General introduction

Marek's disease (MD) is a common lymphoproliferative disease of chickens worldwide, caused by Marek's disease virus (MDV) an oncogenic highly cell-associated alphaherpesvirus that induces T-cell lymphomas in poultry. Originally described in 1907 as a classical paralytic disease associated with polyneuritis (Marek, 1907), MD has shown significant increases in severity in its clinical picture over the last 100 years, particularly in the latter half of this period (Witter, 1997).

The earlier classification of Marek's disease viruses (MDVs) into three serotypes, which was based on antigenic (von Bülow, 1975) properties, has been revised since representative strains of the three serotypes have been sequenced (Lee et al., 2000; Afonso et al., 2001;Izumiya et al., 2001; Kingham et al., 2001; Tulman et al., 2000). The three viruses certainly represent individual and clearly distinct virus species, which correspond to the previous serotypes. A new classification has been proposed and serotype 1 (MDV1) is now also referred to as *Gallid herpesvirus 2* (GaHV-2), serotype 2 (MDV2) as *Gallid herpesvirus 3* (GaHV-3) and serotype 3 of MDV, also known as Herpesvirus of Turkeys (HVT) is now referred to as *Meleagrid herpesvirus 1* (MeHV-1) (Osterrieder and Vautherot, 2004). However, the serotype classification is still commonly used and is convenient, so is used throughout this thesis. MDV1 is the only pathogenic/oncogenic serotype of MDV. MD vaccines are live, often cell-associated, and comprise attenuated MDV1 or naturally occurring MDV2 and HVT strains, or combinations of these.

Despite the widespread use of MD vaccines since 1970, MD remains an important disease threat, partly because of the cost of vaccination and also because of the emergence of new MDV pathotypes of increased virulence over time. Vaccination against MD may protect chickens against clinical MD but it does not preclude infection with wild type MDV or shedding of co-infecting MDV from the feather follicle epithelium (FFE) (Witter, et al., 1971). This is thought to have contributed to the evolution towards higher virulence of MDV since the advent of widespread vaccination. These new pathotypes represent a continuum of

increased virulence and based on extensive pathotyping studies, Witter (1997) proposed a classification of these isolates in mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+). In addition to the increased oncogenic potential of these isolates, the most virulent recent isolates produced acute cytolytic disease with heavy mortality and massive atrophy of the immune organs well before the onset of lympomas.

The primary targets of MDV infection are lymphocytes and consequently, diagnosis, isolation and monitoring of MDVs has been historically based around lymphocytes or lymphocyte-rich tissues, e.g. spleen material or peripheral blood lymphocytes (PBL). These materials are not easily accessible, are of fragile nature and require immediate and laborious processing after sampling. As MDV is a highly cell-associated virus, it is essential that material for *in vitro* isolation of MDV consists of viable cells and this has posed significant technical barriers to the development of routine monitoring systems for MD. However, production of fully infectious virus occurs in the FFE and MDV is shed with feather debris and dander which is the source of natural transmission of MDV and contamination of the environment (Calnek et al., 1970). This material is easily accessible and retains infectivity for long periods, under a wide range of conditions, but is unsuitable for direct inoculation to cell culture without ultra centrifugation to separate virus and additional treatments (Calnek et al. 1970). However, material from the FFE provides a good source of MDV antigens and viral DNA and the quantification of viral load in this material has shown to provide a good indicator of flock MD status in broiler chickens (Walkden- Brown et al., 2005; Islam and Walkden-Brown, 2007).

Recent progress with immunological and molecular biology techniques has enabled more rapid, sensitive and accurate detection of MDV in biological samples, with clear differentiation of the different serotypes and enumeration of viral copy number. In particular, quantitative real-time PCR (qPCR) for differentiation and quantitation of viral nucleic acids offers the opportunity to routinely monitor the MD status from a wide variety of sample materials, e.g. spleen, PBL, feather tips or poultry dust. The latter is an excellent sample material as it integrates information from a whole flock or group of chickens, and is easy to sample, store and process for subsequent qPCR analysis.

This doctoral study was focussed on the development and optimization of MDV serotype-specific qPCR assays and their application under a variety of conditions to investigate aspects of MDV pathogenesis and epidemiology. The ultimate objective of this was to assess the usefulness of different samples in the monitoring of MD status. Another part of the study involved DNA sequence analysis of the MDV1 specific oncogene *meq* to find markers for MD virulence and examine phylogenetic relationships between Australian and international strains of MDV. A final experiment investigated the effect of two environmental enrichment strategies on the well-being of chickens involved in *in vivo* evaluation of MDV in isolators.

The main objectives of this study were:

1. To develop and optimize absolute quantification of MDV2 serotype-specific quantitative real-time polymerase chain reaction (qPCR) assays.
2. To investigate the DNA sequence of the *meq* oncogene in recent Australian isolates of MDV and determine whether sequence variation correlates with virulence.
3. To determine the pathotype of recent Australian isolates of MDV in a pathotyping experiment using commercial layer chickens.
4. To investigate early predictors of subsequent clinical MD status
5. To measure the level of feather dust production in layer chickens, and the shedding pattern of virulent and vaccine isolates of MDVs in feather tips and dust.
6. To improve welfare of experimental layer chickens kept in isolators using environmental enrichment strategies.

The results should advance our understanding of MD epidemiology and pathogenesis and will contribute to future efforts to place the ongoing control of MD onto a sustainable basis in the long term.

Materials from this thesis have been published in international journals and several conference proceedings; a list of these publications is provided on page v.

Chapter 1

Review of literature

1.1 Introduction

Marek's disease virus (MDV) is the aetiological agent of Marek's disease (MD) and a highly cell- associated alphaherpesvirus which was first isolated in 1967 (Churchill and Biggs, 1967). MD is a highly contagious lymphoproliferative disease in chickens inducing paralytic and lymphomatous syndromes, and causing significant losses in the poultry industry worldwide. Three MDV serotypes are classified: Marek's disease virus serotypes 1 and 2 (MDV1 and MDV2) as well as herpesvirus of turkeys (HVT) (van Regenmortel et al., 2000). Soon after identification of MDV, the first vaccine against MD, an attenuated MDV1 was described in the UK (Churchill et al., 1969a). Almost simultaneously the efficacy of HVT as a vaccine was demonstrated in the USA (Okazaki et al., 1970) and the first HVT vaccine was licensed in USA proving to be a great success. In the following years, further serotype 1 and 2 vaccines were developed and shown to have synergistic effects when administered with HVT (Calnek et al., 1983). MD vaccines were the first successful vaccines to be used for protection against cancer (Davison and Nair, 2005) and MD is therefore often considered as a model for the study of viral oncology (Calnek, 1986). The vaccines protect to a greater or lesser extent against mortality, clinical signs and gross MD lesions but none prevent infection, replication and shedding of challenge virus. They are therefore unable to induce sterile immunity, and this is thought to have contributed to the evolution of MDV towards greater virulence (Witter, 1997). This evolution in virulence means that serious outbreaks of MD have occurred periodically around the world at different times, ensuring that MDV remains a persistent threat to the poultry industry worldwide. The last major epidemic of MD in Australia was between 1992 and 1997.

The primary target of infection with MDV is the lymphocyte and early effects are therefore seen in lymphoid organs such as the bursa of Fabricius where B-lymphocytes are produced, the thymus which is the primary producer of T-lymphocytes and spleen (Venugopal and Payne, 1995; Calnek et al., 1998; Calnek, 2001). The tumour inducing oncogenic process in MD, which is characterized by an accumulation of transformed lymphocytes and other immune cells, can involve most organs and tissues including peripheral nerves. The most commonly affected organs and tissues are gonads, spleen, heart, lung, liver, muscle and peripheral nerves (Biggs, 2001). Cytolytic changes, early mortality, severe neurological and atherosclerotic syndromes can also be manifestations of MD as can a clinical syndrome described as transient paralysis. MD can occur at any age from a few weeks old, but is most common between 2 and 6 months of age (Biggs, 2001) although very virulent MDV can induce significant lesions and mortality before two weeks of age in susceptible chickens. In general, involvement of tissues and organs, other than peripheral nerves, increases with increasing incidence of disease. MDV occurs in all poultry- producing countries and, before vaccination was introduced for its control, was responsible for serious economic loss to the poultry industries throughout the world. It is ubiquitous in the domestic chicken and is also present in jungle fowl (Weiss and Biggs, 1972). MD is one of the most fascinating and complex diseases as a number of viral, host and environmental factors interact in complex ways to influence the outcome of the disease. Isolates of MDV vary greatly in their oncogenic potential, ranging from non-oncogenic to highly oncogenic, but viruses not normally considered oncogenic can also be found in the appropriate host environment (Pastoret, 2004).

It is remarkable that it took 60 years after the first description of the disease by Jozef Marek in 1907 (Marek, 1907) for MD to be recognized as a distinct entity and for its causative agent to be isolated. During the early days, the disease was given a variety of names, e.g. polyneuritis, paralysis of domestic fowl, neuromyelitis gallinarum and neuro-lyphomatosis gallinarum. Because of the difficulties of differential diagnosis at that time, a publication by Ellermann (1921) describing lymphatic leucosis led to confusion over recognition of two diseases and it was not until 1960 that MD was finally differentiated from lymphoid leukosis

(Campbell and Biggs, 1961). In the last 40 years, research on MD has achieved numerous successes which have led to better knowledge of the molecular biology of MDV and subsequently, control of MD. However, MDV continues to evolve towards more virulent isolates with changed virulence and tropisms. This is mainly due to the fact that none of the currently available vaccines engender sterile immunity, which has been reported to be a key factor driving pathogens towards greater virulence (Gandon et al., 2001). Therefore, the control and early diagnosis of MD remains an important issue and the novel molecular methods will provide a powerful set of tools to better understand and control MD.

1.2 Brief history of Marek's disease

Marek's disease (MD) was first described by Jozef Marek in 1907 (Marek, 1907), but although being published, it did not stimulate interest for over a decade. The level of interest began to change in the 1920s when the number of recorded outbreaks increased and with them, the number of significant publications. Marek's early description of MD suggested that it was a condition affecting only the nervous system, which was variously described as polyneuritis, paralysis of the domestic fowl and neuromyelitis gallinarum. The presence of lymphoid tumours in MD, and the condition described as lymphatic leukosis by Ellerman (1921) and now known as lymphoid leukosis, led to confusion over separation of the two diseases and it took about 40 years before the two conditions were differentiated and the causative agent, Marek's disease herpesvirus (MDV), was identified (Campbell and Biggs, 1961; Churchill and Biggs, 1967).

As the poultry industry developed, the disease became more and more important and the number of publications increased dramatically thus reflecting the increasing economic impact of the disease to the poultry industry countries, mainly USA and Europe, particularly Great Britain and Germany. In the 1950s, when the poultry industry developed rapidly, MD took on a new form that had devastating consequences for the poultry industry around the world. This lymphomatous form of MD became dominant in the 1950s and 1960s and, since it occurred in younger birds causing higher mortality, it became referred to as the

acute form of MD (Biggs et al., 1965; Payne, 1985) differentiating it from the “classical” paralytic/paretic form.

The acute lymphoid form of MD became particularly serious in the 1960s due to further expansion and intensification of poultry production. A milestone in the fight against the disease was the development of reliable methods for transmitting MD experimentally and cell culture for propagating the causative agent in cell culture. This work eventually led to MDV being identified definitively as the cause of MD in 1967 (Churchill and Biggs, 1967). Another major breakthrough was the introduction of vaccination from 1970, the first demonstration of effective and widespread use of vaccines to prevent a virus-induced cancer in any species (Churchill et al., 1969a; Okazaki et al., 1970; Witter et al., 1970; Rispens et al., 1972a; Schat and Calnek, 1978). The problems of MD morbidity and mortality receded, but vaccination breakthroughs began to be reported and increased virulence of field viruses occurred within ten years (Davison and Nair, 2005). The subsequent introduction of more aggressive vaccines and vaccine regimes has driven MDV to evolve to even greater virulence over the last 30 years (Witter, 1998) and the problem of MD remains a persistent threat to poultry production worldwide.

1.3. MD situation in Australia

As in other parts of the world, MD is endemic in much of Australia and historically had required control (vaccination) in longer lived layers and breeders, but not broiler chickens. The vaccines used were cell free preparations of a locally isolated strain of HVT (NSW 1/70) and a cell-associated MDV2 isolate (MD19) administered subcutaneously (s.c.) at hatch. The importation of live vaccines from overseas was banned. However, the relaxation of Australian quarantine protocols in 1990 paved the way for importation of new strains of both layer and broiler chickens from 1992 onwards and due to improvements in production efficiency, the new chicken strains completely dominated the Australian poultry industry. Conventional Australian vaccines and vaccination programs failed to control MD in the imported genotypes which resulted in a massive MD outbreak in both the layer and broiler industries between 1992 and 1997 with mortalities in the range

of 20-40 % of birds being common in layers and breeder flocks (Cumming et al. 1998; Groves 1995).

The problem in layers and broiler breeders was brought under control by the importation in 1997 of seed for the Rispens CVI988 serotype 1 vaccine and MD remains well controlled by this vaccine at present. Broilers had not traditionally been vaccinated against MD in Australia, but during 1992-97 clinical MD was appearing in birds from 35 days onwards associated with reduced flock productivity, typically around 8 points (0.08) in feed conversion ratio, and increased intercurrent disease (Groves, P., 2005, pers. comm.). These problems were generally responsive to vaccination with HVT and with the production from 1997 onwards of high titre cell-associated HVT vaccine, the immediate problem in broilers was brought under control, although at considerable cost.

Since 1996 imported strains of HVT (FC126, and NBSL S.AR) are now widely used as vaccines for broiler chickens in cell-associated form administered *in ovo*. Layer birds and broiler breeders are all vaccinated exclusively with the introduced CVI988/ Rispens, either alone, or in conjunction with HVT as a bivalent vaccine. The local BH16 attenuated serotype 1 vaccine is not used commercially despite being at least as protective as CVI988/ Rispens (Karpathy et al., 2002, 2003).

HVT vaccine which had been first introduced in 1970, ceased to be protective many years earlier in the USA and other countries, and may do so in Australia. There is clear evidence of this with vvMDV strains (against which HVT confers only partial protection) first identified in Australia in 1985 (McKimm-Breschkin et al. 1990) and isolated from subsequent outbreaks of MD in vaccinated birds (De Laney et al. 1995; Zerbes et al., 1994). In subsequent formal challenge experiments using titrated doses of a local isolate of MDV (MPF57), failure of HVT to provide complete protection has been repeatedly demonstrated (Islam et al., 2002; Islam et al., 2006b; Islam et al., 2007). These findings are consistent with the evolution of Australian MD viruses towards greater pathogenicity in the face of HVT vaccination. This process is likely to have accelerated since the introduction of wider HVT vaccination of broilers in the late 1990s. In Australia, it is estimated that the disease in broilers alone currently costs some $20M in vaccination costs alone and a similar amount in lost production.

Therefore, in order to avoid future outbreaks of MD in Australia and to minimize the evolution of MDV towards greater virulence in the face of 'imperfect' vaccines (Gandon et al., 2001), effective monitoring systems for MD are needed which enable early detection of MDV infection and will predict the behaviour of the disease in chicken populations as vaccine efficacy declines.

1.4 Economic significance

MD has an important economic impact on the poultry industry. The disease almost devastated the poultry industry in the 1960s. According to Farrant (1969), the financial losses in those days due to MD were 20 % of annual farm incomes from eggs and poultry meat with 60 % mortality in layer flocks and 10% condemnations in broiler flocks. The disease was brought under control after the causative agent of MD was identified and vaccines were developed (Churchill et al., 1969a; Okazaki et al., 1970; Witter et al., 1970; Rispens et al., 1972b; Schat and Calnek, 1978). However, none of the current vaccines engenders sterilizing immunity and vaccinated chickens can still be infected with highly virulent MDV that can replicate, be shed and infect other chickens (Witter et al., 1984, 1987; Lee et al., 1999; Islam et al., 2001).

This situation has probably contributed to the problems of field isolates of MDV increasing virulence, as reported by Witter (1997). Gandon et al. (2001) using models of pathogen fitness have shown that non-sterlising or "imperfect" vaccines foster evolution to higher levels of virulence and hence to more severe disease in unvaccinated individuals. Indeed, MD outbreaks still occur and the poultry industry remains concerned about the unpredictability of such outbreaks. Purchase (1985) estimated that losses due to MD totalled about US$ 12 million in the United States in 1984. When combined with economic loss from the costs of vaccine and application and reduced production, however, the total was about US$ 169 million in the United States and US$ 943 million worldwide.

In the last 5 years, the MD situation has been quite stable in most areas of the world (Morrow and Fehler, 2004), but the authors note that it is difficult to obtain a worldwide overview for several reasons including:

- MD is not a notifiable disease
- Low level losses after MD vaccination are generally accepted and treated as normal since it is known that vaccination failures occur at low frequency.

Since prevention of the disease requires optimal hygiene and management, many MD cases are not reported in order to avoid damaging the reputation of the company concerned. Furthermore, the number of such unreported cases is extremely high thus making it difficult to evaluate the worldwide economic losses from MD.

The economic impact of MD on the poultry industry worldwide is thought to be in the range of US$ 1-2 billion annually, although this is a crude estimate (Morrow and Fehler, 2004). According to a recent estimate from the Food and Agriculture Organization (FAO) in 2002, a total number of 45 billion broilers and 57 million tonnes of eggs (corresponding to about 5 billion laying birds) were produced in 2002 which represents a total value of US$ 100-200 billion. In terms of the damage estimate due to MD given above, MD-induced losses correspond to about 1 % of the total value. A significant number of the total population, some 50 billion chickens produced yearly, is vaccinated against MD which indicates the continual global threat of the disease and the cost that producers are willing to expend to prevent it.

1.5 Aetiology of Marek's disease

1.5.1 Classification of MDVs

Marek's disease virus belongs to the family of *Herpesviridae*, subfamily *Alphaherpesvirinae*. Three members of the genus *Mardivirus*, Marek's disease virus serotypes 1 and 2 (MDV1 and MDV2) as well as serotype 3 or herpesvirus of turkey's (HVT) are recognized (Schat, 1987; van Regenmortel et al., 2000; Davison, 2002a). The *Mardivirus* genus was established because accumulated genomic information clearly indicated their affiliation with the *Alphaherpesvirinae*

subfamily of the *Herpesviridae*, but distinguished them from the other genera of the subfamily, the *Simplex-*, *Varicello-* and *Laryngoviruses* (Davison, 2002a, 2002b; Davidson et al., 2002). According to the International Committee on Taxonomy of Viruses (ICTV), the MDVs in the genus *Mardivirus* fall into 3 different species, aligned with serotype. MDV strains belonging to serotype 1 are *Gallid herpesvirus 2* (GaHV-2), those strains belonging to serotype 2 are *Gallid herpesvirus 3* (GaHV-3) and HVT is *Meleagrid herpesvirus 1* (MeHV-1). An overview of the two classification systems is shown in Table 1.1. However, the classification based on the serotype differentiation is widely used and convenient and is therefore used throughout the thesis.

Table 1.1: Current taxonomy and attributes for MDV.

Serotype	ICTV taxonomy	Attributes
MDV1	*Gallid herpesvirus 2* (GaHV-2)	Cause Marek's disease, primarily in chickens. Are oncogenic and spread efficiently between birds.
MDV2	*Gallid herpesvirus 3* (GaHV-3)	Non-oncogenic MDV of chickens. Does not cause disease. Spreads efficiently between birds.
HVT (MDV3)	*Meleagrid herpesvirus 1* (MeHV-1)	Non-oncogenic herpesvirus of Turkeys. Does not spread effectively between chickens.

All strains, apart from attenuated vaccinal strains, belonging to serotype 1 represent the pathogenic (oncogenic) MDV, but they vary greatly in their pathogenic and oncogenic potential (Biggs, 2001). Thus, it has been found useful to further classify MDV1 isolates and strains into categories of pathogenicity or virulence.

The original classification as proposed by Biggs et al. (1965) was based on the terms of classical and acute which were used to distinguish between two distinct forms of MD. These terms became inadequate for the classification of MDV with the isolation of viruses that had characteristics of greater virulence than acute MDV (Witter et al., 1980, Schat et al., 1982). Subsequently, Witter (1983, 1985) proposed an alternative nomenclature. In this system, used for MDV1, formerly classical strains become mild MDV (mMDV), original acute strains became

virulent MDV (vMDV) and strains isolated in the 1980s from MD outbreaks in vaccinated flocks were classified as very virulent MDV (vvMDV). In the 1990s, isolates that were found to be even more virulent than vvMDV could not be classified in this system and Witter (1997) designated them as very virulent plus MDV (vv+MDV) as the high end of a continuum in virulence.

Serotype 2 of MDV is a naturally occurring, infectious virus in chickens, but is non-pathogenic or only weakly pathogenic and nononcogenic in chickens (Schat and Calnek, 1978; Witter et al., 1982; Calnek et al., 1983).

Serotype 3 of MDV, also designated as HVT, is non-pathogenic in chickens, but it does induce a viremia which is associated with induction of protective immune response against MDV 1 and is widely and effectively used as a vaccine, either alone or combined with strains of MDV2 or MDV 1 (Witter et al., 1982; Calnek et al., 1983; Afonso et al., 2001). Table 1.2 below gives an overview of the serotypes and pathotypes of MDV.

Table 1.2: Serotypes and pathotypes of MDV (modified from Witter, 1998).

Serotype	**Origin**	**Pathotype**	**Pathotype abbreviation**	**Serotype abbreviation**	**Disease in chickens**	**Used as vaccine**
1	chicken	attenuated		MDV1	no	Yes
		mild	m	MDV1	Yes	No
		virulent	v	MDV1	Yes	No
		very virulent	vv	MDV1	Yes	No
		very virulent plus	vv+	MDV1	Yes	No
2	chicken	non - oncogenic		MDV2	no	Yes
3	turkey	non - oncogenic	HVT	HVT	no	Yes

1.5.2 Morphology

In general, viruses of all three serotypes have characteristics typical of other *Herpesviridae*.

The unenveloped virion in the cell nucleus measures about 100 nm in diameter and has 162 hollow capsomeres (Churchill and Biggs, 1967; Nazerian and Burmester, 1968). In the nucleus, naked virion particles can appear hexagonal and less frequently, small ring-shaped structures, about 35 nm in diameter can appear in addition (Morgan et al., 1959; Nazerian and Burmester, 1968). The nucleoid measures 50-60 nm and is structured as a torus which is at right angles to and around a less electron-opaque cylindrical mass (Nazerian, 1974). This accounts for the variable shape of the nucleoid seen in section as it depends on, in addition to the stage of development of the particle, the angle of section. More rarely, enveloped particles are found measuring between 130 and 160 nm in diameter in the perinuclear space or in nuclear vesicles. Both naked and enveloped particles are also found in the cytoplasm, but more rarely than in the nucleus.

The 35 nm structures appear first at about 8 h post infection followed about 2 h later by the first appearance of nucleocapsids, and at 18 h post infection the first enveloped virions appear (Hamdy et al., 1974). The nucleocapsids mature to enveloped virions by budding through cellular membranes acquiring their envelope within the process. The enveloping of the nucleocapsid can occur at the inner nuclear membrane or membrane-bound cytoplasmic spaces, the former being more common.

Enveloped virus particles appear as irregular amorphous structures and vary considerably in size due to the lack of rigidity of the envelope. In negatively stained preparations of lysed feather follicle epithelium (FFE), enveloped particles were found to a size ranging from 273-400 nm (Calnek et al., 1970).

The morphology of MDV2 and HVT strains resembles that of MDV1 although in thin sections; nucleocapsids of HVT commonly show a unique crossed appearance (Nazerian et al., 1970).

1.5.3 Genome organisation

The genomes of *Herpesviridae* are double-stranded linear DNA (ds DNA) molecules and the size of the three MDV serotypes ranges from 160 to 180 kb with a buoyant density in neutral CsCl of 1.706 g/ml for MDV1 (Ross, 1985). A total of six different general genome organizations, referred to as classes A through F, are distinguished in the *Herpesviridae* (Roizman, 1996).

Only class D and E genomes are found in the *Alphaherpesvirinae*, and MDV1, MDV2 and HVT represent class E genomes, an organization that is identical to that of the prototype representative of this virus subfamily, herpes simplex virus type 1 (HSV-1) (Roizman, 1996). Class E genomes comprise a unique long (U_L) and a unique short (U_S) sequence. These unique sequences are flanked by sets of identical inverted repeat sequences: the terminal repeat long (TR_L), internal repeat long (IR_L), internal repeat short (IR_S) and terminal repeat short (TR_S), respectively. An overview of the MDV genome organisation is shown in Figure 1.1.

Figure 1.1: Genome organisation of MDV (adapted from Nair and Kung, 2004).

The genomic organisation of the U_L region has been remarkably conserved during the evolution of these herpesviruses. The U_S region, while containing obvious homologues conserved in the different alphaherpesviruses, is more variable in both its organisation and gene content (Kingham et al., 2001). These two regions encode primarily genes involved in replication and virus assembly (Kung et al., 2001).

Entire genomes of representatives of all three members of the *Mardivirus* genus have been sequenced showing that the gene content and linear arrangements of the three viruses are similar in general (Lee et al., 2000; Tulman et al., 2000; Izumiya et al., 2001; Afonso et al., 2001). However, they vary considerably with regard to guanine and cytosine (GC) content and size. The lowest GC content

was found to be in MDV1 with 44.1 %, whereas the highest GC content was 53.6 % in MDV2. HVT has a GC of 47.5 % content that lies in between these two extremes (Tulman et al., 2000; Kingham et al., 2001; Afonso et al., 2001; Izumiya et al., 2001). The total number of genes for the three serotypes varies as well with 103 genes identified for MDV1, 102 for MDV2 and 99 for HVT respectively (Davison and Nair, 2005).

There are two complete genomes from MDV1 sequenced, namely the American strains GA (Lee et al., 2000) and Md5 (Tulman et al., 2000) which were classified as 'virulent' and 'very virulent' pathotypes respectively according to the classification system suggested by Witter (1997). The GA genome is about 174 kb long whereas the Md5 strain is over 3800 bp longer which is mostly due to a longer TR_L and IR_L. The U_S region of both MDV1 strains is similar in length with about 11 kb. The U_L region of these MDV1 strains is similar as well with a length of about 113 kb (Lee et al., 2000; Tulman et al., 2000; Silva et al., 2001).

1.5.4 MDV genes and gene products

The genomic sequences available for *Mardiviruses* clearly identified a total of 103 (MDV1), 102 (MDV2) and 99 (HVT) genes (Osterrieder and Vautherot, 2004) which can broadly be divided into three classes.

The first class represents the vast majority of open reading frames (ORF's) which are encoded by all three viruses and are homologous to those recognized in other alphaherpesviruses, e.g. herpes simplex virus-1 (HSV-1) (Kingham et al., 2001). Largely by analogy to and deduction from the better studied prototype representative HSV-1, 'putative' functions have been attributed to genes and gene products that exhibit homology with the HSV-1 genes (Kingham et al., 2001; Cui et al., 2004; Lupiani et al., 2004; Kamil et al., 2005; Anobile et al., 2006). Within the U_L region of the MDV genomes, homologues of alphaherpesvirus genes corresponding to HSV-1 UL1-UL 55 were readily recognized (McGeogh et al., 1988; Roizman and Sears, 1996) and the organization of the genes UL1-UL54 is collinear with HSV-1 (Kingham et al., 2001; Silva et al., 2001). The function of these ORFs is mainly DNA replication and formation of structural proteins such as those of the capsid (VP5), tegument (UL47 and UL49) and

envelope (glycoproteins, such as gB, gC, gD, gH, and gL) of the mature virus (Lupiani et al., 2001). Transcriptional regulator proteins, such as ICP4, ICP22, ICP27 and VP16 belong to this class as well as enzymes involved in viral DNA synthesis.

To the second class belong the MDV genes that are shared by all three serotypes, but have no homologues in other herpesvirus genomes. These genes are found in the U_L regions adjacent to the TR_L and IR_L in all three serotypes of MDV (Silva et al., 2001). The genes in these regions are most likely to explain why MDV is a lymphotropic virus that infects and replicates in chickens, turkeys and Japanese quail thus differing from other alphaherpesviruses.

The phosphoprotein (pp) 38 is encoded from the opposite junction regions between the U_L and TR_L and between the U_L and IR_L and is associated with MDV1 transformation and pathogenicity (Cui et al., 1991; Ding et al., 2007). However, homologues to pp38 have been reported in both MDV2 and HVT (Afonso et al., 2001; Izumiya et al., 2001; Kingham et al., 2001), but pp38 is not found in other herpesviruses (Silva et al., 2001). The pp38, together with phosphoprotein (pp) 24, forms a complex and is expressed during the cytolytic stages of infection as well as in the feather follicle epithelium (Cho et al., 1998). Deletion of pp38 from the very virulent MDV1 isolate Md5 resulted in a virus that was severely impaired in early cytolytic replication in the chicken, and consequently was virtually unable to cause visceral tumours or nerve lesions, while replication in vitro was unaffected (Reddy et al., 2002). However, studies reported by Xie et al. (1996) indicated that pp38 plays a major role in the proliferation of lymphoblastoid cells.

SORF 1 and SORF 3 and the MDV-specific ORFs in U_L that flank the highly conserved UL1-UL54 are located in all three serotypes (Silva et al., 2001). Adjacent to the TR_L and IR_L, LORFs 1-3 and LORFs 9-11 have been reported only to be present in MDV, however, the function of their encoded proteins is unknown (Silva et al., 2001).

To the third class belong the ORFs that are serotype-specific and these genes are most likely to be involved in the pathogenic and oncogenic potential of serotype 1 of MDVs. In general, genes that appear to be serotype specific are

located in the repeat regions (Silva et al., 2001) and are discussed in the following sections.

1.5.4.1 MDV1-specific genes and gene products

Some of the most important MDV1 specific genes which are located in the repeat regions of the genome are the putative oncogene MDV EcoRI-Q (*meq*), a viral interleukin (vIL)-8 homologue and the phosphoprotein (pp) 38. These genes are exclusive to MDV1 and have been implicated in latency and tumour formation (Nair, 2005). The *meq* gene is the target of intensive investigation. There are two copies of *meq*, one each located in the repeat regions (TR_L and IR_L) flanking the U_L regions (Tulman et al., 2000; Kung et al., 2001). Numerous genetic anomalies have been reported which are associated with attenuation/virulence of MDV1 isolates. The most significant mutation is a sequence expansion of 177 bp within the *meq* gene (Shamblin et al., 2004; Spatz and Silva, 2007).

The major *meq* gene product is a 339 amino acid protein which is encoded by a single exon completely contained in the *meq* fragment (Jones et al., 1992; Kung et al., 2001). Meq has a structure resembling the retroviral oncogenes Jun, Fos, and Maf (Kung et al., 2001). As it shares the hallmarks of these oncogenes such as the ability to induce morphological transformation of cells and the ability to induce lymphocytic tumours (Lupiani et al., 2004; Brown et al., 2006), the *meq* gene plays a major regulatory role in the unique life cycle of MDV. The Meq protein has the propensity to bind to multiple proteins thus resulting in an enormous potential to reprogram viral and host gene expression (Kung et al., 2001; Anobile et al., 2006). Meq dimerizes with c-Jun and forms a heterodimer that is more stable than each homodimer and colocalize with it in the transformed cells (Levy et al., 2005). Same authors found that RNA interference to Meq and c-Jun down-modulated the expression of these genes and reduced the growth of a transformed chicken embryo fibroblast cell culture, suggesting that Meq transforms chicken cells by pirating the Jun pathway (Levy et al., 2005). It is furthermore one of the few genes that is consistently expressed during latency as well as in all tumours (Liu et al., 1998; Kung et al., 2001) and it might thus play in important role in both maintaining latency and inducing transformation (Okada et

al., 2007; Lupiani et al., 2004). The *meq* knockout mutant is able to replicate *in vitro* in chicken embryo fibroblasts (CEF), however, the *in vivo* replication capacity of the *meq* knockout mutant is significantly attenuated and the reduction in growth *in vivo* coincided with the time of entry to latency or reactivation from latency which suggests that *meq* may play a role in the latency process (Nair and Kung, 2004). Another *meq* knockout study indicated that *meq* is dispensable for *in vitro* virus replication and for lytic infection in chickens (Lupiani et al., 2004). Xie et al. (1996) have shown a role for *meq* in the maintenance of the transformed phenotype of MD tumour cells. *Meq* has also an anti-apoptotic function and the potential to stimulate cell growth which also suggests that *meq* contributes to the transformation by MDV1 (Liu et al., 1998).

Another gene, viral interleukin-8 (vIL-8), has a potential role in the pathogenicity of MDV1. The vIL-8 encodes a CXC chemokine and is located within the R_L regions downstream of *meq* (Liu et al., 1999; Parcells et al., 2001). The gene for vIL-8 is a spliced gene product which consists of three exons and its expression may lead to enhanced viral load in chickens and an increased risk for tumour development (Jarosinski et al., 2003). Functional analysis revealed that vIL-8 exhibited a strong chemotactic activity toward chicken peripheral blood mononuclear leucocytes such as lymphocytes and macrophages, but not heterophils (Parcells et al., 2001) and it has been speculated that the function of MDV vIL-8 could be to recruit target cells for infection or to serve as a decoy to cellular immune responses (Lupiani et al., 2001). Recent studies, working with vIL-8 knockout virus, have shown that deletion of vIL-8 results in attenuation of the virus and induction of significantly less gross tumour formation (Parcells et al., 2001; Cui et al., 2005).

SORF2 is also a unique gene of MDV1. SORF2 may play an important role in lytic infection and subsequent immunosuppression (Parcells et al., 1995). Deletion of SORF2 causes a significant reduction in the lytic infection without preventing tumour formation (Parcells et al., 1995). Liu et al. (2001) have shown that SORF2 interacts with growth hormone *in vitro* and *in vivo* which may play a role in MD resistance. However, the function of SORF2 is yet unclear and deserves further study.

The MDV encoded telomerase termed viral TR (vTR) exhibits 88 % sequence identity to chicken TR (chTR) and was likely pirated from the chicken genome (Fragnet et al., 2003). Dysregulation of telomerase has been associated with cell immortalization and oncogenesis and Trapp et al. (2006) have shown that vTR plays a critical role in MDV-induced T cell lymphomagenesis *in vivo*.

1.5.4.2 MDV2 genes

The U_S region of MDV2 is shorter than the U_S region of MDV1 and four unique genes for MDV2 have been identified within this region (Jang et al., 1998). Within the U_L region of MDV2, which is also shorter than the U_L region of MDV1, Hatama et al. (1999) reported one gene (MDV2 ORF273) with no homologous gene product in any other alphaherpesviruses. Recently, Jang (2004) reported another MDV2 specific gene, ORF873, which was mapped to the right part end of the U_L region of the genome.

MDV2 genes found in the long repeats (R_L) have no identifiable homologues in MDV1 (Izumiya et al., 2000). However, Izumiya et al. (2000) identified a homologue of the MDV1 pp38 within the junctions between U_L and repeat sequences of MDV2, but there were differences which are specific to each serotype. Further sequence analysis in the repeat regions of MDV2 showed no homology with those sequences found in the oncogenic MDV1 genome in the respective regions.

However, functional analysis of MDV2 specific genes is rare and more detailed studies on MDV2-unique genes are needed as they are important for understanding the natural nononcogenic phenotypes of MDV.

1.5.4.3 HVT genes

The U_L and U_S genomic regions of HVT are very similar to those of MDV1 and MDV2 with homologous genes showing a high degree of co-linearity, and protein composition show a high degree of identity at the amino acid level. Within the U_L region, there are six genes with homologues in MDV1 and MDV2, but also two genes (HVT068 and HVT070) which are unique for HVT (Afonso et al, 2001).

However, their function and predicted structure remains unclear. The U_S region is shorter than that of MDV1 due to a lack of one MDV1 homologous gene and differences at the U_L/ short repeats (R_S) boundary - HVT contains two homologues of the gene encoding glycoprotein E. The R_S region of HVT is longer than that of MDV1 and with the exception of one homologue to MDV1, the gene content is different from that of MDV1. Another six genes, HVT072-HVT075, HVT079 and HVT096 which are unique to HVT are found in the R_L and R_S region (Afonso et al, 2001), but again, the function of these genes is unknown.

Consistent with its non-pathogenic phenotype, HVT lacks homologues of all MDV1 proteins with putative functions involving viral virulence, host range and oncogenicity. HVT contains no homologues of the *meq* protein, vIL-8 and lacks conserved domains of pp38 (Afonso et al., 2001).

1.5.5 Other Herpesviruses related to MDV

Herpesviruses are widespread in vertebrate species, and more than 120 members of the family *Herpesviridae* have been identified (Davison, 2002b). MDV was initially classified as a Gammaherpesvirus based on its lymphotrophic biological properties (Witter et al., 1969) which MDV shares with the *Epstein-Barr virus* (EBV). EBV is a human pathogen and associated with highly aggressive tumour progression (Perkins et al., 2006). Like MDV, it initially infects B lymphocytes and induces and maintains their proliferation (Altmann et al., 2006). However, MDV was reclassified as an Alphaherpesvirus based on its genetic structure (Buckmaster et al., 1988).

Within the subfamily E of *Alphaherpesvirinae,* the herpes simplex virus 1 (HSV-1), a member of the genus *Simplexvirus*, is a prototype representative with an identical genome organisation to MDV (Roizman, 1996).

The Varicella zoster virus (VZV) is a member of the genus *Varicellovirus* and closely related to HSV-1, sharing much genome homology. VZV and MDV share some aspects regarding requirements for major tegument proteins in replication (Cohen and Seidel, 1994). In addition to the third genus (*Mardivirus)* in this subfamily, a fourth genus is recognized: the genus *Iltovirus.* A member of this genus is *Gallid Herpesvirus 1* (GaHV-1). This virus causes infectious

laryngotracheitis (ILT) in chickens and is another highly contagious disease of chickens which represents a threat to the poultry industry as it causes decreased egg production and mortality (Tong et al., 2001). GaHV-1 is shed in respiratory secretions and transmitted by droplet inhalation. Symptoms include coughing, sneezing, head shaking, lethargy, discharge from the eyes and nostrils and difficulty breathing (May and Tittsler, 1925; Cover and Benton, 1958).

1.6 Pathobiology and epidemiology

1.6.1 Pathogenesis

The pathogenesis of MD is complex life cycle and involves cytolytic, latent and tumour stages of infection (Baigent and Davison, 2004). The feather follicle epithelium (FFE) is the only tissues where fully productive (lytic) infection takes place and infectious virus is produced in the cell-free state (Calnek et al., 1970; Calnek, 2001). This has a very important role in the transmission of the virus, but a limited role in inducing pathology and therefore is described separately. The cytolytic form is characterized by the formation of viral antigens and naked nuclear virions and cell-associated infectivity. This occurs in lymphoid tissues and, to a lesser extent, parenchymatous tissues and results in cell death by apoptosis. During latent and tumour stages the viral genome persists in lymphoid cells that express no viral antigen (latency) or show limited viral expression resulting in the immortalization of the cells (transformation) (Calnek, 1986). The mode of MDV replication changes according to a defined pattern depending on changes in virus-cell interactions at different stages of the disease and in different tissues. All chickens are susceptible to infection with MDV and shed virus, even resistant genotypes which do not develop clinical MD (Hansen et al., 1967; Cole, 1968). The infection *in vivo* can be delineated into four phases and several phases can coexist in different cells in the same bird (Calnek, 1986; Schat, 1987). These are summarised in Figure 1.2 and described in more detail below.

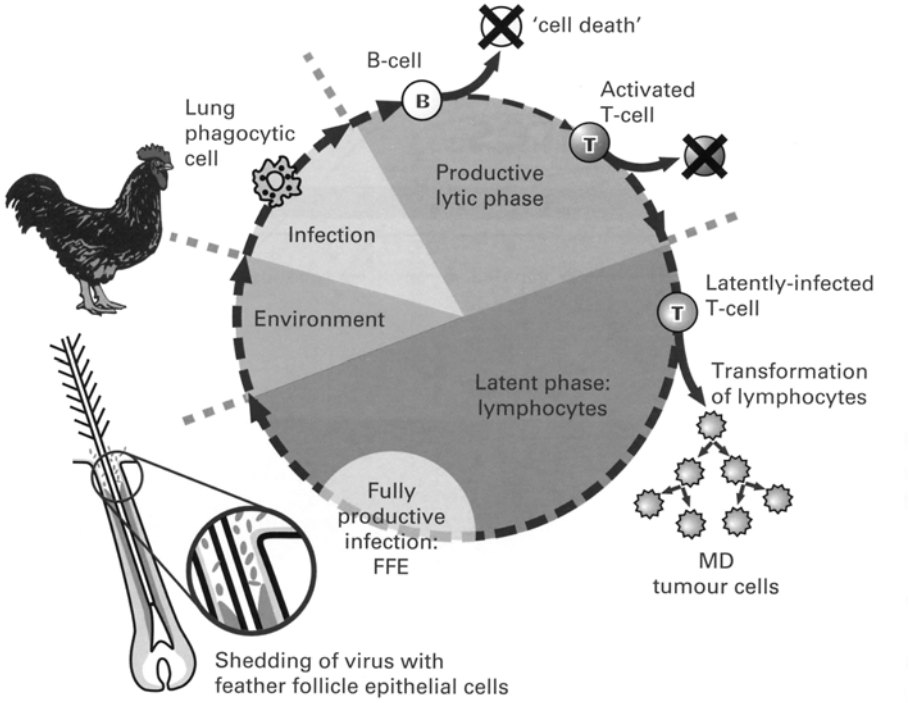

Figure 1.2: Schematic drawing depicting the different stages in the cycle of MD. Birds become infected by the inhalation of infectious virus shed from the feather follicle epithelium (FFE). After initial replication in the lungs, the virus replicates in the lymphoid organs. B-cells (B) undergo a lytic infection, resulting in the activation of T-cells, which are targeted by the virus. During the lytic infection, marked cytolysis in both B and T cells (cells with a cross in it) takes place. T-cells are transformed by the virus to produce tumours in different organs. Infected T-cells carry the infection to the feather follicle epithelium (Baigent and Davison, 1999; Nair, 2005).

1.6.1.1 Entry to the host and infection

Chickens are infected by inhaling particles containing cell-free MDV or infectious keratinized feather follicle epithelial cells which are shed from infected chickens (Johnson et al., 1971; Carozza et al., 1973) and virus crosses the lung epithelium to be taken up by phagocytic cells, mostly macrophages, that transport it to the lymphoid tissues such as the bursa of Fabricius (bursa), the thymus and the spleen where it replicates in the bursa-derived (B)-lymphocytes. These are the primary target of initial viral replication (Shek et al., 1983; Nair, 2005).

Attachment of virus to host cell receptors is likely to involve glycoproteins B, C and D (gB, gC, gD) and initiates fusion to the target cell, then penetration. Uncoating of the virion, by cellular enzymes, releases the viral DNA which circularizes and enters the nucleus. Messenger RNA (mRNA) synthesized in the

nucleus is transported into the cytoplasm for translation (Baigent and Davison, 2004).

Macrophages play a pivotal role in innate immunity and the development of adaptive immune responses by acting as antigen-presenting cells. Macrophages recognize antigen through pattern recognition receptors and release a variety of cytokines as well as nitric oxide. Recent studies have indicated that NO produced through the increased activity of inducible nitric oxide synthase is important for inhibiting MDV replication in the cytolytic phase of infection and continues into the latent phase (Xing and Schat, 2000; Djeraba et al., 2002). Earlier and increased levels of inducible nitric oxide synthase expression and NO production occur in MD resistant genotypes and probably contribute to the reduced MD viraemia and lower levels of clinical disease associated with genetic resistance (Davison and Kaiser, 2004).

Although it is assumed that all chickens within a flock become infected quickly with pathogenc MDV1, only little is known about the MD epidemiology, e.g. daily infection rates or amount of MDV wich needs to be inhaled by a chicken to become infected. This is mainly due to a lack of understanding of the fundamental factors influencing the transmission of MDV from chicken to chicken within populations. With the recent advancements of molecular methods to measure MDV, this should be feasible within the near future and would greatly assist understanding of MD epidemiology with direct implications for current control of MD.

1.6.1.2 Phase 1: early cytolytic phase

Early cytolytic infection is characterised by semi-productive replication of MDV with no fully infectious virus released from the cell or tissue, but expression of the viral genome which may range from the production of virus-specific antigens to enveloped intracellular virions. In the latter case, it is a cytolytic infection with MDV (Biggs, 2001).

An acute, cytolytic infection of lymphoid tissue, notably in the bursa, thymus and spleen appears at 3 days after infection (dpi) and reaches a peak at 5-7 dpi (Payne and Rennie, 1973). This necrotizing infection provokes an acute

inflammatory response resulting in an influx of many cell types including macrophages, thymus-derived (T)- and B-lymphocytes, and various granulocytes. Both uncommitted and committed lymphocytes are present (Payne and Roszkowski, 1973). With activation, T-lymphocytes become susceptible to infection themselves (Calnek, 1986). The activation of T cells during the early cytolytic phase is important for establishing infection in the target cells for latency and transformation (Schat, 1987). This cytolytic infection is only semi-productive, as cell-associated virions can only be spread by direct contact with other lymphocytes or by cell division. This fact may also reflect an inability of these cells to make certain viral structural components, or degradation of mature virus during passage through the outer cell membranes (Baigent, and Davison, 2004).

In the bursa and thymus, these changes are accompanied by severe regression of bursal lymphoid follicles and thymic cortex, resulting in a weight loss of these organs. Thymocytes undergo massive apoptosis (Morimura et al., 1996) which is the primary cause of the weight loss in the thymus. In the spleen, regressive lymphoid changes do not occur and the inflammatory changes result in a weight increase.

The early cytolytic phase lasts approximately 7 days causing a transient or permanent immunosuppression depending on the virulence of the challenge strain. After this MDV becomes latent mainly in T-lymphocytes and most probably in response to developing immune responses (Buscaglia et al., 1988; Davison and Nair, 2005).

1.6.1.3 Phase 2: Latency

During MDV latency, the viral genome persists in the host cell and although there are tumour antigens (MATSA) are expressed, there is no production of infectious virus except after reactivation (Morgan et al., 2001). There are three stages to latency: 1) establishment, 2) maintenance and 3) reactivation (Baigent and Davison, 2004).

The change from cytolytic to latent phase is suggested to occur about 6-8 dpi (Calnek et al., 1984; Buscaglia et al., 1988). At that time, MDV switches from its primary target cell, the B lymphocyte, to activated T cells, mainly of the CD4+

phenotype, in which latent infection is established (Calnek, 1986; Schat, 1985), although CD8+ T cells and B lymphocytes can also be involved. These CD4+ T cells are a subpopulation of T cells that express the CD4 receptor aid in immune responses and are therefore called T helper cells. The CD8+ cells are a subpopulation of T cells that express the CD8 receptor. CD8+ cells recognize antigens that are presented on the surface of host cells by the major histocompatibility complex (MHC) class I molecules, leading to their destruction, and are therefore known as cytotoxic T cells.

The expression of MDV antigens in lymphoid tissue is lost at this stage and tumours are not yet detectable (Ross, 1985; Baigent and Davison, 2004). Buscaglia et al. (1988) showed that host immune responses are crucial for establishment and maintenance of latency. Furthermore, various cytokines, including interferon and soluble mediators such as nitric oxide influence latency and immunocompetence is a requirement for latency to develop and be maintained (Buscaglia and Calnek, 1988; Buscaglia et al., 1988). Another important factor in the maintenance of latency is the oncogene *meq*, since it blocks apoptosis of latently infected CD4+ T cells and transactivates latent gene expression (Parcells et al., 2003).

Latently infected peripheral blood lymphocytes (PBL) disseminate the virus around the body to organs as diverse as the skin, FFE, viscera and nerves (Baigent, and Davison, 2004). In susceptible or suppressed birds, or those infected with a vvMDV pathotype, a second pathological cycle begins 2-3 weeks after primary infection in chickens that have survived the early cytolytic phase. In genetically resistant chickens, infection with MDV does not proceed beyond this phase and the chickens may not develop clinical signs of MD (Witter et al., 1971). However, the MDV infection can persist in these chickens at the latent stage and infective MDV is shed from the FFE in the environment. This may persist for the life of the chicken (Witter, 1971). MDV strains with increased virulence, especially the vv+MDV strains, may not establish latency or establish a fundamentally different type of latency when less virulent strains become latent under identical conditions (Calnek et al., 1998; Jarosinski et al., 2002).

1.6.1.4 Phase 3: late cytolytic phase

The secondary cytolytic infection does not always occur and the development and extent of the secondary cytolytic infection depends on genetic resistance of the host and the virulence of the MDV strain (Cole, 1968; Murthy and Calnek, 1979; Dalgaard et al., 2003). In MD- susceptible chickens, Calnek (1986) reported a second wave of semi-productive infection and cytolysis from 14-21 dpi. This late cytolytic infection affects the thymus, bursa and some epithelial tissues, including the FFE, kidney, adrenal gland and proventriculus (Baigent, and Davison, 2004). At the same time that epithelial tissues are developing focal infections, there is a reappearance of infection in the central lymphoid organs. Necrosis of lymphocytes and epithelial cells is accompanied by pronounced inflammation, infiltration of mononuclear cells and heterophils. The infiltrations can be very intense, especially in the areas of skin around the feather shafts (Calnek, 1986). This is coincident with permanent T cell immunosuppression and subsequent development of lymphomas (Schat and Xing, 2000). Furthermore, bursa and thymus suffer from severe atrophy during this stage of infection.

1.6.1.5 Fully productive infection in the feather-follicle epithelium (FFE)

The replication in the FFE is unique in that it is the only known site of complete cell-free virus replication. It occurs in genetically resistant as well as susceptible birds independently of the virulence of the MDV strain (Calnek et al., 1970).

Virus is probably carried to the skin and feather follicles by latently infected PBL and can be detected from as early as 5 dpi (Purchase, 1970; Baigent et al., 2005a; Islam and Walkden-Brown, 2007). From about 12-13 dpi, virus replication is fully productive, resulting in the release of infectious, enveloped, cell-free virus by passive cell break-up, accompanied by cell death (Johnson et al., 1975). However, the same authors reported that changes in the follicular epithelium after MDV infection did not progress sequentially from small lesions on day 17 post infection. Rather, sections of several follicles on each day contained lesions at various stages of development indicating that the entire epithelium was not infected simultaneously but during a span of several days.

Virus particles first appear in the nucleus of keratinizing cells of the stratum transitivum. Their appearance is followed by the development of a nuclear inclusion characterized by margination of the chromatin and development of a less dense homogenous material in the centre of the nucleus thus displacing the nucleoli and chromatin material to the periphery. Naked and occasionally enveloped immature virions were found either within the inclusion body or in the peripheral regions of the nucleus (Nazerian and Witter, 1970). Immature virions are released through typically enlarged nucleus pores or ruptures in the nuclear membrane into the cytoplasm of epithelial cells where envelopment occurs primarily (Johnson et al., 1970). During the first stages of virus multiplication, virus particles are in the outer two layers of epithelium, the stratum transivativum and stratum corneum. In the keratinized cells of the stratum corneum which contain naked unenveloped and less frequently mature enveloped virions, the virus can undergo complete replication. As the lesion progress, cells of the intermediary and germinal layers become infected and the four layers of epithelium are replaced by acellular material composed of mature virions, inclusion bodies and cell debris (Johnson et al., 1975).

Concerning the natural transmission of MD, the FFE is a virus source of major significance as fully enveloped, infectious, cell-free MDV is shed with the FFE cells sloughing off in the form of dander from about 7 days onwards (Colwell and Schmittle, 1968).

1.6.1.6 Phase 4: Transformation

From 3-4 weeks post-infection, non-productively infected lymphocytes progressively migrate into the visceral organs and peripheral nerves, where they proliferate to form lymphomas. Commonly affected visceral organs are the gonads, liver spleen, heart, proventriculus, bursa and skin. Lymphomas consist predominantly of heterogeneous accumulations of T lymphocytes (75 %) and lymphoblasts; B lymphocytes, macrophages and reticulum cells are present as minorities (Burgess, 2004). Ultra structurally, MD lymphoma cells cannot be distinguished from normal lymphocytes or lymphoblasts (Doak, 1973). The usual target cells for transformation are CD4+ T lymphocytes. However, lymphomas

can also contain smaller, variable numbers of B-cells, macrophages and CD8+ cells (Davison and Nair, 2005). A minority of 1% to ≈35 % of T lymphocytes in MD lymphomas express molecules known as 'MD associated tumour surface antigens' (MATSAs), thought to be markers of neoplastic-transformation (Witter et al., 1975; Burgess and Venugopal, 2002). To date, the CD30 antigen has been identified as a MATSA and CD30 expression by MDV transformed lymphocytes correlates with expression of the *meq* gene *in vivo* (Burgess et al., 2004).

Development of lymphomas depends on the chicken genotype and the MDV pathotype (Burgess, 2004): if chickens are susceptible to the particular MDV, the CD4+ lymphocyte numbers increase and the CD8+ lymphocyte numbers decrease, and gross CD4+ lymphomas form (Burgess et al., 2001). The opposite occurs if chickens are resistant to the MDV pathotype; CD8+ lymphocytes predominate over CD4+ lymphocytes, and lesions regress (Burgess et al., 2001).

The maintenance of the transforming state might be influenced by an altered pattern of transcription of the MDV genome in lymphoma cells as there are only limited regions of the MDV genome transcriptionally active (Silver et al., 1979; Sugaya et al., 1990). Less than 20 % of the MDV genome is transcribed, with approximately 65 % of the resulting RNA being transported to ribosomes. This prevents viral replication and maintains the state of cellular transformation (Baigent and Davison, 2004). One of the major transcripts detectable in MDV tumours is the oncogene *meq* which is associated with the maintenance of transformation and expressed consistently in the nucleus of lymphoma cells and tumour cell lines (Liu et al., 1998).

1.6.1.7 Immunosuppressive effects of MDV infection

Suppression of the immune response by MDV infection is a critical feature of the disease, contributing to the virulence of MDV isolates (Buscaglia et al., 1988; Calnek et al., 1998). In this chapter, immunosuppression is defined as originally proposed by Dohms and Saif (1984) where it is described as 'a state of temporary or permanent dysfunction of the immune response resulting from insults to the immune system and leading to increased susceptibility to disease'.

MDV- induced immunosuppression is often divided into an early transient phase during the initial cytolytic infection followed by a second permanent phase when MDV replication is reactivated and tumours may develop (Schat, 2004). During the early phase, MD-induced immunosuppression can be triggered by apoptosis of CD4+ and CD8+ T cells during early cytolytic infection (Morimura et al., 1996). It has been assumed that this lymphocyte depletion, which results from increased cytolytic replication of MDV, will lead to enhanced oncogenicity. However, studies by Jones et al. (1996) and Witter et al. (1997), suggest that increased levels of cytolytic infection do not necessarily lead to an increase in tumour incidence.

In the bursa, infection with MDV results in initially transient acute cytolytic changes accompanied by atrophy which can be seen at 12-13 days post challenge (dpc) (Jakowski et al., 1969; Witter et al., 1980). Thymic atrophy is also severe and has been recently reported to be present as early as 7 days post infection (dpi) (Islam et al., 2002); lymphocytes are depleted in both cortex and medulla (Witter et al., 1980). However, both bursal and thymic atrophy are significantly influenced by the genetic constitution of the chickens and MDV maternal antibody status.

During the later phases of MDV infection, additional destruction of lymphoid tissues and permanent immunosuppression can occur (Schat, 2004). Islam et al. (2002) showed that the Australian isolate MPF57 caused significant atrophy in bursa and thymus up to 35 dpc in unvaccinated, maternal antibody positive commercial broiler chickens. Several potentially immuno-evasive mechanisms have been described for MD tumour cells. Hunt et al. (2001) suggested that MD tumour cells may have a decreased expression of MHC class I antigens after activation resulting in viral antigen expression. Burgess and Davison (2002) have shown that CD28, a MHC surface antigen, is down-regulated on lymphoma cells, but not in tumour cell lines (Parcells and Burgess, 2004). CD28 is an important co-stimulatory molecule for T-cell activation and the absence of CD28 may be one of the causes of immuno-evasion of the tumour cells (Schat, 2004).

1.6.1.8 Other consequences of MDV infection

In the past 20 years, the character of MD has changed, so that along with the still prevalent visceral lymphoma and neurological symptoms, severe brain oedema and acute deaths, even in fully vaccinated animals, are observed. Most v and vv MDV strains induce transient paralysis in most chicken lines, and the latest clade of MDV strains, vv+ strains, usually isolated from vaccine breaks, can cause atherosclerosis and massive brain lesions that can be fatal (Gimeno et al., 1999; 2001; Witter et al., 1999).

1.6.2 Factors affecting pathogenesis

1.6.2.1 Virus serotype and pathotype

All oncogenic strains of MDV belong to serotype 1 as neither serotype 2 nor serotype 3 have been shown to have any oncogenic potential (Calnek, 2001). Although unattenuated MDV1 strains are all pathogenic and oncogenic, the virus strains can range from weakly oncogenic to hyper virulent and oncogenic (Witter, 1997; Davison and Nair, 2005). The current and commonly used classification system of MDV pathotypes derives particularly from the work of Witter (1983; 1997) and is explained later in detail. This classification is based on the ability of emerging strains of MDV to overcome different vaccination strategies using a standard susceptible strain of chickens. MD vaccines target MDV replication in the cytolytic phase and prevent tumour formation, but they do not induce sterile immunity and vaccinated chickens still become infected with challenge pathotypes which replicate in the host and are shed in the environment (Okazaki et al., 1970; Davison and Kaiser, 2004; Islam and Walkden-Brown, 2007; Islam et al., 2008a). This might have driven MDV to evolve to greater virulence (Witter, 1997; Gandon et al., 2001) as has occurred in the USA (Figure 1.3).

Increasing virulence in this sense appears to be related to increasing oncogenicity although the molecular basis and the mechanisms of viral virulence are not well understood (Payne, 2004). The more virulent pathotypes can cause MD outbreaks in vaccinated chickens and are often associated with central

nervous system (CNS) manifestations, increased visceral lymphoma frequency, increased mortality, early mortality with bursal and thymic atrophy and increased frequency of ocular lesions (Witter, 1983).

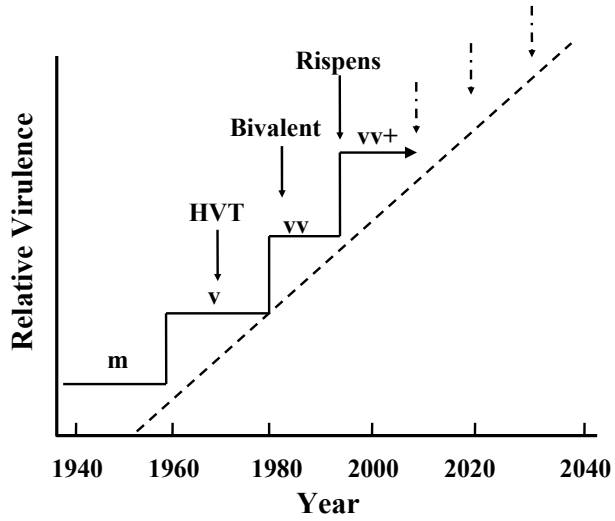

Figure 1.3: Past and projected evolution of Marek's disease virus towards greater virulence in the USA. The introduction of new vaccines is indicated with the arrows, and periods of relatively good control with vaccination by the horizontal parts of the curve. The predominant pathotype of MD (m, v, vv, vv+) during each period is also shown. (Witter, 1998). It is postulated that vaccine based approaches to MD control will invariably break down as the virus evolves under selection pressure from vaccination.

1.6.2.2 Host genotype

The genotype of the host plays an important part in determining the outcome of infection by MDV (Hansen et al., 1967; Cole, 1968). There is a clear genotype – based divergence in pathogenic events beginning at 7 dpi, when the virus enters latency and immune responses first become evident (Shek et al., 1983; Calnek, 2001). All chicken genotypes are susceptible to MDV infection, but they differ greatly in their resistance or susceptibility to clinical MD. Several loci are involved (Calnek, 1985; Vallejo et al., 1998). The chicken major histocompatibility complex (MHC) has a strong influence on MD resistance (Briles et al., 1977; Longenecker et al., 1977; Dalgaard et al., 2003). The MHC linked resistance is thought to involve regulatory components of cell-mediated responsiveness, or to involve differences in the specificity of immune response to MDV or tumour cells (Bacon et al., 2001). Genes within the class I (B-F) region are most influential, with the

B21 haplotype (line N) expressing the lowest levels of class I but possessing the highest order of resistance (Kaufman et al., 1995), whereas the B19 haplotype (line P) expresses the highest levels of class I and represents the highest level of susceptibility (Kaiser et al., 2003). There are other forms of genetic resistance which have been associated with genes outside the MHC, most importantly a cluster of genes (MDV1 locus) on chicken chromosome 1 (Bumstead, 1998). The non-MHC genes are thought to act by influencing cellular interactions, numbers or differences in infected target cells or regulation of cytokines and innate immunity (Bacon et al., 2001). Burgess et al. (2001) show that chickens of both MD resistant and susceptible lines both have predominantly CD4+ thymus cells in tissue lesions during the early cytolytic and latent phases of MDV infection. While CD4+ cells remain predominant in lesions of susceptible strain chickens during the time of tumour development, CD8+ cells become predominant in lesions of resistant chickens (Burgess et al., 2001).

There is a clear genotype-based divergence in pathogenic events beginning at about 7 dpi, when immune responses first become evident. Genetically susceptible chickens develop higher levels of latent infection of T cells (Fabricant et al., 1977; Shek et al., 1983), and lower levels of virus- neutralizing antibodies (Sharma and Stone, 1972; Calnek, 1973). For genetically resistant strains, there are no clinical signs found at all after the first week post infection unless they are infected by virus strains of very high pathogenicity or there are complicating factors such as immunosuppression (Calnek, 1985). Genetically resistant strains develop and maintain a high level of virus- neutralizing antibodies (Sharma and Stone, 1972). The numbers of infected T cells are significantly reduced compared with susceptible birds, and may be due to a superior immunological ability to reject infected or transformed T cells. The early cytolytic lymphoid infection occurs, but lymphomas do not develop (Burgess et al., 2001). There is no secondary phase of cytolytic infection and no permanent immunosuppression. Latent infection in PBL and spleen can be detected and virus continues to be shed from the FFE, however, the level of these infections is low (Calnek, 2001).

1.6.2.3 Host immunity

Acquired immunity also influences susceptibility to MD. This includes passive immunity due to maternal antibodies, which lasts for about 3 weeks, and actively acquired immunity because of either natural infection by MDV of low virulence or MD vaccination, which is of long or permanent duration. These forms of immunity suppress the early cytolytic infection and lymphoproliferation caused by virulent strains (Morimura et al., 1998; Baaten et al., 2004).

Maternal antibodies delay the development of clinical signs of MD, providing some protection against MD morbidity, tumour formation and mortality (Chubb and Churchill, 1969, Calnek, 1972). Witter et al. (1980) showed that bursal atrophy was significantly less in MD resistant and maternal antibody positive chickens whereas necrosis, atrophy, and aplasia of these organs were most pronounced in chickens lacking maternal antibody when exposed to virus (Jakowski et al., 1969, 1970). However, in maternal antibody positive chickens, MD vaccines of all serotypes provide lower levels of protection (Witter and Lee, 1984; Witter, 1982) than in maternal antibody negative vaccinated chickens.

MD vaccines were the first successful vaccines to be used for protection against cancer (Davison and Nair, 2005). Immunization with MDV or HVT vaccines operates at two levels, antiviral and/or antitumour (Schierman and McBride, 1979). Although vaccines present virus structural antigens, also affect infected cell expression which resembles effects seen in pathogenic MDV1 infected cells. Calnek et al. (1979) and Fabricant et al. (1982) reported expression of MATSA in the nerves of chickens and turkeys vaccinated with HVT. Current vaccines of all three serotypes (CVI988, SB-1 and HVT) protect chickens against MD viral tumorigenesis (Okazaki et al., 1970; Rispens et al., 1972a, 1972b; Donahoe and Kleven, 1977; Schat and Calnek, 1978; Schierman and McBride, 1979), most likely by enhancing cell-mediated immunity (CMI) against Marek's disease tumour cells. In addition, HVT has been shown to have antiviral effects by protecting against productive MDV infection in the thymus and cell-associated viremia (Witter et al., 1976). Recent studies have shown that antiviral effects are also present in peripheral blood lymphocytes (PBL) (Islam et al., 2006b), spleen (Islam et al., 2001) and dust (Islam et al., 2005a).

1.6.3 Co-infection with other pathogens

As infection with MDV alters the susceptibility of the host towards other pathogens, it is essential to assure that the MDV strain and experimentally infected chickens are free of other immunosuppressive pathogens (Schat, 2004). Together with other immunosuppressive pathogens such as reovirus, reticuloendotheliosis virus and infectious bursal disease virus, infection with chicken infectious anaemia virus (CIAV) influences the pathogenesis of MD remarkably. CIAV infection has been associated with MDV vaccine breaks or aggravation of MD and it has been reported that co-infection with CIAV can destroy and prevent the development of MDV-specific CD8+ (cytotoxic) T cells (Markowski-Grimsrud and Schat, 2003). As a consequence of CIAV infection, precursor populations of cytotoxic T cells are reduced which results in a greater susceptibility to secondary infections such as MDV. This may affect the interpretation of many of the older studies on the consequences of MDV-associated immunosuppression as many of the papers do not record the CIAV status of the MDV challenge virus used and/ or experimental chickens. If either was true, the immunosuppressive effects of MDV would therefore be overestimated (Markowski-Grimsrud and Schat, 2003; Schat, 2004).

Other factors which may trigger immunosuppression during the cytolytic phase of MDV infection are due to changes in the regulation of immune responses. Nitric oxide (NO) produced by macrophages has been reported to reduce MDV replication *in vivo* (Davison and Kaiser, 2004). However, excessive production of NO in the thymus during the cytolytic phase which is the case during an infection with a vv+ MDV may lead to apoptosis of thymus cells. NO can induce apoptosis by causing mitochondrial dysfunction in these cells (Bustamante et al., 2000). Another factor thought to be involved in MDV-induced immunosuppression is the up-regulation or down-regulation of the production of cytokines which is part of the development of immune responses and as a consequence of cytolytic infection (Schat, 2004). An up-regulation of the pro-inflammatory cytokines IL-6 and IL-8 as reported by Kaiser et al. (2003) in susceptible but not resistant chickens may aggravate MD lesions, leading to immunosuppression.

1.6.4 Transmission

MD is highly contagious and it is assumed that it is readily transmitted among chickens. Regardless of vaccination status, early epidemiological studies report that within 2 weeks after the introduction in a chicken flock, all chickens were positive for MDV using cell culture tests (Nazerian and Witter, 1970) and developed clinical MD within 3-4 weeks after infection (Calnek et al., 1970). However, neither of these early studies was able to determine the daily spread of the virus once introduced in the flock, nor the amount of MDV which each individual chicken needed to inhale in order to become infected. These are important issues which have not yet been investigated fully and demand further research.

The source of infectious virus is the FFE, the only site where fully- productive infection and release of cell-free MDV occurs (Calnek et al. 1970; Nazerian and Witter, 1970). The virus is shed with the debris of dead epithelial cells and also by moulted feathers having infected cells attached to them (Carozza et al., 1973). This infectious dander then can be inhaled by other chickens and the virus may remain infectious for several months in chicken litter and dust and therefore, represents a substantial source for sequential infection (Beasley et al., 1970; Blake et al., 2005).

Carozza et al. (1973) proposed that MDV exists in two different forms in feather dust: cell-free virus and keratin-wrapped particles. Cell-free virus particles associated with skin debris are highly infectious but labile, whereas keratin-wrapped particles are less infectious but more stable in the environment, due to the protection afforded by the cell debris and other components of poultry dust.

The portal for virus entry is the lungs, but little is known about the sequential events following the infection by the natural route, because of problems with delivering a known, precisely timed dose of MDV in its native form in dust (Baigent and Davison, 2004). There may be different mechanisms of uptake of keratin- wrapped MDV and cell- free MDV. Which of these forms of dust-associated MDV is predominantly responsible for initiating infection, remains to be determined (Baigent and Davison, 2004).

1.7 Control of MD

1.7.1 Vaccination against MD

Today, vaccines of all three serotypes are available to control MD in the field. The first vaccine (based on the attenuated oncogenic strain HPRS-16) was described (Churchill et al., 1969a) shortly after identification of the causative agent of MDV in 1967 (Churchill and Biggs, 1967). However, it was quickly replaced by another vaccine which was based on the HVT strain FC126 (Okazaki et al., 1970; Witter et al., 1970; Purchase et al., 1971). This vaccine was initially licensed in the USA in 1971, and because of its great success, it was soon used by the poultry industry worldwide. The HVT vaccine was developed as a cell-associated vaccine (Okazaki et al., 1970), but later it became available as a cell-free vaccine (Patrascu et al., 1972; Eidson et al., 1975, 1978). The cell-free vaccine was mainly used in countries where the necessary cold storage required for the cell-associated vaccine was problematic. HVT- based vaccines are still widely used, either alone or in combination with vaccine strains of MDV1 and/ or MDV2.

In 1978, Schat and Calnek characterized an apparently non-oncogenic strain of MDV2, SB-1 (Schat and Calnek, 1978). The SB-1 derived vaccine itself offered protection against infection with pathogenic strains of MDV1, but when administered in combination with HVT, there were synergistic effects and consequently better protection (Calnek et al., 1983; Witter et al., 1984). The first bivalent vaccine, consisting of HVT and SB-1 was introduced in the USA in 1983 as a response to increasing MD outbreaks in HVT-vaccinated flocks. It is still widely used, although it has not been licensed for the European market (Bublot and Sharma, 2004).

The most widely used strain of attenuated serotype 1 is Rispens/CVI988 which was first isolated by Rispens in 1972 (Rispens et al., 1972a). Because of some safety problems with low-level oncogenicity in highly MD-susceptible chicken lines as reported by von Bülow (1977), the vaccine was launched much later in 1994 in the USA. Present preparations of this vaccine are considered to be the ‘gold standard’ as it offers superior protection even against the strains which are classified as very virulent plus (Witter, 2002; Davison and Nair, 2005).

Recently a novel MDV serotype 1 vaccine was introduced in Australia using strain BH16 which was originally isolated from commercial birds in NSW and attenuated by serial passage in cell culture. This vaccine has comparable efficacy to the CVI988 vaccine (Karpathy et al., 2003) but was only tested against a comparatively mild MDV strain (MPF57). During the last two decades, efforts made on the development of recombinant vaccine have not been very successful. The efficacy of subunit vaccines expressing genes of MDV1 in live vectors, such as HVT and fowlpox virus, was not comparable to the live attenuated and / or avirulent conventional vaccines (Morgan et al., 1992; Nazerian et al., 1992). The latest generation of recombinant MD vaccine candidates based on vaccination with DNA containing the whole genome of MD cloned into a BAC vector also induced only partial protection against vvMDV (Tischer et al., 2002; Petherbridge et al., 2003). Therefore, recombinant vaccines are yet to achieve the improved protection required compared to existing vaccines in order to be widely accepted by the poultry industry. Several approaches towards the improvement of recombinant vaccines are underway (Davison and Nair, 2005) and it is likely that recombinant vaccines will be used in the future.

Vaccines have historically been administered by either the subcutaneous (nape of neck) or intramuscular (leg) routes in 1-day-old chickens. This is still the main method used to vaccinate breeder and layer chickens. The standard dose is usually 2-6x10^3 plaque forming units (pfu) per chicken in 0.2 ml/dose (Witter and Schat, 2003). However today, most major commercial broiler hatcheries perform an automated *in ovo* vaccination, usually around embryonation day 18. There is no apparent adverse effect from *in ovo* vaccination on either the hatchability or the long-term performance of the chickens and as it is done in an automated system, it saves labour cost (Bublot and Sharma, 2004). The early vaccination also provides additional time for an effective immune response to be mounted prior to challenge.

1.7.2 Hygiene and biosecurity

Biosecurity is a set of practices that limit the spread of disease-causing organisms. When teamed with disinfection and sanitation procedures, biosecurity practices can eradicate or reduce pathogens to noninfectious levels.

With regards to MD, the aim of biosecurity measures is the reduction of initial levels of MDV1 on a farm by preventing the entry of MDV1 into a building, and secondly, the prevention of contamination of the environment from that building. Measures to reduce initial MDV1 levels based on all-in all-out systems/single-age building systems have been widely stressed, but air management, and the disposal of chicken carcasses and manure, deserve more attention to prevent environmental contamination (Gimeno, 2004). As well, the of location of poultry farms more than 3.5 km from each other can significantly contribute to the control of MD (Groves, 1995).

1.8 Diagnosis of MD and characterization of MDV

1.8.1 Diagnosis of MD in vivo

The diagnosis of MD, especially in field cases, is mostly based on clinical signs and examination of gross lesions in tissues (Zelnik, 2004). However, the diagnosis of MD under field conditions remains difficult. This is mainly because of the changing epidemiology of MD (Morrow and Fehler, 2004) and other diseases caused by avian leukosis virus (ALV) such as lymphoid leukosis and myeloid leukosis which both can induce tumour formation and may be difficult to distinguish from MD. In 1970, guidelines for the pathologic diagnosis of MD and its differentiation from lymphoid leukosis were described by Siccardi and Burmester (1970). Although these differential diagnostic guidelines were mainly based on visible differences regarding distribution of tumours, and occurring cell type and conformation of the tumours, this publication was the first attempt to address the difficult task of differentiating MD from diseases with similar symptoms like lymphoid leukosis.

MD is a complex disease and it involves, apart from tumour formation in various organs, both neuroplastic and inflammatory components including apparently unrelated conditions that result from inflammatory/ degenerative lesions, e.g. an early mortality syndrome, a transient paralysis syndrome and severe lymphoid organ atrophy with immunosuppression and the attendant consequences (Calnek and Witter, 1997). Furthermore, Fabricant et al. (1978) reported that MDV has been found to be involved in atherosclerosis in chickens. However, in the almost 100 years since the initial description of MD, the clinical picture of the disease has changed - the chronic polyneuritis that was prevalent until 1925 was joined by visceral lymphoma from 1925–1950, and from 1950 onwards more aggressive and faster developing tumours were observed (Benton et al., 1957). During the past 25 years, MDV virulence has continued to increase and the clinical picture of the disease has changed again, so that along with the still prevalent visceral lymphoma and neurological symptoms, severe brain oedema and acute deaths, even in fully vaccinated animals, are observed (Witter, 1998; Osterrieder et al., 2006; Figure 1.4).

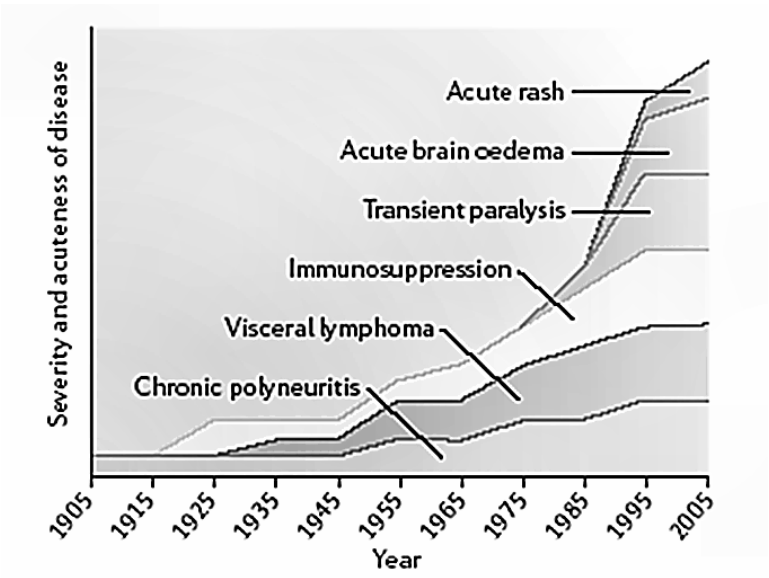

Figure 1.4: The change of the clinical picture of MD over the years (Osterrieder et al., 2006).

1.8.2 Pathotyping of MDV isolates

The term ‘pathotype’ is originally used to designate classes of organisms that induce different types of pathology (Witter et al., 2005). However, in the case of MD, the term is used widely to designate differences in the virulence of isolates as measured (primarily) by the frequency of disease induced and reflect important biological properties that correlate with the breakthrough of vaccinal immunity in the field (Witter et al., 2005).

The method for pathotyping MDV isolates *in vivo* as described by the Avian Disease and Oncology Laboratory (ADOL), Michigan, USA, is regarded as the ‘Gold Standard’ (Witter et al., 2005). The data output is based on the induction of lymphoproliferative lesions in 3 classes of chickens: non-vaccinated, vaccinated with HVT, and vaccinated with a bivalent (HVT + SB-1) vaccine (Witter et al., 2004). The design tests the ability of a test virus to break through immunity induced by these vaccines. The chickens are vaccinated with the respective vaccine at hatch using 2000 pfu. Challenge is performed at 5 days post vaccination using 500 pfu per chicken. In order to prevent inadvertent spread of infection between treatment groups, the chickens are kept in isolators. The test is terminated and all chickens sacrificed at about 56 days post challenge and the gross lesion data of test strains are compared with those of control strains using an interaction chi-square analysis (Steel and Torrie, 1960). Gross lesion data include mortality due to MD lymphoma, early mortality with bursal/ thymic atrophy and frequency of visceral lymphomas or ocular lesions in unvaccinated chickens and were found to be positively correlated with virulence (Witter, 1997). This method has been used to pathotype more than 45 isolates and is the basis for the current pathotype classification of MDV isolates (Witter, 1997). Its limitations include requirements for a specific type of chickens (15x7 ab+), large numbers of animals, and a statistical method to compare lesion responses to those of JM/102W and Md5 control strains (Witter et al., 2004).

A classification system for the pathogenic MDV1 strains as suggested by Witter (1997) is used commonly and divides MDV1 strains into four pathotypes:

- Mild MDV (mMDV): in genetically susceptible chickens these strains cause mainly neural MD and sometimes a low incidence of mainly ovarian lymphomas; their pathogenic effects are preventable with HVT vaccines. The predominant pathotype in “classical” MDV. Classification based on significantly lower pathogenicity than JM/102/W in HVT-vaccinated chickens.
- Virulent MDV (vMDV): these cause a high incidence of visceral and neural lymphomas; their effects can be prevented with HVT vaccines. Reference US strain is JM/102/W. Classification based on lack of significant difference in pathogenicity from JM/102/W in HVT-vaccinated chickens.
- very virulent MDV (vvMDV): these cause high incidence of visceral and neural lymphomas. They are oncogenic in HVT vaccinated birds and in birds genetically resistant to less virulent viruses; their effects are preventable with bivalent vaccines consisting of a strain of MDV2 (SB-1) combined with HVT (bivalent vaccination). Reference US strain is Md5. Classification based on lack of significant difference from Md5 in HVT/SB-1-vaccinated chickens.
- very virulent plus MDV (vv+MDV): these cause a high incidence of lymphomas and are oncogenic in birds vaccinated with bivalent vaccines. Classification based on significantly higher pathogenicity than Md5 in HVT/SB-1-vaccinated chickens.

Another classification system of MDV was established using a statistical approach: Gimeno et al. (2002), proposed the use of the term ‘neuropathotype’ in order to characterize strains that induce distinctly different types of pathology in the CNS. The proposed system is based on analysis of three variables as follows:

- Frequency of birds showing transient paralysis between 9 and 11 dpi.
- Mortality before 15 dpi.
- Frequency of birds showing persistent neurologic disease between 21 and 23 dpi.

Consequently the authors designated MDV1 strains into neuropathotypes A, B and C which roughly correspond to the virulent, very virulent and very virulent plus types of the Witter (1997) system. Gimeno et al. (2002) regard this system as a complementary tool which can be used together with the system suggested by Witter (1997). However, Gimeno et al. (2002) established their pathotyping system in maternal antibody negative chickens whereas Witter (1997) uses maternal antibody positive chickens. Therefore the MD susceptibility may vary in the two systems which make any comparisons between pathotyping results difficult.

1.8.3 Diagnosis of MD in vitro

Laboratory diagnosis has historically involved isolation of MDV possibly followed by identification and characterization of its DNA or antigens (Zelnik, 2004). Detection of MDV-specific antibodies is also used although these tests tend not to be serotypte-specific. Of extremely high importance for successful *in vitro* diagnosis of MDV is the proper collection of infectious material. As MDV is a highly cell-associated virus, it is essential that the samples contain viable cells and therefore, immediate sample processing and cooling is required.

1.8.3.1 Virus isolation and identification

In vitro isolation and propagation of MDV is a prerequisite for further characterization of the MDV isolate. Early studies that mainly focussed on the morphology and structure of MDV used electron microscopic examination of infected material (Nazerian and Burmester, 1968; Calnek et al. 1970; Johnson et al., 1975). However, because it requires specialized and expensive equipment, it is not routinely used for identifying MDV (Zelnik, 2004). The most commonly used method for primary isolation of MDV is the inoculation of susceptible tissue cultures with infectious material such as peripheral blood lymphocytes or lymphocytes isolated from spleen. This only became available in 1967, when Churchill and Biggs isolated MDV successfully for the first time in chicken kidney cell (CKC) culture (Churchill and Biggs, 1967). Shortly afterwards, Nazerian et al. (1968) and Solomon et al. (1968) successfully isolated MDV in duck embryo

fibroblasts (DEF). In both cultures, the presence of infectious virus was confirmed by the development of characteristic cytopathic effects (CPE). In CKC, first MDV foci can be observed as soon as 4 days post infection (dpi) (Schat, 2005).

The CKC and DEF cultures are primary cultures. CKC should be prepared from 14 day old chickens, DEF from 12 day old duck embryos respectively as described by Schat and Purchase (1998). MDV can also be isolated and propagated in other cell cultures such as chicken embryo fibroblasts (CEF) and chicken embryo kidney cell (CEKC). The problem with CEF and CEKC cultures is that they either yield less virus, do not support the replication of MDV or replication is abortive so that the virus would be lost after several passages in these cell cultures (Schat, 1985, Calnek, 1967). Another disadvantage of CEF is the fact that it is not possible to detect foci at 4 dpi which is especially important if vaccine viruses are present in the inoculum (Schat, 2005). The vaccine viruses such as CVI988, HVT and serotype 2 may replicate faster than, and outgrow, wild-type viruses if foci can only be detected at 6 dpi (Schat, 2005). In order to reduce the risk of contamination of the MDV isolates with other extraneous viruses, it is recommended to use specific pathogen free (SPF) chickens and SPF duck eggs for cell culture (Schat, 2005).

Passage of oncogenic and/or pathogenic MDV1 in cell culture results in the loss of oncogenicity and pathogenicity (Churchill et al., 1969b; Rispens et al., 1972a; Witter, 1982). The number of passages required for attenuation is variable, and depends on the isolate of MDV and the criteria used to evaluate attenuation (Witter, 2001a). Complete attenuation has been achieved in as few as 27 (Schat et al., 1985) or 33 (Churchill et al., 1969b) passages in CKC, however, the isolate US isolate Md11 was still virulent after 55 passages, but was attenuated after 75 passages in both DEF and CEF cells (Witter, 1982). In the case of CVI988, the original isolate had low levels of oncogenicity and was attenuated by 35 passages in DEF cells (Rispens et al., 1972b).

Attenuated virus generally grows to higher titres and produces larger plaques in cell culture than its parent (Rispens et al., 1972b; Biggs, 2001). In cell culture, HVT plaques can be readily distinguished from MDV1 or MDV2. However, plaques from MDV1 and MDV2 difficult to differentiate and therefore, serotype

and/or vaccine specific monoclonal antibodies are used for identification (Lee et al., 1983; Witter et al., 1987).

1.8.4 Molecular methods for MDV diagnosis

1.8.4.1 Polymerase chain reaction (PCR)

The isolation and propagation of viruses in cell culture is still the classical approach to identifying a virus. However, it requires expensive equipment and skilled personnel and is laborious and time-consuming (Zelnik, 2004). Methods have therefore been developed for identification of viruses by specific detection of viral DNA or RNA.

The introduction of the polymerase chain reaction (PCR) by Kary Mullis in 1983 has revolutionized molecular biology. The PCR technique is used to amplify a specific region of DNA through repeated cycles of duplication driven by an enzyme called DNA polymerase (Mullis et al., 1986; Mullis and Fallona, 1986). The PCR involves two nucleotide primers, usually between 15 and 30 nucleotides in length, which flank the DNA target sequence that is to be amplified. One of the primers has the same sequence as the so-called ‘sense’ or 5’ strand of the DNA, while the other has the same sequence as the ‘antisense’ or 3’ strand of the DNA. Each of the primers will bind to the complementary strand of DNA and thus initiate the synthesis of a new strand using deoxynucleotide-triphosphates (dNTP’s) which are the basic components of any DNA. The PCR reaction is split into three separate stages (adapted from Reece, 2004):

- Denaturation: The two strands of the target DNA molecule are separated into its component strands by heating which is reversible. This step is usually performed at a temperature of 94 °C.
- Annealing: The two target strands are then allowed to cool in the presence of the oligonucleotide primers. Both primers recognize their complementary binding site on the target DNA and bind to them. The temperature at which annealing of primers to the template DNA occurs

depends upon the length and sequence of the primer and the level of specificity required in a particular PCR reaction. This step is usually performed at a temperature in the range from 45-60 °C.

- Extension: The DNA polymerase binds to the free 3' end of the bound primers and uses deoxyribonucleotide triphosphates (dNTP's) which are also present in the reaction, to synthesize a new DNA strand in a 5' to 3' direction. This step is usually performed at 74 °C as this is the optimum DNA replication temperature for Taq polymerase.

The number of PCR cycles that are performed during an individual experiment depends upon the amount of initial DNA template and the desired amount of DNA required after the amplification process. In general, to avoid replication errors, as few cycles as possible will be performed. This is usually in the range of 25 – 35 cycles, but can be less than that.

1.8.4.2 Real – time PCR

The use of PCR in molecular diagnostics has increased to the point where it is now accepted as the 'Gold Standard' for detecting nucleic acids from a number of origins and it has become an essential tool in the research laboratory (Mackay et al., 2002). Real- time PCR offers the ability to monitor the accumulating amplicon in real time which has been made possible by the labelling of primers, probes or amplicons with fluorogenic molecules (Higuchi et al., 1992, 1993; Mackay et al., 2002).

Threshold cycle and Reaction efficiency

The threshold cycle (C_t) is defined as the PCR cycle in which the gain in fluorescence generated by the accumulating amplicon exceeds a chosen threshold above background fluorescence (Wilhelm et al., 2001). Jung et al. (2000) defined the C_t cycle when the fluorescence generated by the accumulating amplicon exceeded 10 standard deviations of the mean baseline fluorescence, using data taken from cycles 3 to 15. The threshold cycle is when the system begins to detect the increase in the signal associated with an exponential growth

of PCR product during a log-linear phase. This phase provides the most useful information about the reaction (Dorak, 2006). At the beginning of the exponential phase, all reagents are still in excess. This means the low amount of product will not compete with the primers' annealing capabilities and the DNA polymerase is still highly efficient, making the data more accurate.

In a perfectly efficient PCR, the amount of amplified product doubles each cycle. Therefore, a difference of 1 between sample C_t s means that the sample with the lower C_t value had double the target sequence of the other sample; a change in C_t of 2 means a fourfold difference; a change in C_t of 3 means an eightfold difference, and so on: $\Delta C_t = 2^{-\Delta Ct}$ fold change (Valasek and Repa, 2005). The C_t value is inversely proportional to the logarithmic amount of target DNA in the original sample (Valasek and Repa, 2005).

Figure 1.5 shows a typical amplification plot of a tenfold serial dilution of plasmid DNA run in duplicate.

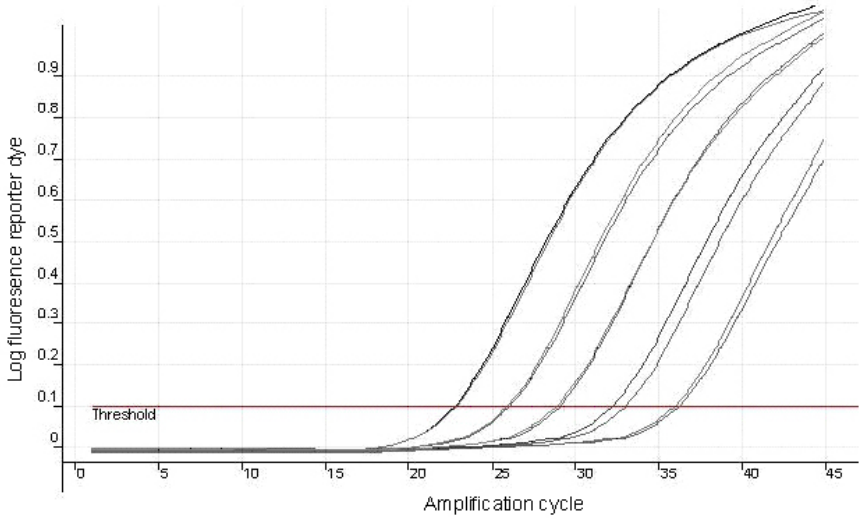

Figure 1.5: Diagram showing the released reporter fluorophore (y axis) plotted as a function of the amplification cycle number (x axis) for a serial tenfold dilution in duplicates of plasmid copies (10^5- 10^1), from left to right (Renz et al., 2006).

Amplicon detection chemistry

There are currently five main chemistries used for the detection of the PCR product during real-time PCR and they can be classified into amplicon sequence specific or non-specific methods of real-time PCR detection (Whitcombe et al.,

1999). The simplest method uses fluorescent dyes, e.g. SYBR Green, that bind specifically to double-stranded-DNA. The disadvantage of these unexpensive dyes is that both specific and non-specific products generate a signal.

Molecular Beacons, adjacent linear oligoprobes, Scorpions and the 5'-nuclease assay (TaqMan®), rely on the hybridisation of fluorescent-labelled oligonucleotides to the correct amplicon. These probes are oligonucleotides with both a reporter fluorescent dye and a quencher dye attached. While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by Förster resonance energy transfer (FRET) through space. These probes are highly specific but expensive.

1.8.4.3 PCR methods for detection of MDV

Conventional qualitative, semi-quantitative and quantitative PCR assays have been developed and are currently used to reliably detect and differentiate the three serotypes of MDV (Davidson et al., 1995; Bumstead et al., 1997; Reddy et al., 2000; Handberg et al., 2001). More recently, quantitative real-time PCR (qPCR) has emerged as an important technique for detection and analysis of nucleic acids due to its improved rapidity, sensitivity, reproducibility and lower risk of cross- contamination (Mackay, 2002). Quantitative real-time PCR assays for all three serotypes of MDV have been developed and are being used to quantify viral load in a variety of samples including spleen, feather tip and dust (Islam et al., 2004a; Baigent et al., 2005a; Islam et al., 2006a; Abdul-Careem et al., 2006). The assays as described by Baigent et al. (2005a) and Islam et al. (2006a) use TaqMan® probes for generating the fluorescent signal whereas Abdul-Careem et al. (2006) use the SYBR® Green chemistry. With these assays, the reliable detection and quantitation of all three serotypes in a single sample is possible.

However, the aim of differentiating reliably between pathogenic and vaccinal strains of MDV1 using the PCR technique, remains unachieved. Several methods have been published, based on standard PCR assays and related techniques, such as Southern blot, but they have not provided consistent or satisfactory results. Based on the earlier findings of Maotani et al. (1986), Silva (1992) reported a difference in the copy numbers of the 132 bp repeat between vaccinal and wild type strains of MDV1 which might be used to identify vaccinal and wild

type MDV1. This 132 bp repeat is located in the IR_L of the MDV genome and several laboratories have reported a strong correlation between the expansion of the 132 bp repeats and the loss of pathogenicity of MDV (Fukuchi et al., 1985; Maotani et al., 1986; Ross et al., 1981; Silva and Witter, 1985). Zhu et al. (1992) as well as Becker et al. (1993) reported similar findings using PCR in combination with Southern blot analysis and radioactive PCR respectively. However, there is no definite proof that the copy number of these 132 bp repeats correlate with the pathogenic potential of MDV1 (Silva et al., 1992) which might be used to differentiate between pathogenic and vaccinal strains of MDV1 and beside, it has subsequently been shown that this marker of attenuation may be lost in as little as one back passage in chickens (Burgess 2003; Young and Gravel 1996), and is not necessarily indicative of attenuation (Niikura et al. 2006; Silva and Gimeno 2007; Silva et al. 2004). Other attempts in Australia have not produced assays that reliably differentiate and quantify wild-type MDV from Rispens vaccine (Burgess, 2003).The development of a a fully quantitative PCR based assay in order to differentiate reliably between pathogenic and vaccinal strains of MDV1 is therefore still a field of ongoing and future research.

1.8.4.4 Advantages and disadvantages of molecular diagnostic methods

The introduction of PCR technology in biological research has revolutionised the rapid detection and quantification of many infectious agents, including MDV. The main advantages of the PCR method are that it is rapid, and DNA isolated even from crude biological materials can be analysed. During the last decade, this technique has been extensively used in MDV research and diagnosis, especially quantitative real-time PCR (qPCR) as it requires no post amplification handling and allows the absolute quantification of virus in several tissues (Islam, 2006; Islam et al., 2006a; Baigent et al., 2005a, 2005b). The qPCR techniques has been shown to be a powerful tool for routine monitoring of MDV under field and experimental condiditions, e.g. monitoring MDV in dust samples from commercial poultry farms (Islam, 2006), investigating the kinetics of all three serotypes of MDV in dust (Islam and Walkden-Brown, 2007). In addtition, qPCR has been shown to be an excellent method in order to establish correlations between viral

load in various tissues, e.g. spleen, blood and dust, and subsequent MD outcome (Walkden-Brown et al., 2006, 2007a, 2007b; Islam et al., 2006b).

However, the drawbacks of using real-time PCR in comparison with conventional PCR include the inability to monitor amplicon size without opening the system and the incompatibility of some platforms with some fluorogenic chemistries.In addition, the start-up expense of real-time PCR may be prohibitive when used in low-throughput laboratories. These shortcomings are mostly due to limitations in the system hardware or the available fluorogenic dyes or fluorophores (Mackay et al., 2002). Another disadvantage of the PCR methods is that none of them measures or differentiates between infectious and non-infectious virus.

1.8.4.5 Genomic analysis of MDV

In the case of MDV research, the complete genomes of all three serotypes have been sequenced: Tulman et al. (2000) reported the complete sequence of the American MDV1 strain Md5, classified as a very virulent strain; Lee et al. (2000) reported the complete genome sequence of the American MDV1 strain GA, classified as virulent; Afonso et al. (2001) described the complete sequence analysis for the HVT strain FC 126 and Izumiya et al. (2001) reported the complete genome organisation of the MDV2 strain HPRS24. Differences in the sequences of the different serotypes have reported recently (Spatz et al., 2007a; 2007b) and have also been discussed earlier.

A further goal is to identify mutations in genes encoded by MDV's of different virulence levels that correlate with the recognized pathotypes as described and classified by Witter et al. (1997). In order to identify pathotypes, Wei and Cui (2002) compared several genes of American and Chinese MDV1 strains which were classified into vMDV, vvMDV and vv+MDV against the gene sequences of the MDV1 vaccine strain Rispens/ CVI988 and the Chinese vaccine strain 814 respectively. These authors reported that there were several amino acid changes and/or deletions in the *meq* gene amongst the investigated strains which are likely to correlate with virulence/ attenuation. Similarly, Shamblin et al. (2004) reported a comparative analysis of MDV genes that appear to correlate with virulence of MDV across the three recognized pathotypes vMDV, vvMDV and

vv+MDV. Amongst the genes these authors investigated were the major surface glycoprotein genes (gB, gC, gD, gE, gH, gI and gL), the major lytic phosphoprotein pp38 and *meq*. However, of the investigated genes, only *meq* showed distinct polymorphisms and point mutations which correlated with virulence (Shamblin et al. 2004). Another possible candidate gene might be the viral lipase homologue (*vLIP*) gene which has been reported to be a virulence factor of MDV (Kamil et al., 2005).

DNA sequencing therefore provides a valuable tool for the research of MDV: the complete genome analysis of prototype strains of each of the three serotypes of MDV together with the DNA analysis of specific genes of further MDV strains, contributes to the understanding of the difference of virulence of MDV strains, MDV pathogenesis and the cellular and viral processes behind MD. However, current methods can directly sequence only relatively short (300-1000 nucleotides long) DNA fragments in a single reaction. The main obstacle to sequencing DNA fragments above this size limit is insufficient power of separation for resolving large DNA fragments that differ in length by only one nucleotide. Another major issue with functional genomics based on DNA sequencing has been shown recently, where it has been discovered that small RNAs can regulate transcription and protein abundance (Vaughn and Martienssen, 2005), and subsequently its function.

Other methods include proteome and transcriptome analysis of the MDV genome which will be a vital method for determining how viral and host proteome changes occurring in Marek's Disease pathogenesis regulate the switch between the lytic and latent phases of the MDV life cycle (Liu et al., 2006; Mohammad et al., 2008).

1.8.4.6 Cloning vectors for sequencing purposes

There are several different types of vector for cloning purposes and the choice of vector depends on a couple of important factors which need to be considered. The most important is the maximum size of insert that the different vectors will accommodate and the absence of chimaeras and deletions (Bolivar and Backman, 1979). However, there are features which are common to all types of vector:

- Sequences that permit autonomous propagation in bacteria or yeast.
- A cloning site to insert foreign DNA; the most versatile vectors contain a site that can be cut by many restriction enzymes.
- A method of selecting for bacteria or yeast containing the vector with foreign DNA; usually accomplished by selectable markers for drug resistance.

Plasmid vectors are widely used for cloning DNA fragments of up to 10 kb. Plasmids are typically circular double-stranded DNA molecules separate from the chromosomal DNA and capable of autonomous replication. They usually occur in bacteria, such as *Escherichia coli* (Bolivar and Backman, 1979). Plasmids often contain genes that confer a selective advantage to the bacterium harbouring them, e.g., the ability to make the bacterium antibiotic resistant and this can be used as a selection criterion for mutants having the desired DNA successfully inserted (Vieira and Messing, 1982). Every plasmid contains at least one DNA sequence that serves as an origin of replication or ori (a starting point for DNA replication), which enables the plasmid DNA to be duplicated independently from the chromosomal DNA. Most also contain a polylinker or multiple cloning site (MCS), which is a short region containing several commonly used restriction sites allowing the easy insertion of DNA fragments at this location (Vieira and Messing, 1982).

Figure 1.7 shows the pGEM®–T Easy plasmid vector circle map. This plasmid vector has been used for the molecular research work in reported in this thesis.

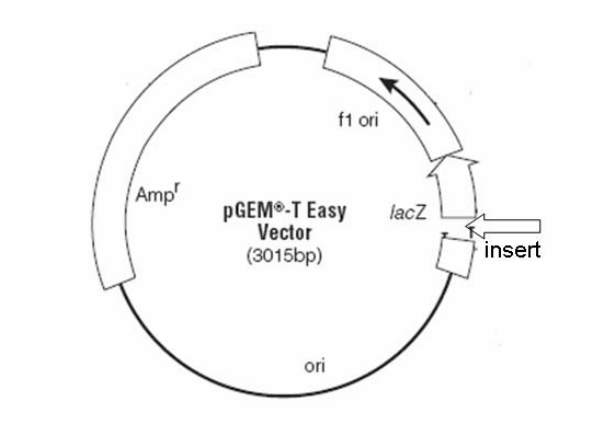

Figure 1.7: pGEM®–T Easy plasmid vector circle map. The arrow marks the insertion site for DNA fragments to be cloned into the vector.

1.8.4.7 Summary of the application and potential of MDV genome analysis

Rapid and reliable diagnosis of MD remains an important issue. Laboratory diagnostic methods such as MDV genome analysis including DNA sequencing and proteome or transciptome analysis will provide viable tools for our understanding of MDV genes and gene products and their function in virus replication. DNA sequencing analysis has shown to be a powerful tool for identifying mutations in the MDV genome which correlate with virulence (Shamblin et al., 2004) and is still widely used in order to investigate the molecular bases for MDV oncogenicity and pathogenicity. More recently, proteomic analysis has emerged and may give answers to the the mechanisms in Marek's Disease pathogenesis which regulate the switch between the lytic and latent phases of the MDV life cycle are still unclear (Liu et al., 2006).

1.9 Conclusions

It has been a century since MD was discovered and first described, but MD is still a continuous threat to the poultry industry, despite widespread use of existing technologies. Sporadic outbreaks of MD continue to occur and have been associated with increased virulence in the field strains of MDV1, the presence of other immunosuppressive infectious agents, and poor flock management.

Therefore, the rapid and reliable diagnosis of MD remains an important issue. Apart from the traditional methods, such as examination of gross pathology, novel laboratory diagnostic methods have gained growing significance.

The quantitative real-time PCR technique (qPCR) is now increasingly the preferred method for rapid, accurate and sensitive quantification of virus. This provides a powerful tool in order to measure virulent and vaccine virus loads and it is highly desirable to determine kinetics and replication of MDV in various genotypes. This method can also play an integral part in establishing association between infection level and disease status, including the substitution of invasive techniques with non-invasive materials for their long-term monitoring. Other molecular methods such as sequencing of whole genomes or selected genes have provided sequence information for all three serotypes of MDV. Recently,

some information has become available about the understanding of MDV genes, their gene product and their functions in viral replication and pathogenicity (Liu et al., 2006; Buza and Burgess, 2007; Mohammad et al., 2008). This new information is helping to elucidate cellular signalling pathways that have undergone convergent evolution and are perturbed by different viruses, and emphasizes the value of MDV as a comparative biomedical mode.l

The aims of this study were the optimization of existing MDV serotype-specific qPCR assays and their application to chicken tissues and dust samples, with a view to improved understanding of the pathogenesis of MD and shedding kinetics MDVs in layer chickens. An additional aim was to sequence the *meq* gene of selected Australian isolates of MDV to determine phylogenetic relationships with other MDVs and to ascertain whether there are markers for virulence in the *meq* gene. Furthermore, the aim was to pathotype recent Australian isolates of MDV1 and investigate early predictors of clinical Marek's disease.

Chapter 2

Absolute quantification of MDV2 genome copy number in dust samples, spleens and feather tips using real-time PCR

2.1 Introduction

Marek's disease virus (MDV) is a double-stranded linear DNA virus belonging to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*. Three members of the genus *Mardivirus*, Marek's disease virus serotypes 1 and 2 (MDV1 and MDV2) as well as serotype 3 or herpesvirus of turkeys (HVT) are classified (van Regenmortel et al., 2000, Davison, 2002). Serotype 1 viruses include the oncogenic MDVs which are further divided into pathotypes, ranging from mild (m)MDV up to very virulent plus (vv+)MDV isolates (Witter, 1997). Attenuated serotype 1 viruses are also used as vaccine isolates. Serotype 2 of MDV is a naturally occurring, infectious virus in chickens, but is non pathogenic or only weakly pathogenic and nononcogenic in chickens (Baigent and Davison, 2004). Several isolates of MDV2 are used as vaccines, mostly combined with either isolates of serotype 1 or 3 of MDV. HVT is infectious for chickens, but does not spread laterally to a significant extent (Tink, 2004). It is used widely as a vaccine for MD. Immunisation with HVT and MDV2 does not prevent productive infection by MDV1, but it offers protection against clinical signs of Marek's disease (MD) which includes immunosuppression, polyneuritis and T-cell lymphoma formation in a wide range of organs and tissues. When HVT is administered with MDV2 there are synergistic effects on protection against MD (Calnek et al., 1983; Witter et al., 1984).

The ability to quantify MDV load accurately in the tissues of infected chickens is important for detailed studies on the virus and the disease (Baigent et al., 2005a). Since the introduction of the polymerase chain reaction (PCR) in 1983, several specific assays have been developed and used to detect and quantify MDV (Bumstead et al., 1997, Reddy et al., 2000, Davidson et al.,

2002a, Zelnik, 2004). However, those methods are labour intensive and require post- PCR handling such as gel-electrophoresis. Real-time quantitative PCR (qPCR) provides a tool for the rapid detection and quantification of MDV and is preferred increasingly to conventional PCR (Niesters, 2001). Real-time PCR assays to detect and differentiate the three serotypes of MDV have already been published (Islam et al., 2004a), as have methods for absolute quantification for MDV serotype 1 (Baigent et al., 2005a, Islam et al., 2006a) and serotype 3 (Islam et al., 2006a).

This chapter describes the development and validation of a plasmid standard for the MDV2 assay reported previously by Islam et al. (2004a) thus allowing absolute quantification of viral copy number. The conversion of the previously used standard for this assay derived from Maravac®, a MDV2 vaccine containing the Australian strain MD19, to absolute standard is demonstrated based on the parallelism with the plasmid standard curve and the determination of viral copy number is described. The method was applied to the absolute quantification of MDV2 in infectious dust samples from poultry farms across Australia, infectious spleen samples and feather tips from chickens vaccinated with an attenuated strain of MDV2. This method allows the determination of the absolute MDV2 genome copy number, thus making comparisons between levels of all three serotypes in the one sample possible.

2.2 Materials and Methods

2.2.1 Development of plasmid standard

A MDV2 specific plasmid standard was developed using part of the sequence of the UL30 *DNApol* gene which is located within the unique long region containing qPCR primers described by Islam et al. (2004a). The gene sequence for MDV2 was obtained from GenBank® accession no. AB024414.

2.2.2 Development of plasmid constructs

A 283 bp fragment of the MDV2 UL30 DNA*pol* gene was amplified by standard PCR using reaction conditions described by Islam et al. (2006a). Primers were designed using Beacon designer 6.0 (PREMIER Biosoft International, Palo Alto, USA) and sequences of the primers are shown in Table 2.1.

Table 2.1: Primers and probes used for quantitative real-time PCR assay and primers used in standard PCR for amplification of fragments of *DNApol* gene for cloning into vector.

Target gene	Primer and probe sequence	Amplicon [bp]
qPCR assay:		
DNApol (Islam et al., 2004a)	Probe: 5'-(ROX)CGCCCGTAATGCACCCGTGACT (BHQ-2)-3'	99
	F-primer: 5'-AGCATGCGGGAAGAAAAGAG-3'	
	R-primer: 5'-GAAAGGTTTTCCGCTCCCATA-3'	
standard PCR assay:		
DNApol	F-Primer: 5'-GTCTGCCCTCGTCTTAGC-3'	283
	R-primer: 5'-ACTCGCTTCCTCCAATTCG-3'	

The PCR products were purified using the Wizard® DNA purification Kit (Promega, Madison, USA) and then ligated into the T-tagged site of the pGEM® T-easy vector according to the manufacturer's protocol (Promega, Madison, USA). After overnight incubation at 4 °C, the ligation mix was transformed into competent *E. coli* (DH5α) and grown overnight on agar plates containing ampicillin/isopropyl-β-D-thiogalactopyranosidase (IPTG) and 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal). Positive transformants were identified using blue-white screening. To confirm the size of the insert in the amplicons, plasmid DNA was extracted from the transformed *E. coli* (DH5α), digested with *Eco*RI and analysed by agarose gel-electrophoresis. The sequence determination of the insert of the generated plasmid pKR-DNA*pol* was conducted by Newcastle DNA

(University of Newcastle, Australia) using universal primers for the pGEM® T-easy vector.

2.2.3 Processing plasmid DNA for standard curve determination

Plasmid DNA was extracted and purified from a 500 ml culture of transfected *E. coli* using a Wizard® plus Maxiprep DNA purification Kit (Promega, Madison, USA) according to the manufacturer's protocol (Promega, Madison, USA). The concentration of plasmid DNA was calculated on the basis of two identical agarose electrophoresis gels stained with ethidium bromide using twofold, fourfold and eightfold dilutions against a lambda standard with known amounts of DNA. In addition, plasmid DNA was quantified using spectrophotometric analysis (VARIAN Cary 50 Conc, UV visible). Plasmid copy number was then calculated from the concentration of plasmid DNA and its molecular weight. Purified plasmid DNA was stored in aliquots at -20 °C. A series of tenfold dilutions, starting at 1x 10^5 down to 1x 10^0 per 5 µl were made from purified plasmid DNA to identify the threshold of detection.

2.2.4 Quantitative real-time PCR (qPCR)

2.2.4.1 Primer/probe sets and qPCR protocol

A TaqMan® real-time qPCR assay was performed using a RotorGene 3000 real-time PCR machine (Corbett Research, Sydney, Australia). The RotorGene was set up as described by Islam et al. (2006a). Briefly, measurements were made on the ROX channel with an exitation wavelength of 585 nm and a detection wavelength of 610 nm. Primer/probe sets used were the same as described by Islam et al. (2004a) and are shown in Table 2.1.

For analysis of the assay results, the RotorGene software version 6.0 was used (Corbett Research, Sydney, Australia). Raw data was analysed using the default settings of the software for determining both the threshold value and baseline. In brief, the (fractional) cycle number, C_t, which indicates where the signal curve crosses an arbitrary threshold intersecting the signal curves in their exponential phases, are determined. The C_t values are proportional to the logarithms of the

initial target concentrations. A calibration curve of the C_t values of the standard dilution series versus the concentrations is calculated and used to determine the concentrations of the unknowns, based on their C_t values. In each assay, a standard curve for the primer set was generated and used to derive the copy number of target sequences in unknown samples.

2.2.4.2 Validation of assays

The sensitivity of the assay for MDV2 was determined by running tenfold serial dilutions of the plasmid DNA with known copy numbers as described under 2.2.3. The lowest dilution in the tenfold dilution series which amplified reliably was defined as the detection limit. The specificity of the primer set was assessed previously as described by Islam et al. (2004a).

The reproducibility of the qPCR assay with the new plasmid derived standard curves was measured by calculating the intra-assay coefficient of variation (CV) by taking the mean CV for duplicate C_t and calculated copy number for plasmid standards in all runs of the same assay. The inter-assay CV was determined by comparing the mean C_t and calculated copy number for each standard in three separate but identical assay runs and determining the CV for each across assays. Each individual assay was performed on separate days and serial dilutions of plasmid as well as the MDV2 vaccine derived standards were prepared freshly on each day.

2.2.4.3 Conversion of previous MDV2 standard

The previously used MDV2 standard derived from Maravac® vaccine and used for relative quantification (Islam et al., 2004a) was quantified in terms of viral copy number in three independent identical assays after parallelism with the plasmid standard curve had been confirmed.

2.2.5 MDV samples

Field dust samples were obtained from broiler chicken farms in Victoria, South Australia, New South Wales, and Queensland. In Australia, broiler chickens are vaccinated with HVT or left unvaccinated. MDV2 has never been used routinely

as a vaccine in broiler chickens. Dusts were collected into sterile sealable 50 ml containers by scratching settled dust from any available surface inside the chicken shed and containers were stored at -20 °C.

Spleen samples were taken from 19 day old broiler chickens which had been vaccinated with MDV2 and HVT or with HVT alone. Both groups had been infected with MDV1 isolate MPF57, 02LAR or 04CRE in addition.

Feather samples came from four month old (day 123 of age) layer chickens (ISABROWN pullets) which had been vaccinated with HVT and MDV2. These chickens were not infected with MDV1. DNA from all samples was extracted using the DNeasy® tissue kit according to the manufacturer's instructions (Qiagen, Clifton Hill, Australia). Extracted DNA was quantified using spectrophotometric analysis (BIO-RAD, SmartSpec TM3000) before the qPCR MDV2 assay was performed.

2.3 Results

2.3.1 Development of plasmid constructs

A 283 bp fragment of the MDV2 specific UL30 DNA*pol* gene within the unique long region was amplified using standard PCR and primers shown in Table 2.1. The fragment included the MDV2 specific sequences described by Islam et al. (2004a) so that later use of the fragment in qPCR was possible without the need for new primer sets. This primer/probe set has been shown previously to not cross-react with MDV1 and HVT (Islam et al., 2004a). The fragment was cloned into the pGEM®–T-easy vector (Promega Corporation) to generate the plasmid pKR-DNA*pol*. To confirm that it contained the correct insert, the DNA was sequenced before further use and the results showed that no base changes had been inadvertently introduced during the generation of the plasmid (data not shown).

2.3.2 Sensitivity

Tenfold serial dilutions of the plasmid pKR-DNA*pol* DNA were made and amplification plots for qPCR assays using the primer/probe set in Table 2.1 are

shown in Figure 2.1. The log-linear standard curve, Figure 2.2, generated from these plots showed a very good fit with R^2 = 0.9994, thus indicating the high accuracy over a wide range of concentrations ranging from 10^1 – 10^5 copies of pKR-DNA*pol* plasmid. The lowest dilution in the tenfold dilution series which amplified reliably was defined as the detection limit, and was 10 copies of pKR-DNA*pol* plasmid as the lowest dilution of 1 copy of pKR-DNA*pol* plasmid did not amplify reliably.

2.3.3 Reproducibility

Standard curves were highly reproducible with no significant difference in slopes ($p>0.05$, Figure 2.3) between individual runs of the same assay for a total of three assays. The mean intra-assay coefficient of variation (CV) for C_t-values was 0.85 % and the mean inter-assay CV for C_t-values was 2.80 %. Calculated plasmid copy number had mean intra- and inter-assay CVs of 20.8 and 21.5 % respectively.

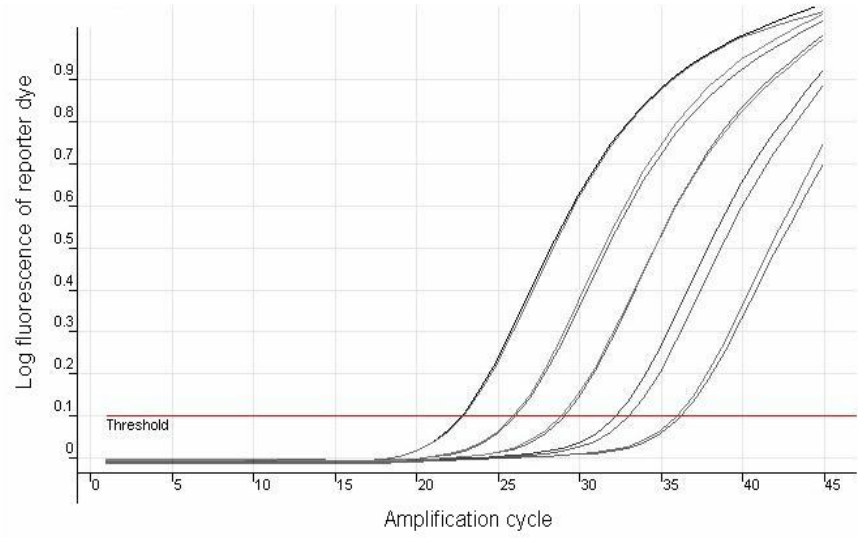

Figure 2.1: MDV2 qPCR assay. The released reporter fluorophore (y axis) is plotted as a function of the amplification cycle number (x axis). A serial tenfold dilution in duplicates of pKR-DNA*pol* plasmid copies is shown (10^5- 10^1), from left to right). The lowest dilution of 10^0 plasmid copies did not amplify.

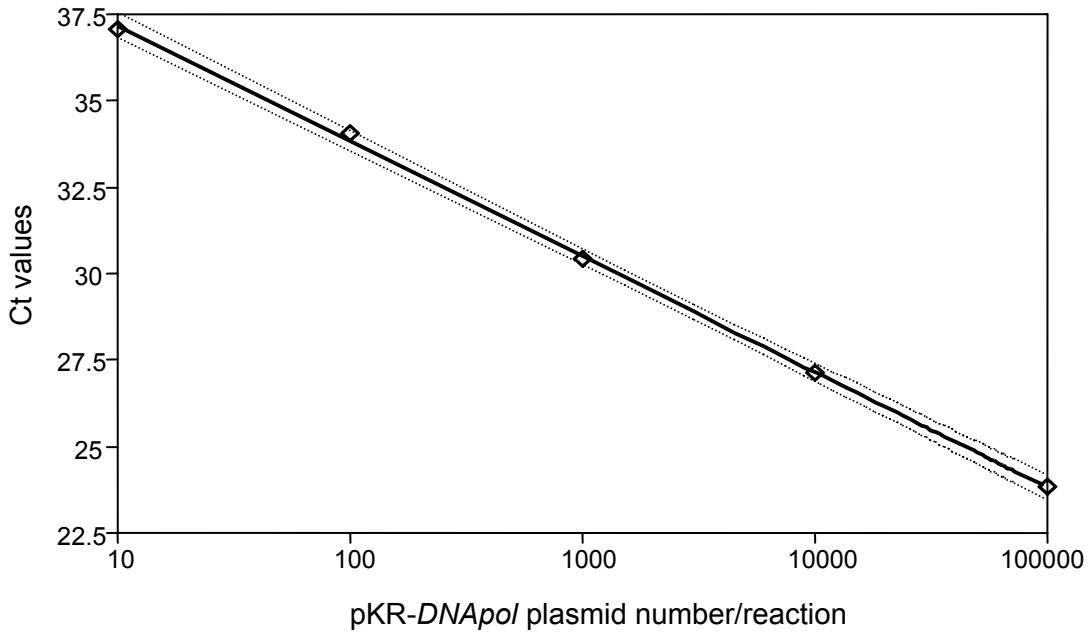

Figure 2.2: Standard curve generated from one of the three qPCR MDV2 assays with the pKR-DNA*pol* plasmid [y = -3.337 log(x) + 40.532, with R^2 = 0.9994]. The dotted lines represent the 95% confidence intervals of the line.

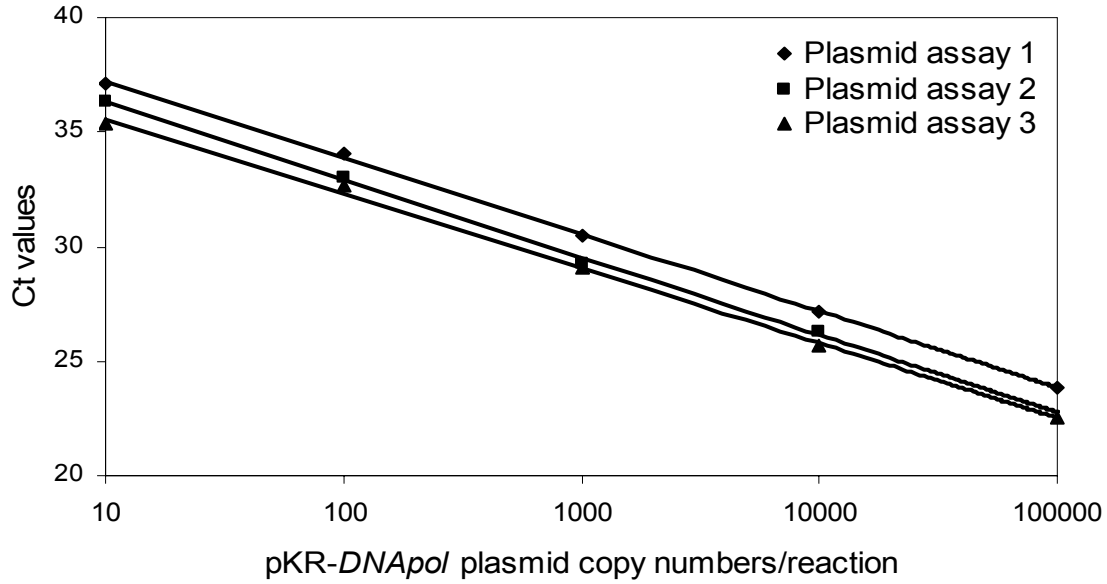

Figure 2.3: Plots of three standard curves generated with pKR-DNA*pol* plasmid in three separate MDV2 assays. The slopes and intercepts did not differ significantly ($p<0.05$).

2.3.4 Conversion of previous MDV2 standards

The reference standard used previously was quantified genomic DNA extracted from Maravac® vaccine, a live cell-associated MDV2 vaccine containing the Australian strain MD19. Figure 2.4 shows that the previous reference standard and the plasmid standard curve generated from the qPCR results form an almost identical parallel line, with no significant difference in slope ($p>0.05$). Figure 2.5 shows a linear regression plot of pKR-*DNApol* plasmid copy number against the previous MDV2 standard curves. With this data, it is now possible to convert previous assays from relative quantification to absolute quantification.

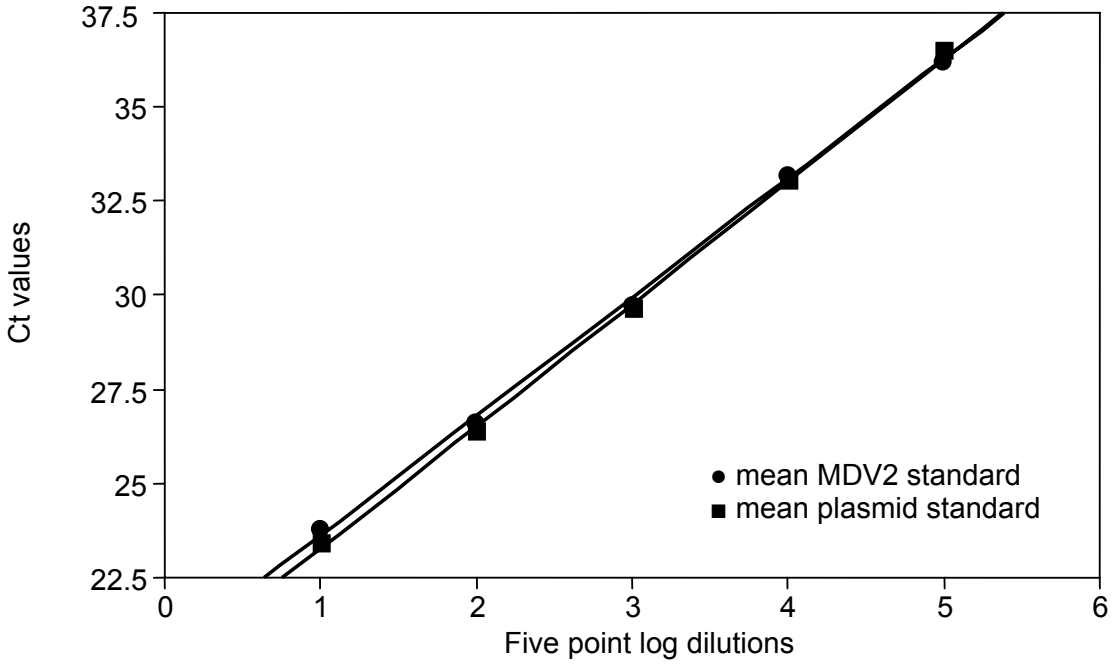

Figure 2.4: Parallelism of standard curves generated from MDV2 positive reference standard and pKR-*DNApol* plasmid standard. All data points display means of three separate assays.

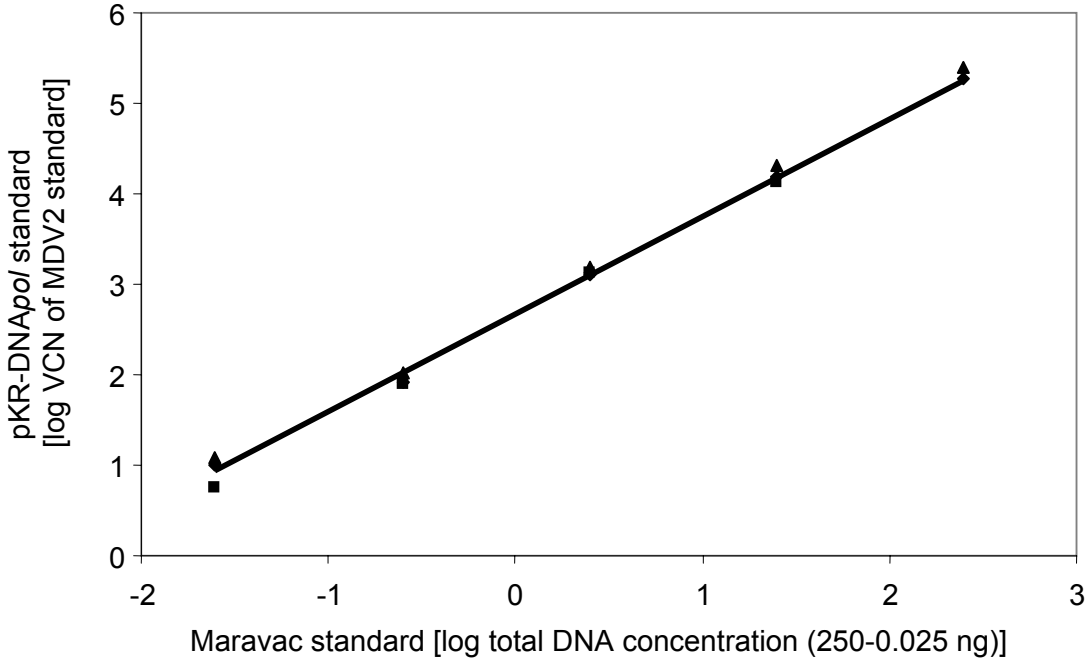

Figure 2.5: Linear regression plot of reference MDV2 concentration and pKR-*DNApol* plasmid number per reaction (y= 1.0812log(x) + 2.6, R^2= 0.9988).

2.3.5 Absolute quantification of MDV2 in field dust, spleen and feather tip

From the thirty dust samples taken from broiler farms across Australia which had been assayed previously for MDV1 and HVT, 13 samples amplified for MDV2 as shown in Table 2.2.

Table 2.2: qPCR analysis of 30 field dust samples (each from a different broiler farm), grouped by the pattern of results for all three MDV serotypes. Data was taken from Islam (2006) and MDV1 and HVT were assayed as described by Islam et al., 2006a. (N.A. = not available, VCN= viral copy number)

No. of samples	Sample	HVT vaccinated	MDV1 VCN/ mg dust [mean]	HVT VCN/ mg dust [mean]	MDV2 VCN/ mg dust [mean]
3	dust	yes	0	0	0
6	dust	yes	0	197977	0
2	dust	yes	0	38256	24054
2	dust	yes	45073	151488	8342
5	dust	no	0	0	0
2	dust	no	0	144576	0
1	dust	no	88749	0	0
3	dust	no	0	N.A.	22933
5	dust	no	495534	0	30469
1	dust	no	7452	13648	2263
Total 30			**9/30**	**13/30**	**13/30**

The mean viral copy number (VCN) per mg dust was $10^{4.3}$. Table 2.3 shows the results for the feather tip and spleen samples. From six feather tip samples obtained from layer pullets vaccinated with HVT and MDV2, all samples amplified in the MDV2 assay with a mean MDV2 load of $10^{4.18}$ VCN/10^6 host cells.The spleens from 5 chickens vaccinated with HVT and MDV2 showed a MDV2 load of $10^{4.2}$ VCN/10^6 host cells. Spleens from 5 chickens vaccinated with HVT only did not contain any MDV2 and did not amplify, as expected.

Table 2.3: qPCR analysis of 6 feather tip samples and 10 spleen samples. (VCN= viral copy number).

No. of samples	Sample	HVT vaccinated	MDV2 VCN/10^6 host cells [mean]
6	feather	yes (HVT/MDV2)	15348
5	spleen	yes	0
5	spleen	yes (HVT/MDV2)	16120
Total 16			**11/16**

2.4 Discussion

This chapter describes the successful development and validation of a MDV2-specific plasmid, pKR-*DNApol,* for the absolute quantification of MDV2 genome copy number in qPCR assays. The assay shows good reproducibility and the detection limit, defined to be the lowest dilution in the tenfold dilution series which amplified reliably, was 10 copies of pKR-*DNApol* plasmid per reaction. As the lower detection limits were determined in a 10-fold dilution series, these values are regarded as conservative estimates. Therefore, the true detection limit lies in between this value and the next lower 1:10 dilution, 1 copy, which did not amplify. With 10 copies of pKR-*DNApol* detected reliably, the sensitivity of the MDV2 assay is greater than the sensitivity of the assay for HVT (Islam et al., 2006a), which amplified reliably down to 75 plasmid copies per reaction and similar to the MDV1 assay (Islam et al., 2006a) which amplified reliably down to 5 plasmid copies per reaction. Thus, the MDV2 assay described here represents another very sensitive method for detecting and quantifying a MD virus. One copy of the *DNApol* gene represents one copy of the MDV genome as the *DNApol* gene is present only in the unique long region.

The generated plasmid standard curve showed parallelism with previous standards used (derived from dilutions of total DNA from 0.025 – 250 ng per reaction) from the Maravac® vaccine indicating the same behaviour over a dynamic range of 10^5. With this data, it was possible to quantify the previously used standards in terms of viral copy number, thus allowing their use in assays providing absolute quantification. The conversion of the previously used standard provides certain advantages over the use of the plasmid standard. The previous standard DNA represents a mixture of both host and viral DNA as is the situation with unknown samples being assayed. Furthermore, from experience with plasmids and DNA samples in qPCR assays, cellular DNA standards are more stable than the plasmid standards, particularly at lower concentrations. As well, there is a significant risk of laboratory contamination when working with plasmid standards. Therefore, future assays will use the vaccine derived standard as before. However, this standard is now quantified in terms of viral copy number per reaction thus allowing absolute quantification of test samples against this standard.

For reasons discussed previously by Islam et al. (2006a) and to standardise the way in which VCN is expressed for the various tissues, the results for the field dust samples are expressed in VCN per mg of dust whereas the results for the feather and spleen samples are expressed in VCN per 10^6 host cells. From 30 field dust samples assayed to analyse MDV2 viral load, 13 samples amplified above the detection limit thus confirming the application of the MDV2 assay to field samples containing a mixture of viruses. It is of interest that MDV2 was detected in 43 % of dust samples from broiler farms where vaccination with MDV2 is not practiced. In 8 samples that were positive for MDV1 from HVT-vaccinated as well as unvaccinated flocks, the viral copy number of MDV2 was higher than MDV1 or at least similar. The same pattern was seen for 2 samples from HVT-vaccinated flocks that were negative for MDV1. This suggests that wild type MDV2 is circulating in Australian broiler farms and warrants further detailed investigation. Of six feather samples from 4 month-old vaccinated layer pullets, all had high MDV2 load, thus suggesting that MDV2 is still replicating and being shed at high concentration from vaccinated birds, even at 123 days of age. Spleens from chickens vaccinated with HVT and MDV2 and challenged with MDV1 amplified reliably above the detection limit as well. Spleen samples from the chickens vaccinated with HVT alone and challenged with MDV1 did not amplify in the MDV2 assay, thus confirming the absence of cross-reactivity with HVT and MDV1 which has been established previously (Islam et al., 2004a).

2.5 Conclusion

A method for the absolute quantification of the MDV2 virus using real-time PCR is described. Together with the assays for the absolute quantification of MDV1 and HVT reported previously (Islam et al., 2006a), it is now possible to perform absolute quantification on samples for all three serotypes of MDV in a range of relevant sample materials. This should lead to improved understanding of the epidemiology and pathogenesis of Marek's disease and offers significant opportunities for improved diagnosis, monitoring and control of the disease.

Chapter 3

Association between Marek's *Eco*RI-Q-encoded (*meq*) gene sequence and virulence in Australian and international isolates of Marek's disease virus

3.1 Introduction

Marek's disease virus (MDV), classified as an Alphaherpesvirus, is one of the most potent oncogenic herpesviruses (Liu et al., 1998). Isolates of MDV can be classified into three serotypes, namely serotypes 1 (MDV1), 2 (MDV2) and 3 (herpesvirus of turkeys or HVT) of which only MDV1 is pathogenic and oncogenic (Okazaki et al., 1970; Schat and Calnek, 1978). MDV1 is the etiological agent of Marek's disease (MD) causing lymphoproliferative lesions and lymphomas in chickens (Churchill and Biggs, 1967). Since the detection of the etiological agent in the late 1960s (Churchill and Biggs, 1967), MD has been controlled to a large extent using vaccines which consist either of attenuated isolates of oncogenic MDVs or the apathogenic HVT or MDV2 serotypes (Witter et al., 1970; Rispens et al., 1972b; Schat and Calnek, 1978).

The Marek's *Eco*RI-Q (*meq*) gene is one of several genes which are unique for MDV1. Based on its transforming properties (Liu et al., 1998) and the results of gene deletion studies (Lupiani et al., 2004), the *meq* gene is most likely to be the principal oncogene for MDV1, with other MDV1 genes serving auxiliary functions. The *meq* gene is 1020 base pairs (bp) long and its gene product, Meq, encodes a 339 amino acid (aa) protein (Jones et al., 1992). On the basis of its structural attributes, Meq can be placed in the immediate family of Jun and Fos- like oncoproteins (Kouzarides and Ziff, 1989). As summarized in Figure 3.1, the Meq protein can be divided into two major domains: the N-terminal basic leucine zipper (bZIP) domain and the C-terminal proline-rich domain (Jones et al., 1992; Qian et al., 1996). Adjacent to the leucine zipper, the basic region of Meq shows 65 % identity with the basic regions of

the regulatory oncoproteins Jun and Fos (Curran and Teich, 1982; Maki et al., 1987). Like that of the Jun-Fos family of bZIP proteins, the leucine zipper of Meq consists of a periodic repetition of five leucines with a heptad spacing and results in an alpha-helical conformation (Qian et al., 1996; Landschulz et al., 1988). The C-terminal domain of Meq resembles that of the Wilm's tumour (WT-1) suppressor protein which also has high proline content (Qian et al,. 1995). Proline-rich domains can function either in transactivation or in transrepression, depending on their specific structures and the context of their binding sites (Wang et al., 1993). In the case of Meq, the C-terminal domain contains a proline rich region which is a potent transcriptional transactivator (Qian et al., 1995; Chang et al., 2002a).

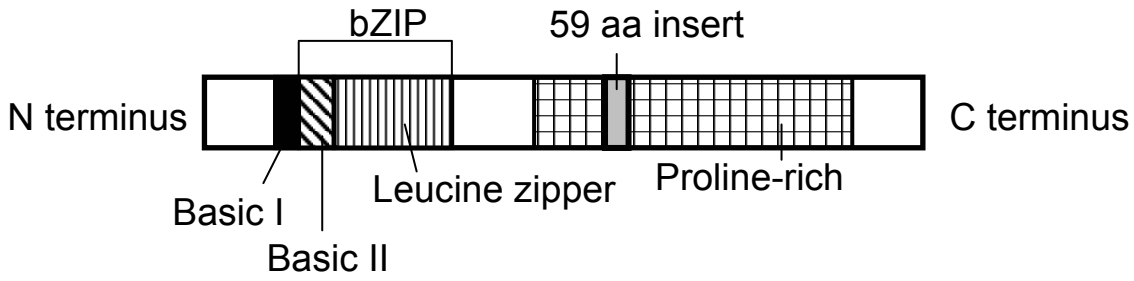

Figure 3.1: Overview of the structure of Meq.

Differences in the *meq* gene between oncogenic and attenuated isolates of MDV1 have been reported with a 177 - 180 bp insertion in the *meq* gene of the attenuated vaccinal isolate CVI988 possibly responsible for its non-oncogenicity (Lee et al., 2000; Chang et al., 2002c; Spatz and Silva, 2007). There are *meq* sequences of several isolates of MDV1 available from GenBank® and when aligned against each other, they show distinct polymorphisms, with point mutations, insertions or deletions that appear to correlate with virulence reported by Lee et al. (2000); Chang et al. (2002b); Wei and Cui (2002) and Shamblin et al. (2004). In particular, Shamblin et al. (2004) found that an interruption at position 2 of the repetitions of four consecutive prolines (PPPP) was correlated with virulence.

In order to investigate whether there are such differences in Australian isolates of MDV1, the *meq* genes from five Australian isolates of MDV1 were analysed by DNA sequencing. The same isolates had been tested for virulence in a pathotyping experiment adapted from the method of Witter (1997) which has been regarded as the 'Gold Standard' for pathotyping MDV. The overall objectives of this study were:

1. To determine whether there is sequence variation in the *meq* gene of Australian isolates of MDV1 and whether this correlates with their virulence as determined in a formal pathotyping experiment.
2. To determine whether there are associations with *meq* sequence and virulence on an international scale.
3. To examine phylogenetic relationships between Australian and international MDV1 isolates on the basis of *meq* sequences.

3.2 Materials and Methods

3.2.1 Sample material for DNA extraction

DNA from six Australian isolates of MDV1, namely MPF57 (De Laney et al. 1998), 02LAR, 04CRE, FT158, MPF132/5 and Woodlands1 (De Laney et al. 1998; Zerbes et al. 1994) was extracted from spleen samples collected at 13 days post infection (dpi) from SPF chicks in a formal pathotyping experiment (Expt 1 of Walkden-Brown et al., 2007a). Briefly, SPF chickens (n=34 per vaccination/challenge combination in 2 replicates) were vaccinated at hatch with HVT (cell-associated HVT 8000 pfu s.c.) or left unvaccinated (diluent s.c.) and challenged intra-abdominally at day 5 of age with 500 pfu of the 6 Australian isolates of MDV1 listed above, with an additional unchallenged treatment receiving diluent only. At 13 dpc, 9 chickens per treatment combination were sacrificed for determination of relative immune organ weight (bursa, spleen, and thymus). Chickens dying or sacrificed during the experiment were examined post-mortem for gross MD tumours. At 56 dpc surviving chickens were sacrificed and similarly examined for tumours. Infection with MDV1 was successful for all MDV1

challenge treatments and absence of virus in unchallenged treatments was confirmed by quantitative real-time PCR (qPCR) of 56 dpc spleens. The putative pathotype was determined by the criteria of Witter (1997) although differences in experimental details mean that these should be seen as broadly indicative. Details of the challenge viruses and the pathotyping results are shown in Table 3.1.

Table 3.1: Details of the Australian MDV isolates used, the incidence of gross MD lesions (%MD) in unvaccinated chickens by 56 dpc, the protective index (%PI) provided by HVT and the putative pathotype based on the classification of Witter (1997) in the pathotyping experiment (Walkden-Brown et al., 2007a).

Details of the challenge MDV						**Pathotyping results**		
Name	**Origin**	**Year**	**Isolated from**	**Vaccination history**	**Batch**	**%MD in unvaccinated chickens**	**%PI**	**Putative pathotype**
Woodlands1	SE Qld	1992	Broiler breeder	Bivalent (serotype 2 & 3)	310804 P14	81	41	vv
MPF57	NSW	1994	Layer	Bivalent (serotype 2 & 3)	140904 P6	84.2	100	v
MPF132/5	NSW	2001	Broilers	Unknown	050904 P5	72%	82.6	v
FT158	North NSW	2002	Broiler breeder	Rispens CVI988	260904 P7	84.2	41.2	vv
02LAR	Victoria	2002	Broiler	Unvaccinated	120904 P6	94.4	38.2	vv
04CRE	Sydney	2004	Layer pullets	Rispens CVI988	260904 P8	52.9	52.7	v

The spleens were stored in sterile 1.5 ml Eppendorff tubes and kept at -20 °C until DNA was extracted using the DNeasy® tissue kit (Qiagen, Clifton Hill, Australia) according to the manufacturer's instructions. Extracted DNA was used as template for a standard PCR to isolate the *meq* gene from each sample using the *meq*- specific primers BCH342 and BCH343 (Table 3.2).

Table 3.2: Sequences of *meq*-gene primers and pGEM® T-easy universal primers used for PCR amplification and sequencing.

Primers	Sequences	Type of primer	Location of primers in Md5 *meq* (GenBank® AF243438)
BCH342	5'-ATTCCGCACACTGATTCC -3'	5'	134786-134803
BCH343	5'-TGCTGAGAGTCACAATGC -3'	3'	135893-135910
M13F-pUC	5'-GTTTTCCCAGTCACGAC-3'	5'	pGEM® T-easy primer
M13R-pUC	5'-CAGGAAACAGCTATGAC-3'	3'	pGEM® T-easy primer
BCMD07	5'-TGAACCTCCCATTTGCACTC -3'	5'	135301-135320
BCH341	5'-CGGAGCGTAGATAATATGG -3'	3'	135460-135478
BCH394	5'-AAAAGGGGAACCTGGCCAAC -3'	3'	135704- 135723

3.2.2 PCR amplification of meq gene

The standard PCR was performed in a 25 µl reaction mixture containing 1 µmol of each primer (BCH 342 and 343, Table 3.2), 1.8 mM $MgCl_2$, 0.2 mM dNTP's, 10x reaction buffer (Fisher Biotec, Perth, Australia), 1 unit of *Taq* DNA polymerase (Fisher Biotec, Perth, Australia) and approximately 1 ng of template DNA. Amplification was carried out over 35 cycles each consisting of 1 min 30 s at 94 °C, 1 min at 60 °C and 2 min at 72 °C, except for the initial 2 cycles in which the period at 94 °C was extended to 5 min. After the final cycle, the elongation phase at 72 °C was extended to 10 min with a consecutive step at 4 °C for 5 min. The amplified fragments with an expected amplicon size of 1124 bp were separated on an agarose gel (1 %), stained with ethidium bromide and visualized with UV light. The PCR products were then purified using the Wizard® DNA purification Kit (Promega, Madison, USA) according to the manufacturer's instructions.

3.2.3 Cloning of PCR fragments

The purified DNA fragment was ligated into the T-tagged site of the pGEM® T-easy vector according to the manufacturer's protocol (Promega, Madison, USA). The ligation mix was transformed into competent *E. coli* (DH5α) and grown overnight on agar plates containing ampicillin/ IPTG and X-gal. Positive

transformants were identified using blue-white screening. The size of the inserts was determined by restriction enzyme analysis using *Pst*I and *Sac*I. Amplicons from each MDV1 isolate were cloned in triplicate and each clone completely sequenced on both DNA strands using primers shown in Table 3.2.

3.2.4 DNA sequencing and analysis

The determination of the sequence of the insert of the generated plasmids was conducted by Macrogen Ltd., Seoul, Korea, using an ABI 3730xl DNA analyser (Applied Biosystems Inc., Foster City, CA, USA) and Macquarie University, Sydney, Australia using an ABI 377 sequencer (Applied Biosystems Inc., Foster City, CA, USA). All primers used for sequencing are shown in Table 3.2. Results from sequencing were analysed using Chromas© 1.43 for analysis of chromatograms and Sequaid™ 3.70 for sequence alignment.

The sequence of each of the Australian samples was aligned against the *meq* gene of the MDV1 isolate Md5 which was completely sequenced by Tulman et al. (2000) (GenBank® accession number AF243438). This MDV1 isolate is classified as 'very virulent' according to the classification system suggested by Witter (1997). In addition, sequences were compared with the *meq* gene of the vaccinal MDV1 isolate Rispens/ CVI988 as published by Spatz and Silva (2007) (GenBank® accession number DQ534538). This isolate had been passaged 27 times on chicken embryo fibroblast cell culture and was obtained from Intervet (Boxmeer, Netherlands) (Spatz and Silva, 2007).

To meet objective two, 12 international sequences of the *meq* gene were obtained from GenBank® and the respective accession numbers are given in Table 3.3. All 9 isolates from the USA have been previously pathotyped *in vivo* using conditions as reported by Witter (1997). The isolates from China and Hungary, however, were not pathotyped according to Witter (1997) and a classification was therefore not possible.

For phylogenetic tree analysis, nucleotide sequences of both the 5 Australian and 12 international isolates were aligned using the PHYLIP software package (Felsenstein, 1989).

3.2.5 Statistical analysis

The association between pathotype classification (attenuated, mild, virulent, very virulent, very virulent +) and proline content (% proline overall) of the Meq protein or the overall number of four consecutive proline repeats in the isolates was analysed using one-way analysis of variance using JMP ™ v 5.1 statistical software (SAS Institute Inc., NC, USA). Significant differences amongst means were determined using Student's T test. Data are presented as least squares means and standard errors.

3.3 Results

3.3.1 Meq gene sequencing and alignment of Australian isolates

The *meq* gene from five isolates tested in the pathotyping experiment were completely sequenced, namely MPF57, 04CRE, 02LAR, FT158 and Woodlands1. The DNA sequences of the *meq* gene of these five isolates were deposited in GenBank® under the accession numbers listed in Table 3.3. Triplicate clones from these five isolates were isolated and purified plasmid DNA was sequenced using the universal primers M13F-pUC and M13R-pUC together with three specific primers designed for this purpose (Table 3.2). The isolate MPF132/5 was included in the pathotyping experiment (Walkden-Brown et al., 2007a), but excluded from the DNA sequence analysis as several attempts to clone the *meq* gene were unsuccessful.

Table 3.3: GenBank® accession numbers of the *meq* gene sequences and the proline content of the derived amino acid sequences of the Meq protein used in this study.

Isolate	Country of origin	Accession number	Size of Meq (aa)	Insertion size (aa)	Proline (%)	Number of PPPP's	Pathotype
MPF57	Australia	EF 523771	398	59	22.9	5	vMDV
04CRE	Australia	EF 523772	398	59	22.2	5	vMDV
02LAR	Australia	EF 523773	398	59	22.2	5	vvMDV
FT158	Australia	EF 523774	398	59	22.9	5	vvMDV
Woodlands1	Australia	EF 523775	398	59	22.2	5	vvMDV
CVI988/Rispens	Netherlands	DQ534538	399	60	23.3	8	attMDV
Md5	USA	AF243438	339	nil	21.3	3	vvMDV
CU-2	USA	AY362708	398	59	23.1	7	mMDV
BC-1	USA	AY362707	398	59	23.1	7	vMDV
GA	USA	M89471	339	nil	21.5	4	vMDV
JM/102W	USA	DQ534539	399	60	23.1	6	vMDV
RB1B	USA	AY243332	339	nil	21.5	5	vvMDV
N	USA	AY362718	339	nil	20.9	2	vv+MDV
New	USA	AY362719	339	nil	20.7	2	vv+MDV
W	USA	AY362723	339	nil	21.2	4	vv+MDV
648A	USA	AY362725	339	nil	20.9	2	vv+MDV
YLO40920	China	DQ174459	339	nil	20.9	3	N.A.
Lmeq	China	DQ453117	398	59	23.1	7	N.A.
ATE	Hungary	AY571784	339	nil	21.5	5	N.A.

N.A. virulence level unknown.

The length of the *meq* gene for all five Australian isolates was 1197 bp. Overall, there was limited sequence variation amongst the five Australian isolates and the sequences of the isolates MPF57, FT158 and Woodlands 1 were identical. At position 239 of the sequences in the isolates 02LAR and 04CRE, there were single nucleotide polymorphisms (SNP's) (data not shown). Both isolates showed an 'A' at this position whereas the other three isolates showed a 'C' at this position. In the isolate 02LAR, additional SNP's were found at positions 558, (T replacing G), 780 (T replacing C) and 1016 (C replacing T). In the isolate 04CRE, there were two additional SNP's found at positions 406 (T replacing C) and 1079 (G replacing A).

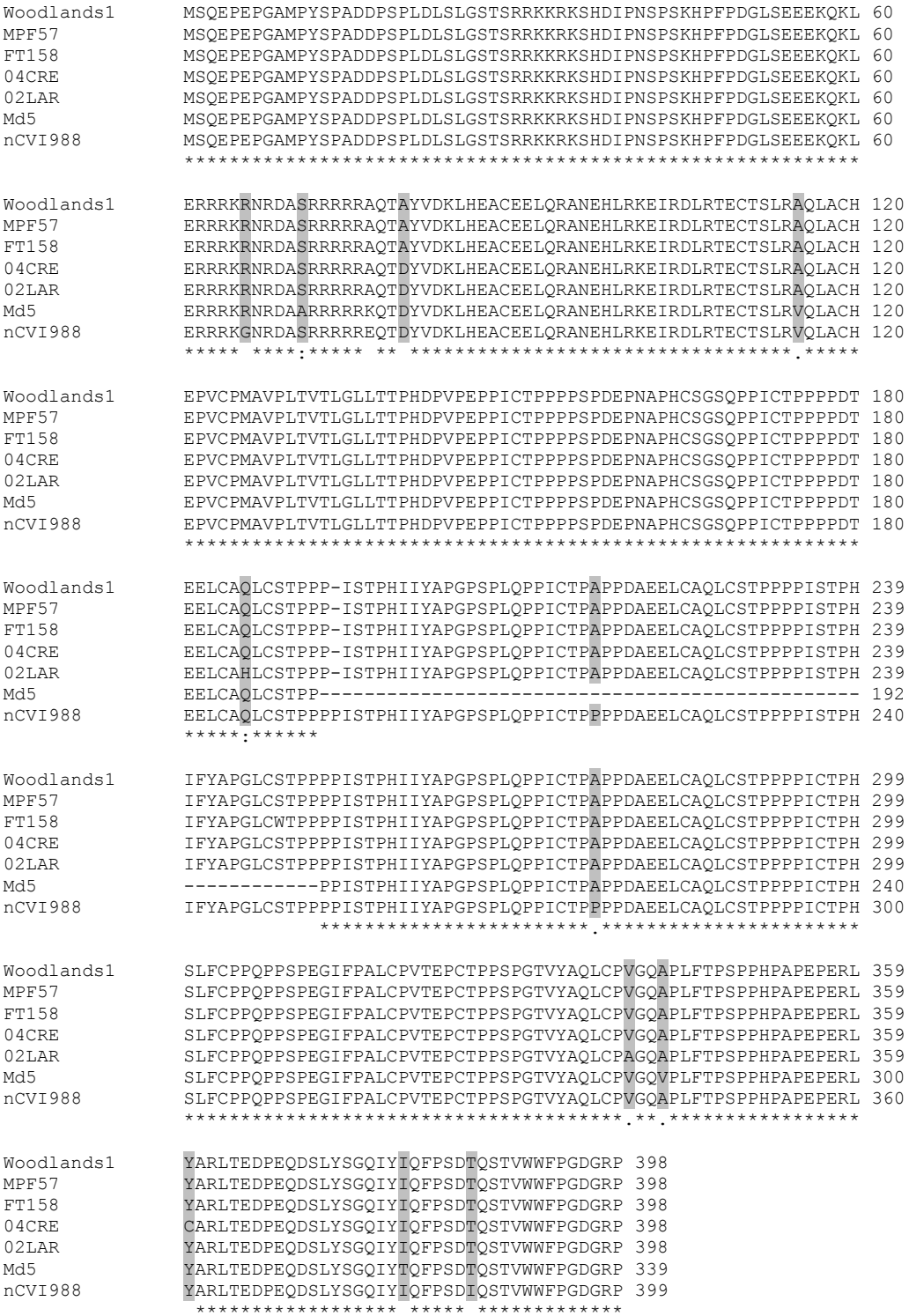

Figure 3.2: Deduced amino acid sequences of the Meq protein for each of the isolates. Identical amino acids are denoted by an asterisk, dashes indicate missing amino acids and shading indicates regions of heterozygosity.

All sequences were translated into amino acid (aa) sequences and aligned as shown in Figure 3.2. The five Australian isolates encode 398 aa. Proline was by far the dominant amino acid encoded by the *meq* gene of all five examined isolates. The isolates 02LAR, 04CRE and Woodlands1 each code proline 90 times (22.2 % of encoded amino acids) while the isolates FT158 and MPF57 code proline 91 times (22.9 % of encoded amino acids). The other encoded amino acids ranging between 1 and 8 % of the total.

The SNP's in the DNA sequence of the isolate 02LAR resulted in a different amino acid at position 186, where 02LAR codes histidine whereas all other isolates code glutamine and at position 339, where 02LAR codes alanine whereas all other isolates code valine. In the isolate 04CRE, one SNP resulted in a different amino acid at position 360, where 04CRE codes cysteine whereas all other isolates code tyrosine. There was no association between sequence variation in *meq* and virulence amongst the Australian isolates. Although all Australian isolates contain the reiterated proline region (PPPP repeats), they show a mutation in the second position of these PPPP stretches (PPPP→PAPP or PQPP).

3.3.2 Comparison of Australian meq sequences with meq of reference isolates Md5 and Rispens/ CVI988

The overall length of the *meq* gene for all five Australian isolates was 1197 bp while for the Md5 and CVI988 reference isolates it was 1020 bp and 1200 bp respectively. All five Australian isolates contained an insertion of 177 bp after position 575 relative to Md5 while CVI988 contained an insertion of 180 bp. The Australian insertions were identical with the CVI988 insertion except that the first 3 bases were deleted and there was a single base difference in the insertion at position 646 in the Australian isolates, aligned to position 649 of CVI988. While the reference isolate CVI988 showed a 'C' at this particular position, all Australian isolates showed a 'G' (Figure 3.3).

```
MPF57           GAGGAACTTTGCGCCCAGCTCTGCTCGACCCCACC---TCCCATCTCTACTCCCCATATT 597
WL              GAGGAACTTTGCGCCCAGCTCTGCTCGACCCCACC---TCCCATCTCTACTCCCCATATT 597
FT158           GAGGAACTTTGCGCCCAGCTCTGCTCGACCCCACC---TCCCATCTCTACTCCCCATATT 597
02LAR           GAGGAACTTTGCGCCCATCTCTGCTCGACCCCACC---TCCCATCTCTACTCCCCATATT 597
04CRE           GAGGAACTTTGCGCCCAGCTCTGCTCGACCCCACC---TCCCATCTCTACTCCCCATATT 597
CVI988          GAGGAACTTTGCGCCCAGCTCTGCTCGACCCCACCACCTCCCATCTCTACTCCCCATATT 600
Md5             GAGGAACTTTGCGCCCAGCTCTGCTCGACCCCACC-------------------------- 575
                ***************** *****************

MPF57           ATCTACGCTCCGGGGCCTTCCCCCCTCCAACCTCCTATCTGTACCCCCGCTCCTCCCGAT 657
WL              ATCTACGCTCCGGGGCCTTCCCCCCTCCAACCTCCTATCTGTACCCCCGCTCCTCCCGAT 657
FT158           ATCTACGCTCCGGGGCCTTCCCCCCTCTAACCTCCTATCTGTACCCCCGCTCCTCCCGAT 657
02LAR           ATCTACGCTCCGGGGCCTTCCCCCCTCCAACCTCCTATCTGTACCCCCGCTCCTCCCGAT 657
04CRE           ATCTACGCTCCGGGGCCTTCCCCCCTCCAACCTCCTATCTGTACCCCCGCTCCTCCCGAT 657
CVI988          ATCTACGCTCCGGGGCCTTCCCCCCTCCAACCTCCTATCTGTACCCCCCCTCCTCCCGAT 660
Md5             ------------------------------------------------------------

MPF57           GCGGAGGAGCTTTGCGCCCAGCTCTGCTCGACCCCACCACCTCCCATCTCTACTCCCCAT 717
WL              GCGGAGGAGCTTTGCGCCCAGCTCTGCTCGACCCCACCACCTCCCATCTCTACTCCCCAT 717
FT158           GCGGAGGAGCTTTGCGCCCAGCTCTGCTCGACCCCACCACCTCCCATCTCTACTCCCCAT 717
02LAR           GCGGAGGAGCTTTGCGCCCAGCTCTGCTCGACCCCACCACCTCCCATCTCTACTCCCCAT 717
04CRE           GCGGAGGAGCTTTGCGCCCAGCTCTGCTCGACCCCACCACCTCCCATCTCTACTCCCCAT 717
CVI988          GCGGAGGAGCTTTGCGCCCAGCTCTGCTCGACCCCACCACCTCCCATCTCTACTCCCCAT 720
Md5             ------------------------------------------------------------

MPF57           ATTTTCTACGCTCCGGGGCTCTGCTCGACCCCACCACCTCCCATCTCTACTCCCCATATT 777
WL              ATTTTCTACGCTCCGGGGCTCTGCTCGACCCCACCACCTCCCATCTCTACTCCCCATATT 777
FT158           ATTTTCTACGCTCCGGGGCTCTGCTGGACCCCACCACCTCCCATCTCTACTCCCCATATT 777
02LAR           ATTTTCTACGCTCCGGGGCTCTGCTCGACCCCACCACCTCCCATCTCTACTCCCCATATT 777
04CRE           ATTTTCTACGCTCCGGGGCTCTGCTCGACCCCACCACCTCCCATCTCTACTCCCCATATT 777
CVI988          ATTTTCTACGCTCCGGGGCTCTGCTCGACCCCACCACCTCCCATCTCTACTCCCCATATT 780
Md5             ----------------------------------ACCTCCCATCTCTACTCCCCATATT 600
                                                   ************************
```

Figure 3.3: Section of analysed DNA of the *meq* gene showing an insertion of 177 bases in all Australian isolates which is similar to the 180 bp insertion in the *meq* of CVI988 except for a deletion of the three initial bases in the Australian isolates (marked by dashes) and a one nucleotide position in the Australian isolates (shaded). In addition, this section shows a single nucleotide polymorphism (SNP) in the isolate 02LAR (shaded). Asterisks on the bottom line indicate bases which are identical for all isolates.

A base change of two consecutive bases in the five Australian isolates compared to both of the reference isolates is shown in Figure 3.4. The Md5 sequence shows an ‘AA’ at position 229 and 230 whereas CVI988 shows ‘GA’. The five Australian isolates have a ‘GC’ at this particular position.

```
MPF57           GAAAGGAGGAGAAAAAGGAATCGTGACGCCTCTCGGAGAAGACGCAGGGCGCAGACGGCC 240
WL              GAAAGGAGGAGAAAAAGGAATCGTGACGCCTCTCGGAGAAGACGCAGGGCGCAGACGGCC 240
FT158           GAAAGGAGGAGAAAAAGGAATCGTGACGCCTCTCGGAGAAGACGCAGGGCGCAGACGGCC 240
02LAR           GAAAGGAGGAGAAAAAGGAATCGTGACGCCTCTCGGAGAAGACGCAGGGCGCAGACGGAC 240
04CRE           GAAAGGAGGAGAAAAAGGAATCGTGACGCCTCTCGGAGAAGACGCAGGGCGCAGACGGAC 240
CVI988          GAAAGGAGGAGAAAAGGGAATCGTGACGCCTCTCGGAGAAGACGCAGGGAGCAGACGGAC 240
Md5             GAAAGGAGGAGAAAAAGGAATCGTGACGCCGCTCGGAGAAGACGCAGGAAGCAGACGGAC 240
                *************** ************** ****************  ******** *
```

Figure 3.4: Section of analysed DNA of the *meq* gene showing differences compared to Md5 and CVI988. Asterisks on the bottom line indicate bases which are identical for all isolates. Differences in sequences are shaded.

The Md5 *meq* gene encodes 339 aa whereas the five Australian isolates encode 398 aa and CVI988 encodes 399 aa. The insertion of 59 aa which is present in all the Australian isolates, is similar to that of CVI988, apart from an additional proline at the start of the insertion for CVI988 (60 aa insertion) and a single amino acid difference at position 216 where an alanine is substituted for a proline molecule (Table 3.4, Figure 3.2). Md5 did not contain this insertion but it, together with the Australian isolates and CVI988 showed a 44 aa sequence after the insertion which was homologous with the initial 44 aa of the insertion. Thus, the bulk of the insertion is a repetition of a proline rich region of Meq.

Table 3.4: Amino acid substitutions in the 5 Australian and the international isolates of the Meq protein.

		Position in Meq																			
Isolate	**Pathotype**	**66**	**71**	**77**	**80**	**88**	**93**	**101**	**115**	**119**	**139**	**153**	**176**	**180**	**186**	**277/ 218***	**337/ 278***	**340/ 281***	**343/ 284***	**361/ 302***	**380/ 321***	**386/ 327***
N	vv+	R	A	K	D	A	Q	K	V	R	T	Q	A	A	Q	A	P	V	A	Y	I	T
New	vv+	R	A	K	D	A	Q	K	V	R	T	Q	A	T	Q	A	L	V	V	Y	T	T
W	vv+	R	A	K	D	A	Q	K	V	C	T	P	P	T	Q	A	L	V	V	Y	T	T
648A	vv+	R	A	K	D	A	Q	K	V	R	T	Q	A	A	Q	A	P	V	A	Y	I	T
RB1B	vv	R	A	K	D	A	Q	K	V	C	T	P	P	T	Q	P	L	V	A	Y	I	T
Md5	vv	R	A	K	D	A	Q	K	V	C	T	P	P	T	Q	A	L	V	V	Y	T	T
02LAR	vv	R	S	A	D	A	Q	K	A	C	T	P	P	T	H	A	L	A	A	Y	I	T
FT158	vv	R	S	A	A	A	Q	K	A	C	T	P	P	T	Q	A	L	V	A	Y	I	T
Woodlands1	vv	R	S	A	A	A	Q	K	A	C	T	P	P	T	Q	A	L	V	A	Y	I	T
MPF57	v	R	S	A	A	A	Q	K	A	C	T	P	P	T	Q	A	L	V	A	Y	I	T
04CRE	v	R	S	A	D	A	Q	K	A	C	T	P	P	T	Q	A	L	V	A	C	I	T
BC-1	v	R	S	A	D	A	Q	K	A	C	T	P	P	T	Q	P	L	V	A	Y	I	T
GA	v	R	A	K	D	A	Q	K	V	C	T	P	P	T	Q	P	L	V	A	Y	I	T
JM/102W	v	R	A	E	D	A	Q	K	V	C	T	P	P	T	Q	P	L	V	A	Y	I	T
CU-2	m	R	S	E	D	A	Q	K	V	C	T	P	P	T	Q	P	L	V	A	Y	I	T
CVI988/Rispens	att	G	S	E	D	A	Q	K	V	C	T	P	P	T	Q	P	L	V	A	Y	I	I
YLO40920	N.A.	R	A	E	Y	A	Q	K	V	C	A	P	P	T	Q	A	L	V	A	Y	I	T
Lmeq	N.A.	R	S	A	D	A	Q	K	A	C	T	P	P	T	Q	P	L	V	A	Y	I	T
ATE	N.A.	R	A	E	Y	T	R	R	V	C	A	P	P	T	Q	P	L	V	A	Y	I	T

*depending on whether or not the isolate has the 59 aa insertion.

The region between the 60th and 120th amino acids showed the most heterogeneity amongst isolates (Table 3.4, Figure 3.2). At position 66, all Australian isolates and Md5 code arginine whereas CVI988 codes glycine. At position 71, all Australian isolates and CVI988 code serine whereas Md5 codes alanine. All Australian isolates code alanine at position 77 whereas Md5 codes lysine and CVI988 codes glutamic acid. At position 80, the isolates Woodlands1, MPF57 and FT158 code alanine whereas 04CRE, 02LAR, Md5 and CVI988 code aspartic acid. At position 115, all Australian isolates code alanine whereas Md5 and CVI988 code valine. At position 194, the CVI988 isolate codes one additional proline molecule.

3.3.3. Association between proline content and repeats and virulence

The proline content and the number of 4 proline repeat sequences of the Australian isolates, the reference isolates and the international isolates are given in Table 3.3. The international isolates include a broader range of American isolates such as CU-2, which was classified as a 'mild' MDV, JM/102W and GA, which were both classified as 'virulent' MDV, RB1B which was classified as 'very virulent' MDV and the N isolate, New isolate, W isolate and isolate 648A which were classified as 'very virulent plus' according to Rosenberger et al. (1997) and Witter (1997). Two Chinese MDV isolates, namely YLO40920 and Lmeq and the Hungarian isolate ATE were also included in this analysis although pathotype information is not available for these.

In the Australian isolates, there were 5 repeats of 4 proline repeats encoded by *meq*, The CVI988 isolate had 8 such repeats whereas the Md5 isolate had only 3. Analysis of variance revealed that the designated pathotypes differed significantly in both the number of 4-proline repeats ($P=0.0016$, $R^2=0.77$) and the total proline content of the Meq protein ($P=0.0055$, $R^2=0.71$) (Figure 3.5).

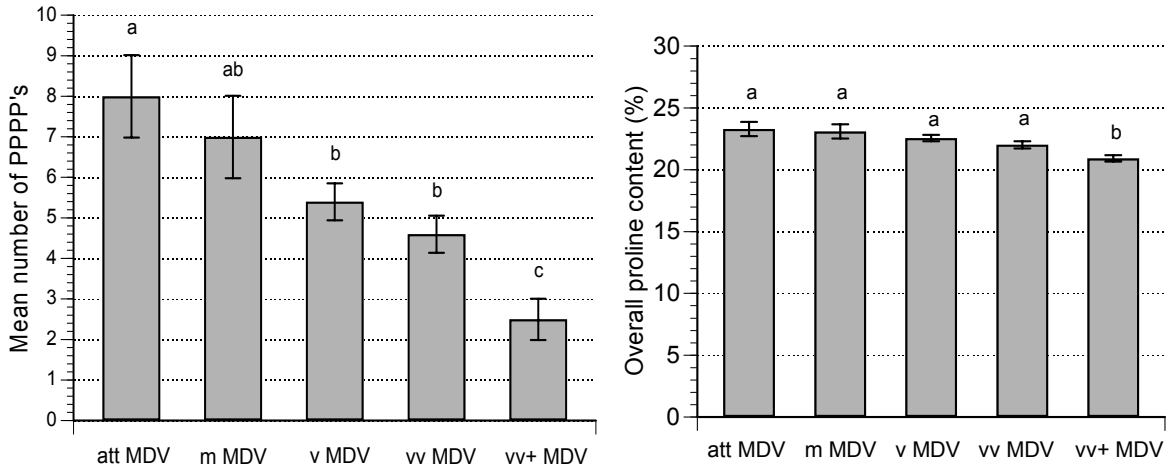

Figure 3.5: Association between proline content and pathotype. Mean (±SEM) number of PPPP repeats (left panel) and overall proline content (%, right panel) grouped by pathotype for MDV isolates used in this study. The two Chinese and the Hungarian isolates were excluded from the analysis as they have not been classified according to Witter (1997). Columns not sharing a common letter differ significantly ($P<0.05$).

3.3.4 Phylogenetic tree alignment

A phylogenetic alignment based on the *meq* sequences of the Australian and international MDV1 isolates is shown in Figure 3.6.

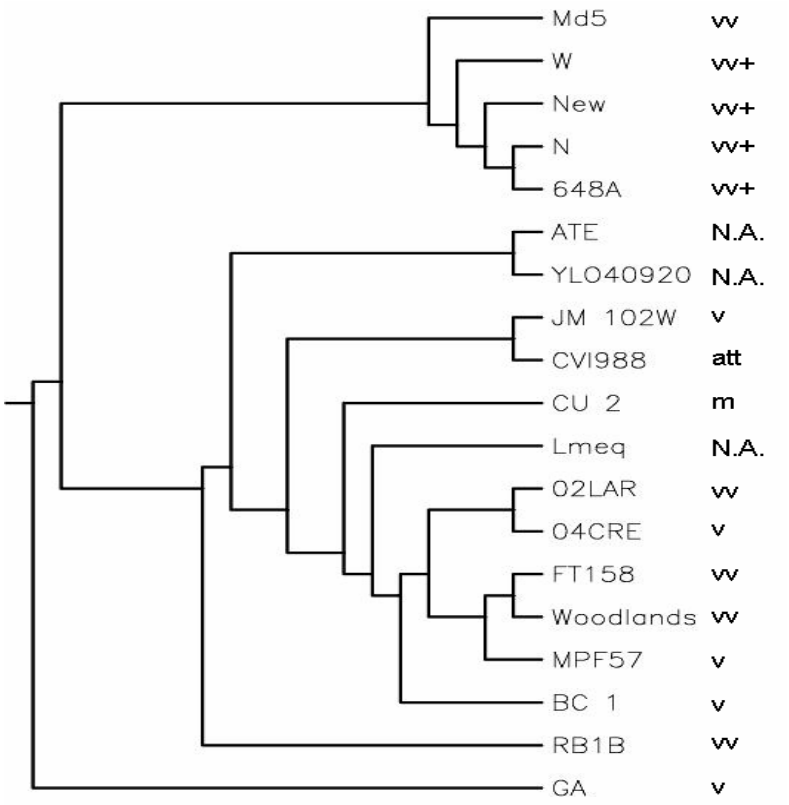

Figure 3.6: Phylogram of the five Australian Meq protein sequences, the two reference sequences and a range of other Meq sequences from GenBank® with their putative classification into pathotypes. N.A. – virulence level unknown, these isolates were not classified according to the system as suggested by Witter (1997).

The tree identifies two major clades with the 177-180 bp insertion being a major differentiating criterion. One clade contains isolates which do not have the 177-180 bp insertion while the other contains isolates which do have the insertion. The clade without the insertion includes a range in virulence types from vv to vv+ containing the very virulent isolates Md5 and the very virulent plus isolates N, New, W and 648A. The virulent isolate GA and the very virulent isolate RB1B both are separate from this group although both isolates do not contain the 177bp insertion. The Chinese isolate YLO40920 and the Hungarian isolate ATE which do not contain the 177 bp insertion were both assigned to a separate subclade. The clade including the 177 bp insertion includes a range of virulence from attenuated to vv, containing the attenuated isolate CVI988, the mild isolate CU-2 and the virulent isolates BC-1 and JM/102W. The five Australian strains form their

own group within this clade, including the vv 02LAR, FT158 and Woodlands 1. The second Chinese isolate Lmeq was also designated to this clade.

A separate phylogenetic alignment where the 177 bp insertion in the Australian isolates had been deleted (not shown) did not reveal major changes to the alignment with the Australian isolates continuing to be included in the clade containing the insert.

3.4 Discussion

The *meq* gene of the five Australian isolates of MDV1 examined exhibited a high degree of identity despite being isolated from widely differing parts of Australia over a decade in time, and despite significant variation in the ability to induce Marek's disease or be protected against it by vaccination with HVT. No consistent mutations which correlated with virulence level were observed within the Australian isolates. All had a 177 bp insertion similar to the 180 bp associated with attenuation or low virulence in reports from other countries and clustered together in a clade of mild and virulent isolates internationally, rather than very virulent or very virulent plus isolates. Across all the international isolates there was a significant negative association between the number of 4-proline repeats in the *meq* protein and the virulence of the MDV1 isolated as determined by the pathotype ascribed using the method of Witter, (1997). Overall the results suggest that the number of 4-proline repeats in the proline rich region of *meq*, rather than the presence or absence of an insertion, is a major determinant of virulence in MDV and has been previously suggested by Shamblin et al. (2004).

3.4.1 Sequence variation in the meq gene of Australian and international isolates of MDV1

With regards to objective one, there was little sequence variation amongst the Australian isolates, despite quite a wide temporal and geographical separation in the origin of the different isolates. While somewhat surprising, it is consistent with the comparatively narrow range of pathotypes encompassed by them. The isolates 02LAR and 04CRE had some substitutions in the amino acid sequence

which would enable differentiation from the other analysed Australian isolates and may help explain the lower virulence of 04CRE and the higher virulence of 02LAR.

With regards to objective two, the sequencing results revealed differences between the *meq* gene sequence and amino acid profile of Australian isolates as a group compared to the Md5 (Tulman et al., 2000) and CVI988 (Spatz and Silva, 2007) isolates of MDV1. All five Australian isolates showed an insertion of 177 bp which was very similar to the 180 bp insertion in the vaccinal isolate CVI988 reported by Spatz and Silva (2007) except for three consecutive bases which code an additional proline in CVI988. The 177 bp insertion has been reported previously by Shamblin et al. (2004) and Spatz and Silva (2007) in the *meq* gene of several American pathogenic isolates as well as a 180 bp insertion in the American pathogenic isolate JM/102W. The insertion is a duplication of a proline-rich domain of the C-terminal of *meq.* Proline-rich domains can function either in transactivation or in transrepression, depending on their structures and the context of their binding sites (Wang et al., 1993). The proline-rich repeats of *meq* resemble the repression domain of the Wilm's tumour suppressor gene WT-1 (Qian et al., 1995). In addition, like WT-1, *meq* appears to contain both activation and repression domains (Wang et al., 1993). Chang et al. (2002b) reported that the Meq proteins containing this duplication exhibit a higher level of repression of transcription than Meq proteins of virulent pathotypes lacking the duplication and this might be the case in the Australian isolates.

3.4.2 Correlation between sequence variation and virulence

The 177 bp insertion in the *meq* gene has been associated with isolates of lower virulence (Shamblin et al., 2004; Spatz and Silva, 2007). However, all five Australian isolates showed an insertion of 177 bp, but based on the results of a pathotyping experiment were classified as "virulent" or "very virulent", according to the classification system of Witter (1997), inducing MD tumours in 53-94 % of challenged chickens (Walkden-Brown et al., 2007a; 2007b). This demonstrates that the 177 bp insertion alone is not necessarily a feature of MDV isolates of

lower virulence. Rather, it appears that the effect of the insertion on the number of 4-repeat proline sequences is what is important, as is discussed below.

Point mutations in the *meq* gene have been reported which are related to virulence (Shamblin et al., 2004). These authors found that higher virulence MDVs had point mutations in the proline-rich repeats that interrupted stretches of four prolines at position two (PPPP to P(Q/A)PP). Their most virulent MDVs had the greatest number of such interruptions (3 for the 'very virulent +' isolates). The *meq* sequences of the five Australian isolates had two point mutations in the proline-rich repeats, including the repeats within the 177 bp insertion that interrupted stretches of four prolines at position two (PPPP to P(A)PP). Following the observation of Shamblin et al. (2004) that interruption of four consecutive proline molecules in the Meq protein is associated with increased virulence, all isolates used in this study were analysed for their proline content and their number of four consecutive proline repeats. The mean number of four consecutive proline molecules within the Meq protein was shown to be significantly correlated with the pathotype classification. The most virulent isolates showed the lowest number of such repeats whereas the attenuated isolates showed the highest number of these repeats (Figure 3.5).

Shamblin et al. (2004) also reported mutations in their isolates in the basic region II, which has been found to be important to nucleolar localization (Liu et al., 1997). These mutations have been found in European isolates which were classified as 'very virulent' (Barrow and Venugopal, 1999) as well as in the vaccinal isolate CVI988. It was therefore suggested that these mutations are virulence-independent and that the very virulent European isolates and CVI988 share common ancestry (Shamblin et al. 2004). The sequencing results of the Australian isolates would support this assumption that these mutations are virulence independent. In all five analysed Australian isolates which showed a range of virulence in the pathotyping study (Walkden-Brown et al., 2007a, 2007b), these mutations appear at the same positions as reported by Shamblin et al. (2004).

Although several early genes have been shown to be essential for early virus replication, which is essential to initiate the pathway to transformation and subsequent development of clinical MD, the *meq* gene is thought to be the

principal oncogene for MDV1, with other MDV1 genes serving auxiliary functions. Jones et al. (1992) were the first authors to report on the *meq* gene as a candidate oncogene of MDV1 and although the *meq* gene is predominantly expressed in the latent state, these authors reported that *meq* may also be expressed in early infection (Jones et al., 1992) which suggests that sequence variation in *meq* may have a significant influence on virulence. Previous studies sequencing genes other than *meq* did not reveal consistent mutations that correlated with virulence (Shamblin et al., 2004; Silva et al., 2004).

3.4.3 Phylogenetic tree alignment

In the phylogenetic tree alignment, the 177 bp insertion appeared to be the main criterion for the designation of MDV1 isolates to each of two major clades. A separate analysis where the 177 bp insertion in the Australian isolates was deleted did not alter their alignment with isolated containing the insert. The *meq* gene sequences of the five investigated Australian isolates of MDV1 form their own group within the clade containing the insert which suggests that the introduction of MDV in Australia has occurred in a single, recent event rather than many introductions over a long time period.

The immediate neighbourhood of the five Australian isolates contained Asian and American isolates of MDV1 which suggests that Australian isolates of MDV1 share comparatively recent common ancestry with overseas isolates. This is further supported by the fact that the Australian isolates have their amino acid substitutions at the same positions as USA isolates as reported by Shamblin et al. (2004). The same pattern has been observed in European isolates of MDV1 (Barrow and Venugopal, 1999).

In conclusion, minor sequence variation in the *meq* gene of five pathotyped Australian isolates was detected. All Australian isolates included a 177 bp insertion in the *meq* gene and these results do not support the association of this insertion with MDV1 isolates of low virulence. Rather, the overall proline content, and the repetition of four consecutive prolines were found to strongly correlate with the pathotype. Typically the insert will increase the number of PPPP repeats and thus lower the virulence of an isolate on average, but we postulate that the

effect of the insert is mediated by its effect on the overall number of PPPP repeats in the proline rich region of *meq*. Although the virulence of a specific isolate of MDV1 is unlikely to be determined soley by mutations in a single gene, the *meq* gene is probably the most suitable candidate gene for epidemiological studies and molecular pathotyping of isolates which could complement the pathotyping of MDV1 isolates *in vivo*.

Chapter 4

Pathotyping of recent Australian isolates of Marek's disease virus (MDV) in commercial layer chickens

4.1 Introduction

Marek's disease (MD) was initially described as a 'polyneuritis', i.e. a cellular inflammation of peripheral nerves (Marek, 1907). Later, the neoplastic nature of MD was recognized (Pappenheimer et al., 1926, 1929) and two forms of lymphomatous MD were then defined: 'classical' and 'acute'. In the classical form of MD, paralytic lesions in the nerves predominated, whereas in the acute form of MD, lymphoid necrosis and lymphomas of many visceral organs were involved (Benton and Cover, 1957; Biggs et al., 1965). The visceral lymphomas typical of acute MD commonly occur in the, ovary, liver, spleen, thymus, heart, intestine, kidney, lung, proventriculus, serosa and skeletal muscle.

The clinical picture of MD has changed dramatically since its initial recognition 100 years ago. The sporadic and chronic nature of the disease has given way to an extremely aggressive, peracute or acute disease in which peripheral neuritis and lymphoma can be absent and protection with previously protective vaccines and immunization regimens can fail (Witter, 1998; Witter et al., 1999). The emergence of this new clinical picture, particularly demonstrated in the USA (Witter, 1997), has been paralleled by the isolation of strains with altered cell and tissue tropisms that can cause >90 % morbidity and mortality in susceptible animal populations, although the relationship between genetic changes in the virus and the clinical presentation of MD remains enigmatic (Witter, 1997; 1998; Witter et al., 1999; Shamblin et al., 2004). The more virulent pathotypes can cause MD outbreaks in vaccinated chickens and are often associated with central nervous system (CNS) manifestations, increased visceral lymphoma frequency, increased mortality, early mortality with bursal and thymic atrophy and increased

frequency of ocular lesions.

Marek's disease vaccines, first introduced in the 1970's, have brought enormous benefits to poultry production and have significantly reduced losses due to MD. However, none of the current vaccines engender sterilizing immunity and vaccinated chickens can still be infected with pathogenic MDV that can replicate, be shed and infect other chickens in the flock. This situation appears to have contributed to the problem of increasing virulence of field isolates of MDV in the USA (Witter, 1997).

To enable systematic classification of MDV isolates on the basis of the pathology they induce, and their ability to overcome the effects of vaccination a standardised MDV pathotyping scheme based on a protective index provided by HVT and HVT/MDV2 bivalent vaccines was developed at the USDA Avian Diseases and Oncology Laboratory (ADOL) (Witter, 1997). The basic method uses challenge groups of 17 genetically susceptible chickens in isolators (Strain 15x7). The chickens contain maternal antibody against MDV (mab+) and are either left unvaccinated, or are vaccinated with 2000 pfu of HVT or HVT/SB-1 bivalent vaccine (HVT/MDV2) at hatch. Five days later chickens are challenged with 500 pfu of the MDV isolate under test and mortality, immune organ atrophy and gross pathology are monitored until the termination of the experiments at 56 dpc. On the basis of induction of lesions such as thymic and bursal atrophy and MD tumours in various organs, each chicken in the test is classified as a MD case or MD negative.

Isolates are ascribed a pathotype class on the basis of their virulence rank (VR) in comparison with reference viruses of each pathotype. In broad terms the pathotype classifications are as follows (Witter, 1997; Witter et al., 2005):

- Mild MDV (mMDV): in genetically susceptible chickens these strains cause mainly neural MD and sometimes a low incidence of mainly ovarian lymphomas; their pathogenic effects are preventable with HVT vaccines.
- Virulent MDV (vMDV): these cause a high incidence of visceral and neural lymphomas (65-100 % MD lesions in unvaccinated chickens, 0-23 % MD lesions in HVT/MDV2 vaccinated chickens (Witter et al., 1997)); their effects are prevented with HVT vaccines.

- Very virulent MDV (vvMDV): these cause high incidence of visceral and neural lymphomas (94-100 % MD lesions in unvaccinated chickens, 6-65 % MD lesions in HVT/MDV2 vaccinated chickens (Witter et al., 1997)). They are oncogenic in HVT vaccinated birds and in birds genetically resistant to less virulent viruses; their effects are preventable with bivalent vaccines containing HVT and MDV2.
- Very virulent plus MDV (vv+MDV): these cause a high incidence of lymphomas and are oncogenic in birds vaccinated with bivalent vaccines containing HVT and MDV2 (100 % MD lesions in unvaccinated chickens, 47-94 % MD lesions in HVT/MDV2 vaccinated chickens (Witter et al., 1997)).

However, the ADOL method is expensive and difficult to implement outside the USA because of its specific requirements for a specific strain of susceptible chicken and reference strains of MDV. To overcome the need for the 15x7 chicken, Witter et al. (2005) proposed a modification of the method that could be used in specific pathogen free (SPF) chickens of different strains, but which still requires the reference pathotype strains. These have since been made available from the American Type Culture Collection.

In the USA HVT has been used almost universally in broiler chickens since its introduction in 1970, alone or in combination with MDV2. The situation in Australia is different. Historically only breeders and layers were routinely vaccinated against MD with only a minority of broilers receiving HVT vaccination (Jackson, 2000). However after a prolonged outbreak of MD in eastern Australia between 1991 and 1996 new strains of HVT, attenuated MDV1 and MDV2 vaccines were introduced and routine *in ovo* vaccination of broilers with cell-associated HVT vaccine became widespread.

Despite the absence of blanket vaccination with MDV in Australia, previous studies have shown that MDVs approximating the vv category against which HVT provides limited protection, were circulating in the Australian poultry industry prior to the outbreak of MD in the 1990s. These isolates were first identified in Australia in 1985 (McKimm-Breschkin et al. 1990) and MDV was subsequently isolated from outbreaks of MD in vaccinated broiler breeders, layers and broilers

(De Laney et al. 1995; Zerbes et al. 1994). These findings are consistent with vaccine failure and evolution of Australian MD viruses towards greater pathogenicity over time. This process may have accelerated since the introduction of widespread HVT vaccination of broilers in the late 1990s.

This pathotyping study in commercial ISABROWN layer chickens was designed to formally pathotype recent isolates of Australian MDVs using an adaptation of the ADOL method. The overall objectives were to confirm the putative pathotype range of Australian MDVs previously ascribed on the basis of less standardised studies (McKimm-Breschkin et al. 1990; De Laney et al. 1995) and to enable comparison of pathotypes reported internationally. The experiment was not powerful enough to determine whether there has been evolution of Australian isolates of MDV towards higher virulence, but trends in this direction or not would be noted and the presence of vv+ isolates would be strongly suggestive of this. Another important objective was to identify early markers of pathogenicity. The experiment was the third of a series of identically designed experiments. The first used SPF chickens and the second commercial Cobb broiler chickens. Thus, the same isolates of Australian MDVs were pathotyped in three genetically different host systems.

The experiment was designed to test the following specific hypotheses:

1. Among the isolates under test will be those pathotyped as vv+ suggestive of evolution towards higher virulence in Australia since earlier studies in the 1980s and 1990s.
2. Thymus, bursal and spleen weights at 14 days post challenge (dpc) will be good predictors of MD incidence and MDV pathotype. The rationale for this hypothesis is that the early cytolytic phase of MDV infection occurs in these immune organs, leading to atrophy (bursa and thymus) and massive infiltration of lymphocytes (spleen) resulting in weight decrease (bursa and thymus) and increase (spleen).
3. MDV load in spleen at 14 dpc will be a good predictor of MD incidence and MDV pathotype. The assumptions behind this hypothesis are that early replication rate in the host is a measure of both the virulence of the challenge virus and the level of host immunity or resistance to MD.

4.2 Materials and methods

4.2.1 Experimental design

The experiment used a restricted 3x3 factorial design with two replicates using 18 isolators with 27 commercial layer chickens per isolator. The two experimental factors were:

- Challenge virus (3 levels) MPF57 (reference isolate), 02LAR and 04CRE, (500 pfu i.a. in 200 µl on day 5 of age).
- Vaccine virus (3 levels) HVT (FC-126, 8000 pfu s.c. in 200 µl), bivalent (containing 63 % HVT FC-126 and 37 % cell-associated MDV2 isolate SB-1 (Vaxsafe SBH vaccine, Batch no. SBH4101, 8000 pfu combined dose per bird, Bioproperties, Ringwood, Vic) and unvaccinated (200 µl diluent) on the day of hatch (day 0).

Two additional isolators contained unvaccinated and unchallenged chickens (diluent injections only) to serve as control groups.

The experiment started on the day of hatch (day 0, 06/06/05) and was terminated at 61 days of age (56 dpc, 09/08/05).

Chickens were maintained and treated according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 2004, the NSW Animal Research Act 1985 and the NSW Animal Research Regulation 1995, and the experiment was approved by the UNE Animal Ethics Committee (AEC No. UNE 05/076).

4.2.2 Experimental time course

On day 0 (day of hatch), the chickens were vaccinated and placed into the isolators. On day 5 of age, chickens were challenged with the respective MDV1 challenge isolate. At 14 dpc, 6 birds per isolator, 120 birds in total, were sacrificed, removed from the isolators and examined post mortem for gross MD tumour lesions, thymic and bursal atrophy. All chickens that died during the experiment were recorded and examined post mortem for gross MD lesions,

thymic and bursal atrophy. Chickens were classified as MD positive when a visible MD tumour was present. All remaining chickens were sacrificed at 56 dpc and examined post mortem for MD gross tumours, thymic and bursal atrophy. Thymus, bursa and spleen weights were recorded from both 14 dpc and 56 dpc kills and spleens were stored at -20 ºC for quantitative real-time PCR (qPCR) analysis.

4.2.3 UNE isolator facility

The experiment was conducted in an isolator facility under physical contamination level 2 (PC2) laboratory conditions. The facility houses 24 isolators (Plate 4.1) and was kept under constant negative pressure with all outgoing air filtered through High Efficiency Particulate (HEPA) filters. Isolators were positive-pressure and soft-bodied with disposable plastic linings, gauntlets and gloves. Each was provided with temperature-controlled HEPA-filtered air via a central air supply system and air was scavenged from each isolator via a series of scavenger ducts and HEPA filtered on exit. Both inlet and outlet air supplies are under manual control via a variable speed controller, giving complete control over air-flow and isolator pressures. There were 12 to 23 changes of air/hour in isolator units depending the settings used which varied with age of the chickens. The dimensions of each isolator were: width 66 cm, length 210 cm, height 88 cm with a stainless steel frame. The floor was made of 2.5 mm thick perforated stainless steel with 12.7 mm holes on 17.45 mm staggered centres (49 % open area) (Plate 4.2). The entire feed supply for the experiment was loaded into a large feed hopper for each isolator and sealed for the duration of the experiment. Water was supplied via automatic and adjustable nipple drinkers.

Plate 4.1: UNE isolator unit. The isolators are aligned in two rows, 12 isolators in each.

Plate 4.2: Individual isolator during construction, prior to fitting the side and gauntlets.

4.2.4 Experimental chickens and their management

A total of 535 day old unvaccinated commercial female ISABROWN chickens from a parent stock vaccinated with Rispens/CVI988 were used, a total of 54 per treatment combination and 27 per isolator. Chickens were kept until 56 days post challenge (dpc), 61 days old. At 28 dpc, all chickens were beak trimmed inside the isolators in response to an outbreak of feather pecking and cannibalism.

Chickens were offered feed (commercial layer starter then finisher, Ridley Agricultural Products, Tamworth) and water *ad libitum* throughout the experiment. Isolator temperatures were set at 34 ºC for the first two days and then decreased by 1 ºC every 2nd day until a temperature of 22 ºC was reached. Faeces accumulated under the floor for the duration of the experiment. Water spillage was collected and drained from the isolator via a water-filled U tube. Lighting was initially 24 h light (days 1-2) followed by 12 h light :12 h dark lighting controlled with an automatic timer.

4.2.5 Vaccination

Vaccination was performed manually sc on arrival at UNE on the day of hatch (day 0) using the recommended dose rates. The vaccines used were cell-associated HVT strain FC-126 (HVT vaccine FC126, Batch no. H02308, 8000 pfu/bird Bioproperties, Ringwood, Vic) and bivalent vaccine containing 63 % HVT FC-126 and 37 % cell-associated MDV2 isolate SB-1 (Vaxsafe SBH vaccine, Batch no. SBH4101, 8000 pfu combined dose per bird, Bioproperties, Ringwood, Vic). Both vaccines were administered in 0.2 ml of diluent supplied by the manufacturer and injected subcutaneously (s.c.). Unvaccinated control chickens received 0.2 ml s.c. diluent as a sham vaccination.

4.2.6 Challenge with MDV1

At 5 days post vaccination, chickens were challenged intra- abdominally with 0.2 ml of infective cell-cultured MDV (in chick kidney cells) kindly provided by Professor Greg Tannock and Ms Julie Cooke of RMIT University, Melbourne. The isolates were free of chicken infectious anaemia virus as confirmed by PCR of the material. A dose of 500 pfu/chicken in 0.2 ml of diluent was used (Minimum essential medium (MEM) with 10 % newborn calf serum). Unvaccinated and unchallenged chickens in two isolators were injected with 0.2 ml diluent as a sham treatment and kept as controls.

Details of the challenge viruses are given in Table 4.1. MPF57 is the standard Australian challenge MDV1 and was included as a reference isolate. It was isolated in 1994 from a bivalent vaccinated flock (De Laney et al., 1998). The

challenge isolate 02LAR was isolated from an unvaccinated broiler flock in 2002 while the isolate 04CRE was isolated from a 6-week-old layer flock which had been vaccinated with Rispens/ CVI988 in 2004.

The vaccine and virus material was stored in liquid nitrogen and thawed at 37 ºC in a water bath immediately prior to subcutaneous or intra-abdominal inoculation using the respective dilution with growth media.

Cell-cultured virus material originated from infectious lymphocyte preparations from a previous experiment in specific pathogen free (SPF) chickens (MD04-C-PT2) which were inoculated onto 24-well plates containing freshly prepared CKC. The plates were incubated for 24 h at 37 ºC; 5 % CO_2 prior to the media being discarded and replenished. Following the change in cell culture media, the inoculated CKC cultures were subsequently incubated at 37 ºC; 5 % CO_2 for 7 days and monitored for cytopathic effect (CPE) every 2-3 days.

Following plaque visualisation using an inverted microscope, the cultures that exhibited high levels of CPE were repassaged into larger flasks until being harvested at about passage 6-8 and crypopreserved. A small amount of culture was retained as a contingency and for future use and was storaged long-term under liquid nitrogen. The titration of MDV virus pools was performed in the following manner.

Preparations of freshly trypsinised CKC were seeded onto a series of 9, 60 mm gridded petri dishes at a cell density of 1:200 of packed cell volume per sample. To facilitate virus plaque visualisation, the titre of each stored virus pool was determined at three different dilutions. Following thawing of the virus pool at 37 ºC, dilutions of 10^{-1}, 10^{-2} and 10^{-3} in cell culture media were made for each virus pool and triplicate aliquots (1 ml) of each dilution were subsequently added to the freshly trypsinised CKC preparations in each gridded Petri dish. The gridded Petri dishes were subsequently incubated at 37 ºC at 5 % CO_2 for 6 days following which time the number of plaques was enumerated. The infectious titre of each virus pool - expressed as pfu/ml –was calculated using the average of the three replicates.

Table 4.1: Details of the challenge isolates used in this experiment.

Virus isolate	Batch number	Passage number	Year of isolation	Bird isolate	Origin	Vaccination history	Dose used
MPF57 B1 (179/6)	P7 200904	7	1994	Layer (14wo ISABROWN)	NSW (Sydney)	Serotype 2 & 3 vaccine	500 pfu
02LAR (179/3)	P6 120904	6	2002	Broiler	Victoria	unvaccinated	500 pfu
04CRE (179/2)	P8 260904	8	2004	Layer (6wo pullets)	NSW (Sydney)	Rispens/ CVI988	500 pfu

4.2.7 Post mortem procedure

Each chicken was weighed immediately after sacrifice or death and standard post mortem examination was carried out for all killed and dead chickens (Bermudez and Stewart-Brown, 2003). Carcasses were checked for nodular lesions on the skin. Breast and thigh muscles were inspected for discrete lymphoid tumours or diffuse infiltration. After opening the carcass, the liver, spleen, gonads, kidney, proventriculus, mesenteries, gastro-intestinal tract, heart and lungs were examined for gross enlargement and discrete or diffuse MD lesions. Tumorous enlargement of the thymus and bursa of Fabricius were recorded as gross MD lesions.

The protective index (PI) provided by HVT for each challenge strain was calculated as:

(%MD in Sham-vaccinated chickens – %MD in HVT-vaccinated chickens) ÷ (%MD in Sham-vaccinated chickens) x 100 (Sharma and Burmester 1982)

where %MD is the percentage of birds “at risk” of exhibiting MD lesions, in which lesions are present. This is generally the population of chickens alive at the time the first gross MD lesion is detected.

Virulence rank (VR) was calculated as 100 – PI (Witter 1997).

A scoring system was implemented for thymic and bursal atrophy ranging from 0-3 in ascending order of severity (0 = normal, 3 = complete or almost complete atrophy). In addition, a severity score for gross MD lesions in all organs was

implemented ranging from 1-3 in ascending order of severity (1 = small foci, 3 = massive tumour). As gross nerve lesions are uncommon with MDV strain MPF57 (Islam, Walkden-Brown and Groves, unpublished observations), nerves were not routinely examined. Iris discoloration or pupil irregularity of the eyes were examined and recorded. Spleens from all killed chickens were removed aseptically using sterile tweezers and thoroughly rinsed with sterile Phosphate Buffered Saline (PBS) before storing them in pre-weighed sterile 5 ml containers. However, the spleens were collected in a contaminated environment in the isolator facility, a source of background contamination for the DNA analysis.

4.2.8 DNA extraction

DNA was extracted from 9-11 mg of spleen using the DNeasy® tissue kit according to the manufacturer's instructions (Qiagen, Clifton Hill, Australia) and stored at –20 ºC. Extracted DNA was then quantified using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop® Technologies Wilmington, USA) prior to analysis by qPCR.

4.2.9 Determination of MDV genome copy numbers by quantitative real-time PCR (qPCR)

All extracted DNA samples were subject to MDV serotype specific TaqMan® qPCR assays. The primer and probe target for MDV1 was the *meq* gene, the *sorf1* gene for HVT and the DNA-*polymerase* gene for MDV2 as published previously by Islam et al. (2004a). MDV genome copy numbers for each serotype were determined by absolute quantification as reported by Islam et al. (2006a) and Renz et al. (2006). The respective TaqMan® real-time qPCR assay was performed using a RotorGene 3000 real-time PCR machine (Corbett Research, Sydney, Australia). The qPCR cycling parameters consisted of: 50 ºC for 2 min, 95 ºC for 2 min, followed by 40-45 cycles consisting of denaturation at 94 ºC for 15 s and annealing/ extension at 60 ºC for 45 s. Each reaction tube contained 0.3 µM of each primer, 0.2 µM of the probe, 12.5 µL of Platinum® Quantitative PCR System-UDG (Invitrogen Australia Pty Ltd.), 5 µL of DNA template (25 ng of DNA) in a total reaction volume of 25 µL.

A standard curve for each primer set was generated in each assay and used to derive the copy number of target sequences in unknown samples. For each assay run, individual standard curves were generated using 4 point 10-fold dilutions of MDV1, MDV2 and HVT standards of known concentration of respective MDV/HVT DNA. No template controls were included in duplicates in each assay and were negative throughout. All samples were randomised across assays to minimise individual assay effects, and samples and standards were amplified in duplicate. Samples which did not amplify or amplified with a C_t value below the lowest standard were treated as negative. The mean intra-assay co-efficient of variation (CV) for all qPCR assay runs was calculated as the mean of the CV values for duplicate samples in all assays. These CVs were based on both calculated viral copy number (VCN) and C_t values.

The mean intra-assay co-efficient of variation (CV) based on C_t values for all qPCR runs was 0.55±0.005 % for MDV1, and 0.46 % for HVT. The CV based on VCN was 10.4±0.09 % for the MDV1 and 10.5 % for HVT assays respectively.

The mean inter-assay co-efficient of variation (CV) for all MDV1 qPCR runs based on C_t values was 12.5±1.75 % and based on VCN was 22.41±0.08 %. There was no calculation of inter-assay CV for HVT assays as only one HVT assay was performed.

4.2.10 Statistical analysis

Analyses were performed using the statistical software JMP 6 (SAS Institute Inc. 2006). Where the chicken was the experimental unit measured, discrete data such as mortality (died/survived), and the presence of MD (positive/negative) were analysed using a generalized linear model with a binomial link function (logistic) fitting the effects of vaccination, challenge treatment and their interaction. Differences between different levels within main effects were tested by specific contrasts within the model. Mortality patterns over time were also analysed using survival analysis (Kaplan-Meier method).

Continuous data (eg bodyweight) were analysed using a general linear model fitting the effects of vaccination, challenge treatment and their interaction directly. MDV load in spleen from 14 dpc was $\log_{10}$ transformed [Log_{10} (x + 1)] prior to

fitting the effects of vaccination, challenge treatment and their interactions in a general linear model.

Relative immune organ weights (thymus, bursa and spleen) were expressed as % of bodyweight (relative organ weight = [(organ weight/body weight) x 100]. and analysed using a similar model but with the effect of operator/dissector fitted, and day post challenge removed (separate analyses were used for each dpc). Birds with tumours of the given organ were excluded from the analysis.

Vaccinal protective index (PI) was calculated as (%MD in unvaccinated chickens - %MD in vaccinated chickens) / (%MD in unvaccinated chickens) x 100. Virulence rank (VR) was calculated as 100-PI and served as a measure of the relative virulence of MDV isolates (Witter 1997). Only chickens at risk of MD were included in the calculation (i.e. alive at the time of the first MD case). For these data (PI and VR) which could only be measured on a whole isolator, data were analysed using a general linear model fitting the effects of vaccination, challenge treatment and their interaction. For this analysis there were only 2 measures per treatment combination (two isolators per treatment combination). For continuous variables, least squares means and standard errors are presented. A significance level of $P<0.05$ is used throughout.

K means cluster analysis was used to analyse relative thymic, splenic and bursal weights of individual birds to identify patterns of association between these organ weights and early prediction of MD.

4.3 Results

This study was led by Prof. S. W. Walkden-Brown and the data on chicken mortality, MD incidence, protective index, virulence and immune organ analyses are presented in this chapter with his permission as they are complementary to the full interpretation and understanding of the experimental results of the following chapters. I was responsible for the studies described in Chapters 5 and 6 but was also deeply involved in the day-to-day execution of this experiment with Prof. Walkden-Brown.

4.3.1 Confirmation of successful application of treatments

Infection with MDV was successful for all MDV challenge and vaccination treatments as determined by serotype specific qPCR analysis. No MD lesions were recorded in any chickens of the two control isolators and the absence of virus in the control isolators was confirmed by serotype specific qPCR analysis of spleens collected at 14 dpc and isolator exhaust dander. Results for the spleens are shown in Table 4.2 below, the results for the dusts are reported separately in Chapter 6.

In spleen samples from 14 dpc, 02LAR and MPF57 induced uniformly high proportions of MDV1 positive chicks (83-100 % overall) but this was not the case with 04CRE which induced 92 % positives in unvaccinated chickens but only 66.7 % and 25 % positives in chickens vaccinated with HVT or Bivalent vaccine respectively (Table 4.2). HVT was detected in 13/13 vaccinated chickens challenged by the different MDVs. No assays for MDV2 were done on spleen samples.

Table 4.2: Summary of number of chicken spleen samples positive for MDV1 and HVT at 14 dpc.

Vaccine	**Challenge**	**qPCR assay**	**Number of chickens positive for qPCR assay (%)**
Unvaccinated	Unchallenged	MDV1	0/12* (0%)
Unvaccinated	02LAR	MDV1	12/12 (100%)
Unvaccinated	04CRE	MDV1	11/12 (91.7%)
Unvaccinated	MPF57	MDV1	12/12 (100%)
HVT	02LAR	MDV1	12/12 (100%)
HVT	04CRE	MDV1	8/12 (66.7%)
HVT	MPF57	MDV1	11/12 (91.7%)
Bival	02LAR	MDV1	12/12 (100%)
Bival	04CRE	MDV1	3/12 (25%)
Bival	MPF57	MDV1	10/12 (83.3%)
HVT	All 3	HVT	13/13 (100%)

* 3 spleens contained traces of MDV contamination close to the threshold of detection. All values in the experiment were adjusted by subtracting the maximum contamination amount.

4.3.2 Mortality/ Survival

Fifteen of 535 eligible chickens died (15/535= 2.8 %) of miscellaneous causes (mostly bacterial infections) up to 5 dpc (i.e. day 10 of experiment) and were not included further in the analysis. Survival and mortality analysis therefore included

393 eligible chickens from 6 dpc onwards. The chickens removed at 14 dpc were not included and neither were 7 chickens removed from the experiment for other uses or which died of accidental causes. The first chicken dying after 5 dpc was at 22 dpc and this was also the date of the 1st detection of MD. This mortality and all subsequent mortality are included in the mortality analysis. The mortality due to MD at 22 dpc was followed by a period of continuous deaths associated with the presence of gross MD lymphomas until the end of the experiment at 56 dpc. Of the 393 chickens at risk of MD, 69 chickens died up to 56 dpc (17.6 %) while the remainder were sacrificed on this date. Table 4.3 shows a summary of mortality by treatment.

Table 4.3: Summary of MD mortality between 6 dpc and 56 dpc.

MDV1 challenge	Vaccine	Total number n	Total mortality n (%)	Mortality with MD lesions n (%)
Unchallenged	Unvaccinated	36	0 (0%)	0 (0%)
02LAR	Unvaccinated	37	18 (48.6%)	17 (45.9%)
	HVT	41	11 (26.8%)	9 (21.9%)
	Bivalent	42	5 (11.9%)	5 (11.9%)
04CRE	Unvaccinated	39	12 (30.8%)	11 (28.2%)
	HVT	40	4 (10.0%)	4 (10.0%)
	Bivalent	38	3 (7.9%)	3 (7.9%)
MPF57	Unvaccinated	36	13 (36.1%)	12 (33.3%)
	HVT	42	1 (2.4%)	1 (2.4%)
	Bivalent	42	2 (4.8%)	1 (4.8%)

There were significant effects of both vaccination ($P<0.0001$) and challenge treatment ($P=0.003$) on total mortality, but the interaction between these effects was non significant ($P=0.27$). Unchallenged and unvaccinated chickens were not included in this analysis as they were not part of the factorial design. Mortality in unvaccinated chickens was significantly higher (38.4 %) than in chickens vaccinated with either HVT (13.0 %) or the Bivalent vaccine (8.2 %) with no difference between the latter two. Mortality in chickens challenged with 02LAR was significantly higher (28.3 %) than in chickens challenged with 04CRE (16.2 %) or MPF57 (13.3 %).

Survival analysis revealed similar results with significant effects of challenge ($P<0.0001$) and vaccination ($P<0.0001$) on the pattern of mortality (Figure 4.1).

Unvaccinated chickens had a significantly higher mortality than chickens vaccinated with either HVT or Bivalent vaccine. Chickens vaccinated with the Bivalent vaccine showed the lowest mortality (Figure 4.1, right panel). When grouped by challenge virus, the isolate 02LAR induced significantly higher mortality than the isolates MPF57 and 04CRE (P<0.05, Figure 4.1, left panel).

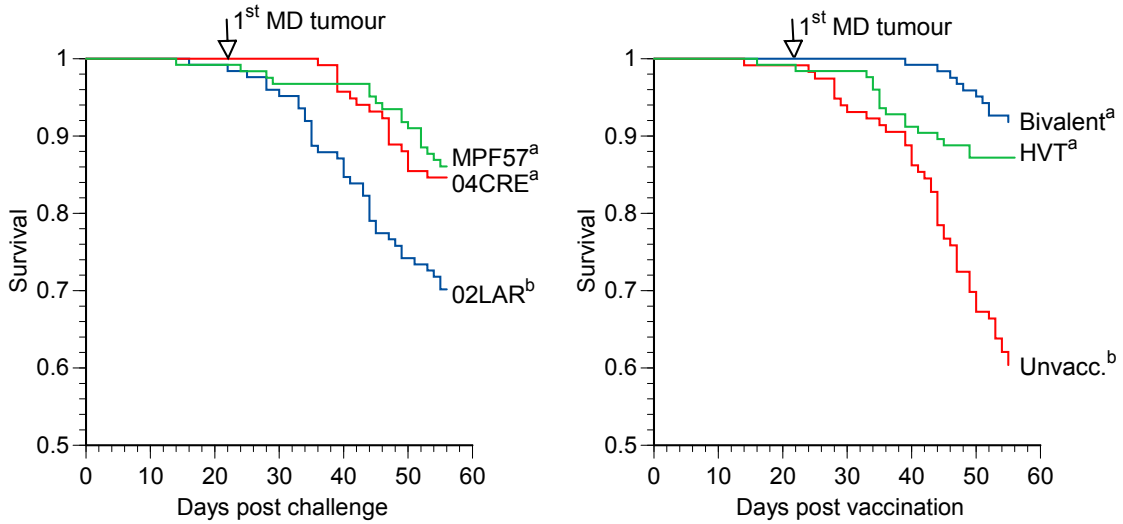

Figure 4.1: Survival patterns from 5 dpc showing the effects of Challenge (Left, P<0.0001) and Vaccination treatments (Right, P<0.0001). Treatments not sharing a common letter in the superscript differ significantly (P<0.05).

4.3.3 MD incidence, protective index and virulence rank

The combined incidence of gross MD lesions in the 393 chickens at risk of MD that died with MD or had MD lesions on post mortem after sacrifice at day 56 is summarized by treatment in Table 4.4. Amongst MDV-challenged chickens there was a significant effect of vaccination (P<0.0001) and challenge virus (P<0.0001) on the percentage of chickens with MD with no interaction between these effects (P=0.67). Unvaccinated birds had a significantly higher incidence of MD (70.5 %) than birds vaccinated with HVT (41.5 %) which in turn had significantly higher incidence of MD than those vaccinated with Bivalent vaccine (24.6 %). For the challenge viruses 02LAR induced a higher overall incidence of MD (57.5 %) than MPF57 (45.8 %) which in turn was higher than that induced by 04CRE (30.8 %).

The definition of the protective index and virulence rank is dependent on the definition of the “population at risk of developing gross MD lesions” and Table 4.4

below summarizes the data for birds at risk being those alive at the time of detection of the first MD gross lesions (22 dpc). Analysis of PI (%) by isolator replicate revealed a significant effect of vaccine virus (P=0.03) but not challenge (P=0.09) with no interaction between these effects (P=0.70). Bivalent vaccine induced significantly higher protection (67.2±6.9 %) than HVT vaccine (43.8±6.9 %) (Table 4.4). While the challenge viruses did not differ significantly there was a strong trend for 04CRE to have higher PI (73.1±8.5 %), than MPF57 (48.4±8.5 %) and 02LAR (45.1±8.5 %) and indeed a specific contrast comparing 04CRE against the other two challenge viruses was significant (P=0.03). As VR is derived from PI the analysis of VR produced the same level of significance for effects as PI. The main finding is that the VR of 02LAR (54.9±8.5 %) and MPF57 (51.6±8.5 %) combined was significantly higher than that of 04CRE (26.8±8.5 %) (Table 4.4). Based on the mean VR, the isolates 02LAR and MPF57 fall into the vv pathotype class whereas 04CRE falls into the v pathotype class according to Witter (1997) (Figure 4.2).

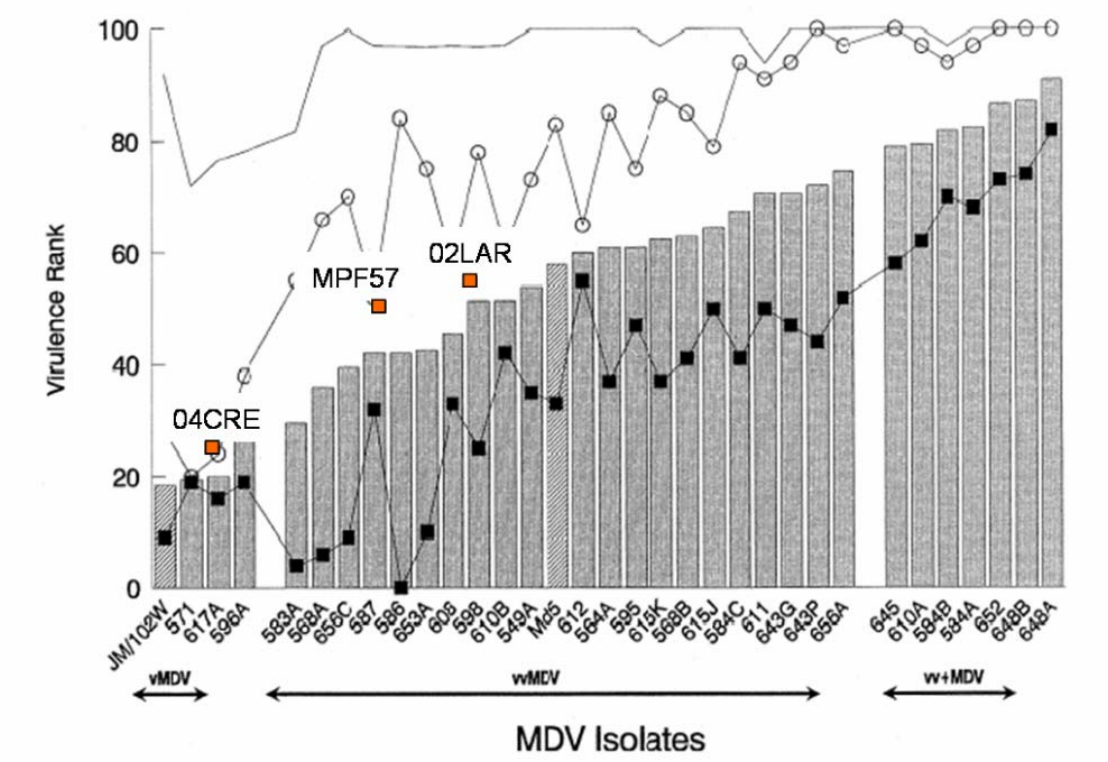

Figure 4.2: Classification of the three challenge viruses into pathotypes based on their mean VR according to the system suggested by Witter (1997). For the isolates examined by Witter (1997), the top line (no points) indicates percent Marek's disease induced in unvaccinated chickens. The middle line (circles) is VR based on data from chickens vaccinated with HVT. The lower line (squares) is VR based on data from bivalent-vaccinated chickens. Bars are the mean of the two values (prototype viruses in hatched pattern).

Table 4.4: Summary of MD incidence, protective index (PI) of vaccines, and virulence rank of the 3 challenge isolates of MDV1.

MDV1 challenge	Vaccine	MD (yes)[d]	MD (no)[d]	%MD	PI	Virulence rank (100-PI)
02LAR	Unvaccinated	31	6	83.8^{ax}		
	HVT	25	16	61.0^{bx}	27.2	72.8
	Bivalent	13	29	31.0^{cx}	63.1	36.9
04CRE	Unvaccinated	23	16	59.0^{ay}		
	HVT	8	32	20.0^{bz}	66.1	33.9
	Bivalent	5	33	13.2^{bx}	77.7	22.3
MPF57	Unvaccinated	25	11	69.4^{axy}		
	HVT	18	24	42.9^{bx}	38.3	61.7
	Bivalent	12	30	28.6^{bx}	58.9	41.1
Overall	Unvaccinated	79	33	70.5^{a}		
	HVT	51	72	41.5^{b}		
	Bivalent	30	90	24.6^{c}		

[abc] Effect of vaccination. Means not sharing a common letter in the superscript differ significantly (P<0.05).

[xyz] Effect of challenge isolate. Means not sharing a common letter in the superscript differ significantly (P<0.05).

[d] number of MD positive or negative birds.

4.3.4 Anatomical distribution, number and severity of MD lesions

Amongst 164 chickens exhibiting MD lesions, gross tumour lesions were most commonly found in the ovary, followed by the liver, spleen and kidneys. The overall distribution of MD lymphomas and the average severity of the lesions (score 0-3 with 3 most severe) are shown in Table 4.5.

The mean number of organs with lymphomas in MD positive chickens was 2.56. This was significantly affected by vaccination (P=0.04) with significantly more organs affected in unvaccinated chickens (2.84±0.16) than those vaccinated with HVT (2.14±0.22). Bivalent-vaccinated chickens were intermediate (2.56±0.28) and did not differ from the other groups. Challenge virus did not affect the number of organs involved (P=0.26; 2.68±0.28, 2.63±0.18 and 2.22±0.20 for 04CRE, 02LAR and MPF57 respectively).

Table 4.5: Anatomical distribution of MD lesions in chickens showing gross lesions.

Organ	Mean severity score	Number and percentage of chickens with MD lesions showing lesions in each organ			
		Un-vaccinated (n=81)	HVT – vaccinated (n=55)	Bivalent-vaccinated (n=28)	Overall (n=164)
Ovary	2.08	68 (84%)	45 (81.8%)	16 (57.1%)	129 (78.7%)
Spleen	1.65	44 (54.3%)	24 (43.6%)	10 (35.7%)	78 (47.6%)
Liver	1.76	46 (56.8%)	16 (29.1%)	12 (42.9%)	74 (45.1%)
Kidney	1.93	17 (21.0%)	13 (23.6%)	10 (35.7%)	40 (24.4%)
Heart	2.00	12 (14.8%)	3 (5.5%)	5 (17.9%)	20 (12.2%)
Eye	1.67	10 (12.3%)	3 (5.5%)	2 (7.1%)	15 (9.1%)
Lung	2.08	9 (11.1%)	1 (1.8%)	3 (10.7%)	12 (7.3%)
Sciatic nerve	1.30	3 (3.7%)	4 (7.3%)	2 (7.1%)	9 (5.5%)
Mesenteries	1.88	4 (4.9%)	3 (5.5%)	1 (3.6%)	8 (4.9%)
Thymus	2.43	5 (6.2%)	0 (0%)	2 (7.1%)	7 (4.3%)
Proventriculus	2.20	4 (4.9%)	0 (0%)	1 (3.6%)	5 (3.0%)
Skin	2.00	2 (2.5%)	2 (3.6%)	0 (0%)	4 (2.4%)
Pancreas	2.75	3 (3.7%)	1 (1.8%)	0 (0%)	4 (2.4%)
Muscle	2.00	1 (1.2%)	2 (3.6%)	1 (3.6%)	4 (2.4%)
Bursa	1.50	1 (1.2%)	1 (1.8%)	0 (0%)	2 (1.2%)

The mean severity score was 1.85 with no overall influence of vaccination (P=0.11) or challenge (P=0.08) although there was significant interaction between these effects (P=0.03). The significant interaction reflected that vaccination had little effect on MD severity in chickens challenged with 02LAR, with increasing effects of vaccination when challenged with 04CRE and particularly MPF57 (Figure 4.3). Overall there were non-significant trends towards lower severity in HVT but not Bivalent vaccinated chickens, and in chickens challenged with MPF57 compared to chickens challenged with 02LAR or 04CRE.

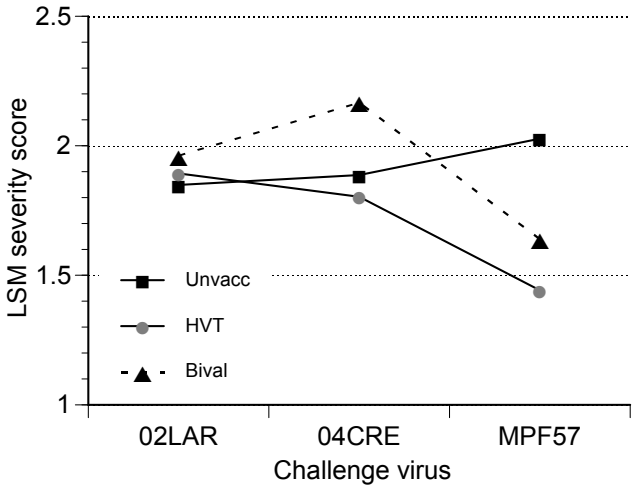

Figure 4.3: Interaction plot showing the differential effect of vaccination on the MD lesion severity score induced by the different challenge viruses.

4.3.5 Body weight, relative immune organ weights and MDV1 load in spleen at 14 dpc and 56 dpc

Statistical analysis of these variables used the complete 3 x 3 factorial design (3 vaccination and 3 challenge treatments in combination) and excluded the "control" unchallenged and unvaccinated treatment. However the means and standard errors for this treatment are presented and included in the figures for reference.

4.3.5.1 Bodyweight

At 14 dpc, 6 chickens from each treatment were sacrificed (total n=120), and their body weights were recorded. The mean bodyweight was 160±1.7 g with no significant overall effect of vaccination (P=0.38) or challenge (P=0.54), however there was significant interaction between these effects (P=0.03) due to a different response to vaccination in 04CRE compared with the other challenge isolates.

In birds challenged with 04CRE bodyweight was lower in vaccinated chickens than unvaccinated chickens whereas in those challenged with the other two isolates BWT was higher in vaccinated chickens (Figure 4.4). The mean weight in unchallenged control chickens that were not included in the analysis was 172±4.1 g, higher than that of any other treatment.

At 56 dpc, there were significant overall effects of vaccination (P=0.02) and challenge (P<0.001) with no significant interaction between these effects (P=0.11) (Figure 4.4). Unvaccinated chickens were significantly lighter (744±13 g) than those vaccinated with either HVT (795±10 g) or Bivalent vaccine (795±9 g). Chickens challenged with 02LAR were significantly lighter (741±11 g) than those challenged with either MPF57 (795±10 g) or 04CRE (797±10 g). The mean weight in unchallenged control chickens that were not included in the analysis was 873±14 g, considerably higher than that of any other treatment.

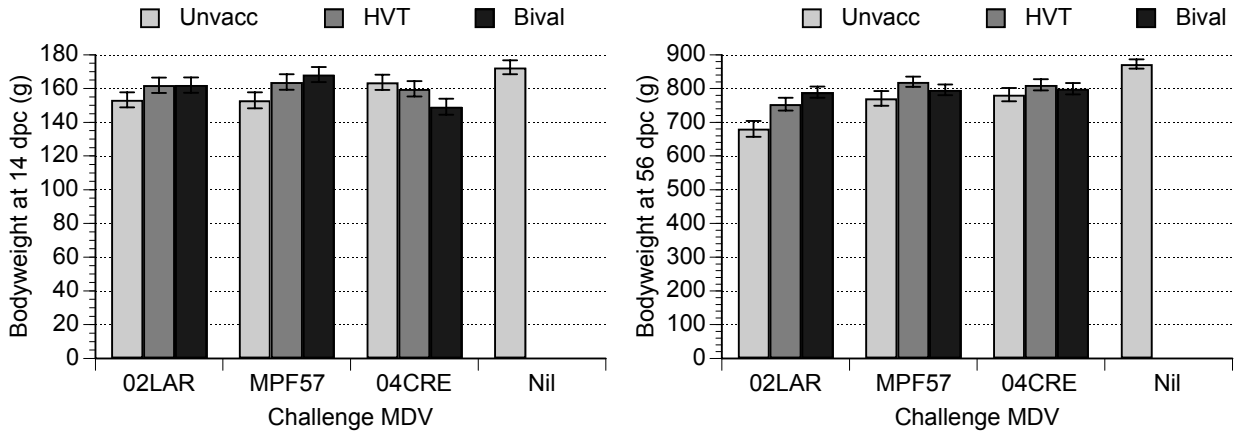

Figure 4.4: Interaction plot showing the effects of MDV challenge and vaccination on chicken bodyweight (LSM±SEM) at 14 dpc (left panel) and 56 dpc (right panel).

4.3.5.2 Bursa of Fabricius

At 14 dpc, the overall mean relative bursal weight was 0.377±0.008 with significant effects of vaccination (P<0.001), but not challenge (P=0.29) or operator (P=0.61) and there was no significant interaction between these effects (Figure 4.5).

Unvaccinated chickens had significantly lower relative bursal weight (0.31±0.01 %BW) than those vaccinated with HVT (0.40+0.01 %BW) or Bivalent vaccine (0.41+0.01 %BW). The mean relative bursal weight in unchallenged control chickens that were not included in the analysis was 0.40±0.02 %BW, which was amongst the higher values although not the highest (Figure 4.5).

At 56 dpc the overall mean relative bursal weight was 0.345±0.006, only slightly lower than the 14 dpc value. There were significant effects of vaccination

(P<0.001) challenge (P=0.002) and operator (P=0.008) with no significant interaction between these effects (Figure 4.5). Unvaccinated chickens again had significantly lower values (0.28±0.01 %BW) than those vaccinated with HVT (0.35±0.01 %BW) or bivalent vaccine (0.36±0.01 %BW). Chickens challenged with 02LAR also had significantly lower values (0.29±0.01 %BW) than those challenged with 04CRE (0.36±0.01 %BW) or MPF57 (0.34±0.01 %BW). The mean relative bursal weight in unchallenged control chickens that were not included in the analysis was 0.36±0.01 %BW, which was among the higher value amongst the treatment groups, and slightly lower than the value at 14 dpc, indicating as with relative thymic weight, that relative bursal weight was relatively constant over this period in unchallenged birds.

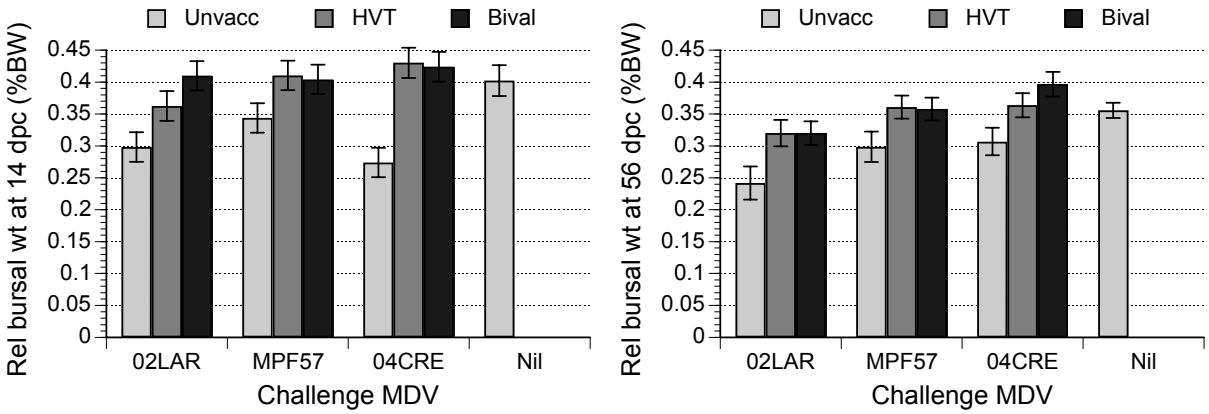

Figure 4.5: Interaction plot showing the effects of MDV challenge and vaccination on relative bursal weight (LSM±SEM) at 14 dpc (left panel), and 56 dpc (right panel).

4.3.5.3 Thymus

Relative thymic weight showed very similar trends as bursa weight.

At 14 dpc, the mean relative thymus weight was 0.350±0.009 %BW and was significantly affected by vaccination status (P=0.01), and operator (P<0.001), but not challenge (P=0.22) with no significant interaction between these effects (Figure 4.6). Relative thymus weight was significantly reduced in unvaccinated chickens (0.32±0.01 %BW) compared to those vaccinated with HVT (0.37+0.01 %BW) or bivalent vaccine (0.37+0.01 %BW).

The operator effect was entirely due to the number of lobes dissected (Figure 4.7, left panel) with no operator effect on mean lobe weight (P=0.65). The mean relative thymic weight in unchallenged control chickens that were not included in the analysis was 0.39±0.03 %BW, which was amongst the higher values. Each thymus was also scored for atrophy on a 0-3 scale (3= severe atrophy). There was a strong linear association between thymus weight and atrophy score (R^2=0.61, P<0.001) as shown in Figure 4.7, right panel.

At 56 dpc, the overall mean relative thymic weight was 0.419±0.013 %BW and was again significantly influenced by vaccination (P<0.001), and operator (P<0.005, Figure 4.6 right panel), but not challenge (P=0.11) with no significant interaction between these effects. Unvaccinated chickens had significantly lower relative thymic weight (0.30±0.02 %BW) than those vaccinated with HVT (0.41+0.02 %BW) or Bivalent vaccine (0.47+0.02 %BW). There was a non-significant trend for 02LAR to have a lower thymic weight than 04CRE (P=0.11). The mean relative thymic weight in unchallenged control chickens that were not included in the analysis was 0.56±0.03 %BW, which was the highest value amongst the treatment groups. The value was considerably higher for the same group at 14 dpc, indicating that relative thymic weight had increased substantially over this period in unchallenged birds.

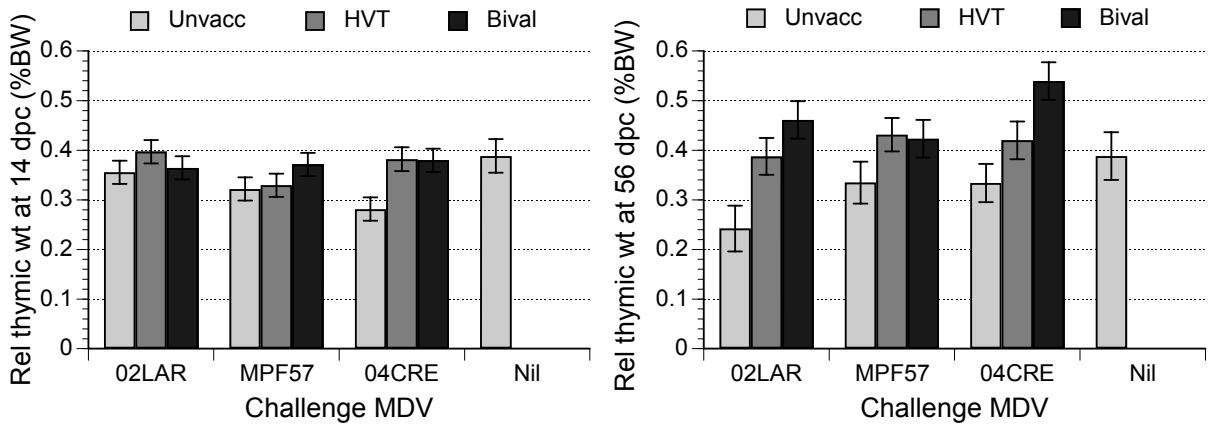

Figure 4.6: Interaction plot showing the effects of MDV challenge and vaccination on relative thymic weight (LSM±SEM) at 14 dpc (left panel), and 56 dpc (right panel).

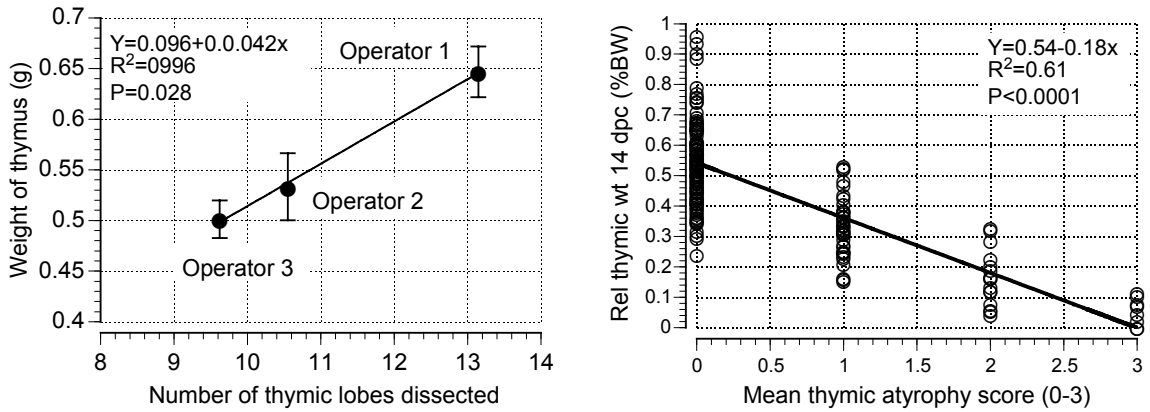

Figure 4.7: Association between mean relative thymic weight and mean number of lobes dissected by each operator at 14 dpc (left panel), and association between mean relative thymic weight and mean thymic atrophy score (0-3) at 14 dpc (right panel).

4.3.5.4 Spleen

At 14 dpc, the overall mean relative weight of spleen (excluding those classed as tumorous) was 0.228±0.004 %BW. This was significantly affected by vaccination (P<0.001) challenge (P=0.02) and operator (P<0.001) with no interaction between these effects (Figure 4.8, left panel). Unvaccinated chickens had significantly higher relative splenic weights (0.25±0.01 %BW) than those vaccinated with HVT (0.21±0.01 %BW) or bivalent vaccine (0.23±0.01 %BW). Chickens challenged with 04CRE had significantly higher values (0.24±0.01 %BW) than those challenged with MPF57 (0.22+0.01 %BW) did. Those challenged with 02LAR were intermediate (0.23±0.01 %BW) and did not differ from the other two. The relative spleen weight of unchallenged control chickens remained constant between 14 dpc (0.212±0.012 %BW) and 56 dpc (0.210±0.005 %BW).

At 56 dpc, there was no longer an overall effect of vaccination treatment (P=0.75) although the effects of challenge virus (P=0.001) and operator (P=0.03) remained significant. There was significant interaction between the effects of vaccination and challenge virus (P=0.03) due to a differential effect of HVT vaccine for the 3 viruses (elevated relative splenic weight in birds challenged with 02LAR, but reduced relative splenic weight in chickens challenged with 04CRE and MPF57) (Figure 4.8, right panel). Chickens challenged with 02LAR had significantly higher relative splenic weights (0.33±0.01 %BW) than those challenged with either MPF57 (0.30±0.01 %BW) or 04CRE (0.28±0.01 %BW).

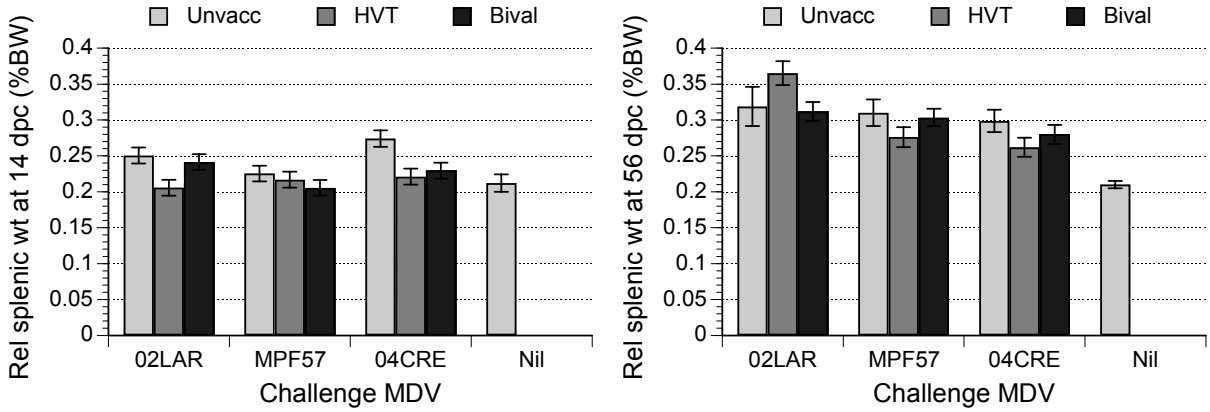

Figure 4.8: Interaction plot showing the effects of MDV challenge and vaccination on relative splenic weight (LSM±SEM) at 14 dpc (left panel), and 56 dpc (right panel).

4.3.5.5 MDV1 load in spleen at 14 dpc

MDV1 load (log_{10}VCN/10^6 spleen cells) at 14 dpc was significantly affected by vaccination status and MDV challenge treatment (P<0.001) with significant interaction between these effects (P=0.02, Figure 4.9). MDV1 load was significantly lower in HVT-vaccinated (4.3±0.25) or Bivalent vaccinated chickens (3.2±0.25) than unvaccinated chickens (5.7±0.59). Challenge treatment also significantly affected MDV1 load (Figure 4.9). In spleens of unvaccinated chickens at 14 dpc, the MDV1 load with 02LAR was 6.1 log_{10}VCN/10^6 spleen cells, with MPF57 5.9 log_{10}VCN/10^6 spleen cells and with 04CRE 4.9 log_{10}VCN/10^6 spleen cells. The same ranking was present in both vaccination treatments (Figure 4.9).

There appeared to be a problem with aseptic collection of spleen samples in the contaminated post-mortem environment which showed basal level contamination with MDV1 (1.94 log_{10}VCN/10^6 spleen cells) and all values in the experiment were adjusted by subtracting this amount.

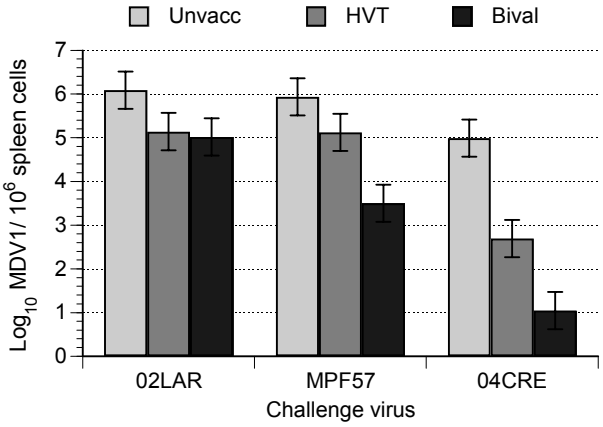

Figure 4.9: Load of MDV1 (LSM±SEM) in spleen of chickens at 14 dpc, by vaccination treatment and challenge virus.

4.3.6 Prediction of MD incidence

4.3.6.1 MDV load in spleen

MDV1 load in spleen at 14 dpc was significantly positively associated with the final incidence of MD in each isolator at 56 dpc, accounting for 64-71% of the total variation between isolators in the incidence of MD (Figure 4.10).

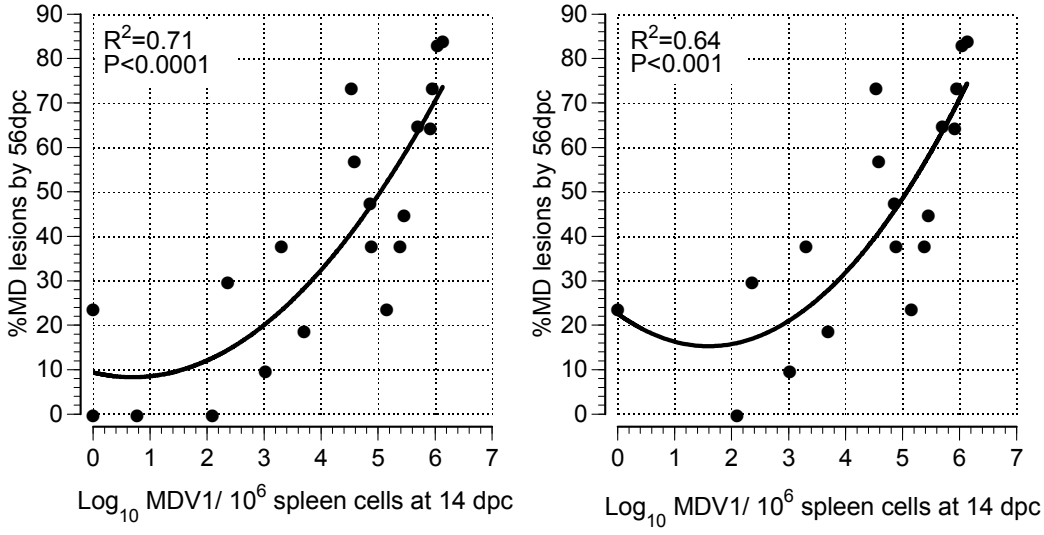

Figure 4.10: Association between MDV1 load in spleen at 14 dpc and the incidence of MD by day 56pc (% birds with gross MD lesions). Each point represents one isolator and the line is a 2nd degree polynomial fit. The left panel includes two unchallenged isolators (each with 0% MD) while the right panel excludes these.

4.3.6.2 Relative immune organ weight

At 14 dpc there was a non-significant negative association between relative thymic weight and the incidence of MD by 56 dpc (P=0.06) but by 56 dpc the association was very strong, for both relative thymic weight and thymic atrophy score (Figure 4.11).

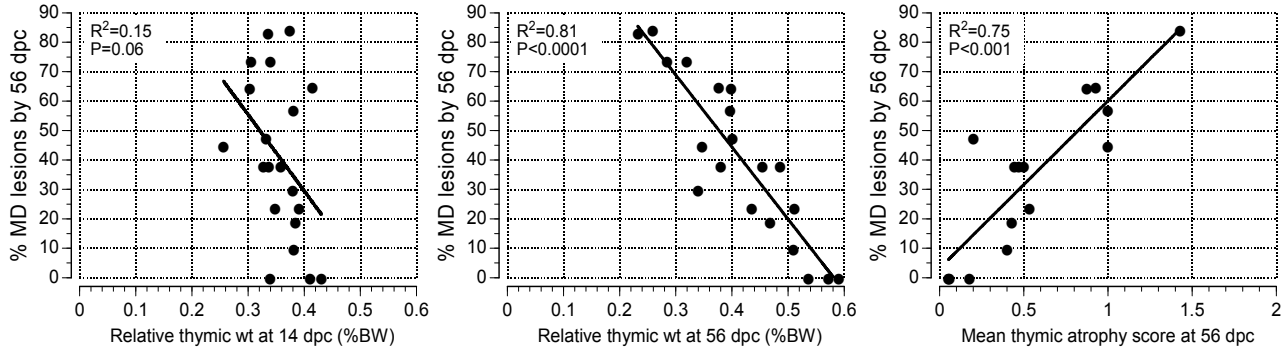

Figure 4.11: Association between relative thymic weight (% of bodyweight) at 14 and 56 dpc (left panels), thymic atrophy score (right panel) and the incidence of MD by 56 dpc (% birds with gross MD lesions). Each point represents one isolator. Unchallenged isolators are included. Chickens with tumours of the thymus are excluded. Relative thymic weights are adjusted for the effect of operator. The line is a linear regression curve.

The situation was similar for relative bursal weight except that there was a significant negative association at both 14 and 56 dpc (Figure 4.12).

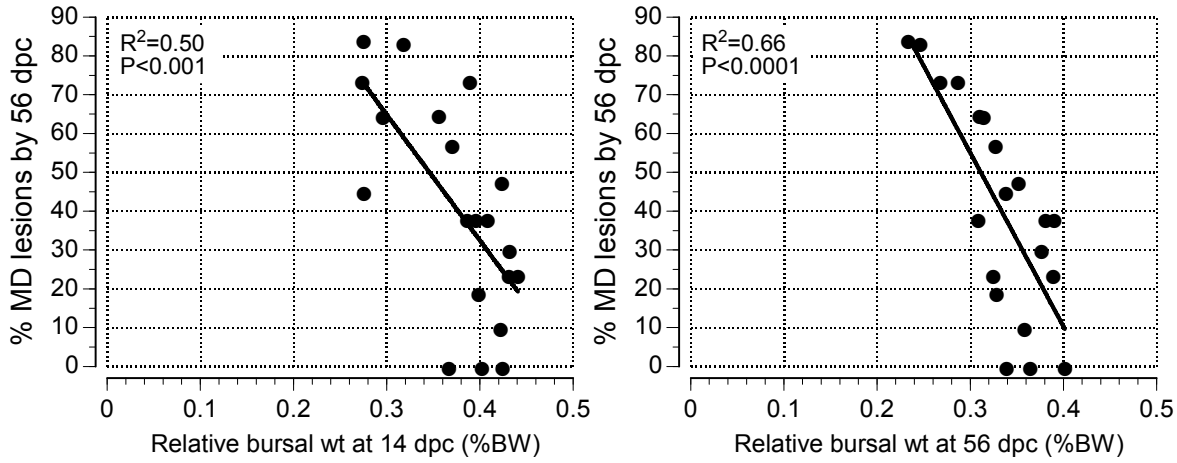

Figure 4.12: Association between relative bursal weight (% of bodyweight) at 14 and 56 dpc and the incidence of MD by 56 dpc (% birds with gross MD lesions). Each point represents one isolator. Unchallenged isolators are included. Chickens with tumours of the bursa are excluded. The line is a linear regression curve.

Unlike the other immune organs, relative splenic weight was positively associated with the incidence of MD although this was only significant at 56 dpc (Figure 4.13). However the associations were weaker than for bursal or thymic weight.

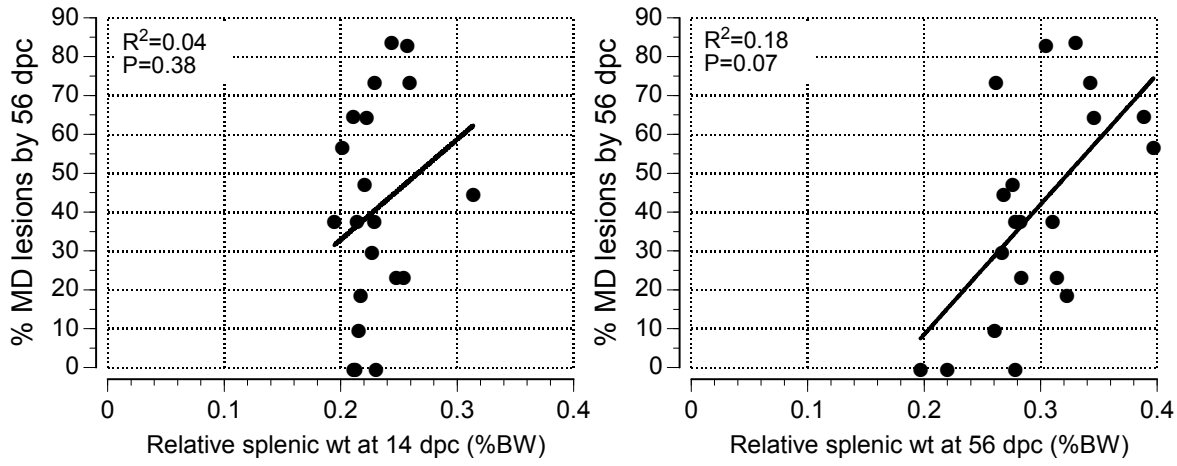

Figure 4.13: Association between relative splenic weight (% of bodyweight) at 14 and 56 dpc and the incidence of MD by 56 dpc (% birds with gross MD lesions). Each point represents one isolator. Unchallenged isolators are included. Chickens with tumours of the spleen are excluded. The line is a linear regression curve.

Use of K-means cluster analysis of relative thymic, splenic and bursal weights of individual birds to identify patterns of association between the 3 organs did not enable more accurate early prediction of MD than individual organ measurement with R^2 values of 0.38 (P=0.006) for a 2 cluster analysis. The two clusters essentially differentiate chickens with relatively large spleens and relatively small bursal and thymic weights with those having the reverse pattern.

Use of the data from all 3 relative immune organ weights at 14 dpc variables in stepwise regression equations did not improve the overall fit with a single variable, relative bursal weight, proving the sole predictor of value.

4.3.7 Association between incidence of MD lesions in unvaccinated and vaccinated chickens

Overall there was a very strong relationship between the incidence of MD induced in unvaccinated chickens, and that induced in HVT-vaccinated chickens

with the same virus (R^2= 0.97, P=0.08, Figure 4.14). However the relationship was less strong for chickens vaccinated with the bivalent vaccine (R^2= 0.77, P=0.31, Figure 4.14). Figure 4.14 clearly shows that the more virulent the isolate (in unvaccinated chickens), the greater the advantage that bivalent vaccine had over HVT in protection.

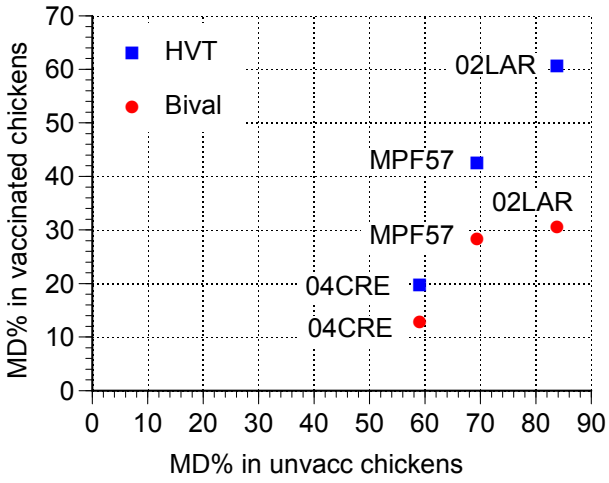

Figure 4.14: Association between the incidence of MD lesions (MD%) in unvaccinated chickens or chickens vaccinated with HVT or Bivalent vaccine (HVT/SB-1).

4.4 Discussion

This study was designed to test recent Australian isolates of MDV in a pathotyping experiment and to identify early predictors of subsequent MD incidence and MD pathotype. Based on the ADOL classification of pathotypes the isolates in this study were classified as vv (02LAR and MPF57) and v (04CRE). A key finding is that none of the viruses were in the vv+ category, which has been the most severe pathotype of MDV in the USA over the last decade. A second important finding of this study was that there was a significant positive association between the MDV1 viral load in spleen and pathotype. This shows that viruses of higher virulence show higher levels of viral replication in the spleen at 14 dpc. However, chickens were challenged with a fixed dose of 500 pfu/chicken by intra-abdominal injection at 5 days of age which is in accordance with the ADOL method. However, under field conditions chickens are often challenged at

placement by the natural route and this should be taken into consideration when interpreting the results of this experiment.

With regards to the first hypothesis, the results of the present experiment suggest that there is no clear evidence that the most virulent recent isolates (02LAR and 04CRE) are more pathogenic than older reference isolate (MPF57) obtained more than a decade ago, or even isolates from more than two decades ago. The first evidence that there are MDV1 isolates of high virulence circulating in Australia was from the mid-1980s (McKimm–Breschkin et al., 1990). Several isolates from this period were classified as vv pathotypes as they induced tumours in up to 90 % of unvaccinated maternal antibody negative (mab-) SPF chickens and more than 80 % in HVT vaccinated SPF chickens (McKimm–Breschkin et al., 1990). Unfortunately none of the strains from this period are available for testing currently due to a lack of cell cultured material. Subsequently in the early 1990s there was further isolation of putatively vv MDV strains (including MPF57) isolated from flocks in which vaccination with HVT or bivalent vaccines provided poor protection (DeLaney et al., 1995; Zerbes et al. 1994).

The Australian reference isolate MPF57 in the present layer experiment induced 69 % MD in unvaccinated chickens, 43 % MD in HVT vaccinated chickens and 29 % MD in bivalent vaccinated chickens. The VR (100-PI) of MPF57 was 67.1 and 41.1 based on PI provided by HVT and bivalent vaccine respectively. In an earlier study, DeLaney et al. (1998) reported that MPF57 induced 60-100 % deaths or gross lesions in unvaccinated mab- SPF chickens. The results of the present experiment are therefore in broad agreement with those reported by DeLaney et al. (1998). However, that study did not investigate the PI provided by HVT and the proposed suggestion to classify this isolate as vv was solely based on incidence of MD lesions. However, given that vaccination with HVT provided poor protection against MD, the present data suggests classifying MPF57 as vv according to the pathotyping system by Witter (1997) which is in agreement with previously published results (DeLaney et al., 1998).

The isolate 02LAR induced 84 % MD in unvaccinated chickens, 61 % MD in HVT vaccinated chickens and 31 % MD in bivalent vaccinated chickens. The VR of this isolate was 72.8 and 36.9 based on the PI provided by HVT and bivalent vaccines respectively. Therefore, despite some reservations about the

experimental method which are discussed in more detail later, this isolate is proposed to be classified as vv according to the USDA pathotyping system (Witter 1997).

The isolate 04CRE induced the least MD incidence in both unvaccinated and vaccinated layer chickens and based on the MD incidence and VR of this isolate, 04CRE is suggested to be classified as a v pathotype rather than vv pathotype in the USDA system (Witter, 1997).

However, it is difficult to compare the results of the present experiment and those of the most comprehensive work of this kind in Australia previously, that of McKimm-Breschkin et al. (1990) and DeLaney et al. (1998). Different chicken strains, virus challenge protocols, and HVT vaccines and doses make the results difficult to compare. Nevertheless it is clear that some of the more virulent viruses in the McKimm Breschkin's study (MPF23, MPF15) produced results comparable with the more virulent virus 02LAR in the present experiment. Vaccination with HVT (250-1000 pfu, probably cell-free) provided very poor protection against MD gross lesions when challenged with MPF23, the most virulent virus in the earlier study (McKimm-Breschkin et al., 1990). Given that MPF23 strains was isolated more than two decades ago, there is no clear evidence that there is ongoing evolution towards MDV isolates with higher virulence in Australia, at least not to that extent and speed as reported from the USA where MDV field isolates have clearly shown to have evolved to higher virulence within a decade (Witter, 1997). This is consistent with the idea that blanket vaccination with an imperfect vaccine is a driver of increased virulence (Gandon et al. 2001) as broiler chickens in Australia have only had widespread vaccination against MD in the last decade, whereas in the USA vaccination has been widespread since the 1970s.

With regards to hypothesis two, bursal weights at 14 dpc showed a significant negative association with the incidence of MD at 56 dpc which remained negative and significant at 56 dpc and was therefore the best predictor of subsequent MD outcome. Similar to bursal weights, thymic weights at 14 dpc showed a non-significant negative association with the subsequent incidence of MD, but was highly significant at 56 dpc. However, the sampling of thymus is labour-intensive and there are significant operator effects which do not occur for bursa and spleen and therefore, its use as a practical means of prediction of MD incidence are

limited. As well, there was no significant difference in immune organ weights for the three different challenge viruses and thus are also poor predictors of pathotype.

The association of spleen weights at 14 dpc and incidence of MD at 56 dpc was positive, but not significant and weaker than for thymic and bursal weights at both 14 and 56 dpc. The associations between relative immune organ weights and subsequent MD outcome are strong in SPF chickens without maternal antibodies in which very great reductions in immune organ weights are observed (Calnek et al., 1998; Walkden-Brown et al., 2006). However the immunosuppressive effect of MDV challenge is much lower in commercial chickens with maternal antibody (eg. this experiment, and Islam et al 2002) and the association with subsequent MD is therefore weaker. Maternal antibody is known to be protective against MD (Chubb and Churchill, 1969, Calnek, 1972; Morimura et al., 1998; Baaten et al., 2004) forming immune complexes with the challenge virus thus providing a protective effect on the immune organs.

With regards to hypothesis three, the MDV1 load in spleen at 14 dpc was highly significantly ($P<0.0001$) associated with subsequent MD with an R^2 value of 0.71 meaning that 71 % of the variation in MD incidence at 56 dpc is explained by MDV load at 14 dpc. Compared to associations between relative immune organ weights and subsequent MD, the R^2 values for relative thymus weight were 0.15, for relative bursa weight 0.5 and relative spleen weight 0.04. Thus, MDV1 load in spleen at 14 dpc explains a much higher proportion of the variation in subsequent MD incidence than relative immune organ weights.

Furthermore, this association revealed clear differences between the effects of vaccination and challenge MDV at this early stage. The most virulent isolate 02LAR showed the highest viral load in spleen, followed by MPF57 and the least virulent isolate 04CRE. This demonstrates that there is an association between pathotype and viral replication in the spleen which has been shown previously (Yunis et al., 2004). It also shows that the host genotype is an important factor for pathotyping experiments as this association was not evident in a similar experiment in Cobb broilers or SPF chickens (Walkden-Brown et al., 2007b). Differences in MDV1 load in spleen at 13 dpc associated with pathotype have also been reported in SPF chickens (Walkden-Brown et al, 2006).

Both the previous pathoyping experiments in SPF chickens and broilers reported a strong positive relation between MDV1 load in spleens and subsequent MD outcome at 13-14dpc 21 dpc and Islam et al (2007) also reported this at 21 dpc suggesting that this variable is an excellent predictor of future MD at any age of the chicken as has been proposed previously.

The measurement of MDV1 load in spleen in the first few weeks after challenge could potentially replace full pathotyping experiments as these measurements can be made prior to the onset of clinical MD which would offer significant ethical advantages and save resources. Other than spleen material, which requires the sacrifice of the respective bird, quantification of MDV load in peripheral blood lymphocytes (PBL) has previously been shown to be a good predictor of subsequent MD outcome in early stages of infection (Islam et al., 2006b). These authors have shown that a mean MDV load below 10^2 viral copy numbers (VCN) per 10^4 PBL, up to 3 weeks following infection, is associated with subsequent protection from MD, while mean MDV load greater than 10^2 VCN per 10^4 PBL is associated with subsequent MD. Compared to spleen samples, PBL would offer the possibility of repeatedly quantifying MDV load in a chicken flock and enable predictions of clinical MD on a flock basis at least 2 to 3 weeks prior to clinical MD and should therefore be considered as another potential tool for early MD prediction.

In conclusion, this study has shown that there is no clear evidence that Australian isolates of MDV have evolved rapidly towards higher virulence over the past two decades. Furthermore, this study has confirmed that viral replication in the spleen is correlated with pathotype and an excellent predictor of subsequent MD outcome. Using MDV load in spleen at 14 dpc as an endpoint in pathotyping experiments allow shortening of these experiments to an extent that measurements can be made prior to the onset of clinical MD.

Chapter 5

Longitudinal quantification of Marek's disease virus serotype 1 (MDV1) and Herpesvirus of Turkeys (HVT) in feather tips and their relationship to clinical Marek's disease in commercial layer chickens

5.1 Introduction

Infection of susceptible chickens with virulent Marek's disease virus (MDV) occurs by inhaling dander shed from MDV-infected birds with the lung being the port of entry for the virus (Calnek et al., 1970; Carrozza et al., 1973). MDV targets a number of different cell types during its life cycle. Lymphocytes are the primary target, although within them, virus production is restricted and free enveloped viruses, is not produced by infected cells (Davison and Kaiser, 2004).

The only site where fully productive infection by MDV occurs with the release of infectious enveloped virus is the feather follicle epithelium (FFE) (Calnek et al., 1970; Carrozza et al., 1973). Earlier reports have shown that the MDV genome can be first detected in FFE and skin 2 weeks post infection (Nazerian and Witter, 1970: Calnek et al., 1970). A more recent study has shown that significant amounts of MDV1 are present in feathers at 6 days post infection (Baigent et al., 2005b). Subsequently cell-free virus is shed with skin and feather debris throughout the life of an infected bird (Calnek et al., 1970). Early studies with both virulent and avirulent isolates of MDV revealed that virulent isolates were more readily found in the FFE and more easily transmitted to contact chickens than avirulent isolates including attenuated MDV1 and HVT (Nazerian and Witter, 1970). Islam et al. (2005a) first reported significant shedding of HVT from vaccinated chickens, but also found that the shedding level of this avirulent strain was far lower than that of virulent MDV1. These findings were confirmed in a separate subsequent study (Islam and Walkden-Brown, 2007) but surprisingly, the avirulent MDV2 shed at a higher rate than either HVT or MDV1. The latter two

studies measured MDV in feather dander, a simple measure which integrates information from a number of birds in a population. However some studies require data from individual birds something that cannot be achieved with a pooled dust sample. It is also ideal to have a measure of MD status that can be made repeatedly on the same chicken. Blood sampling followed by separation of PBL and determination of MDV level in these is the traditional method to achieve this, but this is invasive, laborious and stressful for the chickens and requires extensive immediate post collection processing to separate PBL. Collection of feathers and estimation of MDV content in their tips offers an attractive alternative as has been shown in previous studies (Baigent et al., 2005b; 2007). Widespread use of this method however, requires that it be validated and the association of MDV level in feather tips with clinical MD, and measures of MDV in lymphoid organs be established.

This study was therefore designed to quantify MDV1 and HVT load repeatedly from individual layer chickens and to correlate feather tip MDV1 load with MDV1 load measured in spleens of individual chickens, and group dander samples, and also with subsequent MD outcomes.

The study involved measurements made during pathotyping experiment MD05-C-PT3 (Chapter 4) supported by Australian Poultry CRC project 03-17, although the present study had a separate design nested in the main experimental design. While several measurements in this experiment may appear to duplicate those from Chapters 4 and 6, in this chapter they refer only to the chickens in the 6 isolators included in this study, not the whole 20 isolator experiment.

Objectives:

- To determine the effects of vaccination and challenge virus on MDV and HVT viral load in feather tips (FT), spleen and dander collected from isolator exhausts.
- To relate these data with MD pathology end points up to day 56 post-challenge.
- To determine the level of association between these measures.

Hypotheses:

1. Vaccination with HVT will reduce the incidence of MD and will reduce MDV1 load in feather tip (FT), spleen and dander.
2. MDV isolates MPF57 and 02LAR will differ in the level of MD they induce and the level of MDV1 load in FT, spleen and dander.
3. Chickens which go on to develop MD will have higher levels of MDV1 in FT throughout, than those which do not go on to develop MD. Thus there will be positive association between MDV in feather tips, and subsequent MD.
4. Chickens which go on to develop MD will have lower levels of HVT in FT throughout, than those which do not go on to develop MD. Thus there will be negative association between HVT in feather tips, and subsequent MD.
5. MDV load in feather tips will be strongly and positively correlated with MDV load in spleen and dander at equivalent time points.
6. MDV1 and HVT viral load measured in FT material of layer chickens will be similar to that observed in other recent studies.

5.2 Materials and methods

5.2.1 Experimental design

This experiment was designed as a 2x2 factorial experiment with 48 individually identified chickens in 4 isolators plus 2 control isolators. The two experimental factors were:

- Challenge virus (2 levels): MPF57 and 02LAR, (500 pfu i.a. in 200 µl on day 5 of age).
- Vaccine treatment (2 levels): unvaccinated (diluent only) and HVT (FC-126, 8000 pfu s.c. in 200 µl on the day of hatch (day 0).

Two additional isolators were unvaccinated and unchallenged (diluent injection only) to serve as control groups. The experiment started on the day of hatch (day

0, 06/06/05) and was terminated on 61 days of age (56 dpc, 09/08/05). Chickens were vaccinated at hatch (day 0) and challenged with MDV at day 5 of age.

Detailed background information about the vaccine and challenge material and vaccination and challenge procedure is described in Chapter 4. Chickens in the present experiment were individually wing-banded and also formed part of the main pathotyping experiment described in Chapter 4. While each isolator contained 27 chickens for the main pathotyping experiment, in the four isolators in which feathers were repeatedly sampled for this study, 12 of the 27 chickens were individually wing tagged. Thus the experimental chickens in this study formed a subset of chickens in the isolators.

5.2.2 Experimental chickens

Twelve ISABROWN chickens per treatment combination (48 in total) were individually marked with wing tags and followed throughout the experiment to 56 dpc (61 days of age). Four to six feathers were plucked weekly, starting at 7 dpc (12 days of age), from the axillary tract of individually marked chickens and special care was taken to collect short, newly growing feathers with plenty of pulp. At 14 dpc, 6 birds per isolator (without wing tags), 24 birds in total, were sacrificed, removed from the isolators and examined post mortem for gross MD tumour lesions, thymic and bursal atrophy. Spleens were collected in sterile 1.5 ml Eppendorff tubes and stored at -20 ºC for quantitative real-time PCR (qPCR) analysis.

Chickens were maintained and treated according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 2004, the NSW Animal Research Act 1985 and the NSW Animal Research Regulation 1995, and the experiment was approved by the UNE Animal Ethics Committee (AEC No. UNE 05/076).

Chickens were reared in positive pressure isolators at the UNE isolator facility as described in Chapter 4.

5.2.3 Scoring of MD lesions

Chickens were reared through to 56 dpc when all survivors were sacrificed. All chickens that died during the experiment (prior to 56 dpc) were recorded and subject to the post mortem procedure as described in Chapter 4 which scored for gross MD lesions according to the MD severity scoring (MDSS) system as reported by Islam et al. (2006b) and shown in Table 5.1.

Table 5.1: MD severity score (MDSS). Individual chickens were scored for MD severity from 0 to 2 on the following scale (Islam et al., 2006b).

Score	Description
0	Absence of visible MD tumours
1	MD tumour(s) detected following sacrifice at 56 dpc
2	MD tumour(s) detected following mortality prior to 56 dpc

5.2.4 Feather sample processing for qPCR

Feathers from each individual bird were collected inside the respective isolators into individual sealable (ziplock) plastic bags. Great care was taken to minimize contamination of samples but as they were collected in a contaminated environment it is unlikely that contamination could be completely eliminated. The feather samples were stored at -20 ºC until required for assay.

Prior to DNA extraction, approximately 1 cm of the proximal shaft (the feather tip) was finely chopped to approximately 3 mm lengths using a sterile scalpel blade and transferred into a sterile 1.5 ml Eppendorff tube. DNA extraction for feather tip material, spleen and dust as well as the set up of qPCR assays which were performed on the samples are described in Chapter 4. In order to overcome the inhibitory effect of melanin which is present in feathers of coloured chickens, 10 ug of bovine serum albumin (BSA) was added to the qPCR reaction mix as recommended by Baigent et al. (2005a).

Feathers of 38 of the total of 48 chickens were analysed for MDV1. All feather samples from chickens with MDSS 2 or 1 were analysed as well as 10 randomly chosen samples from chickens with MDSS 0 (Table 5.2).

All samples were randomised across assays to minimise individual assay effects, and amplified in duplicate. In total, 10 qPCR runs were performed for MDV1 and

5 runs for HVT. Mean intra-assay co-efficient of variation (CV) for all qPCR runs were 0.57±0.003 % for MDV1 and 1.67±0.02 % for HVT based on C_t values. The CV based on VCN was 10.16±0.07 % for the MDV1, and 31.94±0.3 % for HVT, assays respectively.

The mean inter-assay CV for all MDV1 qPCR runs based on C_t values was 13.1±0.65 % and based on VCN was 49.18±0.02 %. For HVT, the mean inter-assay CV based on C_t values was 8.79±0.27 % and based on VCN was 77.73±0.59 %.

Table 5.2: Feather tip samples of the treatment combinations which were analysed for MDV1 and HVT by qPCR, grouped by MDSS.

Vaccine	Challenge	Number of birds analysed by qPCR grouped by MDSS			Total
		0	1	2	
Unvaccinated	MPF57	4	4	2	10
Unvaccinated	02LAR	1	4	7	12
HVT	MPF57	4	4	0*	8
HVT	02LAR	1	6	1	8

*none of the chickens in this treatment group had MDSS 2.

5.2.5 Statistical analysis

All data were analysed using JMP™ v 5.1 statistical software (SAS Institute Inc., NC, USA). Data for MDV1 viral load in feathers, spleen and dust from qPCR assays were $\log_{10}$ transformed [$\log_{10}$ (x+1)] to meet the assumptions of analysis of variance. For repeated measures of MDV1 in feather tips a repeated measures REML mixed model was fitted with the individual chicken fitted as a random factor and vaccination (unvaccinated or HVT), challenge (MPF57 or 02LAR) and days post challenge (dpc, 7, 14, 21, 28, 35, 42, 49, 56) as fixed effects. Non-significant interactions between effects were sequentially removed leaving only those with a P value of approximately 0.1 or lower. Repeated measures of MDV1 in dander used a similar model with the isolator rather than the chicken fitted as the random variable. MDV1 in spleen was only measured at 14dpc and so was analysed with a simple model fitting the effects of vaccination, challenge and their interaction.

To determine whether MDV1 load in feathers was influenced by final MD outcome, the data were also analysed in a similar REML model fitting the fixed effects of MDSS, dpc and their interactions.

To determine treatment effects on categorical response variables such as mortality, MD presence or absence and MDSS were analysed using ordinal or nominal logistic regression fitting the treatment effects and their interactions. Mortality data were also subject to survival analysis using the product-limit (Kaplan-Meier) method.

As the HVT data included many zeroes, no transformation was able to normalize the data to ensure that they met the basic assumptions of analysis of variance. Therefore, for the HVT analysis, data were coded negative or positive for HVT and an ordinal logistic model fitted as described above.

Viral load data are presented throughout as $\log_{10}$ transformed least squares means and standard errors. Where more than 2 means were involved the significance of differences between them was determined using Tukey's Honestly Significant Differences (HSD) test. Tukey's HSD does not require a significant F test for the main effect or interaction as a prerequisite for detecting significant differences amongst the means. A significance level of $P \leq 0.05$ is used throughout.

5.3 Results

5.3.1 Application of treatments

Chickens were either vaccinated with HVT or unvaccinated and challenged with two MDV1 isolates. Individual chickens could be tracked reliably throughout the experiment. At 7 dpc, qPCR analysis of feather tips revealed that 36/38 individually marked birds were positive for MDV1. At 14 dpc, all 38 birds were confirmed to be positive for MDV1 by qPCR of feather tip samples and thus, MDV1 treatments were successful (Table 5.3).

Out of 16 vaccinated birds analysed for HVT by qPCR of feather tips, 8 were positive for HVT at 7 dpc and 13 at 14 dpc (Table 5.3). Only one bird was positive

for HVT at 56 dpc, and two birds remained negative for HVT at all measurements throughout the experiment. In one of these birds, HVT vaccination had obviously failed as it was the only chicken with MDSS 2 in the HVT treatment groups and was therefore excluded from all analyses.

Two unvaccinated and unchallenged control isolators were confirmed to be free of MDV1 by qPCR analysis of isolator exhaust dander at 56 dpc, thus demonstrating freedom of cross contamination in the experiment.

Table 5.3: Success of vaccination and challenge treatments determined by qPCR analysis of feather tips at 7 and 14 dpc.

qPCR Assay	qPCR positive chickens at 7 dpc	qPCR positive chickens at 14 dpc
MDV1	36/38 (94.7%)	38/38 (100%)
HVT	8/16 (50%)	13/16 (81.3%)

There were 3 samples assayed for MDV1 where the qPCR reaction might have been inhibited. Two of these samples were from separate unvaccinated birds challenged with 02LAR and occurred at 7 (wing tag ID 3302) and 35 dpc respectively (wing tag ID 3637). The third sample was from a HVT vaccinated bird challenged with MPF57 and occurred at 7 dpc (wing tag ID 3877). The extracted DNA of these samples was much browner compared to other DNA samples which may be a result of high amounts of melanin. The amount of bovine serum albumin (BSA) added may not have been sufficient to overcome the inhibitory effect of melanin in these samples. These samples were regarded as outliers in the analyses and, although they did not have any major effect on the results, were excluded from all analyses of MDV and HVT load in feathers.

5.3.2 Mortality/ Survival

Two of the 48 chickens died due to causes unrelated to MD and therefore, the population at risk of death due to MD included 46 chickens. All 10 chickens that died during the experiment (21.7 %) showed gross MD lesions (Table 5.4). Mortality by treatment is summarised in Table 5.4.

Table 5.4: Mortality in the experiment grouped by vaccination and challenge treatment.

Vaccination	Virus	n	Total mortality n (%)	Mortality with MD lesions n (%)
Unvaccinated	MPF57	12	2 (16.6%)	2 (16.6%)
Unvaccinated	02 LAR	12	7 (58.3%)	7 (58.3%)
HVT	MPF57	12	0 (0%)	0 (0%)
HVT	02 LAR	10	1 (10%)	1 (10%)
All	All	46	10 (21.7%)	10 (21.7%)

Survival analysis revealed significant differences between the patterns of mortality of chickens challenged with MPF57 compared to 02LAR (P<0.05, Figure 5.1, left panel) as well as unvaccinated chickens compared to chickens vaccinated with HVT (Figure 5.1, right panel).

Chickens challenged with 02LAR showed significantly earlier and higher mortality compared to chickens challenged with MPF57 and the same pattern could be observed in unvaccinated chickens compared to HVT vaccinated chickens.

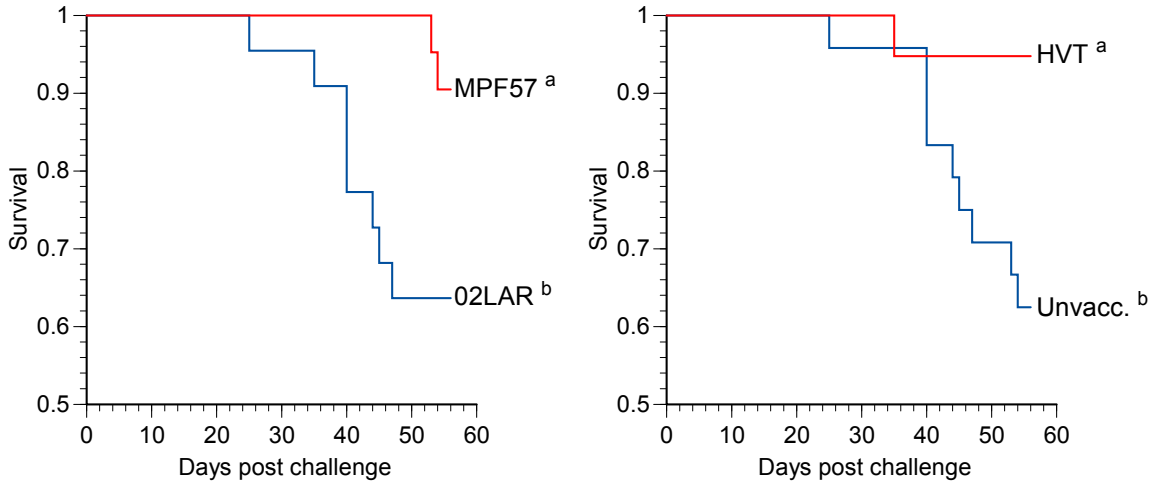

Figure 5.1: Chicken survival grouped by challenge virus (left panel) and vaccination (right panel). Treatments not sharing a common letter in the superscript differ significantly (P<0.05).

5.3.3 Incidence of MD and protective index

Logistic regression analysis for the proportion of chickens with gross MD lesions (% MD) revealed a significant effect of both challenge virus (P=0.019) and

vaccination (P=0.059) with a non significant interaction between these effects (P=0.35, Table 5.5).

Challenge isolate 02LAR induced a significantly higher incidence of MD overall (75 %) than MPF57 (41.6 %). Vaccination with HVT significantly reduced the incidence of MD from 70.8 % in unvaccinated chickens, to 45.8 % in vaccinated chickens.

The protective index (PI) provided by HVT vaccination was low and was similar for the two isolates (36.4 % for 02LAR and 33.4% for MPF57, Table 5.5). The calculation of PI is described in Chapter 4.

Table 5.5: MD incidence (% with gross MD lesions) and protective index.

Vaccination	Virus	MD-Yes	MD-No	MD incidence (%)	PI (%)
Unvaccinated	MPF57	6	6	50	
Unvaccinated	02 LAR	11	1	91.6	
HVT	MPF57	4	8	33.3	33.4
HVT	02 LAR	7	5	58.3	36.4
All	All	28	20		

5.3.4 Effect of vaccination and challenge virus on MDSS

As would be expected given the results above, ordinal logistic regression analysis revealed a significant effect of vaccination (P=0.03) and challenge virus (P=0.009), but not their interaction (P=0.34). Unvaccinated chickens regardless of challenge virus had a significantly higher MDSS than HVT vaccinated chickens (P=0.01). Both unvaccinated and HVT vaccinated chickens which were challenged with the isolate 02LAR had significantly higher MDSS than chickens challenged with MPF57 (P=0.04). The data are summarised in Table 5.6.

Table 5.6: Number of birds in the MDSS categories grouped by vaccination and challenge treatment.

Vaccination	Virus	Total n	MDSS 0 n (%)	MDSS 1 n (%)	MDSS 2 n (%)
unvaccinated	MPF57	10	4 (40%)	4 (40%)	2 (20%)
unvaccinated	02 LAR	12	1 (8.3%)	4 (33.3%)	7 (58.3%)
HVT	MPF57	8	4 (50%)	4 (50%)	0 (0%)
HVT	02 LAR	7	1 (12.5%)	6 (75%)	1 (12.5%)

5.3.5 MDV1 load in feather tips

5.3.5.1 Descriptive overview: Individual chicken profiles grouped by MDSS

Individual chicken profiles grouped by MDSS and vaccination treatment are shown in Figure 5.2. In general, there was a sharp increase of the MDV1 load in feathers until 21 dpc and this was seen in both unvaccinated and vaccinated chickens. In chickens that went on to get MD (MDSS 1 and 2), the patterns were much more erratic, with big reductions of MDV1 load in some samples, especially between days 28-42.

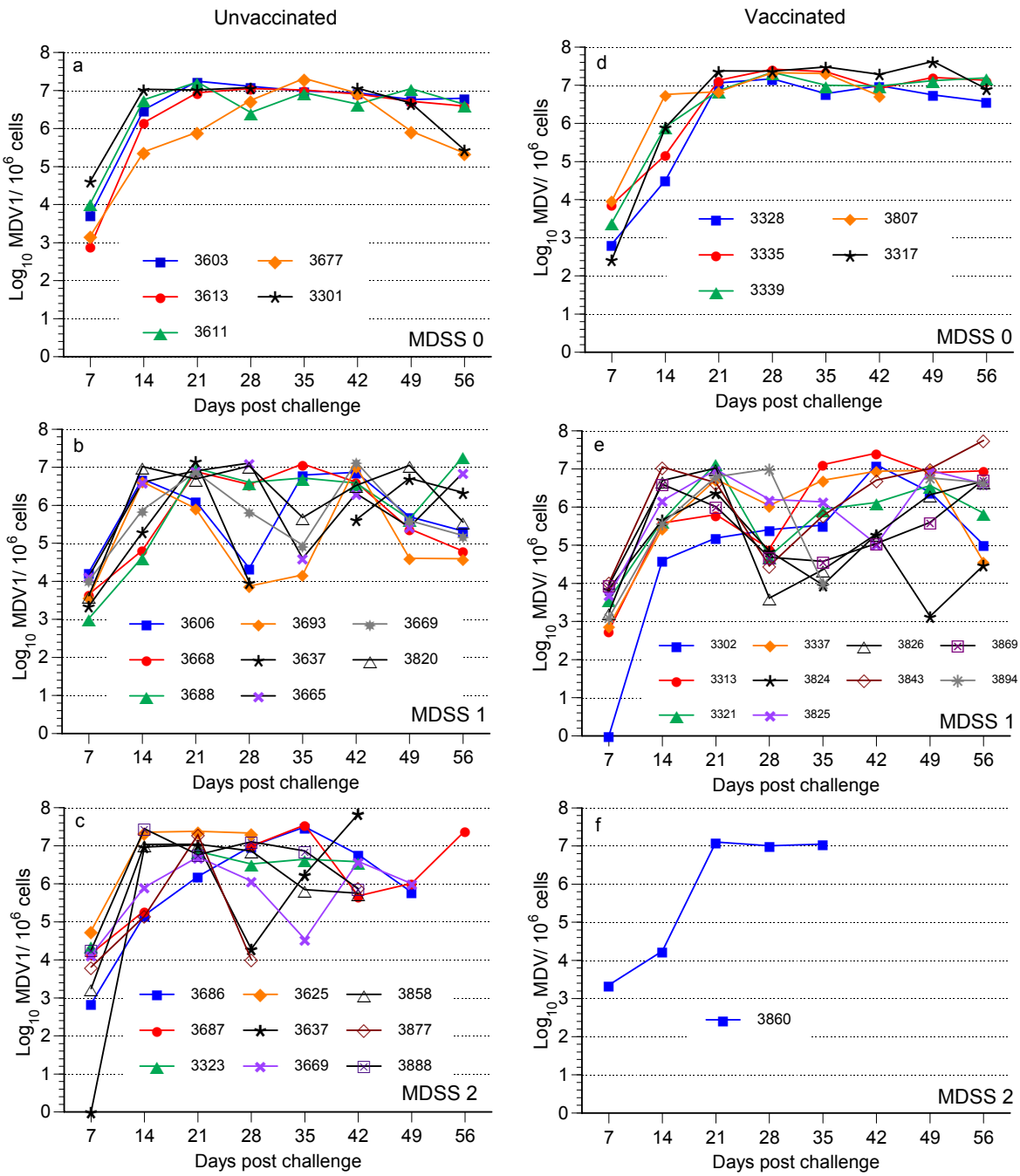

Figure 5.2: Individual profiles of MDV1 load in feather tips over time, grouped by MDSS score. Left panels represent unvaccinated chickens, right panels HVT vaccinated chickens. (a, d) chickens with no MD lesions (MDSS 0); (b, e) chickens with MD lesions at 56 dpc after euthanasia (MDSS 1); (c, f) Chickens dying with MD lesions before 56 dpc (MDSS 2).

5.3.5.2 Descriptive overview: Mean MDV1 and HVT load in individual unvaccinated and HVT vaccinated chickens by MDSS

The mean MDV1 load/ 10^6 cells in feather tips for each individual chicken grouped by vaccination treatment and aligned according to ascending MDV1 load is shown in Figure 5.3.

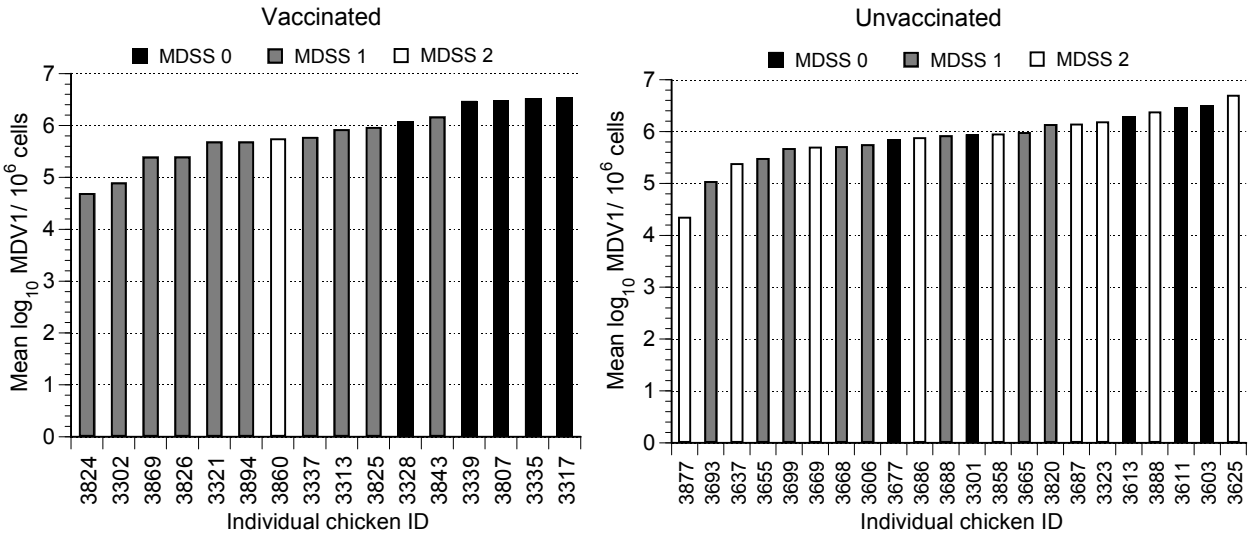

Figure 5.3: Mean MDV1 load in feathers of individual chickens (log_{10} scale) in ascending order, by vaccination treatment (Left panel HVT vaccinated, Right panel unvaccinated) and MDSS.

The mean HVT load together with MDV1 load/ 10^6 cells in feather tips for each individual chicken is shown in Figure 5.4.

Chicken 3894 in Figure 5.4 forms an outlier in the group of chickens with MDSS 1 as it groups strongly with chickens of MDSS score 0. This was the only chicken with MDSS score 1 showing a high level of HVT in feathers. As well, chicken 3860 was the only HVT vaccinated chicken with MDSS 2 and forms an outlier in the group of chickens with MDSS 1.

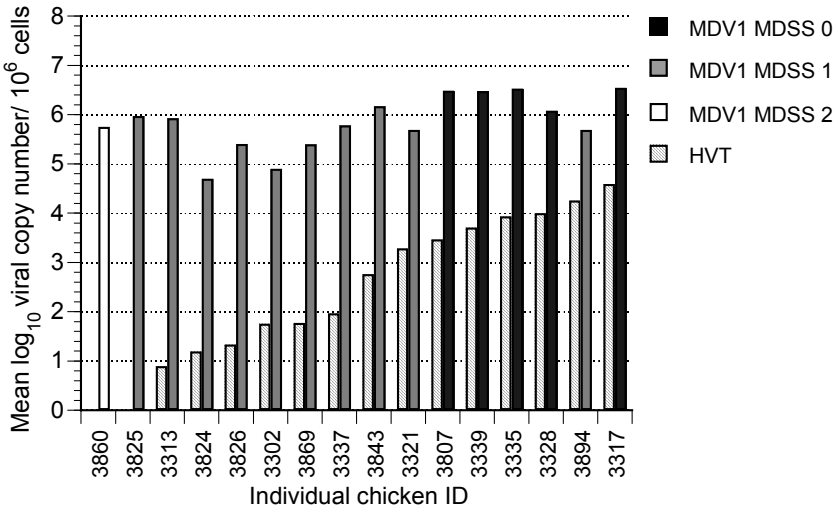

Figure 5.4: Mean MDV1 and HVT load in feathers (log_{10} scale) and MDSS of individual vaccinated chickens sorted by HVT load. There was a strong trend towards higher HVT load in FT in chickens not exhibiting any gross MD lesions (MDSS 0).

5.3.5.3 Effect of challenge virus, vaccination and days post challenge

The MDV1 load in feathers varied significantly due to the effect of days post challenge ($P<0.0001$, Figure 5.5) and its interaction with challenge virus ($P<0.0001$, Figure 5.6, left panel) but not the main effects of challenge virus ($P=0.12$), vaccination ($P= 0.54$, Figure 5.6, right panel) or their interaction.

There was a marked increase of MDV1 in feathers from $10^{3.6}$ MDV1 copy numbers per 10^6 cells to $10^{6.7}$ MDV1 copy numbers per 10^6 cells between 7 and 21 dpc, followed by a decrease after 21 dpc to 35 dpc after which the load of MDV1 in feathers plateaued at $10^{6.2}$ MDV1 copy numbers per 10^6 cells (Figure 5.5).

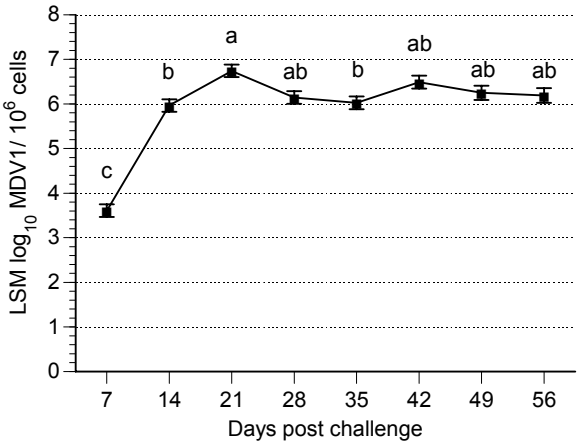

Figure 5.5: Main effect of days post challenge. Least squared means of MDV1 virus load in feather tips (±SEM, log_{10} scale). Means not sharing a common letter differ significantly using Tukey's HSD (P<0.05).

The interaction between days post challenge and challenge virus is shown in Figure 5.6 (left panel). Up to 21 dpc there was significantly higher MDV1 load in feather tips in chickens challenged with 02LAR, but there was a sharp decline (about 1.5 logs) in load between 21 and 35 dpc in chickens challenged with 02LAR but not MPF57 and values were significantly lower than for MPF57 at days 35 and 42. From 49-56 dpc values differed little between the challenge viruses.

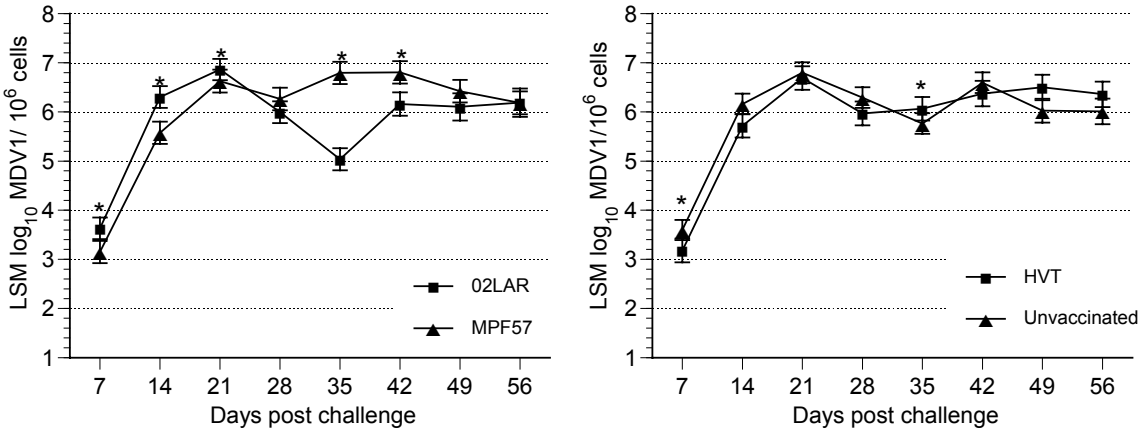

Figure 5.6: (Left panel) Interaction plot of MDV1 virus load in feather tips (LSM±SEM, log_{10} scale) over time for the two challenge viruses. The interaction between challenge virus and days post challenge was highly significant (P<0.0001).

(Right panel) Interaction plot of MDV1 virus load in feather tips (LSM±SEM, log_{10} scale) over time for unvaccinated and HVT vaccinated chickens. The effect of vaccine was not significant (P=0.54). Asterisks mark times when treatments differ using Tukey's HSD (P<0.05).

The non-significant effect of vaccination over time is shown in Figure 5.6 (right panel). Until 28 dpc, the MDV1 load in feathers of HVT vaccinated chickens was up to 5-fold lower than in unvaccinated chickens, however, from 28 dpc onwards, the MDV1 load in HVT vaccinated chickens slowly increased and was eventually 5-fold higher than in unvaccinated chicken at 49 and 56 dpc (Figure 5.6, right panel).

5.3.5.4 Effect of MDSS and days post challenge

The mean MDV1 viral load in feather tips over time varied significantly with days post challenge and MDSS score ($P<0.001$). The interaction between these effects was not significant ($P=0.32$, Figure 5.7).

Chickens not exhibiting any gross MD lesions (MDSS 0) had a significantly higher $\log_{10}$ VCN/10^6 FT cells overall (6.32±0.11; $P<0.05$), than those with MD lesion(s) detected at post-mortem following sacrifice at 56 dpc (MDSS 1) (5.64±0.08) while chickens with MDSS 2 were intermediate (6.03±0.17).

Between 7 and 21 dpc, the amount of MDV1 in feather tips of the chickens assigned to the respective MDSS was not significantly different ($P>0.05$). There was a marked increase from $10^{3.7}$ MDV1 copy numbers per 10^6 cells (mean for all MDSS) at 7 dpc to $10^{6.8}$ MDV1 copy numbers per 10^6 cells at 21 dpc (Figure 5.7). From 28 dpc onwards, the MDV1 load differed significantly between the MDSS classes ($P<0.001$). In chickens assigned to MDSS 0 it plateaued at around $10^{6.9}$ MDV1 copy numbers per 10^6 cells until 49 dpc and decreased to $10^{6.6}$ MDV1 copy numbers per 10^6 cells at 56 dpc.

Chickens assigned to MDSS 1 showed a decrease of MDV1 in feather tips to $10^{5.5}$ MDV1 copy numbers per 10^6 cells at 28 and 35 dpc, before another increase to $10^{6.3}$ MDV1 copy numbers per 10^6 cells at 42 dpc after which the MDV1 amount in feathers was plateauing at around 10^6 MDV1 copy numbers per 10^6 cells.

The MDV1 load in feathers of chickens assigned to MDSS 2 showed a decrease from 21 dpc onwards and were plateauing at around $10^{6.4}$ MDV1 copy numbers per 10^6 cells until 49 dpc before another sharp increase until 56 dpc (Figure 5.7).

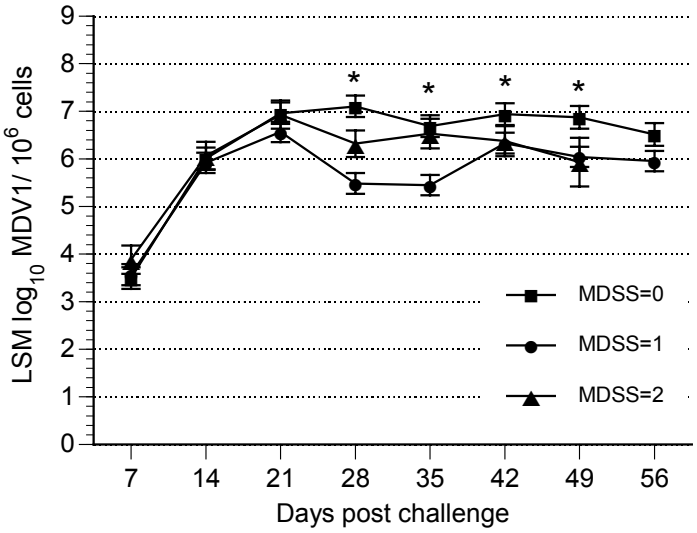

Figure 5.7: Mean (LSM ±SEM) MDV1/ 10^6 cells in feather tips of chickens grouped by MDSS over time (log_{10} scale). The effect of days post challenge and MDSS was highly significant (P<0.001). The interaction between MDSS and days post challenge was not significant (P=0.32).

5.3.6 MDV1 load in spleens at 14 dpc

The MDV1 load (log_{10}VCN/10^6 spleen cells) was significantly affected by vaccination status (P=0.0018) whereas challenge virus had no significant effect (P=0.33) with a non-significant interaction between these effects (P=0.09, Figure 5.8). Overall, the MDV1 load was significantly lower in HVT-vaccinated than unvaccinated chickens (4.9±0.21 versus 6.03±0.21, P=0.05) but this was significant only for the chickens challenged with 02LAR.

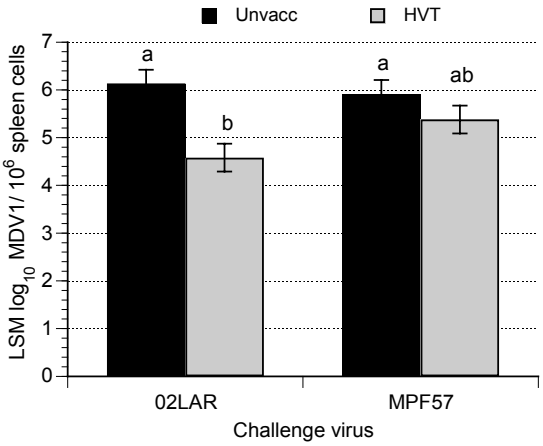

Figure 5.8: Mean MDV1/ 10^6 spleen cells at 14 dpc (LSM ±SEM, log_{10} scale) grouped by challenge virus in unvaccinated and HVT vaccinated chickens. Means not sharing a common letter differ significantly using Tukey's HSD (P<0.05).

5.3.7 MDV1 load in isolator exhaust dander

MDV1 load in the exhaust dust from the 4 isolators (log_{10}VCN/ mg dust) was measured weekly. The effect of days post challenge (P<0.0001) was significant while that of vaccination was very close to significant (P=0.06) and that of challenge virus was not significant (P=0.76). The interaction between challenge virus and days post challenge was not significant (P=0.18, Figure 5.9, left panel) but that between vaccination and days post challenge approached significance (P=0.08, Figure 5.9, right panel) Tukey's HSD test identified significant differences between the treatment groups at different time points as shown in Figure 5.9.

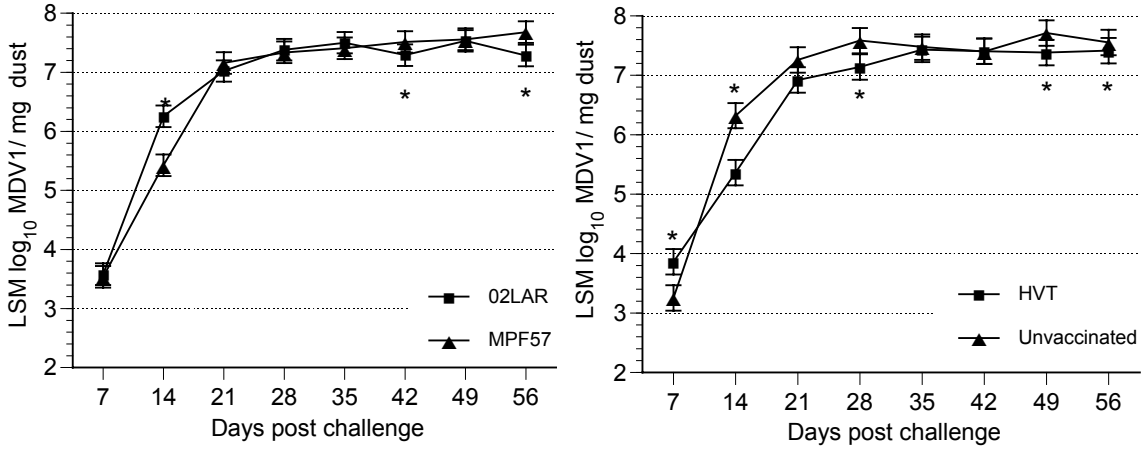

Figure 5.9: Mean MDV1 load in dust (LSM±SEM, log_{10} scale) over time, grouped by challenge isolate (left panel) and vaccination treatment (right panel). Asterisks mark times when treatments differ using Tukey's HSD (P<0.05).

5.3.8 HVT load in feather tips

5.3.8.1 Effect of challenge virus and days post vaccination

The HVT results from qPCR assays contained many negative samples, regardless of dpc. Overall, 73 samples (57.1 %) amplified in the HVT qPCR assay whereas 55 samples (42.9 %) did not amplify. In 23 of these samples; the the C_t value was just marginally lower than the lowest standard in the standard curve (18 viral copies/ reaction which equals 1800 viral copies/ 10^6 cells). These samples were considered false negatives and were subsequently coded as positive and included in the analyses.

The ordinal logistic regression analysis of HVT-positive and negative samples from vaccinated birds revealed a significant effect of challenge virus (P=0.04) but not days post vaccination (dpv) (P=0.59) with a non significant interaction between these effects (P=0.39).

The proportion of chickens challenged with 02LAR which were positive for HVT was significantly lower (0.46) than the proportion of chickens challenged with MPF57 (0.65) which were positive for HVT.

Despite the non-significant interaction with days post challenge there was a consistent trend towards a higher proportion of HVT-positive chickens in the MPF57 challenge treatment between 26 dpv and 54 dpv. Table 5.7 shows the overall percentage of HVT positive and negative samples for each of the challenge viruses.

Table 5.7: Number and percentage of samples with positive/negative HVT qPCR results, grouped by challenge virus.

Challenge virus	**HVT positive n (%)**	**HVT negative n (%)**
02LAR	30 (46.9%)	34 (53.1%)
MPF57	43 (67.2%)	21 (32.8%)
All	73 (57.1%)	55 (42.9%)

Figure 5.10 shows the mean HVT load in feathers over time for all samples and positive samples only. The fragmentary data resulted in large standard errors. After a marked decrease in the positive samples from $10^{3.8}$ to $10^{4.5}$ HVT copy numbers/ 10^6 cells between 12 and 19 dpv, there was a marked decrease to $10^{2.1}$ HVT copy numbers/ 10^6 cells up to 33 dpv. After 33 dpv until 47 dpv, the HVT viral load varied between $10^{2.1}$-$10^{2.7}$ HVT copy numbers/ 10^6 cells. From then onwards, HVT load plateaued until 61 dpv at $10^{2.8}$ HVT copy numbers/ 10^6 cells (Figure 5.10).

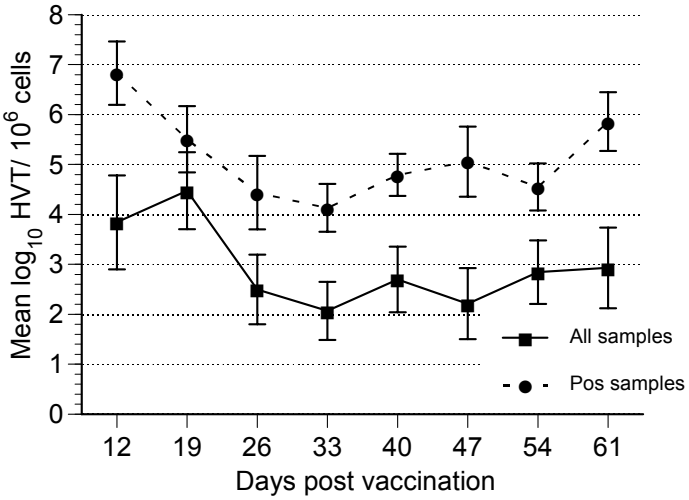

Figure 5.10: Plot of mean HVT virus load in feather tips (±SE, log_{10} scale) over time. The solid line includes all chickens (many zero values) while the dashed line includes only chickens positive for HVT.

5.3.8.2 Effect of MDSS and days post vaccination

The ordinal logistic regression analysis for the proportions of HVT vaccinated chickens positive for HVT in FT revealed that the effect of MDSS approached significance (P=0.08) while the effect of days post vaccination was not significant (P=0.43), but with an interaction between these effects which approached significance (P=0.06, Figure 5.11). There was a strong trend towards higher HVT load in feather tips of chickens with MDSS 0 except at 33 dpv (Figure 5.11).

There was only one chicken (wing tag ID 3860) with MDSS 2 which was only positive for HVT at 19 and 33 dpv.

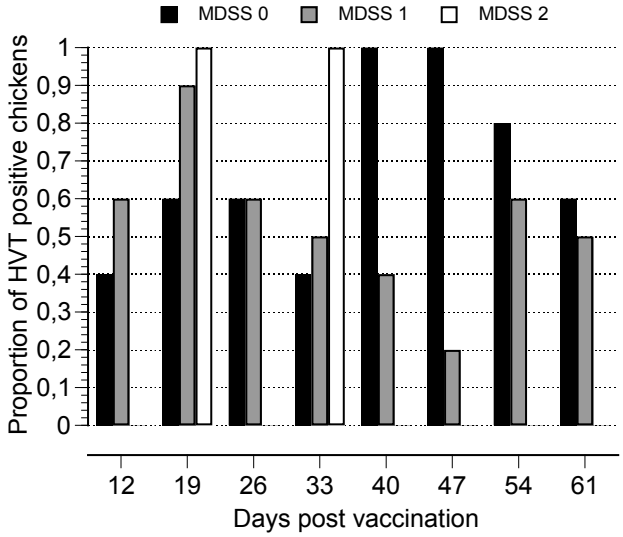

Figure 5.11: Proportion of chickens grouped by MDSS score that were positive for HVT at each time point.

Using a regression approach, there was a non significant negative association between MDSS and mean $\log_{10}$ HVT/ 10^6 cells (R^2= 0.58, P=0.0006, Figure 5.12, left panel). However, there was a trend towards higher HVT load in feathers of chickens given a MDSS 0.

5.3.8.3 Association between MDV1 and HVT load in vaccinated chickens

There was no relationship between MDV1 and HVT load in feather tips of individual chickens, either overall (R^2=0.0088, P=0.18), or at any individual time point (Figure 5.12, right panel).

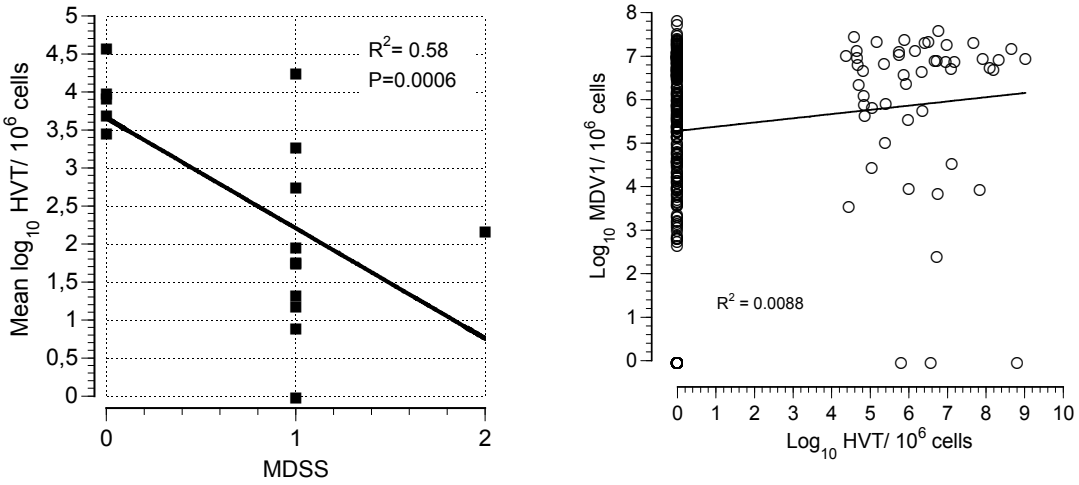

Figure 5.12: (Left panel) Association between MDSS and mean $\log_{10}$ HVT load/ 10^6 cells in feathers of individual chickens. Each data point represents the overall mean HVT value for one individual chicken.

(Right panel) Relationship between MDV1 and HVT copy number at various days post vaccination. Each data point represents one chicken at a specific sampling time, all sampling times are included. The line is a linear regression.

5.3.9 Associations between MDV1 load in feather, dust and spleen and MD outcome on a group (isolator) mean basis

The power of this aspect of the study to detect real effects was comparatively low as it is based upon average values for 4 isolators rather than individual chicken measurements. Table 5.8 shows a matrix of correlations between the different variables. Of most interest, the MDV1 load in dust at 14 was significantly positively associated with subsequent MD ($P<0.05$) whereas MDV1 in feather tips at 14 dpc also had a positive association which approached significance ($P<0.05$).The MDV1 load in spleen at 14 dpc was not significantly correlated with subsequent MD (Table 5.8).

Table 5.8: Matrix of coefficients of correlation amongst isolator mean values for MD incidence at 56 dpc (%MD) and correlates of spleen at 14 dpc.

Variable	**MDV1 in spleen (VCN/10^6 cells)**	**MDV1 in feather tips (VCN/10^6 cells)**								**MDV1 in isolator exhaust dust (VCN/10^6 cells)**								**%MD**
dpc	**14**	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56**	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56**	**56**
spleen 14dpc	1	-0.57	-0.20	0.32	0.86	0.10	0.83	0.78	-0.61	-0.48	-0.33	-0.49	-0.16	-0.32	-0.09	0.02	0.90	-0.03
feather 7 dpc	-0.57	1	0.88	0.43	-0.37	-0.65	-0.66	-0.92	-0.26	-0.33	**0.96**	0.53	0.74	0.89	-0.42	0.59	-0.67	0.84
feather 14 dpc	-0.20	0.88	1	0.81	-0.20	-0.91	-0.55	-0.61	-0.45	-0.43	0.92	0.12	0.57	**0.99**	-0.77	0.44	-0.49	0.92
feather 21 dpc	0.32	0.43	0.81	1	0.10	-0.90	-0.20	-0.04	-0.56	-0.43	0.56	-0.40	0.19	0.77	-0.93	0.14	-0.08	0.72
feather 28 dpc	0.86	-0.37	-0.20	0.10	1	0.32	0.93	0.48	-0.78	-0.75	-0.12	0.01	0.26	-0.35	0.24	0.45	0.94	0.13
feather 35 dpc	0.10	-0.65	-0.91	-0.90	0.32	1	0.60	0.33	0.25	0.15	-0.67	0.29	-0.18	-0.92	0.96	-0.04	0.50	-0.70
feather 42 dpc	0.83	-0.66	-0.55	-0.20	0.93	0.60	1	0.66	-0.49	-0.47	-0.46	-0.07	-0.01	-0.67	0.47	0.20	**0.99**	-0.24
feather 49 dpc	0.78	-0.92	-0.61	-0.04	0.48	0.33	0.66	1	0.02	0.16	-0.81	-0.76	-0.72	-0.65	0.06	-0.57	0.72	-0.60
feather 56 dpc	-0.61	-0.26	-0.45	-0.56	-0.78	0.25	-0.49	0.02	1	0.98	-0.51	-0.18	-0.67	-0.31	0.22	-0.75	-0.52	-0.72
dust 7 dpc	-0.48	-0.33	-0.43	-0.43	-0.75	0.15	-0.47	0.16	**0.98**	1	-0.57	-0.37	-0.79	-0.29	0.08	-0.87	-0.47	-0.74
dust 14 dpc	-0.33	**0.96**	0.92	0.56	-0.12	-0.67	-0.46	-0.81	-0.51	-0.57	1	0.50	0.83	0.89	-0.46	0.72	-0.46	**0.95**
dust 21 dpc	-0.49	0.53	0.12	-0.40	0.01	0.29	-0.07	-0.76	-0.18	-0.37	0.50	1	0.80	0.10	0.53	0.78	-0.19	0.35
dust 28 dpc	-0.16	0.74	0.57	0.19	0.26	-0.18	-0.01	-0.72	-0.67	-0.79	0.83	0.80	1	0.50	0.05	**0.98**	-0.07	0.81
dust 35 dpc	-0.32	0.89	**0.99**	0.77	-0.35	-0.92	-0.67	-0.65	-0.31	-0.29	0.89	0.10	0.50	1	-0.78	0.34	-0.61	0.86
dust 42 dpc	-0.09	-0.42	-0.77	-0.93	0.24	**0.96**	0.47	0.06	0.22	0.08	-0.46	0.53	0.05	-0.78	1	0.15	0.35	-0.54
dust 49 dpc	0.02	0.59	0.44	0.14	0.45	-0.04	0.20	-0.57	-0.75	-0.87	0.72	0.78	**0.98**	0.34	0.15	1	0.14	0.74
dust 56 dpc	0.90	-0.67	-0.49	-0.08	0.94	0.50	**0.99**	0.72	-0.52	-0.47	-0.46	-0.19	-0.07	-0.61	0.35	0.14	1	-0.21
% MD 56 dpc	-0.03	0.84	0.92	0.72	0.13	-0.70	-0.24	-0.60	-0.72	-0.74	0.95	0.35	0.81	0.86	-0.54	0.74	-0.21	1

*Critical values of r (two tailed) for df=2 are 0.95 and 0.99 for P = 0.05 and 0.01 respectively.
Values with P<0.05 are bolded, P<0.01, bolded and undelined.

5.4. Discussion

A major finding of this study was that the MDV1 load in feather tips was significantly reduced (P<0.001) between 28-42 dpc in chickens which subsequently developed MD. This was an unexpected finding. However, a possible explanation is that as the chickens sicken due to MDV infection, there might be changes in the metabolic activity of the skin. This may have a reduction of MDV replication and release from FT as a consequence. Indeed, feathers from sick chickens which died with MD lesions before the termination of the experiment could be more easily removed than feathers from chickens which did not show MD lesions at 56 dpc.

Hypothesis one was that vaccination with HVT will reduce the incidence of MD and will reduce MDV1 load in feather tip (FT), spleen and dander. Vaccination with HVT did significantly reduce the incidence of MD, regardless of the challenge isolate. This was expected as it is the purpose of vaccinating chickens and has been observed in dozens of studies. However HVT only provided limited protection against challenge of around 35 %, indicating that the challenge viruses used had evolved significant resistance to the effects of this vaccine.

Overall, vaccination with HVT did not significantly reduce the MDV1 load in FT of layers although there was a trend towards an up to 3-fold reduction of the MDV1 load in FT until 28 dpc, but not thereafter. This differs from the results of an earlier similar study in broilers (Islam et al., 2008b) In that study, vaccination with HVT consistently reduced the MDV1 load in FT throughout the experiment. Previous studies have shown that vaccine efficacy is influenced by the genetic background of the chicken recipient (Bacon and Witter, 1993; 1994). The decreased efficacy of HVT in terms of reduction of viral replication of MDV1 in feathers of layers may therefore be due to genotype differences between broilers and layers and the challenge isolate.

In spleens at 14 dpc, vaccination with HVT reduced the overall MDV1 load by more than 10-fold which is in broad agreement with similar earlier studies in broilers (Islam, 2006; Islam et al., 2005a, 2007; Islam et al., 2008a).

In dander of the present study, there was a trend towards lower MDV1 load in vaccinated chickens but this was dependant upon time after challenge. At 14 dpc there was significantly lower MDV1 load in vaccinated ($10^{5.3}$ VCN per 10^6 cells) than unvaccinated ($10^{6.3}$ VCN per 10^6 cells) chickens. The reduction of MDV1 load in dander in vaccinated chickens is in agreement with the similar study in broilers (Islam and Walkden-Brown, 2007). In both studies, the most marked reduction of MDV1 load in dander due to vaccination was again present at 14 dpc. As well, an earlier study has reported similar effects of vaccination on the MDV1 load in feather dander (Islam et al., 2005b).

In summary, hypothesis 1 is broadly accepted as the MDV1 load in all tissues was reduced in HVT vaccinated chickens as expected and shown in several earlier studies. The unexpected finding was that in the present experiment, the MDV1 load in FT of HVT vaccinated chickens was only reduced until 28 dpc, but not thereafter, and the magnitude of the effect in FT was small.

Hypothesis two was that MDV isolates MPF57 and 02LAR will differ in the level of MD they induce and the level of MDV1 load in FT, spleen and dander. There was a significant difference in the MD incidence between the two challenge viruses. The challenge virus 02LAR (putatively vv) induced significantly more MD compared to MPF57 (putatively v) (P=0.02) in both unvaccinated and vaccinated chickens. This in agreement to what has been observed in the wider pathotyping study (Chapter 4) and in the similar study in broiler chickens (Walkden-Brown et al., 2007a, 2007b).

The MDV1 level in FT was significantly higher in chickens challenged with 02LAR from 7-21 dpc, but MDV load then fell sharply and was significantly lower than in chickens challenged with MPF57 at 35 and 42 dpc. The fall in MDV load of the more virulent isolate during the period when chickens were developing MD was unexpected. Although there are reports that higher MDV1 replication rates in circulating or tissue-based lymphocytes are associated with increased virulence of MDV1 (Witter, 1991; Rosenberger, 1995; Calnek et al., 1998; Yunis et al., 2004), the situation regarding virus replication in FFE is less clear. Nazerian and Witter (1970), using FFE, found positive correlations between virulence, contagiousness and replication of virulent and attenuated MDV1, and HVT viruses. In contrast, Malkinson et al. (1989) detected quantitative differences in

feather tips of several Israelian MDV isolates, but these differences did not reflect their ability to induce MD and associated mortality. Therefore, although in the present study there was a significant trend towards higher MDV loads in feathers of chickens challenged with 02LAR in the early stages after infection with MDV up to 21 dpc, this association is time dependant, and reverses in direction later in the infection cycle making it an unreliable predictor of the virulence of an isolate.

The MDV1 load in spleens at 14 dpc did not differ significantly between the two challenge isolates. However, the interaction between challenge virus and vaccination approached significance (P=0.09) with a strong trend towards a more marked effect of vaccination with 02LAR. The lack of difference in viral load in spleen between these two isolates is consistent with what was observed in Chapter 4 and the related earlier study in broilers (Walkden-Brown et al., 2007b). However, when isolates vary more in virulence, differences in load in spleen in favour of the more virulent isolate become apparent (eg. 04CRE in Chapter 4; Yunis et al., 2004).

The MDV1 load in dander did not differ significantly between the two challenge viruses. There was a trend towards higher MDV1 load with 02LAR which was particularly evident at 14 dpc. However, in the main dust study which involved 20 isolators (Chapter 6), there was a significant and consistently higher MDV1 load in dander from isolators challenged with 02LAR compared to MPF57.

Overall hypothesis 2 is accepted with spleen and dander showing a trend towards higher levels of MD and MDV1 load with 02LAR compared to MPF57 which was expected and broadly in line with their virulence classification in Chapter 4. However, again, this was less clear in FT, where there was a trend towards higher MDV1 levels with 02LAR, but only up to 21 dpc and not thereafter.

Hypothesis three was that chickens which go on to develop MD will have higher levels of MDV1 in FT throughout the experiment, than those which do not go on to develop MD. This hypothesis is rejected in this experiment. The MDV1 load in FT was actually significantly reduced from day 28 onwards in chickens which subsequently developed MD. This unexpected finding was contrary to what was observed in the parallel study in broilers (Islam et al., 2008b) in which MDV1 in FT was higher throughout in chickens going on to develop MD. In another

longitudinal study in broilers, those going on to develop MDV had significantly higher MDV1 load in peripheral blood lymphocytes (PBL) from 14-35 dpc (Islam et al., 2006b). Furthermore, despite using SPF chickens and a different experimental protocol, Malkinson et al. (1989) reported that surviving birds in their study (which would be about equal to MDSS 0 in the present study) shed less MDV from the FFE than those which developed MD. Although the reason for the unexpected result of the present study is unknown, a reduction on skin metabolic activity, including feather production may be involved as chickens develop MD. Broilers, which much greater reserves of muscle and fat, may be less susceptible to this.

Hypothesis four was that chickens which go on to develop MD will have lower levels of HVT in FT throughout, than those which do not go on to develop MD. This hypothesis is supported by the data in the experiment. This negative association between HVT levels and MD was expected and has previously been observed in peripheral blood lymphocytes (PBL) in a previous study in broiler chickens (Islam et al., 2006b). Interestingly, the proportion of chickens challenged with 02LAR which were positive for HVT was significantly lower (0.46) than the proportion of chickens challenged with MPF57 (0.65) which were positive for HVT. The reason for this is unknown; however, it is likely that there is less HVT in 02LAR birds because of the higher virulence of 02LAR compared to MPF57 which might have a negative effect on the replication of HVT.

Hypothesis five was that MDV load in feather tips will be strongly and positively correlated with MDV load in spleen and dander at equivalent time points. The data do not support this as there was little evidence that MDV1 load in FT, spleen and dander being consistently correlated at equivalent time points. Furthermore, while spleen and feather samples represent virus levels at a fixed time-point, dander samples represent the accumulated shedding of virus over the course of the previous week. However, as these analyses were based on measurements from only 4 isolators, the predictive power was comparatively low. Therefore, a larger study should be performed involving more isolators in order to increase the power of the statistical analyses.

Hypothesis six was that MDV1 and HVT viral load measured in FT material of layer chickens will be similar to that observed in other recent studies. The MDV

pattern in FT in the present study was in broad agreement with an earlier study reported by Baigent et al. (2005b) although in their study, the viral load detected by qPCR at 10 dpv was 1.5x 10^6 VCN per 10^6 cells rising to 3.2x10^7 VCN per 10^6 cells at 14 dpv before decreasing to 2.3x10^6 VCN per 10^6 cells at 28 dpv. These levels were up to 10-fold higher compared to the MDV1 levels in FT in present study. However, Baigent et al (2005b) used a different experimental approach and used maternal antibody negative (mab-) specific pathogen free (SPF) chickens investigating the kinetics of the vaccinal MDV1 isolate CVI988 whereas the present study used commercial layer chickens with positive maternal antibody status (mab+) investigating pathogenic MDV1 isolates. Again, as outlined in Chapter 4, this emphasizes the importance of the genetic background of the host and the presence/ absence of maternal antibodies.

The HVT pattern in FT in the present study was in broad agreement to a similar study in Cobb broiler chickens (Islam et al., 2008b). However, the HVT load in the present study was consistently 1 or more logs higher than in the broiler study. In particular, the initial HVT load at 12 dpv in broilers was $10^{2.2}$ VCN per 10^6 compared to $10^{3.8}$ VCN per 10^6 in layers taking into account only positive HVT samples, but the same was the case when all HVT samples (including those which did amplify intermittently) were taken into account. As well, in broilers, there was a second peak of HVT load at 33 dpv (Islam et al., 2008b), which was not as marked in the present study and occurred at 40-47 dpv.

In conclusion, the main finding of the is experiment was that the MDV1 load in FT was significantly reduced, rather than elevated in chickens that go on to develop MD with the reduction occurring after 21 dpc. This unexpected finding is in total contrast to what happens in MDV1 load in PBL (Islam et al., 2006b). It also contrasts with another study in feather tips in broilers in which MDV was consistently higher in chickens that went on to develop MD, and with what has been observed in dust in which increasing MD incidence is associated with higher rather than lower MDV shedding (Chapter 6; Walkden-Brown et al., 2007b). Furthermore, there was no association between the HVT load in FT and protection against MD. This clearly shows that the value of FT as a determinant of MDV status is limited and is inferior compared to spleen or peripheral blood lymphocytes (PBL) which both have been shown previously to be excellent

predictors of MD outcome (Islam et al., 2006b; Walkden-Brown et al., 2006; Walkden-Brown et al., 2007). This finding might be understandable given the separation of the pathogenic cycle of MD based on oncogenic transformation of lymphocytes in tissues such as spleen and PBL, from the replication cycle associated with disease transmission, which is based on fully productive infection of FFE cells. Unlike feathers, lymphoid tissues such as spleen are the primary target of early cytolytic infection (Calnek, 2001; Baigent et al., 1998; Baigent and Davison, 1999) and previous studies have found high loads of MDV in these tissues in the early stages of infection up to 7 dpc which were highly correlated with subsequent MD incidence (Islam, 2006; Walkden-Brown et al., 2006; Walkden-Brown et al., 2007). In contrast to lymphoid tissues, infection of the FFE occurs later when latently infected lymphocytes, particularly from the spleen, circulate into the bloodstream and thence to peripheral sites including feather tissue. Therefore, kinetics of MDV in feathers does not only follow its own replication cycle associated with viral replication and subsequent shedding into the environment, but also, this cycle lags behind the pathogenic cycle seen in lymphoid tissues which may explain why MDV kinetics in feathers is only poorly correlated with subsequent incidence of MD, especially in the early stages of infection. However, the exact mechanisms of MDV replication and shedding in FT during disease development and progression are still unclear and require further research.

Chapter 6

Shedding profile of three pathogenic isolates and two vaccinal strains (HVT and SB-1) of Marek's disease virus in dander from commercial layer chickens

6.1 Introduction

Under natural conditions, chickens become infected with MDV by the airborne route (Beasley et al., 1970; Calnek et al., 1970; Carrozza et al., 1973). The source of MDV is the feather follicle epithelium (FFE), which is the only site where complete virus replication takes place. This occurs in the keratinizing layer of the FFE and virus is shed in the environment either in the cell-associated or cell-free state from the FFE (Calnek et al. 1970; Nazerian and Witter, 1970). Desquamated epithelial cells or dander are the natural infectious source for other chickens as well as for contamination with MDV of the environment (Witter et al. 1968; Carrozza et al., 1973; Gilka and Spencer, 1993).

HVT is commonly used as live vaccine against MD, either alone, in combination with MDV2, or in combination with attenuated MDV1 vaccines such as Rispens/ CVI988 (Witter et al., 1970, 1984, 1987; Calnek et al., 1983; Schat and Calnek, 1978; Lee et al., 1999). However, none of the current vaccines elicit sterilizing immunity and vaccinated chickens can still be co-infected with pathogenic MDV that can replicate, be shed and infect other chickens in the flock (Witter et al., 1984, 1987; Lee et al., 1999; Islam et al., 2001).

It has been reported that there is a relationship between virulence, contagiousness and replication of the virus in the feather follicle (Nazerian and Witter, 1970). According to these authors, virulent isolates of MDV1 are more readily found in the FFE and easily transmitted to other chickens whereas an attenuated MDV1 isolate and the avirulent HVT were not detected in FFE. Subsequently, these latter two isolates spread very poorly to contact chickens (Nazerian and Witter, 1970). However, Islam, et al. (2005a) reported significant

shedding of HVT from day 9 post vaccination onwards in isolator exhaust dander from HVT vaccinated chickens which were challenged with MDV1. Similarly, Baigent et al. (2005b) detected the MDV1 vaccine isolate CVI988/ Rispens at day 7 post vaccination in feather tips of vaccinated chickens and the numbers of MDV1 genome as determined by quantitative real-time PCR (qPCR) were up to 1000-fold higher in feather tips than in other tissues such as spleen, bursa or thymus.

Existing quantitative data for kinetics of MDV shedding from infected chickens are limited (Handberg et al., 2001; Davidson et al., 2002). The first detailed report on the kinetics of shedding of all three MDV serotypes in broiler chickens was published by Islam and Walkden-Brown (2007). The results of their study defined quantitatively the shedding patterns of pathogenic MDV1, HVT and MDV2 in broiler chickens and revealed that challenge with virulent MDV markedly increased the shedding of vaccinal virus. The study also showed that avirulent MDVs such as MDV2 could shed at rates higher than virulent MDV1.

This study, which is similar to that of Islam and Walkden-Brown (2007), was designed to investigate quantitatively the shedding patterns of all three serotypes of MDV in dander of commercial layer chickens with single or mixed infections. In addition, the study aimed to evaluate the suitability of dander as a substitute for other tissues such as feathers or blood for routine sampling in longitudinal experiments involving infection with MDV.

The study involved measurements made during pathotyping experiment MD05-C-PT3 (Chapter 4) supported by Australian Poultry CRC project 03-17, and represented an additional set of measurements made during that experiment.

The objectives of this study were:

1. To quantify in absolute terms (viral copy number) over time the amount of MDV1, MDV2 and HVT present in dander shed from layer chickens infected with one or more of these viruses using real-time PCR.
2. To determine daily dander production of layer chickens from 1 to 9 weeks of age.

3. To use the data from objectives 1 and 2 to determine daily shedding rates of MDV1, HVT and MDV2 over in layer chickens from 1-9 weeks of age.
4. To determine the effects of vaccination with two MD vaccines on MDV1 shedding rates.
5. To determine whether the virulence of the challenge isolate influences the shedding rates of MDV1.
6. To determine the association between MDV1, HVT or MDV2 load in dander and subsequent MD status in groups of chickens.
7. To compare the results in layer chickens with those reported for broiler chickens by Islam and Walkden-Brown (2007).

6.2 Materials and methods

6.2.1 Experimental design

This experiment was designed as a 3x3 factorial experiment with 2 replicates using 18 isolators.

The two experimental factors were:

- Vaccine virus (3 levels) HVT (FC-126, 8000 pfu s.c. in 200 µl), bivalent (containing 63 % HVT FC-126 and 37 % cell-associated MDV2 isolate SB-1 (Vaxsafe SBH vaccine, Batch no. SBH4101, 8000 pfu combined dose per bird, Bioproperties, Ringwood, Vic) and unvaccinated (200 µl diluent) on the day of hatch (day 0).
- Challenge virus (3 levels) MPF57 (reference virus), 02LAR and 04CRE, (500 pfu i.a. in 200 µl on day 5 of age).

Two additional isolators were unvaccinated and unchallenged. These were given diluent only and served as control groups. The experiment started on the day of hatch (day 0, 06/06/05) and was terminated at 61 days of age (56 dpc, 09/08/05). Chickens were vaccinated at hatch (day 0) and challenged with MDV at day 5 of age.

Detailed information about the vaccine and challenge material and vaccination and challenge procedure is described in Chapter 4.

6.2.2 Experimental chickens

A total of 535 day old unvaccinated commercial female ISABROWN chickens from parent stock vaccinated with Rispens/CVI988 were placed in positive pressure isolators at the UNE isolator facility as described in Chapter 4. A total of 54 chickens per treatment combination were used initially, 27 per isolator, and kept until 56 days post challenge (dpc), 61 days old.

Chickens were maintained and treated according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 2004, the NSW Animal Research Act 1985 and the NSW Animal Research Regulation 1995, and the experiment was approved by the UNE Animal Ethics Committee (AEC No. UNE 05/076). The experiment started on 06/06/05 and ended on 09/08/05.

6.2.3 Collection and processing of dander for qPCR analysis

In order to determine the MDV load in dander, samples were collected weekly from the dust deposits at the 90º bend in the exhaust air outlet of each isolator. To do this, the exhaust air outlet valve had to be closed briefly. Approximately 1 g of dander was scraped into a sterile 1.5 ml Eppendorff tube using a disposable wooden spatula. After each collection, the valve was thoroughly cleaned and disinfected so that the next collection represented the past 7 days of dander accumulation. Dander collection commenced at 7 dpc (12 days after vaccination) and terminated at 56 dpc. The samples were stored at -20 ºC. DNA was extracted from 5 mg of dander and subsequently analysed by serotype specific qPCR following the methods described in Chapter 4.

All samples were randomised across assay runs to minimise individual assay effects, and amplified in duplicate. In total, 8 qPCR runs were performed for MDV1, 4 runs for HVT and 3 for MDV2. The mean intra-assay co-efficients of variation (CV) for all qPCR runs based on C_t values were 0.31±0.004 % for MDV1, 1.14±0.07 % for HVT and 1.45±0.02 % for MDV2. Those based on VCN

were 6.7±0.07 % for the MDV1, 23.1±0.15 % for HVT and 27.64±0.3 % for MDV2, assays respectively.

The mean inter-assay co-efficients of variation (CV) for all qPCR runs based on C_t values were 6.24±0.4 % for MDV1, 19.82±0.88 % for HVT and 23.71±0.40 % for MDV2. Those based on VCN were 16.21±0.003 % for the MDV1, 83.27±0.02 % for HVT and 73.96±0.75 % for MDV2, assays respectively.

6.2.4 Estimation of daily dander production per chicken

Six isolators were selected randomly and dander production was measured weekly throughout the experiment. Dander was collected by passing all of the exhaust air from an isolator through a tared commercial vacuum cleaner filter bags (Menalux T16B, Electrolux Australia- with a tight fit to the isolator / air outlet) for a defined period, then determining the weight of dander in the bag on a dry matter basis. Prior to attachment, dust bags were dried at 55 ºC overnight and weighed individually before being placed over the air outlet of the selected isolators. During weeks 1-4, the filter bags were applied for a period of 48 hrs and from weeks 5-8 for a period of 24 hrs. After collection, the bags were removed and placed in an incubator at 55 ºC overnight and weighed again. The daily dander production per chicken and day was then calculated by determining the difference in weight of dust bag using the 'before' and 'after' weights and taking into account the total dry weight of collected dander, the duration of the collection period and the number of chickens in the isolator during the collection period.

6.2.5 Statistical analysis

All data were analysed using JMP ™ v 5.1 statistical software (SAS Institute Inc., NC, USA). Data for MDV1, MDV2 and HVT viral load in dander from qPCR assays were $\log_{10}$ transformed (Log_{10} Y +1) to meet the basic assumptions of analysis of variance. The transformed data were then analysed for the relevant treatment effects and their interactions in a repeated measures mixed REML model and non-significant interactions sequentially removed leaving only those with a P value of approximately 0.1 or lower. For the MDV1 model, the effects of vaccination (unvaccinated, HVT and Bivalent), challenge (unchallenged, MPF57,

02LAR and 04CRE), days post challenge (dpc) and their interactions were fixed effects in the model with isolator fitted as a random effect. HVT data were similarly analysed for the effects of vaccination (HVT and Bivalent), challenge (three isolates of MDV1 and unchallenged), dpc, and their interactions. MDV2 data were analysed for effects of challenge treatment (three isolates of MDV1 and unchallenged), dpc and their interactions.

Viral load data are presented throughout as least squares means and standard errors. Significant differences amongst means were determined using Tukey's HSD test. A significance level of $P \leq 0.05$ is used throughout unless indicated otherwise.

6.3 Results

Vaccination and challenge with MDV1 was successful for all treatments as determined by qPCR of spleens at 14 dpc. MDV1 was detected in dander from each weekly sampling from isolators challenged with MDV1. In all of the isolators containing chickens vaccinated with HVT or bivalent vaccine, HVT was detected at each weekly sampling and the same was true for MDV2 in isolators vaccinated with bivalent vaccine.

Faint traces of MDV1 were also detected in dander from the control isolators occasionally despite no evidence of infection of chickens with MDV. Trace levels were detected in both control isolators at 7 and 14 dpc, but these isolators were subsequently negative for MDV from weeks 2-8 apart from a trace in one of the isolators at weeks 6 and 7. This occasional low level contamination probably reflects residual non-infective MDV on the exhaust pipes or minor contamination during collection, as the external environment in which the samples are being collected was not free of MDV. Dust data were corrected by subtracting this trace contamination from all values in the experiment.

6.3.1 MDV1 viral load in dander

The presence of MDV1 in dander was detected from 7 dpc onwards with a mean of $10^{3.8}$ VCN/mg dust at 7 dpc. The level of MDV1 in dander then increased

rapidly up to 21 dpc ($10^{6.4}$ VCN/ mg dust) after which it continued to slowly increase to $10^{6.5}$–$10^{7.2}$ VCN/ mg dust between 42 and 56 dpc (Figure 6.1). The MDV1 load in dust varied significantly due to the effect of vaccine (P<0.0001) and days post challenge (P<0.0001), with no significant interaction between these effects (P= 0.35, Figure 6.1, left panel). The effect of challenge isolate was also highly significant (P<0.0001) with no significant interaction between challenge isolate and days post challenge (P=0.42, Figure 6.1, right panel). Overall, the isolate 04CRE showed a significantly lower (5.69±0.13) viral load level throughout the experiment than the other two challenge isolates 02LAR (6.68±0.14) and MPF57 (6.48±0.13) which were not significantly different (Figure 6.1, right panel).

The interaction between challenge isolate and vaccine was also significant (P=0.005, Figure 6.2) due to the fact that vaccination caused a much greater reduction in MDV1 load in chickens challenged with 04CRE, than the other two challenge isolates. HVT significantly reduced MDV1 load relative to unvaccinated chickens (6.6±0.14) by 4.7 % overall (6.29±0.13) and the bivalent vaccine (5.95±0.13) reducing it significantly further, by 9.8 %.

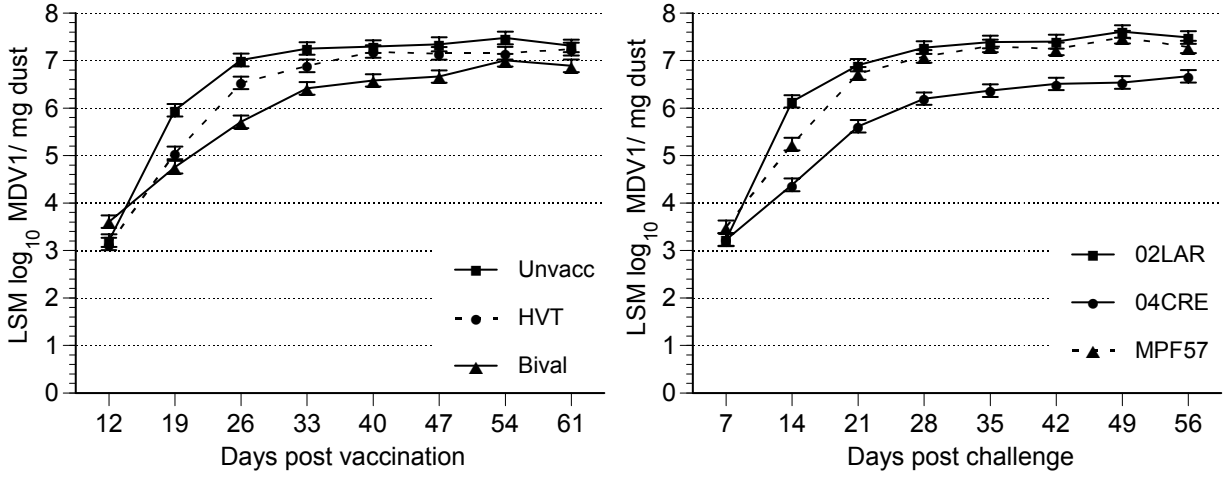

Figure 6.1: MDV1 load in dander (LSM±SEM, $\log_{10}$ scale) over time by vaccine treatment (Left panel) or challenge treatment (Right panel). The effects of vaccine, challenge and day post challenge were significant (P<0.0001).

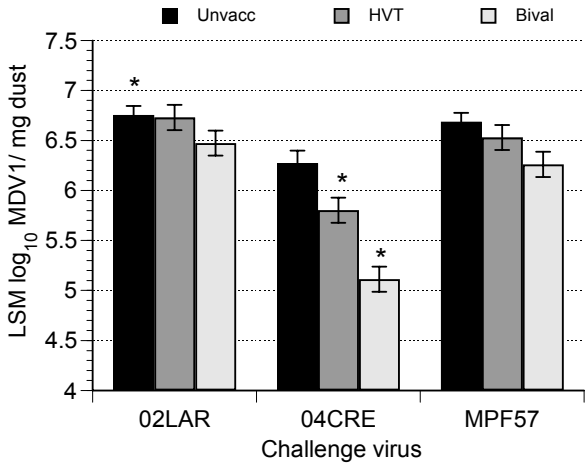

Figure 6.2: Interaction plot of challenge virus and vaccine. The asterisk marks treatments which were significantly different using Tukey's HSD test ($P<0.05$).

6.3.2 HVT load in dander

HVT could be detected from all isolators containing chickens, vaccinated either with HVT alone or bivalent vaccine, from 12 days post vaccination (dpv) onwards. The mean initial HVT load in dander at 12 dpv was $10^{5.2}$ VCN/ mg dust. This increased sharply to a peak at 19 dpv ($10^{6.8}$ VCN/ mg dust) after which it declined to 40 dpv and plateaued at around 10^{6} VCN/ mg dust thereafter (Figure 6.3). There was no significant effect of challenge isolate ($P=0.59$, Figure 6.3, right panel) or vaccine type ($P=0.95$, Figure 6.3 left panel) on the amount of HVT in dander. The interaction between these two effects was also not significant ($P=0.55$). However, there was a significant effect of days post vaccination ($P<0.0001$, Figure 6.3).

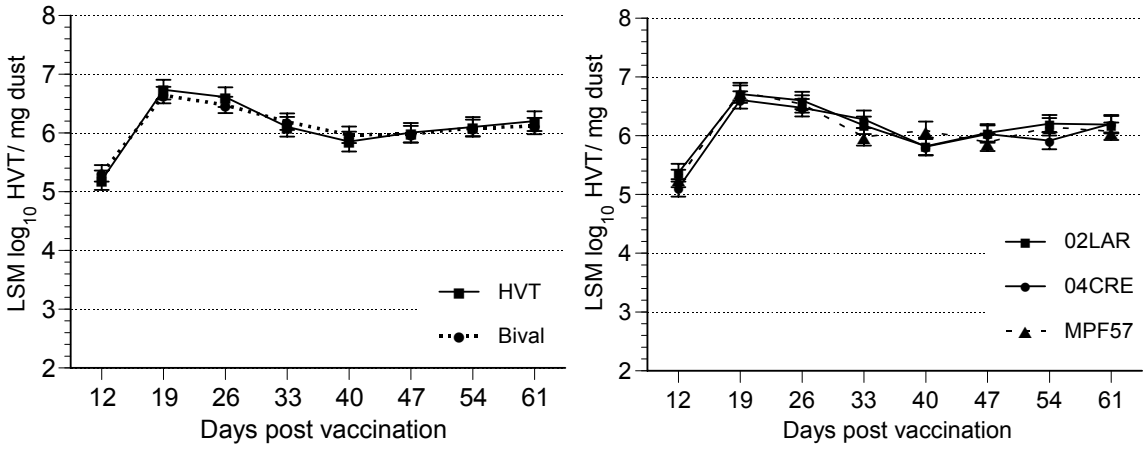

Figure 6.3: HVT load in dander (LSM±SEM, log_{10} scale) over time by vaccine treatment (Left panel) or challenge treatment (right panel).The effects of challenge (P=0.59) and vaccination (P=0.95) were not significant.

6.3.3 MDV2 load in dander

MDV2 was detected in dander from chickens vaccinated with the bivalent vaccine from the first measurement at 12 dpv onwards, with an initial load of $10^{4.7}$ VCN/ mg dust at 12 dpv. It then increased sharply to a peak at 19 dpv ($10^{7.4}$ VCN/ mg dust) after which the MDV2 levels declined slightly to 40 dpv before it plateaued at around 10^{7} VCN/ mg dust (Figure 6.4). There was a significant effect of days post vaccination (P<0.0001, Figure 6.4) but not challenge isolate (P=0.64) with no significant interaction between these (P=0.63).

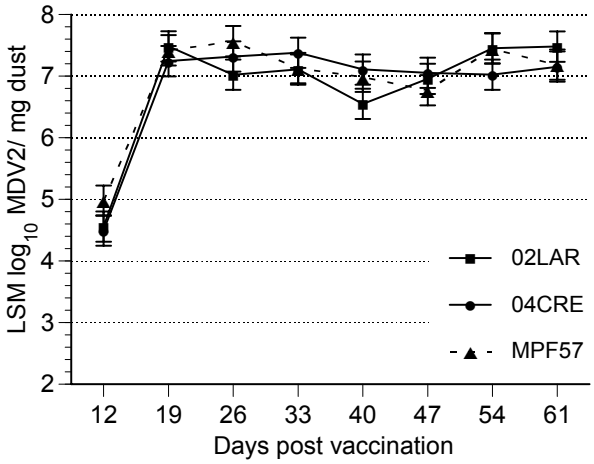

Figure 6.4: MDV2 load in dander (LSM±SEM, log_{10} scale) over time by challenge treatment. Chickens were vaccinated with bivalent vaccine at hatch. Each data point is the mean of two isolators in each challenge treatment. The effect of challenge was not significant (P=0.64).

6.3.4 Viral load of all three serotypes in dander from chickens vaccinated with bivalent vaccine and challenged with MDV1

Dust from 6 isolators containing chickens vaccinated with the bivalent vaccine were analysed for all three serotypes of MDV. The profile of each serotype in the same dust sample is shown in Figure 6.5 (left panel) showing clear differences in the pattern of viral load over time. The effects of MDV serotype, days post challenge with MDV1 and the interaction between these effects were highly significant ($P<0.0001$). The initial level of MDV1 at 7 dpc was at $10^{3.7}$ VCN/ mg dust and this increased in a biphasic way to a maximum of 10^{7} VCN/ mg dust at 49 dpc after which it decreased slightly to $10^{6.8}$ VCN/ mg dust at 56 dpc. The initial level of MDV2 at 12 dpv was at $10^{4.6}$ VCN/ mg dust, followed by a sharp increase to a peak at 19 dpv at $10^{7.4}$ VCN/ mg dust after which it decreased slowly to less than 10^{7} VCN/ mg dust at 61 dpv. At 12 dpv the load of HVT was $10^{5.3}$ VCN/ mg dust. This increased sharply to $10^{6.6}$ VCN/ mg dust at 19 dpv after which it declined to around 10^{6} VCN/ mg dust at 40 dpv before plateauing thereafter (Figure 6.5, left panel).

The overall viral load of MDV2 ($10^{6.87}$/mg dust) was significantly ($P<0.05$) higher than that of MDV1 ($10^{5.93}$/mg dust) and HVT ($10^{6.10}$/mg dust) which did not differ from each other. The interaction between MDV serotype and challenge treatment ($P<0.0001$) is shown in Figure 6.5 (right panel) demonstrating that the level of HVT and MDV2 was not influenced by the challenge virus, whereas the level of MDV1 was significantly influenced by challenge virus with significantly lower MDV1 in chickens challenged with 04CRE relative to the other two challenge viruses.

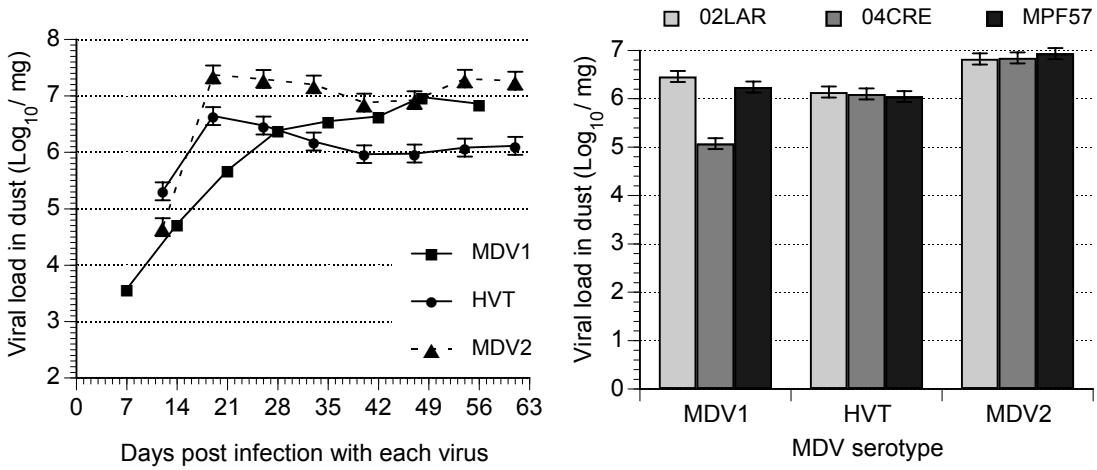

Figure 6.5: (Left Panel) Interaction between the effects of MDV serotype and dpc on viral load in dander from chickens vaccinated with bivalent vaccine and challenged with MDV1 (LSM±SEM) (n=6 isolators).
(Right panel) Interaction between the effects of MDV serotype and challenge MDV on viral load in isolator exhaust dust from the same chickens (LSM±SEM) (n=6 isolators).

6.3.5 Daily dander production per chicken and daily shedding rates of MDV

Daily dander production per chicken over time on a dry matter basis is shown in Figure 6.6. There was no effect of isolator. The mean daily dust production per chicken was 5.19 mg at 12 days of age increasing to 55.04 mg per chicken at 61 days of age in a sigmoidal pattern.

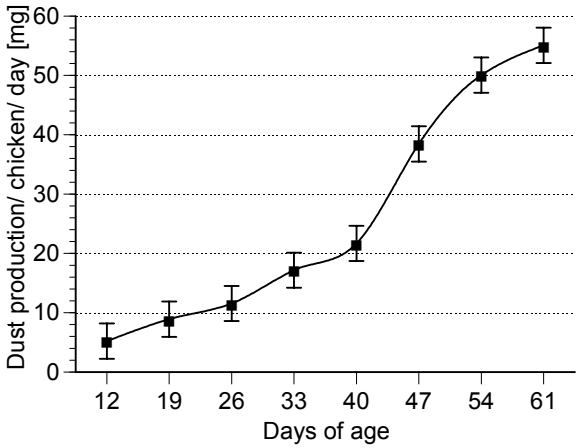

Figure 6.6: Mean 24 h dust production per chicken over time in randomly selected isolators. Each data point represents the mean of six isolators.

By combining dander production data and MDV content in dander, MDV shedding rates per chicken per day can be calculated. The mean MDV1 copy number shed per chicken per day, by vaccination status is shown in Figure 6.7, left panel. The effect of days post challenge was significant (P<0.0001), as was the effect of vaccination (P=0.01) with no significant interaction between vaccination and days post challenge (P=0.93). Throughout the experiment, unvaccinated chickens shed between 5-20 times more MDV1 than vaccinated chickens (Figure 6.7, left panel), although the initial amount of MDV1 at 7 dpc shed from both vaccinated and unvaccinated chickens was similar at about $10^{4.3}$ MDV1 copies per chicken and per day. For both vaccinated and unvaccinated chickens, MDV1 shedding per chicken increased up to 56 dpc through a combination of increasing MDV1 load in dander (Figure 6.1) and increasing dander production (Figure 6.6). At the end of the experiment unvaccinated chickens shed $10^{9.1}$ MDV1 copies per chicken per day, whereas vaccinated chickens shed $10^{8.6}$ MDV1 copies per chicken per day (Figure 6.7, left panel).

The mean viral copy numbers for both MDV2 and HVT shed per chicken per day is shown in Figure 6.7, right panel. The effect of days post challenge was significant (P=0.0004 for MDV2 and P<0.0001 for HVT). The MDV2 viral load in dust was initially almost 7-fold lower than that of HVT at 7dpc ($10^{5.1}$ MDV2/ mg dust versus $10^{5.9}$ HVT/ mg dust), but from then onwards, the MDV2 viral load was about 10-fold higher until the termination of the experiment.

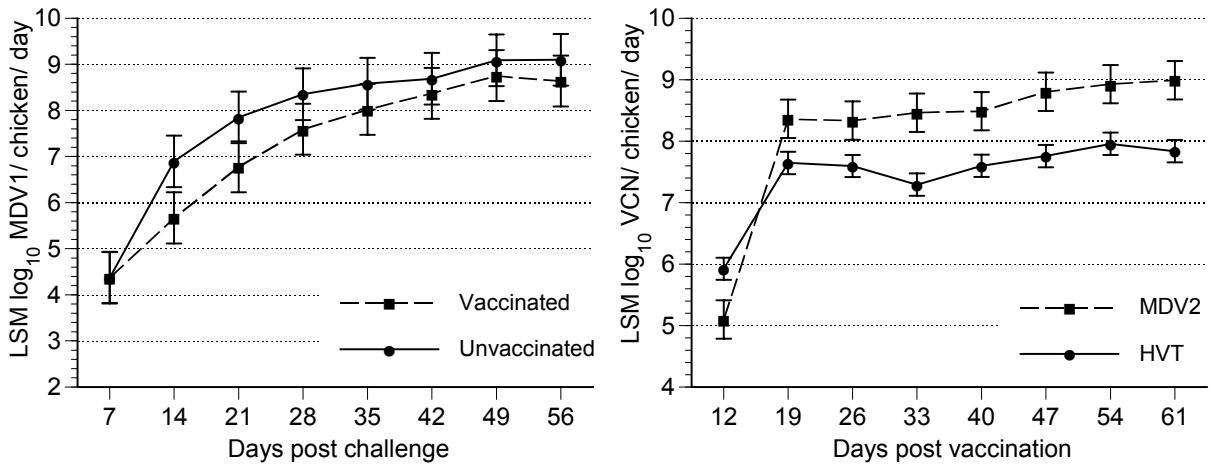

Figure 6.7: (Left panel) Mean 24 h MDV1 shed per chicken over time by vaccination status (LSM±SEM). Each data point represents the mean of six isolators.
(Right panel) Mean 24 h MDV2/HVT shed per chicken over time (LSM±SEM). For MDV2, each data point represents the mean of two isolators, for HVT, each data point represents the mean of three isolators.

The association between mean dust production per chicken per day and mean body weight measured at 19 and 61 days of age was positive and highly significant ($Y=0.0743x-2.66$, $R^2=0.84$, $P<0.0001$).

6.3.6 Prediction of MD incidence

6.3.6.1 Association between MDV load in isolator exhaust dust and the subsequent incidence of MD

MDV1 load in spleen at 14 dpc was significantly positively associated with the ultimate incidence of MD in each isolator, accounting for 64-71 % of the total variation between isolators in the incidence of MD and has already been shown in Chapter 4, Figure 4.9.

MDV1 load in isolator exhaust dust on 14, 21, 28, 35, 42 and 49 dpc was also significantly positively associated with MD incidence by 56 dpc (R^2 0.48-0.66), but at 7 dpc no relationship was evident with a trend towards a negative association (Figure 6.8). The strongest associations were at 14 and 21 dpc.

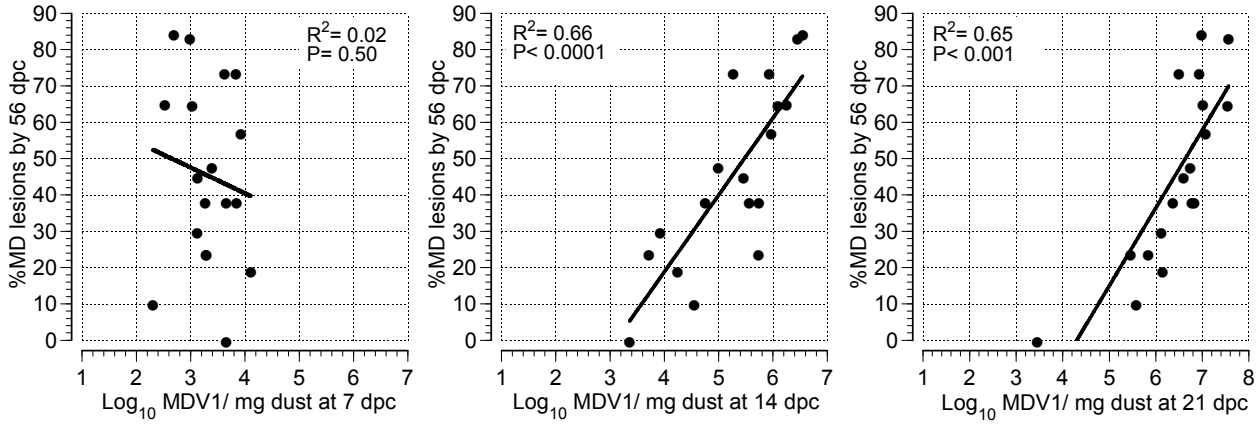

Figure 6.8: Association between MDV1 load in isolator exhaust dust at 7, 14 and 21 dpc and the incidence of MD by 56 dpc (% birds with gross MD lesions) in chickens challenged with MDV1 (unchallenged isolators excluded). Each point represents one isolator. The lines are linear regression curves.

6.3.6.2 Association between HVT and MDV2 load in isolator exhaust dust and the subsequent incidence of MD

Except at 54 dpv where there was a significant and positive association between HVT load in dust and subsequent MD (R^2=0.26, P=0.09), there was no significant association between the mean level of HVT in dust and the incidence of MD, and no trend towards an association. The direction of association was not consistent over time.

For MDV2, despite only 6 isolators being involved, significant associations between MDV2 in dust and subsequent incidence of MD were observed. In the middle part of the experiment (33-47 dpv) there was a negative association between MDV2 and %MD (R^2 range 0.32-0.83) whereas at 54 and 61 dpv the association was positive (R^2=0.62 and 0.67 respectively) (Figure 6.9).

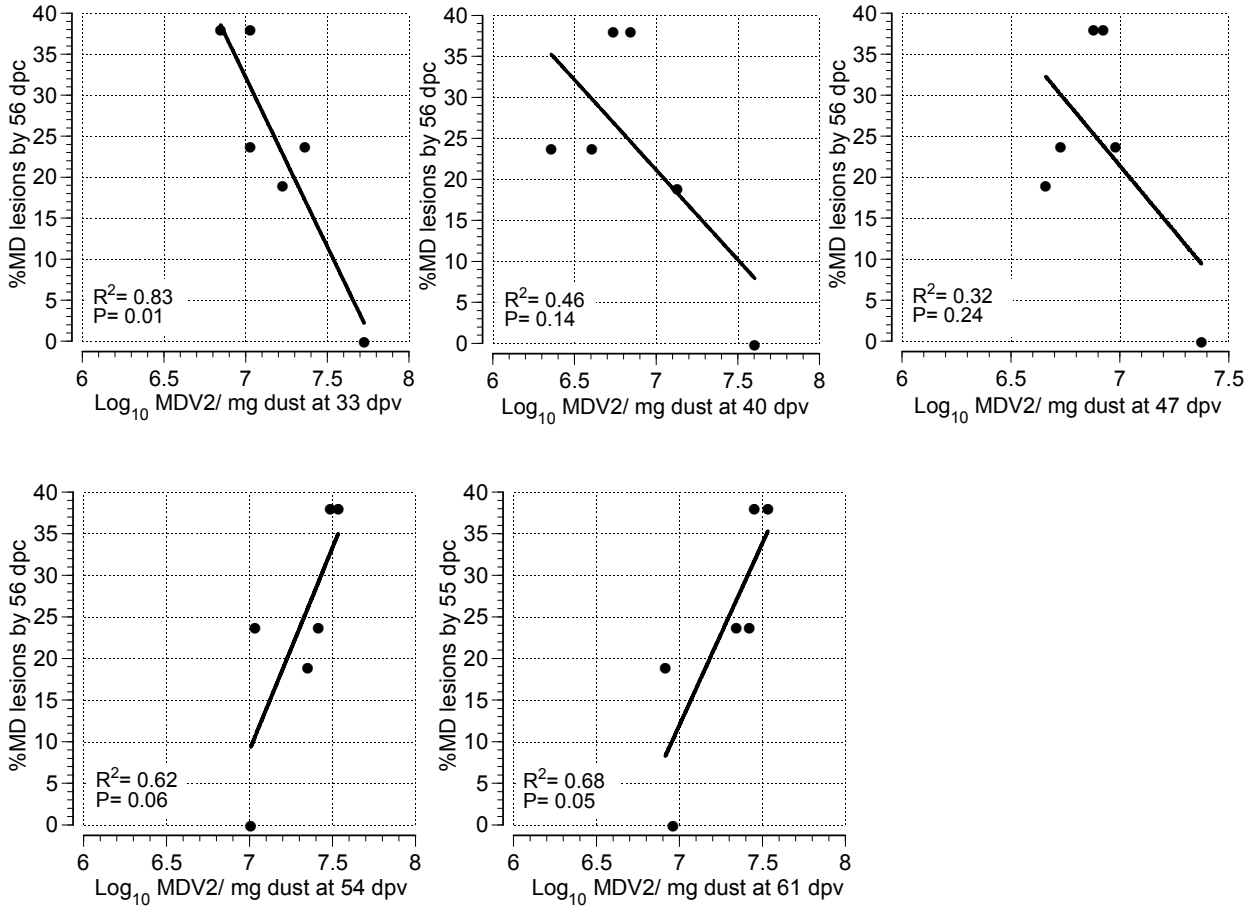

Figure 6.9: Association between MDV2 load in isolator exhaust dust at 33, 40, 47 (upper panel), 54 and 61 dpv (lower panel) and the incidence of MD by 56 dpc (61 dpv, % birds with gross MD lesions) in chickens challenged with MDV1 (unchallenged isolators excluded). Each point represents one isolator. The lines are linear regression curves.

6.4 Discussion

This study is the first report on the kinetics of all three serotypes of MDV in single or mixed infections in commercial layer chickens. It complements a similar study in broiler chickens (Islam and Walkden-Brown, 2007).

With regards to the first three objectives, MDV1, MDV2 and HVT present in dander shed from layer chickens in single or mixed infections were repeatedly measured and quantified. All three serotypes could be detected reliably in isolator exhaust dander from 7 dpc in the case of MDV1 and 12 dpv in the case of HVT and MDV2 throughout the experimental period until 56 dpc or 61 dpv.

There were significant differences in the MDV1 load in dander from birds challenged with three different MDV1 isolates. The viral load in dander from chickens challenged with the isolate 04CRE was up to 10-fold lower compared to MPF57 and 02LAR whereas with 02LAR, the viral load was around 1.5-fold higher than with MPF57 and this ranking was in proportion to the pathotyping data (Chapter 4). This provides evidence that MDV1 viral load in dust is strongly correlated with virulence of the challenge isolate which is a key finding of this study and is later discussed in detail.

The daily dander production of commercial layers between the age of 1 and 9 weeks was successfully determined. Thus, the data enabled calculation of the daily amount of all three serotypes of MDV which was shed from layer chickens into the environment. The pattern of dander production showed a typical sigmoidal growth curve and appears to be closely associated with body weight which shows a similar pattern during this period.

The combination of quantitative determination of viral load in dust and quantitative estimation of dust production by individual chickens enabled determination of the actual shedding rates of all three MDV serotypes in commercial layer chickens under typical vaccination conditions. The rapid increase in viral load in dander during the first 4 weeks of life combined with an increase in dander production by individual birds resulted in a very rapid rise in shedding of all three MDV serotypes over this period followed by a plateauing of viral shedding rates. Another key finding was that the MDV2 isolate examined had very high shedding rates ($10^{6.87}$/mg dust), several fold higher than that of the three MDV1 isolates, particularly up to 4 weeks after vaccination. In contrast, shedding of HVT was about 10-fold lower than that of the other MDV serotypes.

In contrast to the results of an earlier study using cell culture methods where it was reported that HVT and attenuated isolates of MDV1 could not be detected in the FFE and subsequently, only very poorly spread to contact chickens (Nazerian and Witter, 1970), the present study clearly shows that all three MDV serotypes are present in chicken dander as early as 7-12 days after infection. This raises the issue of the efficiency of lateral transmission of vaccinal viruses. It has been reported that HVT exhibits limited lateral transmission in chickens, mainly between older birds (Cho and Kenzy, 1975), and it is possible that the virus

adapted in chickens through natural passage and becomes transmissible between chickens perhaps to a limited extent. However, in a recent study Tink et al. (2005) reported the lack of transmission of HVT virus between broiler chickens in contact with *in ovo* vaccinated flockmates from hatch to 49 days of age as determined by qPCR of spleen tissue. With the CVI988 strain of MDV1, the original uncloned isolate was readily spread by contact (Rispens et al., 1972a) but the clone CVI988/C showed limited transmission between in contact birds (Witter et al., 1987). Thus, the ability to spread is obviously affected by serial passage and attenuation of CVI988 and that this characteristic may vary among different subclones of the virus (Baigent et al., 2005b). Nevertheless, both avirulent strains (HVT and MDV2) shed at levels similar or higher than virulent MDV1.

With regards to objective four, the shedding of MDV1 was reduced in chickens either vaccinated with HVT or bivalent vaccine. This was especially evident in chickens vaccinated with the bivalent vaccine which shed about 3-10 times less MDV1 than unvaccinated chickens. As well the bivalent vaccine also offered superior protection against MD lesions and subsequent MD mortality compared to HVT (Chapter 4) and HVT reduced MDV1 shedding to a lesser extent (1.5-4 fold). A suppressive effect of vaccination on the level of MDV1 in dander was also reported by Islam et al. (2005a) and Islam and Walkden-Brown (2007), but in the present experiment the effects of vaccination were greater, sustained for a longer period and there was a clearer differentiation between the HVT and bivalent vaccines which was not evident in the earlier study reported by Islam and Walkden-Brown (2007). The same authors reported a marked increase (between 10-100-fold) in the shedding rate of the vaccinal viruses HVT and MDV2 in vaccinated chickens challenged with pathogenic MDV1. Unfortunately, this could not be investigated in the present experiment as the respective treatment groups were not included.

Although this shows that vaccination reduces the MDV1 load in dander to varying extents, neither of the two vaccines prevented replication of MDV in the FFE and subsequent shedding into the environment and vaccinated layer chickens continued to shed $10^{8.6}$ MDV1 copies per chicken per day at 61 days of age. Furthermore, both vaccines, though to a varying extent, only offered incomplete protection against clinical Marek's disease. This use of such 'imperfect' vaccines

on a large scale has been postulated to be a significant stimulus for evolution of viruses towards increased virulence (Read, 1994; Gandon et al., 2001). This may well be the case with Marek's disease, particularly in the USA where blanket vaccination against Marek's disease has been practised over decades and has been postulated as a possible cause of the increase in virulence in MDV over this period (Witter, 1997). The current experiment confirms in quantitative terms that vaccinated chickens continue to shed significant amounts of MDV1 into the environment thus it can be expected that MDV1 will continue to evolve towards greater virulence if the theory of imperfect vaccine-driven evolution in virulence is correct.

Although the situation in Australia is different from the USA, there is some evidence of evolution towards greater virulence here with very virulent MDV1 isolates against which vaccination with bivalent vaccines had already failed detected as early as the mid 1980s (McKimm-Breschkin et al., 1990).

With regards to objective five, there is strong evidence that there is a relationship between the MDV1 shedding rate in dust and the virulence of the challenge isolate. Both the isolates MPF57 and 02LAR did not differ in virulence rank (Chapter 4) and both these isolates were shed in significantly higher amounts (02LAR about 1.5-fold higher than MPF57) compared to the isolate 04CRE which also had a significantly lower virulence rank in the pathotyping study (Chapter 4). Furthermore, the MD incidence data of these isolates described in Chapter 4 reflected the same ranking since the isolate 04CRE induced the least MD lesions (13-59 %), followed by MPF57 (29-69 %) and 02LAR which induced the most MD lesions (31-84 %).

This clearly suggests that there is a direct correlation between virulence and shedding rate in dander which is another key finding of this study. Previously, it has been reported that higher MDV replication rates occur in circulating or tissue based lymphocytes which are associated with increased virulence (Yunis et al., 2004). Although the situation regarding virus replication in the FFE is less clear, Nazerian and Witter (1970) reported that there is a positive correlation between virulence, contagiousness and replication of virulent and attenuated MDV1 and HVT viruses.

More recently, Baigent et al. (2005b), using real-time quantitative PCR reported that the avirulent MDV1 vaccine Rispens/CVI988 was readily detectable in the feather tips only 7 days after vaccination, and subsequently rose to levels in this tissue that were almost 1000-fold greater than that in the lymphoid tissues (spleen, bursa of Fabricius and thymus). Likewise, Islam et al. (2005a; 2008) reported significant shedding of HVT from vaccinated chickens challenged with MDV1, as detected by quantitative real-time PCR (qPCR) of isolator exhaust dander, and also significant shedding of MDV1 by chickens vaccinated with HVT. However, none of these latter studies investigated whether there is a correlation between virulence and shedding rate and the present study to date is the most powerful demonstration that MDV1 load in dander varies in proportion to virulence. These differences in shedding rates between challenge viruses were greatest at 14 dpc but were sustained throughout the experiment.

However, in a similar study in broiler chickens, there was no significant difference in the viral load detected in dander from three different MDV1 isolates (Islam and Walkden-Brown, 2007), two of which (MPF57 and 02LAR) were also used in the present study. The most likely reason for this is that there was also no significant variation in virulence between the MDV isolates used, unlike the situation in the present experiment in layers. However differences in host genotype cannot be ruled out as a contributory factor. As well, there are earlier reports that the vv pathotype RB1B-BAC causes high mortality in chickens, but does not spread horizontally (Jarosinski et al., 2007). Thus, virulence and transmission of MDV may be dissociated and not directly related and this has been suggested previously (Dienglewicz and Parcells, 1999).

With regards to objective six, the level of vaccinal virus in dander (both HVT and MDV2), proved less informative for predicting future MD status than MDV1. The level of MDV2 in dust was significantly associated with subsequent MD incidence, with an inverse relationship at 33-47 dpv but a positive relationship at 54 and 61 dpv. This suggests that the more severely infected chickens late in the disease shed higher amounts of MDV2, an observation supported by the report of Islam and Walkden-Brown (2007) that infection with MDV1 greatly increases the shedding rate of HVT and MDV2. Although the exact mechanisms of such enhancement are unknown, it may be that the immunosuppressive action of

MDV1 resulted in reduced immune-mediated inhibition of HVT and MDV2 replication.

In contrast to the vaccinal viruses, strong positive relationships between MDV1 load in dander and subsequent MD incidence were seen at 14-49 dpc, but an unexpectedly poor and negative relationship was evident at 7 dpc. Similar results have been observed in earlier experiments (Islam et al. 2008a; Islam and Walkden-Brown, 2007). The reason for this poor relationship at 7 dpc is likely to be due to a sparing effect of vaccinal virus on the inactivation of MDV1 by maternal antibody (mab). In chicks challenged only with 500 pfu of MDV1, the only target for mab is this virus, while for those also vaccinated with 8000 pfu of vaccinal virus at day 0, it is possible that much of the mab has formed immune complexes with vaccinal virus prior to challenge, thus providing a sparing effect on the challenge virus (Walkden-Brown et al., 2007b).

Measurement of MDV1 load in dust in groups of birds 2-3 weeks after challenge therefore appears to have excellent potential to predict MD outcomes at early stages of infection and the MDV1 load in dust samples has been previously shown to be a good predictor of subsequent MD outcome (Walkden-Brown et al., 2006; Walkden-Brown et al., 2007b). Other than dust, the MDV1 load in spleen at 14 dpc has been previously shown to be another excellent predictor of subsequent MD outcome (Walkden-Brown et al., 2006, 2007b) and in the present experiment, this correlation was also highly significant (R^2=0.71, P<0.0001, Chapter 4). In both spleen and dander, these associations were present from 14 dpc onwards, and therefore measurements can be made prior to the onset of clinical MD, thus offering the possibility of early screening for MDV virulence in chickens with significant ethical advantages over full-length pathotyping experiments. In addition the measurement of MDV load in dust does not require the sacrifice of birds, is considerably more convenient and inexpensive as it integrates information from many chickens. The quantification of MDV viral load in dust has therefore considerable diagnostic and monitoring potential for MD in the industry (Walkden-Brown at al., 2005; Islam et al., 2006a) and is indeed currently being used for this purpose in Australia.

With regards to objective seven, in general, the effects of vaccination and challenge isolate on MDV load in isolator exhaust dust were much greater in the

layer chickens than the broilers with clear demarcation between each of the 3 vaccine treatments, and each of the 3 MDV challenge treatments. Thus, together with the previous study in boilers reported by Islam and Walkden-Brown (2007), this is the first data set clearly showing the effect of genotype on the shedding pattern in chicken dander as the experimental setup was virtually identical for both studies.

The overall level of MDV1 and MDV2 in dander and the patterns of shedding over time were broadly consistent with what was observed in broilers although levels of MDV1 were slightly higher in the layer experiment. While the pattern of HVT load in dander was also broadly similar, it peaked earlier in layers and at a level nearly 10-fold higher than that seen in broiler chickens.

Interestingly, from 14 dpc onwards, MDV2 was the serotype being shed in the highest amounts. This might be understandable given that MDV2 is a quite distinct viral species compared to MDV1 and HVT, but data for MDV2 is rare and requires further research. The MDV2 shedding profile of broilers reported by Islam and Walkden-Brown (2007) was very similar to the profile in layers seen in the present experiment. In broilers MDV2 was also the serotype showing the highest rate of shedding throughout the experiment with viral loads very similar to those observed in the layers. In contrast to the broilers, MDV2 in dust peaked at 14 dpc in the layer experiment which was 14 days earlier than in the broilers.

The shedding rates of dander were much lower in layers than in the broiler study of (Islam and Walkden-Brown (2007). It is of interest to determine whether shedding of dander is directly associated with bodyweight, or some function of bodyweight such as metabolic weight ($BW^{0.75}$) which is closely allied with surface area. A comparison of the data from the two experiments (Table 6.1) shows that in layers the increase in shedding between days 19 and 61 closely paralleled the increase in BW, and in both experiments was more closely associated with BW than $BW^{0.75}$.

Table 6.1: Comparison of bodyweight (BW), metabolic BW ($BW^{0.75}$) and daily dust production between layers and broilers.

Parameter	Age (d)	Layers	Broilers
Mean BW (g)	19	159.4	538
Mean $BW^{0.75}$ (g)		44.82	112
Mean dust production/ bird/day (mg)		8.9	13.5
Mean dust production (mg/kg chicken BW)		56	25
Mean dust production (mg/kg chicken $BW^{0.75}$)		199	121
Mean BW (g)	61	730	3033
Mean $BW^{0.75}$ (g)		140	409
Mean dust production/ bird/day (mg)		51.9	233
Mean dust production (mg/kg chicken BW)		71	77
Mean dust production (mg/kg chicken $BW^{0.75}$)		370	570
Increase BW		4.6	5.6
Increase $BW^{0.75}$		3.1	3.7
Increase dust production/ bird/day		5.8	17.3

However, dust production per unit BW was higher at 61 days of age than 19 days of age. This probably reflects the fact that dander production seems to occur in 2 phases - first a very low production of chick down prior to feathering commencing (first 2-3 weeks) followed by a steady increase associated with full feathering and growth. Thus the comparison between 19 and 61 days of age might be affected by the feathering rate between the two breeds. Unfortunately, in neither of the experiments the chickens were weighed each week during the weekly dust collections which would enable this issue to be fully resolved.

In conclusion, this study has shown that:

a) The MDV1 viral load in dander is in proportion to the virulence of the MDV1 isolate and strongly correlated with subsequent MD incidence from 14 dpc onwards.;

b) Vaccination with HVT alone significantly reduced the MDV1 viral load in dander of infected chickens by up to 4-fold while vaccination with HVT in combination with MDV2 reduced the MDV1 viral load in dander to a much greater extent (up to 20 fold), but none of the vaccines tested in this experiment completely prevented MDV1 replication and subsequent shedding of MDV1. The use of such imperfect vaccines may be a stimulus for MDV to evolve towards greater virulence (Gandon et al., 2001), particularly overseas where blanket vaccination of broiler flocks is practiced (Witter et al., 1997);

c) The MDV 1 viral load in dust continued to increase over a time period of 9 weeks whereas the HVT load in dust peaked at 19 dpv, then declined and plateaued from 33 dpv onwards. A similar pattern was observed with MDV2 which peaked at 19 dpv and plateaued from then onwards;

d) An unvaccinated commercial layer chicken challenged with 500 pfu of MDV1 on day 5 of age sheds approx. 10^9 copies of the MDV1 genome per day from 54 days of age onwards whereas vaccinated chickens shed about $10^{8.8}$ copies of the MDV1 genome per day from 54 days of age onwards;

e) The MDV1 load in dander is an excellent predictor of subsequent incidence of MD, comparable to the predictive power of MDV1 load in spleen and both tissues appear to be superior to use of feather tip or blood material for this. However, dander is a non-terminal sample, integrates information from many chickens, and is more convenient to sample, store and handle than spleen material; and

f) Quantitative real-time PCR assays can play an important role in the monitoring MD status in poultry flock by assaying MDV load in feather dander.

Chapter 7

Evaluation of two environmental enrichment strategies for improved welfare of layer chickens in pathotyping experiments

7.1. Introduction

There are two hypotheses to explain the origin and development of feather pecking. According to Blokhuis and Arkes (1984), feather pecking evolves as redirected ground pecking. However, Vestergaard et al. (1993) consider feather pecking to be the result of an abnormal development of the perceptual mechanism responsible for detection of substrates for dust-bathing. Feather pecking has potentially catastrophic implications for the birds' welfare for several reasons: i) they may suffer pain when they are pecked (Gentle, 1986); ii) the occurrence of pecking-related feather loss increases susceptibility to injury; iii) feather pecking and the removal of feathers can cause bleeding from deeper damage to the skin or follicles and may thereby lead to further cannibalism and the painful death of target birds. Birds which are kept in isolators for research purposes are typically maintained in a relatively bare environment optimised for disinfection rather than bird welfare. They may also be handled intensively. These birds are therefore at risk of developing harmful behaviours including feather pecking. Remedial measures like beak trimming and reduced light intensity are often associated with welfare problems themselves. For example, beak trimming can cause chronic pain (Gentle, 1986) and keeping the birds under low light intensities not only impoverishes the visual environment but it can also result in malnutrition and in the development of eye abnormalities.

In our isolator facility at the University of New England, we have found that feather pecking can be a welfare issue in commercial layer and specific pathogen free (SPF) white leghorn chickens whereas it is rarely a problem in broiler

chickens. The aim of this study was to reduce feather pecking in layer chickens kept in isolators for research purposes by inclusion of bunches of string and/or a sand box in a 49 day experiment in non beak trimmed SPF white leghorn chickens.

7.2. Materials and Methods

7.2.1 Experimental design

The experiment was conducted as a 2x2 factorial design with 6 replicates using 24 identical isolators over a time period of 7 weeks. The two factors in the experiment were the presence or absence of 2 bunches of sterile yellow polypropylene hay baling twine (string) and the presence or absence of a sand bathing box. Each isolator contained 19-20 chickens which were challenged by a variety of Marek's disease virus (MDV) isolates as part of a separate study with a different MDV isolate for each isolator. Bunches of string were suspended from the top of the isolator to approximately 2.5 cm off the floor (Plate 7.1, left). Sand boxes were 47x37x15cm cat litter boxes with in-turned top edges to limit ejection of material by scratching. Each contained 2 kg sterile washed river sand (Plate 7.1, right).

Plate 7.1: Isolator setup with string bunches (left) and with string bunches and sand box (right).

Chickens were maintained at 12D:12L photoperiod with moderate light dimming. Lights were turned up to full strength during working and inspection periods. The 470 experimental chickens were SPF white leghorn chickens (SPAFAS Australia bird, Ex CSIRO WLH line). They were not beak trimmed.

Chickens had access to the string bunches throughout the experiment but access to the sand boxes was provided from 2 weeks of age when the chickens were big enough to get in and out the box.

Chickens were maintained and treated according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 2004, the NSW Animal Research Act 1985 and the NSW Animal Research Regulation 1995, and the experiment was approved by the UNE Animal Ethics Committee (AEC No. UNE 05/076).

7.2.2 Measurements

All measurements were estimated weekly throughout the experiment and were adapted from Bilcik and Keeling (1999). However, unlike in their study, the chickens could not be removed for the measurements and all measurements were made by observing and judging the birds within the isolators. Observation results are expressed per isolator.

7.2.2.1 Feather pecking

The incidence of feather pecking in each isolator was estimated by counting all chickens with any signs of feather pecking per isolator. The number of affected chickens was then divided by the total number of chickens present in each isolator and multiplied by 100.

7.2.2.2 Feather coverage

The extent of harmful behaviour against chicken of the same isolator was expressed as percent of feather coverage for all chickens per isolator, where 100% represented ideal feather coverage and 0% represented no feather coverage of the chickens. The chickens were carefully examined for missing

feathers and given an overall score (100-0 %) per isolator based on the presence and size of bald patches.

7.2.2.3 Feather condition score

Mean isolator feather condition score was estimated using the scoring system of Bilcik and Keeling (1999). Feather condition was scored from 0-5 with 5 being almost all feathers missing and 0 being all feathers intact. All chickens were examined for broken or missing feathers and given a mean score representative of each isolator.

7.2.2.4 Skin injury score

Skin injury was also scored using the scoring system of Bilcik and Keeling (1999) which ranged from 0-4 with 4 representing a wound larger than 2 cm in diameter and 0 representing no injuries and scratches. All chickens were examined for skin injury and given a mean score per isolator.

7.2.3 Statistical analysis

Score and percentage data were not normally distributed and not amenable to transformation so were coded as ordinal and subjected to ordinal logistic analysis using JMP IN 5.1 (SAS Institute, NC, USA). The effects of string, sand box, day of sampling (chicken age) and their interactions were fitted, with non-significant interactions removed from the final model. Because data are not normally distributed, standard errors are not provided with means.

7.3. Results

7.3.1 Incidence of feather pecking

The incidence of feather pecking throughout the experiment is shown in Figure 7.1. It was significantly affected by string ($P<0.001$) and chicken age ($P=0.003$) but not sand box ($P=0.170$) with no significant interaction between these effects,

although there was a trend towards an interaction between the effects of sand box and string (P=0.18, Figure 7.1, left panel). Chickens with string in their isolators had a lower incidence of feather pecking than those without string (2.02 and 7.16 % respectively, P<0.001). The effect of age was manifest mainly as a sharp increase in incidence between weeks 2 and 3 of age as shown in Figure 7.1, right panel. The trend towards interaction between the effects of sand box and string suggests that the effect of string may be greater when a sand box is present.

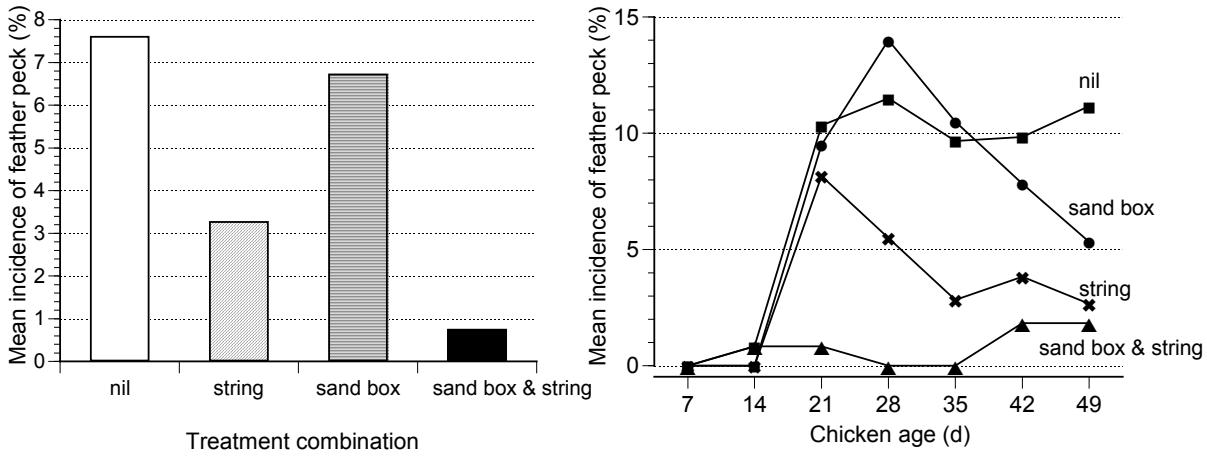

Figure 7.1: Mean incidence of feather pecking by treatment (left panel) and by treatment and week of the experiment (right panel).

7.3.2 Feather coverage

Feather coverage was significantly affected by string (P<0.001) and chicken age (P<0.001) but not sand box (P=0.616) with no significant interaction between these effects (Figure 7.2, left panel). Chickens with string in their isolators had a higher feather coverage score than those without string (97.0 and 89.9 % respectively, P<0.001). The effect of age was manifest mainly as a sharp decrease in feather coverage between weeks 2 and 3 of age as shown in Figure 7.2, right panel.

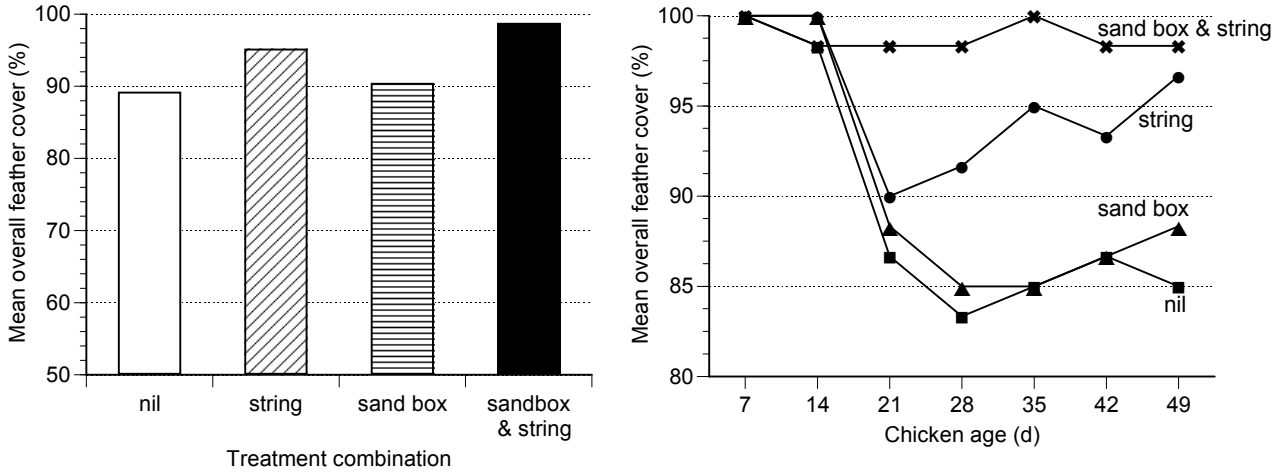

Figure 7.2: Mean feather coverage score by treatment (left panel) and by treatment and chicken age (right panel).

7.3.3 Skin injury and feather condition score

Skin injury score was significantly affected by String (P<0.001) and chicken age (P<0.001) but not sand box (P=0.350) with no significant interaction between these effects although there was a trend towards an interaction between the effects of sand box and string (P=0.196). Chickens with string in their isolators had a lower mean skin injury score than those without string (0.42 and 1.1 respectively, P<0.001, Figure 7.3, left panel). The effect of age was manifest as a small increase in incidence between weeks 1 and 2 of age, followed by a large increase between weeks 2 and 3 of age with no further increase beyond this (Figure 7.3, right panel). The trend towards interaction between the effects of sand box and string is such to suggest that the effect of string may be greater when a sand box is present.

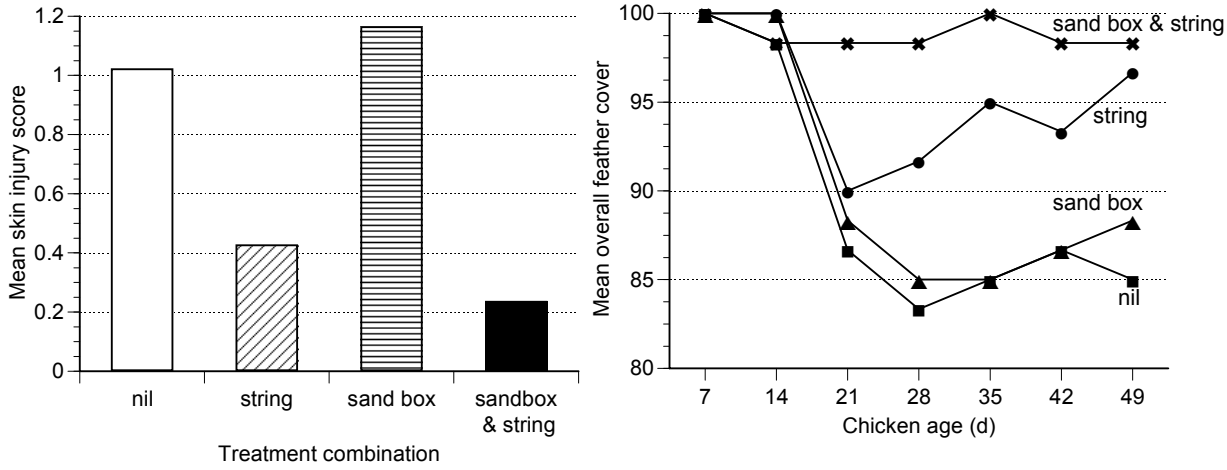

Figure 7.3: Mean skin injury score by treatment (left panel) and by chicken age (right panel).

Feather condition score was significantly affected by string (P<0.001) and chicken age (P<0.001) but not sand box (P=0.547) with no significant interaction between these effects. Chickens with string in their isolators had a lower mean feather condition score than those without string (0.54 and 1.45 respectively, P<0.001). The overall effect of age was manifest as a small increase from 0 to 0.29 between weeks 1 and 2 of age, followed by a large increase to 1.54 in week 3 followed by a gradual decline after this to a value of 0.58 at the end of week 7.

7.4. Discussion

This study has clearly demonstrated the benefits of inclusion of string in isolators in terms of limiting feather pecking and skin damage and improving feather cover and condition. The effects of including a sand box were less clear with no significant effects on these variables although they trended towards more favourable in each case. However, particularly with the string bunches, several chickens got stuck with their tongue in the bunches as the material tended to fray at later stages in the experiment. Although none of the chickens died because of this, it caused major bruising of the tongue. Therefore, it would be worth to test alternative materials to the hay baling twine which would reduce such incidents, e.g. thin leather strings. Future studies could also test other floor materials, such as plastic rather than metal and/or give the chickens access to some hay as environmental enrichment.

For every variable, the most favourable outcome was observed for the isolators in which the sand box and string were both included. Sand boxes were not introduced until day 14 of the experiment, just as feather-pecking activity was escalating. This may have contributed to the lack of significant efficacy. Providing access to a shallower sand box for the initial two weeks is something worth investigating in the future. The larger sand box was too high for chicken access during the early period and this is why access was delayed until day 14. The introduction of sand boxes is unlikely to have been causally associated with the increase in feather pecking observed at the time of introduction as the increase was also observed in isolators without sand boxes. It is more likely that the timing was associated with behavioural changes and the changeover from chick down to proper feathers during this period.

Infection with MDV is a confounding factor in the design and could possibly have affected the incidence of feather pecking as a reaction of the birds suffering from the infection. However, only 2 birds died or were euthanised with evidence of severe pecking injury, one of which showed atrophy of the thymus and bursa typical for infection with MDV. The use of 6 replicates (isolators) for each enrichment treatment combination, with random allocation of MDV isolates to isolators also mitigates against a systematic effect of MDV isolate. Furthermore, the peak of MDV-induced lesions was observed on day 38 post challenge, considerably later than the major rise in feather pecking activity between days 14 and 21, and occurred during a period where there was no increase in severity of feather pecking lesions.

Several birds in all isolators regardless of environmental enrichment had minor feather damage which may have been due to sharp edges on the galvanized steel feeder boxes. This may have acted as a trigger for feather pecking, especially in the isolators with no environmental enrichment. During the last 3 weeks of the experiment, there was no major increase of feather pecking. Instead, feathers of pecked birds started to regrow and skin injuries started healing as well leading to improvement of the overall feather coverage as shown in Figure 7.1, right panel. The findings of this study are consistent with those of Jones (2002) that bunches of string attracted continuous interest from the chickens and reduced feather pecking. The results of the study support the hypothesis of feather pecking as redirected ground pecking as described by

Blokhuis and Arkes (1984) rather than an abnormal behaviour due to lack of substrates for dust-bathing (Vestergaard et al., 1993) as sand boxes did not reduce feather pecking to the same extent as string bunches.

The results of this experiment clearly demonstrate that simple environmental enrichment such as the hanging of bunches of string in isolators can significantly reduce feather pecking in chickens housed in isolators. Further research into the use of sandboxes is indicated, ensuring that access is provided from the time of placement in the isolators.

Chapter 8

General discussion and conclusions

One of the main purposes of the research undertaken in this thesis was to complete the development and optimisation of molecular methods for the absolute quantification of the 3 different Marek's disease virus serotypes. These tests were then utilised to investigate processes important in the pathogenesis and epidemiology of MDV in various tissues in commercial layer chickens. Secondly, DNA sequence analysis of the *meq* gene was performed in order to detect molecular markers for virulence of MDV1 isolates. A third objective was to improve the welfare of experimental chickens kept in isolators by testing two environmental enrichment strategies. These objectives were successfully realized and contribute to an improved understanding in the kinetics of MDV infection in chickens and shedding into the environment. The results from the sequencing study may eventually lead to the development of a real-time PCR test to differentiate between pathogenic and vaccinal MDV1.

While comprehensive discussion has been provided within the relevant chapters of the thesis, this section will review the main findings again in an integrated way and discuss the implications of the work.

8.1 Molecular methods for absolute quantification of MDV2 using real-time PCR

Although specific assays to differentiate all three serotypes of MDV have been available for some years (Becker et al., 1992; Handberg et al., 2001), published methods for absolute quantification using the real-time PCR technique were only available for MDV1 and HVT (Kaiser et al, 2003; Baigent et al., 2005a, Islam et al., 2006a). The development of the MDV2 specific pKR-DNA*pol* plasmid standard and subsequent conversion of the previously used MDV2 assay

rounded off the set of the existing assays. This enabled us to quantify the viral copy number of all three serotypes of MDV separately or in a single sample as has been shown in this thesis and by others using the test (Islam and Walkden-Brown, 2007).

The conversion of the previous MDV2 assay to a fully quantitative assay measuring viral copy number (VCN) required developing a plasmid construct of part of the target UL30 DNA*pol* gene, purifying this and demonstrating parallelism between the existing and plasmid standard curves. The plasmid standards were then used to determine the viral copy number in the tissue based standards which were derived from original MDV2 vaccine material. As plasmid DNA poses a very high contamination risk and is unstable in storage, tissue based standards continued to be used for all real-time PCR assays. Although it has been suggested that plasmid DNA (Kaiser et al., 2003), recombinant DNA, RNA or spiked tissue samples (Baigent et al., 2005a) can be used to derive standard curves, the tissue based standards have some advantages over these. As the accuracy of an absolute real-time PCR assay depends on achieving 'identical' amplification efficiencies for both the native and the target DNA having both the standards and the samples derived from infected tissues ensures similarity and limits the problem of different amplification efficiencies. These standards, which contain a mixture of both of viral and host DNA have also proved to be very stable and reliable during storage compared to plasmid DNA where deterioration and/or loss has been observed (Islam, 2006; Islam et al., 2006a). However, these plasmids were stored in ultra-pure water for sequencing purposes. This is less suitable for long-term storage of any DNA material (Lindahl, 1993) and might have been the reason for the DNA degradation. The recommended buffer for long-term storage of DNA is TE buffer, optional with Ethanol, which minimizes such degradation (Evans et al., 2000). However, TE buffer has inhibitory effects in sequencing reactions (Kieleczawa, 2004) and was therefore not used for elution of the plasmid DNA.

The final units of measurement proposed by Islam et al. (2006a) were adopted for the MDV2 assay. Results for field dust samples are expressed in viral copy number (VCN) per mg of dust whereas the results for the feather and spleen samples are expressed in VCN per 10^6 host cells. Defining the limit of assay

sensitivity was another important step in the assay optimisation. For the MDV2 assay, the detection limit was 10 copies of the MDV2 genome, intermediate in sensitivity compared to the MDV1 and HVT assays which reliably amplify 5 and 37.5 copies of the MDV1 or HVT genome respectively (Islam, 2006; Islam et al., 2006a). The sensitivity of qPCR assays is significantly influenced by the chemistry used for the detection of the amplicon. The assays published by Islam et al. (2006a) as well as the MDV2 assay presented in this study all use a specific TaqMan® oligoprobe. Although this chemistry is more expensive compared to double stranded DNA binding probes such as SYBR® Green I, they offer far greater sensitivity, especially at low initial template concentrations.

The MDV2 assay did not amplify any other MDV serotype thus confirming its specificity which has been shown previously (Islam et al., 2004a). Therefore, the MDV2 assay complements the set of three serotype specific real-time PCR assays showing high sensitivity and enables us to absolutely quantify the MDV2 viral copy number in a variety of sample materials.

Use of real-time PCR in the field of molecular diagnostics has increased to the point where it is now accepted as the gold standard method for detecting nucleic acids from a number of sample and microbial types, replacing a range of non-quantitative and semi-quantitative PCR methods previously developed. The older methods were labour intensive and required post-PCR electrophoresis or other handling steps to quantify the amplicon. Compared to conventional PCR, real-time PCR offers the ability to quantify nucleic acids over an extraordinarily wide dynamic range, is rapid, highly sensitive and yet a robust method and results are easily reproducible. In addition, real-time PCR is performed in a closed reaction vessel that requires no post-PCR manipulations, thereby minimizing the chances for cross contamination in the laboratory.

To date, the absolute quantification of MDV copy numbers in various tissues, particularly spleen, feather and dust, has proven to be a very useful and powerful tool for studying the kinetics of virus infection, responses to vaccination, and genetic resistance of the host (Yunis et al., 2004; Baigent et al., 2005b; Islam et al., 2006a; 2006b; Islam and Walkden-Brown, 2007). Given that MDV2 appears to be common and circulating on many Australian broiler farms despite the absence of vaccination with MDV2 isolates (Renz et al., 2006), the optimisation of

this assay adds a powerful new tool in the study of MD pathogenesis and epidemiology. It also provides the opportunity to determine MDV2 vaccination success on the basis of qPCR of DNA extracted from dust or other samples.

8.2 Association between sequence variation in the *meq* gene and virulence of Australian and international isolates of MDV1 (Chapter 3)

Both biochemical and genetic studies carried out in recent years strongly suggest that *meq* is the principal oncogene of MDV. Perhaps the strongest evidence that *meq* is an MDV oncogene came from *meq* knockout experiments. The *meq*-null mutant viruses replicated well *in vitro*, but had completely lost their *in vivo* oncogenicity (Lupiani et al., 2004). Meq is a versatile protein that binds DNA, RNA and a variety of proteins. Meq has multiple cell cycle dependent subcellular localizations, enabling them to interact with different cell signal molecules. With respect to transformation and oncogenesis, Meq's dimerization with regulatory proteins and transcription factors such as Jun, Fos and ATF, and its binding to AP-1 sites, are likely to be critical in the transformation processes (Levy et al., 2003b; 2005). AP-1 activity is known as a key component in T-cell transformation and activation (Mori et al., 2000; Iwai et al., 2001). The ability of Meq to activate AP-1 and neutralize the activity of p53, a regulatory protein involved in tumour suppression, may also contribute to its strong anti-apoptotic effects (Nair and Kung, 2004).

If *meq* is the principal oncogene for MDV, then it could be expected that mutations in the *meq* gene would result in variation in the oncogenicity and pathogenicity of MDV isolates. Earlier studies overseas had shown that mutations with implications for virulence mainly occurred in the proline-rich region. The most significant were an in-frame 177-180 bp/59-60 aa insertion associated with attenuation and reduced virulence (Lee et al, 2000) and mutations in the section of the gene encoding consecutive proline repeats (PPPP) found in the proline-rich region, which were associated with increased virulence (Shamblin et al., 2004).

Chapter 3 reports the sequencing in triplicate of 5 Australian MDVs in the v to vv category of virulence based on the ADOL method of Witter (1997). The Australian isolates were collected from 3 states over more than a decade. All contained a 177 bp insertion in the proline rich-region and there was limited sequence variation between them. No association between *meq* sequence and virulence was observed amongst the Australian isolates. However, all Australian isolates contained the interruption at the second position of the PPPP repeats (PPPP→PAPP or PQPP).

Phylogenetic analysis, including international strains for which *meq* had been sequenced showed that the Australian strains clustered together within a clade containing MDVs with the 177-180bp insertion. This suggests the introduction of a common ancestor some time in the past. Interestingly there was a considerable range of virulence rankings in both the clade with the insertions, and the clade without the insertions, although the latter were more virulent on average.

In an attempt to identify more reliable markers of virulence and resolve the issue of the effect of the large insertion on virulence, the amino acid profiles encoded by the Australian and international strains were analysed for total proline content and for the number of intact PPPP repeats found in the sequence. Both were strongly and highly significantly associated with virulence. The latter is a particularly good marker with values ranging from 2 PPPP in some very virulent MDVs to 8 in the attenuated vaccine strain CVI988. All the Australian strains had 5 such repeat sequences. This work has clearly confirmed the role of Meq in variation in virulence of MDV, and has clarified that the effect of the major insertion is mediated by its effect on the total number of PPPP repeats encoded by the gene, rather by the presence or absence of the insert itself. This is an important advance. The sequence analysis of Australian isolates also identified unique mutations which could serve as the basis of a specific qPCR test to differentiate between Rispens CVI988 and Australian strains of MDV1 – something that would add a further important test to our armoury for investigating MD.

8.3 Pathotyping Australian isolates of MDV1 in commercial layer chickens (Chapter 4)

This experiment was the third in a series of pathotyping experiments using the same experimental design, based on the ADOL method of Witter (1997). The previous two experiments used specific pathogen free (SPF) chickens and commercial Cobb broilers.

In this experiment there was significant demarcation between the challenge viruses on the basis of virulence, with statistically significant differences between the level of MD induced by each isolate. This was a very good outcome, as it enable testing of various markers of virulence in the experiment, as covered in Chapters 4 - 6. Based on the induction of MD in unvaccinated chickens, and the level of protection afforded by HVT and bivalent (HVT/MDV2) vaccines (Witter 1997) 02LAR could clearly be ranked as a vv pathotype, as could the somewhat less virulent MPF57, with the least virulent isolate) 04CRE falling into the v pathotype category. There was no evidence of the vv+ pathotype which is relatively common in the USA at present. This was reflected in the comparatively good protection provided by the Bivalent vaccine (67 %), which provided significantly better protection than the HVT vaccine (44 %).

These findings do not support a major shift in MDV virulence in Australia, since putatively vv MDVs were reported in Australia from as early the mid 1980s (McKimm-Breschkin et al. 1990). This may be due to the different vaccination strategies and production systems practised in Australia. Unlike in the USA, Australia has only started to routinely vaccinate broilers with HVT about a decade ago and even today around 50 % of broiler remain unvaccinated. This is made possible perhaps by the Australian practice of total removal of litter and disinfection between batches of chickens. The findings of this experiment also suggest that bivalent vaccine will improve control of MD in Australia as it did in the USA. These experiments were conducted prior to release of this vaccine in Australia.

It is beyond the scope of this discussion to fully analyse and discuss the results of the 3 experiments in the series, which are currently published in preliminary form

in a conference paper (Walkden-Brown et al., 2007a) and in reports to funding agencies (Walkden-Brown et al., 2006, 2007b). However, there are several notable differences about the results from the 3 experiments that are worth highlighting here.

The genetic background of the host significantly influenced vaccine efficacy and MD outcomes, confounding pathotype rankings. This was most evident when comparing the experiments in Cobb broiler and Isa Brown layer chickens. The differences between the two experiments in the levels of MD induced and protection provided by vaccines are summarised in Figure 8.1 which emphasises the differences between the two host systems as summarized below.

- 02LAR was more pathogenic than MPF57 in the ISABROWN, while the reverse was true in Cobb chickens (non-significant)
- Vaccinal protection was greater overall in Cobb broilers than ISABROWN layers
- Bivalent vaccine produced superior protection to HVT in the ISABROWN, but not in Cobb chickens. This, together with the point above and Figure 8.1 suggests that the bivalent vaccine provides its greatest advantage over HVT when HVT is failing.
- There was a significant positive association between the level of MD induced in unvaccinated chickens, with that induced in vaccinated chickens in ISABROWN, but not Cobb chickens.

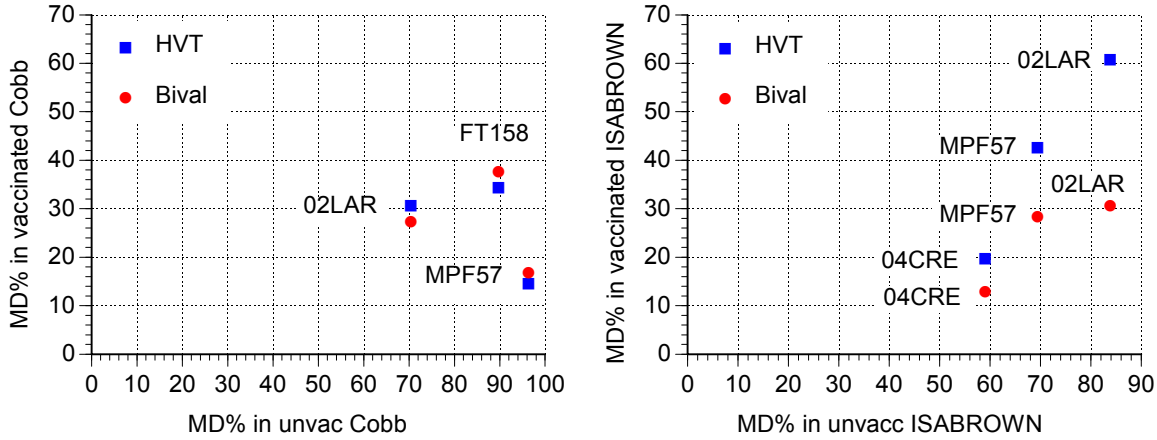

Figure 8.1: Association between MD incidence (%MD) induced in Cobb broilers (Left panel; Walkden-Brown et al., 2007b) and ISABROWN layers (Right panel; Chapter 4) either unvaccinated, or vaccinated with HVT or bivalent vaccine.

The maternal antibody status of the chickens also appeared to affect the results significantly with maternal antibody-negative SPF chickens in the first experiment succumbing to the early paralysis and mortality syndrome on days 9-15 post challenge (Witter et al., 1999), something that was not observed at all in the experiments using maternal-antibody positive chickens. Chickens in the SPF experiment also exhibited marked thymic and bursal atrophy to a far greater degree than that seen in the commercial chickens.

Taken together the results reported in Chapter 4 suggest that Australia is in a comparatively good position regarding vaccinal control of MD, despite severe loss of efficacy of HVT against some isolates. The bivalent vaccine provides relatively good protection in this situation. Neverthess, Australia appears to be at an earlier phase in the evolution of virulence that the USA has experienced, and ongoing evolution in virulence cannot be discounted, particularly if litter reuse increases and reliance on vaccination for control of MD in broilers increases.

8.4 Evaluation of various tissues/ sample materials for MD prediction and routine MD monitoring (Chapters 4, 5 and 6)

One issue which emerged throughout the studies presented in the thesis is the evaluation of the value of various tissues/sample types for MD monitoring and evaluation (e.g. blood/PBL, spleen, feather tips, dander). While a more detailed analysis and results for each of the tissues, except blood, is given in the respective chapters, the advantages and disadvantages of each of these tissues and their value for early and routine monitoring and prediction of MD is discussed in the following and a comparison of the value of spleen, feather tip, dander and blood (Islam et al., 2006b) with regards to predictive power of subsequent MD is discussed below and summarised in Table 8.1.

Feathers are the ideal tissue to study the time course of MDV replication in individual chickens as they can easily and repeatedly be sampled from live birds. Previous studies have reported that viral copy numbers are higher in feather tips than peripheral blood lymphocytes (PBL) for the MDV1 vaccine strain Rispens/ CVI988 over a 28 day period post infection (Baigent et al., 2005b). These authors showed that feather tips are a good predictor of viral load in spleen, the predominant site of immune responses to MDV antigens. As well, viral load in feather tips have been shown to be a suitable tissue in order to determine success of vaccination with CVI988 in individual birds (Baigent et al., 2005b; 2007) and for surveillance of poultry flocks for the presence of pathogenic MDV1 (Handberg et al., 2001).

However, in terms of prediction of subsequent MD outcome the results of Chapter 5 are not straightforward. While there was a significant positive association between isolator group means for MDV1 in FT at 14 dpc and subsequent group incidence of MD, on an individual chicken basis, FT was a poor predictor of MD outcome. In fact it was negatively associated with MD due to reductions in MDV in FT after 21 dpc in birds that went on to develop MD. This is a novel finding, for which there is no immediate explanation and it should be confirmed in further studies. It was not observed in a similar study in broiler chickens (Islam et al., 2008b) in which there was a strong positive association between MDV load in FT

and MD severity score from day 14 onwards. Nor was it reflected in the MDV1 load in dander samples collected in this experiment, for which there was a positive association with MD severity.

In the present study treatments effects such as that of vaccination and challenge MDV were less evident in the FT data than in 14 dpc spleen data or the weekly dust data. Whether this, and the comparative lack of association with MD reflects a true difference between experiments based the use of a layer genotype in this experiment (cf. broilers in Islam et al., 2008b) and the use of virulent MDV rather than the attenuated vaccinal strain used by Baigent et al. (2005b) needs to be resolved by further experimentation. It is possible that contamination of feather samples during and after processing may have masked treatment effects, but this is unlikely as similar precautions were taken in both the layer and broiler experiment, and samples were cleared of superficial contamination prior to analysis. Resolution of these issues is urgent as FT remains an ideal tissue for longitudinal studies on MDV requiring individual chicken data.

Baigent et al. (2005b), using real-time PCR, found that the detection of the vaccinal MDV1 isolate CVI988 in peripheral blood mononuclear cells (PBMC) lagged behind the presence of MDV in lymphoid organs which is consistent with the gradual circulation of lymphocytes, particularly from the spleen, into the bloodstream. While these studies did not correlate the MDV load in PBMC with subsequent MD outcome, Islam et al. (2006b) have shown that chickens which subsequently developed MD lesions had a significantly higher MDV load in peripheral blood lymphocytes (PBL) at 14 and 21 dpc. Therefore, blood is of considerable value with regards to prediction of subsequent MD, and, like FT offers the possibility of repeated measurements from an individual bird. However, the major disadvantages of blood are that its sampling is invasive, time consuming and requires immediate post-collection processing to separate leucocytes.

As would be expected in another lymphoid tissue MDV1 load in spleen at around 14 dpc has been repeatedly shown to be a very good predictor of subsequent MD (Table 8.1; Walkden-Brown et al., 2006; 2007b; Islam, 2006; Islam et al., 2008a), a finding confirmed again in Chapter 4. In the case of spleen, this strong correlation is understandable given that the spleen is the primary site of early

cytolytic infection (Baigent and Davison, 1999) and also a major site of lymphocyte transformation (Schat, 1981). Although the sampling of spleens requires the sacrifice of the respective bird, it offers some advantages over blood due to the ease of collection, lack of post-collection processing, large number of infected cells harvested, and ease of storage.

Dander offers some significant advantages as a material to sample for MDV analysis. It is easy to sampling under field conditions, integrates information from a lot of chickens, does not require the sacrifice of any birds or invasive sampling, and does not require special conditions for transportation and storage as earlier studies have shown that MDV in dander keeps its infectivity over a long time even at high temperatures (Carrozza et al., 1973; Blake et al., 2005). The value of dander as a diagnostic sample for MD was clearly demonstrated in Chapter 6, where the effects of vaccination and challenge MDV virulence were clearly reflected in the MDV1 load in isolator exhaust dust from day 14 onwards. These effects were much more pronounced in the layer chicken study (Chapter 4) than in a similar study in broilers (Islam and Walkden-Brown, 2007), due largely to the greater range of MDV virulence, and vaccine effects observed in the layer experiment. Indeed this study is the first study in which a positive association between virulence of MDV and the rate of shedding of MDV from the host has been demonstrated. The dander study (Chapter 6) has again demonstrated the utility of qPCR for the differentiation and quantification of the different MDV serotypes in feather dander. This will have significant implications for the routine monitoring of MD using qPCR assay of dust, for epidemiological modelling of the behaviour and spread of MDVs in chicken populations and for studies into the evolution of virulence in MDV1. The present study is the most powerful demonstration to date of the utility of this very simple measure which integrates information from a number of birds in a single sample.

Table 8.1: Comparison of various sample materials with regards the value of their MDV1 load as a predictive tool for MD status.

Sample material	Invasive technique	Sacrifice for sampling	Ease of collection	Risk of contamination	Storage	Repeat sampling	Individual bird sample	Prediction of subsequent MD status?	Reference
Spleen	Yes	Yes	Moderate	Medium	-20ºC	No	Yes	Excellent	Chapter 4, (1,2)
Feather	No	No	Very easy	High	-20ºC	Yes	Yes	Variable	Chapter 5 (3)
Dander	No	No	Very easy	High	Room temp, -4 ºC or -20ºC	Yes	No	Excellent	Chapter 6
Blood (PBL)	Yes	No	Requires skill + post-sampling processing	Low	-20ºC	Yes	Yes	Good	(4)

1) Walden-Brown et al. (2007b)
2) Islam et al. (2008a)
3) Islam et al. (2008b)
4) Islam et al. (2006b)

8.5. Kinetics of MDV1 and HVT load in feather tips and dust (Chapters 5 and 6)

The patterns of MDV1 load in dust and feathers were broadly similar. However, in feathers, the MDV1 load peaked at 21 dpc, then declined sharply by about 1 log before plateauing until 56 dpc whereas in dust, the rapid increase to 21 dpc was not followed by a decline and MDV1 load increased slowly until 56 dpc, particularly in vaccinated chickens. Both the MDV1 pattern over time and the level of MDV1 viral copy numbers in dander was in broad agreement with what has been reported in broiler chickens (Islam and Walkden-Brown, 2007; Islam et al., 2008a).

The mean HVT load in feathers compared to dust generally showed the same trends over time with a peak of the HVT load at 19 dpv in both materials followed by a decrease before plateauing from 33-40 dpv onwards until the termination of the experiment at 61 dpv.

Unfortunately, the kinetics of both MDV1 and HVT in spleen were not investigated in the present studies. However, similar previous studies have reported that both viruses increase in spleens of MDV infected and HVT vaccinated chickens until 38-61 days of age (Tink et al., 2005; Islam, 2006; Islam et al., 2008a). Particularly for the HVT loads over time, this pattern in spleen is different from what is seen in feather tips or dust where the HVT load peaked around 14-21 dpc, declined and then plateaued until 56 dpc.

With regards to the effect of vaccination, the MDV1 amount in dust was significantly reduced ($P<0.0001$) in vaccinated chickens, particularly with the Bivalent vaccine. This was not the case in feathers where vaccination with HVT had no significant effect on the MDV1 amount ($P=0.54$, Fig. 5.6). This differs from the situation in the equivalent experiment in broiler chickens in which vaccination with significantly reduced MDV1 load in FT (Islam et al., 2008b) as well as in dander (Islam and Walkden-Brown, 2007). The reasons for the lack of a vaccination effect in FT in the present experiment are unknown but may include differences in chicken genotype, feather sampling method or contamination control. More likely is the fact that, as noted above, chickens that went on to

develop MD in this experiment paradoxically had depressed MDV1 load in FT. These chickens were predominantly unvaccinated and so the depression in MDV in these chickens would have confounded any decline in MDV1 in the vaccinated group which mostly did not go on to develop MD.

With regards to the effect of challenge isolate on the MDV1 load in dust, there was a clear differentiation between the 3 MDV isolates in direct proportion to their virulence – the first demonstration of increased shedding of MDV in more virulent isolates of MDV1. In FT differentiation of the more virulent 02LAR and the less virulent MPF57 was also observed although in a complex interaction with time post challenge that is clearly shown in Figure 5.6. The MDV1 amount in FT of chickens challenged with 02LAR was up to 10-fold higher than for MPF57 between 7 and 21 dpc, but significantly lower on days 35 and 42 dpc. In fact at 35 dpc the difference is more than 50 fold ($10^{5.1}$ VCN per 10^6 cells with 02LAR vs $10^{6.8}$ VCN per 10^6 cells with MPF57). Again, the FT data from this experiment show clear and unexpected differences from the dust data. A possible explanation is that there are differences in the composition of the two sample materials. While dust largely consists of keratinized feather material, the pulp of FT samples which are used for DNA extraction and subsequent MDV detection are a heterogenous mixture of cells, including lymphoid cells.

8.6 Behavioural aspects of chickens kept under experimental conditions (Chapter 7)

The public interest and awareness of the conditions under which chickens are routinely housed has increased dramatically in the past 10 years and continues to increase. However, the welfare issues still faced by many chickens – either broiler or layer chickens – are severe. Fast growth rates, low space allowance and poor environmental conditions can all contribute to major welfare problems. Environmental enrichment can improve poultry welfare and productivity by decreasing harmful behaviours, like fear or feather pecking. Feather pecking is one of the most serious behavioural problems, occurring both in caged birds, birds kept in floor systems as well as in chickens kept under experimental

conditions. The reduction of feather pecking and other harmful behaviour against flock mates in layer chickens kept in isolators was the main aim of this study. Based on previous reports (Jones et al., 2002), string bunches and sand boxes were successfully introduced to white leghorn chickens in a factorial experiment. Providing chickens with bunches of polypropylene string was clearly beneficial for all of the welfare variables under test although there was some entanglement of chickens in the string. Addition of sand boxes at 2 weeks of age had less impact, but appeared to improve the beneficial effects of using string. These results are sufficient to recommend the use of string in isolator experiments where feather pecking is a risk, and they also suggest that evaluation of sandbox introduction at day 1 rather than day 14 should also be investigated. The identification of a string or string substitute which does not fray would also prevent entanglement of chickens in the string.

8.7 Applications of findings

With the many recent advancements in the field of molecular diagnosis of pathogens, and particularly the advent of real-time PCR, it is now feasible to make rapid and comparatively inexpensive molecular tests for detection and quantification of all three serotypes of MDV commercially available to the poultry industry. Thus will enable more routine testing for MDVs and facilitate improved decision making on all MD control issues including vaccination, cleanout and biosecurity.

The qPCR technique has played a major role in the analysis of samples obtained in this study and it has provided novel information about kinetics of all three serotypes of MDV in feathers and dust. DNA sequencing of the *meq* gene of five Australian MDV1 isolates has identified a useful marker of virulence for MD (the number of PPPP repeats encoded), confirmed the relatively narrow range of virulence observed in the tested isolates and demonstrated that there is unique sequence variation amongst the Australian isolates compared to international and vaccinal isolates of MDV1.

The major applications of these studies are:

1. Based on the *meq* sequencing study, it is now feasible to develop a molecular test which will eventually enable to differentiate between pathogenic and vaccinal isolates of MDV1 using real-time PCR technique.
2. The results of the same study suggest that the number of PPPP repeats encoded by the *meq* gene is a sensitive and reliable marker for MDV virulence.
3. Based on the pathotyping study data, it is now feasible to consider short screening experiments for pathogenicity, based on early markers for MDV virulence such as MDV1 load in spleen and dust at 14 dpc. These measurements can be made prior to onset of clinical MD thus offering significant ethical advantages.
4. The series of 3 pathotyping experiments in chickens of different genotypes have demonstrated that this plays an important role for the outcome of pathotyping studies. It is therefore recommended that the tests be carried out in commercial chickens of the industry of relevance, so that the results of the pathotyping experiments have direct relevance.
5. The MDV1 load in dust has again shown to be of excellent value as it provides quantitative information on the status of MDV1 infection and vaccine viruses at a cost which enables constant routine MD surveillance in the field.
6. The data on shedding rates of MDV and the influence of host vaccination status, and virulence of the challenge MDV is the most complete data of its kind to date, and will be of great value when modelling the evolutionary pressures on wild-type MDV1s as they circulated in vaccinated chicken populations. It will also be of considerable use for modelling the epidemiology of MDV and predicting the consequences of different control interventions.
7. Environmental enrichment strategies such as string bunches or sand boxes should be provided for experimental chickens in isolators as they significantly reduce feather pecking and cannibalism.

8.8 Future work

1. Although MDV2 vaccines are only used to a very limited extent in Australia currently, there is evidence that MDV2 is present on many chicken farms. However, the epidemiology of this virus in the Australian poultry industry and its impact on the epidemiology of MD is totally unexplored. Extensive studies are required to define the molecular and biological diversity of available MDV2 isolates and their role in natural protection against MD in the field. These studies may also reveal a new vaccine candidate although 3 vaccinal strains of MDV2 are already available;
2. The sequencing study of the *meq* gene of Australian isolates has shown that there is sequence variation in the Australian isolates compared to other *meq* genes of several international isolates of MDV1. The study should be extended to a wider range of Australian MDVs covering a wide spectrum of virulence. The work should also extend to the sequencing of other promising unique MDV1 genes, e.g. vIL-8 or ICP4;
3. There is currently no qPCR method available to differentiate between vaccinal and pathogenic isolates of MDV1. Based on the sequencing study of the *meq* gene, a qPCR assay is currently under test and first results are very promising. However, further development of the MDV1 qPCR assay for differentiation between avirulent vaccine isolates eg. CVI988/BH-16/HPRS-16 and wild type MDV1 would be very useful for monitoring of vaccination success and MD status in layer and breeder flocks where chickens are routinely vaccinated with MDV1 vaccines;
4. The findings on MDV1 in feather tips of ISABROWN layers need to be confirmed in a follow-up experiment. The reduction in MDV1 load in FT observed in chickens that go on to develop MD needs to be confirmed, and, if so, the underling mechanisms established;
5. There is a lack of information on the rate spread of MDV infection in a chicken flock once a chicken in the flock is infected, either with vaccinal and/or pathogenic MDV of all three serotypes. Using the suite of MDV qPCR assays described in this thesis the daily spread of MDV's in single and mixed infections could be established.

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