AUSTRALIAN POULTRY CRC

FINAL REPORT

Program 2A
Discovery and Development

Project No: 03.33

PROJECT LEADER:
Sandra Sapats

DATE OF COMPLETION: February 2008

Project Title:

Control of Infectious Bronchitis Virus: Improved diagnostics and disease surveillance through the application of genomic technology

ISBN 1 921010 17 7

Control of Infectious Bronchitis Virus: Improved diagnostics and disease surveillance through the application of genomic technology
Project 03-33

Researcher Contact Details
Dr. Sandra Sapats
CSIRO, Australian Animal Health Laboratory
5 Portarlington Rd
East Geelong 3220

Phone: 03 52275770
Fax: 03 53375555
Email:sandra.sapats@csiro.au

In submitting this report, the researcher has agreed to the Australian Poultry CRC publishing this material in its edited form.

Australian Poultry CRC Contact Details
PO Box U242
University of New England
ARMIDALE NSW 2351

Phone: 02 6773 3767
Fax: 02 6773 3050
Email: poultrycrc@une.edu.au.
Website: http://www.poultrycrc.com.au

Published in April 2009

Contents

Executive Summary

Contents of the report

The virulence of a novel variant strain of infectious bronchitis virus (IBV) first isolated from NSW in 2002 is described and the efficacy of currently available commercial infectious bronchitis (IB) vaccines to provide protection evaluated in SPF (specific pathogen free) birds. In addition, selected field strains of IBV are evaluated for their vaccine efficacy in providing broad cross protection against variant IBV strains. The report also compares 2 different methods for evaluating cross protection in SPF chickens birds including traditional isolation of challenge virus in eggs and ciliary activity in the trachea. Lastly, an attempt to localize protective epitopes of IBV is described using a fowl adenovirus vector to express various fragments of the IBV S1 glycoprotein with the aim of developing molecular tools to rapidly assess whether existing vaccines will provide protection against emerging variant strains of the virus.

Background

The continuing emergence of variant strains of IBV poses a serious economic threat to the Australian poultry industry. Over the last 10 years a number of variant strains have been identified in Australia. Recently, a new variant strain of IBV (strain N4/02) was isolated from a number of broiler farms in NSW in 2002/2003 experiencing respiratory disease with elevated mortalities. Genetically, this variant was significantly different to all previously isolated strains in Australia, including vaccine strains. It was uncertain which, if any, of the four commercially available IB vaccines would be the most effective against this particular variant. Hence, the poultry industry requested that the Australian Poultry CRC agree to fund the Australian Animal Health Laboratory (AAHL) to undertake a number of cross protection studies to test the efficacy of current commercial IB vaccines against the N4/02 variant. It was also critical to determine the pathotype of the N4/02 variant in specific pathogen free (SPF) chickens to confirm it's association with only respiratory disease and not nephritis.

Of the four commercial IB vaccines currently used in Australia, 3 belong to the same serotype (Vic S S and I) and are genetically similar, while the fourth vaccine (A3) belongs to a different serotype. In Australia, day old chicks are routinely vaccinated with a single application of IBV vaccine. Chicks are occasionally boosted in the field with a second dose if problems with IBV are suspected. Studies overseas have demonstrated that the use of two genetically different IB vaccines of different serotypes administered 2 weeks apart is highly effective and provides broad cross protection against a range of different IBV variants. Whether a combination of two different local Australian IB vaccines provides broader cross protection has not been scientifically demonstrated. Should this be the case, the development of new vaccines to protect against emerging variants, such as N4/02, may not be necessary.

Cross protection studies in chickens are still the preferred method of evaluating vaccine efficacy. A vaccine is considered effective if, after challenge, it is not possible to re-isolate the challenge virus from the trachea. Unfortunately, this process is very laborious and expensive, requiring the use of large numbers of embryonated SPF eggs for virus culture. Recently, an alternative method has been described which examines the ciliary activity in the trachea of challenged birds to assess vaccine efficacy (Cook *et al*., 1999). In this case, a vaccine is considered effective if the cilia lining the trachea remain intact and "beating" after virus challenge. This method offers considerable savings in terms of time and resources.

Currently it is not possible to determine whether a new vaccine is required to provide protection against a newly emerged variant based upon genetic analysis of the virus sequence. Such a method would greatly facilitate IBV diagnosis, enabling rapid control strategies to be introduced. Previously it has been demonstrated that the spike glycoprotein (S1) of an Australian IBV strain can provide protection against IBV challenge when expressed by a viral vector based on fowl adenovirus (FAdV). Using this technology, it may be possible to locate the protective epitopes within the S1 protein. These regions can then be compared between strains belonging to the same or different "protecto-types" thereby enabling an assessment of the most appropriate vaccine to use for the control of a particular variant strain.

Aims and objectives

- Determine pathotype of novel NSW variant strain N4/02
- Evaluate current vaccines for efficacy against challenge by N4/02
- Evaluate other strains as possible vaccine candidates providing broad cross protection
- Evaluate methods for assessing cross protection
    - traditional re-isolation of challenge virus in eggs
    - ciliary activity as a measure of detecting infection in the trachea
    - molecular approach using fowl adenovirus to express protective proteins of IBV

Methods

To determine the pathotype of N4/02, 40 two-week-old SPF chickens were divided into 2 groups and challenged with a dose of 10^6 egg infectious doses (EID_{50}) of N4/02 per chick. One group of 20 birds was observed daily for 14 days for assessment of clinical signs. The remaining group was sampled at various time points post challenge (3, 5, 7 10 or 14 days post challenge) with tracheal and kidney tissues collected for histological examination.

For assessment of cross protection, day old SPF chicks were vaccinated with one of four commercially available IB vaccines (S, I, Vic S or A3) using 10^3 EID_{50} per chick. Antibody responses were measured and chicks were challenged at 28 days of age with 10^5 EID_{50} of the NSW variant N4/02. Five days post challenge, birds were euthanased and the trachea removed for assessment of virus clearance by either (i) passaging of tracheal scrapings through embryonated SPF eggs (virus isolation) and detection of virus by ELISA (ii) ciliary activity in the trachea using the method of Cook *et al*. (1999) or (iii) quantification of viral load by qRT-PCR. The effectiveness of administering two doses of vaccine given at day 1 and then again on either day 14 or 18, was also assessed using the same or different vaccines at each interval. Two field strains showing superior antigenicity (N25/87 and N3/62) were also included in the animal trials. Similarly, the variant virus N4/02 itself was also tested for its vaccine potential to provide broad cross protection against other previously isolated variant IBV strains (N1/88 and Q3/88).

Recombinant FAdV viruses were constructed encoding different portions of the IBV S1 protein. An attempt was made to detect expression of the truncated S1 proteins in tissue culture using both ELISA and western blot analysis. Chicks were immunised with selected FAdV recombinants (2 doses), sera assayed for the presence of antibodies to the S1 protein and chicks challenged with IBV to assess protection.

Results/key findings

SPF chickens were challenged with NSW variant N4/02. At approximately 4 days post infection 30% of chickens appeared depressed showing signs of respiratory infection. These symptoms were only transient as by day 7 most chicks appeared to be recovering and by day 10 all chicks were fully recovered. No mortalities were recorded and the litter was observed to be dry throughout the bird trial. Histological examination confirmed tracheitis in all chicks, and with the exception of one chicken (1/20), there were no lesions or IBV antigen detected in the kidneys.

Cross protection studies demonstrated that none of the four commercial IB vaccines provided complete protection against NSW isolate N4/02. As a single dose given to day old chicks, VicS appeared to provide the highest level of protection at 40%, while S, I and A3 vaccines provided 20, 27 and 21% protection, respectively. The effect of administering a second dose of vaccine at either 14 or 18 days post the primary vaccination was also investigated using either the same or different vaccine strains for the boost. Overall, the level of protection varied from 20 to 53%, the highest level of protection being observed using 2 doses of the S vaccine administered on days 1 and 14 post hatch. This level of protection was lowered slightly to 43% when the second dose of S vaccine was administered on day 18 post hatch. Other combinations using VicS and A3 (day 14 boost), or A3 and VicS (day 14 boost) were marginally lower with protection scores of 47 and 43%, respectively. Surprisingly, boosting with an increased dose of A3 (10x) after a primary vaccination with VicS appeared to reduce the level of protection to only 20%.

Other field strains were also investigated for their vaccine potential. A single dose of strain N25/87 given to one day old chicks provided the highest level of protection against N4/02 at 53%, while N3/62 (G virus) was significantly lower providing only 24 % protection. However, when the N25/87 strain was used to boost birds vaccinated previously with the S commercial vaccine, a decrease in the level of protection against N4/02 was observed with only 23 % protection.
The vaccine efficacy of the N4/02 variant itself was evaluated. Chicks receiving a primary vaccination of the S vaccine at day 1 followed by a boost with the N4/02 variant showed 93 and 100% protection against subsequent challenge with variant strains N1/88 and Q3/88, respectively. These protective values were significantly higher than those values obtained after a single vaccine dose of either S or N4/02, or 2 doses of the S vaccine.
Two methods were evaluated for assessment of cross protection in birds including measurement of ciliary activity and traditional virus isolation in eggs. There was total agreement between the two methods when comparing the virus challenge (100 % infected) or uninfected controls (0% infected). However, when attempting to compare vaccinated groups of birds (variable levels of protection) measurement of ciliary activity was found to be less sensitive than virus isolation in eggs, failing to detect approximately half of the infected birds.
An attempt to express different fragments of the IBV spike glycoprotein (S1) using FAdV failed to detect any expression *in vitro*. Birds immunized with selected recombinants failed to elicit a detectable antibody response towards the S1 protein and were not protected against subsequent challenge with IBV.

Implications

- NSW variant N4/02 identified as a mild respiratory strain possibly, causing subclinical infection in the absence of any secondary bacterial infection.
- None of the commercially available vaccines in Australia, either individually or in various combinations are able to provide greater than 53% protection against the N4/02 variant. This would suggest the currently available IB vaccines do not have sufficient antigenic diversity to provide broad cross protection.
- Broad cross protection against previously isolated IBV variants can be achieved using a combination of two vaccines, S vaccine strain and N4/02 strain, administered 2 weeks apart.
- Measurement of ciliary activity is less sensitive than virus isolation in eggs and therefore not recommended for assessment of cross protection.
- The FAdV vector utilized in this study was unable to express detectable levels of the IBV S1 protein either *in vitro* or *in vivo*.

Recommendations

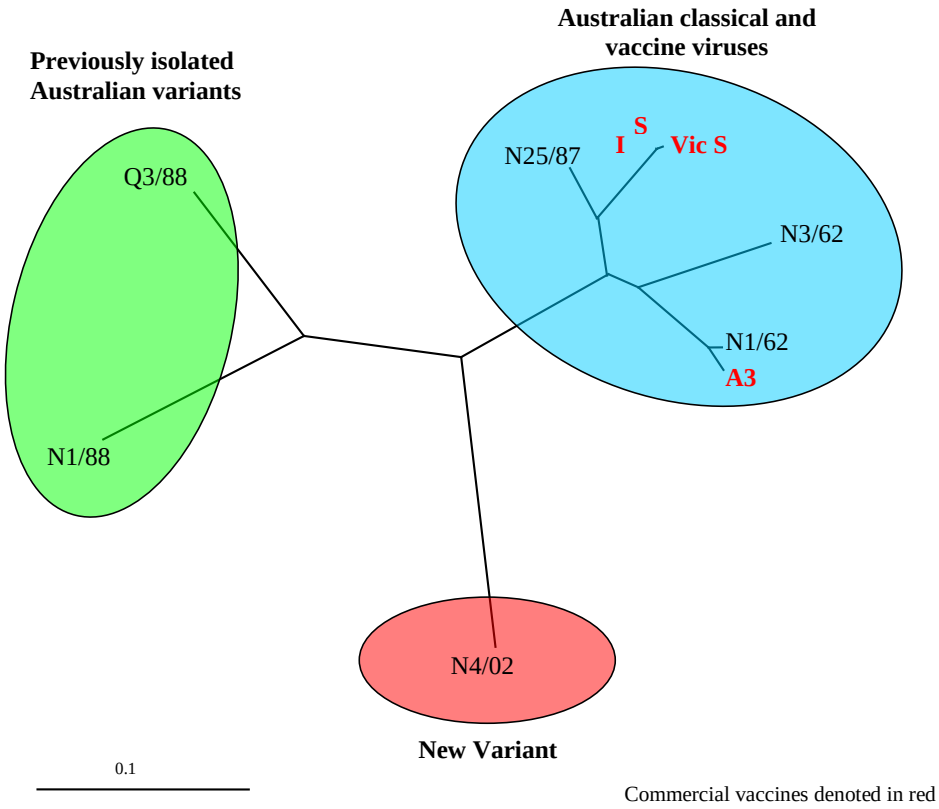
- The potential development of N4/02 as an alternative vaccine to expand the repertoire of IB vaccines currently available in Australia.
- Further evaluation of N4/02 as a vaccine candidate in broilers to confirm suitability in providing broad cross protection against other variant strains of IBV.
- Surveillance of IBV strains circulating in the field, including monitoring of genetic changes occurring in field isolates of the N4/02 strain.
- Continued use of virus isolation for the assessment of cross protection in chickens with the aim of further developing a qRT-PCR designed to specifically detect IBV in the trachea.
- The use of alternative expression systems to localize protective epitopes of IBV, including the development of a reverse genetics system based upon an Australian strain of IBV.
- Pathotype of N4/02 confirmed in commercial birds, including both broilers and layers.

1. Introduction

Infectious bronchitis virus (IBV) is one of the primary causes of respiratory disease in chickens. IBV initially infects the trachea, however some strains can also infect the kidney and oviduct. Infection with IBV reduces the performance of broilers, and is associated with a drop in egg production in layers. Numerous serotypes of IBV have been identified throughout the world, many of which are poorly cross protective (Cavanagh, 2007). Following the first isolation of IBV in Australia in 1962, many different serotypes have been identified, including a number of unique variant strains such as N1/88 and Q3/88 (Sapats *et al*., 1996; Ignjatovic *et al*., 1997).

Recently an outbreak of respiratory disease with elevated mortality was observed across a number of broiler farms in NSW from December 2002 to February 2003. Tracheal and kidney samples were collected from 23 different sites and submitted to CSIRO for analysis. A total of 11 IBV isolates were identified which were genetically almost identical to each other but significantly different from all previously isolated strains in Australia, including vaccine strains (Figure 1). This novel variant strain was subsequently called N4/02. Given the high degree of genetic changes observed within the N4/02 strain, it was predicted that none of the currently available IB vaccines would be capable of providing full protection against challenge with this variant. However, a detailed analysis of cross protections studies in SPF chicks was needed to confirm this suspicion. It was also critical to determine the pathotype of the strain in SPF birds to confirm it's association with only respiratory disease and not nephritis. Hence, the Australian poultry industry approached the Australian Poultry CRC and CSIRO, AAHL to undertake such a study.

Figure 1.1. Phylogenetic relationship of Australian IBV strains used in this study



Of the four IB vaccines currently used in Australia, 3 are genetically and antigenically very similar belonging to the same serotype, while the fourth belongs to another serotype. Common vaccination practice in Australia involves a single application of vaccine administered to day old chicks. Occasionally a boost with a second IB vaccine is administered in the field several weeks later. Based

upon overseas studies, it has been demonstrated that the use of two different IB vaccines of different serotypes administered 2 weeks apart is more effective and provides broader cross protection against a range of IBV strains. Whether a combination of two different local Australian IB vaccines provides superior and broader cross protection has not been determined. Should this be the situation, the development of vaccines to protect against newly emerging variants such as N4/02 might not be necessary. Hence, we propose to test the efficacy of current vaccines in various combinations to determine the optimum combination and timing of vaccination.

Cross protection studies are still the preferred method of evaluating vaccine efficacy. A vaccine is consider effective if after challenge it is not possible to re-isolate virus from the trachea by passaging tracheal scrapings through embryonated eggs. Unfortunately this process is very laborious and expensive. Recently an alternative method has been described which uses ciliary activity in the trachea of challenged birds to access vaccine efficacy (Cook *et al*., 1999). This method offers considerable savings in terms of time and resources, therefore we proposed to compare this method with the traditional method of isolating virus in eggs.

Currently it is not possible to determine whether a new vaccine is required to provide protection against a newly emerged variant based upon genetic analysis of the virus sequence. Such a method would greatly facilitate IBV diagnosis enabling rapid control strategies to be introduced. Our laboratory has previously demonstrated that the S1 protein of an Australian IBV strain provides protection against challenge when expressed within a viral delivery system based upon fowl adenovirus (FAdV). We propose to use this technology to locate the protective epitopes within the S1 protein. Once these regions are identified, it maybe possible to compare these regions among strains of the same and different "protecto-types" thereby enabling an assessment of the most appropriate vaccine to use for the control of a particular variant strain.

2. Objectives

- Determine the pathotype of novel NSW variant strain N4/02
- Evaluate current vaccines for efficacy against challenge by N4/02, both individually and in various combinations
- Evaluate other strains as possible vaccine candidates providing broad cross protection
- Evaluate methods for assessing cross protection
    - traditional re-isolation of challenge virus in eggs
    - ciliary activity as a measure of detecting infection in the trachea
    - molecular approach using fowl adenovirus to express protective proteins of IBV

3. Methodology

3.1 Plasmids, bacteria and viruses

For construction of FAdV recombinants plasmids pJJ1050, pJJ885, pJJ1054 and pJJ925 were obtained from Dr Scott Tyack (CSIRO AAHL, Australia). Plasmid pJJ1050 contained the cytomegalovirus immediate early promoter (CMVie) with a multiple cloning site to enable cloning of the desired gene as well as a SV40 poly adenylation site. Plasmids pJJ885 and pJJ1054 constituted the cfa40 FAdV-8 right hand end (RHE) of the genome and contained unique 2.3 kb and 52 bp deletions, respectively. Plasmid pJJ925 constituted the remainder left hand end (LHE) of the FAdV genome for the FAdV-8 strain, cfa44. The cloning plasmid vector pGEM-T easy was used for the cloning of PCR fragments (Promega, Australia).

For electroporation experiments, *Escherichia coli* (*E.coli*) strain DH5-α was used (Invitrogen, Australia).

Commercial IB vaccines used in the study included: Websters IB vaccine VicS strain (Vic S)(Fort Fort Dodge, Australia), Websters IB vaccine Ingham strain (I) (Fort Dodge, Australia) Websters IB vaccine Armidale A3 strain (A3) (Fort Dodge, Australia) and IB vaccine No 1 (S) (Intervet, Australia). Other strains used included N25/87, N3/63 (G virus), Q3/88, N1/88 and N4/02 (Sapats *et al*., 1996; Ignjatovic *et al*., 1997; Ignjatovic *et al*., 2006).

3.2 Primers

Primers used in this study are shown in Table 3.1 and were synthesised by Micromon (Monash University, Australia).

Table 3.1. List of primers used in this study

Name	Sequence 5'- 3'	Location in virus/plasmid	Use
S1-EcoRV	TAGTTAGATATCATGTTGGTGAAGTCACTGTTTATAG	1-25[#]	PCR of S1 for FAdV
S1(462)BgII	TAGTTAAGATCTTTAAACTGTTAAATTATAAAATA[*]	443-462[#]	PCR of S1 for FAdV
S1(795)BglII	TAGTTAAGATCTTTAATAAACAATAAACTTTTCCTTAAC[*]	772-795[#]	PCR of S1 for FAdV
S1(1227)BglII	TAGTTGAGATCTTTAAGTAATGTAAACCAACAGTCC[*]	1207-1227[#]	PCR of S1 for FAdV
S1(1617)BglII	TAGTTAAGATCTTTATGATCCATTAGTGAGTTTAATATAA[*]	1593-1617[#]	PCR of S1 for FAdV
Snab1F	GGCCGCGTAGAAATGAACGAGACCAGTGTC	pJJ885 8890-8919	PCR confirm FAdV
XbaR	GCCCATCAGAGGGCAAAGGCATAGAAGGAGC[*]	pJJ885 9341-9371	PCR confirm FAdV
SpeF	GGTTCGTGCACGTACGCGGAAACGAGGTCG	pJJ1054 4659-4689	PCR confirm FAdV
SpeR	GGCGGAGCTGCCACGTCAGCAGGTAAATC[*]	pJJ1054 5127-5155	PCR confirm FAdV
IBV-AO1	GGTGAAGTCACTGTTTATAG	6-25[#]	PCR confirm FAdV
IBV 5' GL533	GCCATGTTGTCACTGTCTATT[*]	512- 533[&]	qRT-PCR for IBV
IBV 5' GV391	GCTTTTGAGCCTAGCGTT	391-408[&]	qRT-PCR for IBV

[#]Numbering according to IBV strain N1/62, GenBank accession number U29522.
[&] Numbering according to IBV strain M41, GenBank number accession AY851295 (Callison *et al*., 2006).
[*]Sequence is reverse complementary to published sequence.
Restriction sites used for cloning are shown underlined.

3.3 Isolation of plasmid DNA

Small scale preparations of plasmid DNA were isolated using a Qiagen miniprep kit (Qiagen, Australia) according to the manufacturer's instructions. Large scale preparations of plasmid DNA were prepared using a Qiagen Endo-free plasmid maxiprep filter kit (Qiagen, Australia) according to the manufacturer's instructions.

3.4 Total RNA isolation and first strand cDNA synthesis

Total RNA was extracted from IBV infected allantoic fluid or trachea using a Qiagen RNeasy kit as per the manufacturer's protocol with the addition of a Proteinase K treatment (Qiagen, Australia). Complementary DNA (cDNA) was synthesised using avian myeblastosis virus (AMV) reverse transcriptase (RT) (Promega, Australia). Briefly, 0.5 µg of purified RNA was mixed with 100 pg of random hexamer primer (Promega, Australia) and incubated for 2 min at 100 ^{o}C, then chilled on ice. To this was added 5.0 µl of 5X AMV reaction buffer, 1.0 µl of 10 mM deoxynucleotide triphosphates (dNTP), 20 U RNasin, 20 U AMV RT enzyme and dH_20 to a final volume of 25 µl. Reactions were incubated for 1 h at 42 ^{o}C.

3.5 Restriction enzyme digests, ligations and transformations

All restriction enzyme digestions were performed according to the manufacturer's instructions (Promega, New England Biolabs). Ligations involved the use of vector to insert molar ratios of approximately 1:3 in all cases. DNA fragments, isolated from gels or purified from PCR products, were ligated using T4 DNA ligase (Promega, Australia) according to the manufacturers instructions. The mixture was then used to subsequently transform into electrocompetent *E.coli* DH5-α using procedures described in Sambrook *et al*. (1989).

3.6 Polymerase chain reaction

In general, the polymerase chain reactions (PCR) utilised a PCR mix containing 10 ul of 10 × PCR buffer (Qiagen, Australia), 20 µl of Q-solution (Qiagen, Australia), 1 ul of a 10 mM mix of dNTP (Fermentas, Australia), 100 ng of each forward and reverse primer, 2.5 units of Taq DNA polymerase (Qiagen, Australia) and approximately 10 ng of DNA template and dH_2O to a total reaction volume of 100 µl. Tubes were overlayed with a drop of mineral oil and placed in a DNA thermal cycler (Perkin-Elmer, USA). Samples were subjected to 35 amplification cycles involving denaturation at 94ºC, 1 min, annealing at 50ºC for 1 min followed by extension at 72ºC for a 3 min. The samples were finally subjected to a 72ºC, 15 min extension step.

3.7 Quantitative real time PCR

A quantitative real time PCR was developed based on SYBR Green detection. All reaction were conducted in a Mastercycler® ep realplex real time machine (Eppendorf, Germany) using Real Master Mix SYBR ROX (Eppendorf, Germany). Titration of primers IBV 5' GL533 and IBV 5' GV391 (Table 3.1) was achieved using cDNA derived from strain N4/02 (propagated in eggs). The primers targeted a region located within the 5'untranslated region of the IBV genome and have been described previously (Callison *et al*., 2006). Primer concentrations ranging from 0.3 µm to 0.9 µm were evaluated with different dilutions of cDNA and at different annealing temperatures ranging from 50 - 60ºC. A primer concentration of 0.5 µm for each primer at an annealing temperature of 55ºC gave the highest sensitivity and limited formation of primer dimmers. Another primer pair which targeted the 18s ribosomal RNA, was also utilised as a housekeeping gene to enable normalisation of cycle threshold (CT) values.

For quantification of viral load in tracheal samples RNA was extracted from homogenized tracheal samples and a total of 500ng reverse transcribed into cDNA as previously described. For real time PCR each reaction contained 8 µl of 2.5X PCR mix, 1 µl 20X Sybre solution, 0.5 µm of each primer and dH_2O to a volume of 19 µl. A 1 µl volume of cDNA sample was then added to the reaction (either neat. or diluted 1:5, 1:10). Housekeeping reactions were conducted in separate wells. An initial incubation for 10 min at 95ºC was used to activate the *Taq* polymerase followed by forty cycles of 95ºC, 15 sec (denaturing) and 55ºC, 1 min (annealing and extension) were then conducted. Melting curve analysis was performed and samples were considered positive if both an exponential increase in fluorescence and an IBV specific melting peak were observed.

3.8 Sequence analysis

All DNA sequencing was performed commercially by MicroMon (Monash University, Australia). Amino acid (AA) alignments were performed using the neighbour-joining (NJ) method (Saitou and Nei, 1987) implemented within Clustal X Version 1.62b (Thompson *et al.*, 1997), with 1,000 bootstrapping replicates. The dendrograms were plotted with Treeview version 1.5 (Page, 1996). For phylogenetic analysis the sequences of Australian strains deposited in GenBank were used with the following accession numbers: U29522 (N1/62), DQ059618 (N4/02), U29450 (N1/88), U29451(Q3/88), U29519 (VicS), U29453 (N3/62).

3.9 Outline of cross protection studies in chickens

For all *in vivo* experiments, specific pathogen free (SPF) eggs and chickens were used (sourced from Charles River Laboratories, Australia). Hatched chicks were housed in sterile positive pressure isolators for all experiments. For cross protection studies, one-day old SPF chicks (13-15 per group) were vaccinated with one of four commercial vaccines (Vic S, I, S, A3) or a selected IBV strain as specified. The viruses were administered intraocularly in 0.1 ml of Webster's poultry diluent using $10^3 EID_{50}$ per chick. In some cases a second dose of vaccine was administered either at day 14 or 18 post the primary vaccination. Chicks from each group were wing bled to establish the immune response pre-boost and pre-challenge. At 28 days post vaccination, chicks were challenged with one of three viruses, either N4/02 (10^4 EID_{50}), N1/88 (10^3 EID_{50}) or Q3/88 (10^3 EID_{50}) as a 0.1ml dose intraocularly. Five days later chicks were euthanased and the trachea removed aseptically for subsequent analysis.

All experiments described in this study were approved by the Australian Animal Health Laboratories Animal Ethics Committee in accordance with Australian State and Federal laws.

3.10 Isolation of challenge virus from the trachea

Scrapings of the inner epithelium of each trachea were collected into 2 ml of isolation media (see Appendix), freeze/thawed once and 0.2 ml volumes injected into the allantoic cavity of 5 ten-day-old embryonated SPF eggs. Following 60 hours incubation at 37^{o}C, the allanotic fluid was harvested and pooled for each trachea and clarified by centrifugation for 15 mins at 1500 *g*. The clarified allantoic fluid was then centrifuged at 65,000 *g* for 1 hr to pellet viral antigens. The pelleted material was resuspended in PBSA and assayed for the presence of IBV antigen by ELISA.

3.11 Measurement of ciliary activity in the trachea-

Trachea were placed in transport media (see Appendix) and incubated at 37^{o}C before being cut into rings and examined under a microscope for ciliary activity using methods previously described (Cook *et al*., 1999). For each trachea at least 10 rings were examined and each ring individually scored based upon a scale of 0-4, 0 representing no loss of activity and 4 complete loss of activity. The individual scores for each ring were added together to obtain a ciliostasis score for each chicken (ranging from 0-40).

3.12 Histopathology

Trachea and kidney tissues were fixed in 10% neutral buffered formalin (NBF) and embedded in paraffin wax. Duplicate sections of the tissues were prepared with one being stained with haematoxylin and eosin. The second slide was immunostained for the presence of IBV antigen. The immunostaining utilised monoclonal antibody (MAb) 51-10 directed against IBV, at a dilution of 1:20 (Ignjatovic and McWaters, 1991). After 1 hr incubation, the slides were washed and a secondary HRP-conjugated anti-mouse antibody (Sigma, Australia) was added at a 1:1000 dilution and incubated as before. Finally, the slides were washed and a colormetric signal obtained using 3-amino-9-ethylcarbazole (AEC) (see Appendix). Once mounted, the slides were microscopically examined and scored on a 0 to 4 assessment of severity of histological changes based on the following criteria:

- 0 = nil observed
- trace = very small foci of histological changes or immunoperoxidase staining
- 1 = small occasional foci

- 2 = widespread foci, almost continuous in the case of the tracheas
- 3 = quite advanced
- 4 = very severe

3.13 LMH cell line maintenance

Leghorn Male Hepatoma cells (LMH cells; American Type Culture Collection accession number #CRL-2117) were obtained from Dr Scott Tyack (CSIRO, AAHL). The cells were maintained in LMH growth medium (see Appendix) at 37ºC in the presence of 5% CO_2 . All flasks and/or plates used were pre-treated with filter sterilised 0.2% (w/v) bovine gelatine (Sigma, Australia) in PBSA (see Appendix) at 4ºC for at least one hour to assist adhesion. For passaging, cell monolayers were washed twice with PBSA and a single suspension was obtained by incubation with Trypsin/Versene (Gibco, Australia) for 5 minutes. Two volumes of fresh pre-warmed growth medium were added and the cellular suspension centrifuged at 1000 rpm. Cells were transferred to a fresh flask at a 1:4 split ratio for confluency in 3-4 days.

3.14 Transfection of LMH cells to generate recombinant FAdV

Twenty-four hours prior to transfection, fresh LMH cells were plated into a 24-well tissue culture plate and grown to 80% confluency. For each transfection,, a total of 1.5 ug DNA (1ug RHE containing the S1 fragment and 500ng LHE plasmid pJJ925) in 50ul Optimem (Invitrogen, Australia) was used per well. The combined DNA in Optimem was gently mixed in a polypropolene tube at room temperature for 5 minutes then 2ul lipofectamine 2000 (Invitrogen, Australia) diluted in 50ul of Optimem was added to each tube. The mixture was incubated at room temperature for a further 20 minutes then gently applied to a LMH monolayer which was covered with 500 ul of Optimem. The cultures were. incubated for 18 h at 37ºC with 5% CO_2. Each well was then supplemented with further 500ul of fresh, pre-warmed LMH growth medium. The transfection culture plates were then placed at 37ºC with 5% CO_2, Daily observations of the monolayers were made and after 5 days incubation, the transfected monolayers were serially passaged, up to a total of 4 passages or until positive cytopathic effect (CPE) was observed. In order to passage the potential viruses, the monolayer was frozen at -20ºC, thawed and 200ul of the supernatant was absorbed onto a fresh 24 well LMH monolayer with a confluency of approximately 80-90 %. Fresh growth medium was added following 1hr absorption at 37ºC. Once FAdV like CPE was detected, the recombinant viruses were propagated by passage onto a 75cm^2 LHM cell monolayer and incubated until complete CPE had occurred, usually 2-4 days.

3.15 *In vitro* characterisation of the FAdV-IBV recombinants

In order to detect expression of the IBV S1 protein, LMH cells in 75 cm^3 were grown to approximately 80% confluency, infected with 10^6 $TCID_{50}$ of rFAdV and the cultures observed daily until 80 - 100% CPE was visualized. The cells were then dislodged from the flask into the medium and frozen at -20ºC in 1 ml aliquots. These aliquots were subsequently thawed and tested for expression of S1 fragments by ELISA and western blot analysis. Confirmation of constructs was also performed using PCR to amplify the IBV expression cassettes ensuring that there had been no deletions in the IBV sequence during passage in LMH cells. These PCR products were subsequently sequenced to confirm that the IBV sequences were free of any errors.

3.16 *In vivo* characterisation of the FAdV-IBV recombinants

Initially, 18-day-old embryonated eggs were injected with selected rFAdV via the allantoic route using 10^6 $TCID_{50}$ per egg. Upon hatch, surviving chicks were transferred to positive pressure isolators. At 5 days post inoculation, cloacal swabs (immersed in 1 ml of isolation medium-see Appendix) were taken from chicks and assayed for the presence of virus in LMH cells. Following a single freeze/thaw, the supernatant from the cloacal swabs was passed through a 0.45 µm filter and 200 µl of the supernatant absorbed for 1 hr onto LMH cells at 80% confluency. Fresh growth medium was added and the were monolayers incubated at 37ºC with 5% CO_2. The cells were observed for 5 days for signs of FAdV CPE and serially passaged as previously described if no effect was seen. If CPE was observed, the supernatant and cells were harvested, total DNA was extracted (section 3.3) followed by PCR

confirmation of the recombinant viruses. Sera was also collected 14 days post inoculation and assayed for the presence of antibodies to IBV by western blot analysis and ELISA.
In a second bird trial, the efficacy of selected rFAdV to provide protection against IBV challenge was also evaluated. Chicks were inoculated at one day of age with selected rFAdV's using 10^6 $TCID_{50}$ per chick administered by intracrop gavage. Cloacal swabs were taken at day 5 post inoculation to determine the presence of recombinant virus by passage in LMH cells as described previously. Sera was collected 14 days post vaccination and the chicks boosted with a second 10^6 $TCID_{50}$ dose of rFAdV. Cloacal swabs were again taken 5 days post boost with sera collected 14 days post boost for ELISA and western blot analysis. At day 28, birds were challenged using 10^4 EID_{50} of IBV virus N1/62. Five days post challenge, tracheal scrapings were collected as described previously (Section 3.10). The tracheal scrapings were assayed for the presence of IBV challenge virus by passage in embryonated eggs and subsequent testing in IBV antigen ELISA.

3.17 General test outline of ELISA

For all ELISAs performed in this study, initial coating of the 96 well ELISA plates (Sarstedt, Australia) used reagents diluted in coating buffer overnight at 37^{o}C (see Appendix). Reagents and test samples were diluted in PBS with the addition of 0.05% Tween-20 (PBST) and 5% FCS as a blocking agent (ELISA dilution buffer). Volumes of 100 µl per well of each reagent and sample were added at all stages. Plates were incubated at 37°C for 1 h. All wash steps involved washing the plates three times using PBST. All reactions were detected using 2,2"-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS)(see Appendix). Absorbances were read on a Titertek Multiscan Plus absorbance reader (Titertek, USA) at 405 nm. All samples were tested in duplicate.

(i) IBV antigen ELISA

ELISA plates were coated with one of two anti IBV monoclonal antibodies diluted 1/500 in coating buffer. Monoclonal 16-30 was used for classical and N4/02 strains, while monoclonal 5-28 was used for the variant strains N1/88 and Q3/88 (Ignjatovic and McWaters, 1991). The plates were washed and the concentrated allantoic fluid described in section 3.10 added at a dilution of 1:3. The plates were incubated and washed and purified chicken anti IBV antibody added at a 1:1000 dilution. Plates were incubated and washed then a secondary horse radish peroxidise (HRP) conjugated goat anti chicken antibody (KPL, USA) diluted 1:1000 was added and incubated as before. After a final wash, plates were developed with ABTS and absorbances measured.

(ii) ELISA to detect antibodies against IBV

ELISA plates were coated with IBV strain Q1/76 (derived from concentrated allantoic fluid) diluted 1/400 in coating buffer. Plates were then washed and the test sera added at either 1:10 or 1:50 and serially diluted in log_2 increments across the plate. After 1 hr incubation the plates were washed and a secondary HRP conjugated goat anti chicken antibody (KPL, USA) diluted 1:1500 added and incubated as before. After a final wash, plates were developed with ABTS and absorbances measured.

(iii) FAdV antigen ELISA

ELISA plates were coated chicken derived anti-FAdV serum #18, diluted 1:1000 in coating buffer. Plates were then washed and test samples added either neat or serially diluted in log_2 increments across the plate. After incubation, plates were washed and anti-FAdV ascites MAb 6H2 (TropBio, Australia) added at a 1:800 dilution. Plates were incubated and washed and a secondary HRP conjugated goat anti-mouse antibody (Sigma, Australia), diluted 1:1000, was added and incubated as before. After a final wash, plates were developed with ABTS and absorbances measured.

(iv) ELISA to detect antibodies against FAdV

ELISA plates were coated with the anti FAdV MAb 6H2 (Trobio, Australia) diluted 1: 500 in coating buffer and incubated at 37^{o}C over night. Following the initial incubation, the plates were washed and FAdV added at 1:50 in dilution buffer. Plates were incubated, washed and test sera added either at 1:10 or serially diluted in log_2 increments across the plate. Plates were incubated, washed and a

secondary HRP conjugated goat anti chicken antibody (KPL, USA) added at 1:1500 and incubated as before. After a final wash, plates were developed with ABTS and absorbances measured.

3.18 Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels at 10% were made as outlined by (Sambrook and Russell, 2001) Sambrook *et al*. (1989) (see Appendix). Protein samples and molecular weight markers were diluted 1:1 in 2X reducing loading buffer (see Appendix) and then denatured by incubation at 100°C for 2 min. The protein samples were then electrophoresed in SDS- running buffer (see Appendix) at 170 V constant for 1 h.

3.19 Western blot analysis

SDS-PAGE gels were assembled in a transfer cassette and proteins transferred to nitrocellulose using a Biorad protean II mini gel system (BioRad, USA) according to the manufacturer's instructions. After transfer, the nitrocellulose membrane was placed in blocking buffer (see Appendix) for 1 h at room temperature. The blocking buffer was removed and the primary antibody was added diluted in blocking buffer. Test sera were diluted at 1:20, reagent chick anti IBV antibody was used at 1:1000 and MAb 5-28 specific for the S protein was used at 1:200. The membrane was incubated at room temperature for 1 h, with constant rocking and then washed four times for 5 min with Tris-buffered saline (TBS) containing 0.05 % Tween-20 (TBST) (see Appendix). For detection of chicken antibodies, a secondary horse radish peroxidise (HRP) conjugated goat anti chicken antibody (KPL, USA) diluted at 1:4000 was added, whilst MAb 5-28 was detected using a HRP conjugated goat anti mouse antibody diluted 1:1000 and incubated as before. The membrane was washed four times for 5 min using TBST before being developed using the ECL plus (Amersham Biosciences, England) according to the manufacturer's instructions. The membrane was subsequently exposed to Super RX medical X-ray film (Fuji, Japan) for as long as needed and developed using a Fujifilm FPM-100A X-ray developer (Fuji, Japan).

4. Results

4.1 Pathotype of N4/02 variant

Two week old SPF chickens were challenged with NSW isolate N4/02. No clinical signs were observed on day 2 and 3. Early on day 4 approximately 30% of chicks were depressed and showing signs of respiratory infection i.e. snicking, watery eyes or nasal discharge,. Two chicks in particular appeared to be more affected by the infection than the others with haunched posture observed and depression more severe. On day 5pi (post infection), it was apparent that the clinical symptoms were transient with most chicks appearing brighter than on day 4 although some sneezing persisted. Illness did not progress and the litter was observed to be dry. By day 6, several chicks still showed signs of respiratory illness although most seemed to be recovering. Several smaller chicks were observed to be still a little depressed although recovering by day 7. On days 10 and 14 several chicks remained slightly underweight, however, all chicks appeared to have recovered from the infection. No mortalities were recorded. Hence, N4/02 appeared to be a mild respiratory strain that did not progress, allowing recovery from 7 days post challenge.

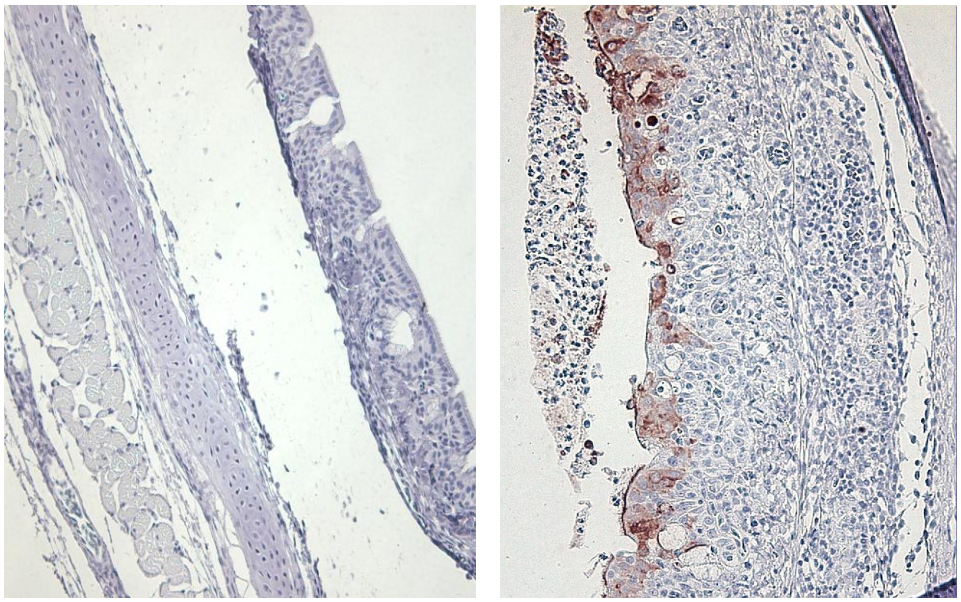
The trachea and kidney were collected at 3, 5, 7, 10 and 14 days post challenge for histological examination and compared to non-infected controls (Table 4.1). At day 3 post infection (pi) two chickens showed early tracheal infection with cellular infiltration and exudate, but only early squamous metaplasia. Two were apparently unaffected. At day 5 pi, all four chickens had mild non-suppurative tracheitis with viral antigen in the epithelium. An example of the type of staining observed in the trachea is shown in Figure 4.1. At day 7pi tracheal lesions persisted but there was substantial reduction in visible antigen detection. One chicken had both intracellular and intratubular detectable viral antigen in its kidney (Figure 4.2). It did not have detectable renal lesions. At day 10pi there was substantial resolution with redevelopment of ciliated columnar epithelium. One chicken was unusual in that there was detectable antigen in its ciliated columnar epithelium. It had more severe cell infiltration and, like the two chickens at day 3, may have missed early infection and then became infected by contact. At day 14pi, epithelial resolution continued and there was no detectable viral antigen. It was noted that there was some cellular infiltration in some kidneys of the non-infected controls (Table 4.1) however these were fewer in number (4 compared with 7 in the challenged chickens) and all were milder than some in the challenged group. Hence, the N4/02 strain caused mild tracheitis in chickens that was resolving from day 7 onwards. Except for one chicken on day 7 pi, there was no indication of N4/02 localising in the kidneys.

Table 4.1 Histopathology of trachea and kidney after infection with N4/02

Day post infection		Trachea				Kidney	
		CI[1]	Exudate[2]	Epith Met[3]	HPR[4]	CI[1]	HPR[4]
N4/02 infected	3	3/4	2/4	2/4	2/4	0/4	0/4
	5	4/4	3/4	4/4	4/4	2/4	0/4
	7	4/4	3/4	4/4	1/4	2/4	1/4
	10	4/4	0/4	1/4	1/4	1/4	0/4
	14	4/4	/4	2/4	0/4	2/4	0/4
No virus control	3	0/4	0/4	0/4	0/4	1/4	0/1
	5	0/4	0/4	0/4	0/1	2/4	0/1
	7	1/4	0/4	0/4	0/1	0/4	0/1
	10	1/4	0/4	0/4	0/1	1/4	0/1
	14	1/4	0/4	0/4	0/1	0/4	0/1

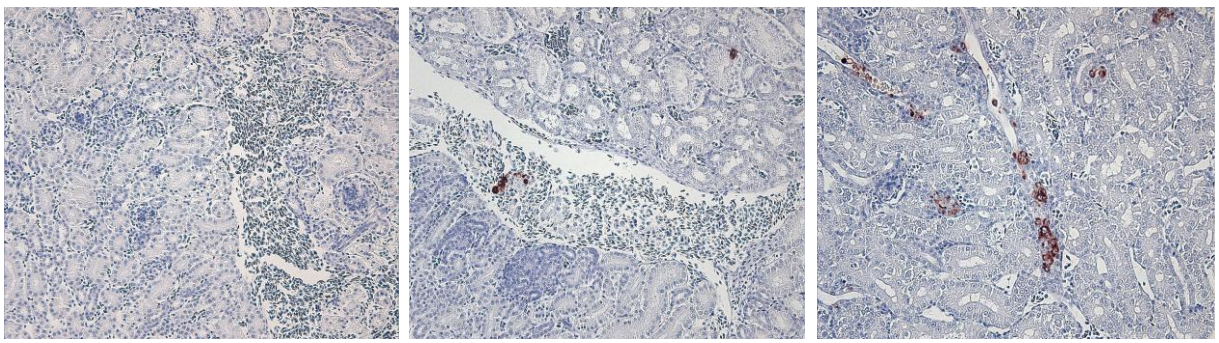
[1]CI = mononuclear cell infiltration, usually submucosal in tracheas and interstitial in kidneys.
[2]Exudate = fibrin and leucocytes in the tracheal lumen.
[3]Epith Met = epithelial metaplasia, usually ciliated columnar epithelium changing to squamous.
[4]HPR = immunoperoxidase staining indicating presence of IBV antigens.

Figure 4.1. Immunohistochemistry of N4/02 in the trachea.



Non-infected N4/02 infected

Figure 4.2. Immunohistochemistry of N4/02 strain in the kidney.



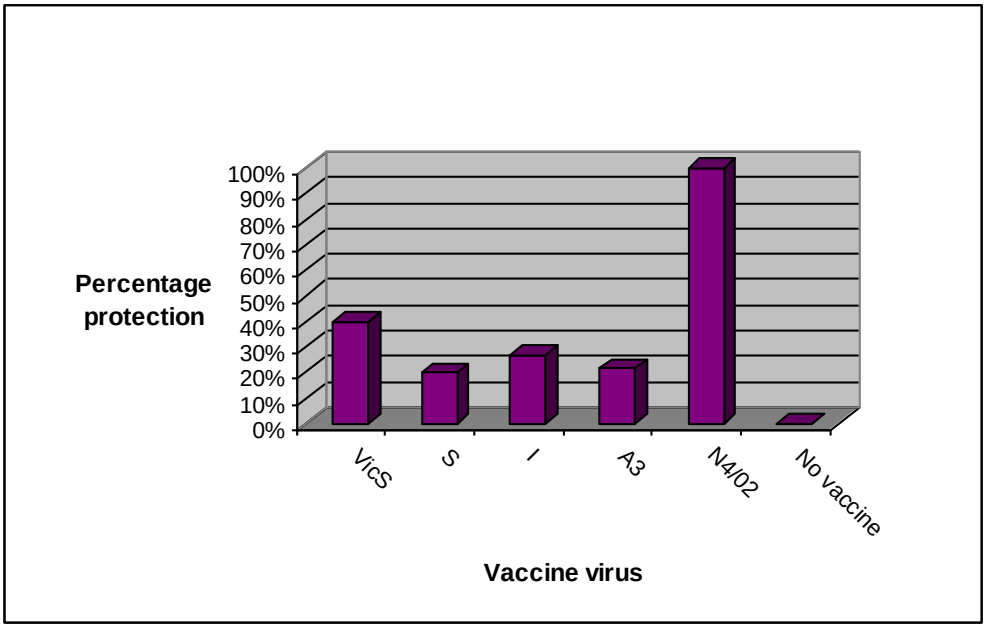
Non-infected N4/02 infected Nephropathogenic N1/62

4.2 Protection afforded by commercial IB vaccines against N4/02

(i) Efficacy of using a single dose of commercial vaccine

One day old chicks were vaccinated with one of four commercially available vaccine strains VicS, I A3 and S. Unvaccinated chicks, and chicks vaccinated with homologous variant virus N4/02 were included as controls. Chicks were subsequently challenged at 28 days of age with the NSW isolate N4/02. Five days after challenge the tracheas were removed and processed in an attempt to reisolate virus through passaging of tracheal scrapings in SPF eggs and subsequent testing of the allantoic fluid for IBV antigen by ELISA. None of the vaccines afforded complete protection against challenge by NSW isolate N4/02 as evidenced by the ability to recover IBV in SPF eggs (Figure 4.3). VicS provided 40% protection, while I, A3 and S provided 27%, 21% and 20% protection respectively. The control group vaccinated with homologous N4/02 virus showed complete protection while the unvaccinated control group showed no protection.

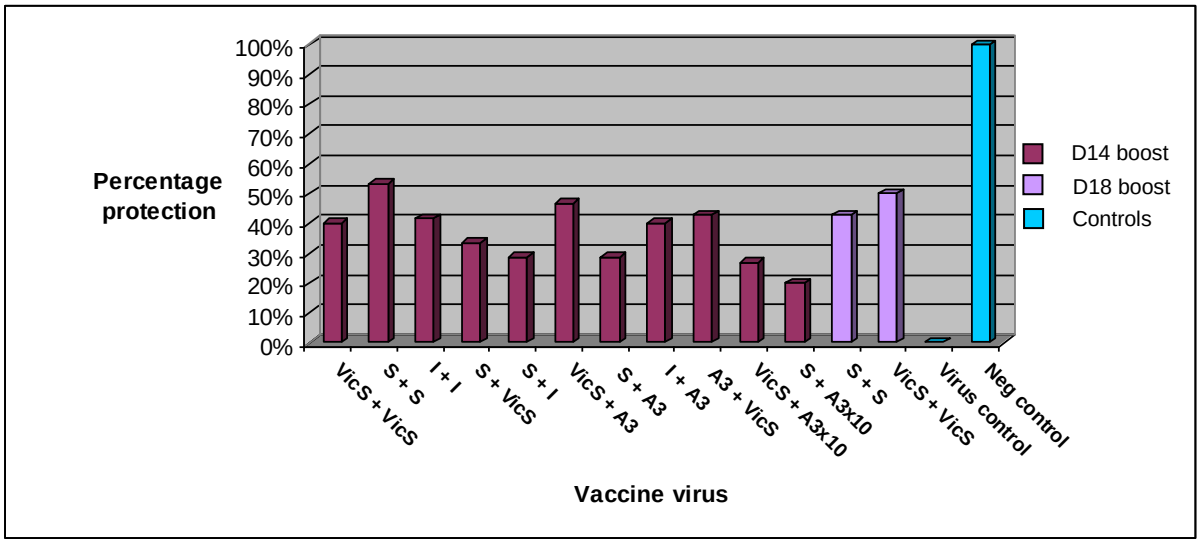
Figure 4.3. Efficacy of different commercial IB vaccines against N4/02 challenge using a single dose



(ii) Efficacy of using a combination of commercial vaccines

The efficacy of using a combination of two existing vaccines (both the same and different) to provide protection against NSW N4/02 variant was investigated. Different parameters were tested, including the influence of factors such as vaccine concentration, timing of second vaccination (day 14 or 18) and the order in which vaccines are administered. Chicks received two vaccinations on days 1 and 14 post hatch with the following combinations of vaccines: VicS + VicS, S + S, I + I, S + VicS, S + I, VicS + A3, S + A3, I + A3 and A3 + VicS. Chicks were subsequently challenged at 28 days of age with NSW isolate N4/02 and the tracheas harvested in an attempt to reisolate virus through passaging in SPF eggs with any resulting IBV antigen detected in ELISA. The level of protection shown by the various combinations are shown in Figure 4.4. None of the combinations tested provided complete protection against the challenge virus N4/02. Various levels of protection were observed ranging from 29 to 53%, the S + S combination providing the highest level of protection, while the S + A3 combination provided the least protection with 29%. In most instances these percentages were only slightly greater than those based upon a single vaccination. In another 2 groups we investigated the effect of vaccinating chicks on days 1 and 18 using the following combinations: VicS + VicS and S + S. Administering the boost at day 18 post hatch appeared to lower the level of protection obtained with S + S to 43%, while marginally increasing the level of protection seen with VicS + VicS from 40% from 44% (Figure 4.4). Initially we titrated each of the 4 commercial vaccines in SPF eggs to obtain the virus titre. Most of the vaccines were of the same titre, however A3 appeared to be approximately 10 fold lower than the other vaccine strains. Hence, we attempted to use a 10 fold higher dose of A3 (A3x10) to test if this increased dose would provide a higher level of protection using the following combinations vaccinating chicks on days 1 and 14: VicS + A3x10, S + A3x10. The levels of protection we observed for these groups was 27% and 20%, respectively (Figure 4.4).

Figure 4.4. Efficacy of different combinations of commercial IB vaccines against N4/02 challenge



The reciprocal antibody titres against IBV were determined for each of the vaccinated chickens (Table 4.2). Overall, there were no significant differences in antibody titres between any of the experimental groups other than the fact that antibody titres were generally higher after a secondary vaccination. There was no correlation between serum titres in birds and the level of protection obtained. Similar observations have been documented by other researchers (Ignjatovic and Galli, 1994; Cavanagh, 2003).

Table 4.2. IBV antibody response in chickens vaccinated with various vaccine combinations.

		Titre at day 28				
		Secondary vaccination with				
Primary vaccination	**Titre at day 14**	**Vic S**	**S**	**I**	**A3**	**A3 (X10)**
VicS	800*	6400			3200	3200
	1600	3200			3200	1600
	800	3200			1600	1600
	400	6400			3200	3200
	800	3200			3200	1600
S	800		3200		1600	1600
	1600		1600		3200	3200
	800		3200		1600	800
	800		3200		3200	1600
	400		1600		3200	1600
I	800			3200	3200	
	800			6400	3200	
	400			1600	6400	
	400			3200	3200	
	1600			3200	12800	
A3	400	12800				
	800	6400				
	400	3200				
	800	12800				
	800	3200				

#Shown as reciprocal titre

4.3 Use of ciliary activity in the trachea to assess cross protection

Ciliary motility in trachea was investigated as an alternative method in evaluating cross-protection. Chicks sampled from cross protection studies described above were euthanased at day 5 post challenge and the trachea removed. A total of ten tracheal rings from each chick were examined and assigned an average ciliastasis score ranging from 1-40; scores lower than 20 were considered protected while scores higher than 20 were considered not protected. Two additional groups of control chicks were included as positive (challenged with N4/02) and negative (unvaccinated, unchallenged) controls. The protection scores obtained using cilastasis were compared to those obtained using virus isolation in eggs (Table 4.3). There was total agreement between the two methods when comparing the virus challenge (100 % infected) or uninfected controls (0% infected). However, when attempting to compare vaccinated groups of birds (variable levels of protection) measurement of ciliary activity was found to be less sensitive than virus isolation in eggs, failing to detect approximately half of the infected birds. Hence, ciliastasis is not as sensitive as virus isolation and is therefore not recommended for evaluating cross protection between different IBV strains.

Table 4.3. Comparison of protection scores based on ciliostasis versus virus isolation in eggs

Vaccine virus	No. trachea infected/no. tested by ciliostasis	Percentage protected by ciliostasis	Percentage protected by virus isolation
Vic S	0/3	100	40
S	1/2	50	20
A3	2/5	60	21
I	0/4	100	27
N4/02	0/5	100	100
S + S D14	0/5	100	53
S + A3 D14	2/5	60	29
S + V D14	4/10	60	33
V + V D18	4/10	60	50
S + S D18	2/10	80	43
Challenge N4/02	9/9	0	0
Unvaccinated/unchallenged	0/8	100	100

4.4 Use of qRT-PCR on the trachea to assess cross protection

A quantitative real time PCR for IBV was developed using SYBR Green detection. Initially the assay was optimized using viral cDNA derived from N4/02 virus propagated in eggs. The assay was found to be highly sensitive (CT value of 18.58) yielding a specific product with a melting temperature of 84ºC which was absent in the negative control. However, an attempt to quantify the viral load of N4/02 in tracheal samples was not possible. The cDNA derived from tracheal samples appeared to amplify a non-specific gene product with a melting temperature of 76-78 ºC in most samples, including the negative controls (un-infected) (Table 4.4). With the exception of 3 tracheal samples, this nonspecific product appeared to be in highest concentration and was therefore responsible for the high CT values observed. Hence, further optimization of this assay is required to accurately detect IBV in the trachea.

Table 4.4. Comparison of ciliostasis and qRT-PCR for assessment of cross protection

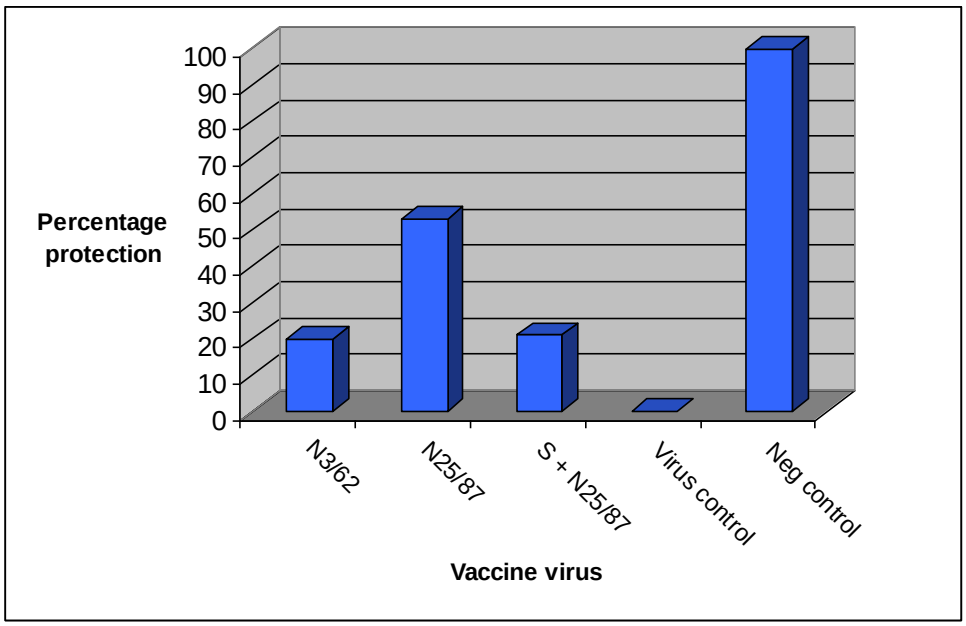
Vaccines used before challenge with N4/02	Ciliostasis* score	Real Time PCR	
		CT&	Tm#
S + S	29	26.79	77
	1	24.77	77
	8	29.00	76
	33	25.35	76
	9	28.34	77
	10	26.31	77
	10	25.44	78
	11	27.33	78
	11	26.29	75
	12	26.14	77
S + VicS	5	25.74	78
	9	29.11	78
	39	29.43	84
	14	27.79	78
	9	28.44	78
	22	24.19	78
	35	24.14	78
	3	27.61	78
	32	27.97	78
	2	26.35	77
VicS + VicS	3	26.95	78
	8	27.29	77
	23	26.44	78
	14	28.21	78
	31	26.11	77
	12	26.41	78
	11	24.64	77
	29	24.31	77
	25	26.12	77
	3	24.03	78
Virus control	40	26.37	78
	40	25.95	77
	40	28.56	78
	39	24.78	77
	40	27.56	78
	40	26.66	84
	40	28.84	77
	40	26.39	77
	40	26.66	84
	40	18.32	75
Negative control	2	25.36	77
	4	25.19	78
	2	25.07	76
	2	24.49	77
	1	29.65	77
Real Time IBV control (egg propagated)	ND%	18.58	84

*Ciliostasis score > 20 = positive. Scores provided are for individual birds.
&CT- cycle time for individual birds
#Tm- melting temperature of 84 indicative of IBV (shown shaded)
%ND not determined

4.5 Identification of other potential IB vaccine candidates

Based upon published data and previous cross protections studies (Sapats *et al.*, manuscript in preparation), two potential vaccine candidates, N25/87 and N3/62 (also known as G virus), were chosen from the repertoire of IBV strains held at AAHL. A single dose of these viruses was first tested for their efficacy to provide protection against N4/02. Of the two strains tested, N25/87 provided the highest level of protection at 53% compared to 20% protection obtained with N3/62 (Figure 4.5). This suggested that N25/87 may be more efficacious than any of the previously tested commercial vaccines, including Vic S which had previously given 40% protection as a single dose. Subsequently, the N25/87 strain was used to boost birds previously vaccinated with commercial S vaccine. However, the level of protection appeared to decrease to 21% (Figure 4.5) indicating that a combination of S and N25/87 was unable to provide complete protection against variant N4/02.

Figure 4.5. Vaccine efficacy of N25/87 and N3/62 against challenge with N4/02



4.6 Protection studies using the N4/02 variant as a potential vaccine against other variants

Overseas laboratories have demonstrated that using a combination of IBV strains for vaccination (e.g. a standard strain and a variant strain) can provide broad cross protection against many different types of variant strains without the need for introducing new vaccines in the face of a new variant emerging. As the NSW variant N4/02 was being considered for the production of a new vaccine in Australia, we wished to determine if N4/02 could be used in combination with the standard S vaccine to provide protection against other previously isolated Australian variants (NSW isolate N1/88 and Qld isolate Q3/88). Chickens were vaccinated at one day of age with S, boosted with N4/02 on day 14 and subsequently challenged with N1/88 on day 28. Birds were euthanased 5 days later and re-isolation of the challenge virus attempted through passaging of tracheal scrapings in SPF eggs. Using a combination of S and N4/02, a protection score of 93% was obtained against N1/88 compared to a protection score of 53% when S or N4/02 were used alone or 46% when two doses of S were used (Figure 4.6). Similar results were obtained when challenging birds with Q3/88 with 100% protection being achieved only when S and N4/02 were used in combination (Figure 4.7). These results therefore demonstrated the potential of using a combination of vaccines (standard and variant) to provide protection against other emerging variant strains in Australia.

Figure 4.6. Vaccine efficacy of N4/02 against challenge with N1/88

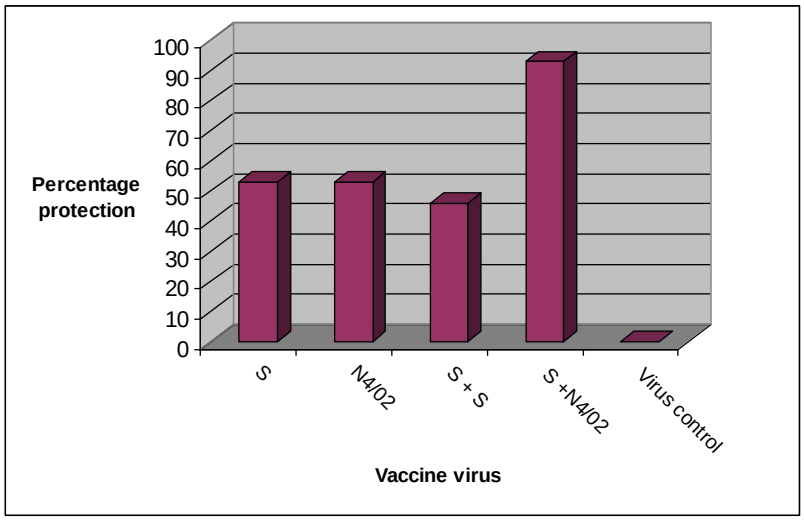
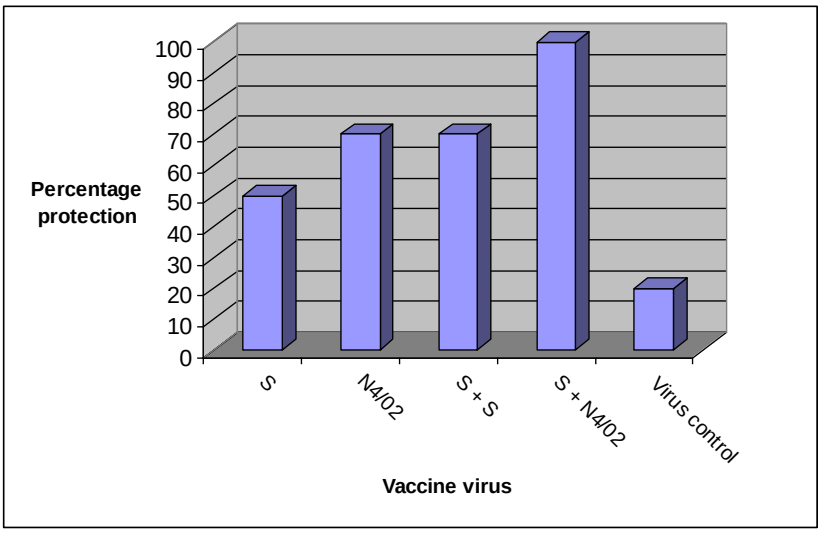


Figure 4.7. Vaccine efficacy of N4/02 against Q3/88



4.7 Construction of rFAdV containing S1 gene fragments of IBV

The Australian strain N1/62 (also know as T virus) was chosen for the expression of the viral S1 protein in FAdV. Randomly primed cDNA was generated against N1/62 and 6 individual PCR reactions set up to amplify the entire S1 gene. These PCR products were cloned and sequenced giving a consensus sequence for the S1 gene. One clone was chosen which most resembled the consensus sequence. This clone was subsequently used for a second round of PCR reactions to amplify smaller regions of the S1 gene containing the coding sequences for the first 154, 265, 409 or 539 amino acids of the S1 protein. These fragments of the S1 protein were chosen based on previously published data which suggested that they may encompass regions involved in protection (Cavanagh *et al*., 1988; Koch *et al*., 1990; Kant *et al*., 1992). Using the *Eco*RV and *Bgl*II restriction sites incorporated by PCR, these S1 fragments were individually cloned into plasmid pJJ1050 containing the CMVie promoter. The nucleotide sequence of these clones was determined and unfortunately a PCR error was detected in two of the clones (introducing a stop codon) requiring a re-amplification of both S1 fragments. These fragments were subsequently sequenced and confirmed to be free of any PCR errors. The second stage involved cloning of these expression cassettes containing the S1 fragments into the RHE of the FAdV genome. For this purpose 2 different RHE's were used for cloning, one contained a 54 base pair deletion (plasmid pJJ1054) and the other a 2,300 base pair deletion (plasmid

pJJ885). The expression cassettes containing IBV sequences were excised from pJJ1050 using *Not*1 restriction sites that flanked the expression cassette and ligated into both of the FAdV-8 RHE plasmids. The orientation of the S1 fragments was confirmed and the plasmids generated where transfected with the LHE of the FAdV genome using the LMH cell line. Transfected monolayers were serially passaged following 5 days incubation for a total of 4 passages. Once FAdV like CPE was detected, the recombinant viruses were propagated in LHM cells and the viral stocks produced. The resulting recombinant FAdV viruses (rFAdV) were designated as follows: rFADV encoding IBV protein fragments 154 to 539 within the large deletion rFAdV vector were designated rFAdV-154_L to rFAdV-539_L, while the corresponding constructs within the small deletion rFAdV vector were designated rFAdV-154_S to rFAdV-539_S. Each of the constructs was tested by PCR to confirm insertion of IBV sequences into the FAdV genome using primers which flanked the entire expression cassette (SnabF aad XbaR for large deletion rFAdV; SpeF and SpeR for small deletion rFAdV) as well as an internal primer with one external primer which amplified the IBV S1 gene (AO1 and XbaR or SpeR). With the exception of construct rFAdV-409_s, all recombinants produced bands of the expected sizes shown in Table 4.5. The recombinant rFAdV-409_s appeared to have a large deletion in the 3' end of the S1 gene and was therefore not studied further.

Table 4.5. PCR confirmation of IBV expression cassettes inserted into in rFAdV

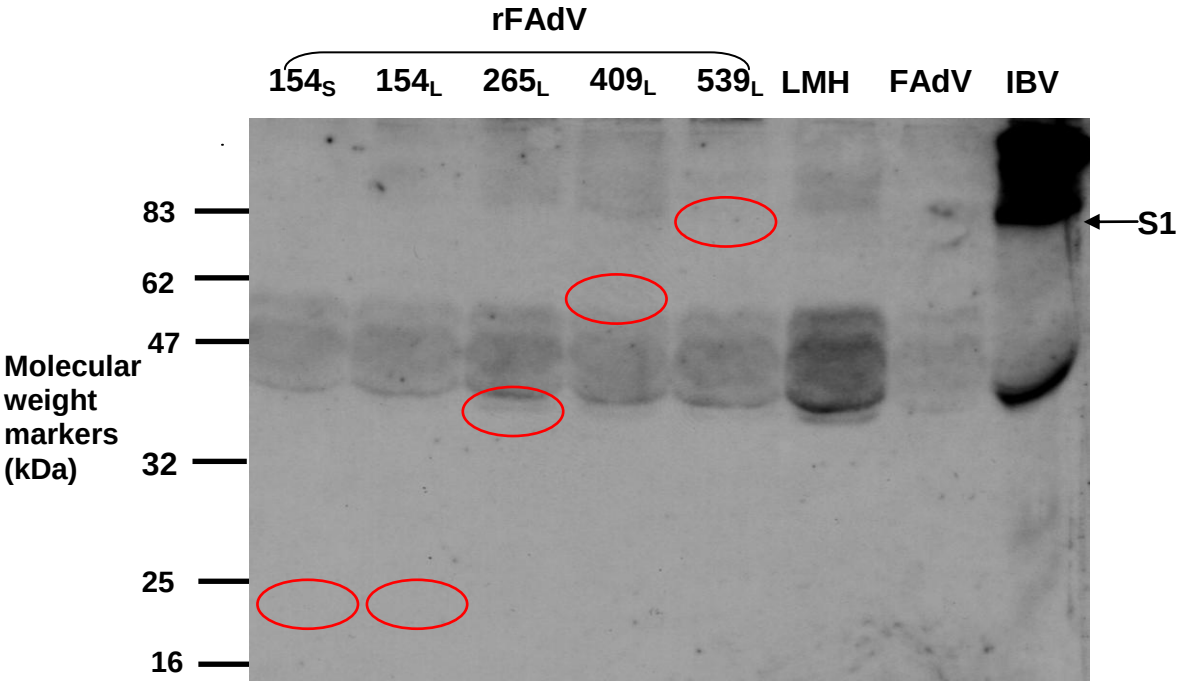
rFAdV construct	**Size of DNA fragment expected by PCR (bp)**	
	SnabF / XbaR*	**AO1 /XbaR&**
rFAdV-154_L	1869	874
rFAdV-265_L	2202	1207
rFAdV-409_L	2634	1639
rFAdV-539_L	3024	2029
	SpeF / SpeR#	**AO1/ SpeR&**
rFAdV-154_S	1884	938
rFAdV-265_s	2217	1271
rFAdV-539_s	3049	2090

*Primers amplify the entire expression cassette within the large deletion FAdV vector
#Primers amplify the entire expression cassette within the small deletion FAdV vector
&Primers based upon one internal and one external primer to specifically amplify the IBV S1 gene

4.8 *In vitro* analysis of rFAdV

In order to detect expression of the IBV S1 protein, LMH cells infected with different rFAdV were harvested (cells and supernatant) once 80-100% CPE was visualized. Cells were disrupted by freeze/thawing and aliquots run on SDS-PAGE and transferred to nitrocellulose for western blotting with hyperimmune anti–IBV sera. None of the recombinants appeared to express detectable levels of the S1 protein. Results obtained for constructs rFADV-154_S, rFADV-154_S, rFADV-154_L, rFADV-265_L, rFADV-409_L and rFADV-539_L are shown in Figure 4.8, with red circles showing the expected location of expressed S1 protein fragments. The blot also contains negative controls (FAdV vector control containing no IBV insert and uninfected LMH cells) and a positive control containing N1/62 virus. The infected cell supernatants were also tested in an IBV antigen ELISA producing a negative result (results not shown).

Figure 4.8. Western blot analysis of IBV S1 fragments expressed by rFAdV



4.9 *In vivo* analysis of rFAdV

Previous studies had also failed to detect *in vitro* expression of the S1 protein by FAdV, however chickens immunized with the recombinant induced an antibody response against the S1 protein and were protected against subsequent challenge with IBV (Johnson *et al*., 2003). Hence, we sought to evaluate selected rFAdV for their ability to induce a similar antibody response and provide protection against challenge with IBV. Initially construct rFAdV-154_L was chosen for *in ovo* administration into 18 day old embryonated eggs using 10^6 $TCID_{50}$. This dose of virus proved lethal for many embryo's with only 2/10 chicks surviving through to hatch. Sera was collected from the surviving chicks and the antibody response against IBV determined by ELISA and western blot. All samples tested negative suggesting that a single dose of rFAdV-154_L may not be adequate to induce an immune response in chickens towards S1 (results not shown). A second animal trial was initiated using constructs rFAdV-265_L, rFAdV-265_L and rFAdV-265_L to vaccinate one day old birds followed by a boost 14 days post the primary vaccination. Five days after each vaccination cloacal swabs were taken and passaged in LMH cells in an attempt to recover the rFAdV. Although rFAdV were readily recovered from swabs taken after the primary vaccination (100%), after the secondary vaccination only 20% of the swabs tested positive for rFAdV. The inability to detect rFAdV in most chickens after the secondary vaccination may have been as a result of the chicken mounting an immune response towards the vector after the primary vaccination. In all cases where rFAdV could be recovered, the virus was shown to contain the entire expression cassette by PCR with no apparent deletions detected. Testing of sera collected on day 14 (after primary vaccination) and day 28 (after secondary vaccination) in ELISA revealed no detectable antibodies to IBV (Table 4.6). However, all chicks produced an antibody response towards FAdV which was significantly higher after the secondary vaccination. Sera were also tested for antibodies to S1 by western blot analysis. None of the sera tested appeared to contain antibodies specific for the IBV S1 protein (approximately 80 kDa). Results obtained for 5 individual chicken sera (Ch 1 - Ch 5) collected on day 28 are shown in Figure 4.9, beside a negative FAdV vector control (containing no IBV insert) and positive controls using hyperimmune anti-IBV chicken sera and MAb 5-28 specific for the IBV S1 protein. At 28 days of age

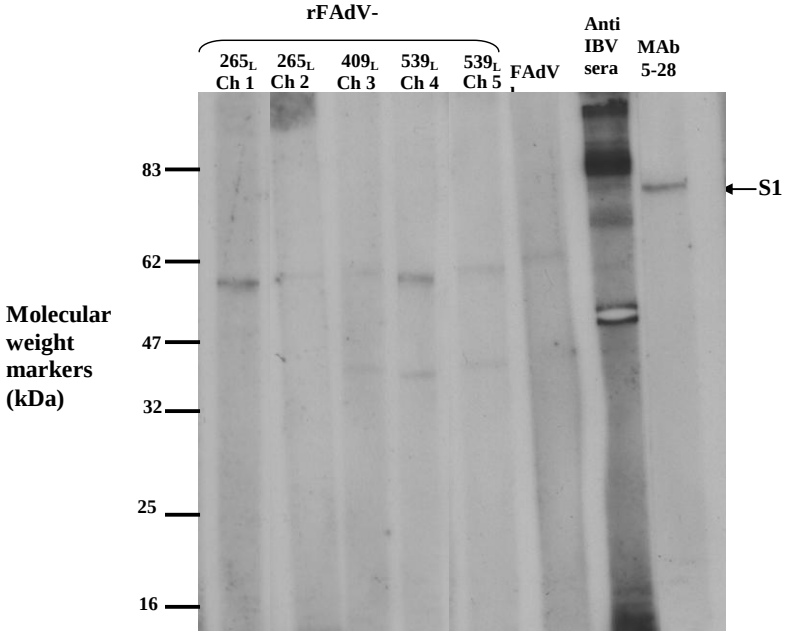
chickens were challenged with IBV strain N1/62. None of the rFAdV constructs was able to provide protection against IBV, with all chickens testing positive for IBV by virus isolation.

Table 4.6. Antibody responses against either FAdV or IBV after vaccination with selected rFAdV

Sera collected	rFAdV construct used for vaccination					
	rFAdV-265_L		rFAdV-409_L		rFAdV-539_L	
	anti-FAdV*	anti-IBV*	anti-FAdV*	anti-IBV*	anti-FAdV*	anti-IBV*
14 days post primary vaccination	160	0	NA#	NA	320	0
	160	0	NA	NA	80	0
	80	0	NA	NA	160	0
	160	0	NA	NA	320	0
	160	0	NA	NA	80	0
	160	0	NA	NA	160	0
14 days post secondary vaccination	320	0	2560	0	320	0
	1280	0	1280	0	1280	0
	2560	0	2560	0	640	0
	640	0	1280	0	640	0
	1280	0	1280	0	1280	0
	640	0	320	0	1280	0
	2560	0	80	0	640	0
	320	0			1280	0

*Reciprocal antibody titres in individual chicken serum against either FAdV or IBV as determined by ELISA.
#NA = not available

Figure 4.9. Western blot to detect anti-IBV antibodies in the sera of birds vaccinated with rFAdV



5. Discussion of Results

The pathotype of NSW variant N4/02 was assessed in SPF birds and the efficacy of different vaccine strategies evaluated. Results confirmed field observations, that that N4/02 was a mild respiratory strain inducing no mortalities in SPF birds. Irrespective of the vaccine used, dose or timing of secondary boost, it was not possible to achieve greater than 53% protection against N4/02 in SPF birds. Given the mild nature of the virus and the partial protection induced by commercial vaccines, it is therefore not surprising that this variant continues to cause intermittent outbreaks in NSW, particularly during the stressful hot summer months when humidity is high and secondary bacterial infections more common. These results also suggest that the commercial vaccines currently available in Australia do not have sufficient antigenic diversity to provide broad cross protection. Of the four vaccines used in Australia, 3 belong to the same serotype and are closely related at the genetic level (Figure 1). Although the fourth commercial vaccine (A3) belongs to a different serotype, using the A3 vaccine in various combinations with the other 3 commercial strains did not significantly improve the level of protection achieved against N4/02. Hence, there is a need to develop alternative vaccines for use in Australia which are antigenically different to those currently available.

Studies overseas have demonstrated that the use of two genetically different viruses of different serotypes administered two weeks apart is able to provide broad cross protection against other variant strains, thus alleviating the need to introduce new vaccines in the face of emerging variants (Cook *et al*., 1999). With this in mind, the efficacy of the N4/02 variant itself as a possible vaccine candidate was evaluated in SPF birds. By using a combination of the S commercial vaccine and the N4/02 strain administered 2 weeks apart, it was possible to achieve 93% and 100% protection against subsequent challenge with previously isolated IBV variants N1/88 and Q3/88, respectively. Although the experiments detailed in this report focus on vaccines which protect the respiratory tract in SPF birds, future experiments would need to be performed using broilers to ensure that the recommended vaccination program is effective at providing protection in both the respiratory tract and kidneys. In addition, the vaccines used in this report were administered by eye drop to ensure that all chicks received an equivalent dose. It would be important to assess if administration by aerosol or drinking water is as effective at providing broad cross protection.

The development of a subunit vaccine containing the S1 glycoprotein of N4/02 would ideally be the vaccine of choice, alleviating some of the concerns surrounding the use of multiple vaccine strains for IBV in the field. Unfortunately, attempts to express the IBV S1 protein using expression systems based on either baculovirus (Song *et al*., 1998) or fowlpox (Wang *et al*., 2002) have not been particularly successful, providing only low levels of protection. Although the use FAdV has shown the most promising results with greater than 90 % protection achieved in SPF birds (Johnson *et al*., 2003), the problems encountered in this study suggest that the system needs further optimization and development. Recently a reverse genetics system has been developed for IBV (Casais *et al*., 2003; Hodgson *et al*., 2004; Cavanagh *et al*., 2007).This has greatly facilitated the ability to accurately modify the IBV genome for vaccine development and for studying the roles of the different IBV proteins in terms of pathogenicity. The development of such a system based upon an Australian strain could greatly enhance our understanding of the virulence of local strains and lead to construction of better vaccines for use in Australia. Recently, an improved IBV vaccine was developed overseas using reverse genetics which allowed *in ovo* administration and provided effective immunity without affecting hatchability (Tarpey *et al*., 2006).

Our work also focused on developing improved methods to assess cross protection between different IBV strains. Of the two methods used to assess cross protection, ciliostasis was found to be less sensitive than virus isolation in eggs, failing to detect approximately half of the infected birds. It has been argued that declaring a chicken "not-protected" due to the ability to recover small amounts of challenge virus from the trachea, when ciliary activity is normal, is being too stringent (Cavanagh, 2003). However, failure to detect low levels of viral replication could lead to subclinical infections such as those observed with the N4/02 variant in NSW. There is also a danger that such low level

infections could allow viruses to slowly adapt into more pathogenic strains through mutation or genetic recombination with other IBV strains. It is somewhat reassuring however, that although we continue to isolate the N4/02 isolate from chicken flocks in NSW (last identified in December 2006), reports from the field suggest that there has been no major changes in virulence. Although we attempted to develop a third method of evaluating cross protection using qRT-PCR (Callison *et al*., 2006), this assay proved unsuccessful. Although it was possible to readily detect IBV that had been propagated in eggs, a non-specific product was amplified using tissues derived from the trachea rendering the test invalid. A number of parameters would need to be further optimized including the modification of the primers used, the use of specific primers to prime transcription of RNA as apposed to the random hexamers used in the study and the use of a specific TaqMan probe for IBV. Recently a number of other qRT-PCR assays have been developed for IBV and these could also be investigated for their efficacy in detecting IBV in the trachea (Jackwood *et al*., 2003; Callison *et al*., 2005; Escutenaire *et al*., 2007).

An attempt was made to express various regions of the IBV S1 protein in FAdV in order to localize protective epitopes within the S1 protein. Unfortunately, it was not possible to detect expression of the S1 fragments, either *in vitro* or *in vivo* and chickens challenged with IBV were not protected. Although a study conducted by Johnson *et al*. (2003) also failed to detect expression of the IBV S1 protein by FAdV *in vitro*, chickens vaccinated with the recombinant produced an antibody response to S1 and showed 90% protection against IBV challenge. There are a number of possible explanations for these differences. The FAdV vector used by Johnson *et al*. (2003) was derived from a hypervirulent strain of FAdV apposed to the mild strain of FAdV used in our constructs. This difference in virulence between the two FAdV vectors could have dramatically influenced the expression levels *in vivo*. The promoters used in the two studies were also different. Johnson e*t al*. (2003) used the endogenous fowl adenovirus major late promoter (MLP) to drive expression of the S1 gene, while in our system we used the CMVie promoter. Although most studies demonstrate that the CMVie promoter out performs the MLP (Goossens *et al*., 2000; de Wilt *et al*., 2001), use of the CMVie promoter in our vector may have had a detrimental effect on the expression levels of S1. The two studies also used S1 genes derived from different IBV strains (VicS versus N1/62 used in our study) which could have influenced expression and/or degradation levels of the S1 protein.

Although the N4/02 strain was identified as a mild respiratory strain in SPF birds it must be emphasised that the results observed in SPF birds may not be typical of results observed in commercial broilers. Indeed, evidence from the field suggests some kidney disease in a small percentage of broilers. With the exception of one bird (1/20), there was no indication of kidney disease in SPF birds. Such differences in host susceptibility to IBV have previously been shown between different genetic breeds of chicken (Otsuki *et al*., 1987). Hence, there is a need to confirm the pathotype in commercial broilers and layers.

6. Implications

- NSW variant N4/02 identified as a mild respiratory strain, causing subclinical infection in the absence of any secondary bacterial infection.
- None of the commercially available vaccines in Australia, either individually or in various combinations are able to provide greater than 53% protection against the N4/02 variant. This would suggest the currently available IB vaccines do not have sufficient antigenic diversity to provide broad cross protection.
- Broad cross protection against previously isolated IBV variants can be achieved using a combination of two vaccines, S vaccine strain and N4/02 strain, administered 2 weeks apart.
- Measurement of ciliary activity is less sensitive than virus isolation in eggs and therefore not recommended for assessment of cross protection.
- The FAdV vector utilized in this study was unable to express detectable levels of the IBV S1 protein either *in vitro* or *in vivo*.

7. Recommendations

- The potential development of N4/02 as an alternative vaccine to expand the repertoire of IB vaccines currently available in Australia.
- Further evaluation of N4/02 as a vaccine candidate in broilers to confirm suitability in providing broad cross protection against other variant strains of IBV.
- Surveillance of IBV strains circulating in the field, including monitoring of genetic changes occurring in field isolates of the N4/02 strain.
- Continued use of virus isolation for the assessment of cross protection in chickens with the aim of further developing a qRT-PCR designed to specifically detect IBV in the trachea.
- The use of alternative expression systems to localize protective epitopes of IBV, including the development of a reverse genetics system based upon an Australian strain of IBV.
- Pathotype of N4/02 confirmed in commercial birds, including both broilers and layers.

8. Acknowledgements

We would like to thank the Poultry CRC and CSIRO for their generous financial support over the last three years.

Special thanks are also due to:

Ms Gaylene Gould for her excellent technical skills and her invaluable assistance with this report.

Drs Vivien Kite, Margaret Mackenzie and Ben Wells for their help in obtaining field samples.

Dr Peter Hooper, for his expert interpretation of histopathology data.

Ms Susanne Wilson for her continuing expert care of chickens in the Small Animal Facilities at AAHL.

9. Appendix

Preparation of buffers, reagents and solutions

Isolation medium-

- Eagles minimal essential medium with Earles salts(EMEM) (Gibco,)
- 5% Foetal calf serum(FCS),
- 200 unit/ml penicillin
- 200ug/ml streptomycin

Transport medium-

- M199 with Hanks salts (Gibco)
- 5% FCS
- 200 unit penicillin
- 200ug/streptomycin /ml)

5-amino-3-ethylcarbazole (AEC) detection solution

- Dissolve 2 mg of AEC powder in 1 ml of dimethyl formamide. Add solution to 19 ml of 0.05 M sodium acetate (pH 5) and mix. Add 10 µl of 30% (v/v) H_2O_2 just before use.

ELISA buffers and solutions

(i) Coating buffer

- 0.795g Na_2CO_3
- 1.465g $NaHCO_3$
- Dissolve reagents in 500 ml of dH_2O, . pH should be 9.6

(ii) ELISA diluent

- 5% FCS in PBST.

(iii) ABTS -2,2'Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma cat A1888)

- ABTS 100mg
- 0.05M citric acid, pH4.0 450ml
- Dissolve ABTS in citric acid. Filter sterilise, Aliquot and store @ 4C

Activate with 36ul H_2O_2/ 21 ml 1 X ABTS immediately prior to application on assay
Read absorbance @ 405 nm

0.05M citric acid, pH4.0
dissolve 10.507g of citrate monohydrate (MW 210.14) in 1 L of distilled water. Adjust pH to 4.0 with 10N NaOH (approximately 6 ml of base may be required)

Gelatine solution (0.1% w/v)

Dissolve 1 g of gelatine powder in 100 ml of sterile PBSA. Heat to 50°C in a water bath and filter through a 0.2 μM pore size into 400 ml of sterile PBSA (pre-heated to 50°C) in a sterile environment. Do not autoclave.

LMH growth medium

- Eagles minimal essential medium with Earles salts(EMEM) (Gibco,)
- 10% FCS
- 10 mM HEPES
- 1 mM/L -glutamine
- 100 U/ml of penicillin
- 100 μg/ml of streptomycin
- 100 μg of fungizone

PBS-Tween 20 (PBST) washing buffer

0.05% (v/v) Tween-20 in PBSA.

SDS-PAGE: Gels and buffers

(i) Gel compositions

Solution component	10 % resolving gel	12 % resolving gel	4% stacking gel
ddH_2O (ml)	2.4	1.9	3
Acrylamide-bisacrylamide (ml)	3.7	4.2	0.66
1 M Tris-Cl, pH 8.8 (ml)	3.8	3.8	N/A
0.5 M Tris-Cl, pH 6.8 (ml)	N/A	N/A	1.25
10% SDS (μl)	100	100	50
TEMED	5	5	5
10% Ammonium persulphate (μl)	100	100	50

(ii) 2 × loading buffer (reducing)

- 125 mM Tris-Cl at pH 6.8
- 20% glycerol
- 4% SDS
- 200 mM of dithiothreitol (DTT)
- 1% bromophenol blue stain

Dissolve all in dH2O.

(iii) **Running buffer**

- 25 mM of tris-hydroxymethylmethane
- 192 mM of glycine
- 170 mM of SDS

Dissolve all ingredients in dH2O.

Western Blotting buffers and solutions

(i) Transfer buffer

- 7.2 g tris-hydroxymethylmethane
- 37.5 g glycine
- 3 g SDS
- 600 ml methanol

Dissolve all in methanol and top up to 3L with dH2O.

(ii) Blocking buffer

Dissolve 5% (w/v) of skim milk powder in TBS.

(iii) Tris buffered saline (TBS)

- 10 mM Tris-Cl at pH 7.5
- 150 mM NaCl

Dissolve all ingredients in dH2O and autoclave

(iv) TBS-Tween 20 (TBST)

0.1% (v/v) Tween-20 dissolved in TBS

10. Glossary

A3	Websters IB vaccine Armidale A3 strain (Fort Dodge, Australia
AA	amino acid
AEC	3-amino-9-ethylcarbazole
AAHL	Australian Animal Health Laboratory
ABTS	2,2”-azino-di-(3-ethylbenzthiazoline-6-sulfonate)
AMV	avian myeblastosis virus
cDNA	complementary DNA
CMVie	Cytomeglavirus immediate early
CPE	cytopathic effect
CT	cycle threshold
dH_2O	distilled/deionised water
dNTP	deoxynucleotide triphosphate
dpi	days post-infection
dsRNA	double-stranded RNA
E. coli	*Escherichia coli*
EID_{50}	mean embryo infectious dose
ELISA	enzyme linked immunosorbent assay
FAdV	fowl adenovirus
FCS	fetal calf serum
IB	infectious bronchitis
IBV	infectious bronchitis virus
I	Websters IB vaccine Ingham strain (Fort Dodge, Australia)
IgG	immunoglobulin class G
LHE	Left hand end
MAb	monoclonal antibody
NA	no information available
NBF	neutral-buffered formalin
NC	no classification
ND	not done
NJ	neighbour-joining
nm	nanometre
NS	no symptoms
NSW	New South Wales
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline containing 0.05% Tween 20
PCR	polymerase chain reaction
pi	post infection
Qld	Queensland
qRT-PCR	quantitative real time PCR
rFAdV	recombinant Fowl Adeno Virus
RHE	right hand end
RT	reverse transcription/transcriptase
S	IB vaccine No 1 (Intervet, Australia).
S1	spike glycoprotein
SPF	specific-pathogen-free
TBS	Tris buffered saline
TBST	Tris buffered saline + Tween 20
T_M	melting temperature
U	units
w/v	weight to volume ratio
Vic	Victoria
VicS	Websters IB vaccine VicS strain Fort Fort Dodge, Australia)

11. References

Callison, S.A., Hilt, D.A. and Jackwood, M.W. (2005) Rapid differentiation of avian infectious bronchitis virus isolates by sample to residual ratio quantitation using real-time reverse transcriptase-polymerase chain reaction. *J Virol Methods* **124**, 183-90.

Callison, S.A., Hilt, D.A., Boynton, T.O., Sample, B.F., Robison, R., Swayne, D.E. and Jackwood, M.W. (2006) Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. *J Virol Methods* **138**, 60-5.

Casais, R., Dove, B., Cavanagh, D. and Britton, P. (2003) Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. *J Virol* **77** , 9084-9.

Cavanagh, D. (2007) Coronavirus avian infectious bronchitis virus. *Vet Res* **38**, 281-97.

Cavanagh, D. (2003) Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis coronavirus. *Avian Pathol* **32**, 567-82.

Cavanagh, D., Casais, R., Armesto, M., Hodgson, T., Izadkhasti, S., Davies, M., Lin, F., Tarpey, I. and Britton, P. (2007) Manipulation of the infectious bronchitis coronavirus genome for vaccine development and analysis of the accessory proteins. *Vaccine* **25**, 5558-62.

Cavanagh, D., Davis, P.J. and Mockett, A.P. (1988) Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. *Virus Res* **11**, 141-50.

Cook, J.K., Orbell, S.J.,Woods, M.A., and Huggins, M.B. (1999) Breath of protection of the respiratory tract provided by different live-attenuated infectious bronchitis vaccines against challenge with infectious bronchitis viruses of heterologous serotypes. *Avian Pathol* **28**, 477-485.

de Wilt, J.H., Bout, A., Eggermont, A.M., van Tiel, S.T., de Vries, M.W., ten Hagen, T.L., de Roos, W.K., Valerio, D. and van der Kaaden, M.E. (2001). Adenovirus-mediated interleukin 3 beta gene transfer by isolated limb perfusion inhibits growth of limb sarcoma in rats. *Hum Gene Ther* **12**, 489-502.

Escutenaire, S., Mohamed, N., Isaksson, M., Thoren, P., Klingeborn, B., Belak, S., Berg, M. and Blomberg, J. (2007) SYBR Green real-time reverse transcription-polymerase chain reaction assay for the generic detection of coronaviruses. *Arch Virol* **152**, 41-58.

Goossens, P.H., Schouten, G.J., Heemskerk, B., 't Hart, B.A., Bout, A., Kluin, P.M., Breedveld, F.C., Valerio, D. and Huizinga, T.W. (2000). The effect of promoter strength in adenoviral vectors in hyperplastic synovium. *Clin Exp Rheumatol* **18**, 547-52.

Hodgson, T., Casais, R., Dove, B., Britton, P. and Cavanagh, D. (2004) Recombinant infectious bronchitis coronavirus Beaudette with the spike protein gene of the pathogenic M41 strain remains attenuated but induces protective immunity. *J Virol* **78**, 13804-11.

Ignjatovic, J., Gould, G. and Sapats, S. (2006) Isolation of a variant infectious bronchitis virus in Australia that further illustrates diversity among emerging strains. *Arch Virol* **151**, 1567-85.

Ignjatovic, J and Galli, L (1994) The S1 glycoprotein but not the N or M proteins of avian infectious

bronchitis virus induces protection in vaccinated chickens. *Arch Virol* **138**, 117-134.

Ignjatovic, J. and McWaters, P.G. (1991) Monoclonal antibodies to three structural proteins of avian infectious bronchitis virus: characterization of epitopes and antigenic differentiation of Australian strains. *J Gen Virol* **72 (Pt 12)**, 2915-22.

Ignjatovic, J., Sapats, S.I. and Ashton, F. (1997) A long-term study of Australian infectious bronchitis viruses indicates a major antigenic change in recently isolated strains. *Avian Pathol* **26**, 535-552.

Jackwood, M.W., Hilt, D.A. and Callison, S.A. (2003) Detection of infectious bronchitis virus by real-time reverse transcriptase-polymerase chain reaction and identification of a quasispecies in the Beaudette strain. *Avian Dis* **47**, 718-24.

Johnson, M.A., Pooley, C., Ignjatovic, J. and Tyack, S.G. (2003) A recombinant fowl adenovirus expressing the S1 gene of infectious bronchitis virus protects against challenge with infectious bronchitis virus. *Vaccine* **21** , 2730-6.

Kant, A., Koch, G., van Roozelaar, D.J., Kusters, J.G., Poelwijk, F.A. and van der Zeijst, B.A. (1992) Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide. *J Gen Virol* **73 (Pt 3)**, 591-6.

Koch, G., Hartog, L., Kant, A. and van Roozelaar, D.J. (1990) Antigenic domains on the peplomer protein of avian infectious bronchitis virus: correlation with biological functions. *J Gen Virol* **71 (Pt 9)**, 1929-35.

Otsuki, K., Nakamura, T., Kubota, N., Kawaoka, Y. and Tsubokura, M. (1987) Comparison of two strains of avian infectious bronchitis virus for their interferon induction, viral growth and development of virus-neutralizing antibody in experimentally-infected chickens. *Vet Microbiol* **15**, 31-40.

Page, R.D.M. (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**, 356-358.

Sambrook, J., and Russell, D. (2001) Molecular cloning: a laboratory manual. 3rd Edition, Cold Spring Harbor Laboratory Press, New York, USA.

Sapats, S.I., Ashton, F., Wright, P.J. and Ignjatovic, J. (1996) Sequence analysis of the S1 glycoprotein of infectious bronchitis viruses: identification of a novel genotypic group in Australia. *J Gen Virol* **77 (Pt 3)**, 413-8.

Saitou, N. and Nei, M. (1987) The neighbour –joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology of Evolution* **4**, 406-425.

Song, C.S., Lee, Y.J., Lee, C.W., Sung, H.W., Kim, J.H., Mo, I.P., Izumiya, Y., Jang, H.K. and Mikami, T. (1998) Induction of protective immunity in chickens vaccinated with infectious bronchitis virus S1 glycoprotein expressed by a recombinant baculovirus. *J Gen Virol* **79 (Pt 4)**, 719-23.

Tarpey, I., Orbell, S.J., Britton, P., Casais, R., Hodgson, T., Lin, F., Hogan, E. and Cavanagh, D. (2006) Safety and efficacy of an infectious bronchitis virus used for chicken embryo vaccination. *Vaccine* **24**, 6830-8.

Thompson, J.D., Gibso, T.J., Plewniak, F., Jeanmougin, F. and Higgins D.G. (1997) The Clustal_X windows interface:flexible strategies for multiple sequence alignment aided by quality

Wang, X., Schnitzlein, W.M., Tripathy, D.N., Girshick, T. and Khan, M.I. (2002) Construction and immunogenicity studies of recombinant fowl poxvirus containing the S1 gene of Massachusetts 41 strain of infectious bronchitis virus. *Avian Dis* **46**, 831-8.