DETECTION OF VIRUSES THAT HAVE THE POTENTIAL TO ALTER THE PATHOGENICITY OF WILD TYPE MAREK'S DISEASE VIRUS

Thesis submitted by

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Declaration

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from published or unpublished work of others has been acknowledged in the text and a list of references is given.

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Statement of the Contribution of Others

This project was supervised by Dr. Graham W. Burgess, Dept. of Microbiology and Immunology, James Cook University, Townsville Australia. The laboratory procedural assistance was obtained from Mr. Ramon Layton, Dept. of Microbiology and Immunology, James Cook University, Townsville Australia. I am thankful to Dr. Graham Burgess for editorial and proofreading of the thesis.

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ABSTRACT

Marek's disease virus serotype one (MDV-1) causes neuropathic, cytolytic and lymphoproliferative disease in poultry. Chicken anaemia virus (CAV) is also a pathogen causing anaemia and immunosuppression in chicken. Marek's disease serotype two virus (MDV-2) is naturally occurring in chicken and apathogenic virus. Natural multiple infection of these three viruses is possible. Presence of CAV and MDV-2 in the poultry flocks is potential to change in the pathogenicity of MDV-1 and also the pathogenicity of the CAV when infected alone.

Three hundred and ten feather samples as three different panels were collected from representative broiler flocks throughout Australia. These broiler flocks had different vaccination history against MDV. These samples were examined to detect the status of MDV-2 and CAV. The results of nested polymerase chain reaction (PCR) demonstrated that these two viruses are present in the broiler flocks. The samples were also investigated for MDV-1 by Mr. Ramon Layton (JCU) and the data from the MDV-1 investigation was analysed together with these two viruses. The flocks, vaccinated with HVT (herpesvirus of turkey) were found to be free from both MDV-1 and MDV-2. However, after the cessation of vaccination, both MDV1 and MDV-2 reappeared, first MDV-1 and then MDV-2. Interestingly when the MDV-2 was increasing progressively, the MDV-1 was decreasing. Although the available data is not sufficient to draw a conclusion, it is strongly suggestive that the presence of MDV-2 in infected birds changes the pathogenicity of MDV-1.The MDV-2 possibly works as a vaccine reducing the clinical MDV despite up to 30% prevalence of wild type MDV-1.

The CAV was present in all the flocks tested with more or less similar pattern of the viral distribution ranged between 50%-100%. Two sets of nested primers were used for detection of the CAV variably reacted with samples. The difference in the reactivity of the primer was then assumed as primer sequence mismatch due to CAV strain differences. This project further aimed to determine the source of CAV in the broilers. The CAV can be transmitted through both horizontal and vertical route. Both parents can transmit the virus to the progeny. To control clinical disease in young chicks, breeding flocks are generally vaccinated with a live vaccine.

Possible source of CAV infection in the broiler flocks and the genetic divergence of the CAV detected in the samples were determined by sequencing the CAV field isolates selected on the basis of reactivity differences of two nested primer pairs and geographical location of the broiler farms. Eight field isolates, vaccine strain 3711 and a reference strain BF4 were sequenced generating 951 bp long sequences covering complete VP3 gene, 3' 86% of VP2 gene and starting 42% of VP1 gene including hypervariable region. The sequence alignment and evolutionary analysis of these data, only Australian isolates and along with other global CAV isolates demonstrated that the Australian CAV field isolates fitted in to three different groups. Vaccine (strain 3711), strain BF4 and one of the field isolates included in a group, six field isolates included in next group together with CIA-1, and remaining one field isolate included in the third group together with the Cux-1.

Although one of the field isolate is included in the group along with vaccines, the isolate had genetic differences sufficient to differentiate from the strain 3711. These results demonstrated that the CAV in the broilers is not the vaccine virus. In addition this group of virus have two non-synonymous mutations in the VP3 gene, not described to dates in the sequences of CAV global isolates except in the sequences of strain CAU/7 (an Australian isolate).

To differentiate possible vertical and horizontal transmission of CAV in the broiler flocks, weekly sequential samples from week one till slaughter from one of the previous test flock was collected and investigated by nested PCR. The nested PCR result demonstrated that the birds were infected from week one. Eighty percent of the samples tested contained CAV DNA in it. This result further indicated that the virus was transmitted vertically. However the infection was sustained to the flock with similar pattern of distribution in the subsequent week samples till slaughter.

Nearly full length genomic identification of the vaccine virus was determined (19 bp shorter). In attempts to sequence full length genome of one field isolates from three genetically different groups of isolates, five different sequence data were obtained. Three overlapping primer pairs were designed to complete the sequences of the remaining 1347/1368 bp long DNA. The same primers were used for amplification

of vaccine strain as well. Two sequences, 723 bp and 438 bp long were identified as chicken genome, one 563 bp long as *Psychrobacter arcticus* 273-4 and two sequences, 1.28 kb and 499 bp long do not have sequence identification more than twenty base pair except primer sequence as CAV at both end of the sequence. These sequence data demonstrated that chicken/other bacteria also have the primer sequence similarity in their genome enough to initiate DNA amplification in that given condition. In addition, these sequences data of chicken genomes or would be chicken genome or the bacterial genome further indicates reliability of the single round PCR results if used as diagnostic tool.

The results from this project demonstrated that MDV-2 and CAV are present in Australian broilers. The presence of MDV-2 infection in the broiler flock prior to MDV-1 infection is likely to decrease the pathogenicity of MDV-1. Every broiler flocks is likely to have CAV infection. At lease three different strains of CAV are circulating in the present Australian commercial broilers. The CAV strains were not the vaccine virus administered to the parental flocks. The CAV strains were wild type virus most likely vertically transmitted to the broilers and they were maintained in the broiler shed environment.

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LIST OF ABBREVIATIONS

µ	Microns
µg	Microgram
µl	Microlitre
A260	Absorbance at 260 nanometres
A280	Absorbance at 280 nanometres
B-cell	B -lymphocyte
Bcl-2	Cancer expressing protein
BFDV	Beak and feather disease virus
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CAV	Chicken anaemia virus
CD	Cluster of differentiation
CEF	Chicken embryo fibroblasts
CIA-1	Chicken infectious anaemia-1
CKC	Chicken kidney cells
CO_2	Carbon dioxide
CPE	Cytopathic effect
CTL	Cytotoxic T lymphocytes
Cux-1	Cuxhaven-1
DEF	Duck embryo fibroblast
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide 5'-triphosphates
dsDNA	Double stranded DNA
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol tetra-acetic Acid
ELISA	Enzyme-linked immunosorbent assay
ERE	Estrogens response element
FAPP	Filtered-air, positive-pressure
FBS	Foetal bovine serum

FFE	Feather follicle epithelium
FREIT	Fluorescence resonance energy transfer
g	Unit of acceleration
g	Gram
GaHV-3	*Gallid Herpesvirus* Type 3
G-C	Guanine and cytosine
HVT	Herpes virus of turkey
IBDV	Infectious bursal disease virus
IFAT	Indirect immunofluorescent antibody test
IFNγ	Interferon gamma
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IL-6	Interleukin 6
IL-18	Interleukin 18
IR_L	Long internal repeat
IR_S	Short internal repeat
IU	International unit
kb	Kilo base
kDa	Kilo Dalton
LB	Luria-Bertani
M	Molar
MATSA	Marek's disease tumour-associated surface antigen
MBP	Maltose-binding protein
MD	Marek’s disease
MDV	Marek's disease virus
MDV-1	Marek's disease virus serotype one
MDV-2	Marek's disease virus serotype two
MSB1	Marek’s disease lymphoblastoid cell lines
mg	Milligram
Mg^{+2}	Magnesium ion
MHC	Major histocompatility complex
ml	Millilitre
mM	Millimolar
NDV	Newcastle disease virus

ng	Nanogram
NO	Nitric oxide
P53	Cancer expressing protein
PBS	Phosphate buffer saline
PBS A	Phosphate buffer saline A
PCR	Polymerase chain reaction
PFU	Plaque formation unit
pH	The negative logarithm of the hydrogen ion concentration
qPCR	Real-time quantitative PCR based serum neutralisation
RECC-CU205	Reticuloendotheliosis virus transformed T-cell line
rpm	Revolutions per minute
RPMI	Rosewell Park Memorial Institute
RNase	Ribonuclease
SN	Serum neutralisation
SOgE-QM7	Recombinant quail muscle cell line
SPF	Specific pathogen free
ssDNA	Single stranded DNA
TAE	Tris acetate EDTA
T-cells	T- lymphocyte
$TCIC_{50}$	Tissue cytotoxic infective dose
TCR+	T-cell antigen receptor
Th	T-helper cell
T_m	Melting temperatures
TR_L	Long terminal repeat
TR_S	Short terminal repeat
U_L	Unique-long
U_S	Unique-short
UV	Ultra violet
vIL8	Viral interleukin-8
VP	Viral protein
vvMDV	Very virulent pathogenic strains of MDV
vv+ MDV	Very virulent plus strains of MDV
w/v	Weight per volume

X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
°C	Degree Celsius

CHAPTER 1

GENERAL INTRODUCTION

Marek's disease (MD) is a neuropathic and neoplastic disease of poultry caused by a highly contagious, cell-associated herpesvirus. The pathogenesis of Marek's disease is complex, involving cytolytic and latent infection of lymphoid cells and tumorigenic transformation of lymphoid cells in infected birds (Baaten *et al.*, 2004). Serotype one, MDV-1 only is capable of producing disease in poultry. The next two MDV, MDV-2 and HVT are naturally occurring in the chickens and in turkeys respectively (Witter, 1998a) and can be used as vaccines for MDV-1. Although this disease has been controlled by vaccination, the poultry industry has experienced production losses due to MDV-1 infection in the past (Witter, 2001).

Chicken anaemia virus is immunosuppressive virus that causes clinical disease in young chickens when chicks free from CAV maternal immunity are infected before three weeks of age. The clinical features of disease are anaemia, haemorrhages, reduction in lymphocytes and immunosuppression (Lucio *et al.*, 1990; Hoop *et al.*, 1992). The subclinical disease is characterised by immunosuppression in all infected birds (Markowski-Grimsrud and Schat, 2003).

Commercial broiler production has been intensified in the past couple of decades to sustain the increasing demand of poultry products. However, this intense production has also introduced genetic imbalance with decreased genetic diversity of the commercial chickens. As a result MDV-1 is able to persist within the host and outside in the poultry house environment producing a constant source of infection (Witter, 2001).

Chicken anaemia virus was first isolated and characterised in 1979 during an investigation of MD breakthrough in MDV vaccinated chickens (Yuasa *et al.*, 1979). In Australia, CAV was first isolated and reported in 1990 (Firth and Imai, 1990). The isolate was designated 704. However, other isolates (CAU/7 and BF4) were isolated before the isolation of 704 by R. McCoy in 1988 (Pallister *et al.*, 1994; Spence,

1996). Later two other viruses 3711 and 3713 (Connor *et al.*, 1991) were isolated. Biological properties of these two isolates and the isolate 704 were studied and were found to be relatively similar (Connor *et al.*, 1991). All of these viruses were isolated from breeding flocks. Genetic studies of isolates 704 (Hamooleh *et al.*, 1996) and CAU/7 (Brown *et al.*, 2000) have been reported.

Under field conditions CAV infection in broilers has unique dynamics in comparison to the infection in egg laying strains in the SPF environment (Sommer and Cardona, 2003). To date, information about the status of CAV in Australian broiler flocks has not been published.

In Australia all three serotypes of MDV have been isolated from clinical samples received from vaccinated flocks (De Laney *et al.*, 1995). However, investigations of the three viruses, CAV, MDV-1 and MDV-2 in the broilers flocks that have no obvious clinical sings of either disease are limited.

Both MDV-1 and CAV are immunosuppressive viruses. Dual infections by these two viruses have the potential to produce synergism and increase pathogenicity. It has been demonstrated that infection of CAV impairs the generation of cytotoxic T lymphocytes with subsequent failure to produce optimum vaccinal immunity (Markowski-Grimsrud and Schat, 2003). In addition the impact of CAV infection in terms of production loses due to MDV can be substantial (Davidson *et al.*, 2004).

Similarly, MDV-2 is an apathogenic virus, naturally occurring in chickens. It has been successfully used as a vaccine either alone or in combination with HVT. The bivalent vaccine is reported to induce increased immunity (Witter *et al.*, 1995). The presence of MDV-2 in the poultry shed is likely to boost the immunity produced by HVT vaccination.

Chicken anaemia virus can be transmitted both horizontally and vertically. Both parents have the potential to transmit infection to their progeny irrespective of the immune status (Cardona *et al.*, 2000a; Cardona *et al.*, 2000b). Horizontal transmission from the CAV contaminated poultry shed environment could be the next important source of CAV infection in broilers. Similarly vaccines so far

produced to control clinical CAV infection are live. Information regarding the replication of vaccine virus in the parental birds and potential transmission to the progeny has not been forthcoming.

Therefore, this project aimed to investigate the prevalence MDV-1, MDV-2 and CAV in representative broiler flocks throughout Australia with different vaccination status for MDV. The sequence of viral CAV isolates was compared with the sequence of the viral strain being used to vaccinate the breeder birds.

It was also planned to speculate on the possible source of transmission of CAV by examination of sequential weekly samples from week one till slaughter from a representative broiler flock. And therefore estimate the time of infection of the birds with CAV.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

The Poultry Industry is one of most important components of the agriculture sector of the world's economy. Birds raised under commercial conditions are vulnerable to environmental exposure to a number of pathogens. Among these pathogens, MDV, Newcastle disease virus (NDV), infectious bursal disease virus (IBDV), CAV and retroviruses have a major economic impact on poultry industries. Marek's disease virus, IBDV, CAV and some retrovirus are viral diseases associated with the immune system causing immunosuppression in chickens, making them more susceptible to other diseases (Miles *et al.*, 2001).

Marek's disease virus is highly contagious and characterised by enlargement of peripheral nerves with infiltrating inflammatory cells and oedema leading to the neural form of the disease with the development of paralytic symptoms (Calnek *et al.*, 1998). Similarly, early immunosuppression and development of malignant T-cell lymphomas in chicks are other important clinical manifestations of MDV infections (Brunovskis and Kung, 1995).

The immunosuppressive viral disease, CAV has the potential to be synergistic with MDV-1 and increase the pathogenicity (Miles *et al.*, 2001). Alternatively MDV-2, an apathogenic MDV is likely to decrease the pathogenicity of the wild type MDV in concurrent infection. In fact MDV-2 has been successfully used as a vaccine against MDV-1 (Witter *et al.*, 1984).

Chicken anaemia virus is the causative agent of chicken infectious anaemia, which is characterised by anaemia, immunosuppression, and secondary infection when chickens are infected before two weeks of age (Miles *et al.*, 2001).

This review will include comments on the pathogenesis of MDV and CAV and will highlight the emergence of highly virulent strain of MDV-1 and the role of the

immunosuppressive viral diseases such as CAV on the pathogenesis of MDV. In addition genomic characteristics of CAV will also be discussed.

2.2 Marek's disease virus

2.2.1 Introduction

The term Marek's Disease was suggested for an avian disease "Fowl Paralysis' at the first conference of the World Veterinary Poultry Association in 1960, though the history of the disease began in 1907 when Dr. Joseph Marek observed paralysis of legs and wings in four adult cockerels. A similar disease manifestation was explained by Kaupp in USA in 1921 and Winkler-Junius in 1924 in The Netherlands arguing that the disease had achieved a worldwide distribution much earlier. The association between paralysis and tumour development was first observed and explained by Pappenhaimer *et al.* in 1922 when an experimental avian flock experienced an explosive outbreak of the disease (Biggs, 2001).

The disease reported from the 1950s onwards appeared to be explosive in nature with mortalities of 30% or more with a high incidence of visceral lymphoid tumours in addition to neural signs as compared with previous outbreaks. Thus, the disease was termed acute MD and it developed its worldwide distribution by the 1960s (Payne, 1985).

Vaccination against Marek's disease became the major target of research and success was achieved in the early 1970s. However, vaccines do not protect against superinfection with wild virus. The problem presented by the appearance of the disease in the vaccinated chickens during the late 1970s and a similar situation of disease reported in the year 1990s could be the consequence of changes in the virulence of the circulating field viruses (Witter, 2001).The disease manifestation was predominantly in the vaccinated flocks, though the disease was essentially the same as that which had been presented since the 1960s. Some outbreaks had unusual clinical and pathological development, such as high incidence of ocular lesions, severe cytolytic changes in the lymphoid organs, or encephalitis which sometimes led to transient paralysis (Witter, 1997).

2.2.2 Taxonomy and viral characterisation

Marek's disease virus belongs to the *Alphaherpesvirinae* subfamily of the family *Herpesviridae* and the genus is *Mardivirus*. The genus *Mardivirus* includes three serotypes of MDV, Gallid herpesvirus 2 and 3 referred to as serotypes 1 and 2 respectively and Meleagrid herpesvirus 1 referred to serotype 3 herpesvirus of turkeys (HVT) (International Committee on Taxonomy of Virus, 2000).

The genome of all of the *Herpesviridae* is double-stranded linear DNA molecules ranging in size from 108 to 230 kbp. The genome of MDV-1 is about 160 kbp. The DNA molecule is further comprised of two unique sequences, a long (unique-long, U_L) and a short (unique-short, U_S), each of which is bracketed by inverted internal (IR_L, IR_S) and terminal repeats (TR_L, TR_S) (Osterrieder, 1999). The MDV genome encodes at least 80 proteins. Most of these genes are common in all three serotypes of MDV and others have homologues in herpes simplex virus. Most of the genes within the IR_L and IR_S fragments are unique to MDV and among these some are only present in the oncogenic strains (Ross, 1999).

2.2.3 Serotypes

On the basis of protein structure and genetic analysis, MDV has been categorised into three serotypes. Serotypes 1 MDV consists of all oncogenic and their attenuated strains, MDV-2 are naturally occurring apathogenic strains, and serotype 3 (MDV-3 or HVT) is a non-oncogenic herpesvirus of turkeys (Schat, 1985). More recently, a new proposal for the nomenclature of MDV-2 has been postulated as *Gallid Herpesvirus* Type 3 (GaHV-3) (Osterrieder and Vautherot, 2004).

The entire genomes of representatives of all these three serotypes, MDV-1, GaHV-3 and HVT have been sequenced. The sequence alignments demonstrated that the genetic constituents and the linear arrangements share many similarities. However, these three viruses consistently vary in constitutions of guanine and cytosine (GC) contents, 44.1%, 47.2% and 53.6% in the genome of MDV-1, HVT and MDV-2 respectively (Lee *et al.*, 2000; Tulman *et al.*, 2000; Afonso *et al.*, 2001; Izumiya *et al.*, 2001; Kingham *et al.*, 2001). In addition, serotype specific genes are also

present in the genome of each virus. The MDV-1 specific genes meq (MDV *Eco*RI-Q), the pp38 and viral interleukin-8 (vIL8) are expressed only by MDV-1. The pp38 and meq genes are indicated to maintain latency and tumour formation (Xie *et al.*, 1996; Ross *et al.*, 1997) whereas the vIL8 is the chemokine encoding gene (Parcells *et al.*, 2001). The HVT specific gene is designated NR-13, the product of which belongs to the Bcl family of proteins that regulate apoptosis (Kingham *et al.*, 2001). The genome of MDV-2 contains neither MDV-1 specific genes nor HVT specific genes.

2.2.4 Epidemiology and pathogenesis

2.2.4.1 Transmission

Marek's disease is highly contagious and readily transmitted among chickens. Fully matured infectious enveloped virions are formed only in the feather follicles. The affected feather follicle epithelium cells slough off with moulted feathers and are the major source of contamination in the environment. Simultaneously, virus is also present in the moulted feathers with infected cells attached (Carrozza *et al.*, 1973). Dust or dander from infected birds is the major source of transmission. Infection could be transmitted either by virus isolated from the dust (cell-free) or by whole dust (cell-associated). The cell-free virions from the skin debris are more infectious and labile whereas virus in the feather follicle epithelium (FFE) enclosed in keratin particles are less infectious but more stable in the environment than a the cell-free virus (Carrozza *et al.*, 1973). Virions may survive for months or even years under temperatures ranging from 20°C to 4°C in the poultry house. Infected birds continue to be carriers for long periods when infected and serves as the source of infection to other susceptible birds. Vaccination can only reduce the excretion/shedding of virus from feather follicles, but it can't be prevented.

Vertical transmission of Marek's disease virus has not been so far reported. It is unlikely to be transferred from the infected dam to its progeny by external contamination of eggs because of poor survivability of the virus at incubation temperatures and humidity (Calnek and Witter, 1991).

2.2.4.2 Pathogenesis

Virus most certainly enters the susceptible birds via the respiratory route after inhalation of virus-contaminated environment in the poultry house. However, no pathological changes have been reported in the lungs during the initial phase of infection. In fact the actual site of virus uptake along the airways and the cellular mechanisms responsible in virus uptake is not yet elucidated. It was suggested that the phagocytic cells in the lungs pick up the virus and transport it to the lymphoid organs such as the bursa of Fabricius, the thymus and the spleen. The virus had been detected in these lymphoid organs between 3 to 6 days post-infection where productive cytolytic lesions are found (Payne and Rennie, 1973). More recently, it is speculated that there is likely to be different virus uptake mechanisms for cell-free virus and the keratin-wrapped virus in the lungs on the basis of size fractions. However, further studies to elucidate this issue are required (Baigent and Davison, 2004).

To date, pathogenesis of MD has been described in different phases, early cytolytic phase, latent infection, late cytolytic phase, and fully productive infection in the feather-follicle epithelium and described as " the Cornell Model" (Baigent and Davison, 2004).

2.2.4.2.1 Early cytolytic phase

After uptake of the virus from the respiratory tissues by macrophages, the virus is brought to the secondary lymphoid tissues via blood circulation. Expression of the viral gene, pp38 at 4-6 days post inoculation in the spleen, gut-associated lymphoid tissues, caecal tonsil, Harderian gland and conjunctiva-associated lymphoid tissue were demonstrated (Baigent and Davison, 2004). It has also been demonstrated that the hypervirulent strain of MDV is capable of infecting macrophages as well (Barrow *et al.*, 2003).

The infections of MDV in primary lymphoid organs, bursa and thymus of the infected birds can be detected after three days post-infection. However, viral load is lower that observed in infections of the spleen (Baigent and Davison, 2004). It was

assumed that the infected B-cells enter these primary lymphoid organs through the general circulation. Consequences of infection in these organs are lymphocytolysis resulting into necrosis and infiltration of inflammatory cells causing severe atrophy of the bursa and thymus. The further consequence of this phase is immunosuppression (Morimura *et al.*, 1995).

It has been reported that B-lymphocytes are the major primary targets for the early cytolytic infection in primary and secondary lymphoid organs. The T-cells are fairly refractory to the infection but that cytolytic infection of B-cells induces activation of T-cells, making these cells susceptible to infection. The majority of the T-cells were CD4+ and CD8+ phenotypes (Calnek *et al.*, 1984b).

2.2.4.2.2 Latent infection

The expression of MDV antigen in the lymphoid tissue is lost at 6-7 days post-infection and shifted to the latent infection. The latent infections are non-productive infection except after reactivation. In the course of latent infection, the viral genome is confined to the nucleus of chicken cells and viral antigen production is not observed (Venugopal, 2000). The study of the latent infection can not be established *in vitro*. *In vivo* study is also not possible due to difficulty in differentiation of the latently infected and transformed infection. In both infections, no cellular lysis is observed.

In this phase, virus predominantly infects T-cells and is responsible for the long-term carrier state where mainly $CD4^+$ cells are infected. Early reports suggest that a minority of B-cells can be latently infected (Calnek *et al.*, 1984a). A more recent study suggested that the latently infected lymphocytes are those that became infected during the cytolytic phase of infection when virus can easily be spread from cell to cell, and then are prevented from completing the explicative cycle by intrinsic and extrinsic factors. In addition it is hypothesised that B-cells are quickly lost because of their short life span in comparison to T-cells, or the latent infection in B-cells is not well induced and/or controlled by external influences as it is in T-cells, or T-cells are intrinsically less likely to support a productive infection (Calnek, 2001). However,

the exact reason behind the significant predominance of T-cells rather than B-cells during this phase is still not explained well.

Host cytokines, IL-6, IL-18 and IFNγ and the soluble mediator, nitric oxide (NO) may have an influence on the establishment and maintenance of latency (Xing and Schat, 2000a; Xing and Schat, 2000b). Another factor associated with upholding latency is the *Meq* gene; it blocks apoptosis of latently infected $CD^{+}4$ T-cells and transactivates latent gene expression (Parcells *et al.*, 2003).

Latent infection in the genetically resistant chicken is suggested to be at low level in the blood lymphocytes and spleen without further considerable effects whereas in the genetically susceptible or suppressed hosts the next sever pathological cycle begins 2-3 weeks after primary infection. These similar consequences can be observed in the birds infected with highly pathogenic strains of MDV (vvMDV). The viruses are then disseminated to the different body organs (skin, viscera and nerves) by the latently infected peripheral blood lymphocytes (Baigent and Davison, 2004).

2.2.4.2.3 The late cytolytic phase

A second wave of semi-productive infection and cytolysis from 14- 21 days post-infection has been reported in MD susceptible chicken. This late cytolytic infection has been reported to affect the thymus, bursa and some epithelial tissues including feather follicle epithelium, kidney, proventriculus and adrenal glands. It is likely that the latently infected cells bring the virus to these organs/tissues where the virus becomes reactivated by the influence of a secondary wave of immunosuppression (Calnek, 1986).

2.2.4.2.4 Fully productive infection in the feather-follicle epithelium

Fully infectious enveloped virions of MDV are found in the FFE of the infected birds after 13 days post-infection. It is suggested that the virus could be carried to the skin by latently infected peripheral blood lymphocytes. This type of infection persists for many weeks after initiation. The virions can be observed in the nucleolus and cytoplasm of the infected cells (Baigent and Davison, 2004). Virions and intranuclear

inclusion bodies are common in the corneous and transitional layers of FFE. Cloudy swellings and hydropic degeneration of the infected cells followed by local perivascular and perifollicular aggregation and proliferation of lymphoid cells are common pathology of this phase of MDV infection. These lesions may develop into subcutaneous lymphomatous tumours in some cases (Payne, 2004).

2.2.4.2.5 Neoplastic transformation

A final consequence of the MDV infection is the neoplastic transformation of the latently infected lymphocytes to lymphoblastoid tumours cells. The initiation of transformations is mostly observed in the spleen. However, development of transformed lesions has been observed in splenectomised birds. After three weeks of infection the splenic T-dependent areas become hyperplastic and these hyperplastic cells are likely to be the precursors of neoplastic transformation (Baigent and Davison, 1999). Transforming infection is reported only in previously activated T lymphocytes ($CD4^+$ and to a lesser extent $CD8^+$ lymphocytes) and has been detected only in association with the virulent serotype 1 MDV. It is presumed that the latent infection is a prerequisite for transformation (Baigent and Davison, 2004).

Lymphomas produced as consequences of MD are of two types, classical and acute. Lymphomas are predominantly observed in the peripheral nerves in case of the classical form of MD whereas in the acute form of the disease, lymphoid necrosis and lymphomas are found in the visceral organs (Biggs *et al.*, 1965). Peripheral nerve enlargement and paralysis due to cellular infiltration is the typical feature of classical MD (Payne and Rennie, 1973). Mild perivascular cuffing in the perivascular space of Virchow-Robin in brain by macrophages and lymphocytes has been observed in both acute and classical forms of MD. The very virulent plus (vv+) strains of MDV inoculated into 18-day-old chickens produced sudden paralysis of the neck and legs referred to as transient paralysis about 9 days later (Witter *et al.*, 1999).

Cytologically the visceral lymphomas are similar to the cellular infiltration in the peripheral nerves. The cellular components in MDV lymphomas have $CD4^+$ $CD8^-$ (helper T cell) phenotype. The characteristic of the lymphoblastoid cell lines are

essentially diploid in the latent phase MDV infection and comprised of a series of antigenic markers. The markers are the MD tumour-associated surface antigen (MATSA), MHC antigen, Forssman antigen and chicken foetal antigens. The brief diagrammatic sequential phases of MD pathogenesis are presented in Figure 2.1.

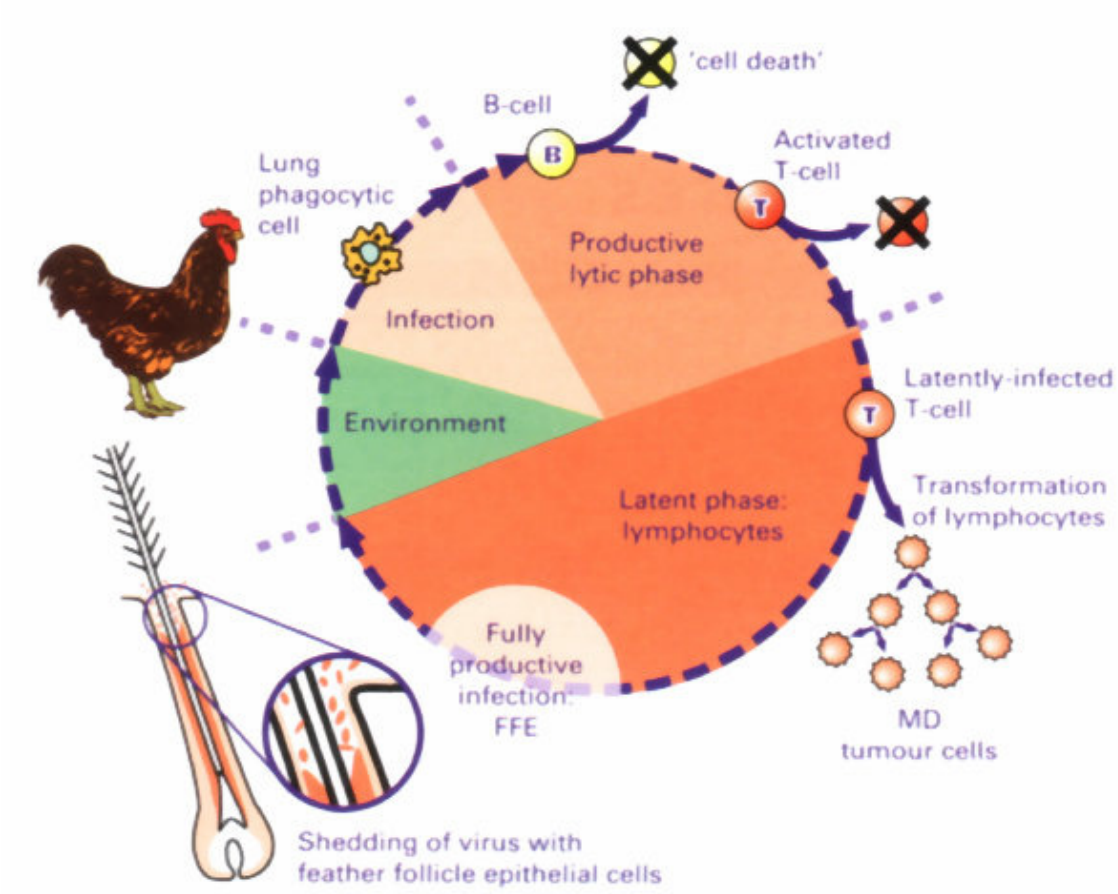


Figure 2.1 Schematic diagram presenting different phases of MD pathogenesis. This picture is adapted from Baigent and Davison, (2004).

2.2.4.3 Clinical findings

It is said to be difficult to determine the incubation period of MD under field conditions. In general the incubation period is 3 to 4 weeks. However, clinical findings may be seen even after several months in some birds. A temporary paralysis can be observed in a few or up to 50% of the infected birds at about four weeks after being exposed to the virus (Calnek and Witter, 1991).

Two clinical forms of the disease are found, classical and acute form. Clinical signs in the classical form are mainly associated with interference with peripheral nerve function. Mortality is generally low not exceeding 20%. Infected birds show flaccid or aspastic paralysis of legs, drooping of one or both wings, torticollis, ptosis, difficulty in respiration and vertical or lateral recumbency with one leg stretched forward the other backward in the advanced stages of disease. Acute forms of Marek's disease are characterised by sudden death without showing the above-mentioned clinical signs and mortality is relatively higher than with the classical form. Some birds may recover after transient paralysis (Purchase, 1985; Venugopal and Payne, 1995; Schat and Xing, 2000).

2.2.5 Impact of chicken anaemia virus infection on MDV infection

Numerous studies have shown that CAV is an important cofactor for a number of avian diseases. Vaccine breakthrough and aggravations of MD infection is often linked to CAV co-infection. An experiment to find out the effect of co-infection of specific-pathogen-free (SPF) chickens with CAV and MDV was performed in chickens inoculated with very virulent MDV strains (vvMDV) and very virulent plus MDV strains (vv+ MDV) in the presence and absence of CAV. The CAV infection exacerbated the infection with the vvMDV. However, the effect of CAV was less prominent in birds infected with the vv+MDV. This experiment indicates that the effect of CAV on the pathogenicity of MDV may be influenced by the pathogenicity of the strain of MDV (Miles *et al.*, 2001).

An interesting characteristic of CAV is that it can infect and replicate in several Marek's disease chicken cell lines such as the MDCC-MSB1 cell line derived from

Marek's disease tumours. A similar trophism may occur in the infected animal. MBS1 cells are mature helper T lymphocytes, which are CD3+, CD4+, CD8+, TCR+, and are used for *in vitro* isolation, replication and titration of CAV (Adair *et al.*, 1993). Chicken anaemia virus has a special affinity to Marek's disease chicken cell lines developed from Marek's disease tumours. In most of the studies of CAV, MDCC-MSB1 has been used as a substrate (Calnek *et al.*, 2000).

The cytopathic effects and detection of viral antigens by immunostaining are the standard criteria used to indicate infection in MSB1 cells. Although these cells appear to be the preferred substrate for *in vitro* infection with many strains of CAV, there are differences in susceptibility associated with other transformed cell lines. The susceptibility to CAV is also dependent on the culture period and loss of susceptibility was detected upon continuous passages (Calnek *et al.*, 2000). However, further explanation or supporting evidence is not reported.

Susceptibility to the secondary infection is increased in chickens infected with chicken anaemia virus, most probably due to the immunosuppressive effect, which leads to impaired development of pathogen-specific T lymphocytes in the host. An experiment in specific-pathogen-free chickens as well as natural infection with CAV and MDV or REV was conducted. MDV or REV-specific CTL were detected at seven days post-infection in chickens that were maternal antibody positive to CAV but the flocks with no maternal antibody to CAV failed to produce pathogen-specific CTL. The mechanism of impairment of CTL generation may have resulted from destruction of lymphoid precursors, or indirectly by alteration in essential cytokines in the infected birds, is not clear because of the unavailability of commercial ELISA kits for the detection of those chicken cytokines (Markowski-Grimsrud and Schat, 2003). However, suppression of IL-2 levels at 8 to 15 days p.i. as well as a remarkable increase in IFN- γ at 15, 22, and 29 days p.i have been reported (Adair, 1991). Infection with MD alone resulted in a 16-fold increase in IFN- γ mRNA levels at 9 days post-infection. The increase in IFN-γ level was maintained by combined infection with MD and CAV in chickens with or without maternal antibodies to CAV (Xing and Schat, 2000a).

2.2.6 Diagnosis

Clinical findings and the pathology in the infected birds may provide provisional diagnosis but confirmatory laboratory diagnosis is required for the differentiation of Marek's disease from other lymphoproliferative diseases such as avian lymphoid leucosis and reticuloendotheliosis (Calnek and Witter, 1991). Proper collection of sample material is most important for the diagnosis. Non-coagulated whole blood, single cell suspensions prepared from lymphoid organs, feather follicle epithelium are considered to be appropriate samples for MDV diagnosis (Davidson *et al.*, 1986; Zelnik, 2004). The collected tissues can be stored in liquid, at 4ºC or -20 ºC depending on the diagnostic methodology selected. For viral isolation, immediate processing is required due to the cell-associated nature of the virus. Feather tips as samples can be stored in cool and dry place for several weeks (Davidson *et al.*, 1986).

2.2.6.1 Viral isolation

Examination of infected materials using electron microscopy was the first tool to study the structure and morphology of MDV (Nazerian *et al.*, 1971). However, it needs expensive equipments and skilled personnel. It is not in routine use. *In vitro* or *in vivo* propagation and isolation of MDV is required for further characterisation. Susceptible chickens, embryonated eggs and recently hatched eggs can also be used to grow the virus. The virus can be grown in different cell lines. For primary viral propagation, chicken kidney cells (CKC) or duck embryo fibroblast (DEF) have been suggested as suitable cell lines (Churchill and Biggs, 1967). Chicken embryo fibroblasts (CEF) are commonly used cells for vaccine production. All three serotypes of MDV can grow in monolayers of chicken embryo fibroblasts producing discrete plaques. Some other continuous cell lines, QM7 SOgE (Schumacher *et al.*, 2000; Schumacher *et al.*, 2002) have also been reported but use of these cells for the primary isolation of MDV from the infected samples has not been described.

2.2.6.2 Serological examination

Agar gel precipitation was the assay used for conformation of MDV after successful isolation in the cell culture in the 1960s. Detection of MDV-specific antigen or antibodies in the clinical samples by direct or indirect immunofluorescence were also used. The development of serotype specific monoclonal antibodies provided tools for the detection and differentiations of MDV and HVT (Lee *et al.*, 1983; Dorange *et al.*, 2000).

The virus can be identified and quantified by immunoperoxidase plaque assay from primary cell cultures. Enzyme linked immunosorbent assay (ELISA) is another technique used for antigen detection and it is 20-40 times more sensitive than the immunofluorescent assays (Calnek and Witter, 1991). Feather tip solid phase ELISA is another simplified assay (Scholten *et al.*, 1990). It is rapid and sensitive. However, it cannot detect latent or transforming infection.

2.2.6.3 Molecular diagnostics

Viral genome amplification and detection by PCR is a rapid, reliable, and sensitive method for the rapid diagnosis and differentiation of all three MDV serotypes (Davidson *et al.*, 1995). However, good laboratory practice is essential in every step from the sample material collection to prevent cross-contamination during DNA amplification.

Differentiation of the vaccine strains and the wild type MDV is necessary to determine the genuine infection in the field. The complete DNA sequences of all three serotypes are documented (Tulman *et al.*, 2000; Afonso *et al.*, 2001; Izumiya *et al.*, 2001). Polymerase chain reaction based on the amplification of the 132 bp repeat sequence, MDV1-specific gene has been developed for the differentiation of wild type and attenuated MDV-1 vaccine viruses. There are only two to three copies of the 132 bp repeats in non-attenuated MDV1 whereas the number of repeats is likely to reach several tens in highly passaged virulent strains of MDV. This property of the virulent MDV-1 further facilitated differentiation of the attenuated MDV1 vaccines as well (Silva, 1992). However, when the vaccine virus is grown in the

birds the number of repeats resembles the wild type virus negating the advantage of the assay (Young and Gravel, 1996). To increase the specificity of the assay nested or semi nested PCR are recommended.

2.2.7 Control measures

Effective vaccination, selection for genetic resistance and strict biosecurity systems are the three major criteria for control of MD. The genetic resistance and the biosecurity are suggested as accessary tools to the vaccination rather than as primary control strategies. All three serotypes of MDV have been as used vaccines.

The first vaccine produced was attenuated oncogenic HPRS-16 strain of serotype 1 MDV which was serially passaged in chicken kidney cells (Churchill *et al.*, 1969). Soon after, another vaccine was developed from MDV serotype-3 or HVT (Okazaki *et al.*, 1970; Witter *et al.*, 1970) and this virus is still widely used. The next MDV vaccine that has become the gold standard is CVI988 also called the Rispens strain which was shown to be protective both in field trials and laboratory experiments (Rispens *et al.*, 1972a; Rispens *et al.*, 1972b). Early reports suggested that the efficiency of the Rispense and the HVT vaccines were comparable (Vielitz and Landgraf, 1972) but later studies confirmed that the Rispens is more efficient in protecting against highly virulent strains of MDV (Witter *et al.*, 1995). Recently, another MDV-1 vaccine was developed from the Australian very-virulent (vv) MDV isolate, BH16 vaccine and it has been claimed to be at least as effective as the Rispens vaccine (Karpathy *et al.*, 2002).

In late seventies, an effort was made to derive vaccine from apathogenic MDV-2 and it was found to be protective against MDV-1. The MDV-2 vaccine was also evaluated in combination with HVT and found to induce synergistic activity with increased protection against MDV-1 compared with the use of either alone (Witter *et al.*, 1984). However, the protection index of this bivalent vaccine was estimated to be somewhere between the protections induced by HVT and Rispens vaccines (Witter *et al.*, 1995). At present, the Rispens, HVT and HVT + MDV-2 (bivalent) are the most widely used MDV vaccines (Bublot and Sharma, 2004).

Vaccines can be administered to day old chicks by the sub-cutaneous, intramuscular, or intra-abdominal routes and the dose rate is commonly 1,000 plaque forming units (PFU) per bird in order to protect against the production of tumours (Ohashi *et al.*, 1999; Xing and Schat, 2000b). This system of vaccination has been progressively replaced by *in ovo* administration at the time eggs are transferred from the incubator to the hatcher (Zhang and Sharma, 2001). Automatic, multiple head injectors have been developed which deliver the recommended amount of vaccine to entire tray of eggs subsequently decreasing the labour cost (Ricks *et al.*, 1999).

In recent years, attempts have been made to produce genetically modified recombinant vaccines which may have superior protective properties. However, they have not yet replaced the traditional vaccines (Bublot and Sharma, 2004).

2.2.8 Emergence of highly virulent wild type Marek's disease virus

By vaccination, the clinical disease can be controlled but not any of the vaccines protect from the subclinical infection of MDV-1, further replication and shedding of the infectious virus occurs. As a result there is the potential to select for ever increasing virulence. The MD outbreak during the 1960s and the early 1990s in vaccinated flocks has been suggested to be due to the emergence of MDV-1 with increasing virulence (Witter, 1997). In addition vaccinations with suboptimal doses of vaccines favour the infection by delaying immunity induction (Nair, 2005).

Existing infection with other immunosuppressive infectious agents like, CAV and IBDV infections may also impair the induction of optimal immunity even though birds are vaccinated with a proven efficient vaccine (Miles *et al.*, 2001; Markowski-Grimsrud and Schat, 2003). The proposed future strategy to control MDV has been summarised in Figure 2.2.

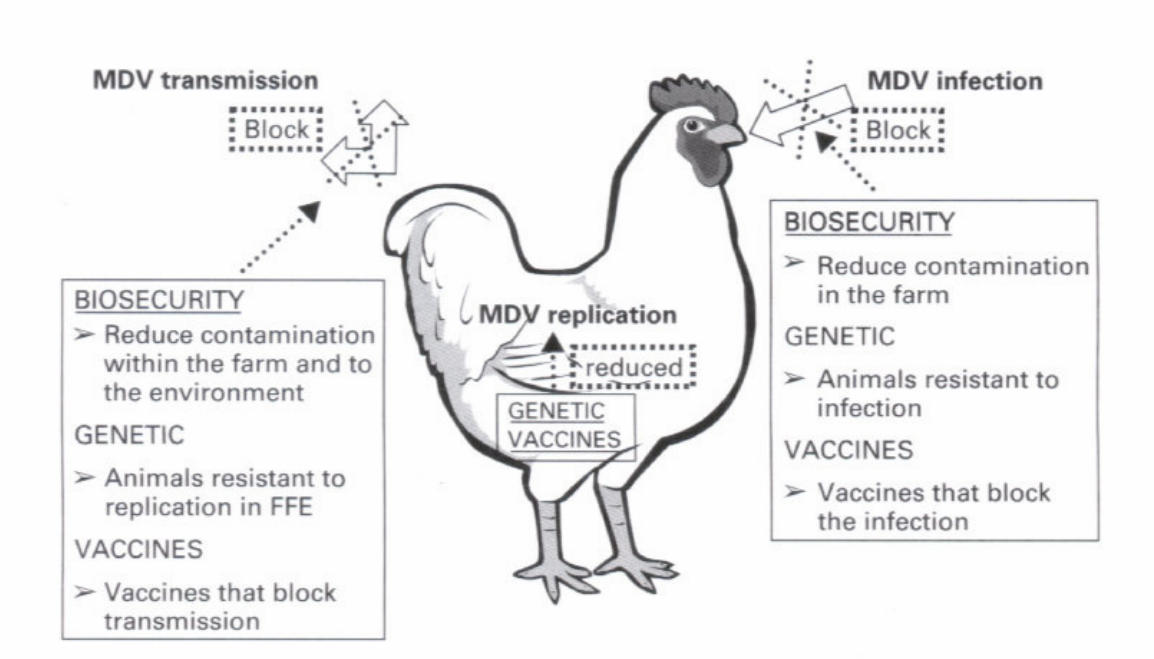


Figure 2.2 Critical points in the epidemiology of MD and possible strategies of control. Those measures that are currently available are underlined. This figure is adapted from (Gimeno, 2004).

2.3 Chicken Anaemia Virus

2.3.1 Introduction

Chicken anaemia virus was isolated for the first time in Japan by Yuasa *et al*. in 1979 during the investigation of Marek's disease outbreaks (Kato *et al.*, 1995). The virus has now been identified in commercial poultry farms in many countries and is considered to have a worldwide distribution (Hoop *et al.*, 1992).

Chickens are reported to be the only susceptible host. The acute disease is characterised by severe anaemia, destruction of erythroblastoid cells in bone marrow, and depletion of primary lymphoid organs leading to immunosuppression with intramuscular and subcutaneous haemorrhages, in the chickens that are infected less than three weeks of age and that have no maternal antibodies to the CAV (Miles *et al.*, 2001). Infection with CAV in the chickens that are more than three weeks of age causes a milder disease with impairment of the production of cytotoxic T lymphocytes (CTL) (Markowski-Grimsrud and Schat, 2003).

2.3.2 Taxonomy and viral characteristics

Chicken anaemia virus, belongs to the family *Circoviridae.* Within the *Circoviridae* family, CAV is placed in the genus *Gyrovirus*. Two other animal circoviruses, porcine circovirus (PCV) and psittacine beak and feather disease virus (BFDV) are now placed in the genus *Circovirus* (International Committee on Taxonomy of Virus, 2000).

The virion of CAV is a non-enveloped icosahedral capsid, 23-25 nm in diameter. The viral genome is circular, 2.3 Kb long, negative sense single-strand DNA (Noteborn *et al.*, 1991). It has three partially overlapping open reading frames encoding VP1 (51.6 kDa), VP2 (24.0 kDa) and VP3 (13.6 kDa), respectively and a 5' nontranscribed regions.

The capsid of the CAV genome contains only VP1. This fragment of genome is 1350 bp long and includes a hypervariable region in the predicted amino acid positions from 139 to 151 (Renshaw *et al.*, 1996). The immunogenic studies of three recombinant proteins (VP1, VP2 and VP3) suggested that not single protein is capable to induce significant level of protective immunity. However, co-expression of both VP1 and VP2 demonstrated a protective level of immune response against CAV and also to their progeny (Koch *et al.*, 1995).

The next protein, VP2 is non structural protein. There is limited understanding of this protein. A study based on immunoprecipitation assay demonstrated that VP1 and VP2 interact directly with each other and suggested that VP2 most likely act as a scaffold protein in virion assembly (Noteborn *et al.*, 1998c). More recent studies on VP2 suggested that it has dual protein phosphatase activity (Peters *et al.*, 2001).

The third protein, VP3 is also non-structural. It has a unique characteristic which initiates apoptosis. Because of this exclusive property, the VP3 is also known as apoptin and the CAV causes apoptosis of the infected hemocytoblasts and thymic precursors in chickens (Noteborn *et al.*, 1994). The apoptotic activity is not limited to chicken thymus, the virus is able to induce apoptosis in various cultured human tumorigenic as well as chicken transformed lymphoblastoid cell lines (Danen-Van

Oorschot *et al.*, 1997). The mechanism associated with the apoptosis of thymocytes has yet to be fully described. The VP2 protein is also said to be capable of producing some apoptotic activity but much weaker than the VP3 (Noteborn, 2004).

The promoter/enhancer region of the CAV genome contains four or five 21 bp direct repeats. After first two direct repeats, a 12 bp insert is located in the genome and is suggested to be essential for efficient viral replication (Noteborn *et al.*, 1998a).

2.3.2.1 Chicken anaemia virus - induced tumour-specific Apoptosis

Several studies had been carried out to highlight CAV-induced apoptosis, a feature of VP3 protein of CAV, hoping to produce tumour neutralising factor *in vitro* (Jin *et al.*, 1999a; Jin *et al.*, 1999b; Oro and Jans, 2004). The VP3 protein is 121-amino acids long. The amino acid constituents are manly serine, proline, and threonine residues and have a positively charged C-terminus (Danen-Van Oorschot *et al.*, 2003). An interesting characteristic of the Apoptin binding have been described. In the cells in which apoptin is likely to induce apoptosis, co-localisation takes place with the condensed DNA whereas with non-apoptic cells VP3 does not co-localise with the chromatin (Noteborn *et al.*, 1998a; Noteborn *et al.*, 1998b; Noteborn *et al.*, 1998c).

Apoptin predominately co-localises with heterochromatin and nuclei in tumour cells and subsequently induce the apoptosis (Leliveld *et al.*, 2003). Recent studies suggested that the proteins fuse to maltose-binding protein (MBP-Apoptin) and subsequently induces the tumour-specific apoptosis (Zhang *et al.*, 2003). The biological basis of its preference for these DNA-dense nuclear substructures is feasible. Furthermore, multimers of the MBP-apoptin (up to 20) accumulate with a continuous stretch of dsDNA into higher-order superstructure in a highly cooperative manner. The polymerisation motif of Apoptin is located in its N-terminal 69 amino acid (Leliveld *et al.*, 2003). However, another study demonstrated that either a C-terminal or N-terminal half is sufficient to induce apoptosis but had less significance compared to full length apoptin. A strong correlation between nuclear localisation and cell death activity can be observed in either case (Danen-Van Oorschot *et al.*, 2003).

Literatures suggest that P^{53} and Bcl-2 are also association with the apoptosis. Apoptin was capable of inducing apoptosis in the tumorigenic or avian or human transformed cells. However, this function was limited to the transformed cells only; no such activity was demonstrated in case of normal cells (Danen-Van Oorschot *et al.*, 1997) and the cells lacking P^{53} , an important mediator of the apoptosis (Zhuang *et al.*, 1995). In addition Bcl-2 is also known as anti-apoptosis protein. However, BCl-2 loses its anti-apoptosis character in the presence of CAV-apoptin in tumorigenic or transformed human cells (Zhang *et al.*, 1999).

2.3.3 Epidemiology and pathogenesis

Chicken anaemia virus is highly resistant to heat, chemical disinfectants and adverse environments. The virus has a worldwide distribution and is present in almost all poultry premises (De Herdt *et al.*, 2001). The chicken is the only the host for CAV. All age groups are susceptible to infection, but clinical disease is developed merely in chickens which are less than three weeks of age and have no maternal antibody against CAV (Miller and Schat, 2004).

2.3.3.1 Transmission

An interesting feature of CAV is its transmission. The virus can be transmitted horizontally as well as vertically. The horizontal transmission is via the oral route. Vertical transmission in commercial poultry flocks is from the hen and probably the rooster to the newly hatched chicks (Cardona *et al.*, 2000b).

Two types of vertical transmission have been described. However, the mechanisms associated with transmission in both cases have not been fully described. The first category is the acutely infected hen that does not transfer antibody to the offspring through the egg. The replication of the virus lasts after development of neutralising antibodies in the chickens (Miller and Schat, 2004).

The next category is much more controversial and involves transmission of the latent virus. The virus can survive for a long time in the reproductive organs of both hens and roosters in spite of the presence of antibodies (Cardona *et al.*, 2000b). Regardless

of the antibody status in the breeding birds, viral DNA can be transmitted to their offspring (Miller *et al.*, 2003). The long-term presence of CAV in the reproductive tract allows the virus to maintain a latency or dormant or inactive phase in those organs and in doing so escapes the host immune response (Miller and Schat, 2004). Therefore, vertical transmission from the antibody positive flocks to their offspring can be expected.

2.3.3.2 Incubation period and clinical findings

Chicks from non-vaccinated parental flock infected with CAV at less than 2 weeks of age develop a disease. The mode of transmission could be vertical or horizontal. The incubation period is suggested to be 8-10 days. The clinical symptoms in the experimental infections are marked anaemia, haemorrhages, pale bone marrow, atrophy of thymus, and increased susceptibility to secondary infection (Lucio *et al.*, 1990; Hoop, 1993). There is an increased susceptibility to secondary infection, as a result of immunosuppression. Destruction of cells are reflected in the blood where hematocrit levels and the number of circulating leukocytes (erythrocytes, lymphocytes and granulocytes) declines (Adair, 2000). The clinical manifestation in the field condition have been described as blue wing disease due to hemorrhagic and necrotic wing tips, development of a hemorrhagic syndrome, anaemia and dermatitis (Engstrom, 1999). The mortality in the clinical disease can be 6%-20% , but in general, mortality ranges from 1%- 5% (Hoop *et al.*, 1992; McIlroy *et al.*, 1992).

The symptomatic scenario in subclinical cases in the newly hatched chicks with maternal antibody and older birds has been described as moderate depression, lower weight gains and increased susceptibility to secondary infections (van Santen *et al.*, 2004a).

2.3.3.3 Infection

Hemocytoblasts in bone marrow and precursor lymphocytes in the thymus are the target cells for CAV infection and replication (Figure 2.3). Hemocytoblasts are the progenitor cells for the production of erythrocytes, granulocytes and thrombocytes which are the main blood components. Development of anaemia in the infected chickens can be the consequence of destruction of hemocytoblasts (Smyth *et al.*, 1993). Similarly, the intramuscular haemorrhages imply the destruction of hemocytoblast and the ultimately reduction of thrombocytes in the infected chicken. Increased susceptibility to the secondary infection and also a decrease in responsiveness to vaccines is the next important consequence of CAV infection which could be explained as the result of decrease in granulocyte and lymphocyte numbers (Miller and Schat, 2004). Restoration of impaired granulopoiesis and erythropoiesis in bone marrow takes place after 16-18 days following the infection (Smyth *et al.*, 1993).

Another major target of CAV is T lymphocyte progenitor cells in the thymus (Jeurissen *et al.*, 1989). Literatures suggest that the virus has minor effects on B-cells and their precursors (Adair, 2000). This suggestion indicates that the common lymphoid progenitor cells in bone marrow, which provides progenitor cells for seeding of the thymus and Bursa, is probably not susceptible to CAV (Adair, 2000) but to elucidate the exact reason behind this fact deserves further study to recognize the viral receptors. A recent study demonstrated that CAV infection impairs the immune response to a second pathogen by interfering with the development of pathogen-specific CTL (Figure 2.4) (Markowski-Grimsrud and Schat, 2002). Immunisation of CAV infected birds for other diseases may not be inducing optimal immunity even when they are vaccinated with appropriate otherwise efficacious vaccines.

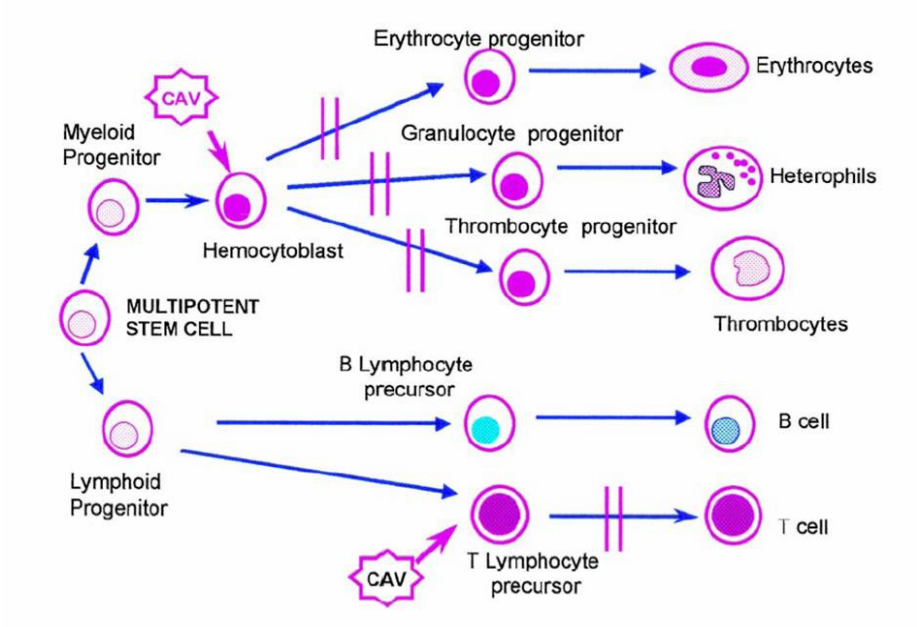


Figure 2.3 Schematic representations of the effects of chicken anaemia virus on haemopoiesis and T cell development. The main target cells are the haemocytoblasts in the bone marrow and T cell progenitors in the cortex of the thymus. Effects of viral replication on the cells are indicated by the parallel lines. This picture is adapted from (Adair, 2000).

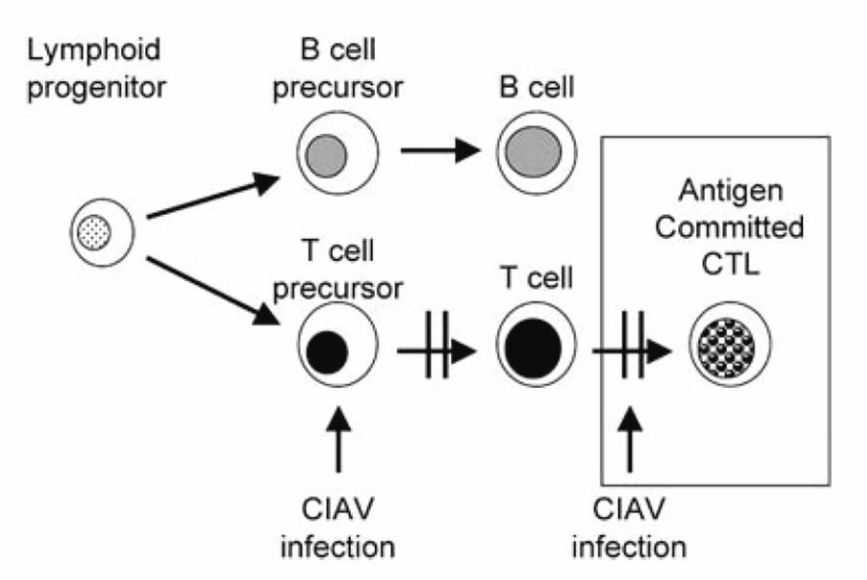


Figure 2.4 Schematic representation of the susceptibility of lymphoblastoid cells to infection with chicken anaemia virus. Effects of viral replication on the cells are indicated by the parallel lines. This picture is adapted from (Miller and Schat, 2004) and was originally based on (Adair, 2000) and the modified (box part) based on (Markowski-Grimsrud and Schat, 2001).

The cortical lymphocytes are the cells in the thymus which are affected in the beginning of the CAV infection (Jeurissen *et al.*, 1989). However, non-lymphoid leukocytes and stromal cells remain unaffected. Studies suggested that among T-cell population, $CD8^+$ cells are more affected than are $CD4^+$ cells suggesting that $CD8^+$ cells are more susceptible (Adair *et al.*, 1993) but other studies so far could not draw a definite significant conclusion regarding lymphocyte destruction. T-cells in the spleen are also found to be infected (Smyth *et al.*, 1993; Adair, 2000) but the exact mechanism is not clearly reported. The mature T-cell in the spleen may become infected in the spleen itself or the infected precursor T cell in the thymus consequently move to the spleen.

Among the CAV strains, differences in pathogenicity have been described. However, the arguments were not backed by strong experimental data. Recently, a putative second serotype has been illustrated (Spackman *et al.*, 2002a; Spackman *et al.*, 2002b) but the importance of the serotype is still to be evaluated. One or both the amino acid differences in the hypervariable region in VP1 at the positions 139 and

144 are important for the differentiation of Cux-1 and CIA-1 in infection and replication in two MDCC- MSB1 cells sub lines (Renshaw *et al.*, 1996). This portion of the genome could also be important for the formation of neutralising epitopes (Miller and Schat, 2004). Another study claims that the VP1 capsid protein, amino acid at position 394 is the major determinant of pathogenicity. The amino acid, glutamine at position of 394 was shown to confer higher pathogenicity. The pathogenicity was decreased when glutamine was replaced by a histidine at this position (Yamaguchi, 2000).

The viral infection and replication are suppressed after production of neutralising antibodies against CAV. Infected chickens produce antibodies and this coincides with the protection of the infected chicken from the development of further lesions. However, immune-compromised birds by embryonal bursectomy (Hu *et al.*, 1993) or chicks co-infection with immunosuppressive diseases like MDV may develop lesions (Imai *et al.*, 1999). The ability of the virus to maintain latency in the breeding flocks and to transmit to their progeny regardless of the antibody status indicates that the development of neutralising antibody does not necessarily eliminates the virus from the infected chicken (Sommer and Cardona, 2003; Brentano *et al.*, 2005).

Hormonal influences on CAV replication is a more recent finding. This was highlighted when SPF birds raised under strict biosecurity became seropositive for CAV at the age of laying. The promoter-enhancer region of CAV DNA contains four consensus cyclic AMP response element sequences (AGCTCA) which are similar to the oestrogen response element (ERE) consensus half-sites (A) GGTCA. The ERE-like sites in the promoter enhancer region of CAV could be recognised by member of the nuclear receptor superfamily and possibly provides a mechanism to regulate CAV activity and maintain very low virus copy numbers under certain situations (Miller *et al.*, 2005).

2.3.3.4 Immunosuppression caused by CAV infection

Chicken anaemia virus infects and replicates in the hemocytoblasts and immature thymocytes. Destruction of these cells by apoptosis impairs the production of erythrocytes, granulocytes, thrombocytes and thymocytes which are responsible for the development of the adaptive as well as the innate immune system (Adair, 2000). Recently, it has been emphasised that the both innate and adaptive immune systems are functionally interrelated. Although the B-cells are not susceptible to CAV infection, elimination of the essential cytokines due to the destruction of T-cells has adverse consequences for the normal function of the B-cell population. The B-cell function is regulated by the Th-cells and their cytokines. Deregulation of Th-cells and the cytokines influences normal antibody responses to infection (De Boer, 1994). In addition, depletion of granulocytes populations due to destruction of precursor cells or impairment of normal function in response to the infection as a result of disruption of cytokine systems collectively leads to the immunosuppression and consequently increases the susceptibility to secondary microbial infection.

Co-infection of CAV with other viruses has been demonstrated to produce synergistic effects on the pathogenicity of either virus alone. An experimental infection of MDV-1 and CAV in specific pathogen free (SPF) chickens resulted in an increase in mortality (Jeurissen and de Boer, 1993; Miles *et al.*, 2001). However, the pathogenicity depended on the virulence of the MDV strain (Miles *et al.*, 2001) or the challenge dose of the MDV (Jeurissen and de Boer, 1993). An increased morbidity and mortality as the consequence of dual infection of CAV with infectious bursal disease virus, reticuloendothelial virus, adenovirus or reovirus has been described (von Bulow, 1991). In addition, CAV infections have often been linked with poor vaccine-induced protection to Marek's disease (Otaki *et al.*, 1988), infectious laryngotracheitis and Newcastle disease (Cloud *et al.*, 1992).

2.3.4 Impact of chicken anaemia virus on the broiler Industry

Although the CAV has been recognised for more than two decades, the exact monetary impact on the poultry industry has not been fully estimated. Recent studies suggest that few if any commercial broiler flocks are free of this virus. A study on the experimental infection of CAV resulted in weight loss and increased mortality in broilers. Subclinical infection in broiler has been reported to increase the mortality and condemnations of birds at the time of slaughter which potentially reduces performance and profitability (McIlroy *et al.*, 1992).

A study of broiler flocks in Northern Ireland reported that the feed conversion ratio, the average weight per bird and the net income per 1000 birds were 2%, 2.5%, and 13% respectively lower in the flocks which were CAV antibody positive (McNulty *et al.*, 1991). In addition, CAV infection is hard to prevent in SPF flocks as well as commercial poultry units. Seroconversion after the onset of egg laying is a big challenge for the SPF industry producing eggs to be used for vaccine production, especially human vaccines like mumps and measles and the poultry vaccines to be used in birds less than three weeks of age (Miller and Schat, 2004).

2.3.5 Molecular characterisation

A search of GenBank data {Basic Local Alignment Search Tool (BLAST) 2.2.12 [Aug-07-2005]} confirms a total of 110 nucleotide sequences of CAV. Of the 110 published sequences, 38 nucleotide sequences of CAV are of full length or near full length genome. Regarding Australian isolates, two full length sequences (Hamooleh, 1996; Brown *et al.*, 2000) and a partial sequence of VP1 (Pallister *et al.*, 1994) are published to date. The genome of CAV is reported to be 2298 bp long with four repeats or 2319 bp with five repeats in the promoter/enhancer region. Most of the wild type CAV genome is reported to have four repeats. It is proposed that the longer genome with five repeats detected in the Cux-1 (N) and 82-2 strains may have been due to the extensive cell culture passage (Noteborn *et al.*, 1991; Kato *et al.*, 1995).

The CAV genome organization so far published suggested that the nucleotide sequence of the different strains of CAV appears to be relatively conserved.

However, the VP1 gene incorporates a hypervariable region from amino acid positions 139 to 151. The amino acid changes in this region might influence the rate of viral replication and the spread of CAV strains in cell culture (Renshaw *et al.*, 1996). However, they failed to demonstrate a relationship between pathogenicity of the strains with differences in amino acid composition at these positions. Some molecularly cloned strains having the amino acid residue H instead of Q at position 394 of VP1derived from a low passage CAV pool (AH9410) were less pathogenic (Yamaguchi *et al.*, 2001). The VP3 protein is reported to be a more conserved protein. However, an amino acid substitution at position 118 of VP3 can influence the nuclear localization of the protein and the development of distinct apoptotic bodies in cell culture (Renshaw *et al.*, 1996).

The amino acid comparison of 13 CAV sequences of VP1 and 10 complete sequences of VP2 and VP3 resulted with the maximal diversity of 4%, 1.4% and 2.3% of VP1, VP2 and VP3 respectively (Islam *et al.*, 2002). Comparable results have been demonstrated from the sequence analysis of 14 CAV strains in Alabama (van Santen *et al.*, 2001). Recently, molecular characterisation of CAV on the basis of the VP1 gene in Nigerian poultry flocks describes a 4.4% diversity at the nucleotide level (Ducatez *et al.*, 2005).

2.3.6 Molecular Epidemiology

The amino acid alignment and phylogenetic analysis of recently published CAV sequence data suggests three major groups of CAV. The phylogenetic tree constructed by aligning the 58 VP1 sequences demonstrated three clusters. The group one (I) included only two Australian isolates. A Malaysian isolate, a Japanese isolate, three other isolates, Nigerian isolates and an Australian isolate were included in group two (II). Group three was further subdivided into IIIa and IIIb. Group IIIa included CIA-1 from the USA, BD-3 from Bangladesh, and Nigerian isolates. Group IIIb included Malaysian isolates, Cux-1-M, Cux-1-N, Nobilis vaccine, Chinese isolates, Nigerian isolates etc. The inter group divergence was reported to be 1.4 to 3.4% (group I-II), 1.2 to 2.5% (group I-III) and 1.8 to 4.1% (group II- III) and the intra- group divergence were 2.9, 1.6, and 2.9% for group I, II and III respectively (Ducatez *et al.*, 2005). However, full length sequence evolutionary analysis

described no obvious grouping among the CAV strains (Brown *et al.*, 2000) except the Australian strain CAU-269/7 that formed a separate group away from the all other CAV strains (Islam *et al.*, 2002).

2.3.7 Diagnosis

Clinical manifestations in the infected birds can lead to a provisional diagnosis. For conformation of the disease, appropriate laboratory procedures are required. The confirmatory laboratory diagnosis could be viral isolation, the demonstration of virus-specific antigen or nucleic acid or serological detection of anti-CAV antibodies or PCR for the detection of viral DNA.

2.3.7.1 Viral isolation and identification

Chicken anaemia virus can be isolated from nearly all infected tissues. In experimental infection in day old chickens, CAV was constantly detected in all the organs including brain up to four weeks post-inoculation. The brain and the rectal content examination at 7 weeks post-inoculation were found to be positive for CAV. However, following inoculation of CAV in older birds (six to seven weeks), maximal viral titre was detected on seven days after the infection in most of the infected tissues except brain or serum and titres rapidly decreased. Specific antibody was detected three weeks post-infection in the bird inoculated at one day of age and at seven days after inoculation in the older chicks (Yuasa *et al.*, 1983).

In vitro propagation was difficult when the virus was identified as an agent inducing anaemia in chickens (Yuasa *et al.*, 1979). Initially day old SPF chickens were used for the propagation of CAV. After four years of detection it became possible to grow the virus in two cell lines, MDCC-MSB1 and MDCC-JP2 and one avian leukosis virus transformed cell line, LSCC-1104X5 (Yuasa, 1983). This discovery facilitated further studies of this elusive virus.

Further studies to support viral propagation were carried out to find the susceptibility of Marek's disease cell lines to CAV suggested that other Marek's disease transformed lymphoblastoid cell lines, MDCC-CU 22 and MDCC-CU147 and a

reticuloendotheliosis virus transformed T-cell line, RECC-CU205 also favoured the replication of one or more strains of CAV. However, MDCC-CU147 is possibly the most susceptible cells line and may produce 10 to 100 fold more virus than MSB1 cell lines (Calnek *et al.*, 2000).

In-ovo propagation of CAV has also been described and suggested that the yield of the virus is relatively moderate after 14 days of virus inoculation (von Bulow, 1991).

For the identification of the virus, there is no CAV-specific cytopathology that could be identified by light microscopy. Therefore, after isolation, identification of CAV has to be verified by either serological investigation (von Bulow, 1991) or molecular procedures.

Viral isolation in specific-pathogen-free chicks (SPF-chicks) and in MDCC-MSB1 is relatively time-consuming and laborious. If viral isolation is not necessary, dot blot hybridisation for the detection CAV-specific DNA (Todd *et al.*, 1991) and *in situ* hybridisation for the detection of CAV in peripheral blood smears (Allan *et al.*, 1993; McMahon and McQuaid, 1996; Sander *et al.*, 1997; Novak and Ragland, 2001) were used prior to the development of PCR.

2.3.7.2 Serology

For the detection of antibody status against CAV, serum neutralisation (SN), indirect immunofluorescent antibody test (IFAT) and ELISA have been successfully used up to now.

Indirect immunofluorescence was the method used for serological examination of CAV by Yuasa *et al* in 1983. Literature indicates potentially conflicting statements regarding the sensitivity and specificity of SNT and IFAT. In comparative studies, SNT was shown to be more sensitive and specific than IFAT for the serological studies of CAV (von Bulow, 1988) but no difference was found when serum dilution was standardised at a different concentration than that used in the first study (McNulty *et al.*, 1988).

To date a few reports regarding the use of ELISA for the detection of serum CAV-antibody are published. Serological studies of 388 sera from SPF and commercial flocks from different countries including Australia were investigated for CAV-specific antibody and using two methods, ELISA and indirect immunofluorescence. Comparable results with 98.5% agreement were found between the two assays but ELISA was simple, easy and superior for the large scale testing (Todd *et al.*, 1990). Later an indirect ELISA was developed and found being better than the IFAT (Otaki *et al.*, 1991). More recently, blocking ELISA (Todd *et al.*, 1999) and a modified blocking ELISA (Tannock *et al.*, 2003) was developed and reported to be a superior technique

An attempt was made to produce CAV protein in a baculovirus vector system and the CAV proteins were tested individually as ELISA antigens for the detection of CAV-specific antibody. The recombinant VP1 protein failed to react with CAV-infected serum in ELISA whereas the two other recombinant proteins, VP2 and VP3, reacted (Iwata *et al.*, 1998). The explanation for the unexpected results of PV1 could possibly be that the antigenic sites were masked by the impurities in the cell lysate system.

Real-time quantitative PCR (qPCR)-based serum neutralisation test for the detection and titration of the neutralising antibodies to CAV is the latest concept in the serological examination of CAV. Comparisons of the conventional assays based on passaging infected cells, the commercial blocking ELISA and the qPCR based serum neutralisation were carried out. The test found that the qPCR-based assay was rapid and equally sensitive for the detection of CAV-specific antibody compared with the conventional viral neutralisation test and more sensitive for the detection of low titre CAV antibodies than was the commercial blocking ELISA (van Santen *et al.*, 2004b). However, qPCR-based assay for the detection of CAV-specific antibody is expensive because of the cost associated with DNA purification. This assay may not be suitable for regular flock monitoring but it could be the method of choice where low antibody titres need to be detected in research or/and for monitoring SPF birds for CAV infection.

2.3.7.3 Molecular diagnostic methods

Polymerase chain reaction is the technique used to amplify and detect viral genomes. It has been demonstrated that the PCR is the most sensitive and reliable technique for the diagnosis of CAV infection. If viral isolation is not required, PCR is the most preferred assay for the detection of CAV. One CAV infected cell or $10^{1.5}$ tissue cytotoxic infective dose ($TCIC_{50}$) of cell free virus (Imai *et al.*, 1998), one femtogram (fg) of CAV replicative form of DNA (Tham and Stanislawek, 1992) or as little as 0.1 fg of target DNA sequence could be detected by standard PCR. The nested PCR is 10 to 100-fold more sensitive than the standard PCR (Imai *et al.*, 1998).

Despite the exquisite sensitivity of the method, nested PCR does not estimate the number of DNA copies in the template. A competitive PCR for detection of CAV-specific DNA was reported (Yamaguchi *et al.*, 2000; Miles *et al.*, 2001) but it is only semi-quantitative. More recently, real-time quantitative PCR using TaqMan technology had similar sensitivity to nested PCR. It has the potential to discriminate between different strains of CAV when strain specific primers were used and is quantitative (Markowski-Grimsrud *et al.*, 2002).

2.3.8 Control measures

So far, vaccination has been suggested to be protective against the clinical chicken infectious anaemia in the poultry industry. After natural infection or experimental inoculation of CAV in the chickens or vaccination, CAV-specific antibody is produced depending on the age of chicken as described earlier. The antibody titre against the CAV is an important parameter in the breeding flocks and SPF flocks. Newly hatched chicks are protected from the clinical disease if antibody against CAV is transmitted to offspring. It has been reported that serum samples from SPF birds maintained in high-biosecurity, filtered-air, positive-pressure (FAPP) houses were positive for CAV antibodies highlighting the difficulty in maintaining freedom from this disease (Takase *et al.*, 2000).

Vaccines against CAV are commercially available. Vaccination of the breeder flocks between 13-15 weeks of age produce protective immunity against CAV to their offspring. Breeding flocks can be monitored for the neutralising antibody against CAV prior to onset of lay and can be vaccinated before fifteen weeks of age if breeding birds are negative in serological screening (Fussell, 1998).

The vaccines so far produced and in use are live vaccines. They can not be administered to birds less than three weeks of age as they are essentially fully virulent viruses (Miller and Schat, 2004). For the development of optimal immunity against CAV, simultanious infection with other immunosuppressive diseases like, IBDV or MDV infections may play an important role. Therefore, development of new CAV vaccines which can be safely used on young chicks deserves attention.

2.3 Conclusion

In conclusion, after reviewing literatures regarding Marek's disease virus and chicken anaemia virus, it is clear that MDV is highly contagious and produces a complex pathogenesis. Marek's disease virus causes neurological disorders; induces tumours and immunosuppression in infected birds. Hemocytoblasts in bone marrow and immature lymphocytes in the thymus are the main targes of CAV infection and infection subsequently results in anaemia and immunosuppression.

Vaccination is the only option to protect the poultry industry from MD. However, periodical sporadic disease outbreaks in the vaccinated flock are a major challenge. Several factors can be associated with vaccination failure and concurrent infection with CAV is one of the most important reasons. The serotype two MDV, a non-pathogenic virus has been used as a vaccine for the protection against MDV-1.

Both CAV and MDV2 have a worldwide distribution producing potential interactions with MDV-1. However, the exact mechanisms and quantification of the cumulative effects of either of these viruses has not been clearly elucidated.

CHAPTER 3
GENERAL MATERIALS AND METHODS

3.1 Cell culture and propagation of chicken anaemia virus

3.1.1 Cell line for viral propagation

The Marek's disease transformed chicken lymphoblastoid cell line MDCC-MSB1 (Akiyama and Kato, 1974) was used for the propagation of chicken anaemia virus. The cell line was obtained from TropBio, JCU. The media used was RPMI-1640 (TropBio, JCU) with variable levels, 2-10% of foetal bovine serum (FBS). The MSB1 cells were stored in liquid nitrogen. Cryovials containing approximately 1.2×10^6 MSB1 cells at the 19^{th} passage level in RPMI-1640 with 10% FBS and 10% dimethyl sulphoxide (DMSO) that were frozen overnight at -80°C and transferred into liquid nitrogen for long-term storage.

The cryovial of MSB1 cells was taken out of the liquid nitrogen and placed into a water bath at 37°C and then transferred into 10 ml of pre-warmed RPMI-1640 with 10% FBS at 37°C. The cells were transferred to a 25 square centimetre cell culture flask with a filtered lid and incubated at 37°C in a 5% CO_2 atmosphere. The flask was observed under an inverted microscope and after approximately 72 hours two thirds of the cells were transferred into a 75 sq. cm culture flask containing approximately 25 ml of RPMI-1640 with 10% FBS RPMI-1640 with 2% FBS being subsequently used as a maintenance media.

3.1.2 Viral propagation in MSB1 cells

Viruses were inoculated into MSB1 cells which were monitored for the presence of viral CPE. Infected cells were passaged every two to three days. Supernatants and cell culture suspensions were collected for further testing. The viruses were passaged up to the tenth passage level. The presence of viral CPE was compared with the control MSB1 cell culture flask.

Supernatants and cell suspensions were stored at -80°C. The control MSB1 cells and some of the MSB1 cells infected with virus were also stored in liquid nitrogen.

3.2 Viruses and its source used in this project

3.2.1 CAV Reference viruses

Two reference viruses were used in this study. A vaccine virus designated CAV strain 3711 and a wild type virus designated BF4 PBY F1 summarised in Table 3.1.

The vaccine virus 3711 was kindly supplied by both the Royal Melbourne Institute of Technology (RMIT) and Intervet Australia. The isolate 3711 was originally isolated from liver and/or bone marrow of SPF bird after inoculation of liver suspension from 18-day-old Queensland broiler breeder with typical CAV infection and further propagation was carried out in MSB1 cell line. The cytopathic effect was observed after 10 sub cultures. The virus was propagated in MSB1 cell lines as described (Chapter 3.1.2).

The wild type virus BF4 was used in a previous study at JCU (Spence, 1996) and supplied by Mr. Rick McCoy of CSIRO Parkville, Victoria. The CAV strain 3711 DNA and Cux DNA was kindly supplied by the RMIT. It was used as positive control in the process of PCR optimisation and viral replication monitoring.

3.2.2 CAV field samples

A total of 310 feather samples from 22 broiler flocks throughout Australia were investigated for presence of CAV by nested PCR. The CAV positive samples were stored at -20°C and used as the source of the virus for molecular sequencing of the field strain.

Table 3.1 Chicken anaemia virus strains used for this project and its source

S.N.	Description	Source
1	CAV Australian strain, BF4 PBY F1	TropBio-JCU
2	CAV strain 3711 DNA	RMIT University
3	CAV vaccine strain 3711	Intervet, Australia and RMIT University
4	CAV field isolates	Detected from field sample investigation

3.2.3 CAV plasmid, pBluescript

The CAV plasmid DNA pBluescript of CIA Δ ORF3, pBCux A and pBCux B was kindly supplied from the Cornell University, USA, summarised in Table 3.2. These plasmid DNAs were dried and stored as pellet. The CIA Δ ORF3 had been mutated in the start codon of ORF3. The pBCux A contained the *Eco*R1-BamH1 fragment of CAV and pBCux B contained the *Bam*HI-EcosR1 fragments.

Table 3.2 CAV Plasmid, pBluescript

S. N. Description	Source
1. CIA Δ ORF3	Cornell University, USA
2. pBCux A	Cornell University, USA
3. pBCux B	Cornell University, USA

3.3 MDV-2 virus strain

For investigation of MDV-2 from the feather samples, known virus was required as control virus. Maravac vaccine strain of MDV-2 was used as control. The cell-associated vaccine virus was obtained from Fort Dodge, Australia. The viral DNA was extracted by Mr. Ramon Layton (JCU) using the High Pure Template Preparation Kit (Roche Diagnostics, USA), according to the manufacture's protocol and was stored at -20°C.

3.4 Oligonucleotide primers used for detection of viruses

3.4.1 Primers used for chicken anaemia virus detection

Two sets of nested primers, Cux (Outside: O3Fand O3R, inside: N3, and N4) and SH-1(Outside: SH-1F and SH-1R, Inside: SH-1 N3 and SH-1N5) (Cardona *et al.*, 2000b) were used for the detection of CAV. The primer sequences, size, length of amplicons, source are summarised in table 3.3. The CAV genomic structure and location of these two nested primers are presented in Appendix 2 Figure 3 A and B.

Table 3.3 Primers used in PCR assay to detect CAV.

Primer name		Sequence (5'-3')	Size (bp)	Position	Amplicon (bp)	References
Cux standard	O3F	CAAGTAATTTCAAATGAACG	20	452 – 471	387	(Cardona *et al.*, 2000b)
	O3R	TTGCCATCTTACAGTCTTAT	20	819 – 838		
Cux nested	N3	CCACCCGGACCATCAAC	17	489 - 505	210	(Cardona *et al.*, 2000b)
	N4	GGTCCTCAAGTCCGGCACATTC	22	677 – 698		
SH-1 standard	SH-1F	CGAACCGCAAGAAGGTGTAT	20	801 - 820	602	(Cardona *et al.*, 2000b)
	SH-1R	GCAGGGTCATTTGTTTAGGG	20	1383 - 1402		
SH-1 nested	SH-N5'	GCGGTATCGTAGACGAGCTT	20	945-964	205	(Cardona *et al.*, 2000b)
	SH-N3"	AGAGATCTTGGCGACTCTCG	20	1130-1149		

The primer sequence consensus with the published nucleotide sequences of two Australian isolates of CAV were established (Appendix 2 Figure 1). Representative CAV sequences were imported from GenBank. Alignments were carried out and primer locations determined using the software GeneDoc.exe (Nicholas and Nicholas. Jrn., 1997).

Searches were carried out using the BLAST to determine the specificity with which the primers were likely to detect CAV sequences. The Cux O3F primer had complete homology (20 bases) with 67 published sequences of chicken anaemia virus. A few17 and 16 base alignments and two 16 base interspecies sequence alignments were observed. Similarly, the O3R primer had perfect alignment (20 bases) with 33 published sequences of CAV, and a limited number of 18 and 16 base interspecies sequence alignments.

The nested primer, N3, had absolute alignment (17 bases) with 47 published sequences of CAV and a few interspecies sequences alignment (16 bases). Likewise, the primer Cux N4 had complete homology with 72 published sequences of CAV and there were no interspecies sequence alignments.

The primer, SH-1F had complete homology with 67 CAV published sequences, 16 bases aligned with 15 CAV sequences and there were a few 16 base interspecies sequence alignments. Primer, SH-1R had perfect alignment (20 bases) with 15 CAV sequences, partial alignment with 17 CAV sequences and few partial alignment with interspecies sequences including human DNA.

The nested inside primer, SH-1N5 had absolute alignment with 89 and one partial alignment with CAV and one partial alignment with a 32 base segment of mammalian orthoreovirus. The primer, SH-1 N3 had absolute alignment with 54 and partial alignments with 30 CAV sequences and six partial interspecies sequence alignments including human DNA.

3.4.2 Primers used for detection of MDV-2

A nested primer set was used for the detection of MDV-2. The outside primer pair were MDV-2 S1FO and S1RO and the inside primer pair were S1FI and S1RI (Burgess and Hartini, 2003). Further description of these primers is summarised in Table 3.4.

Table 3.4 Description of MDV-2 primers

Primer name	Sequence	Size	Product size	gene	References
S1FO MDV2	TGCGTGCGATTAGTGT	16	619	Repeat region	(Burgess and Hartini, 2003)
S1RO MDV2	TCGCAGGAAAGTAAGTTC	18	619	Repeat region	
S1FI MDV2	GCACGCGCATCATAAG	16	304	Repeat region	
S1RI MDV2	TCCTGATTCCGTAGCACT	18	304	Repeat region	

The primer sequence homology with the published nucleotide sequences of MDV-2 were established (Appendix 2 Figure 2) as described above. The outside primers,

S1FO and S1RO had absolute consensus with two published sequences of *Gallid herpesvirus* type 3 nuclear DNA binding protein gene at an inverted repeat region. Similarly, the inside primer set of MDV-2, S1FI and S1RI also had perfect consensus with two published sequences as did the outside primer set detecting the same genomic region of the viral DNA.

3.4.3 Primers used for the detection of MDV-1

A semi nested PCR was used by Mr Ray Layton for the detection of MDV-1. Primers used were: 25 MDV1-R, 24 MDV1-F and 41 MDV1-F (Zhu *et al.*, 1992). The primers 41 MDV1-F and 25 MDV1-R were the outside primer pair and 24 MDV1-F and 25 MDV1-R were the inside primer pair. Further descriptions of these primers are summarised in Table 3.5.

Table 3.5 Description of MDV-1 primers

Primer name	Sequence	Size	Product size	gene	References
41 MDV1-F	CGGGCAAGAACGCATACATCC	21	650	gC	
25 MDV1-R	TGTTTCCATTCTGTCTCCAAGA	22	650/201	gC	Zhu *et al.* , 1992)
24 MDV1-F	CATGCAAGTCATTATGCGTGA	21	201	gC	

3.5 Viral DNA extraction

Viral DNA extraction from cell culture was carried out using the High Pure Template Preparation Kit (Roche Diagnostics, USA), according to the manufacture's instructions. The concentration of extracted DNA was estimated (Chapter 3.5) and it was stored at -20ºC. The working DNA solution was prepared with 2 ng ml^{-1} concentration and was stored at 4ºC.

For regular monitoring of the viral replication and propagation in culture, DNA was extracted by heating the supernatant at 100ºC in a heat block heater for approximately five minutes.

3.6 Estimation of DNA concentration and its purity

The purified DNA was then quantified using either a UV Mini 1240 UV-UVS spectrophotometer (Shimadzu) or a BioPhotometer 8.5 mm (Eppendorf, Hamburg, Germany), with a 10 mm optical path length and a sterile disposable Eppendorf UVette®, Eppendorf biophotomet 8.5 mm light centre height (Eppendorf Hamburg, Germany). The absorbance reading was recorded at wavelengths of 260 nm and 280 nm. The elution buffer was first quantitated as blank and then the reading of the extracted DNA was measured and recorded. The absorbance reading at 260 nm provides the quantity of DNA in nano grams per micro litre (ng μl^{-1}). The ratio of the absorbance at the wavelength of 260 nm and 280 nm is an indicator of the purity of the extracted DNA. A ratio between 1.8 and 2 is an indicative of the highly pure DNA samples.

3.7 Published sequences used to compile a CAV database

Sequences published as GenBank files were downloaded into Vector NTI (Invitrogen) and stored in the database as circular molecules. As several of these files especially the earlier submissions have a variety of starting coordinates the most recent convention of using the *Eco*R1 site or the sequence that aligned with that point as the starting coordinate was adopted. All sequences were numbered from that point.

Aligned sequences were exported as msf files or in FASTA format for further manipulation.

CHAPTER 4

OPTIMISATION AND ADOPTION OF POLYMERASE CHAIN REACTION AND DETERMINATION OF RELATIVE SENSITIVITY OF TWO CAV STRAIN-SPECIFIC PRIMER PAIRS AGAINST STRAIN BF4

4.1 Optimisation and adoption of the polymerase chain reaction

4.1.1 Introduction

The polymerase chain reaction has become a widely used tool for the detection, identification as well as differentiation of pathogens during diagnosis and investigation of human and animal disease. This project aimed to detect and characterise avian viruses; CAV and MDV-2, using PCR as the main method of viral investigation. The oligonucleotide primers were partially selected from the published articles and partially designed according to the requirements. Although some of the oligonucleotide primers used in this project was already published, optimisation and adoption of the methods were required in order to achieve optimum DNA amplification parameters from the equipment available. This also provided an opportunity to become acquainted with the procedures. The primers specially designed for this project needed thorough optimisation.

The magnesium ion (Mg^{+2}) concentration, deoxynucleotide 5'-triphosphates (dNTP), DNA template, enzyme and primers concentrations, as well as the DNA amplification parameters (primer annealing/extension temperature and time, template denaturation temperature and time and, cycle numbers) are the important factors for a successful PCR (Landre, 1995). In this study annealing temperature and Mg^{+2} concentrations were optimised at first for all the primers used in this project.

4.1.2 Materials and methods

4.1.2.1 CAV template for standardisation

Purified DNA prepared from a Cux isolate of CAV as well as the vaccine strain 7311 was generously supplied by Professor Greg Tannock, RMIT, Melbourne. This purified DNA was used to standardise both standard and nested PCRs for CAV.

4.1.2.2 Standard PCR for CAV

All the primers used for the detection of virus as well as sequencing the CAV were optimised for the appropriate Mg^{+2} concentrations and the annealing temperature. Optimisation of $MgCl_2$ concentration was performed at 1.5 mM, 2 mM and 2.5 mM concentrations. Annealing temperature was optimised considering the melting temperature (Tm) of the each primer and applying temperature gradients of 5-10ºC.

Other reagents and primers concentrations used were as follows:

Reagents	**Working concentration**
Molecular grade water	as per reaction volume
Reaction buffer	1 ×
Magnesium Chloride	1.5 mM, 2.0 mM, and 2.5 mM
dNTPs	200 µM
Forward primers	200 µM
Reverse primer	200 µM
Taq Polymerase	0.02 $Units^{-\mu l}$

The PCR reaction volume was 25 µl including template l µl (approximately 1 ng of the DNA) per reaction. Templates used for optimisation were the CAV DNA from RMIT, CAV vaccine strain 3711, CAV reference virus BF4 or DNA from the sample material. The primers, O3Fand O3R and SH-1Fand SH-1R that were optimised are listed in the general materials and methods (Chapter 3.4.1).

The DNA amplification especially for the annealing temperature optimisation was carried out in a Mastercycler ® gradient cycler (Eppendorf Hamburg, Germany). It

had 96 wells, eight rows and twelve columns. Temperature could be fixed at the centre with a nominated gradient on each side. e.g. an annealing temperature of 50°C with a 10°C gradient resulted in an annealing temperature ranging from 40°C on one side of the block to 60°C on the opposite side. for example DNA amplification was carried out with denaturing at 94ºC for 5 minutes followed by 35 cycles of denaturing at 94ºC for 1 minute, annealing at 45-60ºC with 5-10ºC gradients for 1-2 minutes, elongation at 72ºC for 1-2 minutes and a final elongation at 72ºC for 10 minutes.

4.1.2.3 Agarose gel electrophoresis

Electrophoresis of the PCR product was carried out in 1.3-1.8 % w/v molecular biology grade agarose gels (Progen Industries, Australia) in 1× Tris acetate EDTA (TAE) buffer pH 8.0 (Appendix 1.2.2) at different ranges of volts for forty minutes to two hours. Ethidium bromide was dispensed using a dropper bottle cat# 5450 (CPL Continental Lab Production, San Diego, CA) at the rate of 0.5 μg ml^{-1} of agarose gel. The PCR product was mixed with 20% of the 6 × loading dye solution # R0611 (Fermentas Life Sciences, Lithuania) and loaded into a gel. The markers used were GeneRuler™ 100 bp DNA Ladder Plus # SM 0321 or GeneRuler™ 100 bp DNA Ladder # SM 0241 or GeneRuler™ 1kb DNA Ladder # SM 0311 (Fermentas Life Sciences, Lithuania). Image visualisation was achieved by placing the gel into a Gel Doc 1000 (BIO-RAD) transilluminator under ultraviolet rays and the image then, captured and visualised using computer software GeneSnap (SynGene, Synoptics Ltd. Cambridge, England). Estimation of the product size was achieved by matching the sample image with the pattern of the marker.

4.1.2.4 Nested PCR for CAV

The standard PCR was carried out keeping all reagent concentrations including template and the DNA amplification parameters as optimised. For the nested reaction, the PCR master mix was prepared by adding all the reagents and primer concentrations as optimised for the standard reactions. One microliter of the first round PCR product was added as template into the respective tubes as marked in the second round reaction. Primers used for the nested reaction were Cux N3 and N4 and

SH-1N5 and SH-1N3 (Chapter 3.4.1). Thermal cycling parameters, agarose gel electrophoresis and the image visualisation were carried out as described previously for the standard reactions.

In the process of cloning and sequencing of CAV, additional primers were designed. Optimisation of annealing temperature was carried out for each of the primer pair. In addition, dimethyl sulfoxide (DMSO) at the rate of 5%, as co-solvent had been added for the amplification of most difficult segment of CAV genome, with high percentage of G+C in the repeat region.

4.1.2.5 Adoption of PCR for the detection of MDV

The primer sets used for the investigation were S1FO MDV2, S1RO MDV2, S1FI MDV2, S1RI MDV2 (Chapter 3.4.2.); serotype 1 specific oligonucleotide primers were designated 41, 25, 24, F132, and R132 listed in Table (Chapter 3.4.3.). The MDV-1 assays were carried out as part of another project by Mr Ray Layton and results were generously supplied to complement the results of the present project. Adoption of the previously optimised parameters was carried out for standard as well as nested and semi nested PCR reactions.

All the reagents and primer concentrations for the MDV-2 PCR were similar to that described for CAV. The total reaction volume was 25 µl including 1 µl of template. The control viral DNA used was the Maravac vaccine strain in case of MDV-2 (Chapter 3.3.).

4.1.2.6 Sensitivity of the nested PCR for MDV-2

In order to determine the sensitivity of the MDV-2 assay, serial tenfold dilutions of the template DNA were prepared and used as template for the first round of the PCR. An aliquot of l µl of the first round PCR product was used as template in the respective reactions for the second round PCR. Other reagents, primer concentrations as well as DNA amplification parameters were maintained same as first round PCR.

4.1.2.7 Electrophoresis

The electrophoresis of the PCR product was carried out as described above in case of CAV using an agarose gel concentration of 1.5% w/v.

4.1.3 Results

The results demonstrated that all the reagents concentrations and the DNA amplification parameters optimised for the standard PCR were also optimal for the nested reaction for both sets of Cux and SH-1 primers. The same parameters were applicable to the standard as well as for the nested PCR for MDV-2.

4.1.3.1 Standard PCR for CAV

The images of the CAV PCR products after electrophoresis demonstrated that 2 mM and 2.5 mM Mg^{+2} concentrations were appropriate to produce specific PCR products (Figures 4.1 and 4.2). All of the bands appeared to be equally bright indicating that there was no substantial difference between annealing temperatures ranging from 40-60ºC. The DNA bands that were produced using a 1.5 mM concentration of Mg^{+2} and an annealing temperature of 48.3°C were relatively bright. The rest of the bands were faint.

The images with 1.5 mM concentrations revealed that only the annealing temperature of 48.3ºC produced a bright DNA band and the rest of the bands were very faint. A negligible amount of non specific DNA was observed in all three level of Mg^{+2} concentration and gradients of temperature.

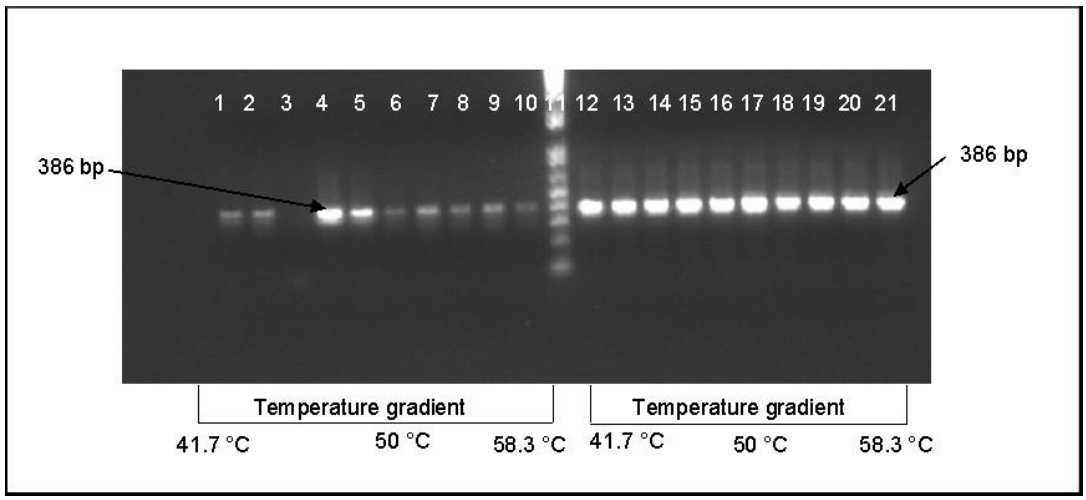


Figure 4.1 Agarose gel electrophoresis of CAV PCR products
Annealing temperature gradient programmed PCR assay with different temperature in each well,
Primer: CAV O3F and O3R, template: CAV DNA of strain 3711,
Lane 1-10: 1.5 mM Mg^{+2} concentrations, Lane 12-21: 2.0 mM Mg^{+2} concentrations,
Lane 11: Marker, 100 bp DNA ladder plus.

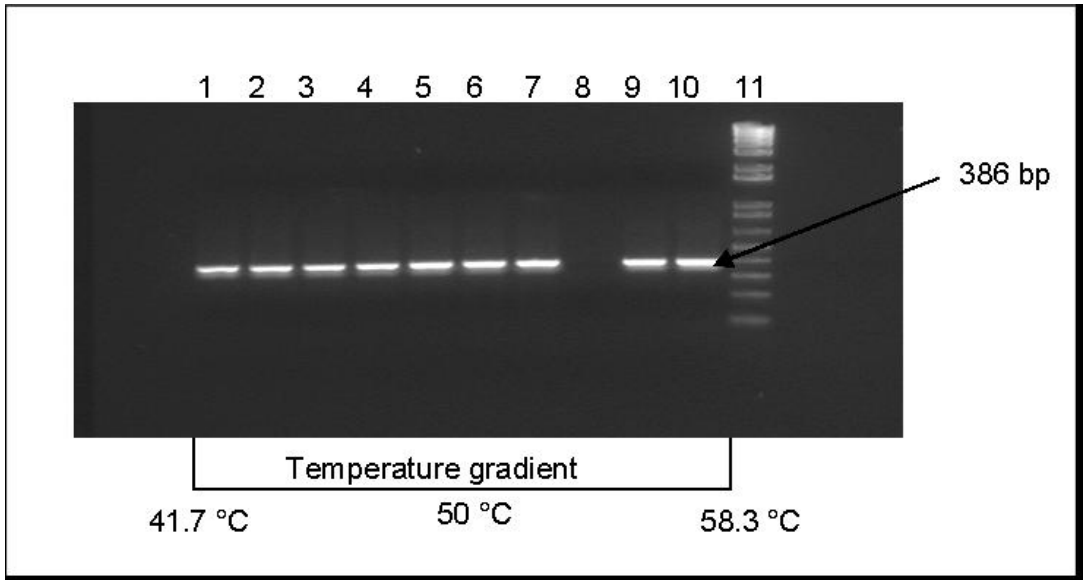


Figure 4.2 Agarose gel electrophoresis of CAV PCR products
Annealing temperature gradient programmed PCR assay with different temperature in each, well Primer: CAV O3F and O3R, template: CAV DNA of strain 3711,
Lane 1-10: 2.5 mM Mg^{+2} concentrations, Lane 11: Marker, 100 bp DNA ladder plus,
In the lane eight, there was no template due to an error in template loading

4.1.3.2 Nested PCR for CAV

Electrophoresis and image visualisation of the PCR products both standard and the nested reaction demonstrated bright DNA bands (Figure 4.3, A and B). A negligible amount of non-specific product was observed in images of standard reaction products and more non-specific DNA was observed in the nested reactions. Negative controls were as expected.

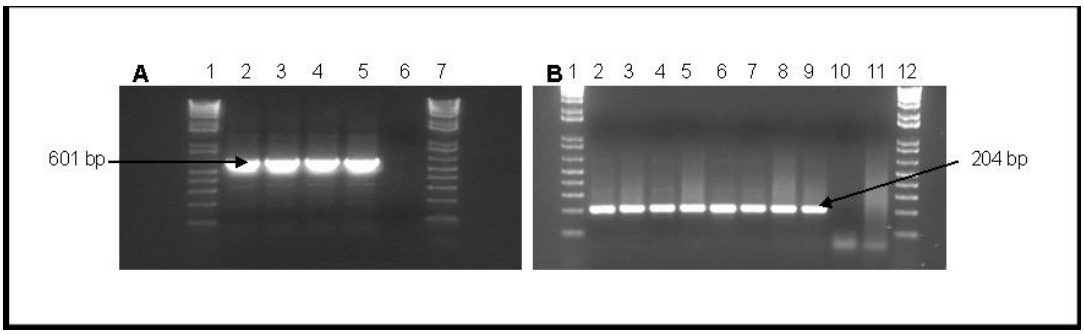


Figure 4.3 Electrophoresis of nested CAV PCR products
Set A:
Product from first round PCR reaction, Template: CAV strain BF4; supernatant collected from the cell culture and DNA extracted by boiling. Primer: CAV SH-1F and SH-1R.
Lane 2-5: product from first round reaction, Lane 6: negative control, Lane 1 and 7: marker, 100 bp DNA ladder plus.
Set B: products from nested PCR assay. Template: 1 µl PCR product from the first reaction,
Primer: SH-1 N3 and N4 (inside primer set).
Lane 2-11: Product from reactions with template of set A. Two reactions from the each outside product were used as template in respective sequential order.
Lane 1 and 12: marker, 100 bp DNA ladder plus.

Annealing temperature optimisation of the VP1 Tail primer set (Figure 4.4) demonstrated clear DNA bands in all the lanes. The ratio between specific products and non specific products with the change in annealing temperature had changed significantly. At low annealing temperatures, the specific to non-specific product ratio was minimum and increased gradually and highest in the fifth reaction onwards.

The non-specific products were gradually decreasing and almost negligible at the highest temperature. However, the specific PCR products were also gradually decreasing from the seventh reaction onwards which indicates that the maximum specific PCR amplification could be achieved within a limited range of annealing temperatures.

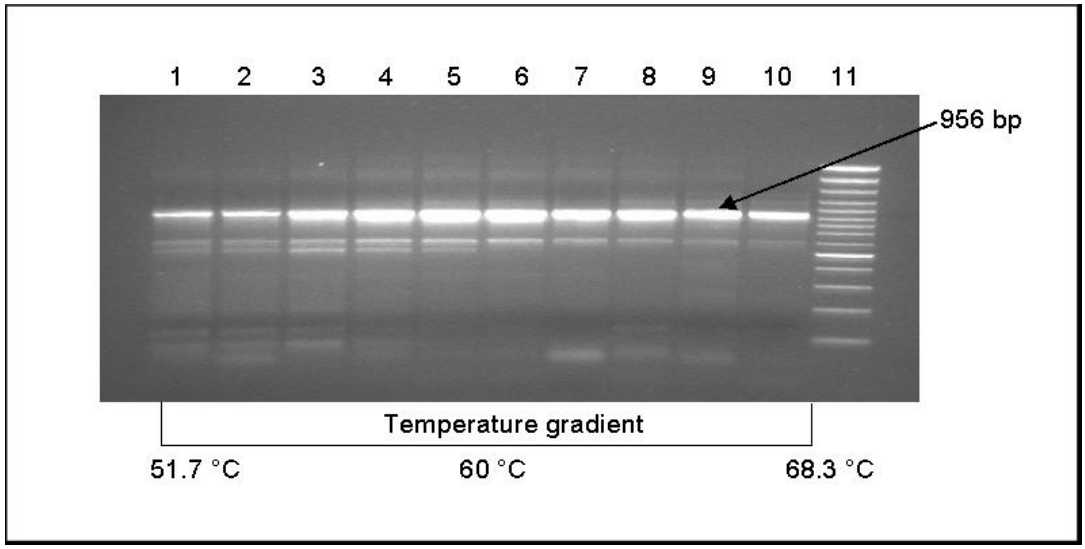


Figure 4.4 Electrophoresis of CAV PCR products
Annealing temperature optimisation, annealing temperature: 60°C with gradient 10°C,
Template: CAV vaccine strain 3711(1 µl in each reaction),
Primers: VP1 Tail set, Images: lane 1-10 PCR products at different annealing temperatures, from 51.7 to 68.3°C and lane 11: marker, 100 bp DNA ladder, Co-solvent: 5% DMSO.

4.1.3 Standard and nested PCR for MDV-2

In the case of MDV-2, DNA bands in both standard and nested PCR were bright at the annealing temperature 60ºC and the Mg^{+2} at the 2.5 mM concentration (Figure 4.5). No non-specific images were observed with standard PCR whereas in the nested reactions, acceptable amounts of non intended DNA were detected above the intended products.

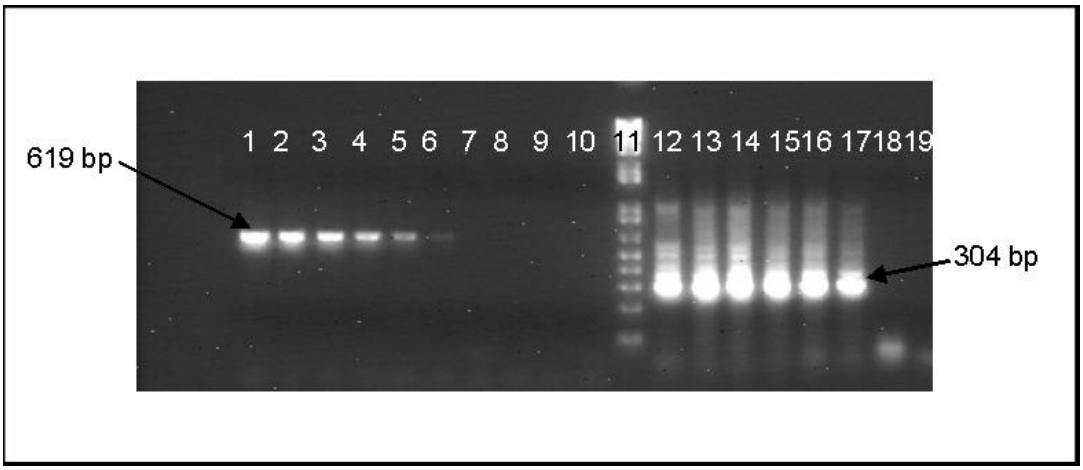


Figure 4.5 Agarose gel electrophoresis of MDV-2 nested PCR products
Lanes 1-10: products of the first round reaction, primer: S1FO MDV2 and S1RO MDV2, Template: ten fold serial dilutions of Maravac vaccine strain of MDV-2.
Lane 11: marker, 100 bp DNA ladder plus
Lanes12-19: product from the nested PCR, primer: S1FI MDV2, S1RI MDV2, Template: 1 µl of PCR product from the first round reaction into respective wells.

4.1.4 Discussion

The results demonstrated that Mg^{+2} concentrations had a significant effect on the success of PCR. The result in Figure 4.1 with 1.5 mM Mg^{+2} concentrations suggested that there could be inefficient activity of Taq DNA polymerase. Taq DNA polymerase is a magnesium-dependent enzyme. Magnesium ions also bind with the template DNA, primers and dNTPs. In addition, Mg^{+2} is also removed by chelators such as EGTA or EDTA which are present in the most of the buffers used in molecular biology procedures as well as for the storage of DNA. Magnesium ions also have an affinity for the negatively charged phosphate groups on the DNA backbones (Markoulatos *et al.*, 2002).

The PCR results of the Mg^{+2} concentration 2.0 mM and 2.5 mM demonstrated optimal PCR products suggesting that template DNA, dNTP and primers concentrations used in this reactions were appropriate for optimal specific DNA amplification. The same concentration was optimal for all of the primers used in this project. Increasing the concentration of Mg^{+2} to more than 2.5 mM could produce larger amounts of non-specific products reducing the intended products. High concentrations of Mg^{+2} stabilise the DNA double strands and interfere with denaturation, ultimately reducing the specific products. In addition, excessive Mg^{+2} concentrations also reduce the specificity of the PCR due to stabilisation of spurious annealing of primer to incorrect template sites (Rychlik *et al.*, 1990).

The results as outlined in Figure 4.4 suggested that annealing temperature had a significant effect on the DNA amplification and generation of specific PCR products. Variations in annealing temperature have significant effect on the quantity of PCR product (Niens *et al.*, 2005). Inappropriate annealing temperatures facilitates primers hybridising to non-complementary sequences which lead to amplification of non-specific products in competition with the specific products, ultimately decreasing the amount of specific products (Wu *et al.*, 1991).

The results of nested PCR suggested that the all the reagents concentrations and the annealing temperature were optimal for both standard and nested reactions. It could

be explained by the T_m valves of the primers. The primer sets used for the standard PCR and nested reactions had a narrow range of melting temperatures (T_m).

The use of co-solvents, dimethyl sulfoxide (DMSO), had demonstrated improvement in the amplification the most difficult part of the CAV genome which had high percentage of G + C. The DNA with high G + C contents are difficult to amplify due to low efficiency of template dissociation (Reysenbach *et al.*, 1992). Addition of co-solvents in PCR enhances the amplification of DNA from the templates with high G + C content and also facilitates amplification at lower denaturation temperatures by lowering the T_m of the template DNA (Landre, 1995).

4.2 Relative sensitivity of CAV primers, Cux and SH-1, with respect to the Australian CAV strain BF4

4.2.1 Introduction

The purpose of testing the sensitivity of these primers with respect to the Australian CAV reference virus (strain BF4) was to determine the relative sensitivity of each the primer sets in relation to a known Australian CAV isolate and speculate on the prevalence of other CAV strains present in the broiler flocks. More than one strains of the virus were expected. While CAV has a relatively conserved genomic structure, two genetically distinct clusters of viruses have been recognised with the type viruses being Cux-1 and CIA-1. The nested primers pair; SH-1 was specifically designed to detect CIA-1 (Cardona *et al.*, 2000b). To date, near full length sequences of two Australian isolates, CAU269/7 and isolate 704 are published. The isolate 704 is genetically close to the CIA-1 and the CAU269/7 is relatively close to Cux-1. The genetic status of the strain BF4 at this stage of the project was unknown.

4.2.2 Materials and methods

Two sets of primers were selected for the detection of the CAV from the published articles taking into consideration that all the CAV strains present in the samples could be detected. The primer set SH-1 F and SH-1R, SH-1 N3 and N5 were the primers specifically designed for the isolation and characterisation of the CAV SH-1

strain (Cardona *et al.*, 2000b). The primer sets Cux O3F and O3R, Cux N3 and N4; had high homology with the most of the published sequence of the CAV genome in BLAST search and alignment using GENEDOC.EXE (Chapter 3.4.1).

The CAV Australian strain was propagated by D.E. Spence at the fifth viral passage level in MSB1 cells in 1993 and the supernatant was stored at -80°C. The virus was again cultured in to the MSB1 cells up to the fifth viral passage level. Viral DNA from the cells and supernatant was extracted using the Roche DNA extraction kit as discussed previously. A tenfold serial dilution of the extracted CAV DNA was prepared. The total volume of PCR was 25 µl including template. PCR master mix for ten reactions for each primer set was prepared with concentration of reagents as optimised previously. One microliter of the serially diluted DNA was added. All Thermo cycling parameters were kept as optimised (Chapter 4.1.3.1. and 4.1.2.4.).

For the nested reaction, again the reaction volume was 25 µl including the template. One microliter of the first round PCR product was added as template into the respective tubes for the next round reaction with inside primers of the both sets of primer. All the reagent concentrations and DNA amplification parameters remained the same as the first round reaction and product was held at 15°C before being run through agarose gel.

4.2.3 Results

The Cux set of outside primers (O3F and O3R) produced products of the expected length of 386 bp. A single band was observed in all lanes with primer dimer obvious in all lanes from the 10^{-3} dilution of the DNA template (Figure 4.6).

The SH-1 set of outside primers (SH-1 F and SH-1R) also resulted in products of the expected length or 601 bp. Bright bands corresponded with the first three dilutions and the band that corresponded with the 10^{-3} dilution was relatively faint. Two additional bands could be clearly seen at the 10^{-1} and 10^{-2} dilutions of the DNA template. Primer dimer could be observed in all lanes. However, it was more prominent from the 10^{-3} dilution of the DNA template.

These results indicate that in the first round reaction the Cux primers produced a PCR product at the 10^{-5} dilution of DNA template while the maximum sensitivity of the SH-1 primers corresponded with a 10^{-3} dilution of template.

The inside set of primers also produced bands of the expected length of 209 bp for the Cux primers and 204 bp for the SH-1 primers. A single band was observed at all dilutions and primer dimer was not in any of the wells in which a specific product was observed (Figure 4.7).

Specific products were observed in seven lanes corresponding to the 10^{-6} dilution of the DNA template for the Cux primers while the SH-1 primers produced specific products in six lanes.

These results suggest that with DNA template prepared from the Australian BF4 isolate of CAV the Cux outside primers are 100 times more sensitive than the SH-1 primers while the inside primers were 10 times more sensitive.

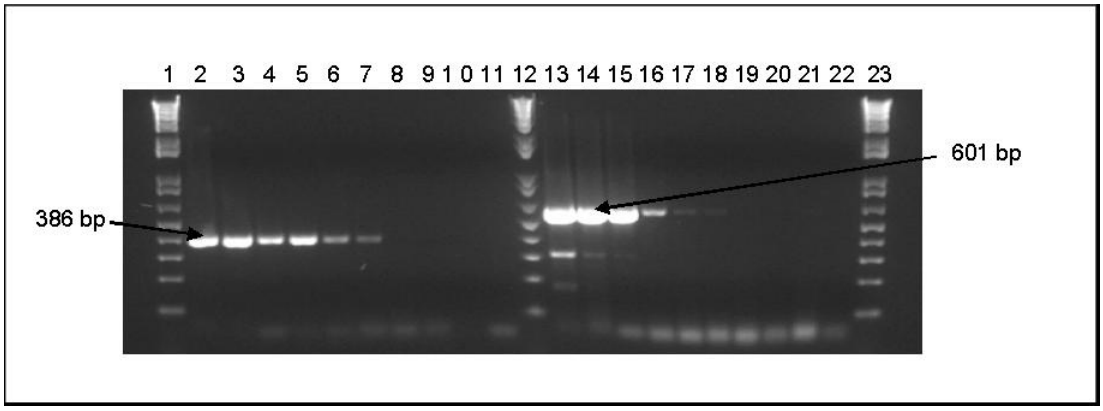


Figure 4.6 Electrophoresis of CAV PCR products

Template: Serial ten fold dilution of Reference CAV DNA from cell culture at five passages after viral infection.

Lane 2-11: primer, CAV O3F and O3R and template, serially diluted in sequential order

Lane 13-22: primer, CAV SH-1F and SH-1R and template serially diluted in sequential order

Lane 1, 12 & 23: marker, 100 bp DNA ladder plus

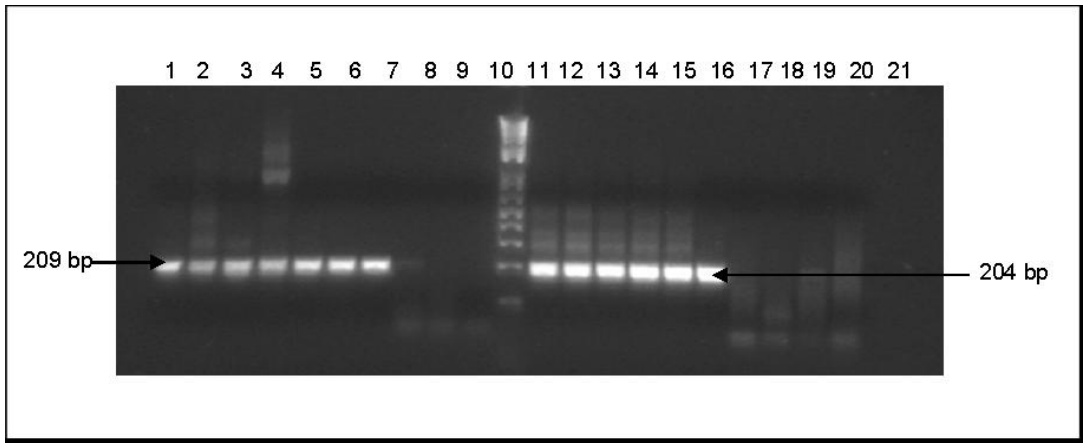


Figure 4.7 Electrophoresis of nested PCR products
Lane 1-10: template, 1µl of the PCR product from the first round reaction with primers Cux O3F and O3R into the respective wells with primers, Cux N3 & N4.
Lane 12-21: Template, PCR product of first round reaction with primers CAV SH-1F and SH-1R into the respective wells with primers, SH-1N5' and SH-1N3
Lane 11: marker, 100 bp DNA ladder

4.2.4 Discussion

The results suggested that there were differences in sensitivity of these two sets of primers with respect to the CAV Australian strain. The Cux primer sets demonstrated better results than the SH-1 primer sets with the isolate. In both standard and nested reactions, images were observed in the reaction with one more ten fold template dilution in case of Cux than with SH-1 primer set. Electrophoresis of the PCR products from the both sets of the primers which were compared were carried out in the same gel so that comparisons could exclude bias associated with the electrophoresis.

The differences in the sensitivity of the primers could be because of primer sequence nucleotide mismatch with the viral genome which could be explained with the fact that the SH-1 primer set was specifically designed from the SH-1 strain sequence (Cardona *et al.*, 2000b; Markowski-Grimsrud *et al.*, 2002).

Clear bright bands were observed in a few more lanes in the nested reactions from dilutions that failed to produce an image in the first round reaction indicating that the nested reactions were clearly more sensitive than were the single round assays.

These results also demonstrated the differences in the relative sensitivity between CAV Cux and SH-1 primers with the Australian CAV isolate. As some variations in CAV genotypes were anticipated in this study it was hoped that these two primer sets would be sufficient to recognise all of the isolates that could be encountered.

4.3 Conclusion

In conclusion, PCR is a very sensitive system. Optimisation of the assay is essential to make maximum use of the laboratory facilities available. The concentration of magnesium ions in the reagents and the annealing temperatures are the two most important parameters for successful PCRs. While the calculated annealing temperature could be used as a guideline, full optimisation is necessary.

The relative sensitivity of two sets of nested primer against the CAV strain BF4 demonstrated differences. This may indicate that there are different strains of CAV infecting Australian poultry. The use of both the primer set for CAV detection in the sample material increased the possibilities of detecting different isolates of CAV.

CHAPTER 5

EPIDEMIOLOGICAL SURVEY TO DETERMINE THE PREVALENCE OF MAREK'S DISEASE TYPES ONE AND TWO AND CHICKEN ANAEMIA VIRUS USING FEATHER SAMPLES COLLECTED FROM BROILER FLOCKS AT SLAUGHTER

5.1 Introduction

Publications describing the prevalence of MDV-1, MDV-2 and CAV in broilers at the time of slaughter are limited and publications specifically describing the presence of any of these three viruses in Australia in broilers had been restricted almost entirely to conference presentations.

Both MDV-2 and CAV have the potential to modify the effects of MDV-1 infection in broilers. It has clearly been shown that MDV-2 can be used as a vaccine to reduce the effects of MDV-1 either by reducing the replication of the virus or suppressing the formation of tumours. If broilers were infected with MDV-2 prior to being infected with MDV-1 there is the potential for the MDV-2 to be acting as a natural vaccine and providing some protection for the infected birds. Conversely CAV is able to replicate in immature T-cells and their bone marrow precursors and this has the potential to be synergistic with MDV-1 producing immunosuppression allowing secondary infection or potentially increasing the production of Marek's disease tumours (Miles *et al.*, 2001).

Marek's disease viruses are reported to be transmitted through infected dander as both MDV-1 and MDV-2 replicate in feather follicle epithelial cells producing enveloped virions (Carrozza *et al.*, 1973). Recently CAV has also been demonstrated in feather samples from infected birds suggesting that this may also be a potential source of virions for lateral transmission (Davidson *et al.*, 2003).

This project used feather samples from broilers collected at slaughter for the monitoring of MDV-1 in a related project at James Cook University. The samples were also tested using PCR to determine the presence of MDV-2 and CAV to

provide data that would allow speculation on the role of these two viruses in modifying the effects of MDV-1 in broilers.

5.2 Materials and methods

5.2.1 Feather samples were available from two separate studies

The first of these was a study of broilers in Victoria. Samples were collected from this flock because of relatively poor performance and a high prevalence of MDV-1 was detected in the samples. Vaccination was commenced with HVT *in ovo* at 19 days incubation. Subsequently 10 feather samples per flock were collected from 10 successive batches of broilers. Vaccination was then suspended and a further three batches of birds were sampled. Feather samples were collected from these birds. In total 130 samples of feathers were collected representing 10 flocks that were vaccinated with HVT and a further three flocks where vaccination was not carried out.

The second series of samples was collected from nine flocks from throughout Australia. A total of 20 samples were collected from each flock. There were two flocks from South Australia, Western Australia, Victoria and Queensland and one flock from New South Wales. All of the flocks were associated with one poultry company and relatively similar management practices were carried out on each of the farms.

Breeder flocks associated with both studies were routinely vaccinated with the 3711 strain of CAV vaccine (Intervet Australia) prior to the commencement of lay.

5.2.2 Sample materials

Feather follicle epithelium (FFE) was the sample materials used for the detection of intended viruses. About 5-6 wing feathers from each of the birds sampled were collected and packed for transport.

5.2.3 Sample processing

The samples were further processed for viral DNA extraction. Two to three feather pulps from the each sample were squeezed into a sterilised Petri dish. The FFE tissues were transferred into 1.5 ml microcentrifuge tubes. Remaining feathers were stored at -20°C for future use if required. The feather pulps were further processed for DNA extraction using the Roche DNA extraction kit or the CAS 1820 ExtractaGene, Corbett Robotics, DNA extractor.

5.2.3.1 Viral DNA extraction from feather follicle epithelium using the Roche Kit

All Victorian (130) samples and eighty samples of the next group were processed for DNA extraction using the Kit (Roche diagnostic, USA) following the manufacturers protocol with little variation. The tissue in the lysis buffer and reconstituted proteinase K were incubated at 55°C overnight either in a dry block heater or in a rotating incubator for tissue digestion instead of one hour incubation. The extracted DNA was stored at 4°C before being processed for PCR.

5.2.3.2 Extraction of DNA using the CAS 1820 ExtractaGene (Corbett Robotics)

Viral DNA was extracted from 100 samples in a second batch using the Corbett automatic DNA extractor. The samples were digested overnight at 50°C in digestion buffer (100 mN Tris Hcl pH 8.0, 200 mM NaCl, 5.0 mM EDTA, 0.2% W/V SDS and 0.2 mg ml^{-1} Proteinase K). The tissue digests were centrifuged at 10,000 *g* for one minute. The clear digests (200 µl aliquots) were placed in 96 well lysis blocks and the DNA extracted in the CAS 1820 ExtractaGene (Corbett Robotics, Brisbane Australia) using a pre-programmed protocol. Briefly, digests were mixed with 600 µl of lysis buffer (5.25 M Guanidine thiocyanate, 10 mM Tris Hcl pH 6.5, 20 mM EDTA, 4.0% V/V Triton X-100 and 64.8 mM DTT) before being incubated at room temperature for 10 min. Lysates were loaded into 96 well glass fibre (GFB) capture plates (Whatman) and the DNA bound under vacuum. The columns were washed twice using 600 µl of wash buffer (100 mM NaCl, 10 mMTris Hcl pH 8.0, 25% V/V isopropanol and 20% ethanol) followed by a single wash in 700 µl of 100% ethanol.

The columns were dried by subjecting the plate to vacuum for 10 min before elution in 150 µl of elution buffer (10 mM Tris Hcl pH 8.5, 0.5 mM EDTA). The eluted DNA in 96 wells plate was sealed with plastic adhesive sheets and stored at 4°C before use.

5.2.4 Oligonucleotide primers

5.2.4.1 Primers used for the detection of chicken anaemia virus

For the detection of CAV, the viral DNA was amplified by nested PCR as optimised (Chapter 4.1.2.2 and 4.1.2.4). Two sets of nested primers, Cux (Outside: O3Fand O3R, inside: N3, and N4) and SH-1(Outside: SH-1Fand SH-1R, Inside: SH-1 N3 and SH-1N5) were used (Cardona *et al.*, 2000b). Detail description of the primers used for detection of CAV has been described earlier (Chapter 3.4.1).

5.2.4.2 Primers used for the detection of Marek's disease type two

For the detection of MDV-2, viral DNA was amplified by nested PCR as optimised (Chapter 4.1.2.5 and 4.1.2.6). The primers used were MDV-2 S1FO, S1RO, S1FI and S1RI (Burgess and Hartini, 2003). Further description of primers is described earlier (Chapter 3.4.2).

5.2.4.3 Primers used for the detection of Marek's disease type one

The data of the MDV1 detection was obtained from Mr. Ramon Layton, JCU.
A semi nested PCR assay was used for the detection of MDV-1. The primers, first round PCR (41 MDV1-F and 25 MDV1-R) and second round PCR (24 MDV1-F and 25 MDV1-R) were used (Zhu *et al.*, 1992). Further description of the primer is described earlier (Chapter 3.4.3).

5.2.5 Detection of Viruses by nested PCR

From the DNA samples, CAV, MDV2 and MDV1 were detected using nested PCR. As large numbers of samples were tested, PCR was carried out using 96 well 0.2 ml

Thin Wall Thermal Cycler Plates, certified RNase and DNase free (Quantum Scientific) so that up to 80 sample reactions could be examined at a time. Template aliquots of 60 µl of each sample were prepared into 96 well heat resistant nunc plates (Nalge Nunc International) in sequential order of identification and were sealed with Adhesive PCR Sealing Sheets so that they could be stored at 4°C and used as required for the detection of viruses.

5.2.5.1 Detection of chicken anaemia virus

5.2.5.1.1 Viral DNA amplification

The required volume of master mix was prepared with the estimated concentration of all reagents as optimised for both standard and nested PCR (ref. chapter). In the first round PCR reaction, the reaction volume in each reaction was 25 µl with two or three microliters of DNA template. The volume of master mix aliquot was adjusted according to the amount of template to be used. Two microliters of template per reaction were added from the entire sample DNA extracted using the Roche extraction kit and three microliters of template per reaction was added from the DNA samples extracted using CAS 1820 ExtractaGene. The volume of the template was adjusted on the basis of chicken genome GAPDH PCR results. The master mix was aliquot into the 96 well PCR plates. The templates were then, added using automatic multi-channel pipettes from the aliquots in the 96 well plates. The plates were sealed with Adhesive PCR Seal (Integrated Science, UK). A 96 well thermocycler (Eppendorf Mastercycler, Hamburg, Germany) was used for DNA amplification.

The DNA amplification parameters were kept as optimised for the both standard and nested reactions. For nested PCR, the product from the first reaction was added as template. Again the reaction volume was 25 µl including one microliter of the template.

5.2.5.1.2 Image visualisation

For the visualisation of image, electrophoresis of the PCR product was achieved either in molecular grade agarose gels with ethidium bromide as optimised or using an E-gel kit (Invitrogen, Australia).

5.2.5.1.2.1 Image visualisation using E-Gel® agarose gel kit

The E-Gel® pre-cast agarose (Invitrogen, Australia) were stored at room temperature. The E-Gel® agarose gels contained ethidium bromide at a concentration of 0.3 µg ml^{-1}, and 0.01% thimerosal added as a preservative. Each E-gel was packed in a sealed plastic package and to be used immediately within 30 minutes of opening. The E-gel contained 96 wells (eight rows and twelve columns) to load samples and eight additional wells for the DNA ladder.

The PCR product was prepared by adding 100 µl of TAE buffer into all wells of the 96 well PCR plate irrespective of the reaction product. Twenty microliter of the PCR product mixed with TAE buffer was loaded into the 96 wells of the E-gel and 6 µl of E-Gel® 96 well Low Range DNA Marker (Fermentas Life Sciences, Lithuania) was added in the eight row of the last column.

Electrophoresis was completed in 12-13 minutes. The image was illuminated with UV using a Gel Doc 1000 (BIO-RAD) transilluminator and the image was then, visualised using computer software GeneSnap 4.00.00 (SynGene, Synoptics Ltd. Cambridge, England) connected to the transilluminator as in agarose gel electrophoresis. Further lane alignment and arrangement of the image was achieved using the E-Editor ™ Software for configuration E-Gel® and E-PAGE™ Gel Images Microsoft Windows® version D (Invitrogen, Australia).

5.2.5.2 Detection of Marek's disease type-2

5.2.5.2.1 Viral DNA amplification

All the reagent concentrations, primers concentrations, amount of template used and the procedures were as described in (chapter optimisation).Viral DNA was amplified in 200 µl PCR tubes as well as in 96 wells PCR plates. Total reaction volume including template was 25 µl.

The thermal cycling parameters for both standard and nested reactions were seven minutes at 94ºC followed by 35 cycles consisting one minute at 94ºC, 50 seconds at 60ºC, one minute at 72ºC and the final elongation for seven minutes at 72ºC.

5.2.5.2.2 Electrophoresis and Image visualisation

Electrophoresis and image visualisation was carried out in 1.5 % w/v agarose gels as described earlier in chapter optimisation (Chapter 4.1.2.3).

5.3 Results

Specific DNA bands stained with ethidium bromide were observed in the images from the nested PCRs for the detection of MDV-2 and CAV using both sets of primers, Cux and SH-1 (Figures 5.1 and 5.2). The results were allocated into four categories (1-4 with 4 being the strongest reaction the clear prominent band). The results observed from the nested PCR of each primer set are listed in Appendix 3 (Tables 1 and 2).

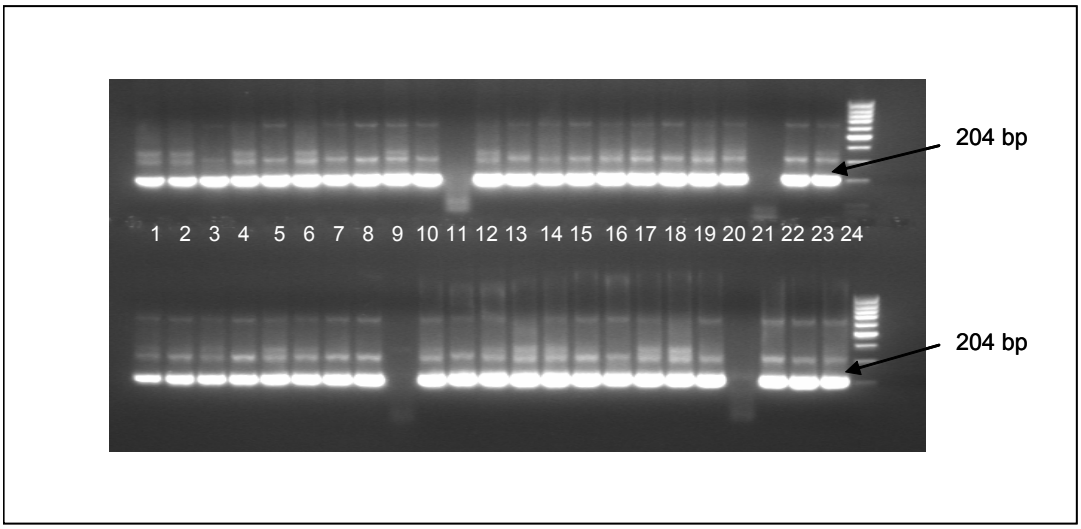


Figure 5.1 Electrophoresis of CAV nested PCR products Primers used were CAV SH-1N3 and SH-1N5 (inside primer set)
Row1: Template: 1 µl PCR product from the first reaction, 1-10, 12-20 and 22-23: samples no. 81-102, lane 11and 21: negative control, lane 24 100 bp DNA ladder.

One sample from samples no. 90 to 100 was missed during loading.

Row 2: template: 1 µl PCR product from the first reaction, 1-8, 10-19 and 21-23: sample no 103-123, lane 9 and 20: negative controls, lane 24 100 bp DNA ladder.

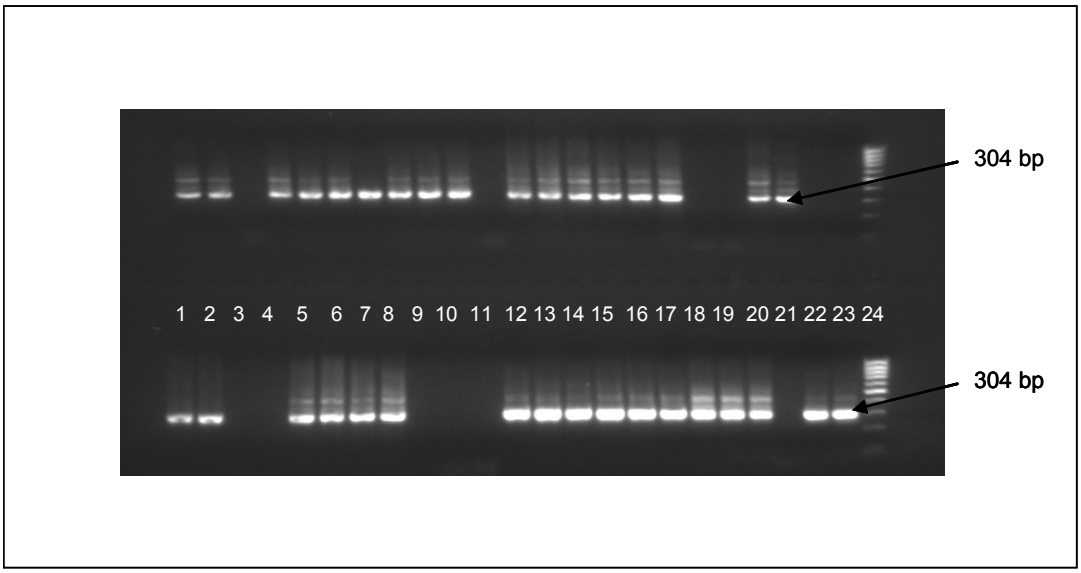


Figure: 5.2 Electrophoresis of nested PCR products. Template: 1 µl PCR product from the first reaction (primer, S1FO and S1RO), Primers: MDV-2 S1FI and S1RI (inside primer set).

Row1: Lanes 1-10, 12 -21 and 23: samples no. 1-21, lanes 11and 22: negative control, lane 24100 bp DNA ladder.

Row 2: Lanes 1-9, 11-20 and22- 23: samples 22-42, lanes 10 and 21: negative control, lane 24 100 bp DNA ladder

5.3.1 Prevalence of MDV-1, MDV-2 and CAV in broiler flocks vaccinated with HVT

The 100 samples from the 10 broiler flocks vaccinated with full dose HVT failed to react in the nested PCRs for both MDV-1 and MDV-2 indicating that the flocks were relatively free from these two viruses while they were receiving the HVT vaccine.

This is in contrast to the CAV results. A total of 90 samples reacted in the nested PCR using the Cux primers while 85 samples reacted in the PCR using the SH-1 primer sets.

The estimated prevalence in the flocks varied between 60% and 100% for the Cux primers and 30% to 100% when the SH-1 primers were used (Figure 5.3).

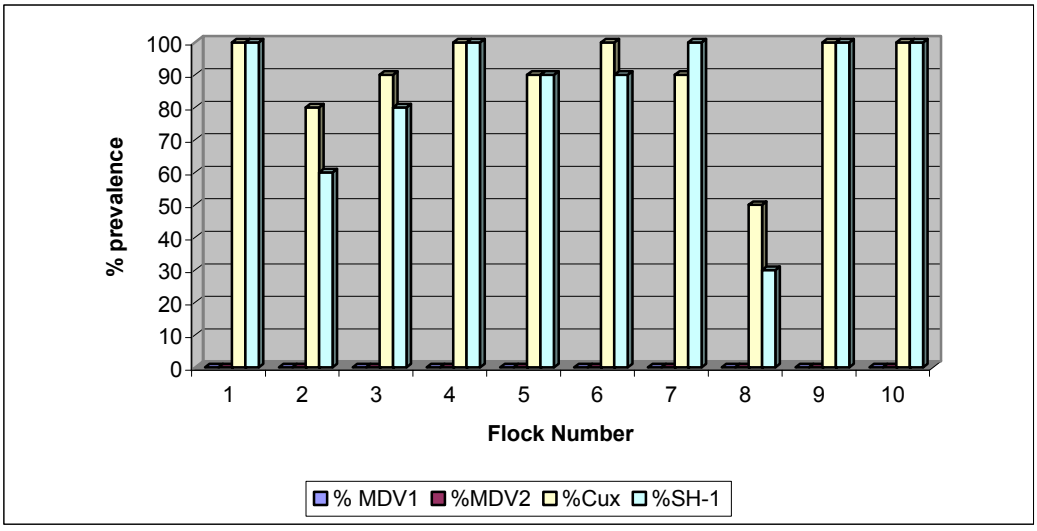


Figure 5.3 Estimated prevalence of three viruses in broiler flocks vaccinated with full dose HVT *in ovo* on day 19

5.3.2 Estimated prevalence of MDV-1, MDV-2 and CAV in broiler flocks after the cessation of vaccination

In this group of samples, 30 samples from three flocks were tested as previously described. Three samples reacted in the MDV-2 nested PCR while nine samples reacted in the MDV1seminested PCR. The estimated prevalence of MDV-1 and MDV-2 had risen to 30% and 10% respectively. All 30 samples reacted in the CAV PCRs with both sets of primers. This suggests a prevalence of approximately 100% at the time of slaughter (Figure 5.4).

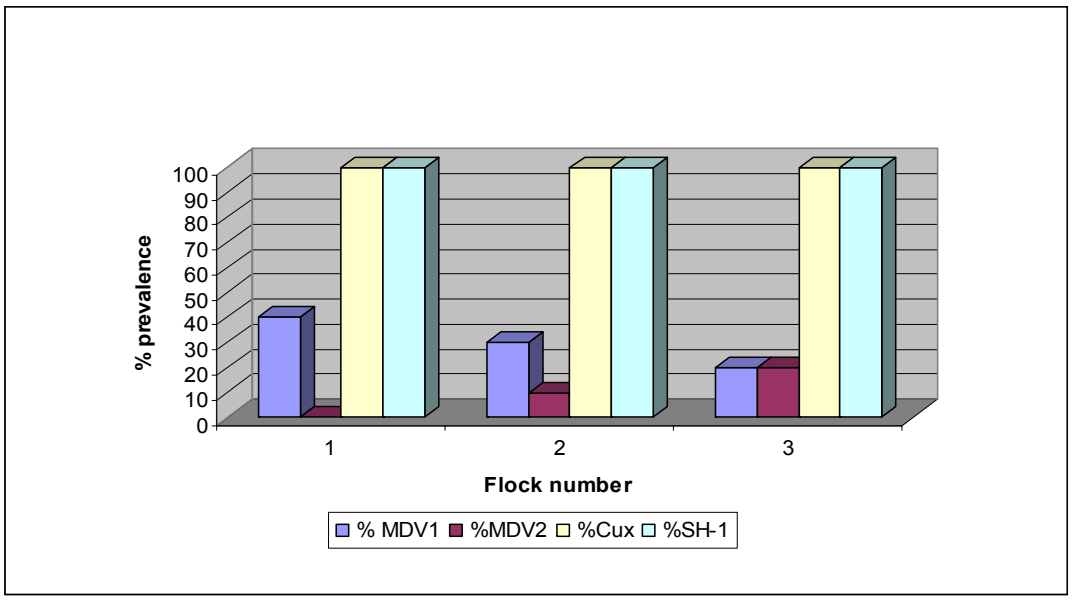


Figure: 5.4 Estimated prevalence of three viruses in samples collected from broiler after the cessation of vaccination

5.3.3 Estimated prevalence of MDV-1, MDV-2 and CAV in a panel of 180 samples from the nine broiler flocks

A total of 23 samples from seven of the nine flocks reacted in the semi nested PCR for MDV-1. This represents an estimated overall prevalence of 13% with an estimated flock prevalence ranging from 0% to 30%. In contrast a total of 110 samples from eight out of the nine flocks reacted in the MDV-2 nested PCR. This represents an estimated overall prevalence of 61% with an estimated flock prevalence ranging from 0% to 100% (Figure 5.5).

The CAV status of this group of samples was similar to that observed for the previous two groups. Estimated flock prevalence suggests that CAV was virtually present in all the test samples (Figure 5.5). While virtually all the samples (99.5%) reacted in the CAV PCR using the CUX primers, only 82% reacted in the CAV nested PCR using the SH-1 primers.

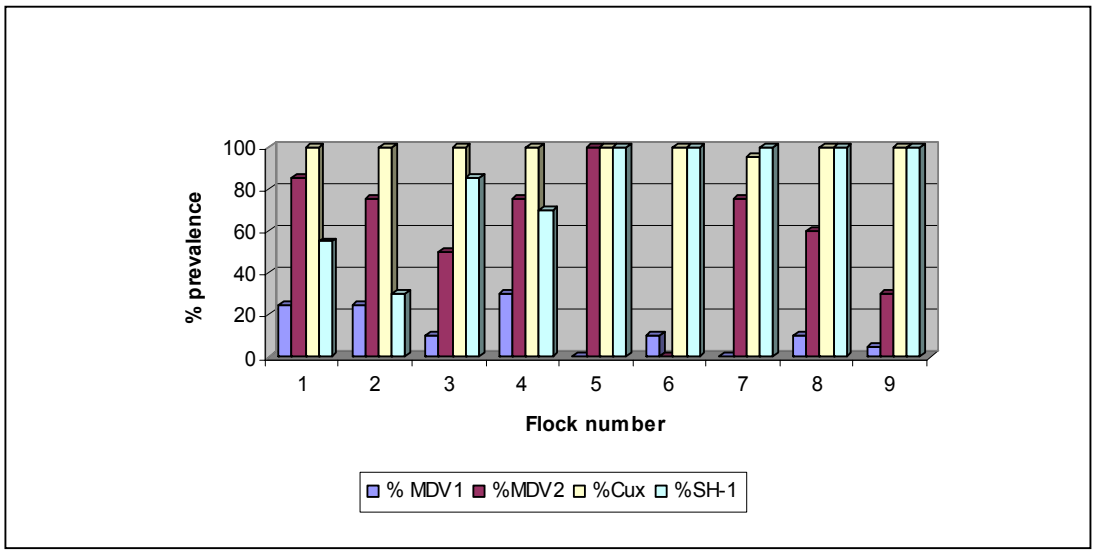
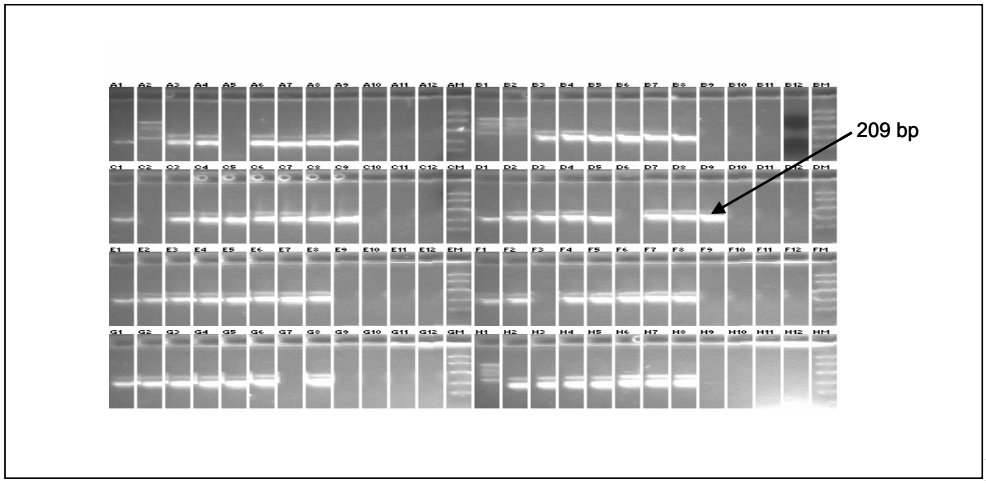


Figure 5.5 Estimated prevalence of three viruses in nine representative broiler flocks in Australia

horesis and image visualisation was carried out in molecular grade agarose with ethidium bromide and alternatively in E-gel kit. Although the E-gel kit was technically advanced, highly efficient and at least twice as fast as the agarose (E-Gel® Pre-cast agarose electrophoresis system, Invitrogen) the bands observed were relatively faint compared with the images in the standard agarose gels (Figure 5.6).



Electrop

Figure 5.6 Gel® agarose gel electrophoresis of PCR products
Primer: Cux nested N3, and N4.
Template: 1 µl of 60 samples PCR products from the first round reaction, negative control after each ten samples and two positive controls at the end,
A1 corresponds to sample no.70, B1 represents to sample no.71….H1 corresponds to sample no. 78 and so on. Marker: E-Gel® 96 well Low Range DNA Marker in the 13th column.

5.4 Discussion

5.4.1 Related to the experimental procedure

The explanation for the faint DNA band could be due to reduced amount of DNA loaded in the E-gel system. In the agarose gel system, the PCR product loaded was 15 µl mixed with 20% loading dye while 20 µl of the 20% PCR product in TAE buffer (100 µl TAE added to 25 µl of PCR product resulting in a one in five dilution) was loaded in the E-gel system.

To use the E-gel system of electrophoresis, the sample aliquots should be prepared in a row wise sequential order for identification. The E-Editor ^{TM} Software produces the row wise image configuration. The analysis of results consumed more time than expected.

Substantial numbers of non-specific image bands were observed in the electrophoresis. Some additional bands were observed in the images produced by the Cux and MDV-2 primers and there were more non-specific bands produced by the SH-1 inside primers. In all cases the non-specific bands were comparatively fewer in all the outside primer PCRs than they were in the nested PCR.

The viral DNA was extracted by two different methods, the Roche DNA extraction kit and the1820 ExtractaGene (Corbett Robotics). The volume of template required for the production of similar result from the template extracted from the Corbett Robotics was 1.5-fold more than using the Roche kit. However, the extraction procedure was automated with as many as 96 samples included in a single run and there were savings in reagents costs.

5.4.2 Related to result interpretation

The results from all three panels of samples tested suggested that all three viruses tested were circulating in poultry sheds throughout Australia. While MDV-1 and CAV can be pathogenic for chickens, these samples were collected from flocks that did not report clinical signs of either disease.
Literature suggests that MDV-1 and MDV-2 have a worldwide distribution (Purchase, 1985). Isolation of MDV-1 can be expected in most poultry production facilities with the potential to cause economic losses (Witter, 1998b). These results confirm that MDV-1 and MDV-2 can be isolated from broiler flocks in Australia.

Early reports described the isolation of a virulent biotype of MDV-1 (Mustaffa-Babjee, 1970) and MDV-1 along with avian leukosis virus (Mustaffa-Babjee, 1971) from clinical samples from Australian poultry flocks. Later MDV-1 was isolated from samples from vaccinated flocks (De Laney *et al.*, 1995). The detection of MDV-1 and MDV-2 in broiler flocks in Australia at the time of slaughter with no obvious clinical manifestation of MDV supports the statement that vaccination may not eliminate the infection. The production losses due to MD have been successfully controlled by vaccination but none of the vaccines eliminate infection, replication and shedding of the challenge virus (Bublot and Sharma, 2004).

In Australia, generally some broiler flocks may be protected from MDV by *in ovo* vaccination at 19 days incubation with HVT vaccine.

The absence of MDV-1 and MDV-2 in samples collected from birds vaccinated with full dose HVT supports the previous studies reporting at least partial inhibition of MDV-2 by HVT *in vivo* as well as *in vitro.* The *in vitro* study indicated that the inhibition can be observed after two or three days post inoculation and is also dose dependent. The maximum inhibition recorded was as high as 91% at high doses (800 PFU) of HVT but no effect was noticed when virus infected buffy-coat was present in the inocula. In *in vivo* studies with the inoculation of both HVT and MDV, the HVT viremia titre was higher than that recorded for MDV. The dose required to infect 50% of the susceptible birds was 13-fold more than when MDV was inoculated alone (Witter *et al.*, 1994). However, previous *in vivo* studies with co-inoculation of MDV-1 and MDV-2 found no inhibition of viremia titres in chickens (Cho, 1975). The interference of MDV-2 by HVT may be serotype 3 viruses specific (Witter *et al.*, 1994).

Subclinical MDV-1 in some poultry flocks with a prevalence as high as 30% may indicate that infection with MDV-2 prior the infection with MDV-1 could possibly have played an important role in suppressing the clinical MD. The enhanced protective effect (synergism) could be expected when more than one serotype of MDV is used as a vaccine. A study of synergism among MDV serotypes indicated that the synergism was significant when birds were vaccinated with MDV-2 and HVT (Witter, 1992). Naturally infection with MDV-2 accompanied by HVT as a vaccine could perhaps produce better protection than does HVT alone could be the explanation for the sub clinical MDV-1 despite a prevalence of up to 30%.

The exceptionally high prevalence of MDV-2 in the third panel of samples possibly needs some further attention.

The observation made in this study is in agreement with other studies describing the prevalence of CAV in chickens including broilers. The presence of virus in broiler at the time of slaughter is as high as 100% in some flocks. A study in the USA that sampled broiler birds weekly until slaughter indicated that CAV infection increased

and was as high as 90% prevalence at the time of slaughter. Infection was detected in all sets of samples with a variable prevalence. The virus was detected in samples collected at one week. There was a decline in the second week onwards and almost no virus was detected by the fourth week of age. The prevalence increased from week five onwards (Sommer and Cardona, 2003).

The source of CAV and the mode of transmission in the broiler flocks included in this study is not clear as these samples were collected only at the time of slaughter. There is no CAV monitoring system in the broiler flocks and no data regarding CAV status in the breeding flocks which supplied the chicks. The infection in the broilers infected the flocks via vertical or horizontal transmission. The presence of MDV-1 and MDV-2 which are exclusively transmitted via the horizontal route in these flocks indicates that conditions favouring horizontal transmission were present.

Alternatively there is the possibility that the virus was transmitted to the newly hatched chicks from either the rooster (Hoop, 1993) or hen (Yuasa and Yoshida, 1983; Hoop, 1992) or both. The third possibility could be that the vaccine virus used to vaccinate the breeders was being transmitted. The CAV vaccine is a live virus. The vaccine virus could have replicated in the hens and could possibly have been transmitted to their progeny. To identify the source of the transmission, molecular characterisation of the field isolates as well as the vaccine virus deserves proper consideration. In addition weekly sequential sampling of broilers from the first week of life until slaughter could possibly generate information to differentiate vertical and horizontal transmission.

On the basis of published data on CAV, isolates can be grouped as two different strains of CAV represented by Cux and CIA-1. Variations in the reactivity of the two sets of nested primers in this study suggest that variations in strains may have been demonstrated. It is likely that the primers have mismatches with some of the CAV sequences.

The current study using feather samples for the detection of CAV may indicate some role of poultry shed dander in the transmission of infection. Feather follicle epithelium is the site of production of infectious cell free virions of MDV and is the

potential source of infection (Nazerian and Witter, 1970). So far there is no clear evidence to indicate the importance of feathers or poultry shed dander in the transmission of CAV. Collection of the poultry shed dander on a weekly basis could be logical for the detection of CAV in dander.

In addition feathers could be more suitable samples for the investigation of CAV infections. Feather tips have been demonstrated to be a favourable source of DNA for the detection of MDV and avian leukosis viruses, subgroup J (Davidson and Borenshtain, 2002). In comparison with the collection of blood samples or the collection of tissue samples as diagnostic samples feathers are relatively easy to collect and handle and they can be collected from live birds. Under favourable conditions the same birds can be used for subsequent sampling at different ages. A critical evaluation of feathers as suitable samples for the investigation of CAV infections compared with traditional samples such as thymus, spleen, harderian gland, reproductive tissues, caecal tonsils and blood is warranted.

All three viruses were present in some flocks. Even though the samples were kindly provided from commercial broiler flocks, information associated with production data was not supplied. Flock performance information is an important parameter for comparing the contribution made by each of the viruses to the performance of the flocks. Some flocks with a prevalence of MDV-1 infection as high as 30% had no performance drop recorded. If these birds were not performing poorly it may have been the result of the presence of MDV-2 which was possibly working as a vaccine subsequently depressing the pathogenicity of the wild type MDV-1. To accurately evaluate the effects of MDV-2 natural infection on the pathogenesis of MDV-1 more extensive sampling accompanied by performance data would be required.

Chicken anaemia virus was present virtually in all flocks. However MDV-1 and MDV-2 were demonstrated only in two groups of samples. This study is not sufficient to estimate the effects of immunosuppression of either CAV or MDV-1. Both CAV (Markowski-Grimsrud and Schat, 2001) and MDV-1 (Calnek *et al.*, 1998) are immunosuppressive diseases. Experimental inoculation of different strains of MDV-1 alone and coinfection with CAV in SPF birds has been carried out. The study found that dual infection with CAV and MDV-1 exacerbated the pathogenicity

of a vvMDV strain RB1B but the effect was less significant when birds were co-infected with CAV and MDV strain 584A (Miles *et al.*, 2001). Further studies to determine the interaction of these three viruses are warranted.

5.5 Conclusions

This study demonstrated that MDV and CAV are present in the Australian broilers at the time of slaughter. Vaccination status of the broilers against MDV is likely to influence the existence of wild type MDV in the broilers. The serotype three, HVT as a vaccine against MDV is likely to suppress replication of MDV-1 and MDV-2. Pre-existence of MDV-2 in the broilers is likely to produce synergism with HVT vaccine to increase the efficiency of immunity responses after vaccination.

Furthermore, CAV has been detected from all the broiler flocks, using two set of nested primers. There have been differences in the primer reactivity in two set of nested primer. The differences in the reactivity between two set of primer is possibly due to genetic differences among the virus detected.

Although the data generated in this project are not sufficient for further explanation of the pathogenicity of dual infection by these two immunosuppressive viruses, it provides the status of these viruses in the Australian broilers at the time of slaughter and subsequently adds to speculation on the possible effect on optimum broiler production. Co-existence of CAV and MDV has been demonstrated to increase the pathogenicity of either virus when infected alone (Miles *et al.*, 2001).

CHAPTER 6

MOLECULAR CHARACTERISATION OF CHICKEN ANAEMIA VIRUS STRAIN 3711 AND THE FIELD ISOLATES TO DETERMINE SOURCE OF VIRAL TRANSMISSION

6.1 Introduction

Chicken anaemia virus was first isolated in Japan in 1979 (Yuasa *et al.*, 1979). The virus was then reported worldwide. Viral isolates from Australian poultry designated 3711, 3713 and 704 have been characterised (Connor *et al.*, 1991). Full-length nucleotide sequence data from one isolate (704) has been published and near full-length sequence from strain CAU/7 (Brown *et al.*, 2000) and one partial sequence spanning the VP1 gene of Australian isolates are available in GenBank (Pallister *et al.*, 1994).

The present study confirmed the presence of CAV in Australian broiler flocks. Viral genome was demonstrated in samples collected at slaughter at a prevalence that approached 100% when both sets of primers were used. The differences in results observed for each of the two sets of primers may indicate the possibility of different genotypes infecting these flocks. To determine the genetic variation of CAV isolates molecular characterisation of selected isolates on the basis of their reaction with each of the two primer sets and the geographical location of the farms was carried out.

Transmission of CAV is poorly understood. Both horizontal and vertical transmission are possible and both parents have the potential to infect the offspring irrespective of antibody status (Cardona *et al.*, 2000b). There is insufficient evidence in the literature to accurately identify the sources of infection in the breeding flock and subsequently infection of the broilers. Potentially broilers can be infected vertically especially from infected breeding hens or from the environment of the poultry shed. Vaccines used to immunise the parent flock are live viruses that have the potential to be transmitted vertically to the offspring. Genotyping the vaccine virus and comparing the sequence of this virus with field isolates would indicate whether the vaccine virus is the source of the infection in the broiler flock.

For the genetic characterisation of CAV, cloning and sequencing of the CAV as described above is required. The CAV genome is a circular, negative sense single stranded DNA. The genome of CAV to date reported is either with 2298 bp with four repeats or 2319 bp with five repeats in the promoter / enhancer region (Noteborn *et al.*, 1991; Kato *et al.*, 1995). Prior to sequencing the genome, propagation of the virus in an appropriate cell line has been described (Todd *et al.*, 1992; Brown *et al.*, 2000; Islam *et al.*, 2002). Sequencing the viral genome directly from the sample materials has also been successful (Cardona *et al.*, 2000b; van Santen *et al.*, 2001; Ducatez *et al.*, 2005). Production of full length clones of the CAV genome and subsequently sequencing with multiple sequencing primers is one of the sequencing strategies (Meehan *et al.*, 1997; Islam *et al.*, 2002). The genome can be amplified by PCR as two *Eco*RI-*Bam*HI fragments and cloned into pBluescript II SK^- (Soine *et al.*, 1993). Alternatively, multiple primers slightly overlapping each other can be designed to produce the entire genome. Each viral DNA fragment then can be cloned into a suitable vector system (Brown *et al.*, 2000; van Santen *et al.*, 2001; Ducatez *et al.*, 2005).

This project aimed to investigate the possible source of CAV infection in the broiler flocks. To achieve this objective, molecular characterisation by sequencing the CAV Australian isolates detected from the broiler flocks in the previous study and the vaccine strain 3711 was carried out. This allowed comparisons to be made between the sequence of the field isolates, the Australian vaccine strain and an Australian reference isolate as well as the published sequences available on GenBank. This information could be valuable to the broiler industry and may provide further information for the establishment of guidelines for the control and monitoring of CAV infections.

6.2 Materials and Methods

6.2.1 Strains of the virus for sequencing

The CAV strains sequenced were CAV 3711, CAV strain BF4 PBY F1BF and the field isolates detected during investigation of samples obtained from broiler flocks (Chapter 5.2).

6.2.1.1 Chicken anaemia virus strain 3711

The Australian vaccine strain 3711 as described in (Chapter 3.2.1) was generously supplied by Intervet Australia and propagated in MSB1 cells.

6.2.1.2 CAV strain BF4 PBY F1, Reference virus

The Australian reference CAV isolate BF4 PBY F1BF as described earlier (Chapter 3.2.1) was also propagated in MSB1 cells.

6.2.1.3 Chicken anaemia virus field isolates

A total of 18 CAV field strains were selected for sequencing. The genome was extracted from the samples collected from broilers at slaughter and the viruses were not propagated. The basis of the selection was the reaction with either set of primers used to demonstrate these viruses and the geographical basis of the flocks from which the samples were collected. The isolates were numbered according to the accession number given to the samples as they were received at JCU.

6.2.1.3.1 CAV field isolate from South Australia

Four samples, SN001, SN014, SN021 and SN033 were selected from this region. The isolates SN001and SN014 were obtained from same flock. Sample number one reacted with both sets of primers while SN014 reacted only with the Cux primers. The next two samples, SN021 and SN033 were selected from the next flock. While SN021 reacted with both sets of primers, SN033 reacted only with the Cux primers.

6.2.1.3.2 CAV isolates from Western Australia

Two samples designated SN045 and SN051 were obtained from the same broiler shed and both samples reacted only with Cux primers. Samples designated SN063 and SN070 were received from next farm with SN063 reacting only with the Cux primers and SN070 reacted with both sets of primers.

6.2.1.3.3 CAV isolates from Victoria

Five isolates were selected from Victoria. Samples SN091 and SN102 reacted with both sets of primers. These isolates were from different flocks. The isolate SN041 reacted equally with both primer sets. The remaining sample SN044 and SN062 were derived from an additional flock and was only detected using the SH-1 primer set.

6.2.1.3.4 CAV isolates from Queensland

Three isolates from Queensland were selected for sequencing. Sample SN129 which reacted with both sets of primers and SN138 which reacted with only the SH-1 primer set were received from the same flock. The remaining sample SN160 was obtained from another flock and reacted with both sets of primers.

6.2.1.3.5 CAV isolates from New South Wales

Two samples SN0161 and SN0171 were selected from this region. These samples were obtained from the same flock and reacted with both primer sets.

6.2.2 Oligonucleotide primers used to produce CAV genome to be sequenced

The first attempts at cloning used primer sets O3F and HS-1R (Cardona *et al.*, 2000b) to produce a 951 bp sequence from the reference strain BF4 PBY F1 and vaccine strain 3711.

A series of attempts were then made to clone a full-length CAV genome using a single pair of primers based on the method described by Islam *et al*. The primer pair

designated Pstl-S and Pstl-AS have extensions corresponding to restriction enzyme sites so that the full-length product can be cloned into pBluescript II SK$^-$ allowing subsequent sequencing using a series of sequencing primers (Islam *et al.*, 2002). Amplification of DNA was attempted using a proofreading enzyme Platinum® *Pfx* DNA polymerase system (GibcoBRL® Life Technologies, Inc.), according to the manufacture's instructions. As this was unsuccessful an additional attempt to produce full-length DNA was made using the primer pair O3F and O3R.

Further attempts to produce full-length DNA were carried out using the primer pair For-O2 nucleotide position 1130 – 1150 and Rev-O2 nucleotide position 1155 - 1176 designed for this project (Table 6.1). The DNA polymerase used was Taq polymerase. The assumption was that the amplicon produced would be either 47 bp or/and full length genome but again the attempt was unsuccessful. The product produced from this primer set was only 47 bp. The reverse primer of this primer set was used in combination of two other forward primers, O3F and CAV Cux-N3 (Cardona *et al.*, 2000b), to examine the reliability of the primer and a clearly specific size amplicon from both combinations were observed. This result indicated that full length genome isolation would be difficult.

Table 6.1 Primers used in the full length sequencing trial

Primer Name	**Sequence (5'-3')**	**Size bp**	**Genomic Position**	**Amplicom size bp**	**References**
Pstl-S	AAAACTGCAGAGAGATCCGGATTG	24	547 - 570	Full length	(Islam *et al.*, 2002)
Pstl-AS	AAAACTGCAGTGAGGGGTTTCCAA	24	537 - 560		
Rev O2	CGCTAGGAGGAACTCTTTCAGG	22	1155 - 1176	47 bp or Full length	This project
For O2	CGAGAGTCGCCAAGATCTCTG	21	1130 - 1150		

The strategy was then changed and concentrated on targeting different part of the genome which could produce sufficient overlaps between successive regions of the CAV genome. Four sets of primer were selected to obtain 2300 bp long CAV circular genome. The first set of primers used was O3F and HS-1R (Cardona *et al.*, 2000b) to produce a 951 bp amplicon. The genomic location, primer sequences and their consensus with other published CAV sequence data for this particular primer pair used for the sequencing are described earlier (Chapter 3.4.1 and Appendix 2

Figure 1). The product produced by this set of primers covered the complete VP3 gene (366 bp) and the 3' portion of VP2 (558 bp out of 651) and the 5' portion of the VP1 gene (571 bp from 1350 bp) of the virus.

This primer pair was used to produce the 951 bp DNA segments of CAV genome from all of the field samples selected for sequencing, the vaccine strain 3711, and the reference strain BF4 PBY F1. These DNA fragments were then cloned and subsequently at least three clones of the each viral strain were sequenced. The assumption was that this portion of genome incorporates the hypervariable region and the sequence data alignment of this region of the genome could be a guideline for the further selection of the field isolates to produce full length sequence.

The next three primer sets were designed for this project using the computer software Oligo 6.60 Software (Molecular Biology Insights, USA) and synthesized by Sigma-Genosys, Australia. These primers were designed on the basis of the sequence consensus produced from the known Australian CAV sequence obtained from the GenBank sequence data and the 951 bp sequence of the reference CAV strain, field isolates and strain 3711 as outlined above.

The VP1 Front primer set (VP1 Front-For and VP1 Front-Rev) was the second set of primers designed to produce a 793 bp amplicon which covered the middle portion of the VP1 gene. The next primer set designed was CAV VP1 Tail, (VP1 Tail-For and CAV VP1 Tail-Rev) which were selected to produce 936 bp products which could cover the terminal portion of VP1 and starting portion of the noncoding region of the genome. The last primer set designed as gap filling primer was CAV-REPfor and CAV-REPrev planned to produce either 467 bp or 488 bp which could span the terminal portion of the non-transcribed region and the starting region of the VP2 gene. The total length of the resultant products could be 3143 or 3164 to produce full length CAV genome of 2298 bp (four repeats) or 2319 bp (five repeats) with sufficient overlap to confidently determine the full-length sequence. Further descriptions of the primers are enlisted in the Table 6.2 and the locations in the sequences are in Figure (Appendix 2 Figure 1 and Figure 3 C).

Table 6.2 Primers used in PCR to sequence CAV

Primer Name	Sequence (5'-3')	Size bp	Genomic Position	Amplicon size (bp)	References
O3F	CAAGTAATTTCAAATGAACG	20	452 - 471	951	(Cardona *et al.*, 2000b)
SH-1R	GCAGGGTCATTTGTTTAGGG	20	1497 - 1477		
VP1F-F	CGAGAGTCGCCAAGATCTCTG	21	1383 – 1402	793	This project
VP1F-R	ATGGTCTCCGTGCCGAGCG	19	1904 - 1922		
VP1T-F	GAGACCCGACGAGCAACAG	19	1635 -1653	936	This project
VP1T-R	GTCACTTTCGCAACGTGGCAGC	22	251 - 272		
REP-F	GTCAAGATGGACGAATCGCTC	21	51 - 71	467/488	This project
REP-R	GGCCTGAACACCGTTGATG	19	499 - 517		

6.2.3 DNA amplification

Optimisation of annealing temperature for the each primer sets was carried out as described (Chapter 4.1.2.2). The DNA polymerase used was Taq DNA polymerase (MBI Fermentas). Amplification of viral CAV DNA was carried out separately for each primer set with little variation of the amplification temperature and addition of co-solvents. Dimethyl sulfoxide at the rate of 5% or sterile glycerol at the rate of 7% was used as co-solvent for the optimal DNA amplification with the primer set VP1Tail-For and VP1 Tail-Rev. The concentrations of the PCR reagents used for each primer were the same as described earlier (Chapter 4.1.2.2). The amplification parameters and annealing temperatures for each primer set are listed in Table 6.3. The denaturing temperature (94°C) and elongation temperature (72°C) were common for all primer set.

Table 6.3 Reagents concentration and DNA amplification parameters used for each primers used for sequencing

Primer name	Annealing (ºC)	Amplicon (bp)	PCR programme denaturation, annealing, elongation, no. of cycles	Primer source
O3F SH-1R	50	951	50s, 60s,90s, 35 cycles	(Cardona *et al.*, 2000b)
VP1 Front For VP1 Front Rev	50	792	50s, 60s, 60s, 35 cycles	This project
VP1Tail For VP1 Tail Rev	60	935	50s, 60s,90s, 35 cycles	This project
CAV-Rep For CAV-Rep Rev	52	466	50s, 50s, 60s, 35 cycles	This project

6.2.4 Viral DNA extraction and purification from agarose gel

The electrophoresis of the PCR product was carried out as described earlier (Chapter 4.1.2.3). The agarose gel was then visualised for specific bands over a UV light in a darkroom. The DNA bands were removed using a separate scalpel blade for each sample. Purification of the DNA from the agarose gel was carried out using the Perfectprep Gel Cleanup kit (Eppendorf, Hamburg Germany), according to the manufacture's instruction.

6.2.5 Quantitation of DNA

The purified DNA was then quantified as described earlier (Chapter 3.6).
The amount of DNA samples was estimated as ng μl^{-1} and the cloning vector measurement was also provided in ng μl^{-1}. The quantity of the DNA required to add to the ligation mixture was a ratio of 1:3 between cloning vector and the sample DNA. The cloning vector size was 2728 bp whereas the DNA insert size was less than 1000 bp in all cases. Therefore, the quantity of the DNA in each sample as well as the cloning vector was converted into femtomols to standardise the concentration of DNA using following formula,

$$\text{DNA (femtomol)} = \frac{\text{Quantity of DNA in ng}}{0.66 \times \text{DNA insert size in Kb}}$$

6.2.6 Cloning of the PCR product

The purified DNA was cloned using the yT & A Cloning Vector Kit Protocol (Yeastern Biotech, Taiwan) or T & A Cloning Vector Kit (Real Biotech Corporation, Taiwan) following manufacture's instructions with minor variations. The ligation of the DNA into the vector was carried out either overnight at 4ºC or at 12ºC for three hours programmed in a thermocycler (Eppendorf Master Cycler Hamburg, Germany).

6.2.6.1 Competent cell transformation

The ligation mixture was transformed into ECOS 101 competent cells (Yeastern Biotech, Taiwan), according to manufacture's instruction with little variation. The amount of cells to be used for each transformation was 50 µl per reaction and the ligation mixture was not more than 10% of the volume of cells but 25 µl or 20 µl of cells and double the amount of the ligation mixture was also used in some reactions. The transformed cells were grown on LB agar (Appendix 1.3.2) with ampicillin, X-Gal and IPTG. Freshly prepared plates were used every time. Plates were incubated at 37ºC for approximately 16-18 hours. Blue/white screening was carried out, white colonies indicating the presence of an insert in the plasmid.

6.2.6.2 Bacterial culture

The white colonies were then cultured into LB (Appendix 1.3.1) broth with ampicillin at a concentration of 100 µl ml^{-1}. A single colony was picked with a sterile loop and inoculated into 5 ml of broth in a sterile universal bottle. At least five colonies from each transformed plate were grown to produce multiple clones of each fragments of DNA cloned. The culture was incubated in a shaking incubator (Bioline 4700, Edwards Instrument Company, Australia) at 37ºC at 125 rpm for approximately 18 hours when the bacterial growth reached maximum level which was indicated by turbidity in the cultured broth.

6.2.6.3 Recombinant plasmid DNA extraction

The plasmid DNA was extracted using either at NucleoSpin® Plasmid kit (Machery-Nagel GmbH & Co.KG, Duren, Germany) or a HiYield Plasmid Mini Kit (Real Biotech Corporation, Taiwan) using the manufacturer's protocols. The bacterial culture was first transferred into a sterile 15 ml centrifuge tube (Falcon tube) and centrifuged at 4,500 *g* for eight minutes. The bacterial pellet was used for plasmid DNA extraction and the eluted DNA was stored at -20°C

6.2.6.4 Bacterial culture storage

All the cultured colonies were not used for DNA extraction. Generally five colonies from each transformed plate were cultured and plasmid DNA was generally extracted from four cultured colonies. Unused bacterial cultures were stored. One and a half millilitre of the culture was added in a microcentrifuge tube. The tube was centrifuged at 10,000 *g* for one minute. The pellet was resuspended in 800 µl of fresh LB media and 200 µl of sterile glycerol and stored at -80ºC.

6.2.6.5 Plasmid DNA purification

The extracted DNA was further purified by ethanol precipitation. The eluted DNA was mixed with 0.1 volume of 7.5 mM ammonium acetate and 2.5 volume of the 100% ethanol and centrifuged at 20,000 *g* for 20 minutes at 10°C. Supernatant was discarded and 70% ethanol was added to the tube which was centrifuged at 20,000 *g* for 10 minutes at 10°C. After discarding the supernatant, the DNA was dried in a vacuum centrifuge (CentriVAp Concentrator, Labconco, USA) for 30 minutes. The dried DNA was carefully resuspended in 40 µl of molecular grade water (Sigma).

6.2.6.6 Quantitation of plasmid DNA

The quantity and the purity of the plasmid DNA was estimated in spectrophotometer as described earlier (Chapter 3.6).

6.2.6.7 Specific size DNA inserts confirmation in the plasmid DNA

The confirmation of the specific size of the DNA insert was carried out by PCR using the same primer sets that were used to produce the inserts.

6.2.7 Sequencing

At JCU sequencing of the plasmid DNA was carried out using Amersham Chemistry DYEnamic ET Dye Terminator Cycle Sequencing Kit for the MegaBase DNA Analysis System (Amersham Biosciences, Sweden), according to the manufacture's instructions. The sequencing reactions were prepared according to instructions specified for 96-well plates. The amount of template used was approximately 300 ng per reaction. The primers used were M13 (-40) either forward or reverse. Two reactions with forward and two with reverse primers for three clones of each isolate were carried out to obtain a full consensus sequence. The DNA amplification was performed in an Eppendorf Mastercycler Thermocycler (Eppendorf, Hamburg, Germany) and the amplification was carried out for 33 cycles of 95°C for 20 seconds followed by 50°C for 20 seconds and 50°C for one minute.

6.2.7.1 Purification of sequencing reaction product

Post-reaction clean up was carried out in a Sephadex Column system using the AutoSeq^{TM} G-50 (Amersham Biosciences, Sweden). Sephadex G50 was prepared from one gram of Sephadex dry powder (Amersham Biosciences, Sweden) to which was added 15 ml of molecular grade water and it was left overnight. Six hundred microliter of the hydrated Sephadex was transferred to a clean column placed in a two millilitre collection tube. The column was then, centrifuged at 2,000 *g* for one minute. The sequencing reaction was loaded onto the surface of the Sephadex with care, not allowing any to flow down the side of the column. The column was then, placed in a collection tube (1.5 ml microcentrifuge tube) and centrifuged again at 2,000 *g* for one minute. The contents of the tube were used for the sequencing reaction.

6.2.7.2 Nucleotide sequence recording and data generation

The samples processed in the Advanced Analytical Centre at James Cook University, Townsville using a MegaBACE Sequence Analyser (Amersham Biosciences, Sweden). Alternatively plasmids were prepared according to the sequencing specification provide by Macrogen (Seoul, Korea). And transported to Macrogen where the samples were sequenced using an Applied Biosystems 3730xl DNA Analyser and M13 primers.

6.2.7.3 Nucleotide sequence clean up

The chromatograms were analysed using the computer software Sequencher™ (Gene Codes Corporation). Plasmid sequences were trimmed and overlapping sequences were assembled to produce a contig.

6.3 Results

6.3.1 Full length DNA amplification

The initial primer pair, Pstl-S and Pstl-AS, used to amplify full length CAV genome failed to produce any products in PCR. A different concentration of reagents and template was used to optimise the PCR in an attempt to produce a specific product even when the proofreading enzyme Platinum® *Pfx* DNA polymerase was unsuccessfully used. This enzyme was used with another pair of primers (O3F and O3R) indicating that the enzyme was unlikely to be the source of the failure.

The next primer pair used in an attempt to amplify full length CAV genome was For O2 and Rev O2. The genome of CAV is circular. It was assumed that a small product of 47 bp and full length genome would be produced. However, only the 47 bp product was observed in electrophoresis. It is possible that the template used for the amplification possibly didn't contain substantial amounts of full length genome. To verify the reliability of the primers, the reverse primer (Rev O2) of this pair was used in a PCR assay in combination either the forward primers, O3F and Cux N3

maintaining the same reactions condition. These combinations produced the expected size DNA bands in electrophoresis.

6.3.2 Amplification of fragments of CAV genome

The primer pair, O3F and SH-1R were initially used in an attempt to amplify and sequence a fragment of the CAV genome. The expected size of amplicon was 951 bp spanning the complete VP3 gene, the terminal 86% of VP2 gene and starting 42% of the VP1 gene including the hypervariable region. In order to ensure that at least 10 field isolates could be sequenced a total of 18 CAV-positive samples were selected. While the original detection of these samples was carried out using nested PCR, samples that produced a prominent band in the first round of the PCR were given preference as it was assumed that these would be more likely to have sufficient genome in the samples to produce the product in single round PCR for cloning and sequencing.

The 951 bp products of 10 field isolates (SN01, SN014, SN051, SN063, SN091, SN0138, SN0160, SN0161, SN041 and SN044), strain 3711 and strain BF4 were selected for cloning. One of the field samples, SN062 failed to react with the Cux primers and in turn failed to produce a product with this pair of primers.

The CAV strain 3711, strain BF4 and eight out of 10 field samples were successfully cloned into a plasmid. The insert was confirmed using the primer pair O3F and SH-1R which was used to produce the 951 bp product. The remaining two samples contained relatively low concentrations of DNA and because of time constraints no further attempts were made to clone these two isolates.

The sequence data had significant similarities as high as 99% with published data of either Cux strain or CIA-1 strain using BLAST. The consensus sequence of the field isolates, Australian published data and Cux strains was produced using the Computer software the GeneDoc (Nicholas and Nicholas. Jrn., 1997) (Appendix 4 Figure 1). The phylogenetic tree of the CAV Australian isolates and the newly sequenced field isolates was produced using the MEGA3 (Kumar *et al.*, 2004). Two predominant clusters were observed among the Australian field isolates (Figure 6.2). The second

cluster could be further subdivided into two smaller groups. One the basis of this result, CAV vaccine strain 3711 and one isolates from each of the groups and subgroups (a total of three field isolates) were selected as candidates for the production of full-length sequence.

The second primer pair used to amplify 793 bp fragments in the VP1 gene of the CAV was CAV VP1 F-For and VP1 F-Rev. Expected sizes of DNA bands were observed in the electrophoresis. The concentration of DNA after purification from agarose gel was satisfactory. The purified DNA fragment of the CAV genome was cloned successfully. The DNA insert was verified using PCR and the results were consistent with the size of band expected.

The sequence data obtained were 793 bp and 723 bp. A BLAST search confirmed that the 793 bp sequences obtain from the strain 3711 and up to 99% homology with published sequences of CAV. The 723 bp sequence had 92% similarity with a 138 bp and 94% with a 96 bp sequence of *Gallus gallus,* derived from messenger RNA (GenBank, XM_430317.1) and primers in the both ends of the sequence. This result could possibly indicate that there may be a relatively high similarity between CAV genome and the chicken genome. However, further investigation of sequences in the chicken genome site suggest that these two sequences are exons from the chicken genome giving further support to the fact that these two primers can recognise either CAV or portions of the chicken genome at least in broiler birds.

The next primer pair used to amplify subsequent portion of the CAV genome was VP1T-F and VP1T-R. The expected amplicon size was 936/957 bp. This fragment of the genome has a high G-C content which was difficult to amplify. When dimethyl sulphoxide was used as a cosolvent PCR produced some products. However, glycerol at a concentration of 7% as a cosolvent had no effect on the PCR. Further cloning was satisfactory.

The PCR product produced using the vaccine strain 3711 as template was approximately the anticipated length and this was successfully cloned into a plasmid. All of the PCR products produced using the field isolates as template were slightly longer. These were also cloned and sequenced.

The sequence obtained from the vaccine strain 3711 was approximately 580 bp with the forward reaction and approximately 352 bases could be resolved using the reverse primer. A BLAST search confirmed that both of these sequences were derived from CAV. However, a gap of 18 bases remained unresolved.

The sequences corresponding to the longer fragments produced a consensus of between 1.18 and 1.28 kb. A BLAST search using these sequences failed to identify anything more than a relatively few short sequences that appear to be irrelevant. Clearly these PCR products were not derived from CAV.

The forth primer pair used to amplify a DNA fragment to fill the gap between the sequence obtained from VP1tail primer pair and the O3F and SH-1R was designated as Rep-F and Rep-R. The expected amplicon length was 467/488 bp (with four or five repeats). There were three separate DNA bands but very close to each other in three samples out of ten (Figure 6.1). It was assumed that these three bands could be of different strains of viral population with different numbers of repeats in those samples. These three DNA bands were purified separately and once again used as template in the same PCR. It was assumed that separation of the three individual DNA bands could be relatively easy and further precaution could be taken to separate the DNA bands for example, increase in concentration of agarose gel and low voltage in electrophoresis. There were no further difficulties encountered in the further cloning and sequencing procedures.

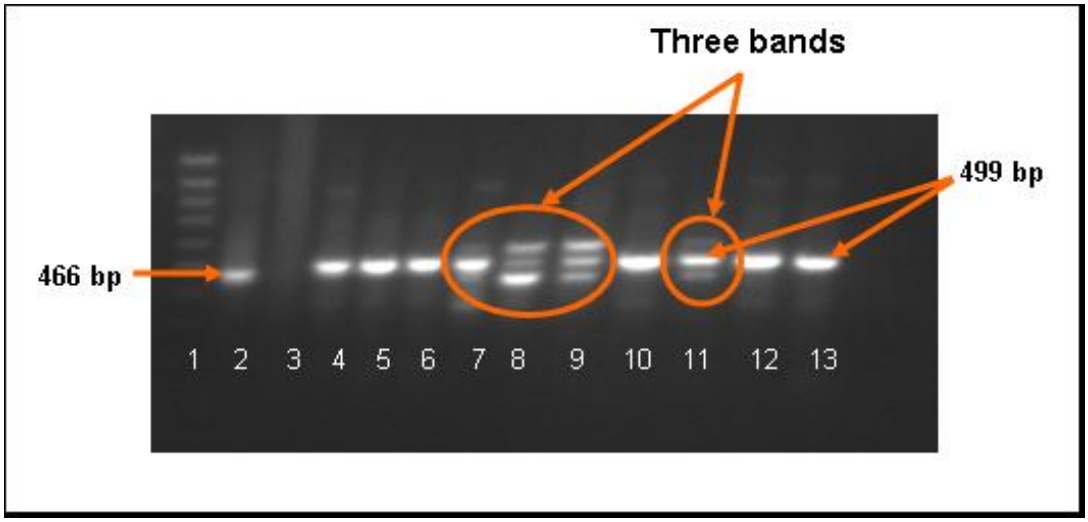


Figure 6. 1 Electrophoresis of PCR product.
Lane 1: marker, 100 bp DNA ladder. Lanes 2 to 13: template, strain 3711, BF4, sample nos. 1, 14, 51, 63, 91,138,160, 161, 41and 44
Primers: CAV REP pair.

Two different patterns were observed. The band from the vaccine virus 3711 was 467 bp and had 99% similarity with CAV using BLAST. The next sequence obtained was 499 bp long that correspond to the middle DNA band in the three bands and from the groups of three bands and it was a similar size to the band produced by strain 3711 (Figure 6.1). The sequences obtained from three different samples and their multiple clones had a similarity higher than 97%. However, these sequences did not have homology with sequences in GenBank using a BLAST search except for the primers on either ends that matched CAV sequences.

The third sequence produced from the upper DNA band of the three bands was 563 bp long. The sequence had 85% homology with *Psychrobacter arcticus* 273-4 and both ends of the sequence had 20 bp primers with complete homology to CAV sequences in a BLAST search. This result probably indicates that the skin of the broilers was contaminated or infected with bacteria that had a high level of sequence homology with *Psychrobacter arcticus* and the bacterial DNA had sufficient homology with the primers to be amplified.

This sequence contained one open reading frame and a protein BLAST search indicated a 97% homology with a fructose-bisphosphate aldolase class II from *Psychrobacter arcticus*.

The fourth sequence obtained from this particular primer pair was 438 bp long and was the smallest DNA band. The sequence had 92% homology with a 240 bp fragment between (20-260 bp) and a 47 bp length with 85% homology in nucleotide positions from 293 to 340 with sequences of *Gallus gallus* ADP-ribosyltransferas in the GenBank sequence data resources using BLAST. This result also indicates that parts of the chicken genome can anneal to primers based on the CAV genome.

6.3.3 Nucleotide bases comparisons of CAV sequences of Australian isolates with the Cux1CAEGFVIR

The outcome of the cloning and sequencing was that a partial sequence of 951 bp was determined for each of eight CAV field isolates and the reference viral strain BF4. In addition a near full length sequence of 2,279 bp was determined for the CAV vaccine strain 3711. This sequence had a 19 bp gap in the G-C rich non coding region of the genome. These sequences along with the previously published Australian sequences were compared with Cux1CAEGFVIR (GenBank accession number M81223) (Meehan *et al.*, 1992). Comparisons were made between nucleotide sequences and protein sequences for the complete VP3 gene and partial sequences of VP1 and VP2.

It was noted that the sequence published for the Australian strain CAU/7 (Brown *et al.*, 2000) also had a 12 bp gap in the same G-C rich region of CAV as was produced in this study in the vaccine strain 3711.

The available sequence data of the Australian isolates demonstrated the limited genetic diversity of the CAV of the 10 new sequences in the three previously published sequences. However, no two isolates were identical at the nucleotide level or the predicted amino acids. The phylogenetic tree constructed from the 951 bp sequence data of the Australian isolates and the Cux1 produced three distinct groups of the isolates (Figure 6.2).

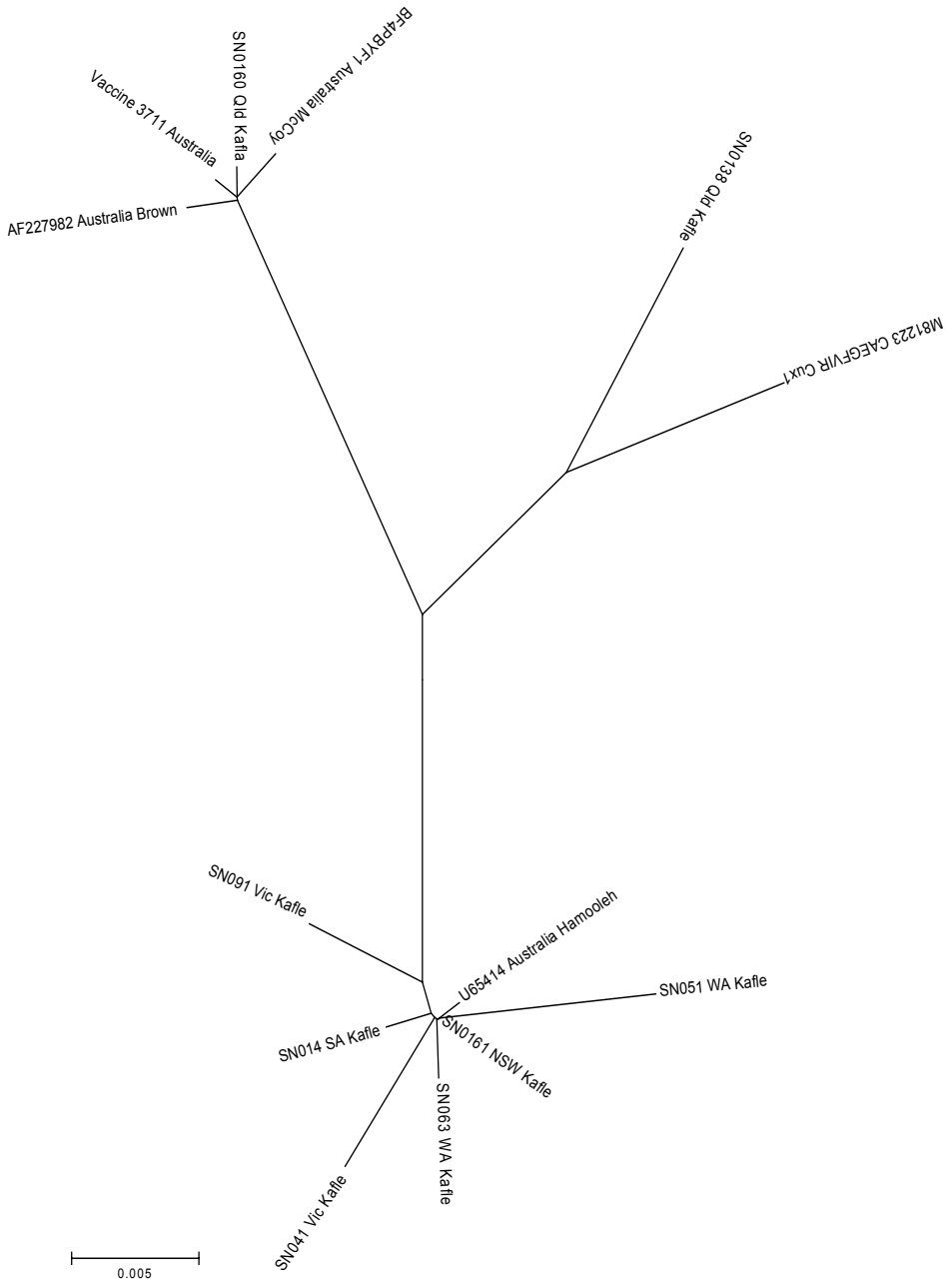


Figure 6.2 Phylogenetic tree constructed from the 951 bp sequence data of Australian isolates using Mega3 minimum evolution, style radiation.

Australian complete VP3 sequences had 15 non-synonymous nucleotides when compared with the Cux1 CAEGFVIR at positions 11,30,48, 64, 74, 124, 161, 200, 209, 217, 135, 241, 271, 273 and 323 (Table 6.4). Common nucleotide substitutions at positions 11, 30, 161 and 200 in the VP3 gene of CAV 3711, SN0160 QLD, CAV BF4 and Australian AF227982 formed a group which is clearly distinct from the Cux1 CAEGFVIR and other Australian isolates.

Table 6.4 Common nucleotide bases substitution in CAV VP3

	Nuclotide position														
Strains	11	30	48	64	74	124	161	200	209	217	235	241	271	273	323
M81223 CAEGFVIR Cux	T	C	C	T	T	A	C	G	C	G	G	C	G	C	C
SN0138_QLD_Kafle	C	.	.	A	.	.	.	.	T	.	.	.	.	.	.
Vaccine_3711_Australia	A	A	.	.	.	.	T	A	T	.	.	.	T	.	.
SN0160_QLD_Kafle	A	A	.	.	.	.	T	A	T	A	.	.	.	.	.
BF4PBYF1 Australia McCoy	A	A	.	.	.	.	T	A	T	.	.	.	.	.	.
AF227982 Australia Brown	A	A	.	.	.	G	T	A	T	.	.	.	.	.	.
SN014_SA_Kafle	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
SN041_VIC_Kafle	.	.	.	.	C	.	.	.	T	.	.	.	.	.	.
SN091_VIC_ Kafle	.	.	T	.	.	.	.	A	T	.	A	.	.	.	.
SN0161_NSW_ Kafle	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
U65414_Australia_Hamooleh	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.
SN063_WA_Kafle	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
SN051_WA_Kafle	.	.	.	.	.	.	.	.	T	.	.	.	.	A	A

Nucleotide differences are compared with CAV **M81223 CAEGFVIR Cux**. Amino acids that are similar in the respective position to M81223 CAEGFVIR Cux are indicated as dots (.).

There were 12 predicted non-synonymous amino acid in all the Australian isolates (Table 6.5). However, common amino acid substitutions at positions histidine (H^4), valine (V^{54}) and asparginine (N^{67}) instead of leucine (L^4), alanine (A4) and threonine (T^{67}) distinguished group one of Australian isolates (CAV 3711, SN0160 QLD, CAV BF4 and Australian AF227982) from group two (SN014 SA, SN051 WA, SN061 WA, SN091 Vic, SN0161 NSW and SN041 Vic) and the Cux1 CAEGFVIR. These are the same groups determined by comparison of nucleotides. The VP3 gene had more than 98% nucleotide sequence homology. These non-synonymous nucleotides had a relatively strong influence on the predicted amino acid composition. Consequently the predicted amino acid diversity was 0.83 - 4.13%.

The field isolate SN0138 QLD was an outlier from both groups. There was a proline (P^4) at position four and a threonine (T^{22}) at position 22. This is consistent with the grouping of this virus along with Cux1 CAEGFVIR in the phylogenetic tree created for 951 bp sequences (Figure 6.2).

Additional amino acid substitutions were noted in SN051 which had two extra amino acid substitutions and SN091 VIC had three substitutions. A common nucleotide differences at position 209 and amino resultant acid substitution at position 70 could possibly be generated by a sequencing error in the original Cux1 CAEGFVIR sequence. Alternately, all the Australian isolates possibly had a common substitution making these isolates different from other strains.

Table 6.5 Common amino acid substitution in CAV VP3

	Amino acid position											
Strains	4	22	25	42	54	67	70	73	79	81	91	108
M81223 CAEGFVIR Cux	L	S	L	I	A	S	S	V	D	P	D	T
SN0138_QLD_Kafle	P	T	.	.	.	.	F	.	.	.	.	.
Vaccine_3711_Australia	H	.	.	.	V	N	F	.	.	.	Y	.
SN0160_QLD_Kafle	H	.	.	.	V	N	F	M	.	.	.	.
BF4PBYF1 Australia McCoy	H	.	.	.	V	N	F	.	.	.	.	.
AF227982 Australia Brown	H	.	.	V	V	N	F	.	.	.	.	.
SN014_SA_Kafle	.	.	.	.	.	.	F	.	.	.	.	.
SN041_VIC_Kafle	.	.	S	.	.	.	F	.	.	.	.	.
SN091_VIC_ Kafle	.	.	.	.	.	N	F	.	N	.	.	.
SN0161_NSW_ Kafle	.	.	.	.	.	.	F	.	.	.	.	.
U65414_Australia_Hamooleh	.	.	.	.	.	.	F	.	.	A	.	.
SN063_WA_Kafle	.	.	.	.	.	.	F	.	.	.	.	.
SN051_WA_Kafle	.	.	.	.	.	.	F	.	.	.	E	N

Amino acid differences are compared with CAV **M81223 CAEGFVIR Cux**. Amino acids that are similar in the respective position to M81223 CAEGFVIR Cux are indicated as dots (.).

The complete VP2 gene of the three Australian isolates including CAV3711 and the terminal 86% of the VP2 gene of the nine field isolates had 22 nucleotide substitutions in their sequences at positions 9, 117, 136, 154,170, 180, 230, 267, 306, 315, 323, 341, 247, 77, 379, 429, 504, 536, 537, 538, 539 and 559 (Table 6.6). However about 50% of the nucleotide substitutions had no influence on predicted amino acids (Table 6.7). Common non-synonymous nucleotide at positions 117, 136, 267 and 306 in sequences of CAV 3711, SN0160 QLD, CAV BF4 and Australian AF227982 formed a group. However, all these substitution had no influence on predicted amino acid composition. The outlier was SN51 with four amino acid substitutions that were not observed in any of the other sequences.

Interestingly, the group two isolates had a common nucleotide substitution at position 504 but this mutation was also silent. In the VP2 gene, 98.77 - 99.46% sequence homology was observed indicating that it is relatively more conserved than is the VP3 gene.

Table 6.6 Common nucleotide bases substitution in CAV VP2 gene

	Nuclotide position																						
Strains	9	117	136	154	170	180	230	267	306	315	323	341	247	377	379	429	504	536	537	538	539	559
M81223 CAEGFVIR Cux	G	T	C	C	T	T	A	C	G	C	G	G	C	G	C	C	C	C	C	A	C	A
SN0138_QLD_Kafle	N/A	C	.	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	G
Vaccine_3711_Australia	A	A	A	.	.	.	.	T	A	T	.	.	.	T	.	.	.	.	.	.	.	G
SN0160_QLD_Kafle	N/A	A	A	.	.	.	.	T	A	T	A	.	.	.	.	.	.	.	.	.	.	G
BF4PBYF1 Australia McCoy	N/A	A	A	.	.	.	.	T	A	T	.	.	.	.	.	.	.	.	.	.	.	G
AF227982 Australia Brown	A	A	A	.	.	.	G	T	A	T	.	.	.	.	.	.	.	.	.	.	.	G
SN014_SA_Kafle	N/A	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	A	.	.	.	.	G
SN041_VIC_Kafle	N/A	.	.	.	.	C	.	.	.	T	.	.	.	.	.	.	A	.	.	.	.	G
SN091_VIC_ Kafle	N/A	.	.	T	.	.	.	.	A	T	.	A	.	.	.	.	A	.	.	.	.	G
SN0161_NSW_ Kafle	N/A	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	A	.	.	.	.	G
U65414_Australia_Hamooleh	.	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	A	.	.	.	.	G
SN063_WA_Kafle	N/A	.	.	.		.	.	.	.	T	.	.	.	.	.	.	A	.	.	.	.	G
SN051_WA_Kafle	N/A	.	.	.	.	.	.	.	.	T	.	.	.	.	A	A		A	.	C	A	G

Nucleotide differences are compared with CAV **M81223 CAEGFVIR Cux**. Nucleotides that are similar in the respective position to M81223 CAEGFVIR Cux are indicated as dots (.).

Sequences not available are indicated as N/A.

Table 6.7 Common amino acid substitution in CAV VP2

	Amino acid position										
Strains	56	77	108	114	116	126	127	170	179	180	187
M81223 CAEGFVIR Cux	F	N	C	R	T	R	P	F	T	T	N
SN0138_QLD_Kafle	Y	.	.	.	.	.	.	.	.	.	D
Vaccine_3711_Australia	.	.	.	.	.	L	.	.	.	.	D
SN0160_QLD_Kafle	.	.	Y	.	.	.	.	.	.	.	D
BF4PBYF1 Australia McCoy	.	.	.	.	.	.	.	.	.	.	D
AF227982 Australia Brown	.	S	.	.	.	.	.	.	.	.	D
SN014_SA_Kafle	.	.	.	.	.	.	.	.	.	.	D
SN041_VIC_Kafle	.	.	.	.	.	.	.	.	.	.	D
SN091_VIC_ Kafle	.	.	.	Q	.	.	.	.	.	.	D
SN0161_NSW_ Kafle	.	.	.	.	.	.	.	.	.	.	D
U65414_Australia_Hamooleh	.	.	.	.	S	.	.	.	.	.	D
SN063_WA_Kafle	.	.	.	.	.	.	.	.	.	.	D
SN051_WA_Kafle	.	.	.	.	.	.	T	I	N	H	D

Amino acid differences are compared with CAV **M81223 CAEGFVIR Cux**. Amino acids that are similar in the respective position to M81223 CAEGFVIR Cux are indicated as dots (.).

The common nucleotide substitutions in the 5' 42% of the VP1 gene of the Australian strain of CAV were at 58 positions. These nucleotide differences in the VP1 gene had clearly generated two groups among the Australian isolates. The strains CAV 3711, SN0160, CAV BF4 and AF227982 (group one) had 13 common nucleotide substitutions at nucleotide positions 186, 198, 270, 282, 345, 372, 420, 435, 441, 489, 525, 534 and 543. The next group (group two) consists of SN014 SA, SN041 VIC, SN091 VIC, SNo161 NSW, U65414, SN063 and SN051 had 12 common nucleotide substitutions at positions 31, 183, 198, 223, 228, 234, 264, 276, 289, 381, 415, and 430. There were 10 common nucleotide substitutions in these two groups at position 86, 195, 201, 222, 252, 318, 339, 373, 555 and 561(Table 6.8). The field isolates, SN0138 QLD had relative more homology with Cux1 CAEGFVIR than they did with these two groups. Although, the field isolate, SN051 WA had greater homology with the second group, it had comparatively more nucleotide substitutions than other members of the group.

Table 6.8 Common nucleotide bases substitution in CAV VP1 gene

	Nuclotide position																			
Strains	31	35	63	64	65	66	86	183	186	195	198	201	210	222	223	228	234	337	240	252
M81223 CAEGFVIR Cux	C	T	C	C	A	C	A	G	G	A	T	T	C	G	G	C	C	G	A	G
SN0138_QLD_Kafle	.	.	.	A	.	.	G	.	.	.	.	.	T	A	.	.	.	.	.	.
Vaccine_3711_Australia	.	.	.	.	.	.	G	.	A	G	C	G	.	A	.	.	.	C	.	A
SN0160_QLD_Kafle	.	.	.	.	.	.	G	.	A	G	C	G	.	A	.	.	.	C	.	A
BF4PBYF1 Australia McCoy	.	.	.	.	.	.	G	.	A	G	C	G	.	A	.	.	.	C	.	A
AF227982 Australia Brown	.	.	.	.	.	.	G	.	A	G	C	G	.	A	.	.	.	C	.	A
SN014_SA_Kafle	A	.	.	.	.	.	G	A	.	G	A	G	.	A	A	T	T	C	.	A
SN041_VIC_Kafle	A	.	.	.	.	.	G	A	.	G	A	G	.	A	A	T	T	T	G	A
SN091_VIC_ Kafle	A	.	.	.	.	.	G	A	.	G	A	G	.	A	A	T	T	C	.	A
SN0161_NSW_ Kafle	A	.	.	.	.	.	G	A	.	G	A	G	.	A	A	T	T	C	.	A
U65414_Australia_Hamooleh	A	.	.	.	.	.	G	A	.	G	A	G	.	A	A	T	T	C	.	A
SN063_WA_Kafle	A	.	.	.	.	.	G	A	.	G	A	G	.	A	A	T	T	C	.	A
SN051_WA_Kafle	A	A	A	.	C	A	G	A	.	G	A	G	.	A	A	T	T	C	.	A

	Nuclotide position																			
Strains	264	270	276	282	289	291	318	321	339	345	372	375	381	406	411	415	420	421	430	432
M81223 CAEGFVIR Cux	C	G	C	A	A	G	T	G	G	C	A	C	C	G	G	A	A	C	G	C
SN0138_QLD_Kafle	.	.	.	G	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	G
Vaccine_3711_Australia	.	T	.	G	.	.	A	.	C	A	G	A	.	.	.	.	G	.	.	G
SN0160_QLD_Kafle	.	T	.	G	.	.	A	.	C	A	G	A	.	.	.	.	G	.	.	G
BF4PBYF1 Australia McCoy	.	T	.	G	.	.	A	.	C	A	G	A	.	A	.	.	G	G	.	G
AF227982 Australia Brown	.	T	.	G	.	.	A	.	C	A	G	A	.	.	.	.	G	.	.	G
SN014_SA_Kafle	T	.	A	.	C	.	A	.	C	G	.	A	A	.	.	C	.	.	C	G
SN041_VIC_Kafle	T	.	A	.	C	T	A	.	C	G	.	A	A	.	T	C	.	.	C	G
SN091_VIC_ Kafle	T	.	A	.	C	.	A	.	C	A	.	A	A	.	.	C	.	.	C	G
SN0161_NSW_ Kafle	T	.	A	.	C	.	A	.	C	G	.	A	A	.	.	C	.	.	C	G
U65414_Australia_Hamooleh	T	.	A	.	C	.	A	.	C	G	.	A	A	.	.	C	.	.	C	G
SN063_WA_Kafle	T	.	A	.	C	.	A	.	C	G	.	A	A	.	.	C	.	.	C	A
SN051_WA_Kafle	T	.	A	.	C	.	A	.	C	G	.	A	A	.	.	C	.	.	C	G

	Nuclotide position																		
Strains	435	441	444	457	465	474	480	486	489	492	507	525	534	542	543	555	558	561
M81223 CAEGFVIR Cux	T	T	T	C	T	C	T	A	G	A	C	C	T	T	C	A	G	G
SN0138_QLD_Kafle	C	.	.	.	.	.	G	.	.	G	.	.	.	.	.	T	A	A
Vaccine_3711_Australia	C	A	.	.	.	.	.	.	T	.	T	A	C	.	T	T	A	A
SN0160_QLD_Kafle	C	A	.	.	.	.	.	.	T	.	T	A	C	.	T	T	A	A
BF4PBYF1 Australia McCoy	C	A	.	.	.	.	.	.	T	.	T	A	C	.	T	T	A	A
AF227982 Australia Brown	C	A	.	.	.	.	.	.	T	.	T	A	C	.	T	T	.	A
SN014_SA_Kafle	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	T	A	A
SN041_VIC_Kafle	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	T	A	A
SN091_VIC_ Kafle	.	.	.	C	.	.	.	T	.	.	T	.	.	.	.	T	A	A
SN0161_NSW_ Kafle	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	A	A
U65414_Australia_Hamooleh	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	A	A
SN063_WA_Kafle	.	.	.	.	C	.	.	.	.	.	T	.	.	.	.	T	A	A
SN051_WA_Kafle	.	.	C	.	.	T	.	.	.	.	T	.	.	.	.	T	A	A

Nucleotide differences are compared with CAV **M81223 CAEGFVIR Cux**. Nucleotides that are similar in the respective position to M81223 CAEGFVIR Cux are indicated as dots (.).

Interestingly, the predicted amino acids substitutions in the VP1 gene were remarkably fewer than would occur if they were randomly distributed suggesting that this protein must maintain a conserved structure. There were 12 predicted amino acids substitutions in this gene (Table 6.9). The two main groups and sub groups corresponded with those predicted by the nucleotide substitutions. There were common amino acid substitutions of arginine (R^{29}), Isoleucine (I^{75}), Leucine (L^{97}), Glutamine (Q^{139}) and Glutamine (Q^{144}) in group two isolates instead of Lysin (K), Valine (V), Methionine (M), Lysine (K) and Aspartic acid (D), and in group one along with SN0138 arginine (R^{29}) and Glutamic acid (E^{144}) were the major amino acid substitutions which differentiate the Australian isolates from the CAEGFVIR Cux1. Both SN0138 and SN051 had additional amino acid substitutions. The rest of other substitutions were scattered and were not shared by more than one isolate.

Table 6.9 Common amino acid substitution in CAV VP1

	Amino acid position											
Strains	12	21	22	29	75	97	136	139	141	144	153	181
M81223 CAEGFVIR Cux	F	H	H	K	V	M	D	K	Q	D	L	F
SN0138_QLD_Kafle	.	.	N	R	.	.	.	.	.	E	.	.
Vaccine_3711_Australia	.	.	.	R	.	.	.	.	.	E	.	.
SN0160_QLD_Kafle	.	.	.	R	.	.	.	.	.	E	.	.
BF4PBYF1 Australia McCoy	.	.	.	R	.	.	N	.	E	E	.	.
AF227982 Australia Brown	.	.	.	R	.	.	.	.	.	E	.	.
SN014_SA_Kafle	.	.	.	R	I	L	.	Q	.	Q	.	S
SN041_VIC_Kafle	.	.	.	R	I	L	.	Q	.	Q	.	.
SN091_VIC_ Kafle	.	.	.	R	I	L	.	Q	.	Q	P	.
SN0161_NSW_ Kafle	.	.	.	R	I	L	.	Q	.	Q	.	.
U65414_Australia_Hamooleh	.	.	.	R	I	L	.	Q	.	Q	.	.
SN063_WA_Kafle	.	.	.	R	I	L	.	Q	.	Q	.	.
SN051_WA_Kafle	Y	Q	P	R	I	L	.	Q	.	Q	.	.

Amino acid differences are compared with CAV **M81223 CAEGFVIR Cux**. Amino acids that are similar in the respective position to M81223 CAEGFVIR Cux are indicated as dots (.).

There were consistent differences in nucleotides in all the Australian isolates in a few positions in the VP1, VP2 and VP3 genes compared with Cux1 CAEGFVIR.

These results clearly indicated that there were different strains of CAV in the samples tested. Although the vaccine strain 3711 was grouped in group one which included one of the field isolates sequenced in this project, none of the isolates were identical to the vaccine virus.

6.3.4 Nucleotide comparisons of CAV Australian isolates with the vaccine strain 3711

In order to determine whether the field isolates were vaccine virus that had been transmitted vertically or horizontally to the offspring a comparison was made between CAV 3711 and the other isolates. The available sequence data for VP1, VP2 and VP3 genes of all the Australian field isolates of CAV were compared with the CAV 3711 at both the nucleotide and amino acid level.

The VP3 gene of CAV Australian field isolates had 14 nucleotide substitutions when compared with strain 3711(Table 6.10). The nucleotide substitutions at positions 11, 30, 161 and 200 were common in the group two and also in the SN0138 QLD. There were common nucleotide substitutions in all the isolates at the position 271.

The predicted amino acids substitutions were most prominent in group two isolates with leucine (L^{4}) replacing histidine (H^{4}), alanine (A^{54}) replacing valine (V^{54}) and in all but one isolate serine (S^{67}) replacing asparagine (N^{67}) (Table 6.11). All of the isolates had a substitution at position 91 while SN138 shared the substitutions at positions 54 and 67 with the group two isolates.

Table 6.10 Common nucleotide substitution in the VP3 gene

	Nuclotide position													
Strains	11	30	48	64	74	124	161	200	217	235	241	271	273	323
Vaccine_3711_Australia	A	A	C	T	T	A	T	A	G	G	C	T	C	C
SN0138_QLD_Kafle	C	C	.	A	.	.	C	G	.	.	.	G	.	.
SN0160_QLD_Kafle	.	.	.	.	.	.	.	.	A	.	.	G	.	.
BF4PBYF1 Australia McCoy	.	.	.	.	.	.	.	.	.	.	.	G	.	.
AF227982 Australia Brown	.	.	.	.	.	G	.	.	.	.	.	G	.	.
SN014_SA_Kafle	T	C	.	.	.	.	C	G	.	.	.	G	.	.
SN041_VIC_Kafle	T	C	.	.	C	.	C	G	.	.	.	G	.	.
SN091_VIC_ Kafle	T	C	T	.	.	.	C	.	.	A	.	G	.	.
SN0161_NSW_ Kafle	T	C	.	.	.	.	C	G	.	.	.	G	.	.
U65414_Australia_Hamooleh	T	C	.	.	.	.	C	G	.	.	G	G	.	.
SN063_WA_Kafle	T	C	.	.	.	.	C	G	.	.	.	G	.	.
SN051_WA_Kafle	T	C	.	.	.	.	C	G	.	.	.	G	A	A

Nucleotide differences are compared with **CAV strain 3711**.
Nucleotides that are similar in the respective position to **CAV strain 3711**are indicated as dots (.).

Table 6.11 Common amino acid substitution in CAV VP3

	Amino acid position										
Strains	4	22	25	42	54	67	73	79	81	91	108
Vaccine_3711_Australia	H	S	L	I	V	N	V	D	P	Y	T
SN0138_QLD_Kafle	P	T	.	.	A	S	.	.	.	D	.
SN0160_QLD_Kafle	.	.	.	.	.	.	M	.	.	D	.
BF4PBYF1 Australia McCoy	.	.	.	.	.	.	.	.	.	D	.
AF227982 Australia Brown	.	.	.	V	.	.	.	.	.	D	.
SN014_SA_Kafle	L	.	.	.	A	S	.	.	.	D	.
SN041_VIC_Kafle	L	.	S	.	A	S	.	.	.	D	.
SN091_VIC_ Kafle	L	.	.	.	A	.	.	N	.	D	.
SN0161_NSW_ Kafle	L	.	.	.	A	S	.	.	.	D	.
U65414_Australia_Hamooleh	L	.	.	.	A	S	.	.	A	D	.
SN063_WA_Kafle	L	.	.	.	A	S	.	.	.	D	.
SN051_WA_Kafle	L	.	.	.	A	S	.	.	.	E	N

Amino acid differences are compared with **CAV strain 3711**.
Amino acids that are similar in the respective position to **CAV strain 3711** are indicated as dots (.).

The VP2 gene of the CAV Australian isolates had relatively fewer nucleotide differences as compared with the VP1 gene. There were 19 substitutions. Common nucleotide substitutions at positions 117, 136, 306, 267, and 504 were noted in

sequences of the group two isolates (Table 6.12). The isolate SN0138 QLD shared four nucleotides substitutions in common with the group two isolates.

The common nucleotide substitutions had no influence on the predicted amino acid substitutions. An amino acid substitution, leucine (L^{126}) with arginine (R) was detected in all the Australian isolates. The isolate SN051 had five amino acid substitutions whereas rest of other isolates had two substitutions (Table 6.13).

Table 6.12 Common nucleotide substitution in the VP2 gene

	Nuclotide position																			
Strains	9	117	136	154	170	180	230	267	306	323	341	247	377	379	429	504	536	537	538	539
Vaccine_3711_Australia	A	A	A	C	T	T	A	T	A	G	G	C	T	C	C	C	C	C	A	C
SN0138_QLD_Kafle	N/A	C	C	.	A	.	.	C	G	.	.	.	G	.	.	.	.	A	.	.
SN0160_QLD_Kafle	N/A	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.
BF4PBYF1 Australia McCoy	N/A	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.
AF227982 Australia Brown	.	.	.	.	.	.	G	.	.	.	.	.	G	.	.	.	.	.	.	.
SN014_SA_Kafle	N/A	T	C	.	.	.	.	C	G	.	.	.	G	.	.	A	.	.	.	.
SN041_VIC_Kafle	N/A	T	C	.	.	C	.	C	G	.	.	.	G	.	.	A	.	.	.	.
SN091_VIC_ Kafle	N/A	T	C	T	.	.	.	C	.	.	A	.	G	.	.	A	.	.	.	.
SN0161_NSW_ Kafle	N/A	T	C	.	.	.	.	C	G	.	.	.	G	.	.	A	.	.	.	.
U65414_Australia_Hamooleh	G	T	C	.	.	.	.	C	G	.	.	G	G	.	.	A	.	.	.	.
SN063_WA_Kafle	N/A	T	C	.		.	.	C	G	.	.	.	G	.	.	A	.	.	.	.
SN051_WA_Kafle	N/A	T	C	.	.	.	.	C	G	.	.	.	G	A	A	A	A	.	C	A

Nucleotide differences are compared with **CAV strain 3711**.
Nucleotides that are similar in the respective position to **CAV strain 3711** are indicated as dots (.).
Sequences not available are indicated as N/A.

Table 6.13 Common amino acid substitution in CAV VP2

	Amino acid position									
Strains	56	77	108	114	116	126	127	170	179	180
Vaccine_3711_Australia	F	N	C	R	T	L	P	F	T	T
SN0138_QLD_Kafle	Y	.	.	.	.	R	.	.	.	.
SN0160_QLD_Kafle	.	.	Y	.	.	R	.	.	.	.
BF4PBYF1 Australia McCoy	.	.	.	.	.	R	.	.	.	.
AF227982 Australia Brown	.	S	.	.	.	R	.	.	.	.
SN014_SA_Kafle	.	.	.	.	.	R	.	.	.	.
SN041_VIC_Kafle	.	.	.	.	.	R	.	.	.	.
SN091_VIC_ Kafle	.	.	.	Q	.	R	.	.	.	.
SN0161_NSW_ Kafle	.	.	.	.	.	R	.	.	.	.
U65414_Australia_Hamooleh	.	.	.	.	S	R	.	.	.	.
SN063_WA_Kafle	.	.	.	.	.	R	.	.	.	.
SN051_WA_Kafle	.	.	.	.	.	R	T	I	N	H

Amino acid differences are compared with **CAV strain 3711**.
Amino acids that are similar in the respective position to **CAV strains 3711** are indicated as dots (.).

The sequence data comparisons and construction of phylogenetic tree on the basis of nucleotide sequence of starting 42% of the VP1 gene clearly demonstrated three groups (Figure 6.3). There were 23 common nucleotide bases non-synonymous in Australian group two (SN014 SA, SN041 VIC, SN091VIC, SN0161 NSW, SN063 WA, SN051 and U65414) in the VP1 gene (Table 6.14). There were minimum differences in SN0160 QLD, BF4 and AF227982. There was no nucleotide differences in sequence of the SN0160 QLD, one and two nucleotide substitutions in BF4 and AF227982 respectively. The highest nucleotide difference in the Australian isolates was in sequence of SN051 (30 substitutions). The SN0138 maintained its differences distinct from both but relatively close to group two on the basis of nucleotide differences but amino acid substitutions demonstrated relatively close to group one.

There were 23 nucleotide bases differences in sequence of SN0138 QLD in this gene. Ten substitutions were common as in group two isolates. However these non-synonymous nucleotides had no influence on the amino acid compositions. There was only one amino acid substitution in SN0138 QLD.

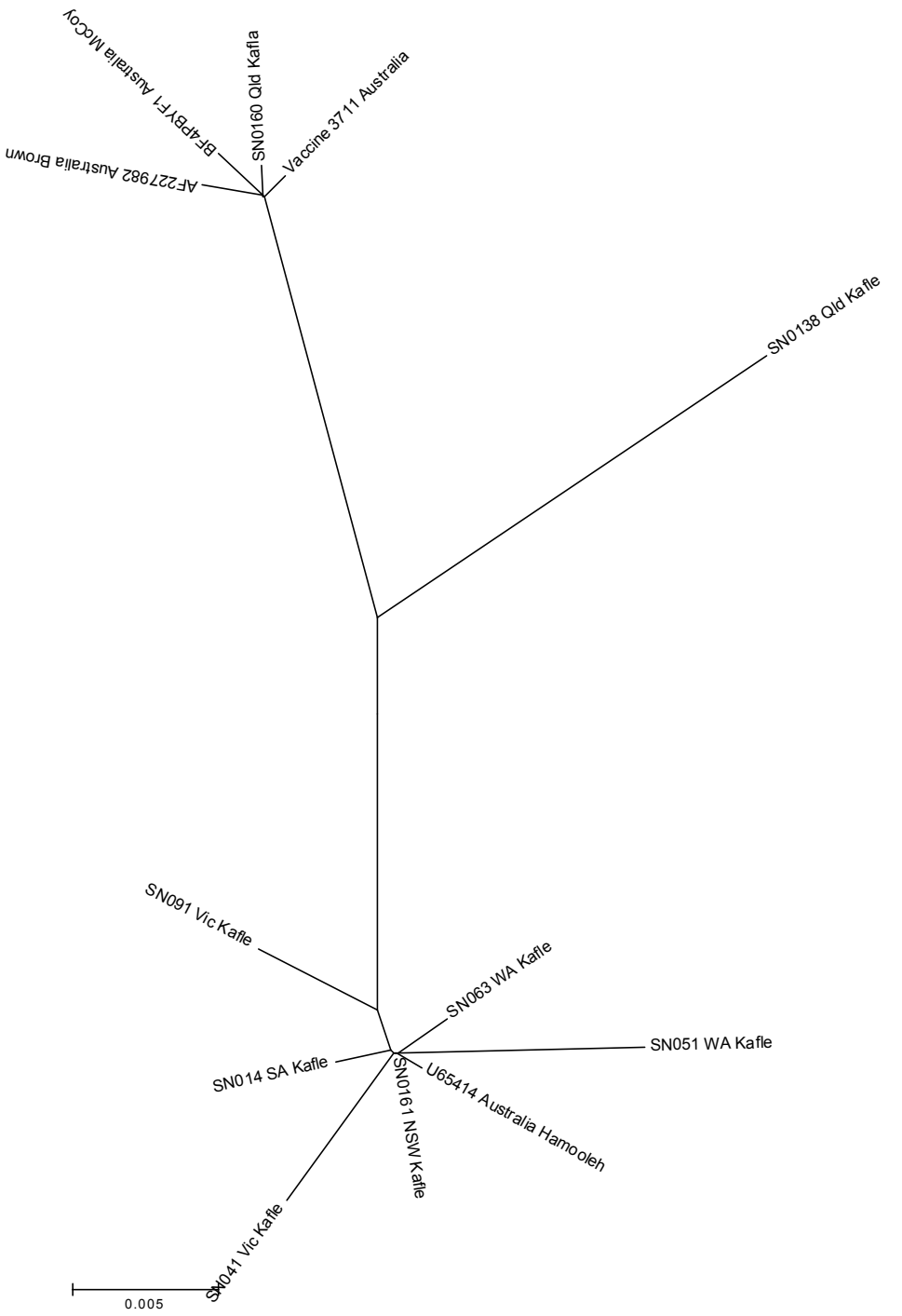


Figure 6.3 Phytogenic analysis of starting 42% sequences of VP1 Australian CAV isolates, MEGA3 (ME, tree/branch style- radiation)

Table 6.14 Common nucleotide bases substitution in the CAV VP1 gene

	Nuclotide position																		
Strains	31	35	63	64	65	66	183	186	195	198	201	210	223	228	234	337	240	252	264
Vaccine_3711_Australia	C	T	C	C	A	C	G	A	G	C	G	C	G	C	C	C	A	A	C
SN0138_QLD_Kafle	.	.	.	A	.	.	.	G	A	T	T	T	.	.	.	.	.	G	.
SN0160_QLD_Kafle	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BF4PBYF1 Australia McCoy	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AF227982 Australia Brown	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SN014_SA_Kafle	A	.	.	.	.	.	A	G	.	A	.	.	A	T	T	.	.	.	T
SN041_VIC_Kafle	A	.	.	.	.	.	A	G	.	A	.	.	A	T	T	T	G	.	T
SN091_VIC_ Kafle	A	.	.	.	.	.	A	G	.	A	.	.	A	T	T	.	.	.	T
SN0161_NSW_ Kafle	A	.	.	.	.	.	A	G	.	A	.	.	A	T	T	.	.	.	T
U65414_Australia_Hamooleh	A	.	.	.	.	.	A	G	.	A	.	.	A	T	T	.	.	.	T
SN063_WA_Kafle	A	.	.	.	.	.	A	G	.	A	.	.	A	T	T	.	.	.	T
SN051_WA_Kafle	A	A	A	.	C	A	A	G	.	A	.	.	A	T	T	.	.	.	T

	Nuclotide position																		
Strains	270	276	282	289	291	318	321	339	345	372	375	381	406	411	415	420	421	430	432
Vaccine_3711_Australia	T	C	G	A	G	A	G	C	A	G	A	C	G	G	A	G	A	G	G
SN0138_QLD_Kafle	G	.	.	.	.	T	A	G	G	A	C	.	.	.	.	A	.	.	.
SN0160_QLD_Kafle	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BF4PBYF1 Australia McCoy	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	.
AF227982 Australia Brown	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SN014_SA_Kafle	G	A	A	C	.	.	.	.	G	A	.	A	.	.	C	A	.	C	.
SN041_VIC_Kafle	G	A	A	C	T	.	.	.	G	A	.	A	.	T	C	A	.	C	.
SN091_VIC_ Kafle	G	A	A	C	.	.	.	.	.	A	.	A	.	.	C	A	.	C	.
SN0161_NSW_ Kafle	G	A	A	C	.	.	.	.	G	A	.	A	.	.	C	A	.	C	.
U65414_Australia_Hamooleh	G	A	A	C	.	.	.	.	G	A	.	A	.	.	C	A	.	C	.
SN063_WA_Kafle	G	A	A	C	.	.	.	.	G	A	.	A	.	.	C	A	.	C	A
SN051_WA_Kafle	G	A	A	C	.	.	.	.	G	A	.	A	.	.	C	A	.	C	.

	Nuclotide position															
Strains	435	441	444	457	465	474	480	486	489	492	507	525	534	542	543	558
Vaccine_3711_Australia	C	A	T	T	T	C	T	A	T	A	T	A	C	T	T	A
SN0138_QLD_Kafle	.	T	.	.	.	.	G	.	G	G	C	C	T	.	C	.
SN0160_QLD_Kafle	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BF4PBYF1 Australia McCoy	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AF227982 Australia Brown	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
SN014_SA_Kafle	T	T	.	.	.	.	.	.	G	.	C	C	T	C	C	.
SN041_VIC_Kafle	T	T	.	.	.	.	.	G	G	.	.	C	T	.	C	.
SN091_VIC_ Kafle	T	T	.	C	.	.	.	T	G	.	.	C	T	.	C	.
SN0161_NSW_ Kafle	T	T	.	.	.	.	.	.	G	.	.	C	T	.	C	.
U65414_Australia_Hamooleh	T	T	.	.	.	.	.	.	G	.	.	C	T	.	C	.
SN063_WA_Kafle	T	T	.	.	C	.	.	.	G	.	.	C	T	.	C	.
SN051_WA_Kafle	T	T	C	.	.	T	.	.	G	.	.	C	T	.	C	.

Nucleotide differences are compared with **CAV strain 3711**.
Nucleotides that are similar in the respective position to **CAV strain 3711** are indicated as dots (.).

The nucleotide substitutions in VP1 are responsible for very few amino acid substitutions (Table 6.). Common amino acid substitutions are Isoleucine (I^{75}), Leucine (L^{97}), Glutamine (Q^{139}) and Glutamine (Q^{144}) in group two instead of Valine (V), Methionine (M), Lysine (K) and Glutamic acid (E). Within group one SN0160 QLD and AF227982 had complete homology with respect to amino acids while CAV BF4 had two animo acid substitutions.

Table 6.15 Common amino acid substitution in CAV VP1

	Amino acid position										
Strains	12	21	22	75	97	136	139	141	144	153	181
Vaccine_3711_Australia	F	H	H	V	M	D	K	Q	E	L	F
SN0138_QLD_Kafle	.	.	N	.	.	.	.	.	.	.	.
SN0160_QLD_Kafle	.	.	.	.	.	.	.	.	.	.	.
BF4PBYF1 Australia McCoy	.	.	.	.	.	N	.	E	.	.	.
AF227982 Australia Brown	.	.	.	.	.	.	.	.	.	.	.
SN014_SA_Kafle	.	.	.	I	L	.	Q	.	Q	.	S
SN041_VIC_Kafle	.	.	.	I	L	.	Q	.	Q	.	.
SN091_VIC_ Kafle	.	.	.	I	L	.	Q	.	Q	P	.
SN0161_NSW_ Kafle	.	.	.	I	L	.	Q	.	Q	.	.
U65414_Australia_Hamooleh	.	.	.	I	L	.	Q	.	Q	.	.
SN063_WA_Kafle	.	.	.	I	L	.	Q	.	Q	.	.
SN051_WA_Kafle	Y	Q	P	I	L	.	Q	.	Q	.	.

Amino acid differences are compared with **CAV strain 3711**.
Amino acids that are similar in the respective position to **CAV strain 3711** are indicated as dots (.).

Subsequently these results have confirmed that none of the eight isolates of CAV detected in the broilers are identical to the vaccine virus.

6.4 Discussion

This project aimed to characterise CAV from selected field samples and the vaccine viral strain 3711 in an attempt to determine the most likely source of infection in the broiler flocks. It is hypothesised based on the PCR results that there could be multiple strains of CAV circulating in Australian broiler flocks.

The sequencing results confirm the presence of three distinctly different groups of viruses infecting broiler flocks throughout Australia. Attempts were made to determine a full-length sequence from one representative of each of the groups as well as the vaccine strain 3711. A 951 bp sequence was achieved from eight of the field strains, a reference strain BF4 and a near full-length sequence was achieved for the vaccine strain 3711. Phylogenetic studies were based on this 951 bp sequence which comprises 42% (190 of 449 codons) of the 5' VP1gene, 86% (185 of 216 codons) of the 3' VP2 gene and the complete (121 codons) of the VP3 gene.

Like most of the sequences of CAV in this particular portion of the genome, 96% to 99% homology was noted when the Australian isolates were compared with Cux 1 (van Santen *et al.*, 2001; Islam *et al.*, 2002; Ducatez *et al.*, 2005). A similar percentage homology was observed between the Australian groups when compared with strain 3711. At least 96%, 98% and 98% sequence homology was observed in sequences of VP1, VP2 and VP3 genes respectively and the predicted amino acid sequences of same fragments of genome had 96%, 98% and 96% homology in VP1, VP2 and VP3 genes respectively when compared with Cux1. The sequence homology of all three genes within the Australian isolates at the nucleotide level was observed to be comparable to the comparisons with Cux1 but the predicted amino acids in two proteins, VP1 and VP3 genes had only 95% homology. This value is 1% lower than the comparisons with Cux1 and indicated that two groups of Australian isolates are relatively more diverse than Cux1.

Although the genome of CAV is relatively conserved, none of the isolates is genetically identical to any other isolate. Absolute homology was observed in the sequence of VP1 and only two nucleotide substitutions in VP2 and VP3 genes of the strain 3711 and the SN0160 QLD. This result indicates that this particular strain of

CAV could be either the vaccine virus transmitted from breeder birds to their progeny or the two viruses at least shared a common recent origin. The isolate SN0160 was obtained from a Queensland broiler flocks. The strain 3711 was obtained from the Intervet Australia but the virus was characterised by Conner and it was originally isolated from the liver of 18-day-old Queensland broiler breeder with clinical symptoms of CAV infection (Connor *et al.*, 1991). The sharing of a common origin is more realistic because only SN0160QLD had this level of homology compared with the sequence of eight isolates which comes from the different geographical region.

If these viruses are not of vaccine virus origin this result clearly indicates that majority of currently existing CAV isolates in the Australian broilers could be transmitted to the progeny either by horizontal transmission from the virus contaminated poultry shed or could be the wild type CAV infecting to the parental flocks and transmitted vertically to the progeny. Further study is essential to differentiate the horizontal and vertical transmission.

There were some common nucleotide differences and subsequently predicted amino acid differences in all the isolates. These differences could possibly be generated by sequencing errors in the original Cux1. Alternatively, Australian isolates were genetically different than the Cux1 CAEGFVIR. Further comparisons between these isolates and sequence of different strains from other part of the world could be useful for speculating on the evolution of these common substitutions. Furthermore, a common synonymous nucleotide base difference corresponds to position 177 of the VP2 gene and to position 271 of VP3 gene is possibly a substitution in the vaccine strain.

The novel amino acid substitutions in group one isolates at positions histidine (H^4), valine (V^{54}) and asparginine (N^{67}) instead of leucine (L^4), alanine (A4) and threonine (T^{67}) respectively, in the VP3 gene are group specific (Table 6.13). The isolate SN0138QLD had predicted amino acids that are indicative of group two isolates in the positions 54 and 67 but at position 4 it had proline (P^4). Publications so far have not discussed these novel amino acids at the above described positions. Further

sequence alignment with the other strains from the different geographical location of the world could be relatively more conclusive.

A portion of the CAV genome is clearly easy to sequence and one segment is relatively difficult. The fragment of noncoding region of the CAV genome has a G-C loop and has been described as being the most difficult for sequencing (Noteborn *et al.*, 1991; Meehan *et al.*, 1992; Brown *et al.*, 2000; Islam *et al.*, 2002). Similar difficulties were encountered in this project and a 19 bp stretch from 2188-2205 remains unresolved.

The sequencing results of the bands produced by the VP1 front, VP1 tail primer pairs and the promoter/enhancer region of the CAV are a constant reminder of potential problems that can be encountered. These primers produced five different bands that were not viral DNA. They were identified as chicken genome, bacterial genome and one band has yet to be identified. BLAST searches of these primers did not predict these results and this is a constant reminder of the deficiencies in the present genome databases. It is also a warning for those who attempt to rely on diagnostic PCR that uses only a single pair of primers.

The sequence obtained from the sample of genomic DNA includes both introns and exons, the published mRNA sequences (GenBank, XM_430317.1 and XM_423531) only include the exons. However, a search of the chicken genome project confirmed the entire sequence as chicken genome. Both ends of the aligned sequences have consensus primer sequences that are recognised as CAV on BLAST. It is now clear that the primers used for the amplification of these fragments of the genome appeared to have enough similarity to initiate the DNA amplification of the chicken genome. Information regarding genetic similarity of the chicken to the CAV is very limited.

The promoter-enhance region of the CAV genome contains four consensus cyclic AMP response element sequences (AGCTCA), which are similar to the oestrogen response element consensus half-sites (A)GGTCA. This similarity influences/ favours the viral replication and is responsible for the seroconversion in the chicken at the age of sexual maturity (Miller *et al.*, 2005). This result further demonstrated

the similarity between chicken genome and the CAV sufficient to initiate DNA amplification.

An interesting point to note is that only clinical samples have produced this type of the result. A similar procedure was followed in each sequencing reaction. In fact most of the sequencing procedures were carried out in batches and the vaccines viral DNA was also included together with the samples. The vaccine virus should have been contaminated with chicken genome because it was grown in MSB1 cells. Variations in chicken genome sequence may have some influence on the results. A similar result has been observed with other diagnostic assays in this laboratory in that additional bands can be recognised in PCR from specific strains of commercial poultry (results not shown).

It would be possible to design additional primers based on the generated sequences and then confirm their reaction with a range of avian samples. However, this was beyond the objectives of this project.

The next two sequences other than CAV were produced using the primer pair VP1-tail and the middle DNA band from the primer set repeat gap filling primers. It is possible that both these sequences are generated from the chicken genome. However, no matches were generated from standard BLAST searches.

The 563 bp sequence obtained from the largest band of three DNA band of Rep- gap filling primer pair was another challenge for this project. This sequence had 85% similarity in the sequence position 34-551 to sequence of *Psychrobacter arcticus* 273-4 on BLAST. This organism is Gram-negative, non-motile, aerobic coccobacillus and psychotropic (cold-living) and was previously called a Moraxella–like organism. It is not surprising that the skin is the normal habitat of the Moraxella group of organism and the sample material used in this project is the feather follicle epithelium. *Psychrobacter* bacteraemia have been described in a cirrhotic patient due to consumption of raw geodic clam (Leung *et al.*, 2005). To date, no literature has described *Psychrobacter* infection in poultry. This sequence contained one open reading frame and a protein BLAST search indicated a 97% homology with a fructose-bisphosphate aldolase class II from *Psychrobacter arcticus*. It was therefore

concluded that the source organism was either a skin pathogen or commensal in the clinical sample.

6.5 Conclusion

The genome of CAV is relatively conserved. However, all the CAV isolates sequenced in this project are genetically different to one another. The majority of the isolates are placed in different groups than the vaccine strain. One field isolate has a high level of homology with the vaccine virus, possibly due to common origin. The detected viruses could be wild type CAV either from the broiler shed or transmitted vertically.

The sequence generated other than CAV and identified partially as chicken genome and bacterial genome which is the next important piece of information obtained during sequencing, questioning the reliability of single round PCR including real time PCR based on CYBR green as diagnostic tools.

CHAPTER 7

MOLECULAR EPIDEMIOLOGY OF CHICKEN ANAEMIA VIRUS IN AUSTRALIA

7.1 Introduction

The molecular characterisation of Australian CAV isolates reported in the previous chapter demonstrated three logical groups of viruses. Isolates in each of these three groups shared common groups of nucleotide and amino acid substitutions.

A striking feature of all three viral proteins was the level of conservation of amino acids especially in VP2. The initial comparisons were made between Australian isolates produced in this study and published sequences of Australian isolates with one of the earliest sequences to be published (Cux1CAEGFVIR) (Meehan *et al.*, 1992) and then with the sequence of the Australian vaccine virus 3711.

Full-length sequences of CAV have been published from a variety of studies and this chapter includes a comparison of Australian isolates with published data from the rest of the world. It was hoped that this analysis would allow speculation on the source of Australian isolates and the extent to which they had evolved in isolation in Australia.

7.2 Materials and methods

This chapter utilises the sequence data of all the Australian isolates discussed in previous chapter (molecular characterisation) and sequences from the GenBank database. The descriptions of the CAV strains used for comparisons are listed in Table 7.1.

The nearly complete sequence of the vaccine strain 3711 was first aligned with the 26 published sequences of CAV.

The 951 bp sequence generated for most of the isolates in this study was then compared with a similar segment from 26 other published sequences. This allowed the nucleotide and predicted amino acid sequence of the complete VP3 gene, the 3' 86% of VP2 and the 5' 42% of VP1 to be compared.

The sequences were aligned using the Clustal W algorithm in GeneDoc (Nicholas and Nicholas. Jrn., 1997). GeneDoc was also used to produce predicted amino acid sequences for each of the three genes. Unrooted phylogenetic trees were produced using MEGA3 (Kumar *et al.*, 2004) and the minimum evolution algorithms.

Table 7.1 CAV sequences used for multiple alignment analysis

Despription	**Strain**	**Geographical region**	**Sequence available**	**References**
M81223_CAEGFVIR_Cux1	Cux1	Germany	2298 bp	Meehan et al., 1992
CAN297684__Scott	Cux-1-33	Germany	2298 bp	Scott et al., 2000
CAN297685_Scott	Cux-1-34	Germany	2297 bp	Scott et al., 2000
AY843527__Liang	TJBD33	China	2298 bp	Liang, 2005
AF313470_Spackman	Del-Ros	USA	2294 bp	Spackman et al., 2000
D10068_Claessens	CAE26P4	USA	2298 bp	Claessens et al, 1991
AB031296_Yamaguchi	A2	Japan	2298 bp	Yamaguchi et al 2000
AY040632_Chowdhury	3-1P60	Malaysia	2298 bp	Chowdhury et al, 2003
AY839944_Liang	LF4	China	2298 bp	Liang, 2005
AF390102_Chowdhury	SMSC-1P60	Malaysia	2298 bp	Chowdhury et al, 2003
AF311892_van_Santen	98D02152	USA	2298 bp	van Santen et al 2001
AF475908_He	China: Harbin	China	2298 bp	He et al , 2002
AY846844_Liang	TJBD40	China	2298 bp	Liang, 2004
AY999018_Liang	SD24	China	2298 bp	Liang, 2005
AB046588_Yamaguchi	AH9410 (C364)	Japan	2298 bp	Yamaguchi et al., 2001
AB046587_Yamaguchi	AH9410 (C140)	Japan	2298 bp	Yamaguchi et al., 2001
AB046590_Yamaguchi	AH9410 (C369)	Japan	2298 bp	Yamaguchi et al., 2001
AB046589_Yamaguchi	AH9410 (C368)	Japan	2298 bp	Yamaguchi et al., 2001
AF395114_Islam	BD-3	Bangaldesh	2298 bp	Islam et al., 2002
L14767_Renshaw	CIA-1	USA	2298 bp	Renshaw, 1996
AF227982_Brown	CAU269/7	Australia	2286 bp	Brown et al., 2000
AF311900__van_Santen	98D06073	USA	2298 bp	van Santen et al, 2001
AB027470_Okamura	TR20	Japan	2298 bp	Okamura et al., 1999
AB119448_Imai	G6	Japan	2298 bp	Imai et al., 2004
AF285882_Chowdhury	SMSC-1	Malaysia	2298 bp	Chowdhury et al, 2003
U65414_Hamooleh	704	Australia	2298 bp	Hamooleh et al, 1996

7.3 Results

Analysis of the available sequence (2279 bp) of the vaccine strain 3711 demonstrated at least 94% nucleotide homology with the published sequences. Some of the published sequences contained insertions or deletions. The Cux-1-34 strain was 2297 bp long with a single deletion at position 2185 (A). The CAV Del-Ros had four nucleotide bases deleted at 2195 (G) and at 2209 - 2211 (CCC) and was 2294 bp long. The strain CAE26P4 was 2298 bp long but it had one base deletion and one insertion at positions 2245 (A) and 2252 (C) respectively. Similarly strain TJ9410 also had one base deletion and insertion at positions 2257 (A) and 2252 (T). The strain CAU269/7 and the vaccine strain 3711 had 12 bp and 19 bp deletions at positions 2193–2205 (GGCTAAAGCCCC) and 2188 - 2205 (GGGGGGGGCTAAAGCCCC). These two strains had a common nucleotide deletion at position 117 (C) and the strain CAU269/7 had one insertion at position 177 (G).

Analysis of the alignment of the 951 bp sequences from the 10 isolates produced in this project and 26 published sequences from GenBank including two Australian viruses demonstrated more than 96% homology. The Australian isolates, SN0161 NSW, SN063 WA, SN041 Vic, SN091 Vic and SN014 SA had 99% homology with two published isolates 98D06073 (USA) and TR20 (Japan). Similarly, the SN0138 Qld had 99% homology with two published isolates 98D02152 (USA) and China:Harbin (China). The group one Australian isolates (SN0160 Qld and BF4 and vaccine 3711) had between 96% and 97% homology with other sequences in the group.

The available sequence of VP1, VP2 and the complete VP3 genes of all of these isolates shared 94%, 98% and 98%, nucleotide identities respectively. Overall it was noted that the CAV isolates had limited genomic variability. However, none of the sequences were identical to any of the published sequences.

The VP1 gene appeared to be the most diverse of the three when nucleotides were compared. The predicted amino acid diversity in the 5’ 42% available sequence of

VP1, the 3' 86% of VP2 and the complete VP3 gene of all of the Australian isolates with the published sequences ranged from 0-5%, 0-2% and 0-7% respectively.

The majority of the nucleotide differences between the isolates were synonymous in the VP2 gene. The non-synonymous mutations in the PV2 gene of all the Australian isolates were scattered and not shared by other isolates (Table 7.2). The Western Australian isolate SN051 stood out with four amino acid substitutions that were not observed in the sequence of any other isolate.

The amino acid substitutions in VP3 appeared to be scattered randomly throughout the gene with the exception of four Australian isolates (CAV strains 3711, BF4, SN0160 and CAU269/7) that had common predicted amino acid substitutions at positions 4 (L-H),54 (A-V) and 67 (S-N). The substitution at position 67 (S-N) were noted in four other isolates TR20, TJBD40, BD3 and SN091 Vic (one of the group two Australian isolates) (Table 7.3).

The VP1 gene appeared to be less conserved; with 20 substitutions out of 190 codons (Table 6.4).

The four Australian isolates SN0160 Qld, 3711, BF4 and SN0138 Qld closely resembled with CAU269/7 (Australia), 98d02152 (USA), Del-Ros (USA) and China:Harbin (China).

The majority of the isolates in this study (SN014 SA, SN041 Vic, SN091 Vic, SN0161 NSW, SN063 WA and SN051 WA) appeared to be relatively homogenous with CIA-1 sharing similar amino acid substitutions with seven other isolates including one previously published Australian isolate 704 (Hamooleh *et al.*, 1996). Other isolates in this group included TR20 (Japan), 98D06073 (USA), CIA-1 (USA), G6 (JAPAN), SMSC-1 (Malaysia), BD3 (Bangladesh). The signature amino acid substitutions were Isoleucine (I^{75}), Leucine (L^{97}), Glutamine (Q^{139}) and Glutamine (Q^{144}) (Table 6.4).

Table 7.2 Non-synonymous predicted amino acids in the VP2 gene

	Amino acid positions																	
strain	5	6	35	54	57	77	108	114	116	126	127	153	170	179	180	186	187	201
Cux1	G	G	S	T	F	N	C	R	T	R	P	V	F	T	T	E	N	E
Cux-1-33	.	.	L	S	.	.	.	.	.	.	.	A	.	.	.	G	D	.
Cux-1-34	.	.	L	S	.	.	.	.	.	.	.	.	.	.	.	G	D	.
TJBD33	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
Del-Ros	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
CAE26P4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	D	.
A2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		D	.
3-1P60	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	D	G
LF4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
SMSC-1P60	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	D	.
98D02152	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
China: Harbin	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
SN0138 QLD	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	D	.
TJBD40	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
SD24	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
AH9410 (C364)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
AH9410 (C140)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
AH9410 (C369)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
AH9410 (C368)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
BD-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
CIA-1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
Vaccine 3711	.	.	.	.	.	.	Y	.	.	L	.	.	.	.	.	.	D	.
SN0160 QLD	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
BF4	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	D	.
CAU269/7	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	D	.
98D06073	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	D	.
TR20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
SN014 SA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
SN041 Vic	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
SN091 Vic	.	.	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	D	.
G6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
SMSC-1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
SN0161 NSW	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
704	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	D	.
SN063 WA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.
SN051 WA	.	.	.	.	.	.	.	.	.	.	T	.	I	N	H	.	D	.

Amino acid differences are compared with CAV **M81223 CAEGFVIR Cux**.
Amino acids that are similar in the respective position to M81223 CAEGFVIR Cux are indicated as dots (.).

Table 7.3 Common amino acid substitution in VP3

	Amino acid positions																			
strain	3	4	19	21	22	25	41	42	54	64	67	70	73	74	79	81	91	103	108	118
Cux1	A	L	P	S	S	L	T	I	A	N	S	S	V	P	D	P	D	S	T	C
Cux-1-33	.	.	A	.	.	.	I	.	.	.	.	F	.	Q	.	.	.	.	.	R
Cux-1-34	.	.	A	.	.	.	.	.	.	.	.	F	.	Q	.	.	.	.	.	.
TJBD33	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
Del-Ros	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
CAE26P4	.	.	.	.	.	.	.	.	.	S	.	F	A	.	.	.	.	N	.	.
A2	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
3-1P60	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	H	.	.	.	R
LF4	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
SMSC-1P60	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	R
98D02152	G	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
China: Harbin	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
SN0138 QLD	.	P	.	.	T	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
TJBD40	.	.	.	.	.	.	.	.	.	.	N	F	.	.	.	.	.	N	.	.
SD24	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
AH9410 (C364)	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
AH9410 (C140)	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
AH9410 (C369)	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
AH9410 (C368)	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
BD-3	.	.	.	.	.	.	.	.	.	.	N	F	.	.	.	.	.	.	.	.
CIA-1	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
Vaccine 3711	.	H	.	.	.	.	.	.	V	.	N	F	.	.	.	.	Y	.	.	.
SN0160 QLD	.	H	.	.	.	.	.	.	V	.	N	F	M	.	.	.	.	.	.	.
BF4	.	H	.	.	.	.	.	.	V	.	N	F	.	.	.	.	.	.	.	.
CAU269/7	.	H	.	.	.	.	.	V	V	.	N	F	.	.	.	.	.	.	.	.
98D06073	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
TR20	.	.	.	.	.	.	.	.	.	.	N	F	.	.	.	.	.	.	.	.
SN014 SA	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
SN041 Vic	.	.	.	.	.	S	.	.	.	.	.	F	.	.	.	.	.	.	.	.
SN091 Vic	.	.	.	.	.	.	.	.	.	.	N	F	.	.	N	.	.	.	.	.
G6	.	.	.	N	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
SMSC-1	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
SN0161 NSW	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
704	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	A	.	.	.	.
SN063 WA	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.
SN051 WA	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	E	.	N	.

Amino acid differences are compared with CAV **M81223 CAEGFVIR Cux**.
Amino acids that are similar in the respective position to M81223 CAEGFVIR Cux are indicated as dots (.).

Table 7.4 Non-synonymous amino acids in VP1

	Amino acid positions																			
strain	12	21	22	29	75	83	89	92	97	98	125	136	139	140	141	144	153	157	181	182
Cux1	F	H	H	K	V	I	T	G	M	Y	I	D	K	S	Q	D	L	V	F	N
Cux-1-33	.	.	.	R	I	.	.	.	.	.	L	.	.		L	E	.	.	.	.
Cux-1-34	.	.	.	R	I	.	A	.	.	.	L	.	.		L	E	.	.	.	.
TJBD33	.	.	.	R	.	.	.	.	.	F	L	.	.		.	E	.	.	.	.
Del-Ros	.	.	.	R	.	.	.	.	.	.	.	.	.		.	E	.	.	.	.
CAE26P4	.	.	.	R	.	.	.	.	.	.	.	.	.		.	E	.	M	.	.
A2	.	.	.	R	.	.	.	.	.	.	.	.	.		E	E	.	M	.	.
3-1P60	.	.	.	R	.	.	.	.	.	.	.	.	.		E	E	.	M	.	.
LF4	.	.	.	R	.	L	.	.	.	.	.	.	.		E	E	.	M	.	.
SMSC-1P60	.	.	.	R	.	L	.	.	.	.	.	.	.		E	E	.	M	.	.
98D02152	.	.	.	R	.	.	.	.	.	.	.	.	.		.	E	.	.	.	.
China: Harbin	.	.	.	R	.	.	.	.	.	.	.	.	.		.	E	.	.	.	.
SN0138 QLD	.	.	N	R	.	.	.	.	.	.	.	.	.		.	E	.	.	.	.
TJBD40	.	.	.	R	.	.	.	.	.	.	L	.	.		.	E	.	.	.	.
SD24	.	.	.	R	.	.	.	.	.	.	L	.	.		.	E	.	.	.	Y
AH9410 (C364)	.	.	.	R	.	.	.	.	.	.	L	.	.	A	E	E	.	.	.	.
AH9410 (C140)	.	.	.	R	.	.	.	.	.	.	L	.	.	A	.	E	.	.	.	.
AH9410 (C369)	.	.	.	R	.	.	.	.	.	.	L	.	.	A	.	E	.	.	.	.
AH9410 (C368)	.	.	.	R	.	.	.	.	.	.	L	.	.	A	.	E	.	.	.	.
BD-3	.	.	.	R	I	.	.	.	L	.	.	.	Q	.	.	Q	.	.	.	.
CIA-1	.	.	N	R	I	.	.	.	L	.	.	.	Q	.	.	Q	.	.	.	.
Vaccine 3711	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	.
SN0160 QLD	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	.
BF4	.	.	.	R	.	.	.	.	.	.	.	N	.	.	E	E	.	.	.	.
CAU269/7	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	.
98D06073	.	.	Q	R	I	.	.	.	L	.	.	.	Q	.	.	Q	.	.	.	.
TR20	.	.	.	R	I	.	.	.	L	.	.	.	Q	.	.	Q	.	.	.	.
SN014 SA	.	.	.	R	I	.	.	.	L	.	.	.	Q	.	.	Q	.	.	S	.
SN041 Vic	.	.	.	R	I	.	.	.	L	.	.	.	Q	.	.	Q	.	.	.	.
SN091 Vic	.	.	.	R	I	.	.	.	L	.	.	.	Q	.	.	Q	P	.	.	.
G6	.	.	.	R	I	.	.	.	L	.	.	.	Q	.	.	Q	.	.	.	.
SMSC-1	.	.	.	R	I	.	.	E	L	.	.	.	Q	.	.	Q	.	.	.	.
SN0161 NSW	.	.	.	R	I	.	.	.	L	.	.	.	Q	.	.	Q	.	.	.	.
704	.	.	.	R	I	.	.	.	L	.	.	.	Q	.	.	Q	.	.	.	.
SN063 WA	.	.	.	R	I	.	.	.	L	.	.	.	Q	.	.	Q	.	.	.	.
SN051 WA	Y	Q	P	R	I	.	.	.	L	.	.	.	Q	.	.	Q	.	.	.	.

Amino acid differences are compared with CAV **M81223 CAEGFVIR Cux**. Amino acids that are similar in the respective position to M81223 CAEGFVIR Cux are indicated as dots (.).

To study the close evolutionary relationship between these isolates phylogenetic trees were derived from the sequence alignment of the full length of vaccine isolate 3711 with the 26 published sequences.

The phylogenetic tree derived from the 27 isolates including isolate 3711 clearly divided these isolates into three major clusters (Figure 7.1). The vaccine isolate 3711 along with next Australian isolate, CAU269/7 (Brown *et al.*, 2000) produced a group distinct from the other clusters.

The next group included isolate 704 (Australia) (Hamooleh *et al.*, 1996), G6 and TR20 (Japan), SMSC-1 (Malaysia) and 98D06073 (USA). The isolate, SD24 (China) (AY999018) was an outlier. The largest cluster included both Cux and CIA-1.

The phylogenetic tree based on the 951 bp sequence of the 36 isolates including 10 current isolates produced three clear clusters (Figure 7.2). The group one isolates included BF4, SN0160 Qld, 3711 and CAU269/7 viruses, all from Australia. The group two isolates included six field isolates from this study, isolate 704 (Australia), SMSC-1 (Malaysia), TR20 (Japan) and 98D06073 (USA). The rest of the isolates were included in a third group and was further divided into two sub groups (IIIa and IIIb). The group IIIa included strains similar to the Cux 1. The subgroup IIIb comprised CIA-1 (USA) and BD3 (Bangladesh). The Australian isolate SN0138 Qld fitted in to the group IIIa, indicating substantial homology with Cux1.

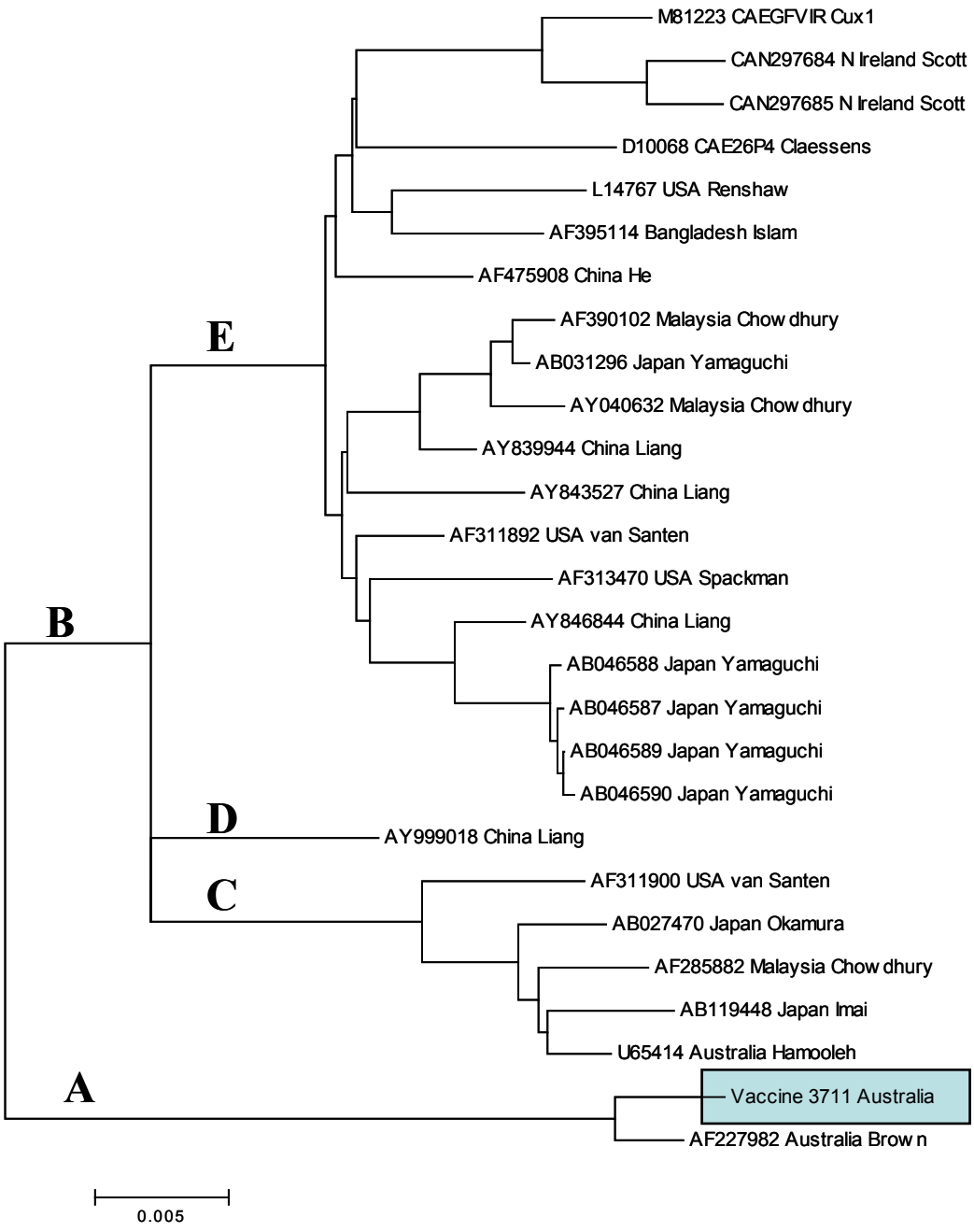


Figure 7.1 Phylogenetic tree derived from the full length sequence of vaccine strain 3711 and 26 published CAV sequences strains using MEGA3 and minimum evolution. The Australian isolate 3711 is enclosed in box. A: P=0.023, B: P=0.005, C: P=0.010, D: P=0.009, E: P=0.007.

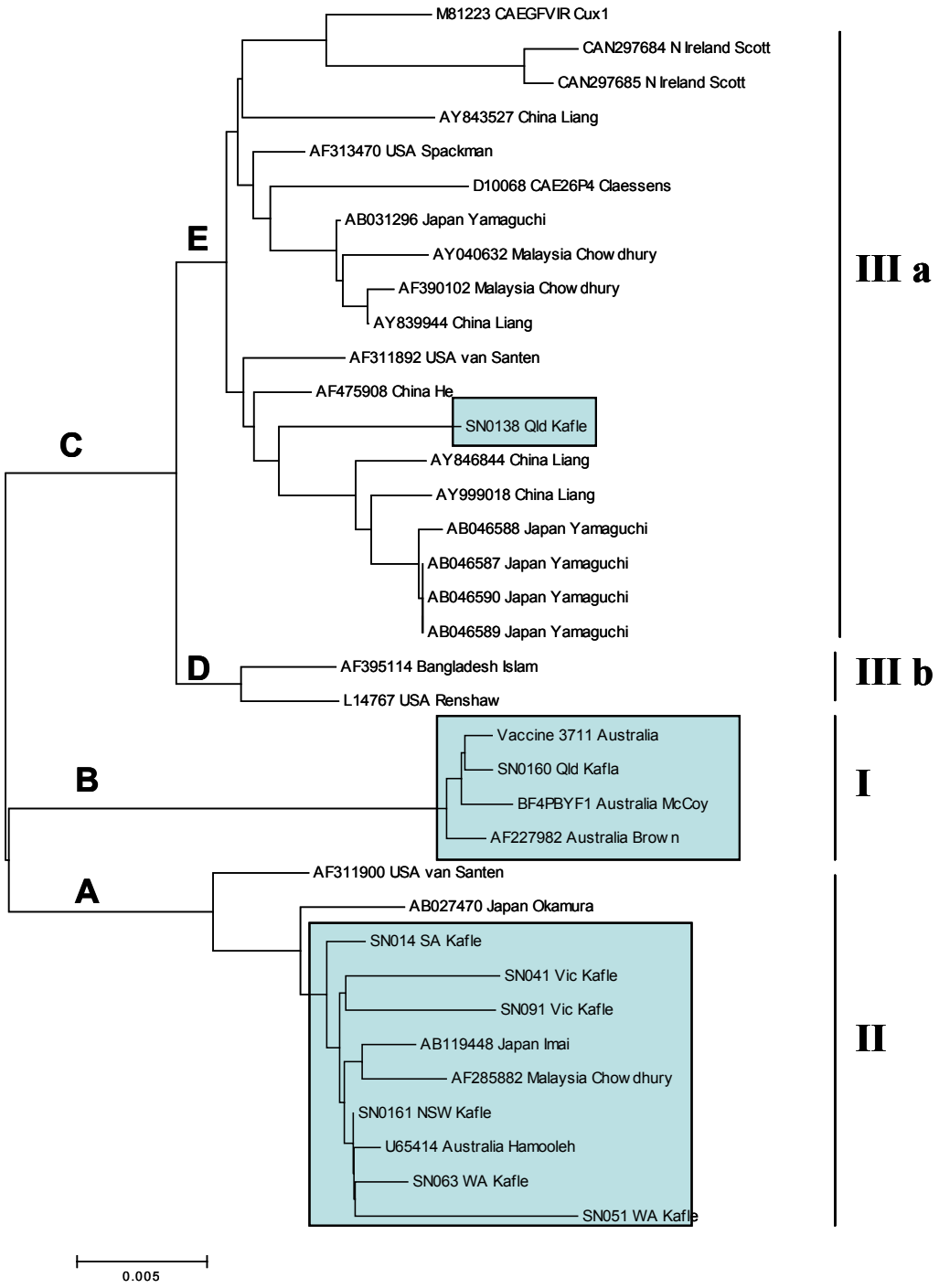


Figure 7.2 Unrooted phylogenetic tree derived from the 951 bp sequence length of 12 Australian strains and 26 published CAV sequences using MEGA3 and minimum evolution. The box enclosed Australian isolates and closely related sequences.

A: P=0.008, B: P=0.017, C: P=0.007, D: P=0.002, E: P=0.002

The VP1 gene was relatively less conserved than VP2 and VP3 at the nucleotide level and the predicted amino acid substitutions were relatively more diverse than the other two genes. Therefore the phylogenetic trees for both the nucleotides and amino acids of the VP1 gene were produced and a tree was produced only for amino acids from the VP3 gene.

The phylogenetic tree derived from the 571 bp sequences of the 10 recent isolates and the 26 published sequences produced three clusters of isolates. The distribution of isolates in the respective clusters was similar to that seen for the 951 bp sequence data.

The phylogenetic tree derived from the predicted amino acid sequence of VP1 of these isolated differs from the tree derived on the basis of nucleotide sequence. The phylogenetic tree based on predicted amino acid sequences generated only two major clusters that coincided with substitutions in the so called hypervariable region (Figure 7.4). The Australian isolates fitted in both clusters. The cluster one included the isolates which contained amino acid residue, glutamine at the positions 139 and 144 in the VP1 gene of the isolates. Six isolates from this study as well as isolate 704 and six published isolates including CIA-1 were included in this group. The next group included five Australian isolates including strain CAU269/7 which had lysine and glutamic acid at the amino acid positions 139 and 144 respectively. The remaining eighteen published isolates (with K and E at positions 139 and 144 respectively) including Cux1 fitted in to this group.

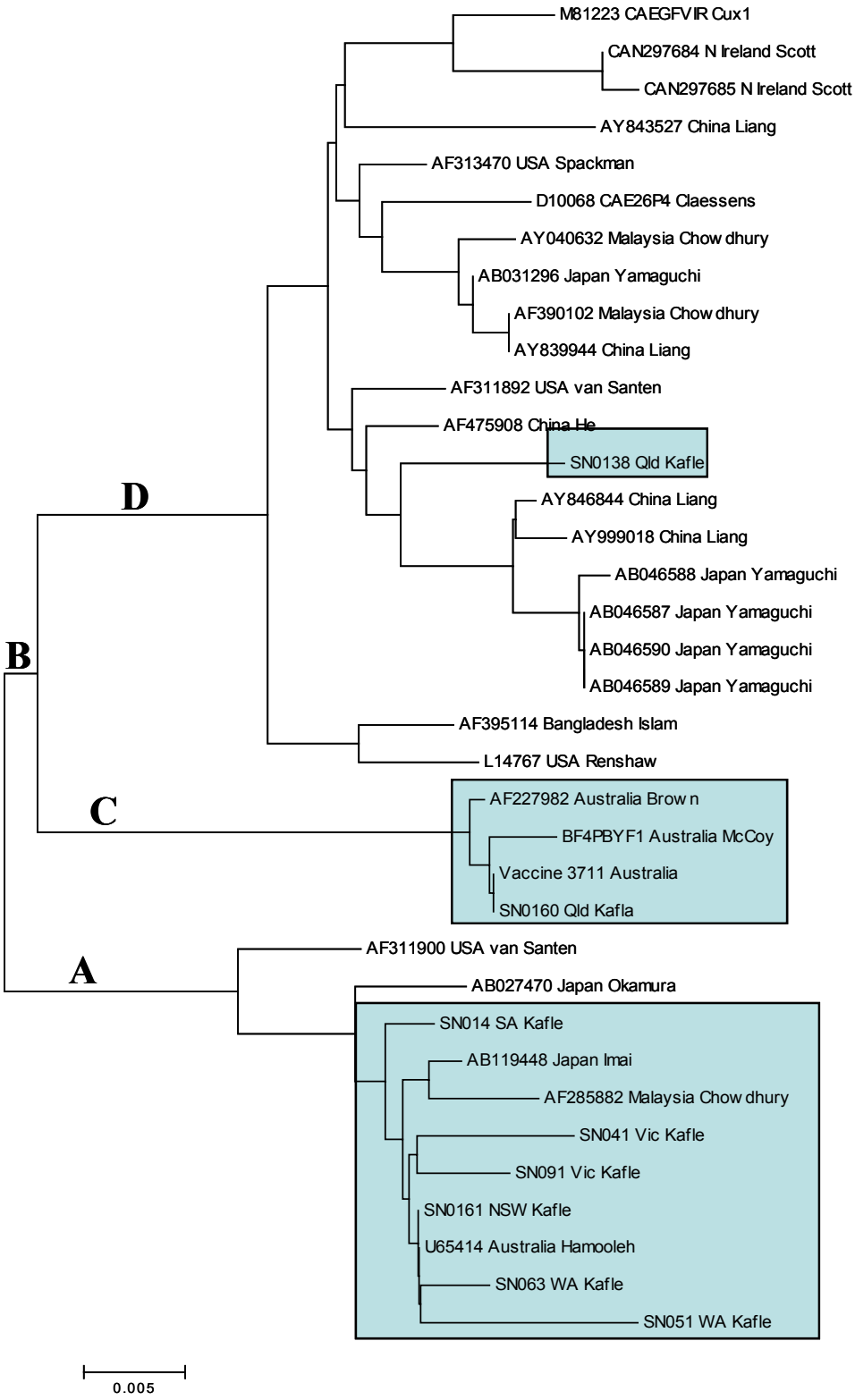


Figure 7.3 Unrooted phylogenetic tree derived from the 571bp sequence of VP1 gene of 12 Australian strains and 26 published CAV sequences using MEGA3 minimum evolution. A: P=0.012, B: P=0.002, C: P=0.021, D: P=0.011.

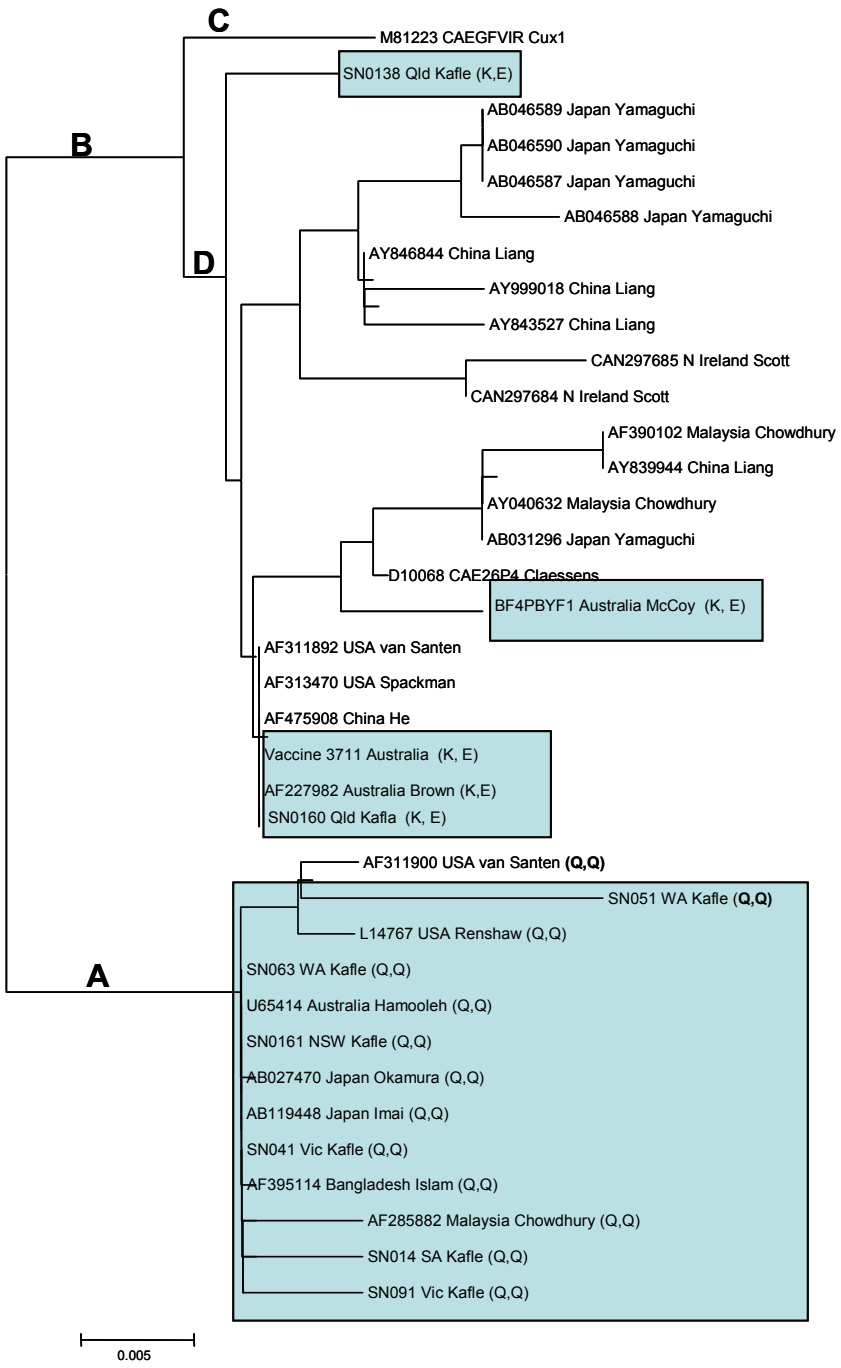


Figure 7.4 Unrooted phylogenetic tree constructed from the190 amino acids of the VP1 gene of 12 Australian strains and 26 published CAV sequences using MEGA3 minimum evolution. The Australian isolates are enclosed in the box along with some very closely related isolates and the amino acid (Q, Q) and (K, E) at the positions 139 and 144 are marked in brackets after each isolates. A: P=0.010, B: P=0.008, C: P=0.008, D: P=0.002.

A phylogenetic tree derived from the predicted amino acid sequence of the complete VP3 gene of the 36 isolates is shown in Figure 7.5. The distribution of the isolates in the tree did not produce a pattern similar to the tree derived from the VP1 gene at either the nucleotide or amino acid level. However, the four Australian isolates including CAU269/7 formed a clear cluster which had amino acid substitutions at positions 4, 54 and 67 (H, V and N respectively). There is another cluster of isolates, SN091 Vic, BD3 and TR20 close to the group of four Australian isolates. These three isolates had an amino acid substitution at position 67 (asparagine instead of serine) similar to the group of four Australian isolates.

In summary the comparisons of the nucleotide sequences and the predicted amino acid sequences of CAV Australian isolates including two published sequences and 24 other published sequences confirmed that the three groups of the Australian isolates were allocated to clusters genetically similar to the other published CAV isolates. The Queensland isolate SN0138 is relatively similar to Cux1. As a group the group one Australian isolates are all share some similarities but in general group with Cux1 and the group two isolates shared amino acid substitutions with and clustered with CIA-1.

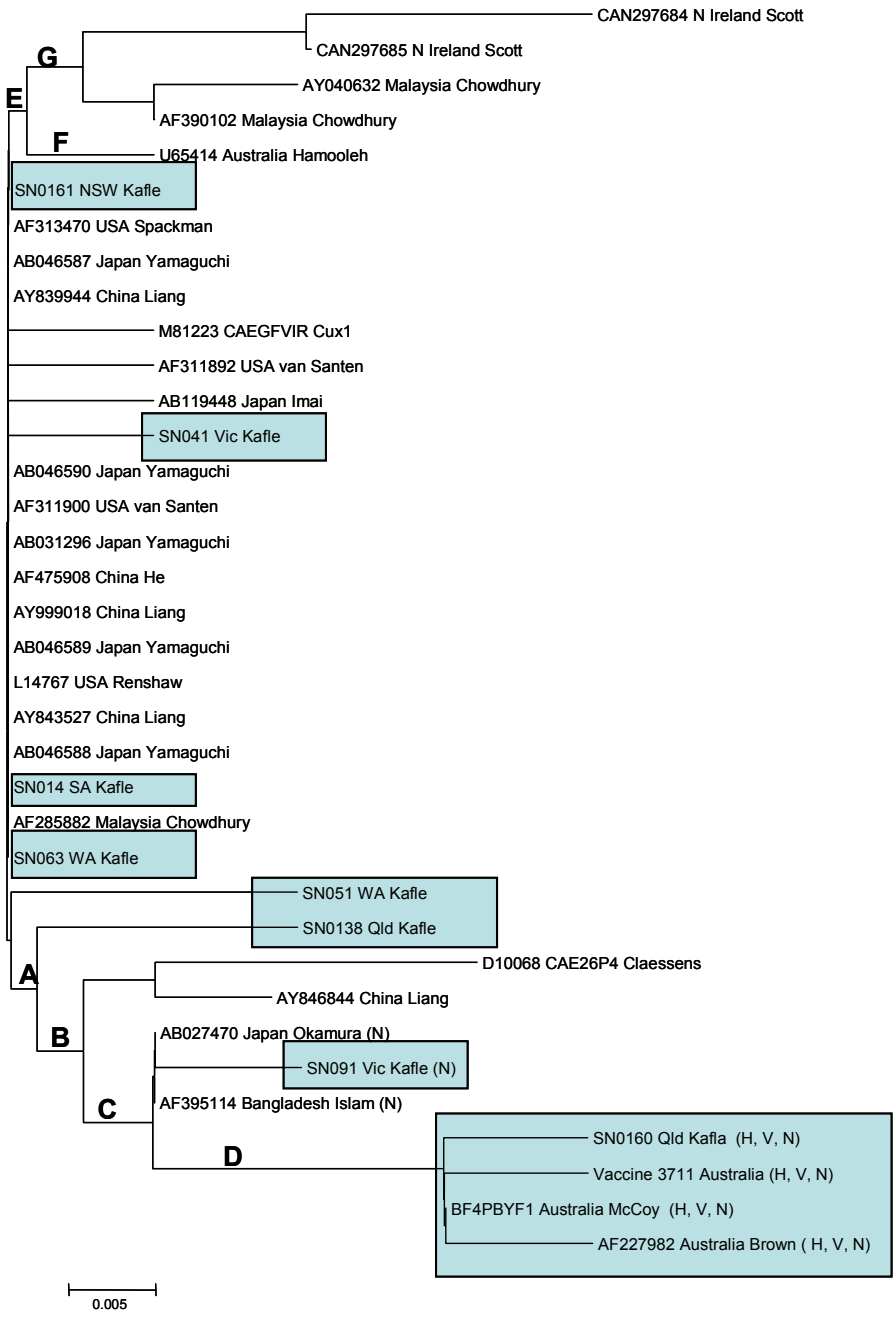


Figure 7.5 Unrooted phylogenetic tree derived from the complete amino acid sequence VP3 of 12 Australian strains and 26 global CAV strains using MEGA3 and minimum evolution. The Australian isolates are enclosed in a box. The group differentiating amino acids (H, V, N) and only (N) at positions 4, 54 and 67 are enclosed in the bracket next to the respective isolates. A: P=0.001, B: P=0.003, C: P=0.004, D: P=0.017, E: P=0.001, F: P=0.007, G: P=0.003.

7.4 Discussion

The 2279 bp sequence of the vaccine strain 3711 was similar to the most of the CAV genomes. Like most of the field isolates, this strain also had four direct repeats in the promoter-enhancer region. The five direct repeats have only been observed in a few isolates (Cux-1 and 82-2) possibly as the result of extensive cell passage (Noteborn *et al.*, 1991; Kato *et al.*, 1995).

Multiple alignments of 2279 bp sequence of vaccine strain 3711 and 26 published sequences determined at least 94% nucleotide sequence identity among the isolates which nearly correlates with earlier findings (Brown *et al.*, 2000; Islam *et al.*, 2002). The transcription regulatory elements in the promoter enhance region were as expected and was similar to other descriptions (Noteborn *et al.*, 1991). The phylogenetic tree derived from the vaccine isolates 3711 and 26 published full length sequences indicated the evolutionary relationship among the isolates as described earlier (Brown *et al.*, 2000; Islam *et al.*, 2002).

The 951 bp sequence alignment of ten current Australian isolates and 26 other isolates including two published Australian isolates demonstrated at least 96% nucleotide homology which is similar to earlier findings (van Santen *et al.*, 2001; Ducatez *et al.*, 2005). The phylogenetic relationship estimation on the basis of 951 bp and the 571 bp 5' fragment of the VP1 gene produced nearly identical patterns of clusters.

The amino acids in the VP1 gene that are indicative of specific clusters are similar to the published sequences. The phylogenetic tree derived from the amino acid sequence and the nucleotide sequences of all these isolates did not produced similar evolutionary relationship among the isolates. The phylogenetic tree derived on the basis of nucleotide sequences produced three clusters whereas the tree based on amino acid sequence produced only two clusters. The phylogenetic tree derived from the amino acid sequences correspond with the amino acid substitutions in the hypervariable region. A similar evolutionary pattern has been descried (van Santen *et al.*, 2001). Differences in the pattern of phylogenetic distribution based on

nucleotide sequence and predicted amino acid sequence is possibly due to fact that the most of the nucleotide mutations did not influence the predicted amino acids.

The predicted amino acid substitutions at position 22 of VP1 gene has described in some of the Alabama CAV isolates. One explanation is that it may have been a recent change (van Santen *et al.*, 2001). The isolates SN0138 Qld and SN051 WA also had amino acid substitutions at position 22 (Table 7.4).

The isolate 704 was isolated by Firth *et al.* and further propagated in the MSB1 cell line along with two other isolates, 3711 and 3713 by Connor *et al*. The cytopathic effect of two isolates 3711 and 3713 was observed after 10 sub cultures whereas isolate 704 produced CPE after only four passages (Connor *et al.*, 1991). The isolates BF4 and the vaccine strain 3711 were propagated in the MSB1 cell line prior to sequencing without any difficulty. This is in agreement with earlier finding that the glutamine (Q) in positions 139 and/or 144 in the VP1 gene affected the rate of viral replication in the MSB1 cell line (Renshaw *et al.*, 1996) as both these isolates had lysine and glutamic acid at positions 139 and 144, respectively.

The isolate L-028, CIA-1 failed to replicate readily in the one of MSB1 sub cell lines and demonstrated variation in the rate of replication in another cell line. The amino acid substitutions at the positions 139 and 144 (Q, Q) in the VP1 gene were suggested to be linked with this trophism (Renshaw *et al.*, 1996). The isolate, BD3 also failed to grow in the MSB1 cell line (Islam *et al.*, 2002). In contrast, the Australian isolate 704 and Malaysian isolate SMSC-1 contained similar amino acid substitutions in 139 and 144 positions as in CIA-1 but isolate 704 and SMSC-1 were propagated in MSB1 cells and a cytopathic effect was observed after four subcultures (Connor *et al.*, 1991; Chowdhury *et al.*, 2003). This perhaps suggests that other factors may also be involved.

Genetic differences in Australian CAV isolates should not be surprising. A previous study focusing on monoclonal reactivity of the Australian isolates 3711 and 3713 and 704 suggested that isolate 704 was reacting in a similar manner to the USA isolate and the two UK isolates, whereas isolates 3711 and 3713 reacted with monoclonal antibody similar to the Japanese isolate. Both patterns were different to that recorded

for Cux1 (Connor *et al.*, 1991). These antigenic differences correlate with the phylogenetic distribution of these isolates predicted from the amino acid of the VP1 gene. The isolate 3711 fitted into the group close to a Japanese isolate. The isolate 704 was included in the clusters of six other Australian isolates and CIA-1 (USA).

It is interesting that the Australian group one isolates are relatively different from both Cux 1 and CIA-1. The amino acid substitution at positions 4 and 54 in VP3 of three isolates sequenced in this study (group one isolates: SN0160 Qld, BF4 and 3711) and CAU269/7 (Brown *et al.*, 2000) are not shared by any other published sequence (Table 7.3). The biochemical consequences of these amino acid substitutions are still unknown. Another amino acid substitution at position 67 of VP3 (Table 7.3), serine (S) to asparagine (N) in the group one isolates, CAU269/7, BD3, TJBD40, TR20 and SN091 Vic may indicate a common ancestry or a random substitution. The isolates BD3, TR20, and SN091 Vic were close to the CIA-1 on the basis of amino acids in VP1 (Table 7.4) whereas the isolate TJBD40 shared greater homology with Cux1.

Recently an alternative vaccine derived from strain CAE26P4 has been introduced to Australia to vaccinate breeding flocks. The previous vaccine, strain 3711 and the recent vaccine strain CAE26P4 have a high degree of homology of predicted amino acids except at positions 141 and 157 in the VP1 gene (Table 7.4). However, there are seven predicted amino acid differences in the respective positions in the VP3 gene of these two strains. The protein coded by this gene is said to be responsible for inducing apoptosis (Noteborn, 2004). There is no indication at this stage that these changes will affect the pathogenicity of the virus.

Based on amino acid substitutions in the VP1 gene, CAV isolates can be placed into two distinct clusters with Cux1 and CIA-1 being the prototype viruses of these two clusters. The Australian isolates have representatives in both of the broad groups. The distinct amino acid changes especially in VP3 in the Australian viruses that are similar to the vaccine strain 3711 may suggest a common ancestry of this group of viruses and to date sequence data on other isolates throughout the world has not identified viruses with similar substitutions.

This ubiquitous virus has only been recognised for a couple of decades. However, it is likely that this virus has been a chicken pathogen for some time and it has coevolved with chickens perhaps for centuries. Genetically and pathologically diverse virus populations have also been noted in CAV isolates that have been passaged many times in cell cultures (Scott *et al.*, 1999; Todd *et al.*, 2001; Chowdhury *et al.*, 2003). The overcrowded environment and continuous availability of susceptible hosts in the commercial broiler industry probably also provides a favourable environment for the generation of new mutants.

7.5 Conclusions

The nucleotide sequence comparisons of the 12 Australian isolates and 24 published sequences confirmed limited variation among the isolates. However, the predicted amino acid sequences of the VP1 and VP3 genes indicated the possibility of three genetically distinct groups among the CAV isolates in Australia. Two predicted amino acid substitutions in the VP3 gene of four Australian isolates make them unique from the rest of the isolates.

CHAPTER 8

DETERMINATION OF TIME OF CHICKEN ANAEMIA VIRUS INFECTION IN A BROILER FLOCK

8.1 Introduction

Chicken anaemia virus is an ubiquitous virus. Our previous study conformed that CAV is prevalent in Australian broiler flocks at the time of slaughter. Almost all of the samples reacted in the CAV PCR. However, the results were not sufficient to determine the source of infection and the possible mode of viral transmission. There were two possibilities of viral transmission. We hypothesized that the viruses were transmitted from either parents to the progeny. Alternatively, the infection could be from the contaminated poultry sheds.

This section of the project aimed to determine the time of CAV infection in a representative broiler flock. Investigation of weekly blood samples from a broiler flock, seven days of age till slaughter would possibly indicate the age of infection. If the infection is present in the first week of age, that would be strongly suggestive of vertical transmission. If the samples are reactive at a later age, that could indicate horizontal infection.

8.2 Material and methods

Ten blood samples collected from the broiler birds at 7, 14, 21, 28, 35 and 42 days of age were generously supplied by a Queensland poultry company. Ten feather samples were also collected from the birds on weeks five and six. Blood samples were collected in capillary tubes (Sarstedt, Germany) with the EDTA dipotassium salt as an anticoagulant. The blood collection using the capillary tube is presented in Figure 8.1. Approximately 260 µl of blood was collected from the wing vein and a further 30 µl (10% v/v) of sodium citrate solution was added. The buffy-coat of the blood samples were separated using the ficol paque (Amersham Bioscience, Uppsala, Sweden) as early as possible and stored at -20°C prior to DNA extraction.

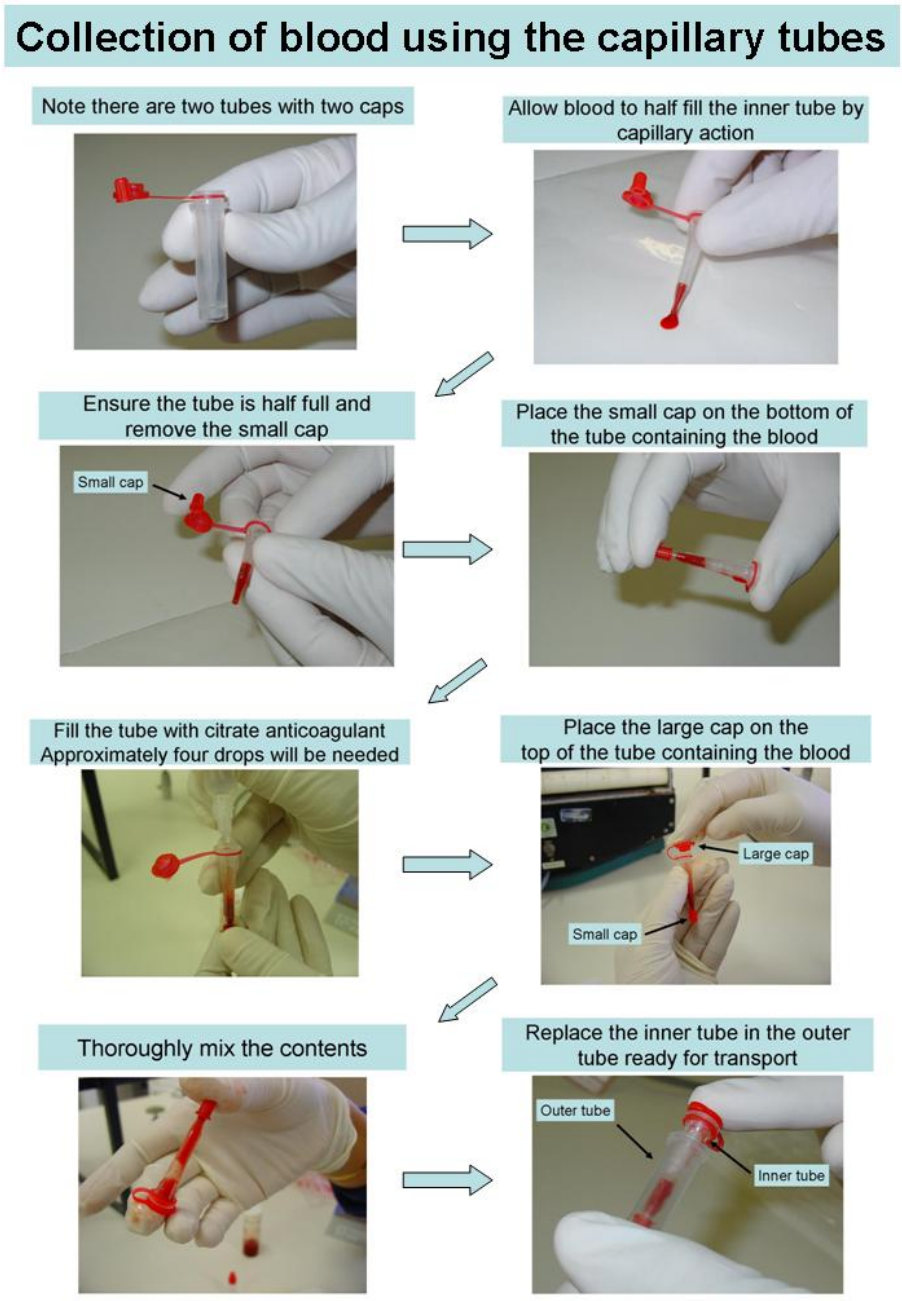


Figure 8.1 Blood collection procedure in sequential order using the capillary collection tube

8.2.1 Buffy-coat separation and storage

The buffy-coat separation was carried out using Ficoll Paque^{TM} (Amersham Bioscience, Uppsala, Sweden). Aliquots of 400 µl Ficoll Paque Plus were prepared in microcentrifuge tubes. The blood in the capillary collection tube was mixed by shaking vigorously and the blood was layered into the Ficoll Paque plus. The tubes were centrifuged at 400 *g* for 25 minutes in a 5804 swing out rotor (Eppendorf, Hamburg, Germany). There were four layers in the most of the centrifuged samples. A clear plasma layer on the top, a cloudy layer of buffy-coat above the Ficoll Paque plus and the layer of RBC at the bottom of the tube. In some samples, a cloudy buffy-coat layer was not obvious. All of the fluid above the RBC layer was collected. The buffy-coat layer was transferred to a microcentrifuge tube which was topped up with PBS then centrifuged at 8,000 *g* for two minutes. The lymphocyte pellets were stored at -20°C after removal of the supernatants. Red blood cells and plasma were also stored at -20°C.

8.2.2 Extraction of viral DNA from buffy-coat and the feather samples

The feather samples were prepared for the DNA extraction as described earlier (Chapter 5.2.3.1). The DNA was extracted from the buffy-coat as well as feather samples using the CAS 1820 ExtractaGene (Corbett Robotics) as described earlier (Chapter 5.2.3.2). The freshly eluted DNA was stored at 4°C before viral detection.

8.2.3 Viral detection

The nested PCR was used for the detection of CAV. The primers used were the Cux-nested primer set as described earlier. All the reagent concentrations, primer concentrations, and the DNA amplification temperatures were as optimised and described in Chapters 4.1.2.2 and 4.1.2.4. The reaction volume in both PCRs was 25 µl including template. The amount of the template used was two microliters per reaction in the first round reaction. One microliter of the first round PCR product was used as template for the nested reaction.

8.2.4 Agarose gel electrophoresis and image visualisation

Electrophoresis of the PCR product was carried out in 1.5 % w/v agarose gels with 0.5 μg ml^{-1} of ethidium bromide. The image visualisation and recording was achieved as described in Chapter 4.1.2.3.

8.3 Results

The specific bands of the nested as well as the first round PCR products stained with ethidium bromide in the gels were observed and recorded from the each weeks samples (Figures 8.2 and 8.3). This indicated that CAV was present in the samples collected from broilers at one week of age. The bands observed from the first round PCR assay were very weak. Some samples which did not have detectable amounts of viral DNA in the first round reaction were found to be clearly reactive in the nested PCR. The results of both first round and the nested PCRs are listed in Table (Appendix 5 Table 1). The weekly estimated prevalence was calculated and found to be 80%, 60%, 70%, 80%, 60% and 60% in weeks 1, 2, 3, 4, 5 and 6, respectively (Figure 8.4).

The results obtained from the feather samples and the buffy-coat samples in the weeks five and six were comparable. Some red blood cells were also used for DNA extraction and found to produce similar result to those produced by the buffy coat samples.

Figure 8.2 Electrophoresis of first round PCR. Template: 2 µl sample DNA, Primers CAV Cux - O3F and O3R

Row1 Lanes 1-9, 11-20 and 22-23: samples from weeks 3, 4 and 5 respectively. Lanes 10 and 21: negative control. Lane 24: 100 bp DNA ladder.

Row 2 Lanes 1-8 and 10 -19: samples from weeks 5 and 6, Lanes 9 and 20: negative control, lanes 21-23: positive controls and lane 24 100 bp DNA ladder

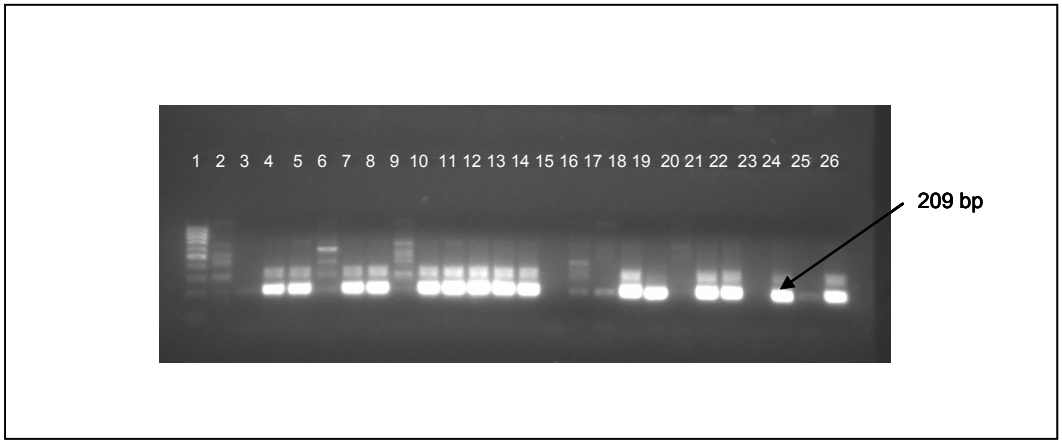


Figure 8.3 Electrophoresis of nested PCR products. Template 1 µl PCR product from the first reaction. Primers: CAV Cux-N3 and N4 (inside primer set).

Lanes 4 -13: week one sample 14: week two sample (odd order due to gel loading disorder), 18 -25: sample week two, lane 26: sample week three, lanes 2, 15 and 25: negative control and lane 1: marker, 100 bp DNA ladder.

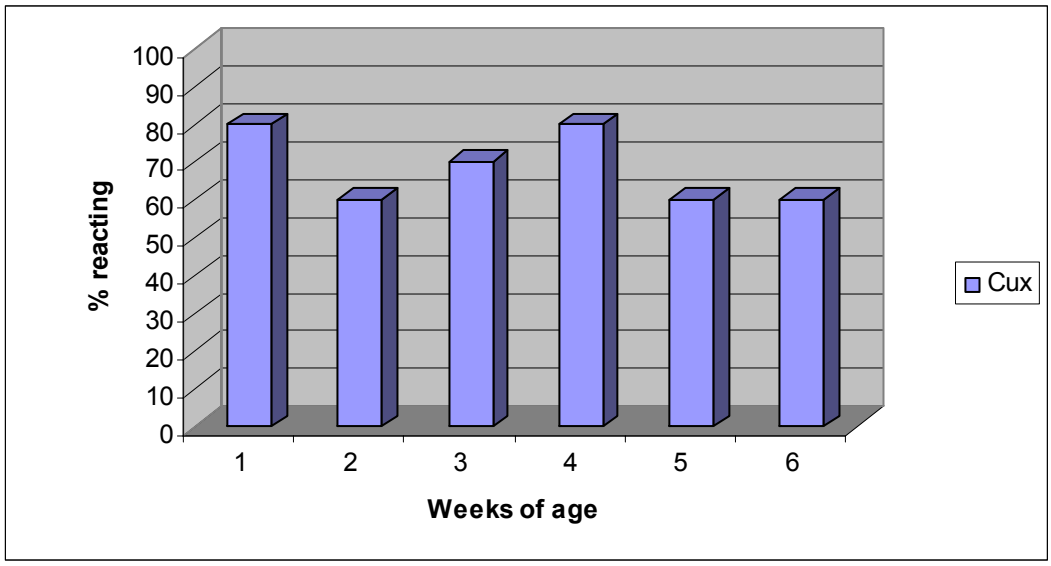


Figure 8.4 Estimated prevalence of CAV in broilers at 1, 2, 3, 4, 5 and 6 weeks of age

8.4 Discussion

The results are based on a limited number of samples from only one flock and therefore should be interpreted with caution.

This study confirmed that CAV infection was present in the broiler flock at one week of age. The incubation period of CAV is reported to be eight to ten days (Taniguchi *et al.*, 1983). This suggests that the virus was vertically transmitted. Infection as early as one day of age could possibly produce detectable amount of DNA at seven days but an 80% prevalence is unrealistic. The maximum viral replication and uniform distribution of the virus in different tissues occurs between 7 to 10 days of infection in birds experimentally infected via the oral route (van Santen *et al.*, 2004a). Therefore the most likely source of infection could be vertical transmission from the parent flock. Both hens and roosters could be potential sources for the transmission of infection to their progeny (Hoop, 1992; Hoop, 1993).

A study to determine the vertical shedding of virus from antibody positive and antibody negative hens was carried out in the US. A variety of samples including egg shell membrane from the growing embryos were tested for CAV DNA. The percentages of the embryos reactive to CAV were 29% for embryos from the antibody negative hens and 36% from the antibody positive hens. The number of

reactors was significantly higher from the egg shell membranes compared with the results from other samples including lymphoid tissues (Miller *et al.*, 2003). The results demonstrated that 29% of the embryos from the antibody negative hens reacted in the PCR indicating that antibody status of the breeding flock is not a reliable indicator that these hens are uninfected. The egg shell membrane may be a very useful sample for monitoring the status of commercial broilers or SPF breeding flocks. In addition egg shell membranes could be relatively beneficial samples in comparison to other tissues (Miller *et al.*, 2003). The egg shell membrane has the distinct advantage that it can be easily collected without sacrificing growing chicks.

The viral DNA detected in blood, gonads, thymus and bone marrow had relatively high percentage than in other tissues, liver, spleen and kidney (Miller *et al.*, 2003). Furthermore this virus demonstrated the special affinity towards the immature lymphocytes, hemoblastoses in the bone marrow which are actively multiplying cells (Adair, 2000). It could be due to reason that CAV, single stranded DNA virus, possibly replicating host cells in these respective organs facilitates the CAV viral replication.

Although the study did not attempt to determine antibody status of the breeding flocks it is assumed that they had high antibody titres to CAV. The breeding flocks had been vaccinated with CAV CAE26P4 (Claessens *et al.*, 1991) supplied by Intervet, Australia. Therefore we hypothesise that the antibody titres of the newly hatched chicks would be sufficient to suppress clinical disease.

A previous study in commercial flocks in the US aimed at detecting CAV genome and antibody to CAV using weekly samples was carried out (Sommer and Cardona, 2003). This study demonstrated that CAV genome as well as antibody against the virus was present in both flocks in all of the samples except those collected at the fourth week. The antibodies were detected in 100% of the day one samples and 18% of the samples contained CAV DNA. Interestingly the number of antibody positive samples at week one dropped to 80% and nearly 80% of the samples reacted in the CAV PCR. After the second week the number of samples positive for both antibody and viral DNA gradually declined and they were not detected by the fourth week. Both antibody and viral titres progressively rose in the fifth week and by week six

90% of samples reacted in both assays. It was concluded that the decrease in viral DNA detection could be explained by the virus not actively replicating because of the presence of maternal antibody. Alternatively it could be the sensitivity of the test or possibly it could be that the infection was eliminated (Sommer and Cardona, 2003). The increase in both the viral and antibody titres in week five onward is possibly due to environmental contamination around four weeks of age. However, the increase in detection of the virus from 20% at day one to almost 80% by seven days while at the same time the antibody titres were decreasing may indicate that there may be some other factors associated with viral replication and maintenance in addition to the antibody status.

The viral replication in the embryonal tissues during incubation period has been suggested to be influenced by hormones involved in organogenesis of the differentiating embryo. Low levels of viral transcription can be detected in the most of the embryo between 0-12 days of incubation (Schat *et al.*, 2003). In addition, intermittent viral shedding irrespective of the antibody status of the bird has been demonstrated (Miller *et al.*, 2003). Multiple samples obtained during 36 days from individual rooster varied in results between 25%-50%. The blastodisk examination to detect CAV in the eggs collected between ten days from CAV antibody positive hens also demonstrated variations in results between 25%-100%. Therefore it is suggested that replication of the virus in those organs may be regulated by reproductive hormones (Miller *et al.*, 2003).

Although we had not estimated the weekly antibody status of the broilers, results from the viral DNA detection demonstrated that the majority of the samples from each of the collections contained viral DNA. We did not examine samples from day old birds but the percentage of samples reacting in the CAV PCR on day seven was remarkably similar to that described by (Sommer and Cardona, 2003). However, the substantial drop that they noted at week four was not recorded (Sommer and Cardona, 2003).

The explanation of CAV infection in this flock is most likely that the virus was vertically transmitted. The birds would have also been exposed to virus from the contaminated environment and this would have been maintained throughout the

growing period. Litter recycling could contribute to horizontal spread exposing the birds to massive viral challenge. A study that placed birds in individual isolators would prevent horizontal transmission. Weekly sample collections with detection of viral genome could then determine the contribution made by vertical transmission. Serological examination at the same time will provide important information and add to our understanding of the role of maternal antibody in CAV replication.

As the number of specimens was small and only one flock was sampled, substantially larger numbers of samples need to be collected to determine the epidemiology of this infection in Australian broiler flocks.

Alternatively, there could be involvement of some age related chicken genetic factors associated with the viral replication. Seroconversion of SPF birds at or near sexual maturity is likely to be regulated by reproductive hormones because of the hormone activated motifs in the promotor region of the CAV genome (Miller *et al.*, 2005). Information in relation to the genetic susceptibility and resistance to CAV particular in broilers so far reported is very limited. It is likely that there are growth associated factors in the broiler which possibly regulates the relatively high feed conversion ratio in the broilers rather than layers. That particular set of genes in the broiler possibly could have an influence on viral replication. Further studies to attempt to determine host viral relationships in the pathogenesis of CAV associated with the chicken genome in these fast growing broiler chickens is essential to understand more about this unique virus.

8.5 Conclusions

Weekly sample investigation to detect the CAV in the broiler demonstrated that the seven day old chicks had detectable amount of CAV DNA. This result further indicated that the chicks were likely to be carrying infection before they were introduced to the broiler shed.

CHAPTER 9

GENERAL DISCUSSION

This project is the first documented report that confirmed the prevalence of MDV-2 and CAV in the Australian broilers at the time of slaughter. The samples were obtained from representative broiler flocks throughout Australia. The management practices are relatively similar in the individual flocks. However, the samples were collected from flocks with different status of vaccination against MDV. The detection of the viruses was carried out using strain-specific nested PCRs.

Optimisation of magnesium ions concentration and annealing temperatures was carried out. This is facilitated the generation of optimal specific DNA amplification strategies for use in this study. Initial evaluation of the relative sensitivity of two sets of nested primers with respect to the known Australian strain BF4 provided some indication of the likely performance of these PCRs.

In the study that included HVT vaccination, samples collected from the birds vaccinated with HVT were free from MDV-2 whereas the non-immunised batches of birds were infected with MDV-2. This result suggested that there is a relationship between vaccination status and the prevalence of the MDV-2 and that HVT is likely to suppress MDV-2 infection. These flocks were also free from MDV-1 (Unpublished data from Mr R Layton, JCU) and it further suggests that the existence of MDV-2 in the flocks may have acted as a vaccine against MDV-1. These findings are not surprising since MDV-2 has been successfully used as vaccine either alone or in combination with HVT and studies have demonstrated that bivalent vaccines have increased efficacy by synergism (Witter *et al.*, 1984; Witter, 1992). Published literature also suggest partial inhibition of MDV-2 by HVT *in vitro* as well as *in vivo* (Witter, 1992).

Two sets of nested primer pairs were used with the assumption that more than one strain of CAV could be present in the samples. The prevalence of CAV in the individual broiler flocks observed was more or less similar with minor variations in the results of the two primer pairs. However, there were some samples that were only

detected by one set of primers and others that were detected exclusively by the alternative set. These moderate variations in the reactivity of two primers were hypothesised to indicate the presence of more than one strain of CAV.

To some extent, these results are likely to have included some biased sampling of the birds and the number of birds included in the sampling. This bias could have been reduced by increasing the number of test samples up to the point where statistically significant findings could have been generated.

Both CAV and MDV are immunosuppressive viruses and pathogenic synergism has been demonstrated experimentally. However, these viruses can be detected with no obvious clinical signs. The impact of their infection on the poultry flocks could be estimated in terms of productivity. However, in this study information on production performance was not supplied nor was it requested.

To determine the genetic divergence of CAV, samples were sequenced. A 951 bp segment that included the hypervariable region of VP1 gene was used for most of the comparisons. The results suggested that the Australian viruses could be placed into three clusters and this is consistent with earlier findings (Brown *et al.*, 2000; van Santen *et al.*, 2001; Islam *et al.*, 2002; Ducatez *et al.*, 2005).

The alignment of the Australian isolates and the 26 published sequences from a variety of countries at both the nucleotide level and predicted amino acid level demonstrated that the three groups of the Australian isolates have substantial homology with viruses represented by Cux1 and CIA-1 and a third group distinct from these two groups but still relatively similar to Cux1. One of the groups of isolates had two predicted amino acid substitutions in the VP3 gene which were not present in any other sequences so far reported except for the Australian isolate CAU/7 (Brown *et al.*, 2000).

One of the objectives of this project was to determine the possible source of the CAV infection in the broilers. Three possible sources had been hypothesised. Broilers could be infected horizontally with wild type viruses from the shed environment or they could be infected vertically either with the virus used to vaccinate the parent

birds or with wild type viruses that had previously infected the parent birds. The sequence data of these Australian isolates demonstrated that the field isolates were not the vaccine virus. The isolate SN0160 Qld shared greatest homology with the vaccine virus 3711 suggesting that they probably had a common origin (Connor *et al.*, 1991).

It was postulated that determining the age at which the birds were infected may help to predict the source of the infection. A study was carried out to collect blood samples at weekly intervals from one Queensland broiler flock. The virus was detected in samples from eight out of 10 birds at week one and by week six CAV DNA was detected from all samples collected. It is unlikely that these birds were infected orally from the shed environment. This result correlates with a study carried out in the US (Sommer and Cardona, 2003) with the exception that the decrease in the detection of viruses at four weeks that they reported was not observed.

The sequential weekly samples were collected from only one flock. Caution should be exercised in interpreting these results as it was simply a pilot study that could be expanded to demonstrate whether these trends are consistently observed. Furthermore we did not test egg shell membranes from the hatching chicks. Egg shell membranes have the advantage of being a non-invasive sample that can clearly identify vertical transmission (Miller *et al.*, 2003).

This is the first documented report of CAV field isolates from the Australian broiler flocks that were sequenced directly from the samples without isolation and adaptation in the cell culture. The ability of CAV to replicate in MSB1 cells has shown to be strain specific (Renshaw *et al.*, 1996; Calnek *et al.*, 2000). Propagation in cell cultures could introduce bias and it provides opportunities for mutations that are not subjected to the selection pressure applied *in vivo*. The amino acid, glutamine at positions 139 and 144 in the hypervariable region in the VP1 gene is associated with restricted growth in MSB1 cells (Renshaw *et al.*, 1996). Six of the field isolates may have been missed if propagation in MSB1 cells had been used as a first step in the study.

This study confirmed the observations of others that part of the CAV genome is relatively easy to sequence and part of the genome is difficult. The promoter/enhance region of the genome with G-C rich loops and 21bp direct repeats was the most difficult to sequence. We were able to produce a near full length sequence of the vaccine strain 3711. However, an 19 bp gap in the sequences was not resolved. Similar difficulties have been noted earlier (Noteborn *et al.*, 1991; Meehan *et al.*, 1992; Brown *et al.*, 2000; Islam *et al.*, 2002) and some published sequences are just shorter than full length (Brown *et al.*, 2000; Scott *et al.*, 2001). The strategy used in this study may not have been the most efficient and other approaches such as cutting the virus with restriction endonucleases or designing primers that efficiently amplify the non-coding region and a second overlapping set for cloning the remainder of the sequence may have been more rewarding techniques.

Results in this study may have some bias as only ten isolates were sequenced and CAV DNA was detected in almost all of the 370 samples tested. Preference was given to those samples that produced a prominent band in the first round reaction and those that failed to react in one of the two nested PCRs. Samples from most states were included.

Three primer pairs used to amplify DNA fragments from CAV also amplified DNA fragments of a similar size to that anticipated. However, they were clearly not CAV sequences. Some were host DNA, some bacterial and some were not identified.

One primer pair readily amplified viral DNA from cell cultures while the products produced from the field samples using the same primers were clearly not CAV DNA. There is not a clear explanation for this difference. The cell cultures and the field samples both contained host genome. However, they were from different genetic strains of birds. The relative concentration of the virus may have also played an important role.

This is a further reminder of the shortcomings of single round PCR as a diagnostic tool. This includes the use of real time PCR based on CYBR green. The additional specificity conferred by nested PCR, PCR ELISA or the TaqMan and FRET

configurations of real time PCR (Markowski-Grimsrud *et al.*, 2002) could reduce the possibility of detecting non-target sequences.

While this project has provided substantial amounts of new information on both MDV-2 and CAV in Australian broilers it further highlights a series of unanswered questions.

It is clear that CAV has close to a 100% prevalence in Australian broilers and the infections are most likely to be vertically transmitted. Further studies could confirm the route of transmission and with sufficient production data it may be possible to speculate on the effect of subclinical infections on these flocks. The implications of strain variations in Australian CAV isolates are not clear. If the major difference is reflected in differences in neutralising epitopes it may be advantageous to commence investigations into the potential advantages and disadvantages of a bivalent CAV vaccine.

The relatively high prevalence of MDV-2 is of interest. There is room for a study that determines the prevalence of these infections along with an estimate of the time at which the birds are becoming infected. This data could then be supplemented with similar data on MDV-1 to determine whether the MDV-2 is playing a significant role as a natural vaccine. Interactions between HVT vaccination and both MDV-1 and MDV-2 infections are likely to occur and MDV-2 should not be ignored when investigations of vaccine efficacy are being carried out.

Unpublished data suggests that Australian birds are infected with at least two distinct genotypes of MDV-2 viruses. The extent of strain variation in these viruses has not been determined. It is assumed that all MDV-2 genotypes are apathogenic in chickens. Each of the genotypes may not behave in an identical manner as a natural vaccine. However, while MD and CAV infection in Australian poultry both appear to be under control it is unlikely that substantial resources will be devoted to studies of these viruses.

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APPENDIX 1

1.1 Reagents used for cell culture and storage

1.1.1 RPMI 1640 media for MSB1 cell

RPMI 1640 (Gibco) with C-glutamine	100 ml
Benzyl penicillin-sodium salt	6 mg
Streptomycin sulphate	10 mg
Fungizone	166 µg
Sodium bicarbonate	200 mg
N/1 HCL	1 ml

1.1.2 Freezing media

Dimethyl sulphoxide (DMSO)	5 ml
Foetal bovine serum	10 ml
RPMI 1640 media	35 ml

1.2 Reagents used for agarose gel electrophoresis

1.2.1 50 × Tris-acetate EDTA buffer (TAE) - Stock solution

0.04 M Tris-acetate	242 g
0.5 M EDTA	100 ml
Glacial acetic acid	57.1 ml

Dissolve in distilled water; adjust pH to 8 and make up to volume of one litre.

1.2.2 1 × TAE Buffer

50 × TAE	200 ml
Distilled water	9.8 L

1.2.3 Gel loading dye

Bromophenol (w/v)	0.25%
EDTA	10 nM
Glycerol (v/v)	30%

1.3 Reagents used for cloning and sequencing

1.3.1 LB Medium

Bacto®-triptone	10.0 g
Bacto®- yeast extracts	5.0 g
Sodium chloride	5.0 g

Dissolve in distilled water; adjust pH to 7.5 and make up to a final volume of one litre. Autoclave and store at 4ºC.

1.3.2 LB agar

Technical agar	1.5 g
Ampicillin	100 µl
IPTG	80 µl
X-Gal	100 µl

Dissolve the technical agar in 100 ml of LB medium and autoclave. Add ampicillin, IPTG and X-Gal before plating.

1.4 Reagents used for blood collection and buffy coat preparation

1.4.1 Sodium citrate buffers

Sodium citrate (w/v)	3%

Dissolve the sodium citrate in required volume of distilled water, sterilise by filtration and store at store at 4ºC.

1.4.2 1 × Phosphate Buffer Solution

0.14 M Sodium chloride	4.0 g
0.01 M di-Sodium hydrogen orthophosphate (anhydrous)	0.71 g
0.002 M Potassium chloride	0.10g
0.001 M Potassium dihydrogen orthophosphate	0.12g

Dissolve in distilled water; adjust pH to 7.2 and make up to a final volume of 500 ml. Autoclave and store at 4ºC.

APPENDIX 2

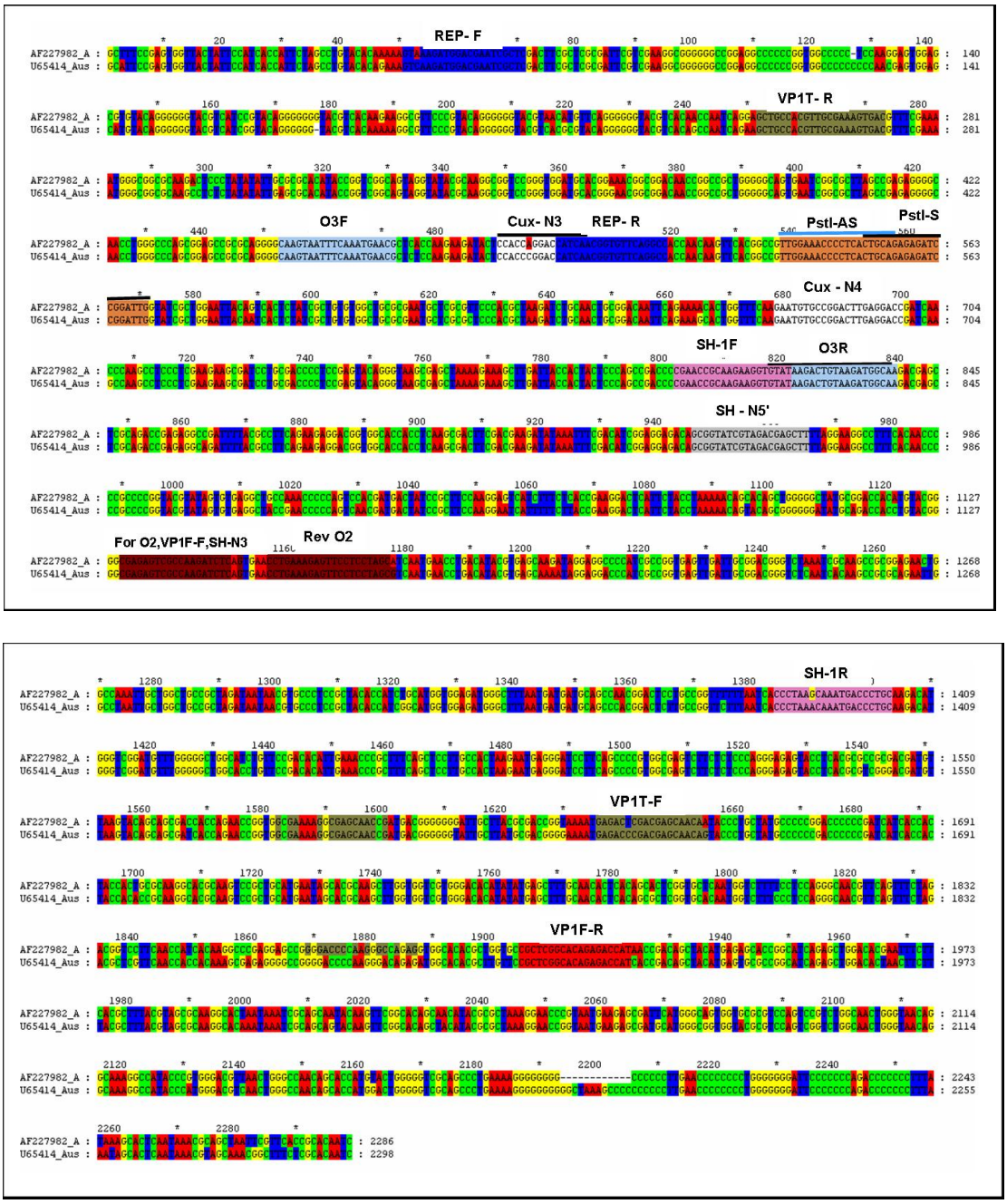


Figure 1 Sequence alignment of CAV Australian strain CAU/7 (gi 6984173), and isolate 704 (gi 1490881).The primers used for detection of CAV from the sample material as well as those used for sequencing are designated. The primer pairs, forward and reverse are indicated with same colour.

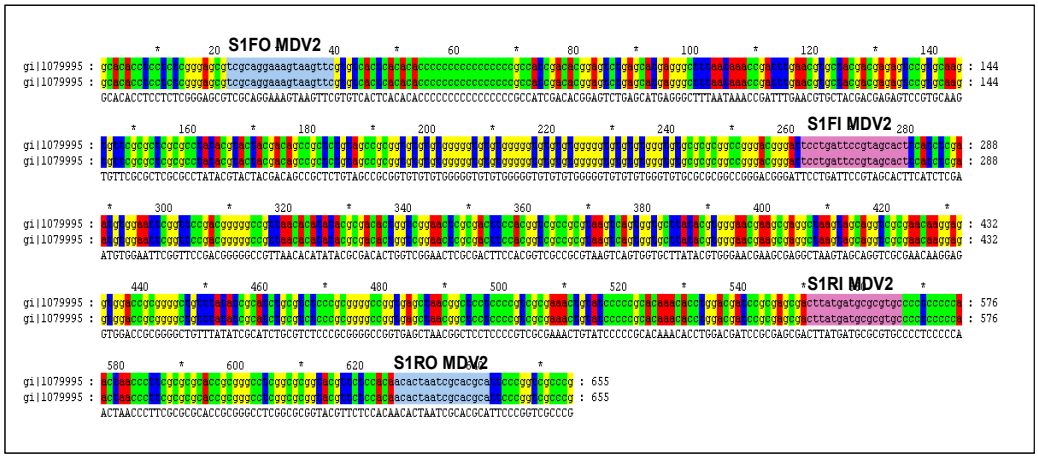


Figure 2 Primer sequence and location in the genome of published sequences of *Gallid herpesvirus* type 3 nuclear DNA binding protein gene at an inverted repeat region.

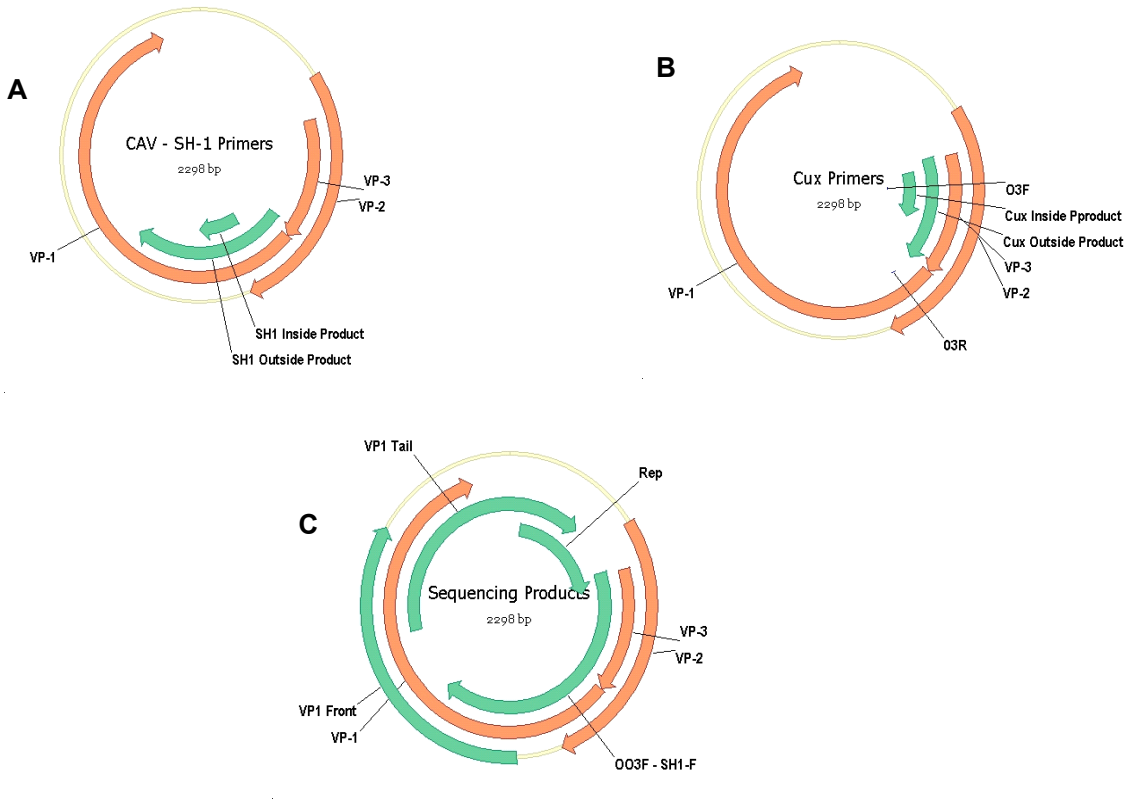


Figure 3 CAV circular genome with VP, VP2, VP3 protein and non-coding region

A Diagnostic nested primer, CAV-SH-1 location in the genome.

B Next diagnostic nested primer, Cux location.

C Primers used for sequencing and their location in the genome.

APPENDIX 3

Results: Table 1

Sample set: One

Total sample no.: 130

Sample material: feather tips

PCR results (1-4)

Score 1 = weak reaction, 4 = strong reaction, no score indicates a negative result

samples			MDV		CAV	
Flock	Number	JCU Number	I"*"	II	Cux	SH-1
	1	1	neg	neg	2	1
one	2	2	neg	neg	2	1
	3	3	neg	neg	2	1
	4	4	neg	neg	3	2
	5	5	neg	neg	3	2
	6	6	neg	neg	4	2
	7	7	neg	neg	4	2
	8	8	neg	neg	4	3
	9	9	neg	neg	4	4
	10	10	neg	neg	4	4
	1	11	neg	neg	4	4
Two	2	12	neg	neg	3	4
	3	13	neg	neg	4	neg
	4	14	neg	neg	4	neg
	5	15	neg	neg	4	4
	6	16	neg	neg	4	4
	7	17	neg	neg	4	4
	8	18	neg	neg	4	4
	9	19	neg	neg	neg	neg
	10	20	neg	neg	neg	neg
	1	21	neg	neg	4	4
Three	2	22	neg	neg	4	4
	3	23	neg	neg	4	4
	4	24	neg	neg	4	4
	5	25	2.5	neg	neg	neg
	6	26	neg	neg	4	4
	7	27	neg	neg	4	4
	8	28	neg	neg	4	4
	9	29	neg	neg	4	4
	10	30	neg	neg	4	neg
	1	31	neg	neg	4	4
Four	2	32	neg	neg	4	4
	3	33	neg	neg	4	4
	4	34	neg	neg	4	4
	5	35	neg	neg	4	4
	6	36	neg	neg	4	4
	7	37	neg	neg	4	4
	8	38	neg	neg	4	4
	9	39	neg	neg	4	4
	10	40	neg	neg	4	4

	1	41	neg	neg	4	4
Five	2	42	neg	neg	4	4
	3	43	neg	neg	4	4
	4	44	neg	neg	neg	4
	5	45	neg	neg	4	4
	6	46	neg	neg	4	4
	7	47	neg	neg	4	neg
	8	48	neg	neg	4	4
	9	49	neg	neg	4	4
	10	50	neg	neg	4	4
	1	51	neg	neg	4	4
Six	2	52	neg	neg	4	4
	3	53	neg	neg	4	4
	4	54	neg	neg	4	4
	5	55	neg	neg	4	4
	6	56	neg	neg	4	4
	7	57	neg	neg	4	4
	8	58	neg	neg	4	neg
	9	59	neg	neg	4	4
	10	60	neg	neg	4	4
	1	61	neg	neg	4	4
Seven	2	62	neg	neg	neg	4
	3	63	neg	neg	4	4
	4	64	neg	neg	4	4
	5	65	neg	neg	4	4
	6	66	neg	neg	4	4
	7	67	neg	neg	4	4
	8	68	neg	neg	4	4
	9	69	neg	neg	4	4
	10	70	neg	neg	4	4
	1	71	neg	neg	neg	neg
Eight	2	72	neg	neg	neg	neg
	3	73	neg	neg	2	3
	4	74	neg	neg	3	neg
	5	75	neg	neg	2	3
	6	76	neg	neg	3	neg
	7	77	neg	neg	3	3
	8	78	neg	neg	neg	neg
	9	79	neg	neg	neg	neg
	10	80	neg	neg	neg	neg
	1	81	neg	neg	2	4
Nine	2	82	neg	neg	2	4
	3	83	neg	neg	2	4
	4	84	neg	neg	3	4
	5	85	neg	neg	4	4
	6	86	neg	neg	4	4
	7	87	neg	neg	4	4
	8	88	neg	neg	4	4
	9	89	neg	neg	4	4
	10	90	neg	neg	4	4
	1	91	neg	neg	4	4
Ten	2	92	neg	neg	4	4

	3	93	neg	neg	4	4
	4	94	neg	neg	4	4
	5	95	neg	neg	4	4
	6	96	neg	neg	4	4
	7	97	neg	neg	4	4
	8	98	neg	neg	4	4
	9	99	neg	neg	4	4
	10	100	neg	neg	4	4
	1	101	4	neg	4	4
Eleven	2	102	neg	neg	4	4
	3	103	4	neg	4	4
	4	104	neg	neg	4	4
	5	105	4	neg	4	4
	6	106	neg	neg	4	4
	7	107	neg	neg	4	4
	8	108	neg	neg	4	4
	9	109	neg	neg	4	4
	10	110	neg	neg	4	4
	1	111	4	neg	4	4
Twelve	2	112	neg	neg	4	4
	3	113	4	neg	4	4
	4	114	neg	neg	4	4
	5	115	neg	neg	4	4
	6	116	4	1	4	4
	7	117	neg	neg	4	4
	8	118	4	neg	4	4
	9	119	neg	neg	4	4
	10	120	neg	neg	4	4
	1	121	neg	neg	4	4
Thirteen	2	122	neg	neg	4	4
	3	123	neg	neg	4	4
	4	124	neg	neg	4	4
	5	125	4	neg	4	4
	6	126	neg	4	4	4
	7	127	neg	neg	4	4
	8	128	4	4	4	4
	9	129	neg	neg	4	4
	10	130	neg	neg	4	4

Results: Table 2

Sample set: Two

Total sample no.: 180

Sample material: feather tips

PCR results (1-4)

Score 1 = weak reaction, 4 = strong reaction, no score indicates a negative result

Sample			MDV		CAV	
Flock	Number	JCU Number	I"*"	II	Cux	SH-1
	1	1	neg	4	4	4
one	2	2	4	4	4	neg
	3	3	4	neg	4	4
	4	4	neg	4	4	4
	5	5	neg	4	4	4
	6	6	4	4	4	neg
	7	7	neg	4	4	neg
	8	8	neg	4	4	4
	9	9	neg	4	4	4
	10	10	neg	4	4	neg
	11	11	4	4	4	neg
	12	12	4	4	4	neg
	13	13	neg	4	4	4
	14	14	neg	4	4	neg
	15	15	neg	4	4	neg
	16	16	neg	4	4	4
	17	17	neg	neg	4	4
	18	18	neg	neg	4	4
	19	19	neg	4	4	4
	20	20	neg	4	4	neg
Two	1	21	neg	neg	4	4
	2	22	neg	3	4	4
	3	23	neg	3	4	4
	4	24	neg	neg	4	neg
	5	25	neg	neg	4	neg
	6	26	4	4	4	neg
	7	27	4	4	4	neg
	8	28	3	4	4	neg
	9	29	neg	4	4	neg
	10	30	neg	neg	4	neg
	11	31	neg	neg	4	neg
	12	32	neg	4	4	4
	13	33	neg	4	4	neg
	14	34	neg	4	4	neg
	15	35	neg	4	4	neg
	16	36	neg	4	4	neg
	17	37	neg	4	4	4
	18	38	neg	4	4	neg
	19	39	4	4	4	4
	20	40	3	4	4	4
Three	1	41	2	4	4	4

	2	42	neg	4	4	2
	3	43	neg	neg	4	2
	4	44	neg	neg	4	3
	5	45	neg	neg	4	4
	6	46	neg	3	4	4
	7	47	neg	neg	4	4
	8	48	2	neg	4	4
	9	49	neg	2	4	neg
	10	50	neg	neg	4	neg
	11	51	neg	neg	4	neg
	12	52	neg	3	4	4
	13	53	neg	3	4	4
	14	54	neg	4	4	4
	15	55	neg	4	4	4
	16	56	neg	neg	4	4
	17	57	neg	neg	4	4
	18	58	neg	neg	4	4
	19	59	neg	4	4	4
	20	60	neg	4	4	4
Four	1	61	neg	4	4	4
	2	62	neg	4	4	neg
	3	63	neg	4	4	neg
	4	64	neg	neg	4	4
	5	65	neg	neg	4	4
	6	66	neg	neg	4	4
	7	67	neg	neg	4	4
	8	68	neg	4	4	4
	9	69	neg	4	4	4
	10	70	neg	4	4	4
	11	71	3	4	4	neg
	12	72	3	4	4	4
	13	73	neg	4	4	4
	14	74	3	4	4	neg
	15	75	3	4	4	4
	16	76	neg	neg	4	4
	17	77	3	4	4	4
	18	78	neg	4	4	neg
	19	79	3	4	4	4
	20	80	neg	4	4	neg
Five	1	81	neg	4	4	4
	2	82	neg	4	4	4
	3	83	neg	4	4	4
	4	84	neg	4	4	4
	5	85	neg	4	4	4
	6	86	neg	4	4	4
	7	87	neg	4	4	4
	8	88	neg	4	4	4
	9	89	neg	4	4	4
	10	90	neg	4	4	4
	1	91	neg	4	4	4
	2	92	neg	4	4	4
	3	93	neg	4	4	4
	4	94	neg	4	4	4

	5	95	neg	4	4	4
	6	96	neg	4	4	4
	7	97	neg	4	4	4
	8	98	neg	4	4	4
	9	99	neg	4	4	4
	10	100	neg	4	4	4
Six	1	101	neg	neg	4	4
	2	102	neg	neg	4	4
	3	103	neg	neg	4	4
	4	104	neg	neg	4	4
	5	105	neg	neg	4	4
	6	106	neg	neg	4	4
	7	107	neg	neg	4	4
	8	108	neg	neg	4	4
	9	109	neg	neg	4	4
	10	110	neg	neg	4	4
	1	111	neg	neg	4	4
	2	112	neg	neg	4	4
	3	113	2	neg	4	4
	4	114	neg	neg	4	4
	5	115	neg	neg	4	4
	6	116	neg	neg	4	4
	7	117	neg	neg	4	4
	8	118	neg	neg	4	4
	9	119	neg	neg	4	4
	10	120	2	neg	4	4
Seven	1	121	neg		4	4
	2	122	neg	3	4	4
	3	123	neg	2	4	4
	4	124	neg	3	4	4
	5	125	neg	neg	4	4
	6	126	neg	neg	4	4
	7	127	neg	3	4	4
	8	128	neg	2	4	4
	9	129	neg	4	4	4
	10	130	neg	4	4	4
	1	131	neg		4	4
	2	132	neg	4	4	4
	3	133	neg	4	4	4
	4	134	neg	4	4	4
	5	135	neg	4	4	4
	6	136	neg	4	4	4
	7	137	neg	neg	4	4
	8	138	neg	3	neg	4
	9	139	neg	4	4	4
	10	140	neg	4	4	4
Eight	1	141	neg	4	4	4
	2	142	neg	neg	4	4
	3	143	1	3	4	4
	4	144	neg	4	4	4
	5	145	neg	4	4	4
	6	146	1	neg	4	4
	7	147	neg	neg	4	4

	8	148	neg	neg	4	4
	9	149	neg	4	4	4
	10	150	neg	4	4	4
	1	151	neg	1	4	4
	2	152	neg	neg	4	4
	3	153	neg	1	4	4
	4	154	neg	neg	4	4
	5	155	neg	1	4	4
	6	156	neg	neg	4	4
	7	157	neg	2	4	4
	8	158	neg	neg	4	4
	9	159	neg	4	4	4
	10	160	neg	4	4	4
Nine	1	161	neg	4	4	4
	2	162	neg	neg	4	4
	3	163	neg	neg	4	4
	4	164	neg	neg	4	4
	5	165	neg	neg	4	4
	6	166	neg	neg	4	4
	7	167	neg	4	4	4
	8	168	neg	3	4	4
	9	169	neg	2	4	4
	10	170	neg	2	4	4
	1	171	1	neg	4	4
	2	172	neg	neg	4	4
	3	173	neg	neg	4	4
	4	174	neg	neg	4	4
	5	175	neg	neg	4	4
	6	176	neg	neg	4	4
	7	177	neg	neg	4	4
	8	178	neg	neg	4	4
	9	179	neg	neg	4	4
	10	180	neg	4	4	4

MDV I and MDV II are MDV serotype one and MDV serotype two, respectively

The samples used for successful in sequencing are highlighted.

Symbol "*" is indicative for the data supplied by Mr. Ramon Layton, JCU.

APPENDIX 4

Figure A4.1 Sequence of the 951 bp products used to compare the Australian field isolates

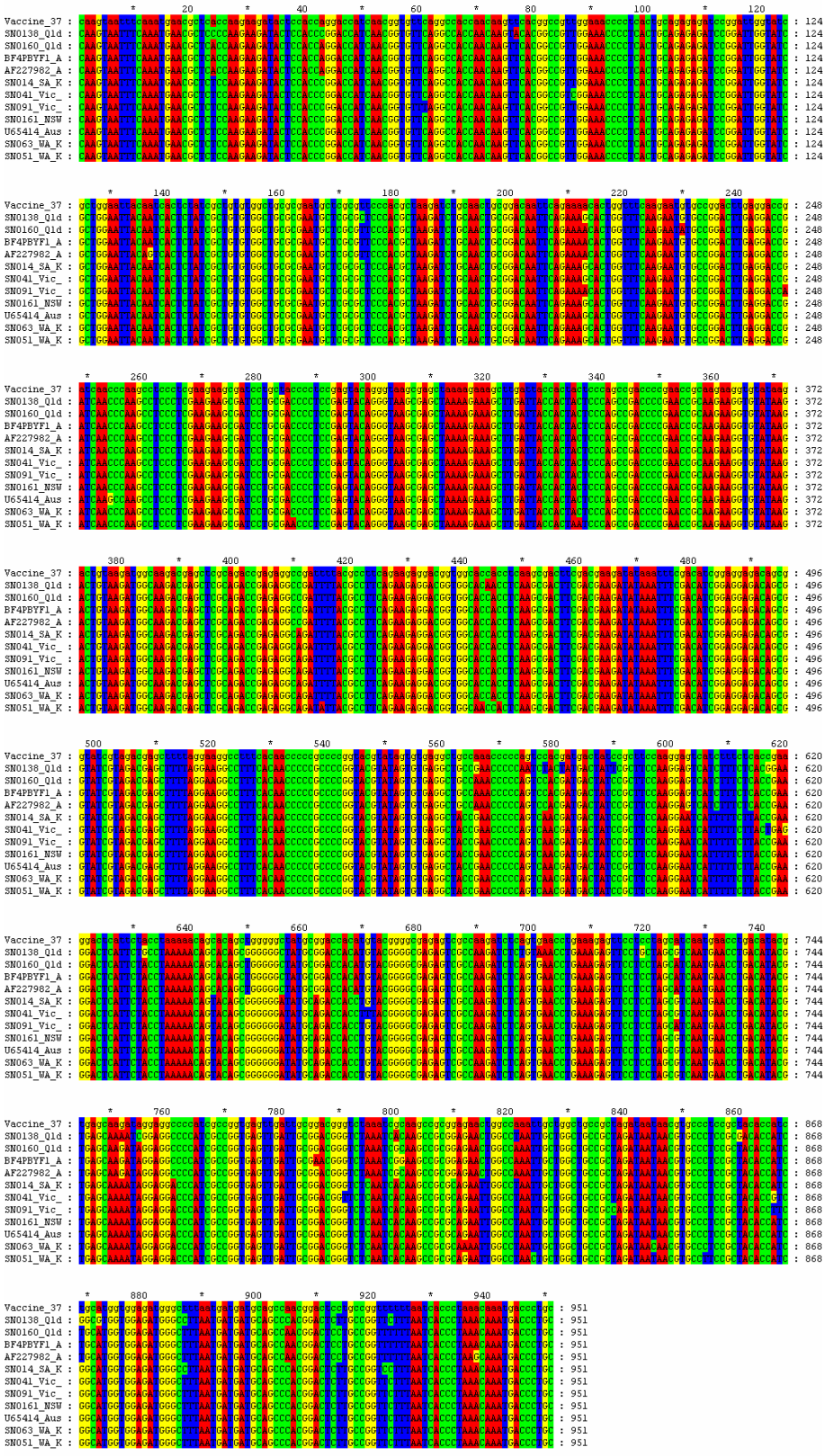
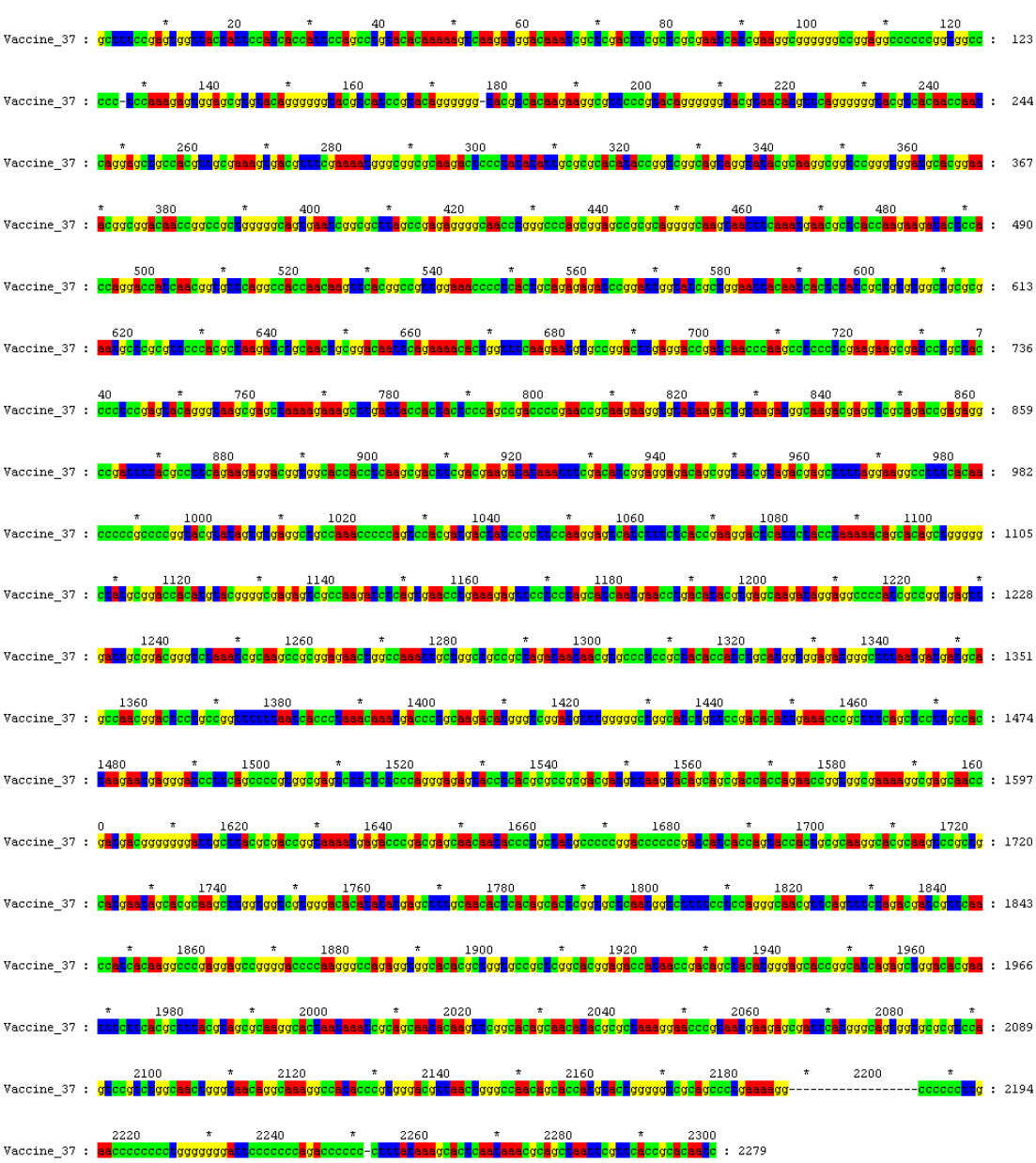


Figure A4.2 Vaccine sequence



APPENDIX 5

PCR results

Sample: Blood with anticoagulant

PCR Results (1-4) 1= weak reaction, 4= strong reaction, neg= negative result

collection week	samples number	JCU number	Cux-out primer	Cux nested primer
	1	1	1	4
	2	2	1	4
week one	3	3	neg	neg
	4	4	1	4
	5	5	2	4
	6	6	neg	neg
	7	7	2	4
	8	8	2	4
	9	9	2	4
	10	10	2	4
	1	11	1	4
	2	12	neg	neg
week two	3	13	neg	neg
	4	14	1	4
	5	15	neg	4
	6	16	neg	neg
	7	17	1	4
	8	18	1	4
	9	19	neg	neg
	10	20	neg	4
	1	21	neg	4
	2	22	1	3
	3	23	neg	3
week three	4	24	1	3
	5	25	neg	neg
	6	26	neg	neg
	7	27	1	4
	8	28	neg	neg
	9	29	1	4
	10	30	neg	4
	1	31	neg	nen
	2	32	neg	nen
	3	33	1	4

week four	4	34	1	4
	5	35	1	4
	6	36	1	4
	7	37	1	4
	8	38	1	4
	9	39	1	4
	10	40	1	4
	1	41	neg	neg
	2	42	1	4
week five	3	43	1	3
	4	44	neg	neg
	5	45	1	3
	6	46	neg	neg
	7	47	neg	4
	8	48	1	4
	9	49	neg	neg
	10	50	neg	4
	1	51	neg	neg
	2	52	1	4
	3	53	1	4
week six	4	54	neg	neg
	5	55	1	4
	6	56	neg	neg
	7	57	neg	4
	8	58	neg	neg
	9	59	1	4
	10	60	1	4