Development of real-time PCR test to quantify infectious bronchitis virus in tissues of chickens

A report for the Australian Egg Corporation Limited

by Juliet R. Roberts and Kapil Chousalkar

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Development of real-time PCR test to quantify infectious bronchitis virus in tissues of chickens

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

Foreword

This study was conducted to develop and evaluate the use of reverse transcriptase real time polymerase chain reaction (RT-PCR) to detect and quantify the viral copy number of infectious bronchitis virus (IBV) from infected tissues of vaccinated and unvaccinated laying hens. The hens were challenged with pathogenic (wild), T and N1/88, strains of IBV and vaccine, A3 and Vic S, strains of IBV when they were in full lay. The hens were killed at regular intervals post-infection. The real time PCR test was designed for rapid detection and quantification of virus from trachea, oviduct and faeces of vaccinated and unvaccinated challenged hens. The level of protection offered by the current IBV vaccines was measured on the basis of viral copy numbers in vaccinated and unvaccinated laying hens. This project was funded from industry revenue which is matched by funds provided by the Federal Government.

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Acknowledgments

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Abbreviations

A3	A3 vaccine administered by eyedrop
BHQ	Black Hole Quencher
bp	base pairs
CSIRO	Commonwealth Scientific and Industrial Research Organisation
Ct	Threshold value
CV	Coefficient of variation
dATP	deoxyadenosine triphosphate
dNTP	Deoxyribonucleotide triphosphate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Thymidine triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EID	embryo infective dose
ELISA	enzyme-linked immunosorbent assay
FAM	6-carboxy fluorescein
IB	infectious bronchitis
IBV	infectious bronchitis virus
IDEXX	IDEXX Pty Ltd.
LNA	Locked nucleic acid
$MgCl_2$	magnesium chloride
mL	Millilitres
NaCl	Sodium chloride
N1/88	a strain of IBV
PCR	polymerase chain reaction
PEG8000	polyethylene glycol 8000
pg	Picogram
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	Reverse Transcriptase Polymerase chain reaction
S.E. (or SEM)	standard error of the mean
TE	tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane
T	a strain of IBV
µL	Microliters
UTR	Untranslated region
Vic S	Vic S vaccine administered by eyedrop
X-gal5-	Bromo-4-Chloro-3-Indolyl-☐-D-Galactoside

Executive Summary

Despite the fact that infectious bronchitis virus (IBV) can cause drops in egg production and quality, very little research has been done regarding the direct interaction of IBV with the oviduct of the laying hen. Production and quality losses are of greater concern than mortality. IBV has always been considered a threat to the Australian layer industry although whether IBV has direct effects on egg and egg shell quality remains speculative. The effects of IBV on the mature oviduct of laying hens in full lay were studied in the USA in 1957 and it is surprising that so few efforts have been made at such studies. In Australia, little effort has been made prior to the current study to investigate the effects of Australian strains of IBV on the mature oviduct of laying hens, to establish its direct relationship with production drops. Also, there is considerable confusion in the industry regarding revaccination during lay for IBV and the factors affecting egg and egg shell quality.

There are several traditional virus isolation and serological methods which can be used to monitor the status of a flock for infectious bronchitis virus infection, although the traditional virus isolation tests are laborious and time-consuming. Hence there is a need for rapid detection of Australian strains of infectious bronchitis virus (IBV) directly from clinical samples, to detect and quantify the viral load in clinical samples. To be able to quickly detect and quantify IBV directly from the oviduct and other clinical samples such as trachea and faeces would be of great advantage.

The present project was conducted to design a real time PCR test to detect and quantitate the IBV viral copy number from clinical samples. This process is complete for the T, A3 and VicS strains of IBV but the quantitative test for the N1/88 strain requires further optimization. The present test was able to detect both the pathogenic (T) and vaccine (A3, VicS) strains of IBV present at as low as 10 viral copy numbers from clinical samples. The test is highly sensitive when compared to the traditional virus isolation techniques.

Although only a small number of samples has been processed so far, it is evident that, based on the viral copy number data, rearing phase vaccination offered protection for the mature oviduct of laying hens. Viral copy number was generally higher for T strain than for the two vaccine strains, suggesting that it may be possible to distinguish between vaccine and wild strains of IBV in tissues, based on quantitative levels. However, it is important to analyse further samples to establish this relationship.

The test was also able to detect the pathogenic strains of virus from faecal samples and vaccination reduced the virus shedding in the faeces.

A non-quantitative PCR has been developed for the strain N1/88 and the RT-PCR product has been successfully purified, cloned and sequenced. However, the real time PCR test for N1/88 requires further optimisation and it is planned to do this during the new project (AECL No. 08/14-UNE). During the new project, we will also process as many additional tissue samples as possible, to further elucidate the relationship between strain of IBV and the quantities found in tissues.

Overall Conclusions

The real time PCR test designed during this project is highly sensitive and can detect as low as 10 viral copy numbers. Along with virulent "T" and vaccine strains A3 and Vic S, the test will also be able to detect at least 7 other Australian IBV strains. Rearing phase vaccination offers good protection for the oviduct of laying hens challenged in full lay. However, the extent of protection offered to the trachea needs further investigation. Also, further work is needed to investigate the persistence of pathogenic and vaccine strains of IBV in trachea, kidney and faecal samples. It is anticipated that the real time PCR test for the IBV strain N1/88 will be fully developed during AECL No. 08/14-UNE and tissue samples will be analysed for this strain of IBV. Also, additional tissue samples will be processed for all IBV strains.

1. General Introduction

1.1 Introduction to infectious bronchitis virus (IBV)

The infectious bronchitis virus (IBV) and its deleterious effects were first recognised in the United States in the early 1930s (Schalk & Hawn, 1931 in Cavanagh & Naqi, 1997). The disease is prevalent in all countries with an intensive poultry industry. Rapid mutation and evolution of new strains makes the study of this virus more difficult. It is an economically important disease associated with mortalities in young chickens and decreased egg production accompanied by inferior egg quality in laying flocks (Broadfoot *et al*., 1956). The commercial egg laying chicken has a level of productivity second to none. However, an outbreak of IBV in developing pullets may have a devastating effect on future productivity. In adult laying hens, IBV has a varying effect on production.

IBV is pleomorphic but generally rounded. It possesses an envelop that is 90-200 nm in diameter with the club-shaped surface projections called spikes being about 20 nm in length (Mcintosh, 1974). All coronaviruses have a high molecular weight single stranded RNA genome polyadenylated at the 3' terminus and with seven polypeptides most of which are glycosylated (Tyrrell *et al*., 1978). One of the unglycosylated polypeptides of molecular weight above 50,000 is associated with the genome and appears to form the nucleocapsid. IBV virions contain three virus specific proteins; the large spike (S or E_2), small matrix (M or E_1) and internal nucleocapsid (N) proteins. The S protein comprises two or three copies of each of two glycopeptides S1 and S2 (Cavanagh, 2004). There are four small non-structural proteins, 3a, 3b, 5a and 5b and a 3' untranslated region. The 3' untranslated region is responsible for initiation of negative strand RNA synthesis and has also been used to access the genotypic variation amongst the newly emerging IBV strains (Williams *et al*., 1993). IBV has the potential to multiply in the epithelial cells of various organs such as trachea, Harderían gland, intestine, kidney and the oviduct of a chicken.

1.2 IBV in layers

In layers, IBV can cause severe declines in egg production and also deterioration in egg shell quality and egg internal quality (Sevoian and Levine, 1957). Such effects may be accompanied by mild (Munner *et al*., 1987) or no respiratory signs (Cook, 1984). The severity of the production decline varies with the period of lay, the virulence of the virus involved and other non-specific factors. The response of individual hens also varies greatly

(McMartin, 1968). Production may start to increase 2 to 3 weeks after infection but, when laying is resumed, egg production remains subnormal (Ignjatovic and Sapats, 2000). Studies regarding the detailed pathogenesis and isolation of IBV in the respiratory tract and kidney of chickens have received much attention. The effect of IBV on egg production has been reported by several workers but associated changes in the different parts of the reproductive tract of adult layer birds have not been elucidated properly.

1.3 IBV in Australia

The presence of IBV in Australian flocks was first identified by the late Associate Professor Robin Cumming, in 1962, as the infectious agent associated with respiratory disease and uraemia. Drs Rob Cumming and Roger Chubb conducted a series of experiments and field trials in the 1960s and 1970s to describe the effects of IB in Australian flocks. These researchers also developed methods for isolating and detecting the virus. In the late 1970s and early 1980s, Wadey and Faragher (1981) used cell culture techniques to identify different subtypes of IBV. This work was furthered in the late 1980s and the 1990s by Dr. Jagoda Ignjatovic and colleagues (Ignjatovic & McWaters, 1991; Ignjatovic *et al.,* 1997; Ignjatovic *et al,* 2006) who developed molecular techniques for characterisation of the different strains of the virus and described their pathogenicity. Ignjatovic and colleagues identified a shift in the prevalent IBV strains from highly nephropathogenic in the 1960s and 1970s to predominantly respiratory in the 1980s and 1990s (Ignjatovic *et al*., 2002). However, more recent isolates investigated were isolated from broiler flocks.

In Australia, 13.175 million poultry layer birds produce 203 million dozen eggs per annum. The gross value of production is $ 288 million per annum (Australian Bureau of Statistics, 2006). The egg industry in Australia loses in excess of $10-15 million dollars per year through downgrading of eggs. Diseases affecting the reproductive tract of adult laying flocks such as IB and egg drop syndrome could contribute to these losses. A better understanding of the pathogenesis of these diseases, which influence internal and external egg quality, will assist development of strategies to reduce the incidence of downgrading. Currently, in Australia, approximately 85 per cent of all eggs produced are sold in shell form, primarily through grocery chains. The balance is processed into liquid, frozen and dried egg products for use in the food service and processed food sectors. In Australia, currently, all pullets reared for egg production are vaccinated by live attenuated strains of infectious bronchitis

virus. It is usual practice to administer three doses in the rearing phase before point of lay. Jolly *et al*. (2005) observed that, when birds vaccinated in the rearing phase were infected with IBV, there were deleterious effects on internal and external quality of eggs laid. Many producers utilise the practice of boosting immunity regularly during the lay cycle. However, questions have been raised about possible negative effects of attenuated vaccines in adult layer birds (Sulaiman *et al*., 2004). Even after the IBV vaccination or revaccination, Australian poultry producers have problems with thin albumen and deterioration of egg shell quality. It is essential to carry out studies of the impact of IBV on egg quality in commercial laying flocks. All around the world, Australia has a reputation for producing food of very high quality. The challenges faced by the Australian poultry industry are reflected in poultry industries around the world. Although the deleterious effect of IBV on egg production and quality is well known, very little research has been conducted on this aspect.

1.4 IBV and oviduct

Isolation and multiplication of infectious bronchitis virus in epithelial linings and tubular glands of the oviduct is still under debate. Despite the fact that the deleterious effect of IBV on the egg industry is well known all over the world, very few studies have been undertaken regarding the details of pathogenesis of IBV in the fully functional oviduct during the time interval between 1957 and 2007. This could be due to the amount time required, maintenance costs and difficulty in keeping birds under strict isolation. The fact that IBV causes a decrease in egg production has been known for at least 56 years, during which time many new strains of IBV have evolved. It is, however, interesting and surprising that very little effort has been made since 1973 to conduct field experiments and that there are many facts such as thin-shelled eggs, temporary or permanent cessation of egg production and watery whites during IBV infection that remain unresolved. Australian strains of IBV have been reported to be uterotropic in unvaccinated Leghorns (Chousalkar *et al*., 2007a) as well as vaccinated HyLine hens (Chousalkar *et al*., 2007b). Australian strains of IBV also have the potential to multiply in the albumen-forming regions of unvaccinated Isa brown hens (Chousalkar and Roberts, 2007a). Virus can also replicate in the shell-forming region of non-laying hens (Chousalkar and Roberts, 2007b). Australian strains of IBV can affect the egg internal quality, however external quality (egg shell quality) was only affected in terms of egg shell reflectivity and shape index (Chousalkar and Roberts, 2007c).

At the present time, the main effects on layer flocks are reduced production ("egg drop") and reductions in egg quality (Cook and Huggins, 1986).

1.5 IBV vaccines and oviduct

The main type of vaccine used in commercial flocks is live attenuated vaccine. Many trials have been conducted using live attenuated or inactivated vaccines, investigating protection offered either to the trachea (Cook *et al*., 1999) or kidney (Ratnasethkul and Cumming, 1983b; Afanador and Roberts, 1994) or both (Ignjatovic and Galli, 1994). IBV vaccine strains have the potential to multiply in oviduct organ culture (Dhinakar Raj and Jones, 1997), however the extent of protection offered by the current Australian strains to the fully functional oviduct of hens under field conditions is speculative.

1.6 Aims and Objectives of the current project

Currently, the reverse transcriptase polymerase chain reaction (RT-PCR) is commonly used for diagnosis of IBV (Ramneek *et al*., 2005). The use of PCR in molecular diagnostics has increased to the point where it is now accepted as a gold standard for detecting nucleic acid from a number of origins and it has become an important tool in research laboratories. PCR for virus detection is displacing all the standard techniques such as cell culture and various serological essays (Niubo *et al*., 1994). For IBV, various genotyping methods include RT-PCR, RNA finger printing (Kusters *et al*., 1987), RT-PCR and restriction fragment length polymorphism (Mardani *et al*., 2006) and S1 or N gene sequencing (Sapats *et al*., 1996a, b). Using RT-PCR, IBV has been also isolated directly from clinical samples such as the trachea or kidney (Mardani *et al*., 2006). However, there are no reports of IBV isolation from the fully-functional oviduct by RT-PCR or any other molecular technique. These days, quantitative PCR or real time PCR has become increasingly popular amongst virologists for detecting and quantifying the viral load in clinical samples. The technique is quick and less laborious compared to other techniques. Real time PCR offers significant improvements to the quantification of viral load because of its enormous dynamic range that can accumulate at least eight $\log^{10}$ copies of the nucleic acid template (Moody *et al*., 2000). Based on the above facts, distribution of virus in trachea, oviduct and environmental samples like faeces after IBV challenge needs to be assessed to

ascertain whether the virus of itself is capable of causing pronounced drops in egg production and quality usually observed in the field.

In the current project, the real time PCR test was designed for the rapid detection of IBV strains from infected tissue samples and also from the faeces of infected hens. The challenge trial also used unvaccinated and vaccinated challenged hens to access the protection offered by the current vaccine in terms of virus shedding (if any) on a quantitative basis.

2.   General Materials and Methods
2.1 Virus strains

Stock virus (allantoic fluid known to contain A3, Vic S, T and N1/88) which was already present in the lab was used for this experiment.

2.2 Single step Reverse Transcriptase PCR (RT-PCR)

2.2.1 RNA extraction from allantoic fluid

Extraction and purification of RNA was conducted according to the manufacturer's instructions using an RNA easy kit (Qiagen) with some modifications. 400 µl of allantoic fluid was mixed with 300 µl of RTL buffer. 5 µl of mercaptoethanol was then added. This mixture was vortexed and kept at room temperature for 10 min. After incubation at room temperature, 700 µl of 70% ethanol was added, and the solution was transferred to a spin column in two steps. Washing and elution was performed according to the manufacturer's instructions. The elution volume was 30 µl. Extracted RNA was quantified and stored at - $70^{o}C$ until used for PCR. Samples were kept at $4^{o}C$ throughout the extraction to avoid degradation of the RNA.

2.2.2 Selection of primers for conventional RT-PCR

For the detection of T-strain IBV from the oviduct samples, a polymerase chain reaction was carried out using the primer pair (UTR1^{-} and UTR2^{+}) as described earlier by Adzhar and coworkers. (1996). These primers amplify a 298 base pair segment of the untranslated region of the IBV genome. The above primers, however, could not detect the IBV strain N1/88. Another primer pair binding within the nucleocapsid gene of N1/88 (nucleotide 668 to 790 of Genebank accession number US52599 Sapats *et al.*, 1996) was designed using Beacon designer 5 (Premier Biosoft International, Palo Alto, USA).

The forward primer (AGATGGCTGAGCGTAAGTAC), and reverse primer (CCTCCTCAATCATCTTGTCATC) amplified a 123 base pair product from strain N1/88

Table 2.1: Oligonucleotides used during conventional RT-PCR reaction.

Name	Sequence	IBV strain detected	Size of PCR product in base pairs (bp)
IBV UTR 1⁻	5'-GCTCTAACTCTATACTAGCCTAT- 3'	T	298 bp
IBV UTR 2⁺	5'- AAGGAAGATAGGCATGTAGCTT- 3'		
(Adzhar *et al*.,1996)			
Forward primer	5'-AGATGGCTGAGCGTAAGTAC-3'	N1/88	123 bp
Reverse primer	5'-CCTCCTCAATCATCTTGTCATC-3'		

2.2.3 Single step reverse transcription polymerase chain reaction

Reverse transcriptase PCR was carried out using a one step RT-PCR kit (Qiagen) as per the manufacturer's instructions. The reaction was carried out in 25 µl of reaction mixture containing 5 µl of 5x reaction buffer, 1 µl of dNTP mix (containing 10 mM each of dATP, dCTP, dGTP and dTTP), 1 µM of each of the oligonucleotides, 1 µl of enzyme mixture (Qiagen single tube RT-PCR kit utilises recombinant heterodimeric enzymes expressed in *E. coli*), 5 ng of RNA template and 8 µl of RNase free water. All the mixtures were kept on ice until transfer to the thermal cycler. The reaction mixtures were amplified for 30 cycles: cycle 1, 30 min at 50 °C, cycle 2, 15 min at 95°C, cycle 3 to 30, 1 min at 94 °C, 1 min at either 56 °C for T strain or 60°C for N1/88 strain followed by 1 min at 72°C. The final extension was performed at 72°C for 10 min. The RT- PCR products were analysed by 1% agarose gel electrophoresis.

2.2.4 Purification of PCR product

PCR products were purified using PCR purification kit (Promega Wizard® PCR Preps DNA purification system as per manufacturer's recommendation.

2.2.5 Sequencing of RT-PCR products

Twelve µl of PCR product at a concentration of 200 ng/µl was sent to the DNA Sequencing Facility (Macquarie University), where reactions were performed and sequencing data was generated. The data was then forwarded by e-mail back to the sender for analysis.

2.3 Cloning

2.3.1 Ligation

A purified 276 bp fragment of the 3' UTR region of the T, Vic S and A3 strains of IBV or 123 bp fragment of the nucleocapsid region of N1/88 strain of IBV was ligated to the T tagged site of pGM T-easy vector (Promega). The reaction was performed as per manufacturer's instructions. The protocol for ligation of DNA into pUC18 was based on methods by Sambrook & Russell (1989). Ligation mix contained 1 X ligation buffer (supplied by Promega), 3 U T4 DNA ligase, 20 ng pUC18 DNA and insert DNA (in a 3:1 molar ratio with vector DNA) made up to 10 µl with MilliQ water. The sample was incubated overnight at 4°C.

2.3.2. Transformation

The *E. coli* DH5-α competent cells were prepared by treatment with calcium chloride (Sambrook and Russell 1989). Competent cells were stored at -80°C. The competent cells were subsequently thawed on ice for 20 min, and gently resuspended using a micropipette. 200 µl of cells were added to 10 µl of ligation mix in a sterile microfuge tube. 5 µl of TE buffer was used as a negative control and 10 ng of uncut pUC18 plasmid DNA as a positive control. The cells were held on ice for 30 min. The bacterial cells were heat shocked at 42°C for 90 seconds and then returned to ice. 1 ml of YT broth was then added and the cells were incubated for one hour at 37°C. 50 µl of X-gal (20 mg/ml in N-N' - dimethyl-formamide) was spread on 2x YTA (2x YT with 100 µg/ml ampicillin) plates for the selection of blue and white colonies. The bacterial cells were centrifuged at 8800 *g* for 30 seconds. 900 µl of the supernatant was removed and cells were resuspended in the remaining 200 µl of YT broth. 50 µl and 100 µl of the culture were plated onto YT plates with the appropriate antibiotics. The plates were incubated overnight at 37°C. Single white colonies of *E. coli* DH5-α containing recombinant plasmids were selected from YTA and grown in 10ml YT broth containing 10 µl of Ampicillin. Extraction of plasmid DNA was achieved by using a rapid boiling method.

2.4 Recombinant Plasmid DNA extraction by boiling method

Single colonies of *E. coli* DH5-α containing recombinant plasmids were isolated and grown in 10 ml 2 X YTA broths at 37°C for 15-16 hours. Cells were pelleted at 1,500 g in a MSE Minor centrifuge for 10 minutes. The resulting pellet was resuspended in 500 µl of STET

(50 mM Tris pH 8.0, 50 mM EDTA, 8% Sucrose, 5% Triton-X100) after which 40 µl of lysozyme solution (10 mg/mL in 50 mM Tris) was added. The sample was then boiled for 90 seconds, cooled on ice and spun at 12,000 g for 10 minutes (Clements Orbital 100). The gelatinous precipitate was removed, 500 µl of isopropanol was added and the sample was held at –20°C for 20 minutes. The plasmid DNA was centrifuged at 12,000 g for 10 minutes (Clements Orbital 100) and supernatant then discarded. The pellet was washed with ice cold 70% (v/v) ethanol by centrifuging for 1 minute at 12,000 g (Clements Orbital 100). The pellet was then dried and resuspended in 50 µl TE buffer (1 M Tris pH 8.0, 0.25 M EDTA pH 8.0).

2.5 Recombinant Plasmid DNA extraction byWizard® Plus Minipreps DNA purification system

Recombinant plasmids in host *E. coli* cells were grown in 10 ml 2X YTA broth overnight at 37°C with shaking. The cells were pelleted using an MSE Minor centrifuge at 1,500 g for 10 minutes. DNA was purified as per manufacturers' instructions, resuspended in nuclease free water and stored at -20°C.

2.6 PEG purification of plasmid DNA

In order to purify DNA for sequencing an equal volume of PEG solution (30% PEG (v/v), 1.6M NaCl) was added to the sample, which was then held at 4°C for 30 minutes. The DNA was centrifuged at 4°C for 10 minutes at 12,000 g (Clements Orbital 100). The PEG solution was removed and the pellet was washed with 70% ethanol and spun for 2 minutes at 12,000 g (Clements Orbital 100). Once the ethanol was removed the pellet was dried and resuspended in 50 µl sterile water. The DNA was precipitated by adding 5 µl 3 M sodium acetate and 125 µl of absolute ethanol and incubating at -20°C for 30 minutes. The DNA was then centrifuged as described above for 15 minutes. The pellet was washed with 70% ethanol, allowed to dry and resuspended in nuclease free water.

2.7 Restriction enzyme digestion

Restriction enzyme digests varied depending upon the concentration of DNA and the optimal NaCl concentration for a given restriction enzyme. For a typical digest, 1 µg of DNA was digested in 1 X TA buffer (33 mM Tris pH 7.9, 66 mM potassium acetate, 10 mM

magnesium acetate, 100 μg/ml gelatin) containing 5-10 U of restriction enzyme (ECOR I) and the appropriate concentration of NaCl in a volume of 20 μl. Plasmid DNA was digested for 1-3 hours. All digests were incubated at 37°C. Agarose gel electrophoresis was carried out to confirm the desired product in the digestion mixture.

2. 8 Sequencing

The purified plasmid DNA samples were sequenced by Sequencing facility, Macquarie University, Sydney, Australia.

2.9 Standard PCR reaction

The recombinant plasmid DNA extracted from the host E coli cells were subjected to the PCR reaction using the primers used earlier during no quantative RT-PCR reaction (Chapter 1, section 1.3.3) to confirm the presence of the desired fragment cloned earlier. The protocol for PCR was modified from Sambrook & Russell (1989). Reaction mixtures contained 1 X reaction buffer (Fisher), 1.8 mM $MgCl_2$, 200 μM dNTPs, 1 uM of each primer, 1 U Taq polymerase, and 100 pg DNA template made up to 25 μl with MilliQ water. Samples were amplified using Eppendorf® Mastercycler.

2.10 Primers and LNA probe for real time PCR

The primers and LNA probe required for the real time PCR were generated within the nucleotide sequence of 3′ UTR region. The 3′ UTR region is involved in the initiation of negative strand RNA synthesis. 3′ UTR region is conserved at a position immediately downstream from the stopcodon of the nucleocapsid gene. 3'UTR sequences from seven Australian strains of IBV were retrieved from Gene bank using following accession numbers: U52596, U52595, U52594, U52597 (Sapats *et al*., 1996); DQ059623, DQ059622 (Ignjatovic *et al*., 2006) and A3 (unpublished sequence). The sequences were aligned using programme Clustal W2 (EBI sequence analysis tool, UK) and resulting alignment is available on request. A region of significant conservation was identified in the 3'UTR region of IBV. The conserved region was used to design the real time PCR primers and LNA probe as shown in Table 1. The primer sets were manufactured by Gene works (Adelaide, Australia) and LNA probe was synthesised by Exiqon (Denmark).

Figure 2.1: Selection of real time PCR primers and probe from 3'UTR sequences of T, Vic S and A3 strains of IBV. Sequences cloned in to the pGM T-easy vector. (* indicates similarity in the sequences).


```
A3    ---------TGATAAAAAACGGCCAGTGA-TTGTAATACGACTCACTATAGGGCGAATTG  50
T     GCGAACATTTATTGGTACGAGGCCAGTGA-TTGTA-TACGACTCACTATAGGGCGAATTG  58
Vic   ------ATTTA--AAAACCGGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTG  52
               *          *      ********* *****  ***********************

A3    GGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCT  110
T     GGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCT  118
Vic   GGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCT  112
      ************************************************************

                        Fwd primer                                LNA probe
A3    TAAGGAAGATAGGCATGTAGCTTGATTACCTATATGTCTATCGCCAGGGAAATGTCTAAT  170
T     TAAGGAAGATAGGCATGTAGCTTGATTACCTATATGTCTATCGCCAGGGAAATGTCTAAT  178
Vic   TAAGGAAGATAGGCATGTAGCTTGATTACCTACATGTCTATCGCCAGGGAAATGTCTAAT  172
      *******************************  ***************************

                                          Rev primer
A3    CTGTCTACTTAGTAGCCTGGAAACGAACGGTAGACCCTTAGATTTTAATTTAGTTTAATT  230
T     CTGTCTACTTAGTAGCCTGGAAACGAACGGTAGACCCTTAGATTTTAATTTAGTTTAATT  238
Vic   CTGTCTACTTAGTAGCCTGGAAACGAACAGTAGACCCTTAGATTTTAATTTAGTTTAATT  232
      ****************************  ******************************

A3    TTTAGTTTAGTTTAAGTTAGTTTAGAGTAGGTATAAAGATGCCAGTGCCGGGGCCACGCG  290
T     TTTAGTTTAGTTTAAGTTAGTTTAGAGTAGGTATAAAGATGCCAGTGCCGGGGCCACGCG  298
Vic   TTTAGTTTAGTTTAAGTTAGTTTAGAGTAGGTTTAAAGATGCCAGTGCCGGGGCCACGCG  292
      ********************************  **************************

A3    GAGTACGATCGAGGGTACAGCACTAGGACGCCCATTAGGGGAAGAGCTAAATTTTAGTTT  350
T     GAGTACGATCGAGGGTACAGCACTAGGACGCCCACTAGGGGAAGAGCTAAATTTTAGTTT  358
Vic   GAGTACGATCGAGGGTACAGCACTTGGACGCCCATTATGGGAAGAGCTAAATTTTAGTTT  352
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Table 2.2: Details of primers and Dual labelled LNA probes used in Real time PCR reaction

Target gene	Sequence	IBV Strain detected	Size of amplicon (bp)
QRT-PCR assay 3'UTR	LNA probe- 5'-(FAM) agAcaTttCccTggcg (BHQ-2)-3' F-primer- 5'- ATAGGCATGTAGCTTGATTACC- 3' R-primer- 5'- GTTTCCAGGCTACTAAGTAGAC- 3'	T, Vic S and A3	76 bp
QRT-PCR assay Nucleocapsid	LNA probe- 5'-(FAM) aaaGgcCaaGctCcaaattt (BHQ-2)-3' F-primer- 5'- AGATGGCTGAGCGTAAGTAC - 3' R-primer- 5'- CCTCCTCAATCATCTTGTCATC - 3'	N1/88	123 bp

2.11 Real time Reverse transcriptase PCR

2.11.1 Standard curve

Plasmid DNA was extracted and purified from a 10 ml culture of transfected E coli using Wizard® plus Miniprep DNA purification kit (Promega) according to manufacturer's instructions. The concentration of plasmid DNA was determined by agarose gel electrophoresis using two, four and eight fold dilutions against the lambda standard with known amount of DNA. Plasmid DNA copy number was then calculated based on the concentration of plasmid DNA and its molecular weight. Plasmid DNA was stored at -20°C. A series of ten fold dilutions starting from 1 X 10^6 to 1 X 10^0 were used to identify the threshold of detection. The reproducibility of the reaction was determined by three independent assays using the plasmid standard. Each individual assay was performed on different days using freshly diluted standard. The inter-assay coefficient of variation (CV) was calculated by comparing the mean of the Ct values of three separate but identical assays.

2.11.2 Real time RT-PCR protocol and validation of test

The LNA probe-based real time RT-PCR was performed using a Rotor Gene 3000 real time PCR machine (Corbett Research, Sydney, Australia) and a one-step RT-

PCR kit (Invitrogen Australia Pty Limited). Each reaction contained 10µmol of each primer and probe, 12.5µl of qPCR supermix and 5 µl of RNA template in a total reaction volume of 25 µl. Duplicate reactions were run for each sample. The cycling parameters were 50^0C for 15 min, then 95^0C for 2 min followed by 45 cycles at 95^0C for 15 s followed by 62^0C for 30s. Raw data was analysed using the default settings of the software for determination of base line and threshold of the reaction. The test sensitivity was determined by running 10 fold serial dilutions of the plasmid DNA with known copy numbers. The limit of detection was determined by three independent runs. In each assay a standard curve was generated and used to derive the viral copy number from infected samples. Each infected sample was analysed in duplicate.

2.12 Animal Trials

2.12.1 Experiment 1 (Chousalkar PhD Project)

One hundred and fifty day-old Isa Brown layers were obtained from a commercial hatchery. At day-old, all the chickens received Rispens vaccine against Marek's disease but no other vaccinations. All birds were beak trimmed at 15 weeks of age. At 25 weeks of age, hens were divided into three groups; one as a control with 48 hens and two IBV treatment groups with 51 hens each. The IBV-free status of the hens was maintained by isolation and strict biosecurity. The hens were raised in isolation sheds at the University of New England campus, Armidale, NSW, Australia. All equipment, sheds and clothing were fumigated or washed with antiseptic or ethanol before use.

2.12.2 Experiment 2 (AECL Project)

Three hundred and ninety day-old Isa Brown layers were obtained directly from a commercial hatchery and raised from day old in isolation houses on the University of New England campus. Birds were beak trimmed at 15 weeks of age. They were transferred to larger isolation houses prior to the challenge experiment. At day-old, 230 chickens received Rispens vaccine against Marek's disease at the hatchery but no other vaccinations. All birds were vaccinated with Vic S on day 1 by the intraocular route at the dose rate of $10^{4.5}$ embryo infective dose (E.I.D.$_{50}$). At 4 weeks

of age, one in four birds were vaccinated with A3 at the dose rate of $10^{3.9}$ E.I.D.$_{50}$. At 12 weeks of age, one in four birds were again vaccinated with Vic S. at the dose rate of $10^{4.5}$ E.I.D.$_{50}$. Vaccines were obtained from Fort Dodge, Australia. All birds were reared on the floor in contact with each other. Rest 160 birds were kept unvaccinated. The hens were raised in isolation sheds at the University of New England campus, Armidale, NSW, Australia under conditions of strict biosecurity. At 25 weeks of age, the hens were divided into ten groups, two control groups with unvaccinated and vaccinated birds (K & VK) with 30 hens each, two IBV treatment groups: unvaccinated and challenged with N1/88 (N) and T (T) strain of IBV with 15 hens each, two IBV treatment groups: vaccinated and challenged with N1/88 (VN) and T strain (VT) of IBV, each with 50 hens, two IBV treatment groups: unvaccinated and challenged with A3 (UA) and Vic S (UV) strain of IBV, each with 50 hens and two IBV treatment groups: vaccinated and challenged with A3 strain (VA) and Vic S (VV) strain of IBV, each with 50 hens. (Details of both the experiments are presented in Tables 2.3 and 2.4).

Table 2.3: Details from Experiment 1

Group	No. of birds	Vaccination			Challenge strain
		1 day old	4 weeks	12 weeks	30 weeks
K	**48**	None	None	None	None
N	**51**	None	None	None	N1/88
T	**51**	None	None	None	T

Details of the Birds (n= 150) from experiment 1

K- Unvaccinated control

N- Unvaccinated and challenged with N1/88

T- Unvaccinated and challenged with T

Table 2.4: Details from Experiment 2

Group	No. of birds	Vaccination			Challenge strain of IBV
		1 day old	4 weeks	12 weeks	30 weeks
K	30	None	None	None	None
VK	30	Vic S	A3	Vic S	None
N	15	None	None	None	N1/88
T	15	None	None	None	T
VN	50	Vic S	A3	Vic S	N1/88
VT	50	Vic S	A3	Vic S	T
UA	50	None	None	None	A3
UV	50	None	None	None	Vic S
VA	50	Vic S	A3	Vic S	A3
VV	50	Vic S	A3	Vic S	Vic S

Details of the Birds (n= 390) from experiment 2

K- Unvaccinated control

VK- vaccinated control

N- Unvaccinated and challenged with N1/88

T- Unvaccinated and challenged with T

VN- Vaccinated and challenged with N1/88

VT- Vaccinated and challenged with T

UA- Unvaccinated and challenged with A3

UV- Unvaccinated and challenged with Vic S

VA- Vaccinated and challenged with A3

VV- Vaccinated and challenged with Vic S

During both the experiments, hens were transferred from floor pens to individual cages in different isolation sheds at 25 weeks of age and remained there until the termination of the experiment. Isolation and strict biosecurity were maintained at all times. All equipment sheds and clothing were fumigated or washed with antiseptic or ethanol before use. Birds were fed commercial broiler starter to 3 weeks of age, chick starter to 5 weeks, pullet grower to 16 weeks and layer mash until the end of the experiment. Hen day egg production was recorded and shell quality was monitored visually.

2.12.3 Source of IBV strains

The two strains of virus used, T and N1/88, were obtained from Dr. Jagoda Ignatovic, CSIRO, Geelong, Australia. Vaccines were obtained from Fort Dodge, Australia.

2.12.4 Experimental procedure

At 30 weeks of age, each bird from N, T, VN and VT groups received N1/88 and T strain of virus at the dose rate of 2×10^5 embryo infective dose (E.I.D.$_{50}$) intraocularly. Each bird from groups UA, UV, VA and VV received A3 strain of virus at the dose rate of $10^{4.9}$ embryo infective dose (E.I.D.$_{50}$), Vic S strain at the dose rate of $10^{5.5}$ embryo infective dose (E.I.D.$_{50}$) and the control birds were sham inoculated with normal saline. Two hens from each challenge group and one hen from each control group were euthanized at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 days post infection (p.i.). At the time that the hens were sacrificed, the position of the egg in the oviduct was noted. Two hens which were out of lay in the T-infected group were sacrificed on the 30[th] day p.i. Hens from the control and infected groups which remained after the experiments were maintained up to 70 days p.i. to monitor prolonged effects on egg production and quality. Trachea, kidney, intestinal contents, and oviduct were collected from every individual hen for virus isolation. Harderian gland, trachea, kidney and different parts of oviduct, infundibulum, magnum, isthmus, and tubular shell gland and shell gland pouch were fixed in formalin for histology. The different parts of oviduct were also collected for ultrastructural examination (these data will be studied during the 1 year period of the new project AECL No 08/14 - UNE). Daily egg production was monitored and eggs were

analysed for external and internal quality parameters. Faeces were collected at weekly intervals from 1 to 10 weeks post infection.

2.12.5 Maintenance of fertile flock

Sixty White Leghorn cross birds (52 females and 8 males) were obtained from a commercial hatchery. At day-old, all the chickens received Rispens vaccine against Marek's disease but no other vaccinations. The birds were raised in separate isolation pens at the University of New England. Birds were fed commercial broiler starter to 3 weeks of age, chick starter to 5 weeks, pullet grower to 16 weeks and layer mash until the end of the experiment. All hens were beak trimmed at 15 weeks of age. The eggs were collected daily. Strict biosecurity measures were followed throughout the maintenance of this flock. The IBV free status was monitored by ELISA as described below.

2.12.6 Enzyme Linked Immunosorbent assay (ELISA)

The IBV free status of the hens which were unvaccinated before viral or normal saline challenge and hens from the fertile flock was monitored by ELISA for IBV antibodies. Blood plasma samples were analysed for infectious bronchitis virus antibodies using a commercially available ELISA kit (IDEXX Laboratories Ltd.). The samples were processed as per the manufacturer's instructions. The microtitre plates were read on a Microplate reader (Benchmark, BIO-RAD) used in conjunction with Microplate Manager ^{TM} Computer program (BIO-RAD). The absorbance values were calculated as an S/P ratio from which the positive/ negative titre endpoint is calculated for each plate assayed.

2.12.7 Virus isolation

Virus isolation was attempted from the trachea, kidney, oviduct and intestinal contents from each individual hen. One gm of kidney sample and 1 gm of intestinal contents was weighed and mixed into 1 mL of nutrient broth containing an antibiotic mixture ((penicillin 10.86 IU/mL, streptomycin 10 mg/mL, gentamycin 250 µl/mL and oxytetracyclin 50 µg/mL). Kidney tissue was forced through a sterile syringe before mixing with nutrient broth. Oviduct samples were scraped using sterile

scalpel blades and 1 gm of oviduct scraping was mixed with 1 mL antibiotic-containing nutrient broth. A 1 cm length of trachea was also scraped and the scrapings were mixed with antibiotic-containing nutrient broth. All the samples mixed in broth were shaken vigorously and centrifuged at 4000 rpm for 15 min in a refrigerated centrifuge. The supernatant was transferred to clean sterile vials and frozen immediately at -20^{o}C. Two gm faecal samples, collected from 1 to 10 weeks post-infection from five hens of each group, were weighed and mixed with antibiotic-containing nutrient broth. The samples were then processed as mentioned above.

The sample extracts were inoculated into the allantoic cavity of 9-day old chicken embryos. Eggs were incubated at 37^{o}C. Two days after virus inoculation, three out of eight eggs were removed from the incubator, chilled and allantoic fluid was collected. The collected allantoic fluid was immediately frozen. Strict aseptic conditions were maintained to avoid any chance of cross contamination. Scissors, forceps and trays were washed with boiling water after processing of each sample and wiped with alcohol before further use. Bench space was wiped with bleach and alcohol after each processing. Sterile gloves were used all the times.

2.13 Isolation of viral RNA for Real time PCR for quantitation of viral copy numbers from clinical samples

2.13.1 Trachea

A 1 cm length of trachea was scraped with a sterile scalpel blade. Extraction and purification of RNA was conducted according to the manufacturer's instructions using an RNA easy kit (Qiagen) with some modifications. The scrapings were mixed with 300 µL of RTL buffer. 5 µL of mercaptoethanol was then added. This mixture was vortexed and kept at room temperature for 10 min. After incubation at room temperature, 400 µL of 70% ethanol was added, and the solution was transferred to a spin column in two steps. Washing and elution was performed according to the manufacturer's instructions. The elution volume was 30 µL. Extracted RNA was quantified using Nanodrop and stored at -70^{o}C until used for real time RT-PCR. The 15ng of RNA was used as a template during real time PCR reaction.

2.13.2 Oviduct

The luminal surface of oviduct tissue was scraped with a sterile scalpel blade. One g of oviduct scraping was mixed with 1 mL of sterile nutrient broth containing an antibiotic mixture. All the samples mixed in broth were shaken vigorously and centrifuged at 4800 x g for 10 min in a refrigerated centrifuge at 4°C. Extraction and purification of RNA was conducted according to the manufacturer's instructions using an RNA easy kit (Qiagen) with some modifications. 200ul of the supernatant was mixed with 400 μL of RTL buffer. 5 μL of mercaptoethanol was then added. This mixture was vortexed and kept at room temperature for 10 min. After incubation at room temperature, 400 μL of 70% ethanol was added, and the solution was transferred to a spin column in two steps. Washing and elution was performed according to the manufacturer's instructions. The elution volume was 30 μL. Extracted RNA was quantified using Nanodrop and stored at -70^{o}C until used for real time RT-PCR. 50 ng of oviduct RNA was used during real time PCR reaction

2.13.3 Faeces

Two gm faecal samples, collected from 1 to 10 weeks post-infection from five hens of each group, were weighed and mixed with antibiotic-containing nutrient broth. The samples were centrifuged at 4800 x g for 10 min and 200 μl of the superannuant was used for RNA extraction. The extraction was performed as mentioned above in section 2.13.2. 20ng of RNA was used during the real time PCR reaction.

3. Results

3.1 Conventional RT PCR

All T, Vic S and A3 strains of IBV were amplified using primers aimed to amply 3' untranslated region and had been shown to generate 276 bp of RT-PCR product. The N/88 strain generated the 123 bp RT-PCR product (Fig 3.1.1). The sequencing of the RT-PCR product confirmed the correct the amplification by the desired primers. The sequences of the resulting RT-PCR product matched with the sequences published by Saptas *et al*. (1996). It was observed that genomic sequence of amplified product of the nucleocapsid region of N1/88 strain of IBV was different to the nucleocapsid region of strains A3, T and Vic S.
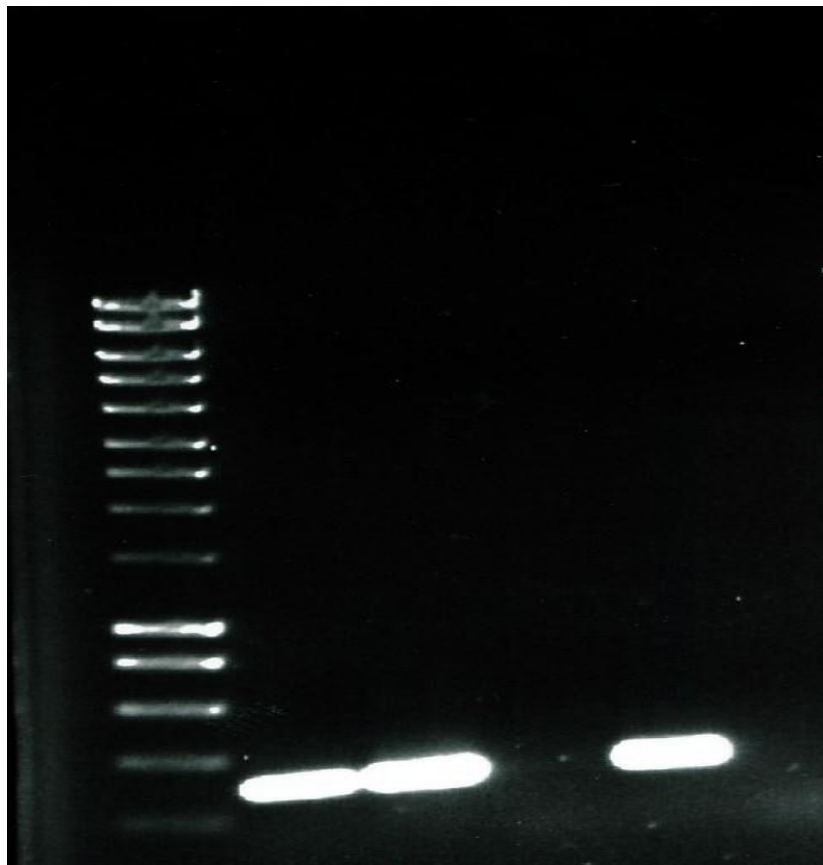
Figure 3.1.1:   **RT-PCR product of infectious bronchitis virus detected from stock allantoic fluid using primers targeting 3′ UTR region. Lane 1- Marker with 200 bp, lane 2- A3 strain of IBV, lane 3- T strain of IBV, Lane 5- Vic S strain of IBV. Note that N1/88 strain of virus (lane 4) did not amplify using these primers.**
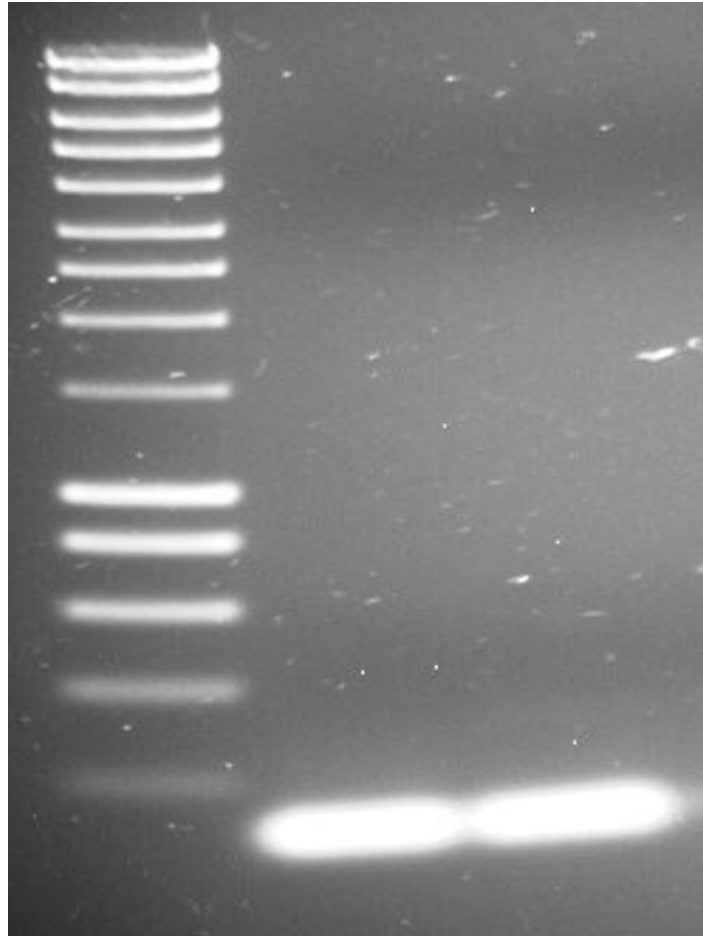
Figure 3.1.2:123 bp RT-PCR product of N1/88 strain of infectious bronchitis virus detected from stock allantoic fluid. Primers were designed to target the area within the nucleocapsid region.

The fragment was cloned into pGMT-Teasy vector (Promega) to generate the plasmid standard. The sequence of the clone indicated that there was no base change during the construction of plasmid. The presence of desired 276 bp fragment was confirmed by digesting the recombinant plasmid with the restriction enzyme ECOR I (Fig. 3.1.3A) and also by employing standard PCR reaction using 3′ UTR primers (Adzhar *et al.*, 1996) (Fig. 3.1.3B). The sequence of this primer pair is given in Table 2.1.

The primers and LNA probe were designed to target the highly conserved region of 3'UTR and amplified 76 bp product (Fig. 2.1). The concentration of the recombinant plasmid was derived by linearising it with suitable enzyme (Fig. 3.1.4A). Using this primer pair, 76 bp PCR product was successfully amplified at 62 ^{0} C annealing temperature (Fig. 3.1.4B).
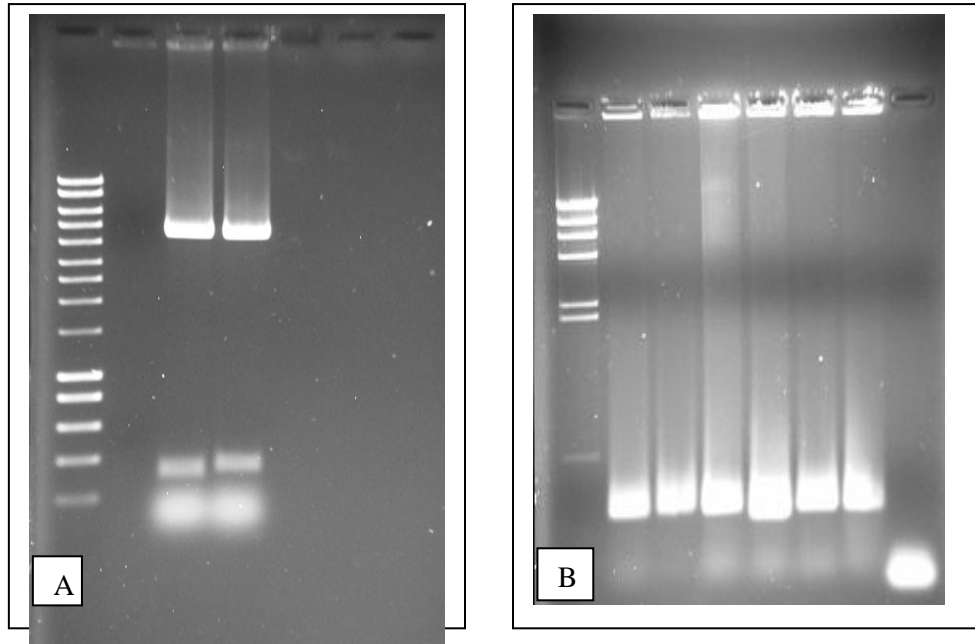
Figure 3.1.3A: The recombinant plasmid digested with enzyme ECOR I. Note the desired cloned fragment of 298 bp.

Figure 3.1.3B: 298 bp amplified product after employing standard PCR using recombinant plasmid as a template and primers for 3′ UTR region. Annealing temperature was 56^0C.
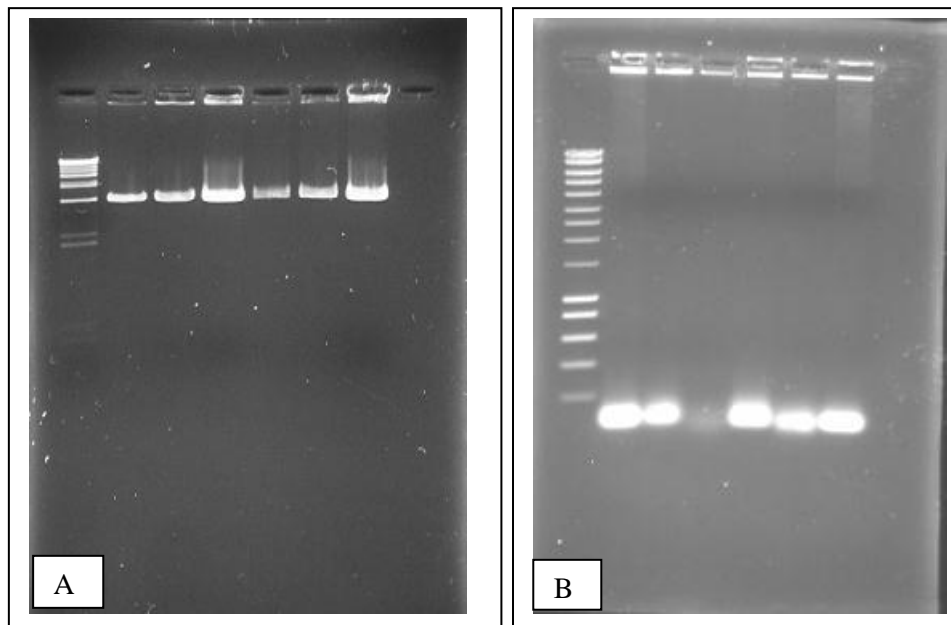
Figure 3.1.4A: The recombinant plasmid linearised with the enzyme BAM HI. The concentration was determined by running three independent gels.

Figure 3.1.4B: 76 bp amplified product after employing standard PCR using real time PCR primers designed to detect T, Vic S and A3. Annealing temperature was 62^0C.
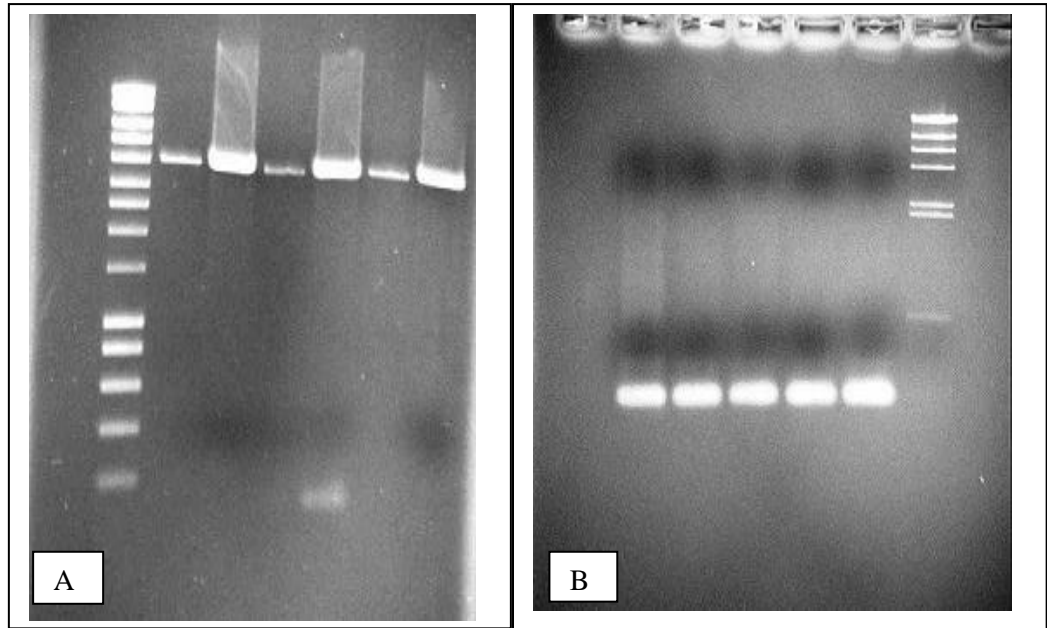
Figure 3.1.5A: **The recombinant plasmid (N1/88) linearised with the enzyme BAM HI. And ECoRI The concentration was determined by running three independent gels. Note the desired 123 bp fragment.**
Figure 3.1.5B: **123 bp amplified product after employing standard PCR using real time PCR primers designed to detect N1/88. Annealing temperature was 64^0C.**

3.2. Real time RT-PCR

Ten fold serial dilutions of the constructed plasmid were used to generate the standard curve for qPCR assays using the primer and probe (Table 2.2). Standard plasmid controls were run on the Rotor-Gene 3000 (Corbett Scientific) by setting up duplicates of 10 fold dilutions of the plasmid molecules ranging from 10^6 (1,000,000 copies), to 10^1 (10 copies) (Fig. 3.1.6). The log-linear standard curve generated from these plots showed high accuracy of the reaction with R^2 value= 0.990 $y = -3.36 \log (x) + 45.86$ with various concentrations containing 10^6 to 10^1 copies.

The efficiency of the reaction was confirmed after three individual runs and cross checking of the Ct values for each dilution between the assays. This result shows that the variation between duplication determinants is low and the assay is linear with respect to Ct value. There was no significant difference ($P > 0.05$) between mean Ct values and mean plasmid copy number of three independent assays (Fig. 3.1.7). The assay was negative below 10 copies, hence the limit of detection and quantification was determined to be 10 copies. Mean intra-assay CV based on Ct value was 0.53 % and mean inter-assay CV based on Ct value was 1.34 %.

For the optimization and quantitation of the exact viral copy numbers from the infected samples, it is very important to generate standard curves. The standard curves are essential to determine the quality of real time PCR assay. Standard curve is created by running a standards of varying concentrations from which the quantity of unknown sample can be calculated. Standard curve is obtained by plotting Ct values against log transformed concentrations of serial ten fold dilutions of the target nucleic acid. In the present experiment to establish the standard curve, the target nucleic acid was amplified and inserted (cloned) in to the vector.
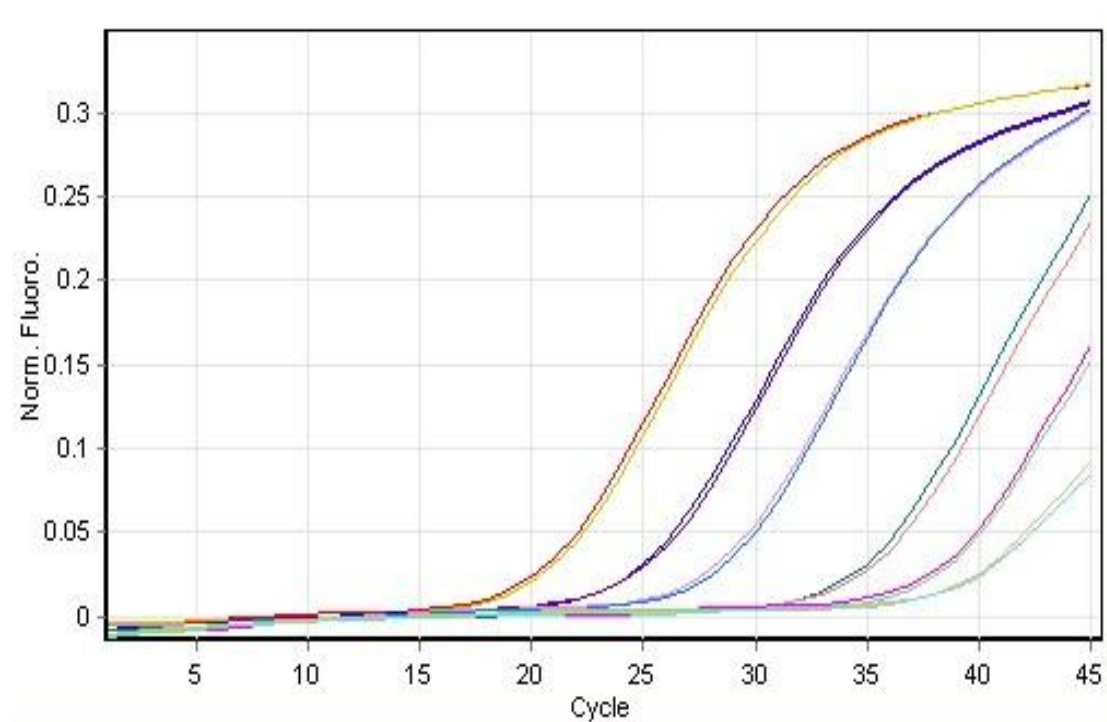
Figure 3.1.6 : IBV QRT-PCR assay The released reporter fluorophore (*y-axis*) is plotted as the function of amplification cycle number (*x- axis*) serial 10 fold dilution of the recombinant plasmid copies (10^6- 10^1) from left to right is shown. The dilution 10^0 did not amplify.
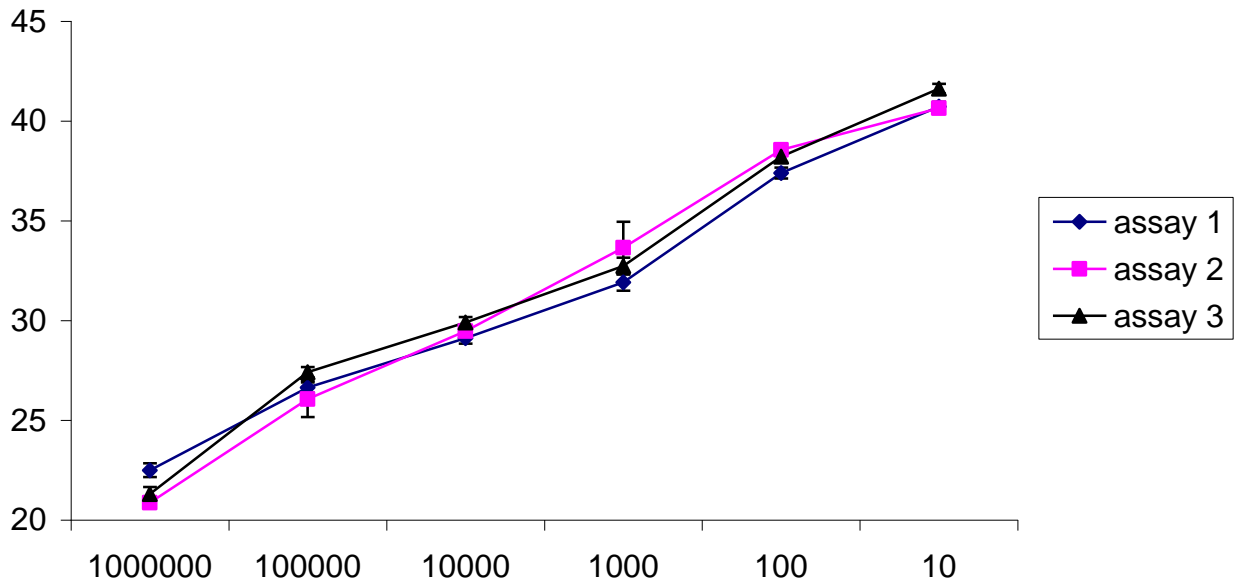
Figure 3.1.7: The comparative Ct values from three independent assays plotted against the IBV-specific plasmid copy number. (Assay for T, Vic S and A3)

3.3 Animal trial

Thirteen hens from the unvaccinated T-infected group and 15 hens from the unvaccinated N1/88-infected group showed coughing, rales and sneezing between the 3rd and 9th days p.i. Twelve hens from the VN group and 8 hens from the VT group showed respiratory signs such as coughing, rales and sneezing between 2 and 8 days p.i. but all respiratory signs had disappeared by day 9 p.i. One hen from UV group showed respiratory signs on day 6 p.i. and respiratory signs were also noticed in group VA on days 8 and 10. All respiratory signs had disappeared after day 10 p.i. (Tables 3.1, 3.2, 3.3). No hens died after infection with either strain of IBV and there was no drop in egg production in any group.

There was loss of shell colour in the first and second week p.i. in the N1/88-infected group and for up to five weeks p.i. in the T-infected group. There were no visible deformities in egg shells in the N1/88-infected group, but occasional occurrence of spotted shelled eggs was noted in the T-infected group. Eggs with yolks that separated from the albumen during egg breakout were observed mostly between the 10 and 16 days p.i. in the N1/88 and T-infected groups.

No symptoms were observed in control group (K).

In vaccinated and challenged hens (groups VN and VT) there was no drop in egg production. In both VT and VN groups, loss of shell colour was observed between 4 and 12 days post infection (p.i.) and eggs were elongated compared to the control group (data not shown). No wrinkled or soft-shelled eggs were recorded during the present trial. One hen from the T infected group killed at day 12 p.i. was an internal layer and had fewer large ovarian follicles. Another hen from the T infected group laid eggs only intermittently from day 10 p.i. and, when it was killed at day 20 p.i., its ovary was atrophied. In both the T infected groups, ovarian cysts were recorded at regular intervals (Table 3.2.1).

Clinical signs were not observed in any of the hens from the vaccinated unchallenged (control) group (VK).

There was no drop in the egg production in vaccinated and unvaccinated and A3 or Vic S challenged hens. However loss of shell colour was observed in unvaccinated Vic S challenged hens (UV).

Change is egg shape was observed in some treatment groups.

Sequences retrieved from GenBank and blast search analysis (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) indicated that, besides the T strain of IBV, the present test would be likely to detect the following strains of IBV: GenBank accession numbers U 52594, U 52595, EU 116941, EF 544168, EF 544165, DQO 59623, DQO 59622, U 52598, AJ 278338, AJ 278337, AJ 278334, AJ 278335, AJ 311362, AY 851295, AY 044186, EU116941.

The real-time RT-PCR assay was used to determine IBV copy number in oviduct samples from the unvaccinated and vaccinated challenged birds. No virus was detected (<10 copies per sample) in oviduct samples from unchallenged birds, indicating that biosecurity precautions were adequate.

In T and VT groups, amongst unvaccinated, challenged hens (T), virus was not detected at 2 or 4 days post infection (Table 3.2.1). However, viral copy number rose rapidly starting at 6 days post infection, to reach a peak of approximately 3×10^6 at 10 days post infection, after which time viral load fell, and virus was undetectable at 22 and 24 days post infection.

For the vaccinated challenged hens (VT), the time course of infection was very similar (Table 3.2.1) but viral load was dramatically reduced and in most cases was two to three

orders of magnitude lower at the peak of infection. However, these results clearly show that the vaccination protocol used does not abolish IBV replication in the oviduct. The viral load in trachea of unvaccinated hens (T) was low compared to the vaccinated hens (VT) at 6 days p.i.

For the UA and VA groups, virus was not detected in the trachea of any of the hens. at day 6 p.i. However, virus was detected in the oviduct of one hen from the UA group and both hens from the VA group at day 12 p.i. The details are presented in Table 3.2.2. In the UV and VV groups, at day 6 p.i., virus was detected in the trachea of both the hens from the UV group although virus was not detected in the trachea of the VV group. Virus was detected in the oviduct of both the hens from UV and VV groups at day 12 p.i. The details are presented in Table 3.2.3.

The virus was detectable in the faeces of unvaccinated and vaccinated T infected hens at 5 weeks p.i. The viral load in the faecal samples of unvaccinated and T challenged hens was high compared to the hens from the VT group. Virus was not detected from the faecal samples of any of the other treatment groups.

Table 3.2.1: Comparison of response of "T" infected Unvaccinated and vaccinated hen. NA is not analysed.

Days post infection	T	Clinical respiratory signs	Viral copy number in trachea	Gross oviduct lesions	Viral copy number in oviduct	VT	Clinical respiratory signs	Viral copy number in trachea	Gross oviduct lesions	Viral copy number in oviduct
2	1	-	NA	-	0	1	-	NA	-	0
	2	-	NA	-	0	2	-	NA	-	0
4	1	+	NA	-	0	1	-	NA	-	0
	2	+	NA	-	0	2	+	NA	-	0
6	1	+	1×10^5	-	294	1	+	3.3×10^5	-	197
	2	-	0	-	0	2	+	0	-	0
8	1	+	NA	-	987	1	+	NA	-	247
	2	-	NA	Cyst	0	2	-	NA	Cyst	315
10	1	-	NA	-	36×10^5	1	-	NA	-	298
	2	-	NA	Cyst	21×10^5	2	-	NA	-	266
12	1	-	NA	-	5×10^5	1	-	NA	-	4×10^5
	2	-	NA	Cyst	16×10^5	2	-	NA	-	79
14	1	-	NA	-	31×10^5	1	-	NA	-	235
	2	-	NA	-	7×10^5	2	-	NA	Cyst	310
16	1	-	NA	-	0.6×10^5	1	-	NA	-	413
	2	-	NA	-	0	2	-	NA	-	264
18	1	-	NA	-	0	1	-	NA	-	253
	2	-	NA	Hard shelled egg in Infundibulum	142	2	-	NA	-	177
20	1	-	NA	-	0.1×10^5	1	-	NA	Cyst	0
	2	-	NA	-	0	2	-	NA	Cyst	157
22	1	-	NA	-	0	1	-	NA	-	0
	2	-	NA	-	0	2	-	NA	-	0
24	1	-	NA	-	0	1	-	NA	Multiple cysts	0
	2	-	NA	-	0	2	-	NA	-	0

Table 3.2.2: Comparison of response of "A3" infected unvaccinated and vaccinated hens. . NA is not analysed.

Days post infection	Group UA	Clinical respiratory signs	Viral copy number in trachea	Gross oviduct lesions	Viral copy number in oviduct	Group VA	Clinical respiratory signs	Viral copy number in trachea	Gross oviduct lesions	Viral copy number in oviduct
2	1	-	NA	-	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA
4	1	-	NA	-	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA
6	1	-	0	-	NA	1	-	0	-	NA
	2	-	0	-	NA	2	-	0	-	NA
8	1	-	NA	-	NA	1	+	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA
10	1	-	NA	-	NA	1	-	-	-	NA
	2	-	NA	-	NA	2	-	-	-	NA
12	1	-	NA	-	0	1	-	NA	-	149
	2	-	NA	-	133	2		NA	-	139
14	1	-	NA	-	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA
16	1	-	NA	-	NA	1	-	NA	Cyst	NA
	2	-	NA	-	NA	2	-	NA	-	NA
18	1	-	NA	-	NA	1	-	NA	Internal layer	NA
	2	-	NA	-	NA	2	-	NA	-	NA
20	1	-	NA	-	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA
22	1	-	NA	-	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA
24	1	-	NA	-	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA

Table 3.2.3: Comparison of response of "Vic S" infected unvaccinated and vaccinated hens. NA is not analysed.

Days post infection	Group UV	Clinical respiratory signs	Viral copy number in trachea	Gross oviduct lesions	Viral copy number in oviduct	Group VV	Clinical respiratory signs	Viral copy number in trachea	Gross oviduct lesions	Viral copy number in oviduct
2	1	-	NA	-	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA
4	1	-	NA	Cyst	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	Cyst	NA
6	1	+	1,69,418	-	NA	1	-	0	-	NA
	2	-	5756	-	NA	2	-	0	-	NA
8	1	-	NA	-	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA
10	1	-	NA	-	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA
12	1	-	NA	-	95	1	-	NA	-	199
	2	-	NA	-	152	2	-	NA	-	167
14	1	-	NA	-	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA
16	1	-	NA	-	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA
18	1	-	NA	-	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA
20	1	-	NA	-	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA
22	1	-	NA	-	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA
24	1	-	NA	-	NA	1	-	NA	-	NA
	2	-	NA	-	NA	2	-	NA	-	NA

4. Discussion

In the present study, we documented the development and evaluation of a single step LNA probe based real time RT-PCR for the detection and quantification of infectious bronchitis virus directly from clinical samples. The assay is based on the 3′ UTR region of the IBV genome, which is highly conserved worldwide (Williams *et al*., 1993). The assay shows good reproducibility and a detection limit of 10 copies, which makes it more sensitive than that of Callison *et al*. (2007). However the assay developed by Callison *et al*. (2007) using 5′ UTR region of IBV genome does not detect the Australian strains of IBV used in this study (based on the sequence). The two tests designed during this study can detect the majority of virus strains from both the subgroups (subgroups 1 & 2 as described by Ignjatovic *et al*., 2006). However, this observation was based on the published nucleotide sequences available on Gene bank. The details and accession number of virus strains detected by our test are described in the Results section. It is possible that the present test could detect additional Australian strains whose sequence is not published on the database and further studies are essential to confirm this.

In the present experiment with vaccinated challenged hens, we did not observe a production drop, or corrugated, soft or misshapen shells during IBV infection, contrary to an earlier report (Munner *et al*., 1987). The difference between our results and previous results could be due to the difference in virus strain or breed of the hens used. Similarly, during our previous studies with unvaccinated Isa Brown hens in full lay, neither of these virus strains caused a drop of egg production or production of corrugated, soft or misshapen shells (Chousalkar and Roberts, 2007). Abnormal egg shells were recorded earlier (Sevoian and Levine, 1957) during infection of unvaccinated laying hens with British and American strains of IBV. Hence it could be concluded that Australian strains of IBV are not responsible for such egg shell quality effects in Isa Brown hens challenged in full lay. There was visual loss of shell colour in both wild and vaccine strain challenged hens. The vaccine strains appeared to offer little protection against loss of shell colour. Further studies are essential to find out the basic cause of loss of shell colour during IBV infection. Loss of shell colour in IBV infected unvaccinated laying hens between 4 to 8 days p.i. was reported in our earlier studies (Chousalkar and Roberts, 2007).

4.1 Respiratory signs and viral load in trachea

The respiratory signs observed during this trial are in agreement with Munner *et al*. (1987) who observed clinical respiratory signs in a small number of vaccinated hens. However, histopathological and virus isolation/detection studies were not conducted by these authors. Such studies on the respiratory system are in progress in our laboratory. In the present study, there was no direct relation between reproductive and respiratory tropism of T strain of IBV. Despite severe respiratory signs, the reproductive performance of some hens remained unaffected. Also, gross pathology was not observed in the oviduct of those hens (Tables 3.2.1). Similar observations were reported earlier during infection with a British isolate of the Massachusetts serotype of IBV (McMartin, 1968). Also, it was interesting that the hens which had virus detected in their oviduct did not demonstrate any respiratory symptoms during virus infection. Both the T and N1/88 strains of IBV varied in their effect on the respiratory tract. Many hens from both the infected groups showed no clinical respiratory signs following virus infection. Similar observations were reported earlier (McMartin, 1968). The lack of uniformity in the respiratory signs in infected hens was recorded in previous studies (Munner *et al*., 1987).

Also it was interesting to note that viral load was high in unvaccinated T (T) infected hens compared to the vaccinated VT group. However it is not possible to draw any firm conclusions based on the viral load in the trachea at 6 day p.i. alone. Day 6 was selected based on our previous histopathological observations, however it is essential to study the viral load at different days p.i.

4.2 Serology

All the hens from the unvaccinated group were negative for IBV antibodies before infection. This proves that it is possible to maintain the birds IBV free long term provided strict biosecurity measures are followed.

All the hens from the fertile flock (which was raised and reared to obtain embryonated eggs for virus isolation) also remained negative for IBV antibodies throughout the experiment.

4.3. Isolation of IBV from clinical samples by chicken embryo inoculation method

The allantoic fluid harvested from the clinical samples was collected and stored at -20. These samples could be tested by real time PCR at a later date.

4.4. Uterotropism of wild and vaccine strains of IBV

Australian strains of IBV have been classified mainly on the basis of their tropism for the kidney and respiratory tract (Ignjatovic *et al*., 2006). Our earlier electron microscopic

(Chousalkar and Roberts, 2007 a & b) and virus detection studies (Chousalkar, 2007) from the oviducts of IBV infected unvaccinated laying hens, together with the findings of the present study, suggest that T, N1/88, A3 and Vic S strains of IBV are able to replicate in the oviduct. However, these strains of IBV have varying degrees of uterotropism and it is possible that other Australian strains of IBV have pathogenicity for the fully-functional oviduct. However, further research needs to be done to confirm this fact. Given the large number of existing IBV strains, it would not be possible to screen all the strains for uterotropism *in-vivo*. However, it may be possible to study this aspect by combining *in-vivo* and *in-vitro* experiments.

T, Vic S and A3 strains of IBV are have been placed in subgroup 1 based on similarity in their nucleocapsid (N) gene sequences (Ignjatovic *et al*., 2006). The N proteins of the T (subgroup 1) strain of IBV share a high level of identity with most of the European and American strains of IBV. In the past, a drop in egg production has been recorded in the vaccinated hens challenged with the American strain of IBV (Munner *et al*., 1987), a finding which was not recorded in the present trial. However it would be interesting to study the persistence of American strains of IBV in the oviduct.

Despite these facts, regular IBV vaccination during the laying cycle is a common practice in Australia. IB revaccination during lay can be disadvantageous for egg shell quality (Roberts, 2004) and the extent to which regular IBV revaccination or intercurrent IBV infection can induce microscopic pathology in the oviduct of the mature laying hen needs further investigation. Some vaccine strains can induce permanent cellular damage to the oviduct of young chickens (Duff *et al*., 1971). However, it should be noted that all the hens vaccinated during rearing in the present study were laying normally and pathology was not recorded in any of the oviducts of control vaccinated unchallenged hens. Hence it could be assumed that neither of the vaccine strains used in the present study induces permanent damage to the oviduct of young chickens although further studies are essential to prove this. Also, whether or not these vaccine strains have the ability to replicate in oviduct tissue needs further investigation.

The test for development of real time PCR for N1/88 strain is in progress and needs further optimization. The test could not be designed during the duration of the present project owing to some technical factors including delay in delivery of the LNA probe from the commercial company and the difficulty of working with this strain of IBV. However during this project a non quantitative RT-PCR test was developed to detect N1/88 strain of IBV from the oviduct

of N1/88 challenged unvaccinated and vaccinated hens (Fig 3.1.2). The RT-PCR product was purified, cloned and sequenced. The sequence of the clone indicated that there was no base change during the construction of plasmid. The plasmid was linearised using suitable enzyme (Fig. 3.1.5A). The desired insert recombinant plasmid was also confirmed by using the conventional RT-PCR using 64^0C annealing temperature. The optimization of the real time PCR reaction is in progress and will be achieved during the 1 year period of the new project AECL No 08/14 – UNE.

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