier protein (Posnett *et al.*, 1988). Thus affinity purification was needed to purify the antibody of interest by immobilising the antigenic peptides to the affinity column. In principle, only antibodies against the peptide and not against the carrier should bind to the column. However, sometimes-unwanted antibodies, such as aldehyde and polyreactive antibodies, do bind (Danbolt *et al.*, 1998). To determine the success of affinity purification, ELISAs were done using affinity purified antibodies. The affinity purified anti-IBDV receptor peptide antibodies showed an increased reactivity with the peptides compared to non-affinity purified anti-IBDV receptor peptide antibodies.

Affinity purified anti-IBDVR-1 antibodies recognised the whole IBDV receptor protein in an ELISA, but recognition by anti-IBDVR-2 antibodies was very weak. Recognition of the whole protein depends on the region of the protein where the peptides are from i.e. if the chosen peptide is buried within the protein, the resulting antibody may not recognise the native protein (Field *et al.*, 1998). The present result matches with this report, because according to Predict7 epitope mapping, IBDVR-2 showed very low values of hydrophilicity, flexibility, antigenicity and surface probability, which are the characteristic features of amino acids buried within the native structure of globular proteins (Van Regenmortel, 1988). This might be the reason why the anti-IBDVR-2

antibodies were not able to recognise the native 40 kDa IBDV receptor protein in an ELISA where the immobilised protein retains at least part of its native conformation.

Neither of the anti-IBDV receptor peptide antibodies recognised the 40 kDa IBDV receptor protein on a nitrocellulose membrane. The reasons for non-reactivity in the western blot could be two-fold. The reducing buffer in SDS-PAGE containing SDS and 2-mercapthethanol may have denatured the epitopes of the receptor protein, or transfer of the receptor protein from the gel to the nitrocellulose membrane could have been incomplete. Receptor proteins on the blot are denatured and conformationally restrained, which may inhibit many protein-protein interactions (Premont and Hall, 2002). This was supported by the finding that an epitope important in IBDV neutralisation was destroyed by treatment with SDS (Fahey *et al.,* 1989). In their study several of the IBDV neutralising monoclonal antibodies failed to react on western blots with viral proteins separated by SDS-PAGE.

The state of folding of proteins is particularly important when monoclonal antibodies, which recognise only a single epitope, are used, because many antibodies recognise assembled topographic determinants, which do not exist on denatured proteins (Dunn, 1986). This may also be applicable to anti-peptide antibodies. There have also been incidences where isolated proteins of low or moderate molecular weight do not electro-elute efficiently from a gel. Such proteins may be fortuitously at their isoelectric point, consequently have no tendency to migrate in the electric field exerted (Gershoni and Palade, 1983). There is also a possibility that certain classes of proteins do not bind to nitrocellulose (Towbin *et al.*, 1979).

Sodium oligooxyethylene dodecyl ether sulfates (DESs), with oxyethylene units of more than four has been found not to cause denaturation of bovine serum albumin. If a protein binds such a detergent, the protein is expected to be efficiently electrophoresed in the presence of the detergent retaining its native structure. This may be worthwhile exploring for analysis or separation of membrane proteins without denaturing them. However, DES is prohibitively expensive (Koide *et al.*, 1987). In addition an attempt was made to retain the native structure of the receptor protein by renaturation. Because protein renaturation prior to blotting can enhance recognition of antigen by some antibodies, a much larger effect can be obtained by transferring the proteins in a more

alkaline blotting buffer without SDS (Dunn, 1986). However, no result was observed in the present study.

The inhibition assay showed that anti-IBDV receptor peptide antibodies successfully blocked the binding of IBDV to its receptor protein on the surface of bursal cells. However, in a parallel assay, despite the fact that IBDV interfered with the binding of the anti-IBDV receptor peptide antibodies to the receptor, the inhibition was not strong enough to block anti-IBDV receptor peptide antibodies binding to the receptor entirely. This result suggests that the binding of IBDV to the receptor was not as effective as the binding of anti-IBDV receptor peptide antibody to the receptor. This could be due to the effects of CsCl during IBDV isolation, as CsCl appeared to affect the stability of viruses during the purification procedure. Chen and Ramig (1992) reported that some viruses such as human rotaviruses appear to lose their outer capsid during CsCl gradient centrifugation.

A competition assay done to determine the affinity of anti-IBDV receptor peptide antibodies and IBDV for the IBDV receptor protein also suggested that the anti-IBDV receptor peptide antibodies had higher affinity for the receptor than for IBDV. To show that the binding of anti-IBDV receptor peptide antibodies to the IBDV receptor is specific, non-immune rabbit IgG was used instead of the anti-peptide antibody but no blocking of IBDV binding to the receptor was observed. A second negative control was included to make sure that there was no non-specific interaction between the IBDV receptor protein and the secondary (detection) antibody (goat-anti-rabbit-IgG-HRPO linked) and no interaction was evident.

The anti-IBDV receptor peptide antibodies raised in rabbits recognised the corresponding peptide sequence and the native IBDV receptor protein in an ELISA tests. Results of the inhibition assay showed that anti-IBDV receptor peptide antibodies blocked IBDV binding to the receptor. In the same assay the affinity of the anti-IBDV receptor peptide antibodies appeared to be higher than that of IBDV. These results suggest that this may indeed be the IBDV receptor, because antibodies against this sequence appear to interfere with viral binding.

CHAPTER 6

GENERAL DISCUSSION

Infectious bursal disease virus is the prototype member of the *Avibirnavirus* genus in the *Birnaviridae* family. It causes considerable economic losses to the poultry industry worldwide and represents a major hazard for various species of wild birds (Caston *et al.*, 2001). IBDV has a tropism for lymphoid tissue of chickens and infects actively dividing and differentiating B-lymphocytes in the bursa of Fabricius. When the infection is not fatal it causes severe immunosuppression and increased susceptibility to secondary infections (van den Berg *et al.*, 2000). Currently the most effective method for IBDV control is vaccination, using either attenuated live or inactivated IBDV vaccines (Juul-Madsen *et al.*, 2002). These commercial IBDV vaccines are selected based on their ability to induce a strong antibody response (Kim *et al.*, 2000). Identification of the IBDV receptor protein on the surface of bursal cells may help to understand the molecular mechanism of IBDV infection and facilitate the design of antiviral agents that can interfere with IBDV binding prior to crossing the bursal membrane.

The IBDV genome is made up of two segments of double-stranded RNA; segment A (3.2 kb) and segment B (2.8 kb). Both segments are confined within a single-shelled icosahedral capsid of 60 nm in diameter. Segment A contains two partially overlapping ORFs. The first ORF encodes VP5 (17 kDa), which is a non-structural protein, whose functional properties are unknown, although it appears to be important in virus release and dissemination. The second ORF encodes for a 110 kDa polyprotein that is autoproteolytically cleaved into three structural proteins of the mature virion, namely VP2 (42 kDa), VP3 (32 kDa), and VP4 (28 kDa) (Boot *et al.*, 2000b). Biochemical analysis of purified capsids from virions revealed that VP2 and VP3 are the major structural proteins of the virus, while VP4, a serine-lysine protease is involved in the proteolytic maturation of the polyprotein (Birghan *et al.*, 2000). VP2 contains major neutralising epitopes, suggesting that it is at least partly exposed on the outer surface of the capsid. While VP3 contains a very basic carboxy-terminal region, which is likely to interact with the packaged RNA, it is believed to be on the inner part of the capsid (Caston *et al.*, 2001). This analysis strengthens the hypothesis that VP2 is the major host protective antigen of IBDV (Lin *et al.*, 1993). Segment B contains an ORF that encodes VP1 (95 kDa), which is assumed to be the RNA-dependent RNA

polymerase that is responsible for the reactions of transcription and replication (Caston *et al.*, 2001).

Although live and inactivated IBDV vaccines are highly efficient, the effectiveness of these vaccines decreases in the presence of maternal antibodies, and some of them cause bursal atrophy because of lymphoid cell depletion. In recent years, the emergence of new virulent strains, cost considerations and risk of reversion to virulent forms have resulted in the effectiveness of live and killed vaccines being reduced (Ho *et al.*, 1999). Therefore, highly efficacious and safe IBDV vaccines are needed (Tsukamato *et al.*, 2002).

Although several studies have been conducted on VP2 and VP3 for use in vaccines, success has only been obtained *in vitro*. In the present study a different avenue was investigated to prevent infection, i.e. one based on interfering with the binding of IBDV to the host receptor proteins. Since cellular receptors play a crucial role in virus pathogenesis, the identification of the receptor is necessary to understand host range, tissue tropism, pathogenesis, penetration, virus replication and the disease symptoms produced by the virus (Wang *et al.*, 1991; Kim *et al.*, 2002). Thus antiviral strategies can be evaluated based on these events (Nieper and Muller, 1998). In order to prove this hypothesis, information needed to be gathered on the IBDV receptor proteins on the surface of bursal cells that IBDV binds to. Previous work in this laboratory identified two possible IBDV receptor proteins on the surface of bursal membranes of 32 and 40 kDa. N-terminal amino acid sequencing of the 32 kDa receptor protein and a Lys-C digestion product was conducted and this provided data to start the present study (Edwards, 2000). The nucleotide sequence coding for this protein was shown to be present in the chicken genome (Edwards, 2000). The difference in size between the PCR products obtained from PCR and RT-PCR in the latter study could possibly be due to the presence of an intron, which are only present in eukaryotic DNA and this suggested that the proteins sequenced were most likely of chicken origin.

The objective of the present study was to isolate IBDV receptor proteins from the surface of bursal cells, prepare anti-peptide antibodies against the receptor sequence previously determined and use the antibodies to prevent IBDV binding to the putative receptor to prove its identity. As

an adjunct to this part of the study, methods for IBDV detection were also evaluated. To this end IBDV was isolated from infected bursal tissue using CsCl density gradient centrifugation and its isolation was analysed using Tris-Tricine SDS-PAGE and electron microscopy. In addition to electron microscopy, several methods can be used for the detection of IBDV; such methods include: virus isolation in cell culture, embryonated chicken eggs, or young specific-pathogen-free (SPF) chickens, immunofluorescent staining technique, agar gel precipitation, antigen capture ELISA, or immunohistochemistry. However, these methods are time consuming, labour intensive, expensive, or non-specific. Besides they lack the ability to detect low levels of IBDV antigens in tissues (Barlic-Maganja *et al.*, 2002). Thus a highly sensitive and specific method was required for diagnostic purposes.

In the present study Titan® one-tube RT-PCR was carried out on the purified double stranded viral RNA and commercially available live IBDV vaccines after the double stranded RNA was isolated. For most viral diagnosis, the optimum sites for PCR primers are within sequences that are unique to the viral genome among all isolates or strains of the virus (Wu *et al.*, 1992). Thus the primers were selected from the highly conserved VP2 genome region of IBDV for the amplification of the VP2 protein gene and conditions were optimised for the RT-PCR reaction. The sequence of primer 1 (anti-sense primer) was 5' - CAA CAG TGT AGT CTC TCC CG -3' and the sequence of primer 2 (sense primer) was 5'- AGA TGT TTG CTG TCA TTG AAG G - 3'. The inter primer spacing for primers 1 and 2 was 110 base pairs. This set of primer specified a 150-base pair sequence located at nucleotide position 1760 to 1910 of the IBDV genome (Wu *et al.*, 1992).

The one-step RT-PCR procedure was used for reverse transcription of IBDV RNA into cDNA and then amplification by PCR under a single set of conditions. Using this technique it was possible to detect as little as 2 ng/μl of viral RNA from infected bursal tissue by ethidium bromide staining after 40 cycles of PCR. This powerful and sensitive technique is based on the principle that IBDV RNA is transcribed into cDNA and the DNA is amplified exponentially by repeating cycles of heat denaturation, annealing, and primer extension (Barlic-Maganja *et al.*, 2002). Because of the minimum hands-on work and low possibility of contamination, Titan® one tube RT-PCR was preferred over the regular RT-PCR technique (Barlic-Maganja *et al.*, 2002).

The presence of the virus was demonstrated by the amplification of a 150 bp band as visualised on 2% agarose and 15% nondenaturing PAGE gels. This product was proved to be correct by restriction digestion with a specific restriction endonuclease, *Bam*HI. This enzyme recognised the duplex symmetrical sequence and cleaved the product between the guanines to produce digestion fragments of the correct size, of 93 and 57 bps (Hensley *et al.*, 1990). Due to the small size of the amplified PCR product (150 base pairs) and restriction digestion fragments (93 and 57 bps) the resolution of the products in a 1% (w/v) agarose gel was very poor. Thus for higher resolution of these bands an increase in gel percentage to 2% (w/v) was needed. Although it was possible to detect the bands on a 2% (w/v) gel, the resolution was still not optimal. Also, because of the higher gel concentration it was difficult to prepare gels since they tended to polymerise before pouring or insoluble agarose granules appeared on the gel, which could affect the mobility of the products. These problems were resolved by using a 15% nondenaturing polyacrylamide gel, although it was time consuming (16 h electrophoresis).

Live vaccines were used in the present study for RT-PCR amplification of the VP2 sequence. The smearing of the amplified band observed in agarose or polyacrylamide gels could be due to contaminating chicken RNA since the vaccine virus is propagated in allantoic fluid.

Infectious bursal disease virus was attached to a Sepharose 4B chromatography matrix for affinity purification of the viral receptor protein from uninfected bursal membranes. In the previous study in this laboratory, Edwards (2000) isolated 40 and 32 kDa IBDV receptor proteins whereas, in the present study the isolated IBDV receptor protein showed two prominent bands of 40 kDa (Chapter 4). For the visualisation of the IBDV receptor protein the same gel system (Tris-Tricine SDS-PAGE) and percentage (10%) was used as was reported by Edwards (2000). This receptor protein was recognised in an ELISA by the anti-peptide antibodies prepared using the 32 kDa receptor protein sequences determined in the previous study (Chapter 5). These results suggest that the 32 kDa receptor protein could be a fragment of the 40 kDa IBDV receptor protein. This fragmentation could be the result of 2-mercaptoethanol, SDS and heating during preparation for Tris-Tricine SDS-PAGE. According to Edwards (2000) there is no sequence overlaps between the N-terminally sequenced 40 and 32 kDa proteins of the bursal membrane

proteins. However, this was very difficult to conclude, because the peptide sequence, especially of the 40 kDa, was very short. Sequence overlaps might have been obtained in relatively longer sequences. Thus, in further studies, more IBDV receptor proteins could be purified for N-terminal amino acid sequencing to provide a longer sequence to determine the sequence overlap of the receptor protein with the N-terminally sequenced 32 kDa protein.

In order to multiply, a virus must first infect a cell. The host range of a particular virus defines both the type of tissue, and the animal species that the virus infects. To infect a cell, the virion must attach to the cell surface, penetrate the cell and become sufficiently uncoated to make its genome accessible to the host machinery for transcription and translation. The attachment step constitutes specific binding of a viral protein (anti-receptor) to a constituent of the cell surface (receptor) (Kim *et al.*, 2002). It seems very promising to interfere with the viral attachment stage before the virus crosses the membrane barrier.

In the present study IBDV appeared to have two prominent receptor bands around 40 kDa. However, a number of viruses use more than one distinct attachment receptor, such as HIV that uses CD4 as its main attachment receptor but also interacts with glycolipid galactosyl ceramide to mediate infection (Kim *et al.*, 2002). Thus, from a prophylaxis point of view, having a single membrane receptor protein may be an advantage over multi-receptors, because it will be difficult to block multi-receptor sites (Varthakavi and Minocha, 1996). Blocking this receptor protein will potentially inhibit IBDV binding and subsequent multiplication (infection). Two of the most commonly used therapies in blocking of virus-receptor interactions are either anti-receptor antibodies or soluble receptors. Furthermore, other chemicals or pharmacologics could be developed that specifically block virus binding either at the level of the cell receptor or the viral attachment protein (VAP) (Bass and Greenberg, 1992). Kim *et al.* (2002) reported that a polyclonal antibody against the 30 kDa Hantaan virus receptor protein blocked binding of Hantaan virus to Vero-E6 cells and reduced virus infection by 70%.

Anti-IBDV receptor peptide antibodies were raised in rabbits against two IBDV receptor protein sequences. ELISA results confirmed that the anti-IBDV receptor peptide antibodies recognised the peptide sequences and the isolated 40 kDa IBDV receptor protein. However, the ELISA

response for the isolated 40 kDa IBDV receptor protein is about 60% that of the peptide sequence (this percentage was taken from the absorbance reading at concentration of 25 μg/ml). This difference in recognition by the anti-IBDV receptor peptide antibodies could be due to (1) impurities in the isolated 40 kDa IBDV receptor protein preparation, or (2) some of the amino acid residues in the peptide sequence could be buried in the native IBDV receptor protein.

Further evidence that the anti-IBDV receptor antibodies probably recognise the native conformation of the receptor were given by the results of the inhibition of virus binding assays and the western blot (no recognition, see below). The inhibition assays showed that the anti-IBDV receptor peptide antibodies effectively blocked the binding of IBDV to the receptor and thus recognise the native receptor conformation. The competition assay also proved that anti-IBDV receptor peptide antibodies had higher affinity for the receptor than for IBDV. In addition, inhibition of IBDV binding by the anti-IBDV receptor peptide antibodies was confirmed to be specific, when inhibition of IBDV binding with non-immune rabbit IgG showed no inhibition. These results strongly suggest that the IBDV receptor isolated here is indeed a receptor for IBDV. For more structural and functional studies IBDV receptor could be purified using the anti-IBDV receptor peptide antibodies as a ligand in the affinity column.

In contrast to the ELISA result, the anti-IBDV receptor peptide antibodies did not recognise the 40 kDa IBDV receptor protein in a western blot. This may be due to epitope denaturation of the receptor protein either by SDS or 2-mercaptoethanol in the reducing treatment buffer used for SDS-PAGE (Premont and Hall, 2002). There was also a possibility of denaturation by detergents such as Na-deoxycholate used during the affinity purification of this receptor protein (Hannam *et al.*, 1998). To avoid these complications an attempt was made to retain the native structure of the receptor protein by renaturation. It was suggested that protein renaturation prior to electro-blotting can improve recognition of antigen by some antibodies (Dunn, 1986). According to Dunn (1986) the use of glycerol in the renaturation buffer enhanced the binding of antibody to all the ATPase subunits of *Escherichia coli*, suggesting this would be useful for other protein antigens. Moreover, incubation of the gel in renaturation buffer and blotting using bicarbonate buffer could improve retention of smaller proteins on the nitrocellulose membrane and enhance folding of the proteins into conformations that more closely resemble the native state of the

protein. However, no recognition of the IBDV receptor protein was obtained using the anti-IBDV receptor peptide antibodies, suggesting that these attempts at renaturation were not successful.

Normally, most of the viral receptors did not evolve to serve as viral receptors, but they may have specific biological functions for the host cells. The biological function of the 40 kDa IBDV receptor protein on the surface of bursal cells is not yet known. However, some of the receptors that have been identified so far are cell surface molecules of known biological functions, including the CD4 molecule for the binding of HIV (Adlish *et al.*, 1990; Thaker *et al.*, 1994). The outcome of the inhibition studies suggests that IBDV infection could be reduced by blocking the IBDV receptor site using anti-IBDV receptor peptide antibodies, but the effect on the natural function of the rceptor will have to be evaluated.

The study could also be directed towards the viral attachment protein (VP2) by sequencing VP2 and raising anti-peptide antibodies in rabbits against VP2. The effectiveness of IBDV inhibition by anti-IBDV receptor peptide antibodies can be compared with that by the anti-VP2-peptide antibody at electron microscopy level using immunofluorescent techniques. Since the IBDV receptor protein could have its own biological function, like CD4 as HIV receptor, blocking the receptor using anti-receptor peptide antibody *in vivo* could affect the biological function of the receptor. Thus it would be more convenient to think of blocking the VP2 from binding to the receptor using anti-VP2 peptide antibodies. Alternatively, the use of virus as a vector to carry the genes for VP2 can be investigated. This would involve inserting the gene encoding VP2 (against which protective responses are generated in the host) into the genome of an avirulent virus. The virus would multiply and present the viral epitope/antigen to the immune system of the chicken. The chicken would mount both a humoral and a cell-mediated immune response to it (Murphy *et al.*, 1999). Using single proteins displaying immunodominant epitopes as vaccines induces effective protective immunity and eliminates the risk of viral replication in the host due the absence of genetic material (Hansson *et al.*, 2000).

In conclusion, a 40 kDa IBDV receptor protein on the surface of bursal cells was isolated. The anti-IBDV receptor peptide antibodies effectively blocked the binding of IBDV to the receptor. The results presented in this study could provide an insight for further studies into the

development of effective IBDV infection controls. Control could be based on interfering with virus-receptor interaction. These include either the use of viral attachment protein (VP2) as a subunit vaccine or production of antibodies against covalently crosslinked complexes of soluble IBDV receptor protein and VP2 to interfere with virus receptor attachment.

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