Chapter 1 General Introduction

Marek's disease (MD) in chickens is a lymphoproliferative disease characterized by early lysis of lymphocytes resulting in bursal and thymic atrophy and immunosuppression, followed in susceptible chickens by T-cell infiltration of nerves and other organs culminating in some cases in lymphocyte transformation and lymphoma development (Calnek 2001b; Calnek and Witter 1991a; Payne 1985b). The causative agent of MD is Marek's disease virus (MDV) a cell associated oncogenic alphaherpesvirus (Churchill and Biggs 1967). MDV is ubiquitous and MD is a very important disease in commercial poultry flocks worldwide with an estimated cost to industry of $1-2 billion (Morrow and Fehler 2004). The disease is named after Jozsef Marek, who was the first describe the disease in the scientific literature (Marek 1907). Domestic fowls are the natural host of the MDV, but quail, geese and turkey are also affected.

After inhalation of MDV, the virus infects lymphocytes and replicates in feather follicle epithelial cells and is shed in large amounts in feather dander, commencing approximately one week after infection (Baigent and Davison 2004; Islam *et al.* 2006). The virus can survive in the environment for as long as several months at room temperature (Witter *et al.* 1968). There is no evidence that vertical transmission of MDV occurs from the hen to offspring through the egg (Solomon *et al.* 1970). MDV is extremely infectious and horizontal transmission is by respiratory route due to inhalation of infected dust particles (Beasley *et al*. 1970). Because the respiratory tract is not the site of virus replication, the virus can then be transported from the lung to the lymphoid organs (Adldinger and Calnek 1973) probably by macrophages and virus can be detected in the lymphoid tissue at 4 days post infection (Adldinger and Calnek 1973).

There are three serotypes of MDV which are now recognized as separate species (von Bülow and Biggs 1975a; von Bülow and Biggs 1975; von Bülow *et al.* 1975). Serotype 1 MDVs (gallid herpesvirus type 2) include all virulent and very virulent oncogenic viruses (Churchill and Biggs 1967; Nazerian *et al.* 1968) and naturally attenuated or non-oncogenic variants, some of which are used as vaccines, such as the Rispens CVI988 strain (Rispens *et al.* 1972a). The serotype 2 MDVs (gallid herpesvirus type 3) are naturally occurring non-oncogenic chicken MDVs (Schat and Calnek 1978a). Serotype 3 viruses (meleagrid

herpesvirus type 1 are the naturally occurring nononcogenic turkey herpesviruses (HVT) (Purchase *et al.* 1971; Witter *et al.* 1970a)..

All three serotypes have been used extensively as MD vaccines, alone and in combination (Okazaki *et al.* 1970; Witter 1982; Witter *et al.* 1987). Vaccine efficacy against early posthatch challenge with virulent MDV is significantly enhanced by injecting HVT *in ovo* (Gagic *et al.* 1999; Sharma and Burmester 1982) at transfer of eggs from setters to hatchers, so is particularly suitable for vaccination of broiler chickens which are produced in the tens of millions daily worldwide. The three serotypes of MDV have many common antigens and are distinguished by serological tests (Lee *et al.* 1983; Ross *et al.* 1975; von Bülow and Biggs 1975b) and more recently by DNA based PCR tests (Islam *et al.* 2004; Renz *et al.* 2006). Virulence of the virus and the genetic background of the chickens determine the consequences of the disease (Nair 2005; Sharma 1976; Weigend *et al.* 2001).

The nature of MD has changed since the disease was first described. The classical form of MD was seen in older chickens and was known as “fowl paralysis”. It involved leg and wing paralysis often with a typical appearance of one leg forward and one leg back. The paralysis was due to lymphocytic infiltration of peripheral nerves, the spinal cord, and associated ganglia (Marek 1907). Mortality was generally low, not often reaching 15%. In the 1950s, as the industry intensified an acute form of MD was observed in adult chickens and sometimes young chickens, with lymphoma formation in the viscera becoming the dominant pathology, in addition to nerve involvement (neurolymphomatosis) (Biggs 1962; Pappenheimer *et al.* 1926), Morbidity in the acute form could be 10–30% and it is possible to reach 70% with high levels of associated mortality. Acute MD threatened the viability of intensive poultry rearing worldwide and resulted in intensive research programs in the UK and USA that ultimately ascertained the cause of MD (Churchill and Biggs 1967) and subsequently produced the first vaccines against MD in 1970 – HVT in the USA (Okazaki *et al.* 1970) and serotype 1 HPRS16 in the UK (Churchill *et al.* 1969b). Also, there was an intensive research program in the Netherlands which led to the development of the CVI988 vaccine (Rispens *et al.* 1972a; Rispens *et al.* 1972b).

MD continued to change and evolve after the introduction of vaccination (Davison and Nair 2005). Change in virulence and tissue tropism has been associated with appearance of other forms of the disease such as transient paralysis (TP) and a more severe form of TP

known as acute transient paralysis (ATP), which is induced by more virulent strains of MDV. In the former case there is an acute onset flaccid paralysis of the neck and wings followed by complete recovery within 24-48 hr (Kenzy *et al.* 1973; Zander 1959) while in the later form the acute onset flaccid paralysis is earlier (acute) 9-10 days post challenge (pc), and most affected birds died in 1-3 days after the onset of clinical signs and recovery was rare (Witter *et al*. 1999). The main lesion of TP is vasculitis and vasogenic oedema in the brain plus perivascular cuffing with mononuclear cells (Swayne *et al.* 1989a; Witter *et al.* 1999). However, unlike the case with classical and acute MD, these newer forms of MD are not observed in the field, and appear to a laboratory phenomenon associated with high-level challenge of chicks free of maternal antibody directed against MD with highly virulent MDV.

MD induced by virulent pathotypes emerging in 1950s was successfully protected against by HVT. However, the vaccines, while protecting against MD, did not prevent infection with MDV (Schat and Baranowski 2007) so it is quite possible to have chickens infected with all 3 serotypes and shedding them simultaneously (Islam and Walkden-Brown, 2007). Such imperfect" vaccines created conditions for natural selection towards higher virulence (Gandon *et al.* 2001). Indeed, in the USA, MDV has been evolving steadily towards higher virulence with breakdowns in the protection induced by various vaccine preparations (Witter 1997). Very virulent MDV (vvMDV) has the ability to break HVT vaccination and very virulent plus (vv+) MDV capable of resisting bivalent vaccine, which is a combination of MDV-2 and HVT. Currently, the CVI988/Rispens vaccine (Rispens *et al.* 1972b) which is a naturally low pathogenic strain belonging to serotype-1, provides the best long term protection against highly virulent MDs of all commercially available vaccines (Chang *et al.* 2011; Witter *et al.* 1995). More recent modeling studies based on experimental findings suggest that both imperfect vaccination and reduced mean lifespan of chickens have contributed to the evolution of virulence of MDV (Atkins *et al.* 2012).

As a result of increases in MD and apparent vaccination failure, a system for pathotyping MDV1 was developed at the USDA-ADOL (Witter 1997; 1998b; Witter *et al.* 2005). The system is founded on the protective index (PI) provided by herpesvirus of turkeys (HVT) and bivalent HVT/MDV serotype 2 (MDV2) vaccines using the presence or absence of gross MD lesions as the diagnostic criterion in vaccinated and unvaccinated SPF chickens against MDV. New isolates have been tested against the reference strains as a means of ranking on

virulence, based on the incidence of MD in vaccinated and unvaccinated groups relative to the reference strain (Witter 1997; 1998b; Witter *et al.* 2005).

MDV isolates have been classified into mild (mMDV), virulent (vMDV), very virulent (vvMDV) and very virulent plus (vv+MDV) pathotypes (Witter 1997; 1998b; Witter *et al.* 2005). In Australia, initial MD pathotyping experiments were carried out in the late 1980s (McKimm-Breschkin *et al.* 1990). Although MDV doses were not fixed and a range of infective material was used to challenge SPF chickens, free of maternal antibody directed against MDV, some very virulent MDVs appear to have been isolated. In the early 1990's, following the introduction of new genotypes of layer and broiler chickens into Australia, there was a failure of vaccinal protection against MD using Australian MD vaccines and vaccination procedures. This led to a major epidemic of MD between 1992 and 1997 when the outbreak was brought under control with the introduction of the Rispens CVI988 vaccine and *in ovo* vaccination of broiler chickens using cell associated HVT vaccine. During the early stages of the epidemic, MDV isolates such as Woodlands 1 (Zerbes *et al.* 1994)) and MPF 57 (De Laney *et al.* 1995) were characterised based on the presence or absence of gross MD lesions and extent of immunosuppression following challenge of maternal antibody-free SPF chicks. These isolates induced 60-100% mortality and gross lesions in unvaccinated SPF chicks and were accordingly pathotyped as vvMDV (De Laney *et al.* 1995; Zerbes *et al.* 1994), despite marked differences in protocol compared to the USDA-ADOL method. Consequently the Australian industry commissioned pathotyping studies based on these methods. This showed that Australian isolates of MDV1, which caused enormous losses in both the layer and broiler industries between 1994 and 2002, induced gross MD lesions in 53 to 94% of unvaccinated chickens, and HVT and bivalent vaccine induced protective indices ranging from 38 to 100% at 56 days post challenge (dpc). This work showed that current Australian isolates of MDV1 were mainly v and vv pathotypes with no clear increase in virulence over time (Renz *et al.* 2012; Walkden-Brown *et al.* 2013b).

In the Australian studies it was observed that, in SPF chickens free of maternal antibody against MDV, significant numbers of birds exhibited an acute paralytic syndrome between days 9 and 15 post challenge with MDV resulting in overall mortality rates of 2-18% (Renz *et al.* 2012; Walkden-Brown *et al.* 2007c). This syndrome which very closely resembled the ATP described by Witter et al. (1999) was not observed in commercial layer or broiler chickens challenged with the same dose and isolates of MDV, presumably due to the

protective effect of maternal antibody. This is consistent with reports on TP and ATP in the literature in which it appears that these syndromes are only seen in maternal-antibody negative chicks under experimental conditions. Therefore these newer syndromes are not a field disease problem, unlike classical and acute MD which were or are major disease threats in commercial populations of chickens. Several studies overseas have shown that antibody (Ab) against MDV has restrictive role on the pathogenesis of MDV (Calnek 1972b; Chubb and Churchill 1969; Kermani-Arab *et al.* 1976). One major aim of this thesis is therefore to test the effect of anti-MDV antibody on the pathogenesis of MD, particularly on the development of TP and ATP.

Relevant to this is the development in the USA, of a pathotyping system for MDV based on the timing and severity of neurological clinical signs following challenge with MDV in maternal antibody negative SPF chicks (Gimeno *et al.* 2002). In this system the incidence of neurological signs after 23 dpc were determined and related to results of longer formal pathotyping experiments using the USDA-ADOL method. This offers opportunity for shorter, cheaper and ethically more acceptable experiments to determine pathotype. Another aim of this thesis is to take the most virulent Australian isolates (MPF23) from the 1980s (McKimm-Breschkin *et al.* 1990) and to evaluate its pathogenicity against the Australian reference strain MPF57 in SPF chickens using Gimeno's neuropathotyping system and compare the results with pathological lesions occurring up to 56 dpc.

The diagnosis of infection with MDV was originally based on isolation and identification of MDV from the infected tissues in cell culture or identification of the infected cells by immunostaining (Churchill and Biggs 1967; De Laney *et al.* 1998). However, recently polymerase chain reaction (PCR) has appeared as a quick and sensitive method of diagnosis of MDV in a variety of tissues and materials. Real-time quantitative polymerase chain reaction (qPCR) assays to measure MDV genome copy number in chicken tissues and environmental samples were developed simultaneously (Baigent et al. 2005; Islam et al. 2004; 2006) and routine moitoring of MDV in broiler shed dust samples has now been practiced since 2005 in Australia (Walkden-Brown et al., 2013b).

In Iraq, there are limited data on the prevalence of MD in the field and there are no data available on the virulence of the MDV isolates currently circulating in Iraq. MD in Iraq was documented for the first time in the late 1970s and early 1980s (Al-Soudi *et al.* 1986; El-

Meligy *et al.* 1988). In an isolation and challenge study, Al-Attar (1997) isolated MDV from MD field cases. There is no clear evidence of MD vaccine failure in Iraq, but outbreaks of MD in vaccinated flocks of commercial farms of layers chickens were reported more recently (Al-Attar 1997; Al-Aubaedi 2000; Zahid 2008). Accordingly, another aim of this thesis was to conduct a molecular survey of MDV prevalence in commercial broiler and local village chickens in Iraq using real-time PCR and to sequence the meq gene for molecular pathotyping as per Renz *et al*. (2012) to ascribe putative pathotypes to these MDV isolates on the basis of *meq* gene polymorphism. There is significant polymorphism in the sequence of the MDV *meq* gene a key oncogene involved in the ability of MDV to induce lymphoid tumours. This may eventually be linked to virulence or be used as a genetic marker for a given isolate.

Chapter 2 Review of literature

2.1 Introduction

Marek's disease (MD) is an immunosuppressive lymphoproliferative disease of chickens caused by the highly infectious cell-associated alphaherpesvirus. Three Marek's disease virus (MDV) serotypes (von Bülow and Biggs 1975b) are classified: MDV serotypes 1 and 2 (MDV-1 and MDV-2) as well as herpesvirus of turkeys (HVT or MDV-3) (Van Regenmortel and Fauquet 2000). The classification of MDV based on the serotype differentiation is widely used. However, 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).

All pathogenic MDVs belong to MDV-1 (Calnek and Witter 1991b; von Bülow and Biggs 1975; von Bülow *et al.* 1975) and constitute a mixture of strains varying in pathogenic and oncogenic potential. After detection of MDV as the causative agent of MD in 1967 (Churchill and Biggs 1967), the work was directed to progress of vaccines. The first vaccine for MD was an attenuated MDV-1, which was developed in the UK (Churchill *et al.* 1969a). Almost at the same time the efficacy of HVT as a vaccine against MD was confirmed in the USA (Okazaki *et al.* 1970) and the first HVT vaccine was licensed in the USA and proved to be highly effective. In the next years, naturally occurring nonpathogenic strains of MDV-1, MDV-2, and HVT or MDV-3 have been used alone or together as vaccines against MD (Calnek *et al.* 1983; Hirai *et al.* 1986; Witter 1982; Witter 1997). At the same time in the Netherlands, Rispens and his colleagues describe the use of the naturally attenuated strain of serotype-1 of MDV, which also commonly called Rispens (CVI988) (Rispens *et al.*1972), derivatives of which have been serving recently as the best protecting MD vaccines.

Despite the extensive use of MD vaccines since 1970, MD remains an important disease threat (Morrow and Fehler 2004). Moreover, MD is a rather unique disease in that vaccination against MD may protect chickens against clinical MD but it does not prevent infection or shedding of co-infecting MDV from the feather follicle epithelium (FFE) (Calnek

et al. 1970; Davison and Nair 2005; Islam *et al.* 2005a). This could cause not only maintain MDV transmission and reservoirs but also provide a basis to the suggestion of increasing the virulence of MDV. Thus, there are concerns that newly evolving strains circulate in chickens, resulting in breaks in vaccine protection, hence threatening the effectiveness of the existing MD vaccines (Eidson *et al.* 1978; Gimeno 2008; Schat and Baranowski 2007; Witter 1998a).

Studies have shown that field isolates in the USA appear to have evolved over time towards greater virulence (Witter 1997) based on a formal pathotyping system developed by (Witter 1997) and subsequently refined (Witter *et al.* 2005). MDV-1 strains are classified as mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+)) (Witter 1997).
In a similar manner the clinical and pathological pictures of the disease have changed since the first description of disease in 1907 by Marek (1907), who reported a generalized polyneuritis in chicken. In the early 1950s, a “classical” form of the disease manifested itself paralysis and paresis in older chickens with predominant gross enlargements of the peripheral nerves, causing partial or complete paralysis of the legs and wings while lymphomas were noted infrequently mainly in the ovary. The morbidity and mortality rarely exceeds 15%. The duration of the disease may be over a few weeks or many months (Campbell 1956; Marek 1907; Pappenheimer *et al.* 1926).

During the late 1950s and through the 1960s an acute form of MD with high mortality was described in layers causing 40% mortality in layers 6-16 weeks old. Lesions were also frequently observed in up to 10% of broilers with outbreaks sometimes involving up to 70% of the flock. Chickens were severely depressed and some died without clinical signs. Lymphoma formation was most frequent in the skin, skeletal muscles and visceral organs in addition to involvement of peripheral system (Benton and Cover 1957; Benton *et al.* 1962). This disease syndrome was designated as “acute MD” to differentiate it from the earlier form named “classical MD” (Purchase and Biggs 1967).

Increasing virulence of MDV strains causes vaccine failure in the USA (Witter 1997), Europe (Barrow and Venugopal 1999; Powell and Lombardini 1986) and Australia (De Laney *et al.* 1995; McKimm-Breschkin *et al.* 1990). With the more virulent MDV strains, acute transient paralysis (3-7) days post infection (dpi) with superior cytolytic action, unexpected tissue tropism, increased atrophy of lymphoid organs and immunosuppression, has a greater capability to transform T cells, and induce earlier mortality of their host (Barrow and

Venugopal 1999; Calnek 1998; Witter 1997). Vasogenic oedema in the brain was the typical finding. Recently, more virulent strains of MDV have been described with higher frequency in vaccinated flocks (Baigent *et al.* 2006; Gimeno 2008; Witter 1983; 2001b). Experimentally, many of these recent MDV isolates produce high early mortality syndrome (EMS) (Walkden-Brown *et al.* 2007a; Witter *et al.* 1980). (see section 2.8.2.6) with an acute early cytolytic disease in the form of severe atrophy of the lymphoid organs, thymus and the bursa of Fabricius in SPF chickens free from Mat-Ab (Witter *et al.* 1980). Part of this syndrome is a transient paralysis (TP), first described by Zander (1959). The typical signs were an acute onset of flaccid paralysis of the neck and wings followed by a sudden and complete recovery within 24-48 hr. In contrast, a more severe acute TP was identified experimentally (Gimeno *et al.* 1999; Witter *et al.* 1999). It was characterised by severe neurologic signs 9-10 dpi generally followed by death 1-3 days subsequent to the onset of neurological signs (Witter *et al.* 1999).

Studies have shown that field isolates in the USA appear to have evolved over time towards greater virulence (Nair 2005; Witter 1997). Based on a formal pathotyping system developed by (Witter 1997) and subsequently refined (Witter et al. 2005), MDV-1 strains are classified as mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+) (Witter 1997).

Because lymphocytes are one of the main target cells of MDV (Calnek *et al.* 1979; Payne and Renine 1973), diagnosis, isolation and monitoring of MDVs has historically been based around lymphocytes or lymphocyte-rich tissues. However, the other key target cell for the virus is the feather follicle epithelium (FFE) and high levels of virus are found in this tissue, and keratinized epithelial cells shed as dander during chicken growth. QPCR tests to detect and quantify MDV in this stable material are now proving useful to detect viral replication at this site (Islam *et al.* 2006; Renz *et al.* 2006; Walkden-Brown *et al.* 2013a).

2.2 MD History

The early reports of MD were descriptive and suggested that the clinical picture was linked mainly to pathological changes in the peripheral nervous system. Moreover, the causal agent of MD remained obscure to the earlier investigators. MD was first described in 1907 by Josef Marek (Marek 1907), who identified the syndrome in four cockerels that were affected

with paralysis of the legs and wings. Marek determined chickens as being affected whenever thickening of the sacral plexus was present, and the thickening was due to monocellular infiltration. He referred to the disease as “neuritis interstitialis” or “polyneuritis”, but he failed to determine the causative agent of the disease. Marek regarded the degeneration of the nerve fibres to be secondary to the cellular infiltration (Payne 1985a).
The next description of the symptoms was by Kaupp (1921), who found no pathological changes in the cerebellum, medulla or in the spinal cord segment related to the paralysed part, but he recognized congestion in the peripheral veins. The finding of Kaupp was also supported by May *et al.* (1925) who stated that microscopic lesions of brain and spinal cord provided little information to support the diagnosis of the disease. However, Doyle (1926) described the symptoms and the pathology of the disease, and he stated that symptoms depended upon the portion of nervous system involved, and attributed the lethargic symptoms to the occurrence of encephalitis and iritis causing a contracted, non responsive pupil.

In 1926, the disease was recognized as a neoplastic disease producing tumors in various visceral organs when Pappenheimer *et al.* (1926) found that some chickens had lymphoid tumors in visceral organs in addition to the lesions in the central and peripheral nervous system; these authors gave a good description of the histopathology of the brain, cord and peripheral nerves. Efforts to demonstrate the infectious agent of the disease were still unsuccessful at this stage.

Early nomenclature of the disease lacked clarity because these names either identified the histological character or the clinical picture of the disease (Sevoian 1967); they were insufficient to discriminate MD from other diseases with similar histological lesions, thus creating an issue in differential diagnosis. Initially, the name fowl paralysis was the common name used to refer to the syndrome (Pappenheimer *et al.* 1926). Other names used included polyneuritis, which indicated an inflammatory nature of the lesions, and this was the original description of the disease provided by Marek (1907). Further synonyms used in the early years were leukosis of chickens which led to confusion between lymphoid leukosis (LL) and MD.

In the late of 1940s and early 1950s, coinciding with the development towards industrial poultry production, MD took on a new form that caused high mortality in broiler chickens at a

young age of around 4 weeks due to an increasing incidence of visceral lymphomatosis (VL) in several visceral organs. In the late 1950s, the paralytic syndrome occurred at a relatively low frequency but there was a great diversity of tissues involved in VL, including the skin and muscle (Benton and Cover 1957).

In the late 1960s, losses from the acute lymphomatous form of MD reached their peak and the disease had spread to most countries with industrialized chicken production. In 1970 the financial losses from carcass condemnations in the US, mainly from MD, increased to US$200 million per annum, with a condemnation rate of 1.6 % of all the broilers inspected. The most significant discovery during this period was the isolation of the causative agent of the disease (Churchill and Bigg: Solomon *et al.* 1968). On the basis of virus isolation and serological reactions (Chubb and Churchill 1968; Nazerian *et al.* 1968; Purchase 1969), a herpesvirus was considered to be the etiological agent of MD. This was also when finally the longstanding confusion with regards to nomenclature of MD and other neoplastic diseases was resolved; retroviruses were found to be the causative agent of avian leucosis and reticuloendotheliosis (Calnek *et al.* 1970; Churchill and Biggs 1967).

After the isolation of the causative agent of MD, Churchill and his colleagues attenuated a virulent MDV-1 isolate to produce a vaccine (Churchill *et al.* 1969a). However, it was the HVT vaccine that firstly got licensed for commercial use in the USA and has proven to be a great success (Okazaki et al., 1970; Witter et al., 1970). Since the introduction of vaccines against MD, economic losses due to MD have been drastically reduced, and vaccination has since been used successfully around the world. However, the introduction of more aggressive vaccination strategies over recent decades appears to have driven MDV to evolve to strains with higher virulence (Atkins *et al.* 2012; Davison and Nair 2005) and thus MD continues to threaten the poultry industry around the world.

2.3 MDV the causative agent of MD

2.3.1 MDV classification

Marek's disease viruses belong to the family of *Herpesviridae*, subfamily *Alphaherpesvirinae*. On the basis of the genomic organization, MDVs are placed within the

genus *Mardivirus*. Members of Herpesviridae are double stranded, enveloped DNA viruses. MDV was classified on the basis of DNA sequence as an alphaherpesvirus homology and genome organization (Lee *et al.* 2000; Tulman *et al.* 2000). Three members of the *Mardivirus* were initially categorized based on serological characteristics. The pathogenic serotype 1 (MDV-1), the nononcogenic serotype 2 MDV from chickens (MDV-2), and the related nononcogenic herpesvirus of turkeys (serotype 3; HVT).

The complete genomes of all three serotypes have been sequenced completely in recent years (Afonso *et al.* 2001; Tulman *et al.* 2000) that has led to a re-classification of MDV serotypes as separate but closely related species. The official nomenclature of the three serotypes according to the International Committee on the Taxonomy of Viruses (ICTV) is shown in Table 2.1. As the serotype classification is still widely used and convenient, this classification system is used throughout the thesis.

Table 2.1: Taxonomy and attributes for MDV

Serotype	ICTV taxonomy	Attributes
MDV-1	Gallid herpesvirus 2 (GaHV-2)	Oncogenic and cause MD in chickens mainly. They spread efficiently between birds.
MDV-2	Gallid herpesvirus 3 (GaHV-3)	Non-oncogenic non-pathogenic MDV of chickens. Spreads efficiently between birds.
HVT (MDV-3)	Meleagrid herpesvirus 1 (MeHV-1)	Non-oncogenic herpesvirus of Turkeys does not spread efficiently between chickens.

2.3.2 The MDV virion

Structurally, MDV resembles other herpesviruses. Members of the Herpesviridae are double stranded, enveloped DNA viruses with roughly spherical morphology. The MDV virion includes an envelope, comprising a complex, loose, lipid sac incorporating the viral glycoproteins, which surround an amorphous tegument (Davison and Nair 2004). Within the tegument, an icosahedral capsid encloses a linear double-stranded DNA core (Figure 2.1). In MDV, as for all herpesviruses the icosahedral capsid is composed of 162 capsomers (Homa and Brown 1997). Because of the envelope, the virion is somewhat pleomorphic and

can range in diameter from 120-200nm (Fenner *et al.* 1993). The morphology of the other viruses of the MDV group resemble MDV-1 with few but potentially important differences (Silva *et al.* 2001).

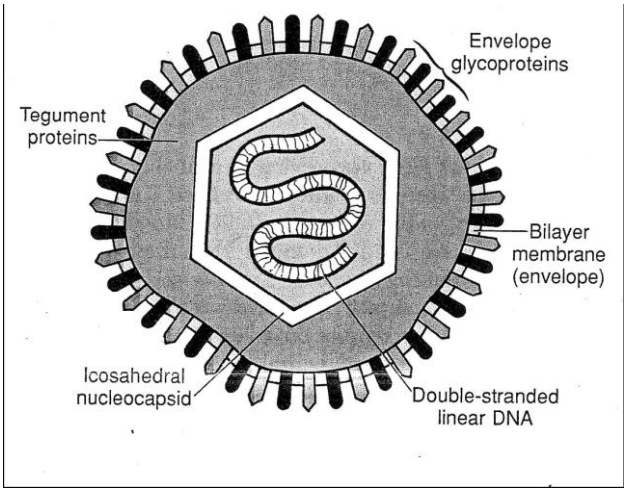

Figure 2.1: Schematic diagram showing the structure of the enveloped herpesvirus (Straus 1993).

Typical naked particles, which are without the outer envelope, were seen in negatively stained preparations of concentrated culture fluids. These particles measured 95-100 micron and had 162 capsomeres. All virus found in extracellular fluids has been naked (Nazerian and Burmester 1968; Schat 2005).

2.3.3 The MDV genome

The genomes of herpesviruses are all double-stranded linear DNA molecules that range in size from 108 to 230 kbp (Damania 2004; Maotani *et al.* 1986). A total of six different general genome organizations are distinguished in the herpesviruses (Roizman and Pellett 2001). Only class D and E genomes are found in the *Alphaherpesvirinae.* All three members of MDV have a general genome structure that resembles the herpes simplex virus genome (Figure 2.2); that is, unique long (UL) and unique short (US) regions are flanked by terminal and internal, long, and short repeat regions: (TR_L, TR_S, IR_L, and IR_S) (Cebrian *et al.* 1982).

MDV DNA has a sedimentation constant of 56*S* corresponding to a molecular weight of 1.2 x 10^8 daltons. In the alkaline gradients, the largest and most prominent band contains a DNA sedimenting at 70*S* corresponding to 6.0 x 10^7 daltons in molecular weight (Lee *et al.* 1971). The gene contents and linear arrangements of the three viruses are generally similar, but vary considerably with regard to guanine and cytosine (GC) content and size. MDV-1 has a GC content of 44.1%, while that of HVT and MDV-2 are 47.5 % and 53.6 % respectively (Afonso *et al.* 2001; Izumiya *et al.* 2001; Kingham *et al.* 2001; Lee *et al.* 2000; Tulman *et al.* 2000). The number of genes encoded by the three serotypes of MDV varies also with 103 for MDV-1, 102 for MDV-2 and 99 for HVT (Davison and Nair 2005).

The majority of genes identified in the gene repertoire of all three viruses consists of ORF that are homologous to genes found in other *Alphaherpesvirinae (Osterrieder and Vautherot 2004)*. Genus- and type-specific genes are also present in the three genomes. Among these, the most prominent are the MDV-1-specific *meq* (MDV EcoRI-Q) and the *pp38* (phosphoprotein-38) genes that both of which have been implicated in MDV-1 latency and tumour formation. In addition, a chemokine-encoding gene, *vIL8* (viral interleukin-8), is expressed by MDV-1, but is absent from the avirulent MDV-2 and HTV genomes (Parcells *et al.* 2001).

Genes encoding proteins involved in T-cell transformation (*meq*) and others with potential involvement in tumorigenicity, viral virulence, and host range (pp24, pp38, IL-8, and SORF2) have been described (Brunovskis and Velicer 1995; Cui *et al.* 1999; Ikuta *et al.* 1985; Tulman *et al.* 2000), but are absent from the avirulent MDV-2 and HTV genomes (Parcells *et al.* 2001).

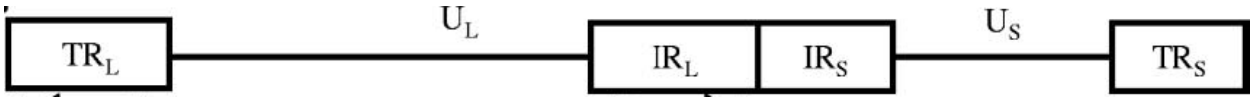

Figure 2.2. Genome organisation of MDV. Adapted from (Jarosinski *et al.* 2005). TR_L = terminal long repeat region; (UL) = unique long region; IR_L =internal long repeat region; IR_s =internal short repeat region; US= unique short region; TR_S = terminal short repeat regions.

The genomic sequences available for *Mardivirus* suggest production of approximately 100 proteins by each of the three viruses (Kingham *et al.* 2001). These are broadly divided into

three classes. The first class consists of the MDV genes that are homologous to other known alphaherpesviruses. The MDV homologues of unique long region of the MDV genome (UL); UL1 to UL54 fall into this class. These genes play similar roles in alphaherpesvirus replication (Silva *et al.* 2001).

A second class consists of the MDV genes that are shared by all three serotypes, but are not found in other herpesvirus genomes. Presumably, these are the genes explain why MDV is a lymphotropic virus that infects and replicates in chickens. Open reading frame (ORF); SORF, ORF 1 and SORF 3 and the MDV-specific ORFs in UL that flank the highly conserved UL1 – UL54 are located in all three serotypes (Silva *et al.* 2001). The third class contains the ORFs that are serotype-specific and are located in the repeat regions. These genes are expected to be involved in the pathogenic and oncogenic potential of MDV-1 strains.

2.3.3.1 MDV-1

MD is a lymphoproliferative viral disease caused by the pathogenic strains of MDV serotype-1 (MDV-1). Chickens are the main species affected. MD has been reported in some other types of birds; turkeys, pheasants, ducks, swans, partridge and quail (Biggs 1967).
Recent increases in MD-related mortality among vaccinated chickens have occurred in different parts of the world (Buscaglia *et al.* 2004; De Laney *et al.* 1995; Kross *et al.* 1998; Witter *et al.* 1999). Vaccination of newly-hatched chicks with live vaccines has been widely used to control MD, but vaccinated chickens become infected and shed MDV (Islam and Walkden-Brown, 2007). Vaccine breaks have occurred with regularity and there is evidence that the use of MD vaccines could be driving MDV to greater virulence (Davison *et al.* 2005; Hirai *et al.* 1986; Witter 1997). These viruses were characterized by higher cytolytic activity, unusual tissue tropism, increased atrophy of lymphoid organs, immunosuppression, enhanced capacity to transform T cells, and induced earlier host death (Barrow and Venugopal 1999b; Calnek et al. 1998b; Islam et al. 2000). Increasing virulence of MDV1 has also been associated with induction of MD in hosts other than chickens. Clinical outbreaks of MD in commercial turkey flocks, with mortality from tumours reaching 40-80% between 8-17 wk of age, have been reported in France, Israel, and Germany (Davidson et al. 2002a; b; Voelckel et al. 1999).

The molecular basis for this evolution is still unknown, although there is evidence that there are sequence changes in parts of the MDV-1 genomes. However, functional studies with mutant viruses are required to identify the determinants of altered virulence of these MDV pathotypes (Spatz *et al.* 2008; Spatz and Silva 2007). Currently, there are different methods available for virulence testing; a pathotypic classification of the Avian Diseases and Oncology Laboratory (ADOL) of the USA based on tumor response in vaccinated and control chickens has been proposed for pathotying classification. The ADOL protocol involves comparison of the tested viruses to prototype MDVs of known pathotypes within each challenge study of a susceptible genotype of chicken with maternal antibody (Mat-Ab) against MDV (Witter, 1997; Witter *et al.*, 2005) and viruses are classified under this as shown in Table 2.2.

As the ADOL procedure is slow, costly and difficult to replicate in different laboratories, a system for neuropathotyping MDV-1 strains is developed and is somewhat complementary to the ADOL pathotyping. The system uses the neurologic responses from SPF chickens free of Mat-Ab inoculated with strains of MDV-1 as a basis of classification. Such signs included transient paralysis (TP), and persistent neurological disease (PND) (Gimeno et al 1999). Accordingly, on the basis of the timing and severity of the neurologic signs, a tested strain of MDV-1 can be classified in one of three neuropathotypes A, B, and C, which is roughly compatible to the pathotypes virulent, very virulent, and very virulent plus, respectively (Gimeno *et al.* 2002). A description of the neuropathotypes is given below:
Neuropathotype A. Induces TP in a low to medium percentage of chickens (<50%), with very low mortality from 9-15 d.p.i (<10%). PND observed in few chickens between 21 and 23 d.p.i (<15%). Neuropathotype B. Induces both TP and PND at higher frequencies than Neuropathotype A (TP 33 - 96%, PND 18 - 58%). Mortality rate remains low (< 25%).
Neuropathotype C. Induces the most severe neurologic responses. The frequencies of TP and PND are very high, up to 100%. There is also a high rate of mortality (17 - 100%).

Table 2.2. USDA-ADOL classification of MDV pathotype (Witter, 1997)

Classification	Description
mMDV (mild)	Induces mainly paralysis and nerve lesions with little or no mortality in pathotyping experiments. Vaccination with HVT confers good protection. The predominant pathotype in "classical" MD. Classification based on significantly lower pathogenicity than JM/102/W.
vMDV (virulent)	Causes low levels of mortality by day 56pc, but induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT vaccination confers good protection. The reference US strain is JM/102/W and classification is based on lack of significant difference from JM/102/W in HVT-vaccinated chickens.
vv MDV (very virulent)	Causes moderate levels of mortality by day 56 post challenge (pc) and induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT vaccination is only partially protective but HVT/MDV-2 vaccines provide a high level of protection. The reference US strain is MD5 and classification is based on lack of significant difference from MD5 in HVT/SB1-vaccinated chickens.
vv+MDV (very virulent plus)	Causes high levels of mortality by day 56pc and induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT and HVT/MDV-2 are only partially protective. Classification based on significantly higher pathogenicity than MD5 in HVT/SB1-vaccinated chickens.

On a molecular basis, genes which are unique for MDV-1 strains have been used in attempt to pathotype MDV-1 strains recently. One major gene of interest is the *meq* (MDV *Eco*RI-Q) gene. The *meq* gene is is constantly expressed through latency and in all tumours (Kung *et al.* 2001; Liu *et al.* 1998). The *meq* knockout mutant is able to replicate *in vitro* in chicken embryo fibroblasts (CEF), but the *in vivo* replication ability of the *meq* knockout mutant is considerably attenuated and the reduction in growth *in vivo* coincided with the time of entry to latency or reactivation from latency, indicating that *meq* may play a role in the latency process (Nair and Kung 2004). However, some *meq* knockout mutants render the virus non-oncogenict but still induces lymphoid organ atrophy (Lee *et al.* 2012). *Meq* has also an anti-apoptotic function and the potential to stimulate cell growth and transformation by MDV-1 (Liu *et al.* 1998). The generation of a rMd5ΔMeq virus by deleting both copies of the ***meq***

gene from the genome of a very virulent strain of MDV-1, confirmed that Meq is involved in transformation but not in lytic replication in chickens (Lupiani *et al.* 2004). It is now also accepted that *meq* is a major oncogene of MDV-1 strains, as it closely resembles the *jun*/*fos* oncogene family in the N-terminal portion whereas mutations are mostly present in the proline-rich C-terminus of *meq* (Jones *et al.* 1992).

Differences in the *meq* gene between oncogenic and attenuated isolates of MDV-1 have been reported with a 177 to 180 bp/59-60 aa insertion in the *meq* gene of the attenuated vaccinal isolate CVI988 being possibly responsible for its non-oncogenicity (Chang *et al.* 2002c; Lee *et al* 2000; Shamblin *et al.* 2004 Spatz and Silva 2007). Some other strains of low virulence, i.e. JM10, MKT1 share this feature. However, several Australian isolates included a 177 bp insertion in the *meq* gene and these results do not support the association of this insertion with MDV-1 isolates of low virulence (Renz *et al.* 2012).

Rather, the number of PPPP repeats, independent of the presence of the insertion, is a better indicator of pathogenicity because the Australian isolates of MDV-1 had been previously pathotyped *in vivo* using methods based on the ADOL method of Witter (1997). Subsequently, the Australian isolates of MDV-1 were reported to vary significantly in pathogenicity (v to vv) (Renz *et al.* 2012; Walkden-Brown *et al.* 2007c). Also, Shamblin et al. (2004) suggested that an interruption at position 2 of the repetitions of four consecutive prolines (PPPP) was correlated with virulence (Figure 2.3).

By using the overall proline content and the repetition of four consecutive prolines (PPPP), it has been found that more virulent strains contain lowest repetition of four prolines and less virulent strains contain highest repetition of (PPPP)(Renz *et al.* 2012; Shamblin *et al.* 2004).

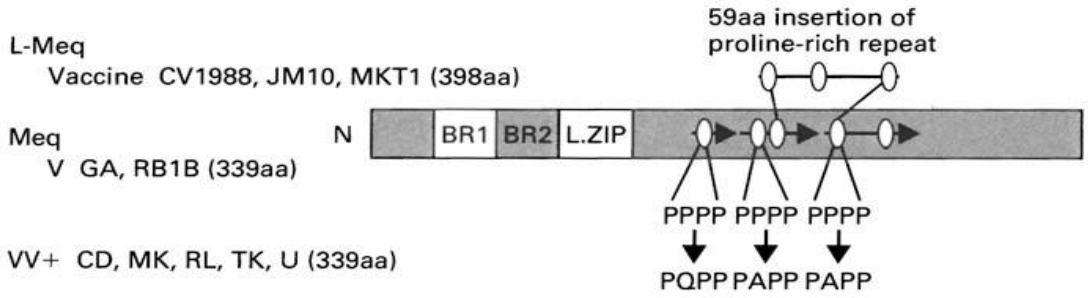

Figure 2.3. Natural variants of the Meq protein. Wild-type Meq, represented by the GA strain (Meq (339aa) carries two and a half of the proline-rich repeats (arrows) with multiple PPPP motifs (oval shape). L-Meq, represented by the vaccine strain CVI988, has a 59aa proline-rich repeat insert (top). The very virulent plus (vv+ MDV) isolates CD, MK, RL, TK and U carry mutations at the second position of the PPPP motif (Nair and Kung, 2004).

2.3.3.1 Transmissibility and Environmental survival of MDV-1

MDV replicates the fully infectious form in the epithelial cells of the keratinizing layer of the feather follicle epithelium (FFE), which then slough off and are shed as highly infective dander. Thus, dust of poultry houses and feather dander serve as the source for inhalation of infectious MDV. Natural infection is via inhalation of infectious dust and dander from MDV infected chickens (Beasley *et al.* 1970; Calnek *et al.* 1970; Jurajda 1972). Nazerian and Witter (1970) found that virulent strains of MDV1 were more readily detected in the feather follicle epithelium and more easily transmitted to contact chickens than avirulent strains including attenuated MDV1 and HVT. Baigent *et al. (*2005) reported that vaccine strain of MDV1 (CVI988 Rispens) was readily detectable in the feather tips after only 7 days while Islam *et al. (*2007) detected MDV1 and HVT viruses in dander filtered from isolator exhaust air from day 7 and MDV2 from day 12 after infection.

2.3.3.2 MDV-2

Some flocks of chickens around the world appear to be infected with MD viruses at some stage in their life, and they may show little clinical evidence of the disease when compared to identical groups with MD mortality of chickens. The suggestion was that the flocks

became protected through infection with a mild immunizing strain of MD virus (Zander *et al.* 1972). Serotype 2 MD viruses were first observed by Biggs and Milne who isolated several such viruses that differed from virulent MDV in pathogenicity, plaque size, affinity for growth in chicken embryo fibroblast cultures, and other characteristics (Biggs and Milne 1971). MDV-2 is comprised of apathogenic strains of MDV isolated from clinically normal chickens. They include strains such as SB-1 (Schat and Calnek 1978a), 301B/1 (Witter 1987) and HPRS-24 (von Bülow *et al.* 1975). The nononcogenic strain, SB-1 (Schat and Calnek 1978a) provided protection against MDV challenge on its own, but when administered with HVT it had synergistic activty, providing improved protection.(Calnek *et al.* 1983; Schat and Calnek 1978b; Witter and Lee. 1984). This prompted the introduction of combined serotype-2 and HVT (bivalent) vaccines, which had a higher protection index than HVT (Calnek *et al.* 1983; Witter 1982).

These avirulent serotype 2 MDVs could be differentiated from virulent serotype 1 MDVs and HVT (serotype 3) on the basis of type-specific antigens (Bülow and Biggs 1975). The development of monoclonal antibodies against the type specific antigens of all three serotypes by Lee *et al.* (1983) confirmed the existence of the three serotypes and made it possible to serotype isolates with ease and accuracy at that time since it was the most available and reliable method. More recently, molecular biology techniques such as PCR are more commonly used to separate the different serotypes/species (Walkden-Brown *et al.* 2003; Wang and Wei 1993).

Many studies have demonstrated that MDV-2 strains are nononcogenic and they have been widely used as vaccines alone and in combination with other strains providing significant protection against subsequent exposure or challenge with virulent virus (Witter 1976; Yao *et al.* 2007). This grew from the observation that the presence of MDV-2 isolates in HVT vaccinated flocks ameliorated the excessive losses sometimes seen in these flocks (Witter *et al.* 1984). Compared with HVT, the MDV-2 strains had lower growth rates, smaller plaque sizes, and larger syncytia sizes; they also grew to higher titres on CEF than DEF and did not grow as well on QT3 cells. The MDV-2 strains could also be differentiated from HVT by the relative titres of their antisera against HVT and SB-1 antigens (Witter 1983). MDV-2s produce extremely small amounts of cell-free virus (recoverable from sonicated cultures). Even after serial passage *in vitro*, the yield of cell-free virus is less than that of HVT. The serotype 2 viruses replicate readily in chickens, spread efficiently by contact, and produce

antibodies detectable by agar gel precipitation tests (indicating the presence of A antigen) (Schat and Calnek 1978b; Witter *et al.* 1990). SB-1, MDV-2 strain, can induce early protection against MDV challenges with virulent MDV after a few days of SB-1 inoculation (Schat and Calnek 1978b; Witter *et al.* 1990). Therefore, it was used as vaccine for MDV prevention. The combination vaccine of HVT and SB-1 was better for the growth rate and the general survivability of the birds when it is administrated *in ovo* rather than post hatching (Sarma *et al.* 1995). On the other hand, it is shown that serotype 2 viruses of MDV are endemic in most chicken flocks (Schat and Calnek 1978b; Witter *et al.* 1990). As a result freedom from prior infection seems important in the epidemiology of MD.

2.3.3.3 Herpesvirus of Turkeys (HVT)

HVT is a nononcogenic herpesvirus and antigenically related to MDV. It has been isolated from normal turkeys (Anderson *et al.* 1969; Kawamura *et al.* 1969; Witter *et al.* 1970a) which are its natural host.

HVT is classified as the third serotype within the (MDV) group. HVT is non pathogenic in chickens, but it does induce a viraemia which is associated with induction of protective immune responses against MDV-1 infection. Chickens infected with HVT become persistently infected and maintain long-lasting immunity (Purchase *et al.* 1972; Witter and Solomon 1972). HVT can be (highly) capable of spreading horizontally among turkeys (Witter et al., 1970). However, only very limited lateral transmission was reported in older chickens; transmission between young chickens infected early in life is not thought to occur (Cho and Kenzy 1975). This was confirmed in a recent study using molecular methods (Tink *et al.* 2005) indicating that HVT shed by chickens is largely non-infective for other chickens. The role of egg transmission of HVT in chickens has been extensively studied and there is good evidence that HVT is not egg-transmitted (Colwell *et al.* 1973; Paul *et al.* 1972).

In the USA, inoculation of chicks with HVT has been shown to induce immunity against MD (Okazaki *et al.* 1970). The development of immunity has been demonstrated by the protection of inoculated birds against artificial or natural challenge with virulent MDV and also by the appearance of antibodies, demonstrable by enzyme linked immunosorbent assay (ELISA) test (Cheng *et al.* 1984). The presence of anti-MDV maternal antibody apparently did not interfere with the establishment of an active immunity, as shown by the

development of serum neutralizing (SN) antibodies in all inoculated animals (Witter *et al.* 1971). There was no evidence that HVT spread from vaccinated birds to the sentinels or that cell-free infectious HVT was present in the feather follicles of vaccinated birds (Nazerian and Witter 1970). However, some scientists suggest that HVT might shed insufficient virus to spread effectively and this is supported by more recent studies (Islam *et al.* 2008b) . The lack of effective spread probably accounts for some visible MD lesions in vaccinated chicks that have not received an adequate amount of vaccine (Purchase and Okazaki 1971). Subsequently, the HVT vaccine was the first vaccine against MD approved in the USA and has since been a great success. In the UK, it was a MDV-1 vaccine which was the first licensed vaccine against MD (Churchill *et al.* 1969a). Synergistic effects were found when serotype 1 and 2 vaccines were administered with HVT (Calnek 1982; Witter and Schat 2003). In the Netherlands, Rispens (CVI988) was tested as a vaccine for the first time in 1971; it was found that CVI988 gave a high protective efficacy against MDV challenge (Rispens *et al.* 1972a; Rispens *et al.* 1972b). It provided superior protection, than the attenuated HVT, HPRS-16and R2/23 serotype-1 vaccines (Rispens *et al.* 1972a; Rispens *et al.* 1972b; Tan *et al.* 2007; Witter *et al.* 1995).

Challenge with pathogenic MDV-1 increased the total shedding rate of the vaccinal virus HVT and subsequently higher vaccine virus loads in dander may not relate to vaccine quality or vaccination efficacy, but rather to coinfection with MDV-1 and/or MDV-2 (Abdul-Careem *et al.* 2007; Eidson *et al.* 1971; Islam and Walkden-Brown 2007).

2.4 Biology of MD

2.4.1 Source of virus and route of infection

MD is highly contagious as the virus released in cell-free form from the desequmated feather follicle epithelium of infected chickens transmits very effectively via the respiratory route to non-infected birds directly and indirectly (Cho and Kenzy 1972; Sevoian *et al.* 1963). From day 7 post-infection MDV commences fully productive replication in epithelial cells of the germinative layer of the feather follicle of the infected chicken, and large numbers of enveloped infectious virions are released into the environment with feather dander, persisting throughout the life of an infected bird (Calnek *et al.* 1970; Witter *et al.* 1971).The shedding profile of MDV-1, MDV-2 and HVT in co-infected commercial broiler chickens was

determined by measuring MDV copy number per mg of feather dander using qPCR, and the daily rate of production of feather dander from chickens housed in isolators dander, with peak shedding of approximately 10^9 virus copies per chicken per day (Islam and Walkden-Brown 2007). The continuous shedding of the virus from infected birds leads to contamination of the environment and as a consequence poses a serious risk to flocks especially on farms with poor hygiene or on farms where chickens of different ages are reared at the same farm or on neighbouring farms (Baigent and Davison 2004; Davidson 2009a; Witter and Solomon 1971). MD can be induced by intra-tracheal insufflations of 1-5 mg of infective poultry dust (Walkden-Brown *et al.* 2007b) supporting the bulk of available reports that the main route of infection with MDV is by inhalation of dust particles containing cell free viruses (Baigent and Davison 2004; Schat and Baranowski 2007; Sevoian and Chamberlain 1963).

2.5 Pathogenesis

MDV infection occurs through the respiratory route by inhalation of cell-free of MDV-1 in feather dander (Baigent and Davison 2004; Beasley *et al.* 1970). Some MDV is carried to lymphoid organs,by infected phagocytic cells (Barrow *et al.* 2003). MDV is lymphotropic with incompatible type of virus-cell interaction, ranging from fully productive and semi-productive infection to non-productive neoplastic and non-productive latent infection (Baigent and Davison 2004; Payne 1985b).

The primary target cell for MDV pathology is the lymphocyte. The final outcome of infection with MDV is strongly affected by the epidemiological triad (agent, host and environment). The pathogenesis of infection with MDV can be divided into four phases (Burgess *et al.* 2001; Calnek 2001a; Calnek *et al.* 1989; Morgan *et al.* 2001; Nair and Payne 1995; Witter *et al.* 1971).

Early cytolytic phase. After MDV particles are delivered to the lymphoid organs, the virus undergoes cytolytic infection in these organs. Bursa-derived (B) lymphocytes and a small percentage of activated T lymphocytes are infected between 3 and 6 dpi and are the primary target cells for the first phase of lytic infection (Abdul-Careem *et al.* 2008; Baigent and Davison 1999; Calnek 1986; Calnek *et al.* 1984; Davison and Nair 2004; Shek *et al.* 1983; Witter and Schat 2003). In the early pathogenesis, the importance of B-cells is highlighted in

the observations that birds deficient in B-cells have reduced viraemia; no evidence of cytolysis; very low levels of viral internal antigen in the lymphoid tissues; normal spleen weights; and the mean time to death is extended (Schat *et al.* 1981)

An inflammatory response associated with an increase in the reticulum cells and macrophages also occurs. Profound immunosuppression is present in lymphoid organs such as spleen, the bursa of Fabricius and thymus (Calnek 2001a; Islam *et al.* 2000; Jarosinski *et al.* 2005), resulting in lymphocytolysis and inflammation (Biggs 2001). Due to inflammatory processes, the spleen shows weight increase while bursa and thymus undergo weight losses due to cytolysis (Davison and Nair 2004; Payne and Renine 1973). Cell-associated viraemia during this period is believed to be a result of virus spreading throughout the body (Davison and Nair 2004). The cytolytic phase is important for establishing infection in the target cells and inducing subsequent latency and transformation (Schat 1987). Cytolytic infection of B-cells activates T-cells, rendering them susceptible to infection as the cytolytic phase declines (Calnek 1986). T-cells become infected late in the cytolytic phase, continuing their infection into latency (Shek *et al.* 1983). However, only activated T-cells (expressing surface MHC class II) are thought to be susceptible to MDV infection (Calnek *et al.* 1984). This early cytolytic infection is only semi-productive, as cell associated virions only spread by direct contact with other lymphocytes or by cell division.

Latency. During this phase, viral replication typically decreases and a latent infection is established between 7 and 10 dpi in activated $CD4^+$ T cells. Latency is characterised by presence of the MDV genome in infected lymphocytes but viral antigens are not or only to a very limited extent expressed. There are factors that play roles in the maintenance of latency such as the oncogene *meq*, since it blocks apoptosis of latently infected CD4+ T cells and transactivates latent gene expression (Parcells *et al.* 2003). 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 (Abdul-Careem *et al.* 2009; Djeraba *et al.* 2000; Xing and Schat 2000).

Late cytolytic phase. This phase is characterized by reactivation of MDV replication between 14 and 21 days post infection with cytolytic productive-restrictive infection and permanent immunosuppression. This phase and the next phase are only observed in susceptible chickens following virulent MDV infection while, in resistant chickens, late cytolytic infection may persist for the rest of the host's life (Baigent and Davison 2004; Witter *et al.* 1971). This

late cytolytic infection affects the thymus, bursa and some epithelial tissues, including the FFE, kidney, adrenal gland and proventriculus. Infected lymphocytes usually harboring the MDV genome with limited expression of viral antigens, migrate through the bloodstream to visceral organs and peripheral nerves where they become neoplastically transformed in susceptible chickens, and proliferate to form gross lymphomas 3–4 weeks post-infection (Baigent *et al.* 2005b). These lesions are responsible for the symptoms of the disease and eventual mortality from MD.

Transformation phase. In susceptible chickens, the infection progresses to transforming infection in a small fraction of T lymphocytes and neoplastic lymphomas are produced in various organs from about 28 dpi (Witter and Schat 2003).The phases of infections are summarised in Figure 2.4.

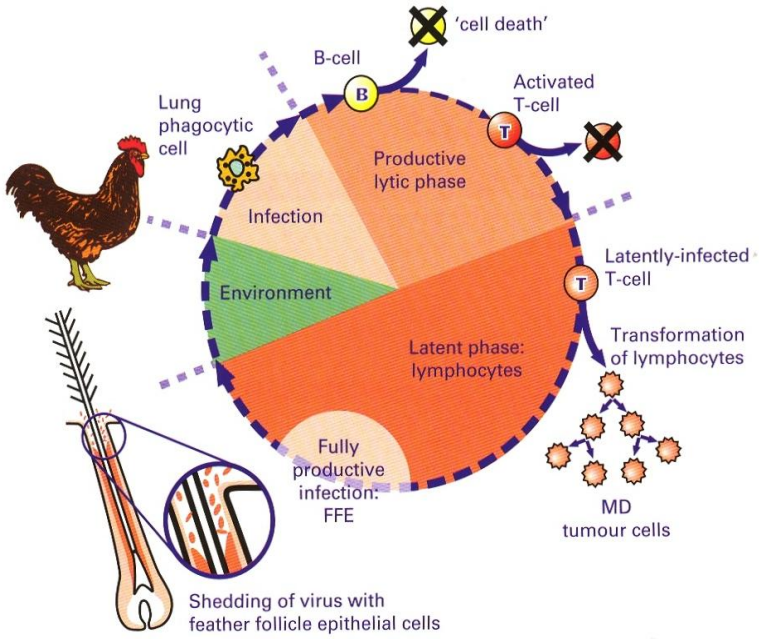

Figure 2.4. Schematic of key stages of MD pathogenesis (Baigent and Davison 2004).

Fully productive infection in the feather follicle epithelium (FFE). Virus is probably carried to the skin and feather follicles by latently infected peripheral blood lymphocyte (PBL) and can be detected there from as early as 5 dpi (Baigent *et al.* 2005b; Purchase 1970). Latently infected resistant chickens as well as vaccinated chickens can shed virus throughout their life without showing any clinical MD (Witter *et al.* 1971). The FFE infection commences 7-14 dpi and productive replication in FFE with high level expression of MDV antigens occurs

(Gilka and Spencer 1993). At 7-14 dpi, cell-free virus is shed in feather dander into the environment (Baigent *et al.* 2005a; Beasley *et al.* 1970; Carrozza *et al.* 1973; Islam and Walkden-Brown 2007). This virus is the source of infection for other chickens, via the respiratory route (Beasley et al. 1970; Davidson 2009b; Islam and Walkden-Brown 2007a).

2.6 Susceptibility of the virus to chemical and physical agents

Virus infected feather material and poultry dust retained infectivity at room temperature for several months and for at least 7 years at 4°C but the viruses in form of crude skin extracts from MD-infected birds were inactivated by a variety of chemicals and disinfectants after a 10 minute treatment period (Carrozza *et al.* 1973; Mare and Graham 1973).

Cell free MDV obtained from the skin of infected chickens was inactivated when treated for ten minutes at pH 3 or 11 and stored for 2 weeks at 4°C, 4 days at 25°C, 18 hours at 37°C, 30 minutes at 56°C, or 10 minutes at 60°C (Calnek and Adldinger 1971). Dander, litter and feathers from infected chickens are infectious and presumably contain cell free virus from the feather follicle epithelium bound to cellular debris. However the infectivity of such material is much longer than that of cell frees MDV, and was retained for 4-8 months at room temperature (Hlozanek *et al.* 1977; Witter *et al.* 1968). Cell free MDV and HVT can be lyophilized without loss of titre in the presence of a stabilizer containing sucrose, phosphate, glutamate and albumen (SPGA) (Calnek *et al.* 1970). HVT passes filters of 220 nm and stability of freezing and thawing, sonication and temperature is increased in the presence of SPGA.

The use of disinfectants has been an integral component for of infectious disease control programs, but the appropriate disinfectant needs to be selected based on the susceptibility of the target virus. The denaturants and oxidant group of disinfectants are most commonly used in veterinary medicine for inactivation of viruses on surfaces such as cages, floors, and feed trays. Various investigators have reported attempted disinfection with 1% Persteril (36-40% peracetic acid, 7-10% hydrogen peroxide, 1% sulfuric acid) (Jurajda and Klimes 1970), phenolics and iodine (Solomon et al. 1970), or formaldehyde fumigation. Formaldehyde has a broad spectrum of antimicrobial activity and can be used to decontaminate chicken sheds.

However, thorough physical cleaning prior to formaldehyde fumigation is another important element to combat MDV in poultry facilities (Shulaw and Bowman 2001).

2.7 Economic significance

MD is an economically important disease worldwide. MD suppresses the immune system, thus making its host more susceptible to secondary infections resulting in aggravated losses amongst infected chickens (Lee *et al.* 1978; Schat and Baranowski 2007; Wong *et al.* 2000). Over recent years, MDV isolates have continuously evolved towards greater virulence in the face of blanket vaccination which is particularly obvious in the USA (Witter 1997). Infection with MDV results in the loss of lymphoid cells which may account for a decreased or delayed antibody response and as a consequence resistance to other diseases may be decreased in these chickens (Calnek 1972a).

Other economic impacts include condemnation of poultry meat due to skin leukosis, and reduced productivity due to lower daily body weight gain and reduced egg production (Wu *et al.* 2009). There are several reports that regard MD as a highly significant disease on a worldwide scale (Nair 2005; Schat and Baranowski 2007). Indeed it is one of the most costly viral diseases in the chicken industry and is thought to have an annual cost of over US$1 billion to the poultry industry (Morrow and Fehler 2004) Even in the absence of MD outbreaks, the cost of vaccination, the main form of MD control, is high with more than 20 billion doses of vaccine used every year. Cell associated MD vaccines and vaccination methods are some of the most expensive used in the poultry industry. In spite of the success of vaccination, the occurrence of hypervirulent pathotypes of MDV has led to serious outbreaks in some countries, and vaccine failures, which are common and have resulted in serious economic losses (Schat and Baranowski 2007).

2.8 Epidemiology of MD

Many factors can determine the spread, incidence and development of MD. Some are associated with MDV itself and the method of its spread (pathogen factors). Others are external influences (environmental factors), including exposure to infected litter (dirty brooding), environmental temperatures, humidity, and air circulation from infected litter and

their effect on the MDV survival (Zander 1972). Other factors are related to the host (host factors) and include: genetic susceptibility, immune status of the bird and the age of exposure to MDV (Sevoian and Chamberlain 1963; Witter 1970; Witter *et al.* 1973).

2.8.1 Pathogen factors affecting MD

The pathogenicity and oncogenicity of MDV-1 isolates vary widely (Witter 1997). The other two serotypes; MDV-2 and HVT (Schat and Calnek 1978a; Witter *et al.* 1970a), serologically related but separate groups, are non-oncogenic and are used in live vaccines to MD, either alone or in combination with attenuated MDV-1 strains (Witter and Schat 2003). The virulence of MDV is a reasons for a wide variation in the incidence of the disease (Biggs 1997). All strains from serotype 1 are pathogenic and they were classified according to the virulence rank of the serotype 1 MDV strains (Witter 1997) whereas, strains from serotype 2 and 3 are apathogenic (Schat and Calnek 1978a; Witter *et al.* 1970a). Some isolates from serotype 1 can cause a high incidence of MD while other isolates were prepared as useful vaccines, e.g. CVI988 (Rispens *et al.* 1972b)

Marek's disease viruses over the past years have been evolving greater oncogenicity. Formerly described as a paralytic disease (Marek 1907), today MD is mostly manifested as an acute disease with tumours in multiple visceral organs (Nair 2005). Infection with more virulent viruses can cause high mortality, more lesions in tissues in genetically resistant, unvaccinated birds or in genetically susceptible and HVT vaccinated chickens. For instance RB-1B strain of MDV is a highly oncogenic strain that consistently induces a high incidence of MD with rapid-onset tumors in visceral organs (Schat *et al.* 1982) while infection with the CU-2 isolate (low virulence) caused neural lesions without lymphoma (Smith and Calnek 1974). CVI988 MDV initially caused minimal lesions only in very susceptible birds but after further passages in tissue cultures became completely avirulent (Rispens *et al.* 1972b).
In Australia, infection with the highly pathogenic MPF23, putatively classified as a very virulent isolate according to Witter (1997), caused a high incidence of MD in susceptible and HVT vaccinated chickens compared to the isolate MPF57, classified as a virulent isolate according to ADOL developed by Witter (1997), in a challenge experiment (Renz *et al.* 2012; Walkden-Brown *et al.* 2007a).

Increased virulence of MDV has been associated with induction of disease at progressively younger ages. For instance, in experimental infection of chickens induced with highly virulent strains, death due to MD occurred as early as 8-10 days (Witter *et al.* 1980). Such very virulent plus isolates occur in the USA, have a superior ability to transform T-cells, increased immunosuppression and atrophy of lymphoid organs, kill their host earlier and show an altered tissue tropism. The appearance of a new clinical picture of MD which is due to the emergence of new MDV-1 strains with unusual tissue tropism that can cause higher morbidity and mortality in susceptible animal populations over time is shown in Figure 2.5.

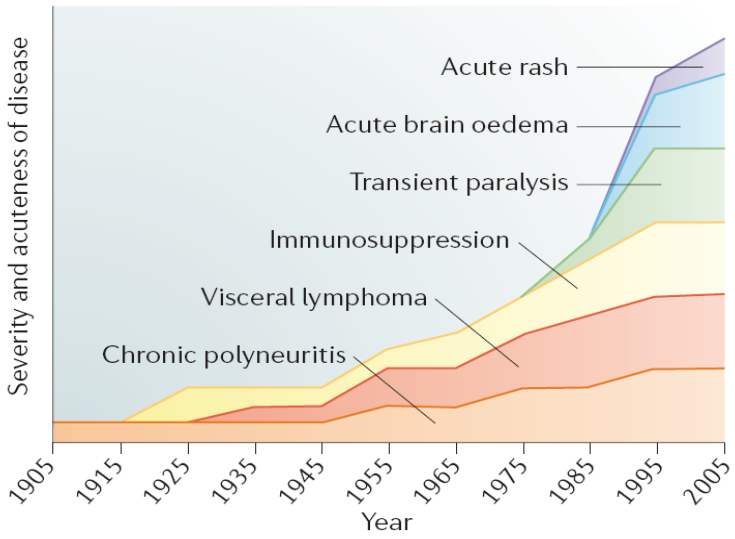

Figure 2.5. The rise in severity and variability of clinical signs after infection Marek's disease virus pathogenicity (Osterrieder *et al.* 2006).

The vv+ MDV isolates emerged in the 1990's and their relative proportion increased between 1990-1992 to 21%, and then up to 33% between 1993-1995. Based on these findings, Witter (1997) supposed that the evolution of field virus pathogenicity was related to the vaccination programs used by the poultry industry as shown in Figure 2.6. This hypothesis is coherent with the suggestion that blanket vaccination with an imperfect vaccine is a driver of increase in virulence for pathogens (Gandon *et al.* 2001).

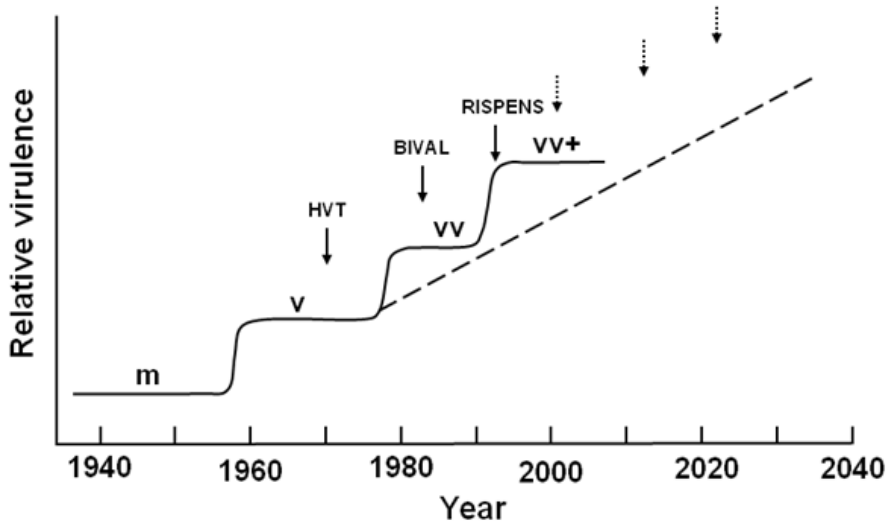

Figure 2.6. Past and projected evolution of MDV towards greater virulence in the USA. The introduction of new vaccines is indicated with the arrows, and periods of relatively good control with vaccination by the horizontal parts of the curve. The predominant pathotype of MD (m, v, vv, vv+) during each period is also shown (Witter 1998a).

2.8.2 Environmental factors affecting MD

Because MDV survives in dust (Carrozza *et al.* 1973) and MDV can be recovered from dried feathers maintained at ambient temperature for a long time (Schat 1985), it is very important to diminish the contact of young chickens to virulent MDV, using sufficient cleaning to remove all organic material and appropriate virucidal disinfectant for all surfaces in chicken houses.

In spite of the transmission of the infectious agent of MD can be mediated by the environment factors such as temperature and humidity, but the effect of environmental factors on mortality rate of MD outbreak are limited. However, a higher dietary protein level is coupled with high MD mortality (Proudfoot and Aitken 1969). It has been mentioned that occasionally lower MD mortality is observed when young chicks are reared on litter on which a previous flock infected with MD (Proudfoot 1970). However, there was a conflict with the observation of (Lapen and Kenzy 1975), who used airborne decomposition products from chicken litter in the rearing environment. Disinfectants were applied to chickens and their environments, and formaldehyde fumigation of eggs and newly hatched chicks was

undertaken but, they found no significant differences in the incidence of gross MD lesions between treated and untreated groups.

Recently, in an epidemiological survey of MDV in Australian broiler flocks (Groves *et al.* 2008), factors identified as significant risk factors for the presence of MDV-1 in dust were season of hatch of birds hatch (birds hatched in summer-autumn odds ratio 3.73), farms with more than 4 sheds (3.02) and farms with another chicken farm within 2 km (2.98), and using wood-based litter was more protective compared to straw or rice hulls) (0.65) were found to be protective against the presence of MDV1 in dust, and the provision of clothing for visitors on farm (odds ratio 0.48). Factors which were significantly associated with higher viral load detected in dust samples were chicken strain, sexed flocks, proximity of another poultry farm and higher average live weight (>2764 gm), while *in ovo* HVT vaccination, wood-based litter material and processing at an older age were associated with lower viral loads (Groves *et al.* 2008). Also there is a significant association between chicken density within large farms and greater virulence because there is less of a disadvantage in killing a host if there are other hosts to infect (Atkins 2010).

2.8.2.1 Host factors affecting MD

2.8.2.2 Host range

The natural host of MDV-1 is the chicken. MD has also been found in and isolated from quail (Kenzy and Cho 1969), and reported to infect ducks (Cottral and Winton 1953) and owls (Halliwell 1971). Lesions resembling those of MD have been noted in turkeys and MD has been diagnosed in commercial turkey flocks (Davidson *et al.* 2002; Hafez *et al.* 2002; Voelckel *et al.* 1999). These recent findings suggest that the host range of MDV serotype-1 has apparently expanded to include turkeys (Fadly *et al.* 2004).

2.8.2.3 Host genotype

The response to the deleterious effects of MD infection differs greatly between chickens depending on their resistance or susceptibility to clinical MD. Due to the genetic background of various chicken breeds, there is some degree of resistance to MDV infection in some breed compared to other, more susceptible chicken breeds when challenged under the same conditions (Calnek 1985a; Sharma and Stone 1972; Witter *et al.* 1971). This

phenomenon was also recognized by Pappenheimer *et al.* (1926) who mentioned that the characteristic lesions of MD do not always reveal themselves in typical paralytic symptoms, and suggested that diagnosis cannot be made from symptoms alone. In fact these chickens were classified as genetically resistant to clinical MD. The development of extensive lymphoproliferation does not occur in genetically resistant chickens even if challenged with extremely pathogenic strains of MDV (Sharma and Stone 1972). That is mean MDV infection can occur in genetically resistant chickens but chickens are more likely to tolerance of the virus infection.

Genetic resistance has been found to be both associated and non-associated with the major histo-compatibility complex (MHC). However, all chickens are in some way susceptible to MDV (Davison and Kaiser, 2004). The chicken MHC haplotype affects disease outcome significantly (Liu *et al.* 2001a; Liu *et al.* 2001b; Taylor 2004). Chickens with the MHC B^{21} haplotype are highly resistant to MD and those with the B^{19} haplotype are highly susceptible with the alloalleles of the MHC (Bacon *et al.* 2001; Briles *et al.* 1977; Hansen *et al.* 1967).

Prior to the introduction of vaccines for MD, the poultry industry used genetic selection as an important tool to control MD during rearing. When vaccines became available the use of genetics became more a concern of researchers than industry (Gavora *et al.* 1974; Liu *et al.* 2001a; Spencer *et al.* 1972; Vallejo *et al.* 1998). However, more recently, with the development of good markers for genetic resistance to MD, it is again used by the breeding industry, particularly the layer industry. Early selection for MD resistance was based on differences in MD susceptibility following challenge (Cole *et al.* 1973; Cole 1968). However, more recent approaches include the use of marker-assisted selection based on major histocompatibility (B) complex (MHC) polymorphism or detection of quantitative trait loci (QTL) associated with genetic resistance to MD (Liu *et al.* 2001a).

Calnek (1972a) noted that precipitin antibody appeared early in both resistant and susceptible chickens, whereas neutralizing antibody occurred in nearly 100% of the resistant strains but was rare in the susceptible strains. Other studies revealed that the level of viremia and the distribution of viral antigens in the tissues of genetically resistant chickens were much lower compared to genetically susceptible chickens that were simultaneously exposed to MDV (Sharma 1973; Sharma and Stone 1972). Powel (1986) and Powel(1982) suggested that the higher vulnerability of some chicken breeds was because lymphocytes of

susceptible chickens have more receptors for MDV, since these lymphocytes are more susceptible to infection *in vitro* than are those of resistance breeds. Additionally, the T lymphocytes of susceptible chickens may be more susceptible to transformation. The susceptibility to MDV was transferred to resistance chickens by adoptive transfer of T lymphocytes in the form of thymus fragments (Powell 1986; Powell *et al.* 1982). And a later study considered the difference in susceptibility of the two lines of chicken to MD is associated with differences in number of MDV-infected lymphocytes. The mean number of MDV-infected lymphocytes was greater in susceptible chickens than in resistance line (Baigent *et al.* 1998). Kaiser *et al.* (2003) found that MDV loads were only different after 10 dpi with an increase in susceptible lines and a decrease in resistant chickens.

2.8.2.4 Age of the birds at the time of MDV infection

The age of the chicken at infection is a factor influencing the response of chickens to MDV infection and tumour formation. Burmester *et al.* (1959) found an almost exclusive incidence of the visceral form of MD in older birds several months after MDV infection. Benton and Cover (1957) demonstrated that the host response of chickens of various ages inoculated simultaneously with the same isolate resulted in a gradient decrease of neural involvement, from a high incidence in young chicks to a low incidence in mature birds. This paralleled, both clinically and pathologically, spontaneous cases from the field. Although no paralysis was seen in the affected mature birds, use of a larger number of birds could have resulted in an occasional case of paralysis. Pappenheimer *et al.* (1926) noticed that the highest incidence of MD at that time was in younger adult birds and the earliest symptoms in spontaneous cases were observed at 12 weeks of age. Later, Sevoian and Chamberlain (1963) showed that day-old chicks were more susceptible than their dams to MD with the neural form dominating in young chickens and the visceral form in mature birds. Calnek (1973) noted that reports published between 1936 and 1955 stating that birds acquired resistance to MD with age were not entirely supported, He conducted 7 experiments involving chickens from 1 day to 4 ½ months of age and free of MD. A strong age resistance developed consistently in genetically resistant line but it was weak or absent in genetically susceptible line chickens, indicating that this type of ”genetic resistance” is probably synonymous with so-called age resistance.

Also the possibility of resistance at older ages due to previous exposure of older birds to virus of low virulence, should be taken in consideration. It is possible to confuse age resistance with natural or genetic resistance to MD. However, several authors have suggested that true age resistance is associated with lesion regression in older chickens in contrast to inhibition of lesion development in birds of all ages in genetically resistant chickens (Sharma 1976; Sharma *et al.* 1973; Witter *et al.* 1973). Age related resistance was confirmed to be unrelated to tumour resistance caused by prior infection or genetic constitution (Sharma and Witter 1975). Increased virulence of MDV has been associated with induction of disease at progressively younger ages. For instance, in experimental infection of chickens induced with highly virulent strains, death due to MD occurred as early as 8-10 days (Witter *et al.* 1980).

2.8.2.5 Sex of the birds

Females were more susceptible to the disease than males (Biggs and Payne 1967).. MD lymphoid tumours principally involves the ovary of chickens, but treatment with female and male sex hormones had no significant effect on the incidence of MD (Biggs and Payne 1967). In Australia, during the early paralysis syndrome it was found that males died at a greater rate than females between 11-16 dpc (Walkden-Brown *et al.* 2007a; Renz *et al.* 2012) but the overall effect of sex on survival was not significant. In contrast, between 34-55 dpc females died at a greater rate than males. This indicates a significant sex difference in susceptibility to the early paralysis and the later oncongenic forms of MD (Walkden-Brown *et al.* 2007a).

2.8.2.6 Immune status of the birds

Chicken serum contains three major immunoglobulin components; IgG, IgM, and IgA. Because chicken IgG differs in its immunochemical properties from the mammalian IgG, It has been termed IgY (Leslie and Clem 1969). Immunoglobulin is transferred to the egg yolk during yolk formation, and then is transmitted to the embryo through blood circulation (Tressler and Roth 1987; West *et al.* 2009). This passive immunity is crucial to new hatched chickens for survival. The amount of IgY in the egg yolk (100-400 mg) corresponds to the amount found in 30 ml of blood (Carlander 2002). In the last 3 days before hatching, a

striking increase in IgY uptake by the embryo takes place. The newly hatched chicken has 1-2 mg/ml of maternal IgY in its plasma (Kowalczyk *et al.* 1985).

Maternal antibody levels show abrupt variations in the first week of the chicken's life, and then fall to an undetectable level between the 2nd and 4th weeks of life. Calnek and Adldinger (1971) identified two types of MDV antibodies: neutralizing and precipitin. Neutralizing antibody binds to the virus and interferes with its ability to infect a cell. Precipitins react with MDV antigen to produce a precipitate in agar gels (Ikuta et al. 1984; Onuma et al. 1975). Comparison of isolates from both classical and acute MD showed that the line of precipitation was common to all with no antigenic differences being detected (Chubb and Churchill 1968).

Witter et al. (1971b) evaluated the antibody status of individual chickens naturally exposed to MDV under field-type conditions. Titres of MD precipitins peaked prior to the 28th week and persistence of precipitins was related to persistence of viral antigens. They also noticed that birds with high virus titres died more frequently of MD than other birds. Other studies have shown that neutralizing Abs occur in nearly 100% of MD resistant strains compared to rare occurrence in MD susceptible strains (Calnek 1972a; Calnek and Hitchner 1969).

Anti-MDV maternal antibody passively acquired from the dam is protective during the first 3 weeks of chickens' life (Chubb and Churchill 1968; Chubb and Churchill 1969; Witter *et al.* 1969). Inoculation of chicks with anti-MDV antibody is also protective (Burgoyne and Witter 1973; Calnek 1972b).

The reported magnitude of protection provided by anti-MD maternal antibody at the time of exposure varies widely. Among the published work, it is more pronounced in some lines of chickens than in others. The effects of Mat-Ab on the development of MD were summarized as (Burgoyne 1972 ; Calnek 1972b; Payne and Renine 1973):

1- Lower chickens' mortality due to MD.
2- Retard MD onset and increased latent period to death.
3- Lower the relative frequency of tumor creating by MDV.
4- Lower levels of viral antigens and infected cells below the common limits.
5- Potent drop of the destructive effect on hamopoietic and lymphoid tissues.
6- Delayed latent periods for precipitin antigen and cell-free virus production in the skin, and lower frequency of virus isolation from buffy coat cells.

The exact mechanism by which maternal antibodies inhibit or delay the development of MD is not well understood. Calnek (1972b) attributed the protective effects to a reduction in virus replication as evidenced by a lower viraemia and reduction in antigen production in lymphoid organs tissues. So an early sparing of lymphoid tissue by maternal Abs could protect the immune system during the lytic phase, and also reduce the level of latent infection and subsequent transformed lymphocytes.

Both MDV and HVT infections stimulate the production of humoral antibodies, which can have a significant effect on infection with the homologous virus serotype (Calnek and Smith 1972; Witter and Burmester 1979). The presence of maternal antibodies against HVT have also been indicated as possible reasons for vaccination failures (Calnek and Smith 1972). Cell-associated MD vaccines are less susceptible to the adverse effects of chick maternal antibody than cell free vaccines, and homologous antibody has a greater adverse effect than heterologous antibody. King et al (1981) describes the effects of heterologous antibodies on vaccination with different serotypes. Heterologous antibodies had little effect on vaccine responses and inhibition of vaccinal immunity compared with homologous antibodies. For this reason, MDV vaccines often vary by serotype between generations. For example, parent stock may typically be vaccinated with a serotype 1 MDV vaccine, while their progeny are vaccinated with MDV2 and/or HVT (Eidson *et al.* 1978; Witter and Burmester 1979).
One focus of this thesis is the protective mechanism of maternal antibodies involved in an early mortality syndrome which was reported previously by Witter et al. (1980). After experimental infection of day old chickens without maternal antibodies with very virulent isolates (Md5 and Md11), an early mortality syndrome (EMS) at 8-13 dpi was induced in 66-94% of the chickens. The birds were depressed or paralyzed with severe atrophy of the bursa and the thymus, but no lymphomas or lymphoproliferative lesions were observed (Witter et al., 1980).

In Australia, several MDV isolates derived from field outbreaks in vaccinated chickens (De Laney *et al.* 1995) were also reported to induce EMS in significant numbers of chickens with severe atrophy of the bursa of Fabricius and thymus in genetically susceptible chickens without maternal antibodies (Islam *et al.* 2001; Islam *et al.* 2002; Walkden-Brown *et al.* 2007a). In these studies, the EMS was repeatedly induced between 9 - 20 days after MDV challenge, usually with a mortality peak around 9-12 dpi. Figure 2.7 shows young birds

affected with paralysis which is usually seen with the EMS. It was noticed that male chickens were significantly more susceptible to EMS than females, which were in turn significantly more susceptible to lymphoma later on. However, the EMS was never observed in commercial chickens with maternal antibodies to MDV, thus suggesting that maternal antibodies, particularly against MDV, provide significant protection against the devastating effects of the EMS (Walkden-Brown *et al.* 2007a).

There are some previous reports showing that maternal antibodies against MDV protected against bursal atrophy, weight loss, and early mortality when birds were challenged with the very virulent MDV isolate Md5 (Lee and Witter 1991). In another study, it was reported that the incidence of microscopic lesions and mortality was very low in chickens with maternal antibodies and was significantly lower when compared to chickens without maternal antibodies when exposed to the virus at 1 day old (Sharma 1976).

Figure 2.7. Photographs of chickens exhibiting early paralysis syndrome induced by the Australian isolate of MDV (Walkden-Brown *et al.* 2007a).

2.9 Immunnity against MDV

In response to MDV infection, both non-specific (innate) and specific (adaptive) host responses are stimulated. Innate resistance provides an immediate protective response

using a range of mechanisms to deactivate the invading pathogen (Stewart, 1997), while adaptive immune responses usually appear around 5 to 7 dpi and include the development of MDV-specific antibodies and cytotoxic T lymphocytes (CTL) (Davison and Kaiser 2004). The outcome of infection is decided by the virulence of the virus and result of the interaction with the host immune system (Schat and Markowski-Grimsrud 2001). Because the nature of MDV is cell-associated and lymphotropic, T cell-mediated immune response has been detected as an effective immune response. On the other hand, antibodies play an important role only when the virus is present in a cell-free form or when MDV antigens are expressed on cell surfaces (Omar *et al.* 1998; Sharma and Witter 1975b)

2.9.1 Innate defense mechanisms

The innate immune response is a non-specific immediate and temporary immune response against a spectrum of molecular signals. It possesses many elements, such as macrophages and natural killer (NK) cells, secretion of type I interferons (IFNs) and pro-inflammatory cytokines. Some of these components are developed upon MDV infection, and they exert a role in host protection from the virus (Schat and Xing 2000; Sharma and Witter 1975b).

Macrophages have phagocytic, microbicidal and tumoricidal functions (Qureshi *et al.* 2000). Macrophage cells respond immediately upon infection with any pathogen. After infection with MDV, the macrophage phagocytic activity increase (Powell *et al.* 1983). Activated macrophages manage their role in defence mechanisms via phagocytosis and the release of various mediators such as nitric oxide (NO) and cytokines. MDV infection induces the expression of inducible NO synthase (iNOS) resulting in increased production of NO, which may inhibit MDV replication (Djeraba *et al.* 2000; Jarosinski *et al.* 2002; Xing and Schat 2000). However, macrophages may transport MDV from the respiratory site of infection to primary lymphoid organs including the bursa of Fabricius (Calnek *et al.* 1970; Schat *et al.* 1982b). Barrow et al. (2003) detected MDV in macrophages and suggested that the virus may replicate in these cells as well as being transferred from the lungs to the bursa of Fabricius.

2.9.2 Adaptive immune responses

The adaptive immune response consists of cell-mediated immune responses and production of neutralizing antibodies against various MDV proteins by plasma cells (Davison and Kaiser 2004).

Cell mediated immune responses include $CD4^+$ T helper (Th) or $CD8^+$ CTL against virus-infected or tumor cells (Kindt et al. 2007). Cell mediated immune responses are initiated by the interaction of CD4+ and CD8+ T cell receptors (TCR) with the major histocompatibility complex (MHC) molecules loaded with MDV antigens. The cell mediated immune response is regarded more effective than antibody mediated immune response because MDV is a highly cell-associated virus (Markowski-Grimsrud and Schat 2002; Omar *et al.* 1998). However, antibodies play a role in the formation of immunity against MD (Davison and Kaiser 2004). Presence of maternal antibodies alters MDV pathogenesis as they reduce clinical signs of MD, tumour formation, morbidity and mortality (Calnek 2001a; Davison and Kaiser 2004 ; Witter and Lee. 1984). But the presence of maternal antibodies also interferes with vaccination against MD, especially in the case of cell-free vaccines (Calnek 1982). Similarly, passive antibody transfer has been shown to delay the onset of clinical disease in the chicks (Chubb and Churchill 1969), and to interfere with day old vaccination efficacy (Calnek and Smith 1972).

Precipitating and virus-neutralizing (VN) antibodies develop within 1-2 wk after infection, and of these, the VN fraction correlates highest with bird survival. Non-neutralizing antibodies were proposed to coat infected cells and abrogate cell-to-cell spread of the virus. These antibodies might induce antibody-dependent cell mediated cytotoxicity (ADCC) that further aids the lysis of MDV-infected cells (Schat and Markowski-Grimsrud 2001)

2.10 Pathology of MDV

2.10.1 Clinical signs

The clinical signs of MD have changed remarkedly over time due to variation in the virulence and tissue tropism of the isolates. Chickens may become persistently infected with MDV

without developing clinical disease or die without showing any signs of lymphoma or lymphomatous lesions.

Initially, the classical clinical signs of MD were a marked paralysis and paresis mainly affecting the limbs of adult chickens. Paralysis and paresis are the end result of lymphocytic infiltration of the peripheral nerves, spinal cord, and associated ganglia (Marek 1907; Pappenheimer *et al.* 1926). Chickens exhibited asymmetric paralysis of one or more limbs; drooping of a wing, lack of coordination in walking, followed by a lame or hopping and staggering gait. Infrequent gasping and dilation of the crop may occur if the vagus nerve is involved. The paralysis progressed to a generalised body paralysis and eventually death (Calnek and Witter 1984; Purchase and Biggs 1967). With classical MD, the frequency of lymphomas was low and their distribution was limited to visceral organs, mainly the ovary (Pappenheimer *et al.* 1926).

During the 1950-70's, MDV isolates affected chickens at a much earlier age (4-8 weeks young), causing poor performance, paralysis, and a high rate of mortality. Lymphoma of the viscera and other tissues including the skin of chickens were frequently present (Benton and Cover 1957), and consequently caused increased condemnations due to skin lesions in meat chickens (Eidson and Schmittle 1968). Visceral lymphoma emerged between 6-20 weeks of ages under field conditions. For this reason, it was named acute form of MD (Biggs 1966; Eidson 1982; Sevoian 1966; Sevoian and Chamberlain 1963; Sevoian *et al.* 1962) The ocular form was seen occasionally across all age groups (Sevoian and Chamberlain 1963). Experimentally, acute MDV induced bone marrow aplasia, anaemia, severe atrophy of bursa of Fabricius and thymus, and chickens sometimes died without showing any lymphoma (Gilka and Spencer 1995).

Both previous and current MDV isolates are also capable of inducing a transient paralysis (TP). The chickens develop a generalised paralysis at 8 -12 dpi, including a flaccid paralysis of the neck and limbs. The neurologic signs disappear within 24 to 96 hours. After recovery, many chickens with previous TP develop the typical signs of MD (Kenzy *et al.* 1973; Zander 1959). More recently, a very virulent plus MDV isolate was reported to induce an acute onset of neurologic signs at 9-10 dpi similar to TP under experimental conditions in chickens without maternal antibodies. Most affected chickens died within 1-3 days after the onset of

neurological signs Spontaneous recovery was rare and the syndrome was termed acute TP (Witter *et al.* 1999).

The tissue tropism of recent MDV isolates has also changed, e.g. in the early days it was associated with mainly neurological signs, but rarely any lymphoma presence in the visceral organs. The current MDV isolates, apart from being capable of inducing early mortality, an increased frequency of ocular lesions, and atrophy of thymus and bursa, show a high incidence of lymphoma formation, particularly in the gonads, but also spleen, liver,kidney, muscle and lungs (Witter 1983; 1997). Other clinical signs such as swelling of the face and comb in addition to diarrhoea have been seen under field conditions (Ekperigin *et al.* 1983).

2.10.1.1 Differential diagnosis based on clinical signs.

The clinical signs of MD and that of other avian oncogenic viruses overlap, e.g. weight loss, paleness, occasional paralysis, and abnormal feathering are also signs of reticuloendotheliosis virus (REV) infection. REV is not as ubiquitous as MDV or avian leukosis, and it has a broader host range, including ducks, geese, turkeys, quail and infection of other species of birds is possible while MD infects chickens, and the disease is less common in turkeys and quails.

Paralysis or incoordination is also seen in chickens infected with infectious bursal disease virus (IBDV). However, IBDV is often accompanied by petechiae and haemorrhages (Cosgrove 1962; Jackwood *et al.* 2011). Chickens infected with IBDV usually also show signs of severe prostration, watery diarrhoea, soiled vent feathers, vent picking, and inflammation of the cloaca which are different to the clinical signs of MDV infection.
Lymphoid leukosis (LL; also termed avian leukosis) only occurs in chickens. Chickens with lymphoid leukosis have few typical clinical signs which makes it difficult to differentiate from MD based on clinical signs. Chickens infected with LL may show inappetence, weakness, diarrhoea, dehydration, and emaciation. Infected chickens become depressed before death. Death rarely occurs before 14 weeks of age and is more frequent around the time of sexual maturity.

2.10.2 Macroscopic lesions of MD

In the classical form of MD, the most prominent feature is the enlargement of one or more peripheral nerves. The most affected nerves were the sciatic nerve, brachial plexus, abdominal vagus and the intercostal nerves. The normal gross striation and glistening appearance of the nerve is lost and the nerve's colour changes to a greyish or yellowish colour and becomes oedematous. Lymphomas generally are typically white-grey discrete swellings in the visceral organs and gonads, especially the ovary, liver, spleen, kidney and heart. Ocular lesions were described in chickens between 1929 and 1943, consisting of pupil irregularities and loss of iris pigmentation (grey eye), it was common in older birds (16 -18 weeks old) (Nelson and Thorp 1943; Pappenheimer *et al.* 1926).

Recently evolved highly virulent MDV isolates frequently induce extensive, diffuse lymphomas. The lymphomas may involve the skin and muscles and can also cause ocular lesions (Purchase and Biggs 1967; Renz *et al.* 2012; Walkden-Brown *et al.* 2013b; Witter 1997). Unusual ocular lesions from infection with a highly virulent MDV isolate have been reported in vaccinated chickens (Ficken *et al.* 1991; Spencer *et al.* 1992; Witter 1997). Most virulent MDV isolates induce acute cytolysis, leading to the (sometimes complete) atrophy of the thymus and bursa.

2.10.2.1 Differential diagnosis based on macroscopic lesions.

At necropsy, the location, form and size of the gross lesions can provide some indication of MDV infection, but histological or molecular examination is, in most cases, essential for accurate diagnosis. Single or dual infections of chickens with REV, IBDV, LL and/or MDV complicate the differential diagnosis (Biggs and Payne 1976).

MDV lymphomas are very similar to those observed with LL. Table 2.3 summarizes some characteristics of MDV and LL lymphomas. The difference between MD and LL is that, in LL, gross lymphomas commonly occur in the bursa of Fabricius (Fadly and Venugopal 2008; Peterson *et al.* 1966). The localization of the tumours in the bursa is pathognomonic for LL, where various-sized lymphomas can be detected (Neuhybel 1989; Siccardi and Burmester 1970). The enlarged bursa can be detected by palpation by around 14 weeks of age (Watts 2013). In MD, tumours may occasionally occur in the bursa, and if tumours occur in the bursa, the tumors are diffuse in nature, involving the entire plicae, involving the entire plicae

However, bursal lymphomas are also known to be induced by REV.

Table 2.3: Selected characteristics of LL and MD tumours, which may be used for differential diagnosis (Siccardi and Burmester 1970).

Variable	LL	Marek's Disease
Age of occurrence	16 weeks or older	6 weeks or older
Neural signs and lesion	No	Yes
Tumour location;		
Bursa of Fabriccius	Yes	Occasionally*
Visceral organs	Yes	Yes
Eye	No	Yes
Skin	No	Yes
Muscle	No	Yes
Cell type	Lymphoblast	Small, medium and large lymphocytes; few lymphoblast and plasma cells

* Tumors may occasionally occur in the Bursa in MD. These tumors are diffuse in nature, involving the entire plicae

Chickens with REV infection show a runting syndrome and lesions include bursal and thymic atrophy, enlarged nerves, anaemia; the birds may be unthrifty, stunted and have abnormal feathering (Kawamura et al. 1976; Koyama et al. 1976) which may be of diagnostic value. Experimentally REV induced enlargement of peripheral nerves in chickens 3 to 6 weeks post inoculation (Witter *et al.* 1970b). REV naturally induced lymphomas in turkeys (Paul *et al.* 1977) and quail (Carlson *et al.* 1974).

Involvement of peripheral nerves and visceral lymphomas are common findings in MD. However, while there is variation in the enlargement of peripheral nerves by histological assessment in MD, involvement of peripheral nerves in LL infection can be excluded (Siccardi and Burmester 1970). Thus, enlarged nerves could be considered as specific lesions for MD if the less extensive nerve lesions seen with REV infection are ruled out (Witter *et al.* 1970b).

The early MDV infection of lymphoid organs usually presented itself as an acute lymphoreticulitis (Payne and Rennie 1976). The pathological picture included necrosis of the cells of the thymic cortex and bursal follicles, sometimes with formation of cyst-like vacuoles due to cytolytic damage. This was followed by invasion by macrophages and granulocytes with hyperplasia of reticulum cells, which is dominant in the spleen resulting in increased organ weight and it may play a role of in the induction of immunosuppression. In the bursa of Fabricius, cystic changes in the bursal follicles and epithelialisation has been reported with MDV infection. In the inter-follicular areas, lympocytic proliferation may occur causing the bursa to eventually exhibit gross enlargement and diffuse tumours (Abdul-Careem *et al.* 2008).

The heterogeneity of cells is characteristic of MD; there is variation in size and morphology of the lymphoid cells from small lymphocytes to large lymphoblast. The nuclei of most cells are not detectable in the presence of large number of lymphoblast cells, indicating a high level of malignancy. Heterogenic lymphoid cells were reported in some parts of the ciliary body, sclera and choroid membrane (Ficken *et al.* 1991; Pandiri *et al.* 2008; Smith *et al.* 1974). The heart muscle fibers are usually influenced by infiltration and proliferation of heterogenic lymphoid cells as diffuse or focal cell accumulations amongst the muscular fibers or the pericardium.

In the proventriculus, lymphoid cell infiltration was frequently found in the muscularis mucosa and glandular lobules. Slight cell infiltration was usually observed around the small blood vessels of the mucous membrane, muscle layers and subserosa (Witter *et al.* 1980). Proliferations of lymphoid cells have been observed in the peripheral and centeral nervous systems in addition to visceral organs (Payne 1972). Three main types of nerve lesions were identified (Payne 1985a):

1- Proliferative (Type A): frequently observed in chickens that died early with MD and lesions consist of masses of different sized lymphocytes, reticular cells and lymphoblasts with lymphoid tumour development.

2- Inflammatory (Type B): Type B lesions are observed in chickens which are clinically affected by MD over a longer time period. They are characterised by infiltration of small lymphocytes, plasma cells and small numbers of macrophages.

3- Chronic minor lesions (Type C): Type C lesions are small and comprise with little aggregations of lymphocytes and plasma cells. Chronic minor lesions are seen in bird with no clinical signs of MD.

Along the spinal cord the cellular infiltrations are almost limited to the white matter and the nerve roots (Gimeno *et al.* 1999). Lesions observed in the CNS are mild perivascular cuffing proliferative lesions and include the meninges, chorioid plexuses, mid-brain and medulla oblongata consisted chiefly of large lymphoid cells. Perivascular cuffing is usually present in all parts of the brain. Some of the lesions are associated with microglial proliferation and some astrocytes. The cuffs consist of various degrees of cell layers. Some are small consisting of only one or two layers of loosely arranged cells. The others were large consisting of several cell layers and mitoses were frequently observed (Kenzy *et al.* 1973; Zander 1959). However, chickens inoculated with recent highly virulent (vv+) MDV strains (648A) developed severe vasculitis and perivascular vacuolization. Vasculitis was transient and disappeared. The perivascular cuffing remained and diffuse cellular infiltration of the neurophil cells tended to increase. The paralysis often observed was attributed to oedema (vacculation) (Gimeno *et al.* 2001; Gimeno *et al.* 1999; Kornegay *et al.* 1983; Swayne *et al.* 1989c; Witter *et al.* 1999). Some less common lesions reported include the necrotizing vasculitis associated with vv MDV that caused lesions with fibrinoid necrosis of blood vessels (Cho et al. 1998).

2.10.2.1 Differential diagnosis based on microscopic lesions.

A characteristic of MD is that the lesions may occur in a wide variety of tissues plus there is heterogeneity of lymphoid cells. Heterogeneity is due to variation in size and morphology of the lymphoid cells from small lymphocytes to large lymphoblasts. Nuclei of most cells are not detectable. Presence of a large number of lymphoblast cells within heterogenic lymphoid cells in MD lesions is an indicator of high level of malignancy. This is in contrast to LL lesions, where the tumour cells are uniformly large immature lymphoblasts with a vesicular nucleus and a prominent nucleolus (Siccardi and Burmester 1970).

In the lymphoid tumours in the bursa of Fabricius seen after infection with LL, one or more follicles of the bursa are transformed 5-8 weeks after infection. Microscopically the follicle(s) distend(s) with lymphoblasts (intrafollicular proliferation) and tumours of the bursa are not

observed grossly until 16-22 weeks after infection, when neoplastic cells spread and cause metastatic tumours. In contrast, with MD bursal tumours the lymphocytic proliferation is interfollicular, causing gross bursal enlargement. The bursal follicles in MD also may show characteristic cystic changes and epithelialisation. Primary and metastatic tumours of LL are composed of homogenous populations of neoplastic lymphoblasts compared to the heterogeneous lymphoid cells of MD tumours.

With MD, the bursa of fabricius generally undergoes atrophy in young birds due to cytolysis of the bursal follicles. This finding is not pathognomonic for MD, since it may occur in many other diseases, e.g. IBDV (Cosgrove 1962; Jackwood *et al.* 2011), but it is different from bursal lesions seen with LL infection.

The proliferation of lymphoid cells after MDV infection has been observed in the peripheral and centeral nervous systems in addition to visceral organs (Cho *et al.* 1970; Gimeno *et al.* 2005; Payne 1972). The accumulation of lymphoid cells in various parts of the nervous systems is common to many bacterial and viral infections, thus it cannot be considered pathognomonic for MD. However, it may be a useful tool to at least differentiate MD from LL lesions.

Nerve lesions induced by REV are characterized by copious plasma cells, but they can not always be differentiated from lesions of MD (Payne and Biggs 1967; Witter *et al.* 1970b).
Also with REV-infected birds, the bursa is involved in the B-cell lymphomas similar to that of LL. Nonbursal (T-cell) lymphomas can be induced with REV and they may resemble those seen with MDV infection microscopically (Witter *et al.* 2005). REV lymphomas are both B- and T-cell induced (Davidson 2009b; Santos *et al.* 2009; Witter and Crittenden 1979; Witter *et al.* 2005 ; Witter *et al.* 1981) and cannot easily be discriminated from MD and LL except by virus isolation studies (Witter and Fadly 2003; Witter *et al.* 1986). Because REV is not yet as ubiquitous as LL and MDV, the demonstration of infectious virus, viral antigens, and proviral DNA in tumor cells has diagnostic value (Davidson 2009b; Davidson *et al.* 1995a).

Transformed T-cells within MD tumours have been shown to express a surface antigen that reacts with the monoclonal antibody AV37 (Burgess and Davison 1999; Burgess *et al.* 1996; Ross *et al.* 1997). A variety of Marek's-associated tumour surface antigens or MATSA have also been described (Sharma 1981; Witter *et al.* 1975). However, these MATSA are host,

not virus antigens, they are not MDV-specific. MATSA+ and AV37+ can be detected in the spleen from as early as 5 dpi (Burgess *et al.* 1996; Murthy and Calnek 1979), suggesting that host-cell changes associated with transformation occur soon after infection. The tumours induced by MDV show pleomorphism of T-lymphoid cells, and Witter and colleagues suggested determination of the T-cell and B-cell frequency in the tumours as an additional diagnostic tool (Witter *et al.* 2005). Neumann and Witter have used a microscopic immunofluorescent technique to differentiate lymphoid leukosis and MD, using MSB-monoclonal antibody and the anti-MATSA and anti-IgM sera. MD lymphoma cells had largely (70%) T-cell antigen markers and MATSA (4.3%), whereas more than 90% of LL lymphoma cells had B-cell and cell surface IgM antigen (Neumann and Witter 1978; Neumann and Witter 1979).

2.11 MDV diagnosis and monitoring

Diagnosis is usually made on the basis of clinical signs, typical MD gross lesions in individual chickens or in the flock with confirmation by histopathology and/or positive identification of MDV by virus isolation or molecular methods. Immunological methods such as ELISA are also available but tend to suffer from cross-reaction between MDV serotypes. Each of these will be considered in detail in the subsections below.

2.11.1 Virus isolation

Isolation, culture and identification of MDV-1 from infected tissues such as whole blood, PBL, splenocytes or tumour cells is an important component of the diagnosis of MDV-1 infection. However, this procedure is increasingly expensive and time consuming and thus is being replaced by PCR detection and quantitation of MDV-1. Isolation of MDV involves inoculation of intact infected cells (e.g. PBL, splenocytes or tumour cells) onto cultures of various chicken cell types for approximately 7 days followed by identification by plaque type (inaccurate) or immunofluorescence (IF) using serotype-specific monoclonal antibodies (Cui *et al.* 1987; Lee *et al.* 1983). The cell types used include chick embryo fibroblasts (CEF), duck embryo fibroblasts (DEF) or chick kidney cells (CK). The use of monoclonal antibodies is necessary as all three serotypes may exist together in the same host and the characteristics of MDV-1 plaques can change due to serial passage. Isolation in cell culture

followed by immunostaining is a lengthy, complex and costly procedure so tends to be restricted to research laboratories (De Laney *et al.* 1998; Lee *et al.* 1983; Silva *et al.* 1997). Thus diagnosis of MD is often based on pathological confirmation of the disease together with demonstration of viral DNA or antigens in tissues, and detection of antibodies (Witter and Schat 2003). However, the emergence of hypervirulent pathotypes of MDV-1, which induce non-classical signs of MD experimentally, provide a further challenge in the diagnosis of MD (Zelnik *et al.* 2004).

2.11.2 Molecular methods

In recent years, nucleic acid-based diagnosis of viral diseases of chickens, including MD, has become popular because of the high sensitivity and specificity of these methods (Cavanagh 2001). *In situ* hybridization has been used for the detection of the MDV genome in infected tissues (Endoh *et al.* 1996; Ross *et al.* 1997), but this is too laborious to be a routine diagnostic assay compared with the polymerase chain reaction (PCR)-based diagnostic techniques. PCR enables sensitive and accurate detection of MDV in biological samples, with clear differentiation of the different serotypes (Becker *et al.* 1993; Burgess and Davison 1999; Davidson *et al.* 1995b). Early PCR methods were mostly qualitative (Davidson *et al.* 1995b; Handberg *et al.* 2001; Walkden-Brown *et al.* 2003) or at best semi-quantitative (Bumstead *et al.* 1997; Burgess and Davison 1999; Reddy *et al.* 2000). However, with the development of real-time quantitative PCR (qPCR), the MDV copy number can now be precisely determined in a given sample (Baigent *et al.* 2005b; Islam 2006; Islam *et al.* 2004; Yunis *et al.* 2004).

The development of these methods was greatly facilitated by the complete DNA sequencing of the MDV-1, MDV-2 and HVT genomes (Afonso *et al.* 2001; Izumiya *et al.* 2001; Kingham *et al.* 2001; Lee *et al.* 2000; Renz *et al.* 2010; Tulman *et al.* 2000). This has enabled the design of MDV serotype-specific primers and probes. The PCR utilizes *in vitro* enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA (Erlich 1989). The entry of PCR technology into biological research has revolutionised the rapid sensing and quantification of many infectious agents, including MDV. The main benefits of the PCR technique are rapidity, ease and high levels of accuracy, sensitivity and specificity (Baigent *et al.* 2005b; Bumstead *et al.* 1997). DNA isolated even from crude biological such as poultry dust (Islam *et al.* 2006; Walkden-Brown *et al.* 2013a) and litter materials (Walkden-Brown *et al.* 2013c) can be analysed.

2.11.3 Serological assays

Serological methods for identifying MDV antigens or detecting MDV-specific antibodies in sera from infected birds were generally used after successful virus isolation in the 1960s (Zelnik 2004). Following infection, MDVs replicate persistently in the host and thus anti-MDV antibodies continue to be produced. Immuno-assays used to detect MDV antibody/antigen include agar gel precipitation (AGP) (Chubb and Churchill 1968), immunofluorescence (Purchase and Burgoyne 1970), virus neutralisation tests (Calnek and Adldinger 1971), and enzyme linked immunosorbent assay (ELISA) (Cheng *et al.* 1984). ELISA based on polyclonal antibodies has produced variable results when applied to detect MDV antibodies in diseased chickens and monitor vaccinal antibodies in vaccinated flocks (Adeniran and Oyejide 1995; Davidson *et al.* 1986).

However, these tests have been ineffective in separating antibodies of the three serotypes (Witter and Schat 2003). The specificity and sensitivity of immunofluorescent assays using monoclonal antibodies is very high. Although the monoclonal antibodies are generally serotype specific, some exhibit some cross reaction. For example, the monoclonal antibody H19 against pp38 can be used to discriminate between CVI988 and other MDV-1 isolates because CVI988 contains a mutation in pp38. The same antibody can also be used to differentiate between the other 2 serotypes (Cui *et al.* 1999). Because of the limited availability of these monoclonal antibodies, the use of the ELISA is limited, generally to research laboratories (Lee *et al.* 1983).

ELISA has not found widespread diagnostic use because MDV vaccines contain live infected CEF or other chicken cell lines. Thus they induce antibodies against CEF antigens, causing high background readings in ELISA. Often the titres of MDV antibody are low due to the immunosuppressive effect of MDV, which depletes Ab producing B cells. Therefore the ELISA test is more efficient if used to chickens not vaccinated to MDV. Also, most ELISA tests can not differentiate between MDV serotypes so cannot differentiate between responses due to vaccination and natural infection (Davison and Nair 2004). This can partly be resolved by using monoclonal antibodies in the ELISA tests. Monoclonal Abs against MDV are readily available (Lee *et al*. 1993) and in addition to being used to detect antibody, monoclonal Ab can be used to detect the presence of MDV antigens (MDV-Ag) in different

infected samples either in *vitro or in vivo.* For example, monoclonal Ab have been used to detect the presence of MDV in tissue, and the test enable more specific detection of MDV-Ag in tissue (Cho *et al.* 1999; Gimeno *et al.* 2001).

2.12 Control of MD

Prior to vaccination, the control of MD was principally based on the thoroughly cleanout, limited re-use of litter and segregation of young stock away from the older birds to reduce the chance of their early exposure to poultry dust (Bankowski *et al.* 1970). Newly hatched chicks were placed all at once in thoroughly cleaned and disinfected premises and kept separated from other age groups. This 'all in-all out' system has proven to be very successful and, previously, chickens could be maintained free from clinical MD and from MD precipitin antibodies up to 58 weeks old (Burmester 1969).

The introduction of vaccines was a welcomed tool to the armoury to combat MD, they became the primary means of control of MD, apart from the selection for genetically resistant chickens (Bacon and Witter 1992; Calnek 1986). Vaccination is protective against clinical MD, but does not prevent infection with MDV (Purchase *et al.* 1971; Witter 2001a). Thus, chickens remain carriers of the virus and continue to shed virus into the environment for the rest of their life (Witter 1997; Witter *et al.* 2005). However, vaccination has shown to reduce susceptibility to infection (Crabb *et al.* 2009; Islam *et al.* 2008a), and reduces the rate of shedding of MDV (Islam and Walkden-Brown, 2007).

MD vaccines are live vaccines, cell free but mostly cell associated and are administered at hatch, or in the case of HVT, *in ovo* at days 17-18 of incubation (Sarma *et al.* 1995). Unfortunately *in ovo* vaccination is only effective with HVT, but not MDV-2 or MDV-1 vaccines which need to be administered at hatch. The first vaccine was based on the attenuated UK oncogenic strain HPRS-16 (Churchill *et al.* 1969b), but it was quickly replaced by HVT strain FC126 (Okazaki *et al.* 1970; Purchase *et al.* 1971; Witter *et al.* 1970a) developed in the USA, which later was also develope d into a cell-free vaccine to avoid the requirement for storage in liquid nitrogen (Eidson *et al.* 1978; Eidson *et al.* 1975; Patrascu *et al.* 1972). The non-oncogenic MDV-2 strain SB-1 was discovered later as a vaccine strain against MD (Schat and Calnek 1978b; Witter *et al.* 1990). The protection was significantly

enhanced when SB-1 was administered in combination with HVT (Calnek 1982; Witter *et al.* 1984). The 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 a widely used vaccine (Bublot and Sharma 2004). In 1972 a mildly pathogenic serotype 1 virus Rispens/CVI988 was isolated (Rispens *et al.* 1972a) and has since become the most widely used strain of attenuated MDV-1 vaccine. It was introduced to the USA in the early1990s and Australia in 1996.

Today, vaccines of all 3 serotypes are available, and are used either alone or in combination with each other. In ascending order of efficacy the main general MD vaccine formulations are (Witter and Lee 1984; Witter *et al.* 1985).:

1) HVT alone,
2) HVT plus MDV-2, and
3) CVI988 (Rispens) with or without serotypes 2 or 3

The CVI988 (Rispens) strain appears to be the most efficacious vaccine at present and is widely used in layer and breeder chickens. Unfortunately, MDV-1 has evolved in virulence over time and part of this process has been failure of control by vaccination (Witter, 1997; 1998). Failure was first observed against HVT, then bivalent HVT/MDV-2 vaccines. This is due to the non-sterilizing nature of the MDV vaccines and has been reported with other pathogens (Gandon *et al.* 2001).

The presence of maternal antibody can interfere with the development of immunity following vaccination with live virus. In general, cell-free MDV is more susceptible to the effects of maternal antibody than cell-associated MDV, and heterologous antibody is less inhibitory than homologous antibody. For the latter reason, breeder chickens are typically vaccinated with a vaccine of different serotype to that of their progeny (Calnek and Smith 1972; Chubb and Churchill 1969; King *et al.* 1981; Patrascu *et al.* 1972; Witter 1984). Because all commercial breeder and layer chickens are vaccinated against MDV, all commercial chickens are hatched with maternal antibodies against MDV.

In addition to presence or absence of antibodies, the protective efficacy of MD vaccines depends on many factors including the genetic background of the chickens (Bacon *et al.* 2001), virulence of the infecting virus strain, and the vaccine dose administered (Witter 2001b). Traditionally, vaccines were administered subcutaneously immediately after hatch.

However, in the last decade or so there has been a change in the route of administration towards *in ovo* to prevent early exposure to MDV (Sharma *et al.* 1995). Also there are many reasons responsible for vaccine failure other than the appearance of biological variants of MDV. If chickens are reared in farms without cleaning or disinfection, heavy and very early exposure to infection can be a reason for vaccine failure. Inaccuracies in the preparation,of vaccines such as poor titres in vaccine batches or contamination of the vaccine during the preparation with viruses such as chicken infectious anaemia can lead to vaccine failure. Similarly, storage, handling and administration of vaccines can determine whether or not MDV vaccination program can successfully protect aginst MD (Zanella 1982).

The factors that may favour vaccine breakdown include evolution of viral pathogenicity and emergence of more virulent strains (Gimeno 2008). The emergence of strains of MDV with increasing virulence can be attributed to several events that have occurred over the last few decades, such as the intensification of poultry production, changes in host genetics, and vaccination pressure (Schat and Baranowski 2007; Witter 1997).

2.13 MD in Australia

In Australia control of MDV-1 infection prior to the mid 1980s was largely by vaccination of long-lived layers and breeders against MD with no vaccination of broilers. Vaccination of layers with locally isolated HVT started in 1971 followed by the introduction of the local serotype 2 vaccine (Maravac) which was introduced in 1978. Layer birds typically receive bi-valent vaccines containing either HVT, or MDV-2 and HVT.

Broilers typically receive cell-associated HVT vaccine (Jackson 2000a; Jackson 2000b). These provided satisfactory protection until the mid 1990s, when low potency vaccine batches were associated with vaccination failures. At this time, very virulent strains of MDV-1 were isolated from layers flocks that were poorly protected against MD by HVT and bivalent vaccines in Eastern Australia (McKimm-Breschin *et al.*, 1990). The new very virulent MDV strains also appeared to cause high mortality in Australian-breed meat parents and imported layer and meat strains vaccinated with HVT and/or serotype 2 Maravac vaccine (De Laney *et al.* 1995; Groves 1995; Jackson 1996). Importantly, in 1992 there were major imports of new layer and meat chicken genotypes into Australia and Australian MD vaccines failed to

protect the imported genotypes against MD (Cumming *et al.* 1998). Consequently there was a major outbreak of MD in Australia 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), and elevated mortality, intercurrent disease and reduced feed conversion efficiency evident in commercial broilers.

The problems in broilers were largely overcome by the introduction of *in-ovo* vaccination with high potency cell-associated HVT vaccine in 1997. The problem in layers and broiler breeders was brought under control by the importation in 1997 of master seed for the Rispens CVI988 serotype 1 vaccine. Since 1996 imported strains of HVT (FC126, and NBSL S.AR) (Karpathy *et al.* 2003; Karpathy *et al.* 2002) 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 Rispens CVI988 vaccine either alone, or in conjunction with HVT as a bivalent vaccine. In addition a highly effective local serotype 1 vaccine has been developed, which was shown to be equally effective as Rispens CVI988 (Karpathy *et al.* 2003 ; Karpathy *et al.* 2002).

Faced with vaccination failure and an apparent increase in virulence in MDV isolates, isolation of MDV and virulence or pathotype testing became common in the 1990s and 2000s in Australia. Initially this involved non-standard methodologies using SPF chickens (Zerbes *et al.*, 1994; De Laney *et al*., 1995; 1998) but later an adaptation of the USDA ADOL pathotyping method was used in SPF, broiler and layer chickens. The outcomes of all of these experiments are summarised by Walkden-Brown *et al.* (2007a) with individual reports being published for the SPF studies (Renz et al., 2012), the studies in COBB broiler chickens (Walkden-Brown *et al.* 2013b) and those in ISA Brown layer chickens (Renz 2008). The main findings from these studies were:

1- The disease outcome for a given MDV isolate at the same dose varied widely depending on the host chicken used. Some differences were clearly due to differences in maternal antibody presence (none in SPF chickens) and others probably due to host differences in genetic resistance to MD; An acute early syndrome was observed between days 9 and 15 post challenge with very virulent strains of MDV in maternal antibody negative SPF chickens. The syndrome was neurological and characterised by tremors, ataxia, torticollis, paresis, paralysis and death in severe cases. It occured two or more weeks prior to any tumour formation

associated with classical or acute MD (Walkden-Brown *et al.* 2007a). Affected chickens also had extreme thymic and bursal atrophy, so presumably were very immunosuppressed. The syndrome has never been previously reported in Australia although it is probably identical to the acute transient paralysis syndrome reported in the USA in maternal antibody negative chickens (Witter et al., 1999). The syndrome has never been observed or reported in commercial chickens, which all are maternal antibody positive for MD.

2- There was no clear evidence of evolution in virulence amongst the isolates and time period tested; Australian isolated were pathotyped as vMDV or vvMDV, with no vv+MDV found. There was a poor overall relationship between “virulence” (the ability of the virus to induced disease in unvaccinated chicks) and “vaccine resistance” (the ability of the virus to overcome the effects of vaccination, suggesting that these are different traits of the virus. The ADOL pathotyping method places greater emphasis on “vaccine resistance” so is probably a measure of this trait, more than of “virulence”. The Australian reference MDV strain, MPF57 is a case in point, being highly pathogenic in unvaccinated birds, but with limited effect in vaccinated chickens, thus being ascribed a vMDV rather than a vvMDV pathotype.

Detection and quantitation of MDV-1, HVT, and MDV-2 in poultry house dust using qPCR is sensitive, reproducible, and meaningful, both biologically and commercially. Tactical vaccination based on monitoring of MDV-1 rather than routine vaccination may reduce selection pressure for increased virulence in MDV-1 (Walkden-Brown *et al.* 2013a).

2.14 Conclusions

MDV, an alphaherpesvirus, upon its replication in its natural host the chicken, produces a number of different disease syndromes depending on host susceptibility and the level and virulence of the challenge virus. MD varies in its clinical presentation; the classical form characterised by paresis and paralysis in older chickens, the acute form characterised mainly by visceral lymphoma in younger birds, skin leukosis, lymphodegenerative syndromes characterised by extreme thymic and bursal atrophy, acute neurological syndromes usually in younger chickens accompanied with encephalopathy termed transient

paralysis (TP), which is either reversible (classical TP) or irreversible or what was called as acute TP, and even a syndrome characterised by atherosclerosis.

In the early 1970s, vaccination of 1-day-old chicks became common practice, greatly reducing economic losses associated with MD. However, regardless of advanced technologies to control MD by vaccination in *ovo,* and good facilities and management practices, sporadic outbreaks of MD continue to occur and have been associated with increased virulence in the field strains of MDV-1, particularly in the USA. This tendency of MDV to shift towards increased virulence and greater resistance to the protective effects of vaccination ensured ongoing economic impact and requires vigilance to maintain effective control. The annual cost of MD is estimated to be $1-2 billion worldwide through costs of vaccination and control, morbidity and mortality, and through increased condemnations, decreased growth rates and increased susceptibility to other pathogens (Morrow and Fehler 2004).

Australian MDV isolates vary widely in virulence with several falling into the vvMDV category. Between 1990 and 1992 there was an uncontrolled epidemic of MD in vaccinated flocks in Australia. The occurrence of MD in vaccinated Australian flocks was associated with vvMDV. These new isolates when used experimentally in unvaccinated, maternal antibody negative SPF chickens induce severe early paralysis, marked early cytolytic effects in lymphoid organs, reduced body weight and high early mortality between days 9-15 post challenge, well before development of tumours and in the absence of nerve enlargement. This syndrome appears to be very similar or identical to “Early Mortality Syndrome” reported in USA in 1980 or “acute transient paralysis” reported in 1999 (Witter *et al.*, 1999). However in the Australian studies it was noted that vaccination was completely protective against this early paralysis/mortality syndrome, and that it could not be induced in commercial layers or broilers having maternal antibody directed against MDV. This was strongly suggestive of a complete protective effect of maternal antibody, something supported by a careful analysis of the published literature on early paralysis and mortality syndromes for MD. However this has not been formally tested experimentally in the same genotype of chicken, differing only in maternal antibody status. One of the main goals of this thesis is to conduct such experiments to formally test the hypothesis that maternal antibody is protective against the early paralysis induced by vvMDV.

The importance of this point is that many authors ascribe the reports of early paralytic/mortality syndromes to increases in the virulence of MDV visible under field conditions (eg. Osterrieder et al., 2006) when in fact they remain an experimental rather than a field phenomenon and their occurrence is dependent far more on host immune status than any property of the challenge virus. They appear to be a product of using maternal antibody negative SPF chickens in experiments to test the virulence of MDV. Having stated this, it is clear that for a given SPF host system, reproducible differences in early paralytic syndromes can be induced by MDV isolates of different virulence as described in the neuropathotyping system of Gimeno *et al.*, (2002).

Chapter 3 General Materials and Methods

3.1 The experiments

A total of five experiments was conducted during the PhD project. The first experiment was the production and maintenance of chicken flocks with and without maternal antibodies against MDV. Two MDV challenge experiments were conducted in the isolator facilities at UNE. One of the remaining two experiments was a field survey of a current MDV outbreak in Australia while the other experiment was a molecular study to characterize and determine the prevalence of MDV in spleens and dust of broiler farms and local layer breeds in Iraq.
The title, timing and location of the experiments are listed in Table 3.1.

Table 3.1. The title, timing and location of the experiment carried out during the PhD project.

Code	Name of experiment	Timing	Location	AEC approval number
MD09-SW-Mab1	Production of fertile eggs with and without maternal antibodies against Marek's disease virus (MDV).	7Jul 2009 to 20 Dec 2009	Isolation Penson Western campus	AEC 09/094
MD09-SW-Mab2	Effect of challenge dose, age of chickens and specific maternal antibodies status on the incidence of acute early Marek's disease syndrome.	25 Nov 2009 to 30 Dec 2009	UNE PC2 Isolator,W33	AEC 09/145
MD10-SW-Field Outbreak	A field study to detect Marek's disease virus in an outbreak at broiler farms by serological and pathological investigation.	5 Jun 2010	Western NSW	Outbreak
MD10- SW-Survey	Molecular study of the epidemiology of Marek's disease virus-1 in broiler flocks and indigenous layers in Iraq.	15 Jul 2010	Iraq and UNE Laboratories	AEC 10/064
MD12-SW-NPT1	Neuropathotyping of MDV isolates MPF23 and MPF23	15 Mar 2012 to 10 Apr 2012	UNE PC2 Isolator, W33	AEC 11/129
MD12-SW-Mab3	Effect of inoculation of SPF chickens with IgY inoculation	21 Dec 2012 to 11 Jan 2012	Animal house and UNE PC2 Isolator,W33	AEC 09/178
MD12-SW-Mab4	Response of commercial chickens to anti-MDV IgY derived from eggs of immunized hens.	12Jun 2012 to 12 Apr 2012	UNE PC2 Isolator, W33	AEC 09/178
MD12-SW-Mab5	Protective effect of anti-MDV IgY derived from eggs of immunized hens on the pathogenesis of MD	17 Aug 2012 to 17 Oct 2012	UNEPC2 Isolator, W33	AEC 11/129

3.1.1 Handling of experimental animals

All chickens of all experiments 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. The Animal Ethics Committee of the University of New England (UNE) approved all the experimental protocols.

3.1.1.1 Source of experimental animals

Male and female specific pathogen free (SPF) layer chickens were used in the majority of the experiments. The source of SPF chickens was Australian SPF services (Melbourne, Australia) and the chickens were certified to be free of antibodies against MDV amongst a range of other poultry pathogens.

Commercial ISABrown chickens from an MDV vaccinated parent flock were used in experiment 5. In experiment 4 and 6, ISABrown chickens were used as positive controls for measuring anti-MDV antibodies. Other chickens used in the study were commercial broiler chickens from Baiada Poultry Pty Ltd (Tamworth, Australia).

For the work in Iraq, indigenous layer chickens as well as Ross commercial broilers which are commonly used on most Iraqi farms were sampled for this study.

3.2 Animal rearing at UNE

3.2.1 Isolation pens, Western campus

The chickens were placed in four isolation pens containing wood shavings with *ad libitum* food and water, and hover brooders (Figure 3.1, left panel). They were kept under the natural daylight period. The pens (Figure 3.1, right panel) were kept under quarantine conditions throughout the experimental period to prevent the spread and the shedding of vaccine virus to unvaccinated groups. Each group consisted of about 33 chickens; ten hens per one cock per isolation pen.

To achieve good biosecurity and to ensure that unvaccinated chickens remained free from vaccinal MDV until they produced eggs, each isolation pen was provided with its designated

tools and protective clothing. The feed and litter were handled with precautions to prevent contamination.

Figure 3.1: (left panel). Isolation pens on Western campus of UNE.
(right panel). Typical setup of each isolation pen.

3.2.2 UNE isolator facility

Two experiments were conducted in the UNE isolator facility under physical contamination level 2 (PC2) laboratory conditions. The facility can house 24 isolators (Figure 3.2) 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 isolator 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 were 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 on 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 canters (49 % open area).

Figure 3.2: The UNE isolator facilities. The isolators are aligned in two rows, 12 isolators in each row.

The entire feed supply for each 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. Chickens were offered feed (commercial layer starter then finisher, Ridley Agricultural Products, Tamworth) and water *ad libitum* throughout the experiments. Isolator temperatures were set at 34 ºC for the first two days and then decreased by 1 ºC every second 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 hours light (days 1-2) followed by 12 hours light: 12 hours dark lighting controlled with an automatic timer.

To prevent the spread of the infection to other animals in other locations and to reduce environmental contamination, disposable suits, boots, head caps and gloves were worn. Care was taken to decontaminate the boots, necropsy instruments, cages, and vehicles.

Immediately after each experiment, the rooms including all appliances and isolators units were disassembled and physically cleaned with detergent followed by high pressure steam cleaning. Prior to each experiment, the facilities were fumigated using 40 % formalin and potassium permanganate, then ventilated with filtered air.

3.2.3 Source of chickens with MD antibodies only

To obtain offspring chickens with maternal antibodies against MDV only, 53-56 day old male and female SPF chickens, without maternal antibodies against MDV were vaccinated with the attenuated MDV1 vaccine Rispens/CVI988 (Bioproperties Ltd., Australia). The vaccination was performed manually at UNE using the recommended dose rate administered subcutaneously in 0.2 ml of diluent supplied by the manufacturer.

3.2.4 Egg collection for hatching

Fertile eggs from the parent flock described above immunized against MD were collected two times daily for five days before the incubation start, scheduled for 9th October 2009. The nest litter material in each pen was replaced daily after the second egg collection to avoid egg soiling. Collected eggs were put into commercial egg carton racks. Eggs were stored with the small end pointed downwards in a cool-humid storage room kept at 18 °C with 75 % relative humidity following recommendations by North and Bell (1990) Once sufficient fertile eggs were collected, they were transferred to the hatching incubator which was cleaned and fumigated before incubation.

3.2.5 Hatching of Eggs

Eggs were allowed to warm slowly to room temperature before placing them in the incubator. The eggs were placed with the blunt ends upwards in a commercial incubator which was set at 37.5 °C, with a relative humidity of 59 % and hourly egg turning. On the 10th and 14th day of incubation, eggs were candled to estimate the number of chickens to hatch. After the chicks were hatched in the incubator, they were transferred to the UNE isolator facility.

3.3 Sampling procedures

3.3.1 Collection of blood for ELISA

All chickens were bled from the wing vein, and 0.5 - 3 ml of blood was collected using a 3 ml syringe with a 23G needle. The blood was transferred immediately into either a 4 ml Z serum

clot activator tube (Greiner Bio-One, Germany) or 1.5 ml microfuge tubes. Each tube was labelled with the number of the wing tag and collection date. The blood samples were taken to the laboratory where they were kept at room temperature for approximately 1 hour and then centrifuged at 2,000 × g for 15 min at room temperature using either a benchtop centrifuge (Biofuge 13, Heraeus, Germany) or an Allegra X15R centrifuge (Beckman, USA). The serum samples were transferred into sterile 1.5 ml microfuge tubes, labelled and stored at −20 °C until further analysis.

3.3.2 Collection of blood for peripheral blood lymphocytes (PBL)

Blood samples for PBL were taken from wing veins by stabbing the wing vein with a sterile 21G needle. Resulting blood was collected using a sterile plastic bulb pipette into 1.5 ml microfuge tubes preloaded with 300 µl of 3 % sodium citrate as anticoagulant. The separation of PBL is described further below.

3.3.3 Collection of dust samples

Dust samples were collected from sheds of different farms during an outbreak on commercial broiler chicken farms in Western NSW in order to confirm MDV presence. Dust was also collected from commercial broilers farms located in seven provinces in the middle and south of Iraq. The dusts were scraped from various surfaces using a sterile wooden spatula of every shed that was visited.

Dust samples were also collected in experiment 4 weekly up to day 49 days post challenge (dpc) from 15 isolators and commenced at 7 dpc. The dust was collected from the dust deposits at the 90° bend in the exhaust air outlet duct of each isolator. After each collection, the outlet duct of each isolator was cleaned thoroughly. The collected dusts were then stored in disposable sterile plastic bags at –20 °C until further analysis. Dusts were collected for detection and quantification of MDV by qPCR, and for isolation of the *meq* gene for sequencing from the Iraqi dust samples.

3.4 Source of vaccine and challenge MDV viruses

In Australia, a vaccination breakdown in 1985 (Reece *et al.* 1986) resulted in subsequent outbreaks of MD in vaccinated birds (McKimm-Breschkin et al. 1990) and MDVs were isolated from these outbreaks (De Laney et al. 1995; Zerbes et al. 1994). Among these viruses were 02LAR, MPF23 and MPF57 that were used throughout the thesis's experiments Table 3.2. Recent formal pathotyping experiments classified 02LAR and MPF23 viruses into very virulent (vv) and MPF57 as virulent (v) according to (Renz *et al.* 2012; Walkden-Brown *et al.* 2007c).

Table 3.2: The MDV isolates used in the thesis.

Strain	Serotype	Uses	Supplier
CVI988 (MRIS6111P)	attenuated MDV1	vaccine	Bioproperties Ltd
02LAR (P2181109)	vv MDV1	challenge	UNE collection
MPF23 (P021209)	vv MDV1	challenge	UNE collection
MPF57 (P4181109) (P7040810)	v MDV1	challenge	UNE collection

3.5 Chicken vaccination and challenge protocols

3.5.1 Vaccination

In experiment 1, chickens were vaccinated manually with Rispens/CVI988 (Bioproperties Ltd, Australia). After the vaccine was thawed at 37 °C in a water bath and diluted, recommended doses of vaccine (4000 PFU/0.2 ml) were administered to chickens subcutaneously in the loose skin at the top of the neck.

3.5.2 Titration of MDV isolates in cell culture and calculation of PFU

Infective stock materials of pathogenic MDV1 isolates were cryopreserved in liquid nitrogen at UNE. Prior to each MDV challenge, the viruses were removed from liquid nitrogen and were immediately thawed in a 37 ºC waterbath.

Each of the viruses had been adapted, passaged and titrated on chicken embryo fibroblasts (CEF). The titration was done on 6 well plates with confluent CEF seeded 24 hours prior. A serial dilution from 10^{-1} to 10^{-4} from the original vaccination material was prepared and 200 µl of each dilution added to duplicate wells of CEF. The cultures were incubated at 38.5 °C and 5 % CO_2 for 3-5 days until plaques became visible. Plaques were counted under an inverted microscope at the dilution that gave the easiest distinction between plaques where a guide of 10-60 plaques per well was appropriate. The titre was calculated by using the following equation: Counts x 5 x dilution factor = titre (pfu/ml).

3.5.3 MDV challenge

The challenge MDV isolates were diluted in M199 cell culture media containing 10 % fetal calf serum and 1 % antibiotic/antimycotics (Invitrogen, Australia) to the desired doses for each experiment and used within 30 min of thawing. The timing of thawing and use was recorded. Chickens of experiment 2, 3 and 6 were challenged, and each bird received 500, 2000 or 8000 PFU in 0.2 ml. Control birds received sterile PBS or M199 cell culture medium only. Three Australian MDV strains were used to challenge the chickens used in the experiments of this thesis. The viruses were pathotyped as virulent or very virulent of MDV1 (Delaney *et al.* 1998; McKimm-Breschkin *et al.* 1990; Renz *et al.* 2012; Zerbes *et al.* 1994). The viruses used in this study had been passaged previously 2-7 times on CEF monolayers Table 3.3.

Chickens were challenged by intra-abdominal (IA) injection except in experiment 6, where both, IA. and subcutaneous administration of the virus were used.
Chickens used in experiment 1 were inoculated at 0 or 7 day old to test the effect of MD-maternal antibodies in different ages while chickens of experiment 6 were inoculated at 5 day of age.

Table 3.3: MDV1 challenge isolates of the experiments, passage no., dose (PFU)/bird and the delivered volume and delivery route to the birds

Experiment	Challenge isolate	Passage no	Batch no	Dose (PFU)/bird	Dilution media	Dose volume (µl) and route of administration
1	02LAR	2	181109	500,1000, 2000	M199 growth media DP115	0.2 ml i.a.
1	MPF57	4	181109	500,1000,2000	M199 growth media DP115	516 µl i.a.
2	MPF23	4	021209	500, 2000, 8000	M199 growth media	200 µl s.c.
2	MPF57	7	040810	500, 2000, 8000	M199 growth media	200 µl s.c.
5	02LAR	4	181109	500, 2000	M199 growth media	1 ml (200 µl sc and 800 µl i.a.)

3.6 Euthanasia of chickens

At the termination of experiments, the surviving birds were euthanized by cervical dislocation (Zander *et al.* 1997). In brief, the legs are held in a fixed position by one hand. The thumb and index finger of the other hand circle the base of the skull and the middle and ring finger under the beak. Cervical dislocation is attained by the rapid extension of the arm holding while the head is in dorsal flexion. Chickens were handled with care to maintain complete separation of the cervical vertebrae.

3.7 Post mortem procedure

Immediately after chickens were euthanized, body weights were determined before starting the post-mortem examination. For each chicken that was euthanized or died during the experiments, a standard post-mortem examination was carried out (Bermudez and Stewart-Brown 2003; Sutherland *et al.* 2004). Suitable dissection instruments, disinfectant, and containers of fixation medium (10 % neutral buffered formalin) were kept available for organ sample collection for histopathology. Detailed recording sheets were used to record the necropsy findings and the individual identification information for each chicken, such as age, sex, identifying marker colour and wing tag number. For the field work, a sequential order number was used for the post mortem records. Carcasses were checked for nodular lesions on the skin and breast and thigh muscles were inspected for discrete lymphoid tumours or diffuse lymphoid infiltration. After opening the carcass, the internal organs were examined for gross MD lesions.

A detailed necropsy sheet was used to document the observations. All samples were marked with the experiment code, sampling date, ID number of the farm and shed and the sequence of the chicken which corresponded with the post mortem record number.

3.7.1 External examination and examination of brain

The general condition and the size of the birds were recorded and an external examination was conducted to detect presence or absence of external lesions, and then an incision was made through the skin from the vent to the beak in the ventral midline, the skin was raised

away from chicken chest and abdomen to expose the pectoral muscle in the midline to observe the subcutaneous adipose lesions The coxofemoral joints were then disarticulated to facilitate the exposure of the internal organs. To identify the external surface of trachea and to observe the thymus along both sides of the neck, the skin at the neck region was reflected. Thymus is five pairs of pale pink lobes lies just superficial to the jugular veins (McLelland 1990), and was routinely examined for atrophy due to the cytolysic effect of MDV. A transverse cut was made across the upper beak to examine the sinuses, hard palate and tongue. Eyes were examined for any abnormalities. The brain was examined while in the skull, for swelling and haemorrhage in the meninges or brain tissue. The brain was removed and fixed in 10 % neutral buffer formalin solution.

The muscles and bones of the legs were examined. The size, the gross appearance and colour of the sciatic nerves on both sides were compared. A piece of the sciatic nerves was removed and fixed in 10 % neutral buffer formalin solution for the histopathological study.

3.7.2 Examination of visceral organs

The sternum and rib cage was lifted upwards, and the chickens' air sacs, lungs, visceral organs in both abdominal and thoracic cavities were observed.
Because the liver completely covers the spleen under its dorsal surface, sampling the spleen for extraction of MDV DNA or for cultivation of MDV on tissue culture, requires care to avoid contamination of the spleen. The spleen is readily visible at the junction of the proventriculus and gizzard after it has been exposed by moving the liver to right side.

The normal colour of the liver varies with the diet and the age of the chickens from yellow in 0 day old and young chickens due to yolk absorption. Adult birds usually have a dark red to red brown coloured liver, but they have a yellow-tan liver if on a high fat diet and the organ may be soft. The gallbladder was examined. Oesophagus, proventriculus, gizzard, intestine, caeca, rectum and cloaca were opened.

Examination of the heart included observing the overall shape of the heart, pericardium, fluid or the fibrinous exudation in pericardial sac, thickness of ventricle walls, and the state of valves. In normal chickens, the left ventricle of the heart of is usually thicker than the right

ventricle. After the gastrointestinal tract was removed, the aorta and smaller connecting arteries were examined.

The trachea was opened with scissors from the larynx to the thoracic inlet and to the bifurcation of the trachea. The lungs are segmented and lie between the ribs on both sides of the spine. The lungs were examined and palpated.

The kidney lobes, ureters, the adrenal glands are and gonad that is located near the kidney were all examined for abnormalities.

3.7.3 Collection of spleen samples

The spleen was excised with clean forceps and transferred into a sterile plastic tube of suitable size after washing with sterile PBS. All samples were marked with the experimental code, sampling date, ID number of the farm and shed and the sequence of the chicken which corresponded with the post-mortem record. For the work done in Iraq, detailed data were recorded containing farm details, province and exact location within the province.
The collected spleens were placed in a box containing ice and transferred from the site of work to the laboratories, where the spleens were stored at – 20 °C until further analysis using qPCR and for isolating the *meq* gene for sequencing (Iraqi samples only).

3.7.4 Weighing and scoring of lymphoid organs

Lymphoid organs from normal control chickens and MDV infected chickens were inspected. Spleen including its capsule, bursa, and thymus were removed and weighed using a Mettler analytical balance (Zurich, Switzerland). Bursas were fixed in 10 % buffered formalin solution and stored for histopathology. The extent of the histopathologic lesions was scored on the basis of lymphoid necrosis and/or depletion of the organ.

3.7.5 Scoring system

As mentioned in 3.6, all surviving chickens from the experiments were euthanized and all dead and euthanized chickens were examined at post-mortem for the presence of gross MD tumours. These chickens were scored 0 to 4 for severity of lesions.
A scoring system was implemented for thymic and bursal atrophy ranging from 0-4 in ascending order of severity (0 = normal, 4 = complete or almost complete atrophy).

3.8 General materials

3.8.1 Buffers and Media

3.8.1.1 ELISA buffer

3.8.1.1 Carbonate buffer pH9.6

1.59 g/litre sodium carbonate ($NaCO_3$)
2.93 g/litre sodium hydrogen carbonate ($NaHCO_3$)

3.8.1.2 Phosphate buffer saline Tween (PBST)

1 litre phosphate buffer saline pH7.4 (Sigma Cat. no. 1000) with 0.5 ml Tween 20 per 1 litre of distilled water
For dilution samples, standards, and blanks after addition of 1% skim milk powder. Also used for dilution of conjugate**.**

3.8.1.3 Washing PBST: pH 7.2 to 7.4 in distilled water plus 0.5 ml Tween 20 per litre

8 g/litre	sodium chloride (NaCl)
0.2 g/litre	potassium chloride (KCl)
1.15 g/litre	sodium hydrophosphate (Na_2HPO_4)
0.2 g/litre	potassium dihydrogen phosphate (KH_2PO_4)

3.8.1.4 Citrate Phosphate buffer pH 5.0

7.3 g/litre citric acid ($C_6H_8O_7$)

9.648 g/litre sodium hydrophosphate (Na_2HPO_4)

3.8.1.5 Substrate

100 ml citrate phosphate buffer

34 mg OPD (O-phenylenediamine; $C_6H_4(NH_2)_2$.

50 µl hydrogen peroxide (H_2O_2; 30 % w/v)

OPD is sensitive to light; it was covered with aluminium foil during mixing for 1 hour and hydrogen peroxide was added just before using the substrate mixture.

3.8.1.6 Stopping solution

1 M sulphuric acid (H_2SO_4)

98 ml H_2SO_4 (98 %) per litre distilled water

3.8.2 Histopathological reagents and stains

3.8.2.1 Tissue fixative (10 %, buffered neutral Formalin)

10 % neutral buffered formalin was prepared as below:

100 ml formaldehyde (CH_2O) 37 %

900 ml distilled water (H_2O)

4.0 g anhydrous monobasic sodium phosphate (NaH_2PO_4)

1.5 g sodium pyrophosphate (Na_2HPO_4)

The solution was mixed until the ingredients were dissolved.

3.8.2.2 Harris' Haematoxylin

5 g Haematoxylin ($C_{16}H_{14}O_6$)
50 ml absolute alcohol (ethanol; C_2H_2OH)
100 g potassium alum $KAl(SO_4)_2$
1000 ml distilled water (H_2O)
2.5 g mercuric oxide (HgO)
40 ml glacial acetic acid (C_2H_4O)
The haematoxylin was dissolved in the absolute alcohol, and then added to the alum that was previously dissolved in warm distilled water in a 2 L flask. The solution was rapidly brought to boil. Afterwards, the mercuric oxide was added and the mixture was allowed to boil a few more min, while stirring. When the solution turned dark purple, the stain was rapidly cooled by putting the flask into a box containing ice. When the solution was cold the acetic acid was added. The solution was left to stand overnight, then filtered and used
.

3.8.2.3 The differentiating solution

Acid alcohol was prepared as follows:
12 drops of 1 N hydrogen chloride HCl
50 ml 70 % alcohol (ethanol)

3.8.2.4 Eosin solution

10 g Eosin Y
200 ml dd$H_2$0
800 ml ethanol (95-100 %)
The eosin was dissolved in water, and then ethanol (95-100%) was added. Just before use 0.5 ml glacial acetic acid was added to each 100 ml of the stain.

3.8.3 Reagents and bacterial media for molecular work

3.8.3.1 Ampicillin (Sigma-Aldrich A0166), 100 mg/ml, 10 ml

1 g ampicillin, sodium salt of ampicillin was weighed into a sterile 10 ml centrifuge tube and made up to 10 ml with sterile distilled water. After it was dissolved by mixing, 1 ml each was dispensed into sterile microfuge tubes and stored at -20 °C.

3.8.3.2 Gel loading dye, 25 ml

10 g Sucrose ($C_{12}H_{22}O_{11}$)
63 mg bromophenol blue ($C_{19}H_{10}Br_4O_5S$)
63 mg xylene cyanol FF ($C_{25}H_{27}N_2NaO_6S_2$)
The reagents were dissolved in approximately 15 ml distilled water, then made up to 25 ml, and dispensed in 1 ml aliquots in microfuge tubes.

3.8.3.3 PBS (phosphate buffered saline), 1 litre

8 g sodium chloride (NaCl)
0.2 g potassium chloride (KCl)
1.44 g sodium pyrophosphate (Na_2HPO_4)
0.24 g potassium dihydrogen phosphate (KH_2PO_4)
The reagents were dissolved in about 800 ml of distilled water. After the mixture was adjusted pH to 7.4 with hydrochloric acid, the volume was made up to 1 litre with distilled water and autoclaved.

3.8.3.4 RNase (DNase free), 1 ml

10 mg RNase (Sigma R 5503)
10 μL Tris, pH 7.5
15 μL 1 M NaCl
975 μL distilled water
The solution was boiled for 30 min.

RNase was added to a 1:10 dilution of loading dye, and 1 μL was added just before loading the restriction digesting reaction in gel.

3.8.3.5 STET, 100 ml

Lysis buffer for boiling minipreps (50 mM Tris, pH 8.0; 50 mM EDTA; 8% sucrose; 5% Triton X-100)

5 ml 1 M Tris, pH8.0
20 ml 0.25 M EDTA
16 ml 50% Sucrose
5 ml Triton X-100
54 ml distilled water

The buffer was autoclaved and stored at room temperature.

3.8.3.6 TE Buffer, 100 ml

1 ml 1 M Tris, pH 8.0
0.4 ml 0.25 M EDTA (pH 7.5-8.0)
98.6 ml distilled water

The buffer was autoclaved and stored at room temperature

.

3.8.3.7 TEAC Buffer pH 7.6

4.85 g Tris base
0.75 g EDTA

Distilled water was added up to approximately 800 ml. The pH was adjusted to 7.6 with glacial acetic acid.

3.8.3.8 0.5 M Tris, pH7.4 / 1.5 M NaCl, 1 L

60.55 g Tris base
87.66 g NaCl

Distilled water was added up to approximately 800 ml. The pH was adjusted to 7.4 with concentrated hydrogen chloride (HCl). Finally, the volume was adjusted to 1 litre.

3.8.3.9 Freshly prepared lysozyme (10mg/ml)

10 mg lysozyme
1 ml 50 mM Tris, pH8
The solution was mixed until completely dissolved.

3.8.3.10 YT, 2X, liquid medium, 500 ml

8.0 g tryptone
3.0 g yeast extract
2.5 g NaCl
Approximately 400 ml of water was used to dissolve the constituent. Once dissolved, the volume was adjusted to 500 ml. 10 ml aliquots were dispensed in McCartney bottles and autoclaved. The McCartney bottles containing YT medium were stored at room temperature.

3.8.3.11 YTA, 2X Plates, 500 mL

8.0 g tryptone
5.0 g yeast extract
2.5 g NaCl
7.5 g agar (bacteriological agar; OXOID LP0011)
Approximately 400 ml of water was used to dissolve the ingredients. The volume was adjusted to 500ml. was added. The media was autoclaved, and then cooled to 55 °C in a water bath After cooling, 0.5 ml of Ampicillin 100 mg/ml was added and after mixing, the media was poured into sterile Petri dishes, and then stored at 4 °C until used.

3.8.3.12 X-gal for blue/white selection (50 mg/ml)

1 ml X-gal (Promega, Madison, USA)
1.5 ml dimethyl formamide

3.9 General laboratory procedures

3.9.1 Separation of (PBL)

Isolation of lymphocytes from whole blood was carried out using Ficoll-Paque (Amersham Pharmacia, Biotech, Sweden). 600 µl of citrated blood sample was layered carefully over 300 µl of Ficoll-Paque that was previously loaded in a 1.5 ml microfuge tube.

After centrifugation at room temperature at 9000 x g for 30 min, the lymphocytes appeared as a greyish white layer. The white band of lymphocytes was collected using a sterile plastic pipette and transferred to a new microfuge centrifuge tube containing 500 µl sterile PBS, and then centrifuged at 400 x g for 10 min to wash the lymphocytes. The supernatant was discarded, leaving the lymphocyte pellet, which was stored at -20 ^{o}C until further processing.

3.9.2 DNA extraction from PBL, spleen and dust

DNA was extracted using the Bioline Isolate DNA Kits (Australia) according to the manufacturer's instructions. The DNA samples were extracted either from the entire PBL pellet, 10 mg spleen or 5 mg dust and stored at -20 °C. The DNA from tissue samples that were collected in Iraq were extracted at the Central Health Laboratories and the laboratory of the Hospital of Human Chest Disease at Samawa province- Iraq, while all other samples were processed at UNE laboratories. Samples processed in Iraq were initially stored at -20 °C, and shipped in a cool box with ice bricks.

All DNA samples were quantified using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop® Technologies Wilmington, USA) and diluted to a fixed concentration of 5ng /µl before use as a template in the qPCR assays.

3.9.3 Enzyme-linked immunosorbent assay (ELISA)

3.9.3.1 MDV ELISA

The concentration of antibodies directed against MDV antibody in the chickens' sera were tested using an indirect ELISA procedure adapted from that described by Zelnik *et al.*, (2004).

The MDV antigen was derived from Rispens/CVI988 (Vaxsafe; Bioproperties Ltd, Australia). The protein concentration of the Rispens antigen stock material was 1.1209 mg/ml and was diluted 1:100 in 0.05 M carbonate buffer, pH 9.6. The microtitre plates (Steriline) were coated with 100 µl per well and incubated overnight at 4 °C. The serum samples were diluted 1: 100 with PBS-Tween-20 (PBST) containing 1 % skim milk.

The coated plates were washed three times with PBST prior to blocking them with PBST containing 1 % skim milk for 1 hour at room temperature. After removal of the blocking solution, 100 µl per well of previously diluted serum samples in duplicate, including a negative and positive control sample were loaded onto the plate and incubated at room temperature for 1 hour. Each plate also contained duplicate wells with a 10 point standard curve prepared as a 2 fold serial dilution series from positive standard serum with known titres obtained from breeder broiler chickens vaccinated against MDV with Rispens vaccine. Details for the standard are given in Table 3.4 below

Table 3.4: Standard details for MDV ELISA

Standards	Dilutions	Titre units
S 01	1:16	512
S 02	1:32	256
S 03	1:64	128
S 04	1:128	64
S 05	1:256	32
S 06	1:512	16
S 07	1:1024	8
S 08	1:2048	4
S 09	1:4096	2
S 10	1:8192	1

The plates were washed again three times with PBST prior to adding 100 µl per well of Anti-chicken IgG (Sigma-Aldrich A9046) conjugated to horseradish peroxidase used at a 1 : 5000 dilution in PBST containing 1 % skim milk. The plates were then incubated at 37 °C for 1 hour before washing them again three times with PBST. The activated OPD substrate was then added, 100 µl per well, and incubated for 10 min at room temperature under a lightproof

cover. The reaction was stopped with 50 µl of 1 M H_2SO_4 per well. The plates were read at 492 nm on a microplate reader.
The amount of MDV antibodies was calculated as the percentage of reactivity when compared with the reaction of the positive and negative control sera that was obtained on the same plate.

3.9.3.2 Chicken infectious anaemia virus (CAV) ELISA

The control chickens of both immune statuses of experiment 1 were screened for CAV by ELISA antibody tests to ensure their freedom from CAV infection. Sera of extra chicks from the hatches on day 0 were also tested for the presence of CAV antibodies by ELISA, using the Flokchek CAV Antibody Test kit (IDEXX Laboratories 99-08702, Westbrook, USA). Sera were diluted 1: 10 according to the manufacturer's instructions. Absorbance was read at 650 nm in a Microplate EL310 Autoreader (Bio-Tek Instruments, Inc., Winooski, VT). The sample-to-positive control (S/P) or sample-to-negative control (S/N) ratios calculated as recommended (Idexx Laboratories, Inc., Westbrook, ME).

3.9.4 Pathological study

3.9.4.1 Gross lesions

As already mentioned, a routine necropsy was performed on all birds dying or euthanized at the end of the trial. After opening the carcasses, Skins were checked for nodular lesions, breast, thigh muscle, the peripheral nerves, including sciatic, and brachial plexus, were examined for gross enlargement. The visceral organs were examined for gross changes including enlargement or discrete or diffuse MD lesions in the thymus, bursa, liver, spleen, gonads, G.I.T, proventiculs, and brain. Suspect tissues from these organs from chickens with MD clinical sigs of EMS and TP were submitted to histopathological examination along with sections of tissue were also sampled from uninfected controls. In gross and histological tissue, scores grades were given to indicate the degree of bursal or thymic atrophy for e.g. score of 0 if there was no atrophy to 4 when there was complete atrophy present.

3.9.4.2 Laboratory technique for histology

3.9.4.1 Fixation

For histopathological evaluation, tissues were freshly collected from euthanized chickens. Brains were removed immediately as possible after necropsy. Tissues were fixed in 10 % buffered formalin. Pieces of tissue (nerve, bursa, thymus, liver, kidney, lung, etc.) of approximately 1 cm^3 and the whole brains were rapidly removed from freshly euthanized chickens and put in 10 % buffered formalin. The buffered formalin was ten times the volume of tissue to allow adequate penetration of the fixative. The containers were labelled with the identity of the chickens. Specimens were left in the fixative for at least 48 hours, and then the tissues were trimmed (Figure 3.3, left panel), and sections placed in small plastic histology embedding cassettes (Figure 3.3 right panel).

Figure 3.3: (left panel). Tissue trimming after 48 hrs in 10% neutral buffered formalin (right panel). Trimmed tissue was put in a plastic cassette, labeled with a pencil.

3.9.4.2 Dehydration

Fixed, trimmed, and washed tissues in the labelled plastic embedding cassettes were transferred to the automatic Histokinette tissue processor (Figure 3.4, left panel).

The blocks of tissue are transferred sequentially to 30 %, 50 %, 70 %, 80 %, 95 %, and 100 % ethanol for about two hours each. The blocks are then placed in a second 100 % ethanol solution.

3.9.4.3 Clearing

Xylene, an organic solvent, was used as the clearing agent to enable removal of alcohol and allow infiltration of paraffin wax. The blocks were moved by the tissue processors sequentially into a 50:50 mixture of absolute ethanol: xylene (initially) for two hours and then moved to pure xylene (two jars) for another two hours.

3.9.4.4 Paraffin wax infiltration

Paraffin wax is an infiltrating agent to replace the xylene and to provide a support matrix medium after it solidifies to allow thin sectioning. Paraffin wax (melting point 56 °C) was used for embedding.

3.9.4.5 Vacuum

The vacuum option in processing is useful to speed up wax penetration especially in those organs containing micro spaces such as lungs. These tissues were infiltrated for 20 min under vacuum and given a second infiltration for 20 min in fresh wax.

3.9.4.6 Blocking Tissues in Embedding System

The Leica embedding system (Figure 3.4 right panel) was preheated for at least two hours before the emergence of specimens from the Histokinette tissue processor. The cassettes, in a pot of melted paraffin wax were moved quickly from the Histokinette to the Leica embedding system to a basin with molten wax. The basin is to store cassettes of tissue samples. One plastic cassette was opened at a time and transferred from the basin to the hot plate of the embedding centre. Paraffin wax was added to fill the casting cup.

The tissue was oriented into a desired position in the embedding cast using the bottom half of the labelled plastic cassettes as a solid matrix frame. Hot wax was added until it nearly reached the top of the plastic cassette. Finally, the cast cup or dish was transferred to the cooling plate (35 – 50 °C), of the embedding centre. On the cooling plate surface, the embedding medium solidifies quickly, and then the cups are warmed in the hand before the removal of the wax block.


Figure 3.4: (left panel).The automatic tissue processor (Histokinette) that moves the tissues from the beginning to the end through the various reagents on a time scale that set is in advance.
(right panel). The Leica wax embedding system where the tissues were oriented in paraffin blocks.

3.9.4.7 Microtome

The extra wax on the sides of tissue wax blocks was cleaned to achieve proper block stability and prevent mobility in the microtome block's frame. After cleaning, the blocks of tissues were cooled in the refrigerator (4 °C) before sectioning, and then a tissue block was fixed in the block's frame of the specimen holder of the microtome (Figure 3.5). A disposable microtome steel blade (Reichert Jung USA) was mounted in the microtome and remained stationary when the microtome was running, and initially, tissue blocks were trimmed, then the tissue thickness was altered to 5-6 µm thick and serial sections were cut.

Figure 3.5: Microtome for sectioning of paraffin tissue block; it consists of a stationary knife

holder/blade and a specimen holder.

Ribbons of sections were picked up directly from the microtome and floated in an adjacent water bath that was hot enough to allow sections to flatten completely. Tissue sections then collected carefully from the water bath on a glass microscope slide, labelled and packed upright at 37 ºC for several hours. The dry and labelled sections slides were put on a hot plate at 60 ºC for 30 min to melt wax and improve tissue adhesion to the glass slides.

3.9.4.8 Staining

To achieve a good quality of staining, the remaining wax was removed from the slides using two (number 1 and 2) xylene jars at the beginning of the series of Coplin jars where sets of xylene and alcohol were used for steps before and after staining. Before staining the slides went through de-waxing by xylene and then went through rehydration by sets of descending concentration of alcohols then to water. After removal of paraffin and hydration of the tissue, the staining series were started from step 9 to 11 of Coplin jars, using haematoxylin solution for 8 min, then differentiation in 1 % acid alcohol for 30 sec. Finally the post staining step that consist of another series of Coplin jars, where slides were taking from water back to another two jars of xylene (number 3 and 4).The routine stain is haematoxylin and eosin (H and E). The slides were left in the solution for a set period of time as shown in Table 3.5, and then taken through a solution such as acid-alcohol that removed part of the stain. Tap water was used for bluing when the slides were left there for 15 min; whereas the staining with haematoxylin was differentiated in acid alcohol for two quick dips.

Table 3.5: The reagents of staining procedures, their function and the time was spent by the slide in each series of Coplin jars

No. of steps	reagent	aim	time
1	Xylene 1	De-waxing and clearing	10 min
2	Xylene 2	De-waxing and clearing	2 min
3	Absolute ethanol	Remove clearing agents	2 min
4	Absolute ethanol	Remove clearing agents	2 min
5	95% ethanol	rehydration	2 min
6	80% ethanol	rehydration	2 min
7	50% ethanol	rehydration	2 min
8	Distilled water	rehydration	2 min
9	Haematoxylin	staining	12 min
10	Acid alcohol	Stain differentiating	2 very quick dips
11	Running tap Water	wash and bluing	15 min
12	2% Eosin	stain	5 min
13	Tap Water	wash	Rinse well
14	50% ethanol	dehydration	1 min
15	80% ethanol	dehydration	2 min
16	95% ethanol	dehydration	2 min
17	Absolute ethanol	dehydration	2 min
18	Absolute ethanol	dehydration	2 min
19	Xylene	clearing	5 min
20	Xylene	clearing	5 min

3.9.4.9 Cover slipping

After staining the section of tissue on the slides, the cover slip and DPX as a mounting medium was applied over tissues to keeps the tissue on the slide from degrading.

3.9.5 Scoring of histopathologic lesions

All sections were assigned a lesion severity score. A standard 4-point grading system was followed in assessing the severity of lesions in sections from individual birds. For all tissues, a lesion score of 0 represented no lesions. The grades were defined as follows: 1= minimal; 2 = mild to moderate and 3 = extensive. Overall lesion scores for a chicken were calculated by the sum of score numbers for lesions in different tissues. For bursal sections, 1 was defined as mild variation in follicle size where less than 5-25 % of the follicles was affected, 2 as moderate variation in size of follicles if changes > 25 and up to 50 % of the follicles were undergoing lysis, and 3 as either necrosis or follicle atrophy (due to lysis of lymphocytes). For thymic sections, 1 was defined as mild cortical thinning, 2 as moderate cortical thinning, and 3 as absence of cortical lymphocytes with acute inflammatory infiltration.

For the brain the scoring grade was also given to the degree of infiltration of mononuclear cells in which the lymphocyte was the predominant cells in the meninges, choroid plexus and the perivascular cuff, 0= no lesions, 1=minimum changes, 2= mild to moderate, 3= extensive changes. Scoring grade also used in assessing the severity of brain vacuolation necrosis from 0-3, meningeal oedema. For sciatic neuritis the inflammatory changes were scored as A, B, C.

3.9.6 Real-Time PCR Analysis

Extracted and quantified DNA samples and mastermix for the real-time PCR assays were prepared using the liquid handling station CAS-1200 (Corbett, Sydney, Australia). The assay was described in detail previously (Islam *et al.*, 2006). Briefly, each reaction contained 0.3 µM of each primer, which are shown in Table 3.6, and 0.2 µM of the corresponding probe, 12.5 µl qPCR Supermix (Kapa Biosystems, Australia), 5 µl of DNA template (25 ng) in a total reaction volume of 25 µl. Duplicate reactions were run for each sample.

Table 3.6: TaqMan® probe and primer for MDV1 real-time PCR assays (Islam *et al.*, 2006).

Probe & primer	Sequences
Probe	5'-(FAM)CGTCTTACCGAGGATCCCGAACAGG(BHQ-1)-3'
F primer	5'-GGAGCCGGAGAGGCTTTATG-3'
R primer	5'-ATCTGGCCCGAATACAAGGAA-3'

A Rotor Gene 3000 instrument (Corbett Research, Sydney, Australia), was used for quantitative real-time amplification and analysis. The cycling parameters were: 50 °C for 2 min, then 95 °C for 2 min followed by 40-50 cycles consisting of denaturation at 94° C for 15 s and annealing/extension at 60 °C for 45 s (Islam *et al.* 2006; Islam *et al.* 2004). Results of the qPCR assays were analysed using software supplied with the Rotor Gene instrument (Corbett Research, Sydney, Australia). The default settings of the program were used to define both the threshold value and baseline for analysis of the raw data. A standard curve was generated in each assay and used to determine the viral copy numbers in unknown samples.

3.9.7 Standard PCR

Spleen and dust DNA samples from Iraq which amplified in the real-time PCR assay and gave positive results were used as a template for a standard PCR to amplify the complete *meq* gene from each sample using the primers BCH342 and BCH343 (Table 3.7), reaction buffer, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, 1 µmol of each primer and 1 unit of the hot-start DNA polymerase Taq (Fisher Biotec, Perth, Australia). The final volume of the reaction mixture was 25 µl and contained 1 ul of the template DNA. The PCR was carried out over 35 cycles and the detailed cycling program is given in Table 3.8.

Table 3.7: Sequences of *meq*-gene primers used for PCR amplification

Primers	Sequences	Orientation of primer	Location in MD5 genome*	Expected amplicon size (bp)	Use
BCH342	5'-ATTCCGCACACTGATTCC -3'	5'	134786-134803	1125 (with BCH343)	Amplification, sequencing
BCH343	5'-TGCTGAGAGTCACAATGC -3'	3'	135893-135910	1125 (with BCH342)	Amplification, sequencing
M13F-pUC	5'-GTTTTCCCAGTCACGAC-3'	5	2958–2972*		Sequencing
M13R-pUC	5'-CAGGAAACAGCTATGAC-3'	3'	175–192 *		Sequencing
BCMD07	5'-TGAACCTCCCATTTGCACTC -3'	5'	135301-135320		Amplification, Sequencing
SJW1	5'-CTAACGCTCCACATTGCT -3'	3'	135345–135362	600 (with BCH342)	Amplification, Sequencing
SJW2	5'-AATGCGGATCATCAGGGTCT-3'	3'	135878–135897		Sequencing

*The location of M13-pUC primers in pGEM-Teasy is given.

Table 3.8: thermo-cycler program for standard PCR assays

Temperature	Time	Cycles
94 °C	5 min	2x
94 °C	1 min 30 s	35x
60 °C	1 min	=
72 °C*	2 min	=
4 °C	Infinite	hold

*After the final cycle, the elongation phase at 72 °C was extended to 10 min.

3.9.8 Visualization and analysis of standard PCR products and plasmids

The PCR products were analysed on a 1 % (w/v) agarose gel along with a 100 bp molecular-weight marker or lambda *Hind*III standards (Axygen Biosciences,Fisher Biotec, Australia). Fourhundred mg agarose powder (Promega, Madison, USA; V3121) was dissolved in 40 ml electrophoresis buffer (1 x TEAC) in a microwave oven for about 30 sec. The completely molten gel was poured onto the centre of the casting tray, which was sealed with tape and the comb was put in place. The gel tray was left for 30 min to cool and solidify.
Electrophoresis was usually performed using 96 V for 1 hour before staining in ethidium bromide and visualization using a UV scanner.

3.9.9 PCR product and gel fragment purification

The Promega Wizard® PCR Clean-Up System (Promega, Madison, USA) was used to extract and purify DNA fragments from PCR products following the manufacturer's instructions and the eluted purified DNA was stored at –20 °C.
The Promega Wizard® Gel Clean-Up (Promega, Madison, USA) kit was used to extract and purify DNA fragments from gels following the manufacturer's instructions. The eluted DNA was stored at –20 °C.

3.9.10 Construction of *meq* plasmids for sequencing

The purified DNA fragments were ligated into the pGEM® Teasy vector (Promega, Madison, USA). The instructions of the manufacturer were followed to set up the ligation reaction. The components of the ligation mixture were added to each tube as shown in Table 3.9.

Table 3.9: set up for the ligation reactions

Reaction component	volume (μl)
2 x Rapid buffer	5
pGEM vector (50 ng/μl)	1
PCR product (15 - 50 ng)	2
T4 ligase	1
Sterile MilliQ water	1
Total volume	10

The ligation mixes were subsequently used to transform competent *E.coli* DH5α (BioLabs, New England, USA) and blue white screening was performed to identify colonies containing the target plasmid. Cultures from selected colonies were grown overnight at 37 °C in YT medium containing X-gal and Ampicillin. X-gal is a dye and a substrate for β-galactosidase, turns blue when hydrolyzed by β-galactosidase and therefore it is suitable for blue/white screening.

X-gal was spread across surface of agar plates with a sterile glass spreader, and was allowed to absorb for 20 min under a lightproof cover before they were plated with the transformation broth.

3.9.11 Boiling lysis mini-prep protocol

Prior to extracting DNA from overnight cultures, glycerol stocks were prepared from each culture, and 0.5 ml of 80 % glycerol was added to 0.5 ml of the culture in a sterile 1.5 ml microfuge tube and stored at -20 °C as stock material.

The remainder of the culture was then transferred to 10 ml centrifuge tubes, which were centrifuged at 2,500 x g for 10 min. The supernatant was discarded and 500 µl STET as well as 40 µl freshly prepared lysozyme added to the sample. The samples were boiled for 90 sec, and immediately afterwards cooled for a few seconds on ice. After centrifugation at

10,000 x g for 10 min, the gelatinous precipitate was removed and 700 µl isopropanol was added to each tube and mixed by inversion. The mixture was then kept at -20 °C for 20 min to allow the DNA to precipitate. The samples were then centrifuged at 10,000 x g for 10 min and the supernatant discarded. One ml of ice cold 70 % ethanol was added to each sample and centrifuged again at 10,000 x g for 1 min. The supernatant was discarded and the pellets were allowed to dry on the bench for 10 min and were resuspended in 50 µl TE. Afterwards, 4 µl of each sample plus 1 µl of loading dye containing RNase was analysed on a 1% agaroase gel by electrophoresis using 96 V for 1 hour. A lambda-*Hind*III marker was used to determine size and concentration of plasmid DNA.

3.9.12 Restriction enzyme digestion of purified plasmid recombinants

The plasmid DNA was also analysed using restriction enzyme analysis to determine the size of the fragment of the inserts. The plasmid DNA was digested with *Sac*I, *Pst*I and with *EcoR*I. The enzyme restriction reactions were set up as follows:

12.8 µl	H_2O
2 µl	10x Buffer
0.2 µl	BSA
4 µl	plasmid DNA
1 µl	restriction enzyme (2-10 U)

Total volume: 20 µl

The reactions were incubated for 1 hour at 37 °C in a water bath and 5 µl of each digestion reaction was analysed on a 1 % agarose gel using electrophoresis at 96 V for 1 hour.

3.9.13 DNA sequencing and analysis

The determination of the sequence of the insert of the generated plasmids was conducted by Macquarie University (Sydney, Australia) using an ABI 377 sequencer (Applied Biosystems Inc., Foster City, CA, USA). The primers used for sequencing are shown above in Table 3.7Table 3.7. For analysis of the sequencing results, Chromas© 1.43 was used for analysis of chromatograms, Sequaid™ 3.70 for sequence alignment, trimming and assembly of the fragment. Also ClustalW2 (EMBL-EBI, Cambridgeshire, UK) was used for multiple sequence alignment and for translation of sequence nucleotides to amino acids.

Chapter 4 Prevalence of Marek's disease virus in different chicken populations in Iraq and indicative virulence based on sequence variation in the EcoRI-Q (*meq*) gene

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Abstract

A cross sectional survey was conducted in six provinces in southern Iraq to determine the point prevalence of Marek's disease virus (MDV) in different chicken populations followed by sequencing the *meq* gene for phylogenetic analysis and virulence-associated polymorphisms. A total of 109 samples from unvaccinated flocks were analysed comprising 52 dust and 30 spleen samples from commercial broiler farms and 27 spleens from local layer chickens purchased in the town markets. The overall prevalence of MDV was 49.5% with no significant differences between provinces (P=0.08) or sample types (P=0.89). Prevalence ranged from 36.8% in Karbala and Nasiriyah to 65% in Amarah. The percentage of positive samples was 59.1%, 46.7% and 48.1% in broiler dust, broiler spleen and layer spleen respectively. The overall mean (±SEM) Log^{10} MDV viral copy number per mg of dust or spleen as determined by quantitative PCR was 1.78±0.19 with no significant differences between provinces (P=0.10) or sample types (P=0.38). In positive samples only, the overall mean was 3.43±0.18.

Sequencing of the *meq* gene from samples that showed high levels of MDV target in qPCR testing was attempted. Nine samples were sequenced. These sequences were compared with *meq* sequences of MDVs of different pathotype. All the Iraqi MDVs had a short *meq* gene of 897 base pairs due to deletion of 123 bp relative to the reference strain Md5. The Iraqi *meq* sequences also contained single nucleotide polymorphisms resulting in differences in the amino acid sequence. All of the nine Iraqi *meq* genes encoded two repeats of 4-proline sequences. The published negative association between 4-proline repeat number and MDV virulence suggests that the Iraqi MDVs are likely to be highly virulent, but this needs to be confirmed by *in vivo* testing.

Taken together these results indicate that MDV is common in unvaccinated commercial and village chickens in southern Iraq, that there is limited *meq* gene sequence variation, that all sequenced samples had a short *meq* with two 4-proline repeats and that this is consistent with a high level of virulence.

Key words: Marek's disease virus, Iraq, *meq* gene, sequencing, pathotype, virulence
Abbreviations: aa= amino acid; dNTP's = Deoxyribonucleotide triphosphate; (GaHV-2) = gallid herpesvirus 2; HVT = herpesvirus of turkeys; MD= Marek's disease; MDV= Marek's disease Virus; m = mild; $MgCl_2$ = magnesium chloride; *meq* = Marek's EcoRI-Q (meq) gene; Meq= the protein products of Marek's *Eco*RI-Q (meq) gene; UL = unique long region; US =unique short region, v = virulent; vv = very virulent; vv+ =very virulent plus; ORF = open reading frame; PPPP = proline repeats; qPCR = Real-time quantitative PCR; viral copy number =VCN; RL = repeat long region; RS = repeat short region regions

4.1 Introduction

Marek's disease (MD) is a lymphomatous disease of chickens caused by an alpha herpesvirus gallid herpesvirus 2 (GaHV-2), which is commonly referred to as MDV serotype 1 (MDV-1) (Osterrieder and Vautherot 2004). Other related MDVs in the genus *Mardivirus* include Gallid herpesvirus 3, an avirulent chicken herpesvirus commonly referred to as MDV serotype 2 (MDV-2); and Meleagrid herpesvirus 1, a naturally occurring herpesvirus of turkeys commonly referred to as (HVT or MDV serotype 3, MDV-3). MDV-1 includes all the pathogenic and oncogenic strains and some vaccine strains of naturally attenuated oncogenic MDV including the Rispens CVI988 vaccine (Rispens *et al.* 1972a). Chickens are infected by inhalation of infective virus shed from the feather follicle epithelium in dander (Calnek *et al.* 1970) and this is a useful material for molecular detection of MDV in chicken flocks (Walkden-Brown *et al.* 2013a). The pathogenic and oncogenic strains can induce T-cell lymphoma in chickens within weeks of infection (Calnek 1985b). The pathogenic strains of MDV1 can be pathotyped using standardised *in vivo* methods into mild (m) MDV, virulent (v) MDV, very virulent (vv) MDV and very virulent plus (vv+) MDV strains based on the level of disease induced in unvaccinated and vaccinated chickens (Witter 1997; Witter *et al.* 2005). All of the three MDV species are used in MD vaccines, alone or in combination.The vaccine strains produce non-sterile immunity, preventing or delaying lymphoma development, but failing

to prevent infection with pathogenic MDV. Thus vaccination is “imperfect” (Gandon *et al.* 2001), and vaccinal and pathogenic MDV strains persist together in the host (Witter and Schat 2003; Witter *et al.* 1971), simultaneously shedding virus in dander . Vaccine failure occurs due to continuous evolution of field strains possibly due to strong selective pressure generated by extensive vaccination with such imperfect vaccines (Davison and Nair 2005; Islam and Walkden-Brown 2007; Witter 1983; 1997).

The length of MDV genome is about 174-178 kilo base pairs (kb) (Lee *et al.* 2000; Tulman *et al.* 2000). The genome is similar to that of herpes simplex virus-1, comprising unique long and short regions as well as repeat long and short (UL, US, RL and RS) regions (Fukuchi *et al.* 1984; Lee *et al.* 2000). The genes in the repeat (RL and RS) regions are specific for MDVs, and the Marek’s *Eco*RI-Q (*meq*) gene is the most prominent gene amongst them. *Meq* is an oncogene and is present in MDV-1, but not MDV-2 or HVT (Afonso *et al.* 2001; Izumiya *et al.* 2001; Levy *et al.* 2005). Deletion of the *meq* gene results in failure of transformation of T-cells in chickens (Lupiani *et al.* 2004). The *meq* gene of the US isolate GA which is one of the first complete genome sequences available is 1020 bp long and its gene product is a 339 amino acid (aa) protein (Jones *et al.* 1992). However, the *meq* gene of the attenuated MDV-1 vaccinal isolate CVI988 differs from that of oncogenic isolates of MDV-1 with a 177 to 180 bp insertion (Chang *et al.* 2002a; Lee *et al.* 2000; Spatz and Silva 2007). Similarly, five Australian isolates of MDV-1 also have an insertion of 177 bp, but they have been pathotyped as v and vv depending on the indices of lymphoma and protection in unvaccinated and HVT-vaccinated specific pathogen free chickens (Renz *et al.* 2012).

Comparative molecular analysis of the DNA and deduced amino acid sequence of *meq* genes from v, vv, and vv+ pathotypes of MDVs have revealed changes and point mutations in the *meq* gene due to insertions or deletions that correlated with virulence (chang *et al.* 2002a ; Shamblin *et al.*2004) and now it is widely accepted that the *meq* gene is one of the most important genes involved in the determination of virulence of an MDV isolate (Lee *et al.* 2008; Lupiani *et al.* 2004). There are two reports showing that that the published pathotype of an MDV is associated with the number of uninterrupted 4 proline repeats (PPPP) in the C terminal domain of the Meq protein. The number of such repeats varies from 2 to 8 with highly virulent MDV-1 strains generally having the fewest and attenuated strains having the most (Renz *et al.* 2012; Shamblin *et al.* 2004).In the most recent of these papers the association was shown to be statistically significant.

The diagnosis of infection with MDV was originally based on isolation and identification of MDV from the infected tissues in cell culture or identification of the infected cells by immunostaining (Churchill and Biggs 1967; De Laney *et al.* 1998). More recently, real-time quantitative polymerase chain reaction (qPCR) has appeared as a rapid and sensitive method of diagnosis with the added advantage of quantification of MDV genome copy number (Baigent *et al.* 2005b; Islam *et al.* 2004) and ability to detect virus in a wide range of materials including poultry dander or dust (Islam *et al.* 2006; Islam and Walkden-Brown 2007; Renz *et al.* 2006).

In Iraq, there are very limited data available on the prevalence of MD and no data available on the putative virulence of the MDV isolates currently circulating. There were many outbreaks of MD across all regions of Iraq in the late 1970s and early 1980s (Al-Soudi *et al.* 1986; El-Meligy *et al.* 1988). In a retrospective study at the University of Mosul, MD was the most commonly diagnosed viral disease of poultry during the period of 1977-1986 (Al-Sadi *et al.* 2000). Currently MD vaccination is used only in commercial layers and breeder chickens. Serotype 1 CVI988 (Rispens) is used either alone or in combination with HVT while broiler chickens and indigenous village chickens remain unvaccinated. There is no clear evidence of MD vaccine failure in Iraq, but outbreaks of MD in vaccinated flocks of commercial farms of layers chickens have been reported (Al-Attar 1997; Al-Aubaedi 2000; Zahid 2008).
To better understand the MD situation in Iraq we designed an epidemiological study to test the following hypotheses:
MDV will be commonly found in samples of spleen and dust from unvaccinated commercial and village chickens.
The prevalence of MDV will vary between regions and types of chickens.
Sequencing of *meq* gene of Iraqi MDVs for molecular pathotyping will reveal significant polymorphism.

4.1.1 Materials and Methods

4.1.1.1 Site and ethical approval

Sample collection and DNA extraction occurred in Iraq while qPCR analysis, *meq* gene sequencing and data analysis occurred in Australia. The study was approved by the Animal Ethics Committee of the University of New England, Australia (AEC10/064).

4.1.2 Survey design

The survey estimated the point prevalence of MDV infection as detected by qPCR analysis of samples collected in a cross-sectional study with a 6×3 factorial design. Experimental factors and levels were: 6 provinces of southern Iraq: Hillah, Karbala, Samawah, Nassiriya, Al Kut and Amarah) and 3 sample types: (i) spleens from local layers (purchased from market); (ii) spleens from commercial broilers; and (iii) dust from commercial broiler farm sheds. All samples were from unvaccinated chickens or chicken populations. The target numbers of samples of different types to be collected in each of the six provinces were 5, 5 and 10 for layer spleen, broiler spleen and broiler dust respectively.

4.1.3 Extraction of DNA from the collected samples

The DNA was extracted from the samples in the Central laboratories of Samawa province, Iraq, using the Isolate DNA Kit (Bioline, Australia) according to the manufacturer's instructions. The extracted DNA was sent to Australia for further analysis under an approved quarantine permit (AQIS permit IP10013784).

4.1.4 Real-time PCR (qPCR)

The extracted DNA was quantified by using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop® Technologies Wilmington, USA) prior to analysis by qPCR. The DNA of the samples was diluted to a concentration of 5 ng/µl and analyzed by qPCR to quantify viral load (Islam *et al.* 2006).

4.1.5 PCR amplification of meq gene for DNA sequence analysis

PCR amplification of the *meq* gene and subsequent sequencing was attempted on samples from different regions containing high levels of MDV target and ceased after 9 samples had been sequenced. The extracted DNA was used as template for a standard PCR from each sample using the *meq*-specific primers, which are shown in Table 4.1. For sample 3A (layer spleen, Samawah), the entire *meq* coding region was amplified using the BCH342 and BCH343 primers. For the 6F (layer spleen, Hella), 10A (broiler dust, Samawah), 42C (broiler spleen, Amarah), 51C (layer spleen, Amarah), 52C (layer

spleen, Amarah), 57C (broiler dust, Amarah), 59C (broiler dust, Amarah), and 95E (broiler dust, Karbala) samples, two overlapping fragments of the *meq* gene were amplified using the primers pairs BCHM342/SJW1 and BCMD07/BCHM343. This was necessary as the amount of DNA in the original samples was very low and the primer pair BCH342/BCH343 did not amplify sufficient amounts of DNA for purification and sequencing. The standard PCR was performed in a final volume of 25 µl reaction mixture including 1 µmol of each primer, 10x reaction buffer, 0.2 mM dNTP's, 1.8 mM $MgCl_2$, 1 unit of Taq DNA polymerase (Fisher Biotec, Perth, Australia) and 1 ng of template DNA. Amplification cycling was over 35 cycles. Thermocycling conditions were as described previously by (Renz *et al.* 2012). All PCR products were separated electrophoretically in an agarose gel (1%) and visualized after staining with ethidium bromide. The target PCR products were then purified using the Wizard® DNA purification Kit (Promega, Madison, USA) according to the manufacturer's instructions.

4.1.6 Cloning of PCR fragments

The purified DNA fragment of one sample (3A) was ligated into the T-tagged site of the pGEM® T-easy vector according to the manufacturer's protocol (Promega, Madison, USA). Competent *E. coli* (DH5α) was used for transformation with the ligation mix and grown overnight on 2x YT agar plates containing 100 µg/mL ampicillin and 20 mg/mL X-gal, followed by blue-white screening to identify transformants containing recombinant plasmids. Restriction enzyme analysis was used to determine the size of the inserts in the plasmids.

4.1.7 DNA sequencing and analysis

The sequencing of the 3A *meq* gene clones and 6F, 10A, 42C, 51C, 52C, 57C, 59C, and 95E PCR products was performed by Macquarie University, Sydney, Australia using an ABI 377 sequencer (Applied Biosystems Inc., Foster City, CA, USA). The primers used for sequencing in this study are shown in Table 4.1. Results from sequencing were analysed using Chromas© 1.43 for analysis of chromatograms, Sequaid™ 3.70 and ClustalW© 1.83 (European Bioinformatics Institute) software for sequence alignment.

4.1.8 Statistical analysis

The ratio of positive to negative samples for MDV was analysed using a generalized linear model with a logit link function, fitting the effects of province (six provinces) and sample type (bird and sample type combined) and the interaction between the two. The MDV load in samples (virus copy number per mg of sample) was log transformed [$log^{10}(y+1)$] and fitted in a general linear model testing the effects of chicken type, sample type (bird and sample type combined) and their interaction. The output subjected to analysis of variance. Analyses were performed using JMP ™ v 9 statistical software (SAS Institute Inc., NC, USA) with a significance level of $P<0.05$ used throughout.

4.2 Results

4.2.1 Prevalence of MDV as determined by qPCR (%)

There was a trend towards an influence of province (P=0.08) but not sample type (P=0.89) or their interaction (P=0.13) on the proportion of MDV-positive samples as shown in Table 4.2. Of 109 samples tested, 54 were positive giving an overall prevalence of MDV1 of 49.5%. The prevalence in different provinces ranged from 36.8% in the Karbala and Nassiriya to 65% in Amarah There was little difference in prevalence between commercial broiler and village layer chickens, or between dust and spleen samples, being 59.1%, 46.7% and 48.1% for broiler dust, broiler spleen and layer spleen respectively.

4.2.2 MDV load

There was no significant influence of province (P=0.10), sample type (P=0.38) or their interaction (P=0.40) on mean Log^{10} MDV viral copy number per mg of dust or spleen sample Table 4.3. The overall mean (±SEM) of 1.78±0.19 when back transformed gives a mean viral copy number (VCN) of 59.8 viral copies per mg of spleen or dust. In positive samples only, the overall mean was 3.43±0.18. The Log^{10} viral load in different provinces ranged from 0.64±0.47 in the Karbala to 2.51±0.45 in Amarah. There was little difference in viral load sample types being 1.86±0.30, 1.30±0.35 and 1.94±0.8 for broiler dust, broiler spleen and layer spleen respectively. There was no significant difference in viral load between broiler dust and broiler spleen (P=0.23) or between broiler spleen and layer spleen (P=0.22).

4.2.3 *meq* gene sequencing of Iraqi samples

The meq gene from nine samples from four different provinces was sequenced. It was cloned in duplicate from one sample (3A) while the sequences of eight other samples (6F, 10A, 42C, 51C, 52C, 57C, 59C, and 95E) were obtained from purified PCR products. The length of the meq ORF (897 bp) was identical in all nine Iraqi field samples. Sequence analysis showed that each of the nine samples contained a meq open reading frame (ORF) of 897 bp nucleotides, which encoded a 298 amino acid polypeptide. To confirm that the length of the meq gene obtained from overlapping PCR products was 897 bp, the entire gene was amplifed from the nine samples (Figure 4.1). The size of the product (approx. 1000 bp), which includes 81 bp upstream of the meq start codon and 11 bp downstream from the stop codon, is the size predicted from the sequence generated with the overlapping fragments (897 bp + 92 bp = 989 bp).

The *meq* sequence of the 3A clones (from layer spleen, Samawah) contained five bp differences when compared to the eight *meq* gene sequences obtained from PCR products. The sequences obtained from 6F (layer spleen, Hella), 10A (broiler dust, Samawah), 42C (broiler spleen, Amarah), 51C (layer spleen, Amarah), 52C (layer spleen, Amarah), 57C (broiler dust, Amarah), 59C (broiler dust, Amarah), and 95E (broiler dust, Karbala), were identical except at position 502 which gave an ambiguous sequence off the same PCR template. Thus, it is not certain that the difference at position 502 represents a polymorphism.

4.2.4 Comparisons within and between Iraqi and international *meq* sequences

The sequences obtained from the Iraqi samples was aligned with the sequences of the *meq* gene of the Australian reference strain MPF57 (v) and USA reference strains of different pathoptype: JM/102/W (v), Md5 (vv), 648A (vv+) and Rispens CVI988 (att). These sequences were obtained from GenBank® and respective accession numbers are given in Table 4.4. Compared to the *meq* sequences of either the US isolate Md5 (1020 bp), the Australian isolate MPF57 (1197 bp) or the vaccine isolate CVI988 (1200 bp), the Iraqi *meq* sequences at 897 bp were shorter and contained a 123 bp in-frame deletion in the sequences encoding proline-rich region. In comparison to the sequence of Md5,

there were also several single nucleotide polymorphisms (SNP) in the Iraqi *meq* sequences.

4.2.5 Amino acid alignment of Iraqi sequences and international sequences of the Meq protein

The deduced Meq amino acid sequence from the Iraqi samples and MDV isolate Md5 were aligned as shown in Figure 4.4. The Iraqi *meq* genes encode 298 aa, the Md5 *meq* gene encodes 339 aa whereas the Australian reference strain MPF57 and the vaccinal strain CVI988 encode 398 and 399 aa respectively (Table 4.4.). All the Iraqi samples showed a deletion of 41 aa in the proline-rich domain when compared to Md5. As a consequence of the SNP's in the DNA sequences of the Iraqi samples there were a number of changes in the amino acid sequences of the Iraqi Meq proteins compared to Md5. These differences were at the positions 71, 77, 80, 119, 168, 242 and 279 in the Iraqi Meq protein. All Iraqi samples shared amino acid polymorphisms at position 77 (K →E), 242 (V→A) and 279 (T→I).

4.2.6 Number of 4 proline (PPPP) repeats

The deletion mutations within the Iraqi *meq* genes occurred in the region encoding the proline-rich domain and, as a consequence, the number of 4-proline repeats (PPPP) in the proline-rich domain of the protein of the Iraqi Meq was reduced to only two. This is similar to the US isolate 648A (vv+) which also contains only two PPPP repeats. In contrast, Md5 contains three, MPF57 contains five and Rispens/CVI988 contains eight PPPP repeats. In the Iraqi Meq protein, the overall proline content was 19.6 %, less than Md5 (21.3%), MPF57 (22.9%), or Rispens CVI988 (23.3%).

4.3 Discussion

The overall prevalence of MDV in the surveyed regions of Iraq as determined by qPCR was 49.5% supporting our hypothesis that MDV would occur commonly in unvaccinated chickens in Iraq. This prevalence is somewhat higher than the 26.1% MDV positive dust samples from 249 known unvaccinated broiler farms in Australia (Walkden-Brown *et al.* 2013a). This may reflect the higher level of vaccination against MDV used in the Australian broiler industry where approximately 50% of flocks are vaccinated.

Interestingly there were few differences in the prevalence of MDV or the viral load measured in spleen samples collected from commercial broiler or village layer chickens, or between dust and spleen samples collected from broilers. The former does not support our second hypothesis while the latter does not support the greater sensitivity of testing dust compared to spleen, reported by (Walkden-Brown *et al.* 2013a). However it does confirm the utility of using farm dust samples to monitor MDV.

The extent to which the high prevalence of MDV detected in Iraq translates into clinical MD is unknown. Broilers in Iraq are killed at a relatively early age (approximately 40 days) and it is unlikely that clinical MD would be manifest by this age. However the immunosuppressive effects of MDV are evident from the first week of infection (Witter *et al.* 1980), and could be expected to impact adversely on chicken performance in infected flocks. Village chickens live to older ages and clinical expression of MD is therefore more likely in these birds. Indeed, Al-Sadi *et al.* (2000) reported that in Mosul, northern Iraq, MD was the most commonly diagnosed viral disease of poultry. However, clinical MD is most commonly seen where there are high concentrations of poultry and associated high levels of early challenge, and these conditions may not be met for village poultry. Furthermore susceptibility to MD is under strong genetic control (Bacon *et al.* 2001) and it is possible that indigenous long-lived chickens have faced natural selection for MD resistance for long periods.

Sequence analysis of the *meq* gene of MDV from nine Iraqi samples revealed limited variation. This result did not support our third hypothesis that sequencing of the *meq* gene would reveal significant polymorphism. It is unclear why there was such limited variation in *meq* gene sequence. Significant polymorphism is evident within the USA (Shamblin *et al.* 2004) and between countries (Renz *et al.* 2012) but other studies have shown limited polymorphism within countries. For example Renz *et al.* (2013), found limited variation in *meq* gene sequence in 5 Australian MDVs isolated from very widely separate regions over a 10-year period.

When compared with well-characterised international isolates of MDV the Iraqi samples had a shorter *meq* gene comprising 897 bp due to a deletion in the proline-rich domain. The proline-rich domain is crucial for the transactivation activity of Meq in transformed T-cells (Liu and Kung 2000; Qian *et al.* 1996). Previous studies have also reported diversity in *meq* due to the differences in the proline-rich repeat region. Chang *et al.* (2002b) reported that MDV-1 strains that have duplicated proline-rich motifs and a longer meq (L-*meq*) exhibit a higher level of trans-repression than do *meq* variants containing the single

proline-rich motif short *meq* (S- *meq*), which is found in virulent strains. While the *meq* gene of the Iraqi samples is amongst the shortest reported, even shorter *meq* sequences have been lodged in GenBank, including what was nominated as very short *meq* gene with an overall length of 744 bp (GenBank accession AB087744.1). The very short *meq* gene also has a deletion in the sequences encoding the proline-rich region. The Meq product of the very short *meq* gene was 247 aa while, the products of the Iraqi *meq* genes were all 298 aa.

The attenuated vaccinal isolate CVI988 has a longer L *meq* gene due to a 177 - 180 bp insertion relative to MD5 which was thought perhaps responsible for its non-oncogenicity (Chang *et al.* 2002a; Lee *et al* 2002; Spatz *et al.* 2007). However insertions of 177 bp, resulting in L *meq* gene have also been reported in Australian isolates of pathogenic MDV ranging in pathotype from virulent or very virulent (Renz *et al.* 2012). The Iraqi *meq* gene, which encodes 298 aa, was shorter than the clusters of L-*meq* that encode ≥398 aa and S-*meq* that encode 323-339 aa, but longer than a reported very short or VS-meq encoding 247 aa (Renz *et al.* 2012). These and other polymorphisms in the sequence of *meq* genes have been linked with virulence in a number of studies (Chang *et al.* 2002a; Lee *et al* 2002; Shamblin *et al.* 2004 Spatz and Silva 2007). It has been reported that MDV1 strains with low copies of the proline-rich repeat region of *meq* tended to be highly virulent strains (Chang *et al.* 2002b; Shamblin *et al.* 2004) and Renz *et al.* (2012) identified a significant statistical association between the number of 4-proline repeats in the sequence and reported pathotype. More pathogenic MDV was associated with a lower overall proline content and fewer 4-proline repeat sequences.

In this study, *meq* sequences of the Iraqi MDV samples had a low proline content of 19.6% and only two 4-proline repeats, the same number as reported for the most virulent MDV isolates that have been pathotyped such as the vv+ MDV1 strains including 648A (24). Based on this the Iraqi MDV strains are likely to be highly virulent but this requires confirmation in formal *in vivo* pathotyping experiments.

4.3.1 Conclusion

A molecular survey of MDV presence in chicken spleen and farm dust samples has revealed a high prevalence of MDV in commercial broiler and local layer chickens from 6 provinces in southern Iraq. Sequencing the *meq* gene from 9 samples revealed limited polymorphism and a uniformly short length of 897 bases. Amino acid alignment revealed

a low proline content of 19.6% and only two 4-proline repeats (PPPP). This suggests that Iraqi MDV isolates are potentially highly virulent, but this needs to be confirmed by formal pathotyping *in vivo*. These findings demonstrate the potential of molecular epidemiological methods to investigate the prevalence and potential virulence of MDV.

4.4 Acknowledgements

The authors would like to thank the following people who assisted with the experiment: In Iraq, Dr. Emmad Al-Kammas at Baghdad Veterinary College, Mr.Ali Hussun, Head of the Central Medical Laboratories (Samawah), and also Dr. Abu-Khaila and the laboratory staff of the Samawah Tuberculosis Hospital. We also thank Mrs. Sue Burgess and Megan Sutherland for their assistance and technical support at the University of New England, Australia.

Table 4.1: Primers used for amplification and sequencing of the *meq* gene

Primers	Sequences	Orientation of primer	Location in MD5 genome*	Expected amplicon size (bp)	Use
BCMD07	5'-TGAACCTCCCATTTGCACTC -3'	5'	135301-135320	600bp (with BCH343)	Amplification, Sequencing
BCH343	5'-TGCTGAGAGTCACAATGC -3'	3'	135893-135910	1125 bp (with BCH342)	Amplification, Sequencing
BCH342	5'-ATTCCGCACACTGATTCC -3'	5'	134786-134803	1125 bp (with BCH343)	Amplification, Sequencing
SJW1	5'-CTAACGCTCCACATTGCT -3'	3'	135345-135362	600bp (with BCH342)	Amplification, Sequencing
SJW2	5'-AATGCGGATCATCAGGGTCT-3'	3'	135878-135897		Amplification, Sequencing
M13F-Puc	5'-GTTTTCCCAGTCACGAC-3'	5'	2958-2972		Sequencing
M13R-Puc	5'-CAGGAAACAGCTATGAC-3'	3'	175-192		Sequencing

*The location of M13-pUC primers in pGEM-Teasy is given.

Table 4.2: Prevalence of MDV as determined by qPCR (positive/total) by sample type and province. The effects of sample type, province and their interaction were not significant.

Code	Province	Ratio of MDV positive/total samples (%)			
		Broiler dust	Broiler spleen	Layer spleen	All sample types
A	Samawah	6/10	2/5	2/5	10/20 (50.0%)
B	Nassiriya	4/10	2/4	1/5	7/19 (36.8%)
C	Amarah	6/10	2/5	5/5	13/20 (65.0%)
D	Kut	4/9	5/6	3/5	12/20 (60%)
E	Karbala	6/10	1/5	0/4	7/19 (36.8%)
F	Hillah	1/3	2/5	2/3	5/11 (45.5%)
All provinces		27/52 (51.9%)	14/30 (46.7%)	13/27 (48.1%)	54/109 (49.5%)

Table 4.3: Mean MDV-1 viral load (Log^{10} VCN/mg of sample) by sample type and province (least squares means ± SEM). The effects of sample type, province and their interaction were not significant.

Code	Province	Log^{10} MDV-1 VCN/mg of sample (LSM±SEM)			
		Broiler dust	Broiler spleen	Layer spleen	Province LSM
A	Samawah	2.45±0.60	1.33±0.86	2.23±0.86	2.00±0.45
B	Nassiriya	2.11±0.64	1.27±0.96	0.67±0.86	1.35±0.48
C	Amarah	2.76±0.61	1.37±0.86	3.41±0.86	2.51±0.45
D	Kut	0.91±0.64	2.17±0.78	2.56±0.86	1.88±0.44
E	Karbala	1.60±0.61	0.32±0.86	0.00±0.96	0.64±0.47
F	Hillah	1.36±1.11	1.37±0.86	2.75±1.11	1.83±0.59
Type	LSM	1.86±0.30	1.30±0.35	1.94±0.38	

Table 4.4: GenBank® accession numbers of the *meq* gene sequences of selected well characterised international isolates of MDV and other variables derived from the sequences.

MDV Isolate	Country of origin	Accession number	Meq length (aa)	Insertion size (aa)	Proline (%)	Number of PPPP's	Pathotype*
CVI988/Rispens	Netherlands	DQ534538	399	60	23.3	8	attMDV
JM/102W	USA	DQ534539	399	60	23.1	6	vMDV
MPF57	Australia	EF 523771	398	59	22.9	5	vMDV
RB1B	USA	AY243332	339	nil	21.5	5	vvMDV
Md5	USA	AF243438	339	nil	21.3	4	vvMDV
648A	USA	AY362725	339	nil	20.9	2	vv+MDV
Iraq3A	Iraq	KC243262	298	nil	19.6	2	NP
Iraq6F	Iraq	KC243263	298	nil	19.6	2	NP
Iraq10A	Iraq	KC243264	298	nil	19.6	2	NP
Iraq42C	Iraq	KC243265	298	nil	19.6	2	NP
Iraq51C	Iraq	KC243266	298	nil	19.6	2	NP
Iraq52C	Iraq	KC243267	298	nil	19.6	2	NP
Iraq57C	Iraq	KC243268	298	nil	19.6	2	NP
Iraq59C	Iraq	KC243269	298	nil	19.6	2	NP
Iraq95E	Iraq	KC243270	298	nil	19.6	2	NP

* NP= Not Pathotyped

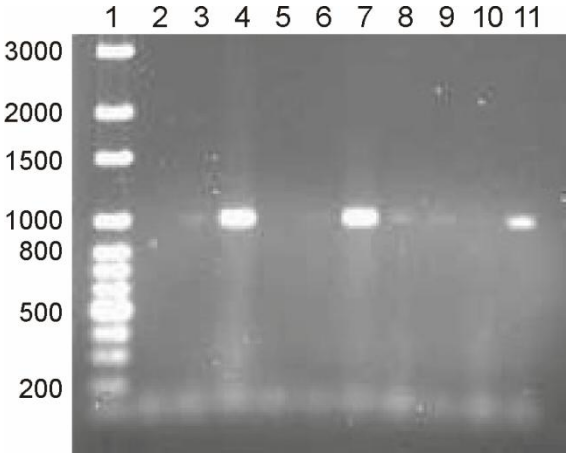

Figure 4.1: Confirmation of meq gene size in Iraqi samples. The meq gene was amplified from 3A (lane 3), 6F (lane 4), 10A (lane 5), 42C (lane 6), 51C (lane 7), 52C (lane 8), 57C (lane 9), 59C (lane 10), and 95E (lane 11) using primers BCH342 and SJW2. These primers are predicted to generate a product of 989 bp if the meq ORF is 897 bp in length. No template DNA was added to the reaction in lane 2. Lane 1 contains a 100 bp ladder.

```
IRAQ1   MSQEPEPGAMPYSPADDPSPLDLSLGSTSRRKKRKSHDIPNSPSKHPFPDGLSEEEKQKL 60

IRAQ2      MSQEPEPGAMPYSPADDPSPLDLSLGSTSRRKKRKSHDIPNSPSKHPFPDGLSEEEKQKL 60
IRAQ3      MSQEPEPGAMPYSPADDPSPLDLSLGSTSRRKKRKSHDIPNSPSKHPFPDGLSEEEKQKL 60
Md5        MSQEPEPGAMPYSPADDPSPLDLSLGSTSRRKKRKSHDIPNSPSKHPFPDGLSEEEKQKL 60
648A       MSQEPEPGAMPYSPADDPSPLDLSLGSTSRRKKRKSHDIPNSPSKHPFPDGLSEEEKQKL 60
           ************************************************************

IRAQ1      ERRRKRNRDASRRRRREQTDYVDKLHEACEELQRANEHLRKEIRDLRTECTSLRVQLACH 120
IRAQ2      ERRRKRNRDASRRRRREQTDYVDKLHEACEELQRANEHLRKEIRDLRTECTSLRVQLACH 120
IRAQ3      ERRRKRNRDAARRRRREQTYYVDKLHEACEELQRANEHLRKEIRDLRTECTSLRVQLARH 120
Md5        ERRRKRNRDAARRRRRKQTDYVDKLHEACEELQRANEHLRKEIRDLRTECTSLRVQLACH 120
648A       ERRRKRNRDAARRRRRKQTDYVDKLHEACEELQRANEHLRKEIRDLRTECTSLRVQLARH 120
           **********:*****:** ************************************ *

IRAQ1      EPVCPMAVPLTVTLGLLTTPHDPVPEPPICTPPPPSPDEPNAPHCSGSQP---------- 170
IRAQ2      EPVCPMAVPLTVTLGLLTTPHDPVPEPPICTPPPPSPDEPNAPHCSGPQP---------- 170
IRAQ3      EPVCPMAVPLTVTLGLLTTPHDPVPEPPICTPPPPSPDEPNAPHCSGPQP---------- 170
Md5        EPVCPMAVPLTVTLGLLTTPHDPVPEPPICTPPPPSPDEPNAPHCSGSQPPICTPPPPDT 180
648A       EPVCPMAVPLTVTLGLLTTPHDPVPEPPICTPQPPSPDEPNAPHCSGSQPPICTPAPPDA 180
           ****************************** **************.**

IRAQ1      --------------------------------PICTPAPPDAEELCAQLCSTPPPPICTPH 199
IRAQ2      --------------------------------PICTPAPPDAEELCAQLCSTPPPPICTPH 199
IRAQ3      --------------------------------PICTPAPPDAEELCAQLCSTPPPPICTPH 199
Md5        EELCAQLCSTPPPPISTPHIIYAPGPSPLQPPICTPAPPDAEELCAQLCSTPPPPICTPH 240
648A       EELCAQLCSTPPPPISTPHIIYAPGPSPLQPPICTPAPPDAEELCAQLCSTPPPPICTPH 240
                                          ****************************

IRAQ1      SLFCPPQPPSPEGIFPALCPVTEPCTPPSPGTVYAQLCPVGQAPLFTPSPPHPAPEPERL 259
IRAQ2      SLFCPPQPPSPEGIFPALCPVTEPCTPPSPGTVYAQLCPVGQAPLFTPSPPHPAPEPERL 259
IRAQ3      SLFCPPQPPSPEGIFPALCPVTEPCTPPSPGTVYAQLCPVGQAPLFTPSPPHPAPEPERL 259
Md5        SLFCPPQPPSPEGIFPALCPVTEPCTPPSPGTVYAQLCPVGQVPLFTPSPPHPAPEPERL 300
648A       SLFCPPQPPSPEGIFPALCPVTEPCTPPSPGTVYAQPCPVGQAPLFTPSPPHPAPEPERL 300
           ********************************** *****.****************

IRAQ1      YARLTEDPEQDSLYSGQIYIQFPSDTQSTVWWFPGDGRP 298
IRAQ2      YARLTEDPEQDSLYSGQIYIQFPSDTQSTVWWFPGDGRP 298
IRAQ3      YARLTEDPEQDSLYSGQIYIQFPSDTQSTVWWFPGDGRP 298
Md5        YARLTEDPEQDSLYSGQIYTQFPSDTQSTVWWFPGDGRP 339
648A       YARLTEDPEQDSLYSGQIYIQFPSDTQSTVWWFPGDGRP 339
           ****************** ******************
```

Figure 4.2: Deduced amino acid sequences of the Meq protein of the nine Iraqi MDVs compared to those of USA reference strains Md5 and 648A. Dashes indicate missing amino acids. Sequences which differ from the Md5 sequence are shaded. Asterisks on the bottom line indicate complete agreement in amino acid identity. IRAQ1 includes samples 6F, 51C, 57C, 59C, 95E; IRAQ2 samples 10A, 42C, 52C and IRAQ3 sample 3A.

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(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

	Author's Name (please print clearly)	**% of contribution**
Candidate	Salih Jabir Wajid	70
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Chapter 5 *In vivo* characterisation of two Australian isolates of Marek's disease virus including pathology, viral load and neuropathotyping based on clinical signs.

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Abstract

Objective To evaluate the pathogenicity of Australian Marek's disease virus (MDV) isolate MPF23 (1985) against the reference strain MPF57 based on pathology, viral load and neuropathotyping on the basis of clinical signs.

Procedure Two MDV challenge isolates (MPF57 or MPF23) were administered to unvaccinated SPF layer chicks on day 5 after hatch at three challenge doses (500, 2000 or 8000 plaque forming units (PFU)/chick). Mortality, body weight, immune organ weights, MDV load in peripheral blood lymphocytes (PBL) and clinical signs were measured to 56 days post challenge (dpc).

Results MPF23 was the more pathogenic of the two viruses inducing higher mortality (81% cf. 62%) and incidence of MD lesions (100% cf. 76%). MPF23 induced earlier, more sustained and more severe neurological signs in the period 26-56 days post challenge (dpc). However, there were few differences during the period 0-23 dpc used in the neuropathotyping classification under test. The observed pattern during this earlier period classifies both viruses as neuropathotype B consistent with a very virulent pathotype. MDV load in PBL at 7 and 44 dpc did not differ between virus isolates but load at 7 dpc was significantly and negatively associated with time to euthanasia or death.

Conclusion MPF23 appears to be as, or more, virulent than MDV strains isolated over the subsequent two decades. The neuropathotyping system developed in the USA did not clearly differentiate between the two isolates under test, however extension of the period of assessment of clinical signs beyond 26 dpc did reveal clear differences.

Key words: Marek's disease virus, neuropathotyping, pathotyping, virulence, immunosuppression, Australia, viral quantification.

Abbreviations: ADOL, Avian Diseases and Oncology Laboratory; CTP, classical transient paralysis; CEF, chick embryo fibroblasts; dpc, days post challenge; H&E, hematoxylin and eosin; HVT, herpesvirus of turkeys; MD, Marek's disease; MDV, Marek's disease virus; PBL, Peripheral blood lymphocyte; PVC, Perivascular cuffing with lymphocytes; PFU, Plaque forming unit; qPCR, Real-time quantitative polymerase chain reaction; SPF, Specific pathogen free; UNE, University of New England; vMDV, virulent MDV pathotype; vvMDV, very virulent MDV pathotype; vv+, very virulent plus MDV pathotype**.**

5.1 Introduction

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by the oncogenic cell associated alphaherpesvirus MDV). The clinical picture, tissue tropism and pathological lesions induced by MDV have changed over time. MD was described for the first time as polyneuritis by Marek (1907) in 1907, when the disease manifested itself clinically as a sporadic and chronic paralytic syndrome with swelling of the peripheral nerves in older fowls. This is known as classical MD. Later, occasional occurrence of lymphomas in the viscera in addition to polyneuritis was recognized. By the 1950s and 1960s as the poultry industry intensified, the severity and importance of MD increased, characterised by a high incidence of visceral lymphomas and gross enlargements of the peripheral nerves. This form of MD, now classified as acute MD was also seen in younger chickens including broilers, and posed a major threat to the chicken industry. A significant research effort in the USA and UK resulted in clear differentiation of the causative agents of MD and lymphoid leucosis and development of effective vaccines to control MD (Churchill *et al.* 1969a; Okazaki *et al.* 1970). Initially herpesvirus of turkeys (HVT) vaccination was widely used to protect chickens against MD. However, the increase in virulence of MDV continued and break through disease in HVT-vaccinated chickens was observed in the USA (Eidson *et al.* 1981; Schat *et al.* 1982). The development of the acute form of MD and breakdown of vaccinal control were attributed to evolution of MDVs to greater virulence. Subsequently, the bivalent HVT+SB-1 (a nononcogenic serotype 2 MDV strain) (Schat and Calnek 1978b; Witter *et al.* 1990) was introduced. Also it was found that HVT provides only partially protective but HVT+SB-1vaccines provide a high level of protection against vv MDV strains (Schat *et al.* 1982).

However, again new more virulent MDV strains appeared, overcoming the protective effects of this vaccine (Baigent *et al.* 2006; Gimeno 2008; Schat and Baranowski 2007; Witter 1997) and exhibiting higher cytolytic activity, unusual brain tropism, increased atrophy of lymphoid organs, increased lymphoma induction and earlier host death (Cho *et al.* 1998; Gimeno *et al.* 1999; Witter *et al.* 1999). In light of these developments, methods for pathotyping of MDV to differentiate between isolates of different virulence became indispensable, and it became important to determine whether the evolution of MDV virulence observed in the USA (Witter 1997), was also occurring in other countries including Australia.

Early reports of virulence testing of Australian isolates of MDV used a variety of methods (De Laney *et al.* 1998; McKimm-Breschkin *et al.* 1990; Zerbes *et al.* 1994). However, differences in chicken genotype, maternal antibody status, challenge dose (if specified), vaccine type and dose make comparisons of isolate virulence amongst these and international reports difficult. To overcome this, more recent characterisations of MDV isolates (Renz *et al.* 2012; Walkden-Brown *et al.* 2013b; Walkden-Brown *et al.* 2007c) have used an adaptation of a standard pathotyping protocol developed at the Avian Diseases and Oncology Laboratory (ADOL) of the USA (Witter 1997; Witter *et al.* 2005).This method, designed for use in chickens with maternal antibody directed against MDV, classifies MDVs into mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+) pathotypes. In the Australian studies it was observed that in SPF chickens free of maternal antibody against MDV, significant numbers of birds exhibited an acute paralytic syndrome between days 9 and 15 post challenge with MDV resulting in overall mortality rates of 2-18% (Renz *et al.* 2012). This syndrome was not observed in commercial layer or broiler chickens challenged with the same dose and isolates of MDV, presumably due to the protective effect of maternal antibody (Walkden-Brown *et al.* 2013b). Based on studies in similar maternal antibody-free SPF chickens Gimeno and colleagues (Gimeno *et al.* 2002) developed an alternative pathotyping system for MDV based on the timing and severity of neurological clinical signs following challenge with MDV.

The main purpose of this study was to evaluate the virulence of Australian MDV isolate MPF23 against the reference isolate MPF57, as determined by the incidence of MD and the neuropathotyping classification of Gimeno *et al.* (2002) based on neurological signs. MPF23, a particularly pathogenic MDV was isolated in 1985 and induced tumours in more than 80% of HVT vaccinated SPF chickens and more than 55% of chickens vaccinated with MDV2 or HVT/MDV2 vaccine (McKimm-Breschkin *et al.* 1990). MPF57, the Australian standard challenge isolate was isolated in 1994 and induced 60-100%

mortality and gross lesions in unvaccinated SPF chickens (De Laney *et al.* 1998). Both isolates were initially classified as vvMDV, but more formal pathotyping by the ADOL method has seen MPF57 re-classified as vMDV (Renz *et al.* 2012).

5.2 Materials and Methods

5.2.1 Experimental design

The experiment utilised a 2x3 factorial design with an additional external negative control group which was not challenged with any virus. The experimental factors in the factorial part of the experiment were MDV challenge isolate (MPF57 or MPF23) and challenge dose [500, 2000 or 8000 plaque forming units (PFU)/chick] administered on day 5 after hatch. The experiment lasted for 61 days (to 56 dpc). The experiment was approved by the Animal Ethics Committee of the University of New England.

The experimental chickens comprised 51 SPF white leghorn type (Lohmann LSL classic) chickens hatched at University of New England (UNE) from SPF eggs provided by Australian SPF Services Pty Ltd, Woodend Vic, Australia. As the chickens were from unvaccinated SPF parents they would be free from maternal antibody to MDV. The chicks were also unvaccinated.

At hatch chicks were individually identified by wing tag and toe mark and transferred to three positive pressure isolators. One isolator held 9 unchallenged chickens, while the other two isolators held 21 chickens challenged either with (MPF57 or MPF23). Each challenge MDV was administered via subcutaneous (s.c.) route at doses of 500, 2000 and 8000 PFU in 200 µl diluent (tissue culture Medium199) to 7 birds in each isolator. Unchallenged control chickens were administered 200 µl of the diluent only.

5.2.2 Challenge viruses

The challenge viruses were grown and titrated at UNE in chick embryo fibroblasts (CEF). Batch numbers were MPF23_P4_021209 and MPF57_P7_040810 respectively. CEF were inoculated with splenocytes from SPF chickens challenged with low passage virus and the isolates titrated for PFU after passages 4 and 7 respectively. The original infective MPF23 material was kindly provided by Prof. Greg Tannock of RMIT University and this is the first time MP23 has been grown successfully in cell culture since the first

report of this isolate by McKimm-Breschkin *et al.*, (1990). It was the most virulent of the isolates tested in that study. MPF52 was isolated in 1994 and has been extensively characterised since (De Laney *et al.* 1995; Renz *et al.* 2012; Walkden-Brown *et al.* 2007a; Walkden-Brown *et al.* 2013b).

5.2.3 MD diagnosis and scoring

Clinical monitoring of individual chickens to enable classification of chickens into the 5 clinical patterns described by Gimeno *et al.* (1999) commenced at 5 days post challenge (dpc). Birds were scored 0-3 for severity of paresis/paralysis of the neck, paresis/paralysis of limbs, ataxia, torticollis and nervous tics (0 = no signs, 3 = severe). These were summed to provide an overall clinical score. Chicks were euthanized if clinical signs became severe and recovery appeared unlikely.

Diagnosis of MD was based on gross pathology observed on post-mortem examination, or in the case of a small number of chickens, clinical paralysis accompanied by mild gross signs such as thymic or bursal atrophy. Carcasses were checked for nodular lesions on the skin. Breast and thigh muscles were inspected for discrete lymphoid tumours or diffuse infiltration. The thymus was inspected for atrophy and scored 0-3 in ascending order of severity (0 = normal, 3 = complete or almost complete atrophy). After opening the carcass, the internal organs were examined for gross enlargement and discrete or diffuse MD lesions. The bursa of Fabricius was examined and scored for atrophy as for the thymus. The sciatic nerve and plexus were examined for enlargement, change of colour or loss of striations, or asymmetry in size. All MD lesions were scored 1-3 subjectively for severity based on the size and extent of the lesion.

From some chickens with neurological signs, brain and peripheral nerve (right and left sciatic nerves) were collected for histological examination. The tissue samples were fixed in 10% neutral-buffered formalised saline solution for 48 hr, dehydrated in ascending graded ethanol solutions and embedded in paraffin wax, sectioned into 5 micron sections, mounted on glass slides, and stained with haematoxylin and eosin (H&E). Marek's disease lesions of the sciatic nerve were classified as type A-C or mixtures as described by Payne and Biggs (1967). Type A is characterised by proliferation of lymphoid cells, presence of characteristic Marek's disease cells, and sometimes demyelination and Schwann cell proliferation. B-type lesions comprise diffuse infiltration of plasma cells and mainly small lymphocytes, and interneuritic edema while

demyelination and Schwann cell proliferation. C-type lesions are characterized by light infiltration of plasma cells and small lymphocytes.

5.2.4 Blood sampling and qPCR of lymphocytes for MDV

Blood samples were collected from all birds at 7 and 44 dpc. Blood samples were collected into 1.5 ml microfuge tubes pre-loaded with 150 µl of 3 % sodium citrate prior to separation of peripheral blood lymphocytes (PBL) using Ficoll Paque^{TM} PREMIUM (Amersham Biosciences, Sweden). DNA was extracted from PBL using the ISOLATE genomic DNA mini kit (Bioline, Australia). All DNA was quantified using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop® Technologies Wilmington, USA). Taqman real-time PCR assay (qPCR) for MDV1 was performed using a Rotor Gene 3000 real-time PCR machine (Corbett Research, Australia) using the method described previously (Islam 2006; Islam *et al.* 2006).

5.2.5 Statistical analysis

Mortality patterns were investigated using survival analysis (Kaplan-Meier product-limit method) and the chi square test of independence. The latter was also used to compare the ratio of MD positive and negative chickens between groups. Treatment effects on live weight relative immune organ weights and qPCR data were investigated by analysis of variance testing the effects of sex, MDV challenge isolate, challenge dose and two-way interactions. MDV viral load data were transformed using a Log^{10} (y+1) transformation to stabilise the variance. Association between variables was analysed using linear regression models. Analyses were performed with JMP10 (SAS Institute, Cary NC, USA). Data for continuous variables are generally reported as least squares means and standard errors. A statistical significance level of $P<0.05$ is used throughout.

5.2.6 Results

5.2.6.1 Clinical signs

No neurological signs were observed in the control chickens. In the MDV-challenged groups signs commenced at 5 dpc and showed a biphasic pattern with an initial period of clinical signs between 5 and 17 dpc followed by a period of no clinical signs, then a second period of clinical signs starting on 25 dpc for MPF23 and 41 dpc for MPF57

(apart from signs from a single bird that showed clinical signs on 25 dpc before dying on 26 dpc) and continuing until the end of the experiment at 56 dpc (Figure 5.1). The incidence of clinical signs peaked at 8 dpc for both MPF23 (12/21 birds) and MPF57 (9/21 birds). Clinical signs typically commenced with depression or unwillingness to move followed by leg dragging/knuckling over, drooping of wings, mild opisthotonos and then paralysis. Paralysis during the later episodes typically involved chickens having one leg stretched forward and the other backward, together with flaccid paralysis or paresis of necks and wings.

There was no significant effect of challenge virus ($P < 0.47$) or dose ($P = 0.88$) on the percentage of chicks exhibiting early neurological signs between 5 and 17 dpc (81 and 71 % for MPF23 and MPF57 respectively; and 79, 71 and 79 % respectively for 500, 2000 and 8000 PFU). On the other hand, there was a highly significant effect of challenge virus ($P = 0.006$) but not dose ($P = 0.70$) on the percentage of chicks exhibiting late neurological signs between 21 and 56 dpc (90 and 52 % for MPF23 and MPF57 respectively; and 64, 79 and 71 % respectively for 500, 2000 and 8000 PFU. There was also a significant effect of challenge virus ($P = 0.01$) but not dose ($P = 0.21$) on the number of days for which a chicken exhibiting clinical signs was recorded with clinical signs (9.3 and 5.0 days for MPF23 and MPF57 respectively; and 8.6, 8.0 and 5.0 days respectively for 500, 2000 and 8000 PFU.

There was no significant effect of challenge virus ($P = 0.38$) or dose rate ($P = 0.89$) on the proportion of chicks in the neurological classifications of Gimeno *et al*., (1999) up to 23 dpc. Overall 1 chick (2.4%) showed acute transient paralysis (ATP) leading to death, 45% showed classical transient paralysis (CTP) with signs of 4 days duration or less, 29% showed neurological signs over a span of more than 4 days (PND) and 23% exhibited no clinical signs in this period (N). The percentages of chickens in each category that went on to show later neurological signs were 0, 84, 75 and 50 % respectively ($P = 0.1$).

5.2.6.2 Mortality and MD lesions

There was no mortality in the control group. The first death was at 7 dpc followed by a delay to the second death at 23 dpc and there was a significant difference in the survival curves with MPF23-challenged chickens showing a greater and more rapid late mortality rate ($P=0.03$; Figure 5.2). The overall mortality rate was higher in the MPF23 group (81%) than the MPF57 group (62%) but the difference was not significant ($P = 0.17$;

Table 5.1). There was a significant overall effect of challenge dose with significantly lower mortality rate in chickens challenged with 500 PFU (43%) than 2000 (79%) or 8000 PFU (93%) (P = 0.01). More females (77%) than males (65%) died during the experiment but the difference was not statistically significant (P = 0.38).

The first MD lymphoma was observed at 29 dpc. MD was diagnosed in 100% of birds in the MPF23 group compared with 76% in the MPF57 group (P = 0.01), with no significant effect of dose (P = 0.2, Table 5.1). A higher percentage of females (95%) than males (80%) were diagnosed with MD during the experiment but the difference was not statistically significant (P = 0.12). Gross MD lesions were most prevalent in sciatic nerve, spleen, ovary and liver with lesions observed in 12 different locations or tissues (Figure 5. 3).

The two virus strains induced a similar histopathological pattern in chicken brains characterised by perivascular infiltrates of the mononuclear cell or cuffing (PVC) with variable degrees of vacuolation. PVC was observed in all brain regions including the cerebellum (Figure 5. 4a and 4b), optic lobes, cerebrum as well as the meninges (Figure 5. 5a). The PVC consisted of lymphoid cells with very few plasma and blast-type cells, characterised by an indistinct cytoplasm and a round or oval vesicular nucleus containing dark clumped chromatin (Figure 5. 5b). Vasculitis, hypertrophy of the vascular endothelium and intramural vacuolation were observed in the white matter of the cerebrum and the cerebellum in many sections from paralysed birds. Chickens exhibiting clinical signs during the early period (5-17 dpi) tended to have atrophy of thymus and bursa and histological lesions in the brains with or without MD lesions in the sciatic nerve. Gross enlargement of the sciatic nerve was encountered in chickens with clinical signs in the second episode (after 25 dpi), and they were type A, B or mixed type of A and B, (Figure 5. 6a and b) while C type (Figure 5. 7) was detected in chickens without gross nerve enlargement.

At 56 dpc there were significant effects of MDV challenge on bodyweight, and relative splenic, but not bursal weights (Table 5.2). Challenge with MDV reduced bodyweight and increased relative splenic weight with a non-significant reduction in relative bursal weight. Males were significantly heavier than females, but did not differ in relative bursal and splenic weights (Table 5.2).

5.2.7 MDV load in PBL

MDV load in PBL [Log^{10} (VCN/$10^{\wedge 6}$ cells +1)] at day 7 was significantly higher in female (2.96 ± 0.55) than male (0.94 ± 0.54) birds (P = 0.034) but there were no significant effects of challenge virus (P = 0.74) or challenge dose (P = 0.17) (Table 5.3). At 44 dpc viral loads were nearly 4 logs higher and the effects of sex (P = 0.48), challenge virus (P = 0.98) and challenge dose (P = 0.70) were all non-significant (Table 5.3). Samples from control chickens collected at days 0, 44 and 56 dpc were negative. In challenged birds, MDV load at 7 dpc had a significant negative linear association with days to euthanasia or death after challenge (P = 0.03, R^2 = 11.9%). There was also a non-significant trend towards a negative association with days to onset of clinical signs (P = 0.12, R^2 = 7.4%). Associations with other variables were non-significant. MDV load at 44 dpc was weakly positively associated with MDV load at 7 dpc (P = 0.12, R^2 = 9.7 %) but there were no significant associations with other measured variables.

5.3 Discussion

MPF23 was clearly the more pathogenic of the two viruses, as they were assessed by mortality and induction of MD. This difference was also clearly manifested in the clinical pattern observed (Figure 1). However, the difference in clinical pattern was only evident after 25 dpc with the MPF23 group showing earlier, more sustained and more severe clinical signs in the period 26-56 dpc. This lies outside the 0-23 dpc window used by Gimeno *et al.*, (1999; 2002) for neuropathotyping of MDV isolates. In the present experiment no difference between groups challenged with the two Australian MDV isolates was evident during this period, with the observed pattern classifying both viruses as neuropathotype B (Gimeno *et al.* 2002), consistent with a vv pathotype under the ADOL system of Witter (1997).

This classification is consistent with earlier *in vivo* evaluations of MPF23 (McKimm-Breschkin *et al.* 1990) and MPF57 (De Laney *et al.* 1998) in unvaccinated maternal antibody negative SPF chickens. However, subsequent studies on MPF57 in SPF white leghorn (Renz *et al.* 2012) and Cobb broiler (Walkden-Brown *et al.* 2013b) chickens have shown that while MPF57 is highly pathogenic in unvaccinated chickens as in the present experiment, vaccination with HVT bivalent HVT/SB1 vaccine provided a very high level protection, resulting a pathotype classification of MPF57 as v rather than vv. As discussed in both of these papers, such results suggest that the ADOL pathotyping

method adopted in the studies may confound two distinct traits that may or may not be closely associated. The first of these is classical virulence, as evidenced by disease induction in unvaccinated susceptible chickens and the second is vaccine resistance, or the ability to withstand the protective effects of vaccination with a particular vaccine. The proposed neuropathotyping method of Gimeno et al., (2002) is a test of virulence alone, without a vaccine resistance component. The isolate MPF23 is more likely to be a vv pathotype under the ADOL method based on the failure of HVT and or serotype 2 MDV vaccinations to provide significant protection in the initial report on this isolate (McKimm-Breschkin *et al.* 1990). However, confirmation of this would require a more formal pathotyping test using defined dose rates of challenge virus.

The bimodal clinical and mortality patterns observed in the current experiment are in concordance with the bimodal patterns of mortality reported earlier for MPF57 in SPF chickens (De Laney *et al.* 1998; Renz *et al.* 2012). However the early mortality occurred later in the present experiment, perhaps reflecting a recent change in the genotype of the SPF chickens to the current Lohmann LSL classic bird. This may exhibit greater genetic resistance to MD than earlier SPF genotypes used in Australia. While it is clear that the early clinical signs were due primarily to neurological lesions, given the lack of MD lymphomas observed during this period, clinical signs during the later periods may be confounded with general depression associated with widespread lymphoma formation. The neuropathotyping approach of Gimeno et al., (2002) avoids this confounding by concentration on the initial wave of clinical signs and does offer some advantages in terms of early characterisation of virulence. However, our results suggest that it may not be sensitive in differentiating MDV isolates of similar but clearly different, virulence.
Microscopic lesions of the brains of birds clinically affected with progressive paralysis during the early clinical stages of the experiment were characterized primarily by a perivascular cuffing, vasculitis, vacuolation (compatible with oedema) of the cerebrum and cerebellum, and scattered infiltration of lymphoid cells in the neuropil together with frequent meningitis. These CNS lesions were very similar to those described in previous reports (Gimeno *et al.* 2002; Gimeno *et al.* 1999; Kornegay *et al.* 1983; Swayne *et al.* 1989a; Swayne *et al.* 1988), where it was suggested that vasogenic brain oedema is perhaps the primary lesion resulting in paralysis associated with vv MDV strains.

MD nerve lesions encountered in this study were also consistent with earlier reports. Lymphocytic infiltration of peripheral nerves has been reported in chickens with neurological signs of TP (flaccid paralysis, ataxia, or torticollis) (Cho *et al.* 1970; Wight 1968; Zander 1959) and also classical MD (Marek 1907; Pappenheimer *et al.* 1926;

Wight 1962b)TP, which was observed in the present experiment was characterized by an acute onset of flaccid paralysis of the neck and wings followed by a rapid recovery within 24-48 hr. Classical MD (fowl paralysis), also observed in the experiment was characterized by spastic paralysis of the limbs and lameness due to lymphocytic infiltration of peripheral nerves, spinal cord, and associated ganglia.

Early evaluation of MDV load in spleen or PBL is an attractive alternative predictor of MDV virulence (Yunis *et al.* 2004) and MDV load in spleen at 7 dpc is a good predictor of subsequent MD status (Walkden-Brown *et al.* 2013b). In the present experiment, MDV load in PBL at 7 dpc was significantly and negatively associated with days to euthanasia or death after challenge with a non-significant trend (P = 0.12) towards a negative association with days to onset of clinical signs. However the prediction equations were not strong, accounting for a maximum of 11% of variation in the target variable. Neither challenge isolate, nor dose of virus significantly influenced MDV load in PBL at 7 and 44 dpc. Interestingly female chickens had significantly higher MDV load than males at 7 dpc. This is consistent with the oft-reported greater susceptibility of female chickens to MD but not with the reported higher incidence of early MD mortality in male than female chickens (Renz *et al.* 2012).

These results support an earlier report of very virulent MDVs circulating in Australia in the mid 1980s (McKimm-Breschkin *et al.* 1990) and show that MPF23, the most virulent isolate from that period is as, or more, pathogenic than MPF57, one of the most virulent Australian isolates of the following decade. Taken together with other studies involving MPF57 and later isolates (Renz *et al.* 2006; Walkden-Brown *et al.* 2013b), there is little evidence in Australia of a systematic increase in the virulence of MDV over the last 3 decades unlike the reported situation in the USA (Witter 1997). The results of the present experiment also showed that the neuropathotyping approach of Gimeno et al., (2002) did not differentiate between the isolates MP57 and MPF23 despite some differences in pathogenicity evident only later in the experiment. Both were ascribed neuropathotype B, broadly consistent with a vv pathotype under the ADOL system. These results indicate that, if the clinical observation period is extended, a more sensitive measure of virulence may be obtained. However the practicalities of such prolonged observation and the confounding of neurological signs with the onset of lymphoma's limit this approach and suggest that pathotyping based on the induction of gross MD lesions remains a more sensitive test of pathogenicity.

Table 5.1: Mortality rate and incidence of MD to day 56 dpc by challenge treatment and dose.

Treatment	Overall treatment effect		Challenge dose (PFU)	Effect of dose	
	Mortality	MD		Mortality*	MD
MPF57	13/21 (62%)[a]	16/21 (76%)[a]	500	3/7 (43%)	4/7 (57%)
			2000	4/7 (57%)	7/7 (100%)
			8000	6/7 (86%)	5/7 (71%)
MPF23	17/21 (81%)[a]	21/21(100%)[b]	500	3/7 (43%)	7/7 (86%)
			2000	7/7 (100%)	7/7 (100%)
			8000	7/7 (100%)	7/7 (100%)
Control	0/10 (0%)	0/10 (0%)			

[ab] Means not sharing a common letter in the superscript differ significantly (P<0.05)

* Overall effect of dose was significant (P = 0.01). 500 PFU mortality < 2000 and 8000 PFU.

Table 5.2: Effects of MDV challenge and sex on body weight, relative bursal weight and relative splenic weights of chickens surviving to 56 dpc.

Effect	Level	n	Least squares means (± SEM)		
			Bodyweight (BW, g)	Relative bursal weight (% BW)	Relative splenic weight (% BW)*
Virus	Nil (Control)	9	792 ± 36[a]	0.303 ± 0.038	0.196 ± 0.021[a]
	MPF57	8	681 ± 39[ab]	0.278 ± 0.041	0.418 ± 0.026[b]
	MPF23	4	600 ± 53[b]	0.171 ± 0.057	0.377 ± 0.038[b]
	P-value		0.02	0.18	0.001
Sex	Female	9	591 ± 37[b]	0.231 ± 0.039	0.333 ± 0.027
	Male	12	791 ± 34[a]	0.270 ± 0.036	0.328 ± 0.020
	P-value		0.001	0.48	0.88

* 2 outlier birds with grossly enlarged spleens removed.

[ab] Means not sharing a common letter in the superscript within effect and variable differ significantly (P<0.05)

Table 5.3: Least squares means (± SEM) for MDV load in PBL at 7 and 44 dpc, by challenge virus and dose. These effects were non-significant. P values are provided in the text.

Treatment	Challenge dose (PFU)	MDV load in PBL [Log^{10} (VCN/$10^{\wedge 6}$ cells +1)] (Least squares mean ± SEM)	
		7 dpc	44 dpc
MPF57	500	1.43 ± 0.95	5.25 ± 0.39
	2000	2.45 ± 0.95	5.95 ± 0.35
	8000	1.93 ± 0.85	5.77 ± 0.39
	Overall	1.93 ± 0.53	5.65 ± 0.34
MPF23	500	0.20 ± 0.91	5.80 ± 0.39
	2000	1.46 ± 0.91	5.99 ± 0.83
	8000	3.40 ± 1.05	5.21 ± 0.92
	Overall	1.67 ± 0.53	5.66 ± 0.34

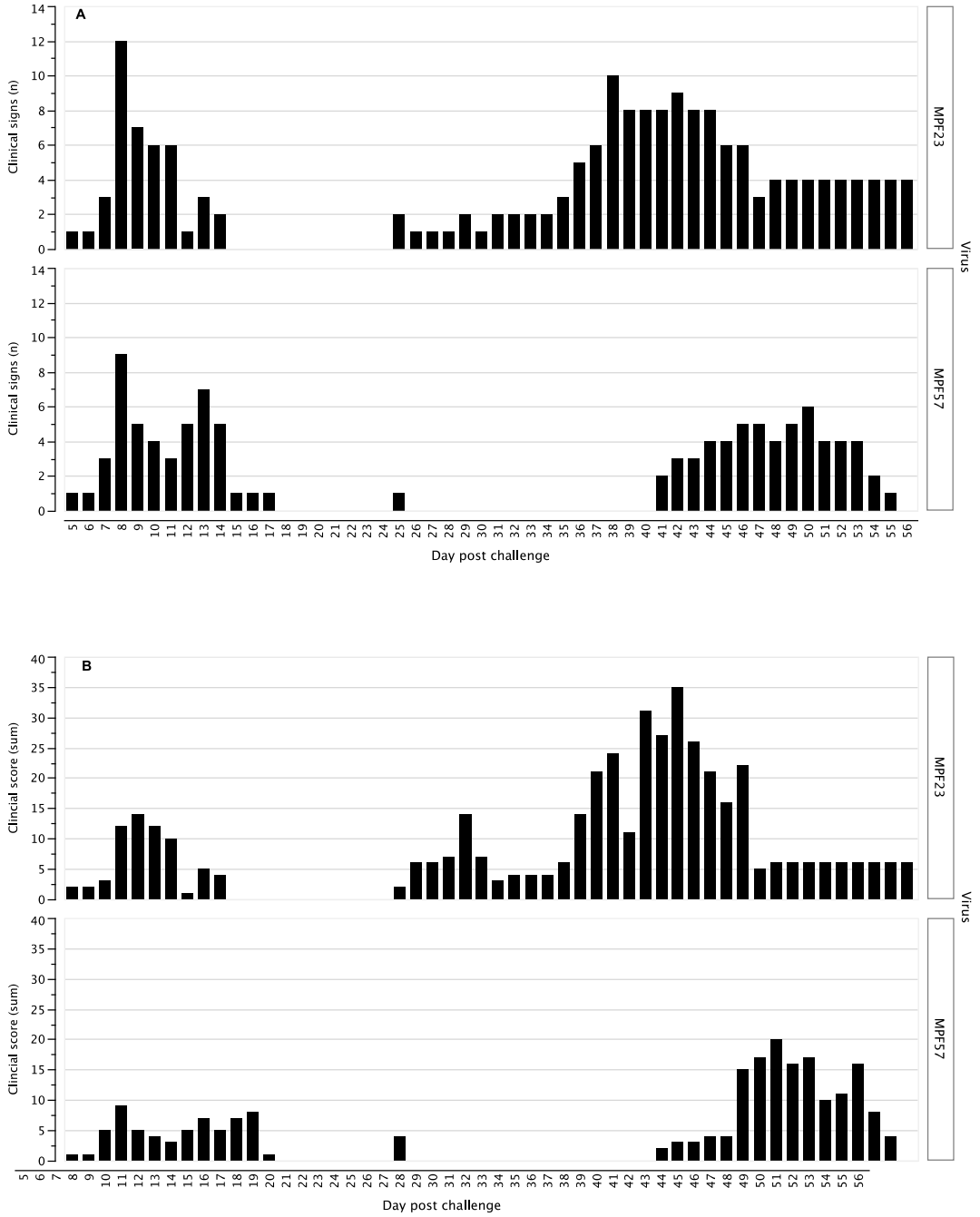

Figure 5.1: Number of chickens exhibiting clinical signs (A) and sum of clinical severity score (B) by challenge virus and day post challenge. There were 21 chickens in each virus group.

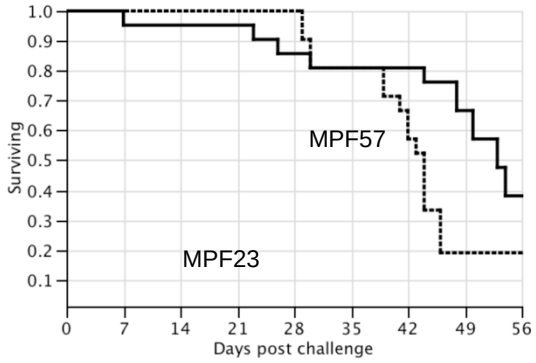

Figure 5.2: Effect of challenge virus on chicken survival to 56 dpc ($P < 0.05$). No control chickens died during the experiment.

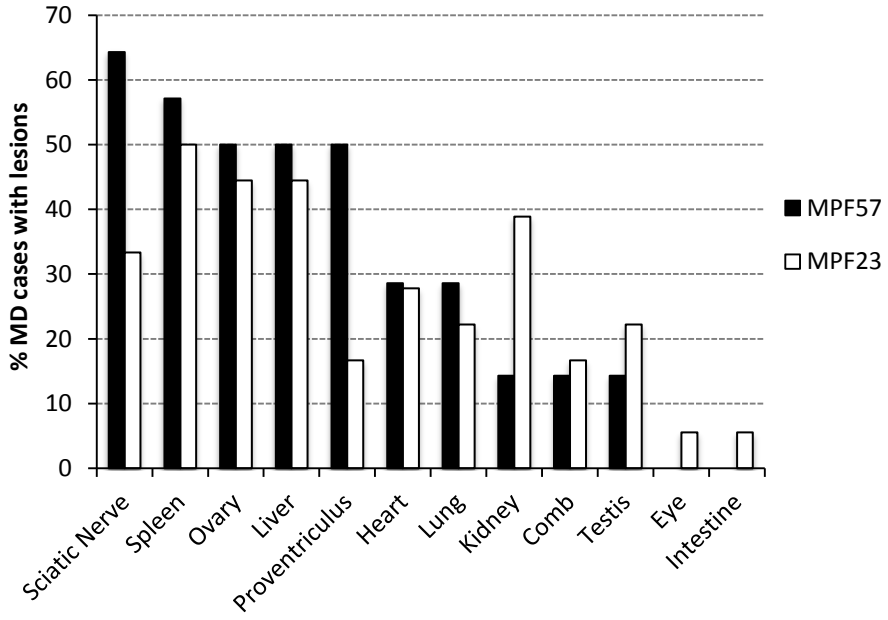

Figure 5.3: Incidence of gross MD pathology in chickens diagnosed with MD by organ and challenge virus.

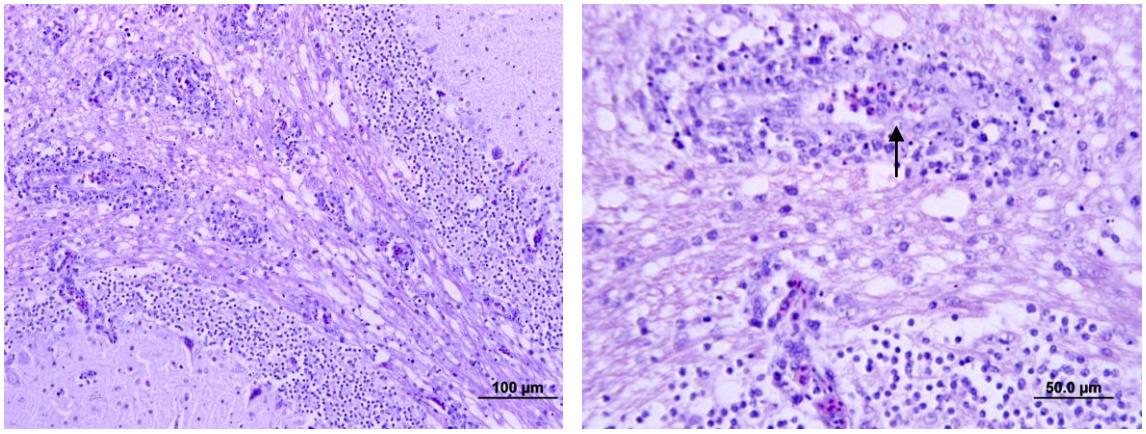

Figure 5.4: a) Section of cerebellum from a chicken euthanized at 29 dpc following a progressive paralysis. Note PVC and the diffuse vacuolation of the white matter and the granular layer. (H&E stain; × 200 magnification). b). High power view showing diffuse linear and round vacuolation of the perivascular region in white matter and the molecular layer of the cerebellum near the lower border of the micrograph. Note hypertrophied endothelial cells of the blood vessels (arrow) and infiltration with lymphoid cells and heterophils. (H&E stain; × 400 magnification).

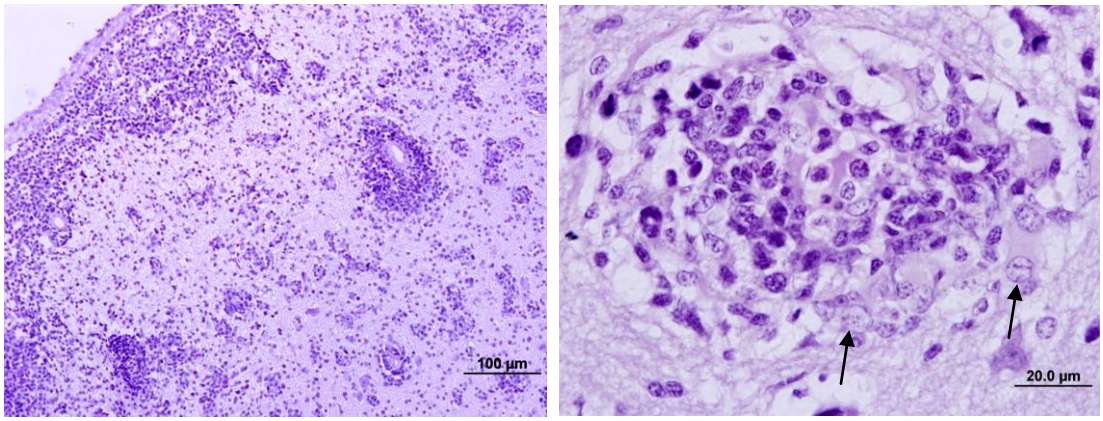

Figure 5.5: a) Section of cerebrum from a comatose chicken in the MPF23 challenge group euthanized at 29 dpc. It shows mononuclear PVC and meningitis with sparsely distributed lymphoid cells in the parenchyma. (H&E stain; × 200 magnification).
b). High power view showing PVC with lymphoid cells and blast cells (arrow) plus intramural vaculation (H&E stain; × 1000 magnification).

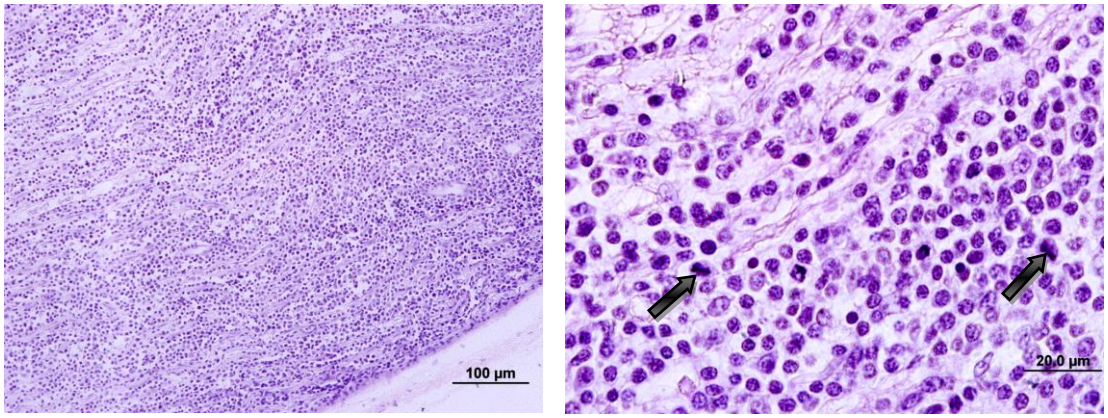

Figure 5.6: a) Section of sciatic nerve from a bird euthanized at 38 dpc with gross enlargement of the nerve, showing oedema and extensive lymphocytic infiltration (mixed A and Btypes). (H&E stain; × 200 magnification).
b) Higher magnification showing infiltration of small and large mononuclear cells between the sciatic nerve fibres with mild oedema between the individual nerve fibres (upper left corner). The arrowed cells exhibit mitotic figures (H&E stain; × 1000 magnification).

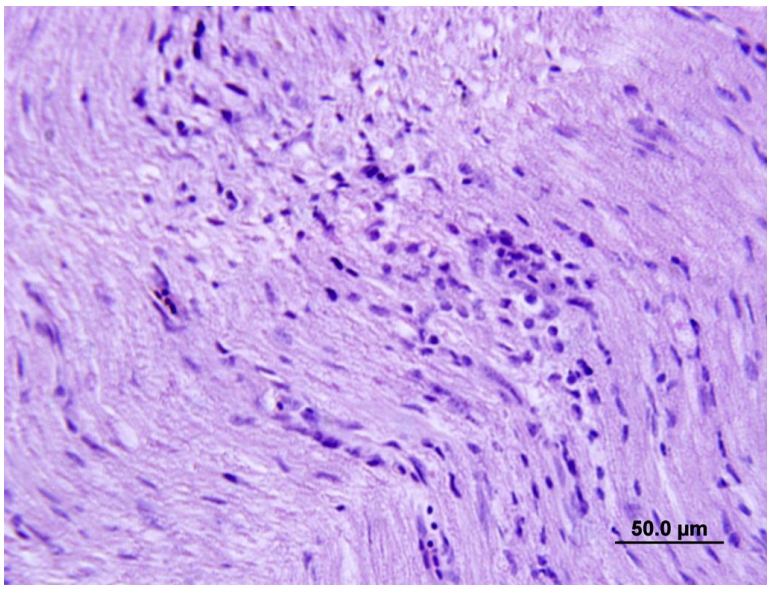

Figure 5.7. Section of sciatic nerve from a bird euthanized at 28 dpc of MPF23 showing mild lymphocytic infiltration (C-type). (H&E stain; × 200 magnification).

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STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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Chapter 6 Effect of antibody directed against MDV on the development of Marek's disease acute transient paralysis and lymphomas.

6.1 Introduction

Previous studies have shown the restrictive effects of anti-MDV antibody on the pathogenesis of MDV infection despite the avidly cell-associated nature of the MD virus. Chubb and Churchill (1969) reported that anti-MDV maternal antibody (Mat-Ab) blocks the early destructive lesions caused by MDV, delays the development of humoral MD antibody, and renders chickens more resistant to tumorigenesis. In addition Payne and Renine (1973) found that Mat-Ab lowered the level of MDV antigens detectable in the tissues of infected chickens. Calnek (1972b) concluded that anti-MDV antibody in serum was efficacious in reducing the incidence and severity of MD when injected 4 but not at 7 days after virus challenge. Both congenital Mat-Ab or artificial injection of anti-MDV Ab prior to, or at the time of, virus inoculation reduced the incidence and severity of the acute necrotizing changes and lowered MD mortality and viral antigen detection in tissues (Calnek 1972b). Similarly, anti-MDV Ab delayed virus production in the skin, and lowered the frequency of virus isolation from buffy coat cells (Burgoyne and Witter 1973). The effects of IgY extracted from the egg yolks of MDV-exposed chickens, on pathogenesis of MDV, were also examined by Kermani-Arab *et al.* (1976), who found that viraemia and development of lesions were both markedly delayed by administration of anti-MDV IgY.

In Australia, isolation of vv MDV from natural outbreaks in vaccinated chickens (De Laney *et al.* 1995) induced an early mortality syndrome (EMS) in significant numbers of SPF white leghorn (WLH) chickens free from Mat-Ab (Renz *et al.* 2012; Walkden-Brown *et al.* 2007c). However, EMS was not observed in commercial chickens with Mat-Ab to MDV, suggesting that MDV Mat-Ab provide protection from the acute transient paralysis syndrome (Walkden-Brown *et al.* 2007a). The EMS syndrome, which was repeatedly induced between days 9 and 20 after vv MDV challenge is similar to the acute transient paralysis (ATP) induced by highly virulent MDV isolates reported by in the USA, which also induced in chickens free from Mat-Ab (Witter *et al.* 1999). For the remainder of this chapter the EMS syndrome will therefore be referred to as ATP.

In the literature on MD, the development of ATP is most commonly portrayed as a consequence of the on going evolution in virulence of MDV and thus is a property of the MD virus for (e.g. Osterrieder *et al.* 2006). It is generally overlooked that ATP has been demonstrated only in chickens free of Mat-Ab and therefore is only induced experimentally, as all commercial chickens contain Mat-Ab directed against MDV. Thus it is an experimental rather than a field phenomenon, unlike the other key manifestations of MD such as classical paralysis and lymphoma formation. However, while examination of various published reports provides strong evidence that Mat-Ab provides complete protection against ATP, the various experimental results are confounded by different chicken genotypes and protocols and there have been no studies directly comparing the induction of ATP in chickens differing only in Mat-Ab status.

The broad aim of the series of experiments described in this chapter was to make this comparison and test the general hypothesis that the presence of Mat-Ab directed against MDV is completely protective against ATP. Four experiments were conducted to test this and other subsidiary hypotheses.

6.1.1 . Materials and Methods

In order to study the effect of anti MDV-Abs on the pathogenesis of MDV, four experiments were conducted as summarized in Table 6.1.

Table 6.1: Key details of the experiment conducted during the effect of MDV Ab

Expt.	Purpose	Location	Chicken no. and type
1	To compare the protective effects of Mat-Ab in WLH chickens differing naturally in Mat-Ab to MDV	Isolator facility	140 White leghorns with and without natural Mat-Ab
2	IgY extraction and evaluation of anti-MDV titres following inoculation with extracted IgY	Animal house isolators	50 SPF free from Mat-Ab and 20 ISA Brown with Mat-Ab
3	IgY extraction from 2000 eggs and evaluation of anti-MDV titres following inoculation with extracted IgY	Isolator facility	44 ISA Brown with Mat-Ab
4	To compare the protective effects of Mat-Ab in WLH chickens inoculated with different doses of anti-MDV IgY	Isolator facility	280 SPF WLH SPF chicks

6.1.2 Experiment 1: Effect of challenge dose, chicken age and natural anti-MDV maternal antibody (Mat-Ab) status on the incidence of acute transient paralysis (ATP) in white leghorn (WLH) layer chickens.

Experiment 1 was designed to compare the protective effects of naturally acquired maternal antibody (Mat-Ab) against development of ATP in WLH layer chickens with or without naturally acquired Mat-Ab. The experiment was approved by the UNE Animal Ethics Committee (Approval AEC09/145).

The following main hypotheses were under test:

1- Mat-Ab will be protective against ATP, particularly when challenge is at day 0. More ATP will be induced by the vv MDV strain 02LAR than the v MDV strain MPF57.

2- There is a dose difference in the range of 500 to 2000 PFU in the induction of ATP. Severity of ATP will be positively correlated with MDV viral load in peripheral blood lymphocytes (PBL

3- Mat-Ab will be protective against bodyweight loss and immunosuppression caused by MDV challenge as determined by body weight and relative bursal and thymic weight.

6.1.2.1 Experimental design - Expt 1

The experimental design was an incomplete 2 × 3 × 2 x 2 factorial design. The four factors were two levels of Mat-Ab (+ve and -ve), three MDV challenge doses (500, 1000, or 2000 plaque forming units per chick), two Australian MDV strains (02LAR and MPF57), and two ages at challenge (0 and 7 days of age, day 7 only for MPF57). The experiment was conducted in 4 isolators in the UNE isolator facility in building W33 with challenge viruses and dates of challenge in different isolators, but chicks of each level of Mat-Ab status and challenge dose kept within each isolator and identified individually. The experiment had a duration of 28 days. The arrangement of treatments is summarised in Table 6.2.

Table 6.2: Experiment 1. Arrangement treatments in isolators and chick numbers

Isolator No	Treatment (virus)	Chick type	Anti-MDV antibody status	Challenge dose (PFU)	Challenge age (d)	N
4	02LAR	UNE	+Pos	500	0	5
				1000	0	5
				2000	0	5
		SPF	-Neg	500	0	5
				1000	0	5
				2000	0	5
1	02LAR	UNE	+Pos	500	7	5
				1000	7	5
				2000	7	5
		SPF	-Neg	500	7	5
				1000	7	5
				2000	7	5
2	MPF57	UNE	+Pos	500	7	5
				1000	7	5
				2000	7	5
		SPF	-Neg	500	7	5
				1000	7	5
				2000	7	5
3	Control	UNE	+Pos	Nil (diluent)	0	25 (15)*
		SPF	Ab-	Nil (diluent)	0	25 (15)*
Total						140

*10 birds of each type euthanized at day 0 for serum collection leaving 15 birds.

6.1.2.2 Experimental chickens - Expt 1

In order to test the effects of MDV-Ab on the pathogenesis of MDV, a preliminary experiment was conducted to generate chicks with and without Mat-Ab by breeding SPF WLH chickens (1 male: 10 females) with and without vaccination against MD. At 8 weeks of age, 130 SPF chickens raised in isolators had a mild trim of the top beak, and were placed in four isolation sheds on wood shavings, which is more likely to have been rice hulls. The chickens had *ad libitum* access to feed and water, under natural photoperiod as described in Chapter 3 (section 3.2.1 and Figure 3.1). At 76 days of age chickens in

two sheds were vaccinated against MD with Vaxsafe®RIS CVI988 (Batch MRIS 6111p, Bioproperties, Vic Australia) according to manufacturer's instructions using the diluent provided by the manufacturer. Vaccination was performed manually in a volume of 0.2 ml/bird subcutaneously at the back of the neck. The remaining chickens in the other two sheds were left unvaccinated. On 120 day post vaccination blood samples were collected from 10 hens from each shed to determine of MDV-specific antibody titre in serum samples, using indirect ELISA adapted from that of Zelnik *et al*. (2004). Following detection of IgY antibodies in the sera of unvaccinated hens, they destroyed. Only vaccinated hens were used as a source of Mat-Ab positive chickens in experiment 1.

The experiment was initiated with 140 newly hatched chickens providing 30 birds per isolator and 20 chickens euthanized at day 0 for sera collection. The chickens were SPAFAS Australia line, (ex CSIRO Hyline White Leghorn, WLH) used by (SPAFAS Australia Pty. Ltd., Melbourne). Chicks containing anti-MDV Mat-Ab were hatched at UNE from fertile eggs produced from Rispens-vaccinated hens.

The two chicken types were hatched at the same time and then held in positive pressure isolators at UNE for the 28-day duration of the experiment. Chickens were individually identified and assigned randomly, with stratification on Mat-Ab status, into four experimental groups of 30 chickens, each in a single isolator. One group was an unchallenged control group, while the other three groups were challenged with MDV (02LAR day 0, 02LAR day 7 and MPF57 day 7). Each challenged group was divided into 6 subgroups of 5 birds comprising the 3 challenge doses and two maternal Mat-Ab levels in the experimental design. In addition to individual wing tags, all the birds for each subgroup were identified by different toe web marks indicating the dose of the virus they received and Mat-Ab status. One isolator was challenged with MDV isolate 02LAR at 0 day of age while the other two challenge groups were challenged with either 02LAR or MPF57 at 7 days of age. Birds in the control isolator were sham-challenged with diluent only.

6.1.2.3 MDV isolates and challenge details of Expt 1

The viruses used in this study were O2LAR (Batch P2 181109) and MPF57 (Batch P4 181109) which are cell culture adapted Australian strains of serotype 1 MDV. MPF57 was isolated in 1994 from a flock vaccinated with serotype 2 and 3 vaccine (Delaney *et al.* 1998) while 02LAR was isolated in 2002 from an unvaccinated broiler flock on the

Mornington Peninsula in Victoria (Renz et al., 2012). In formal pathotyping studies MPF57 has been pathotyped as v MDV while 02LAR has been pathotyped as vv MDV (Renz *et al.* 2012; Walkden-Brown *et al.* 2013b). Both viruses were grown and titrated on chick embryo fibroblasts at UNE prior to storage in liquid nitrogen and subsequent use. Chickens were inoculated via the intra-abdominal route with 0.2 ml of 02LAR and MPF57 that diluted to one of 3 dose levels (500, 1000, or 2000 PFU).

6.1.2.4 Observations, measurements and laboratory methods - Expt 1

Details of all methods used are presented in Chapter 3. Following MDV inoculation, clinical signs, mortality or signs of recovery were monitored twice per day. Clinical signs of ATP were scored mild, moderate or severe on the basis of the extent of signs including depression, ataxia, tremor, torticollis, paresis and recumbency. A routine necropsy was performed on all birds dying or euthanized during the course of the experiment. The atrophy of bursa and thymus were scored from 0-3 (0 = normal size, 1 = mild reduction, 2 = moderate decrease, 3 = tiny or complete disappearance).

Blood was sampled on days 0 (terminal), 7, 14, 21 and 28 from unchallenged control birds (Mat-Ab positive and negative) to determine anti-MDV titre in sera and at 16 days of age (16 or 9 dpc) from all birds for PBL extraction. MDV antibody titre was determined by MDV-specific ELISA in sera from unchallenged control birds at 0, 7, 14 and 21 and 28. Sera from the 20 chicks euthanized at day 0 were also tested for chicken avian infectious anaemia (CAV) antibodies, by ELISA, using the Flokchek CAV Antibody Test kit (Idexx Laboratories, Westbrook, USA). Sera were diluted 1: 10 according to the manufacturer's instructions. At room temperature, aborbence was read at 650 nm wavelength using a model EL310 Microplate Autoreader (Bio-Tek Instruments, Inc., Winooski, VT).

6.1.3 Expt 2: Extraction of MDV-specific antibody from eggs and effect of administration to chicks on anti-MDV titres (MD09-SW-Mab3)

The experiment aimed to test antibody extraction from egg yolks containing specific anti-MDV antibody and the effect of such antibody on anti-MDV antibody levels following inoculation into chicks, in comparison with chicks having natural anti-MDV Mat-Ab.
The hypotheses under test were:

1- Anti-MDV antibody titres of one day old SFP chickens will increase after immunization with the extracted IgY, and this increase will be dose dependent.

Inoculation of Mat-Ab negative SPF chickens with anti-MDV1 IgY doses up to the equivalent of that contained in 6 eggs will increase the anti-MDV1 titre to levels comparable with those of commercial chickens with maternal antibodies. The intra-abdominal (i.a.) and subcutaneous (s.c.) routes of IgY inoculation will provide similar increases in titre.

2- The titre of the inoculated IgY will decrease in similar fashion to the curve decay of the Mat-Ab of Mat-Ab positive control chickens, from breeder hens immunized with Rispens vaccine.

The experiment was approved by the UNE Animal Ethics Committee (Approval AEC09/178).

6.1.3.1 Experimental design - Expt 2

The experiment was designed to test the effect of parenteral administration (s.c. or i.a.) of IgY extracted from egg yolks from hens vaccinated with Rispens CVI988, on the level of anti-MDV Ab in the sera of SPF white leghorn chickens and compare this with natural levels of antibody in ISA Brown chicks, which were hatched from the same batches of the eggs used for IgY extraction. Specific aims were to test (i) the concentration of antibodies in the extracted IgY before injection (ii) the effect of parenteral administration of IgY (s.c. and i.a.) on the level of IgY in the sera of chickens and (iii) differences between the titre of MDV-Ab acquired passively by congenital or artificial inoculation.

The experiment had 2x4 factorial design with five SPF chicks per treatment, plus an external positive control (10 ISA Brown chickens hatched from the same parent flock as that used to supply the eggs for IgY extraction). Experimental factors and levels were: Immune status and inoculated IgY dose. Immune status levels were chickens free from Mat-Ab (SPF) or chickens with maternal anti MDV-Ab (ISA Brown). Inoculated IgY dose levels were the amount of IgY extracted from 0, 2, 4, or 6 egg(s). The experiment had a duration of 21 days.

6.1.3.2 Extraction of antibody and administration to chickens - Expt 2

IgY was extracted from 120 eggs from layer breeders vaccinated against MDV with Rispens CVI988 (Vaxsafe® RIS-Bioproperties Pty Ltd-Victoria, Australia). Extraction and

purification of IgY from the yolk was by water extraction followed by caprylic (octanoic) acid and ammonium sulphate precipitation (Akita and Nakai 1992; McLaren *et al.* 1994) and dialysis as described by Kim and Patterson, (2003). After freeze drying, the extracted IgY was re-suspended in PBS (120 µl/egg extracted) and administered s.c. or i.a. at day 0 old (hatching). Inoculated IgY doses were equivalent: 0, 2, 4, or 6 egg(s)/chick.

6.1.3.3 Expt 2. Experimental chickens and experimental design

This study used 50 SPF chickens free from MDV-Ab and 20 ISA Brown chickens. The twenty ISA Brown chickens were generated from parents that vaccinated with CVI988. Moreover, 120 eggs for IgY extraction also were from the same parent flock. Thus, twenty ISA Brown chickens would have anti-MD Mat-Ab. Out of 50 SPF chickens, 40 were randomly assigned into two groups of 20 chickens in an isolator each. The remaining 10 chickens were bled for serum collection at day 0 of age and then euthanized immediately by cervical dislocation. Similarly, out of 20 ISA Brown chickens 10 were randomly selected and held in a 3rd untreated control group. The remaining 10 ISA Brown chickens were bled for serum at day 0 of age and then euthanized. Chickens were identified individually by wing tags, and by treatment using toe web marks. The arrangement of treatments in isolators is shown in Table 6.3.

Table 6.3: Experiment 2. Arrangement of chicks and treatments in isolators

Isolator	Chick no.	Type	Immune status	Treatments
1	10	ISA Brown	Mat-Ab +ve	Non-inoculated control
2	20	SPF	Mat-Ab -ve	IgY from 0, 2, 4 or 6 eggs (5 birds each)
3	20	SPF	Mat-Ab -ive	IgY from 0, 2, 4 or 6 eggs (5 birds each)

6.1.3.4 Measurements and laboratory methods - Expt 2

Sera were obtained on day 0 before the first inoculation (10 SPF and 10 ISA Brown, terminal), and on days 7, 14, and 21 dpi from all remaining birds. MDV antibody titre was determined in these sera and in extracted egg yolk material using the MDV-specific ELISA as described in section 3.9. 3.1 in Chapter 3.

6.1.4 Large-scale extraction of IgY from eggs and testing in commercial ISA Brown chicks (MD12-SW-Mab5)

The results of experiment 2 indicated that a large number of eggs would need to be extracted to provide enough IgY to achieve physiological levels following administration by inoculation in a large challenge experiment. This experiment therefore involved IgY extraction from 2000 eggs and subsequent testing to confirm the efficacy of the different batches in elevating IgY levels *in viv*o. The hypotheses under test were;

1- Injection of the extracted anti-MD IgY into Mat-Ab positive chicks will raise the anti-MDV titre above natural levels and this will be dose responsive.

2- The elevation in titre induced by injection with extracted IgY will persist for at least 14 days.

This experiment was conducted under UNE Animal Ethics approval AEC11-129.

6.1.4.1 Antibody extractions - Expt 3

Extraction of IgY from 2000 eggs from commercial Hyline Brown hens vaccinated with Rispens CVI988 was performed in 16 batches using the method previously described. Each batch was reconstituted in PBL using a standard dilution that resulted in a mean concentration of 1.64 eggs/ml of IgY (range 1.1-2.8 eggs/ml).

6.1.4.2 Experiment design and chickens

The experiment used 44 freshly hatched ISA Brown chicks from parents vaccinated with the Rispens CVI988 strain of MDV. The chicks were distributed among 3 isolators; 10 as a non- inoculated control group and two treatment isolators of 17 chickens each. Chicks in one treatment isolator were administered a 1 ml per chicken dose of IgY for each of 17 batches of Ig-Y, which were 16 new batches of IgY, plus the IgY batch that was prepared and used in the previous experiment. Chicks in the other treatment isolator were administered a 2 ml dose. In both cases the doses were split between s.c. and i.a. administration. Chickens were held in positive pressure isolators and maintained for the 28-day duration of the experiment. The experiment was designed to use SPF chickens with the ISA Browns as positive controls, but the hatch of SPF chickens failed and so the experiment was modified to use only the ISA Brown chicks.

6.1.4.3 Measurements and laboratory methods

Sera were obtained on days 0, 5, 14, 21 and 28 dpi. MDV antibody titre was determined in these sera and in extracted egg yolk material using the MDV-specific ELISA described in section 3.4.3.1 in Chapter 3.

6.1.5 Experiment 4: Effect of anti-MDV IgY derived from eggs of immunized hens on the pathogenesis of Marek's disease (MD12-SW-Mab4)

This experiment was designed to test the effect of different doses of administered IgY and different challenge doses of MDV on the incidence of acute transient paralysis and later tumorigenesis in SPF chickens challenged with vvMVD isolate 02LAR. Hypotheses under test were:

1- Challenge of SPF chicks with 3 doses of vvMDV will induce ATP in a dose-dependent manner. In contrast, tumor development is dose independent.

2- Inoculation of SPF chicks with extracted IgY will reduce the incidence, and delay the onset of ATP in a dose-dependent manner as determined by clinical signs.

3- Inoculation of SPF chicks with extracted IgY will protect against weight loss and immunosuppression in a dose-dependent manner.

4- Inoculation of SPF chicks with extracted IgY will reduce the incidence, and delay the onset of MD in a dose-dependent manner as determined by lymphoma presence.

The work was conducted under UNE Animal Ethics approval AEC11-129.

6.1.5.1 Experimental design and chickens – Expt. 4

The experiment used a complete 2×3 factorial design with two replicates using 12 isolators (2×3×2=12) plus external control groups in 3 isolators. The negative external control consisted of two replicates (isolators) of SPF chickens left unchallenged and a

Mat-Ab positive external control consisted of ISA Brown chickens in one isolator. The two experimental factors and their levels were challenge doses of 02LAR virus (500 or 2000 PFU) and IgY doses equivalent to IgY extracted from 0, 1 or 4 eggs. Each treatment combination was replicated in two separate positive-pressure chicken isolators.

The trial used 224 SPF chickens free of anti-MDV Mat-Ab and 20 ISA Brown chicks containing anti MDV antibody. The SPF chicks were hatched at UNE from eggs supplied by Australian SPF Services P/L (Woodend Vic). The strain of SPF chicken was the Lohmann LSL Classic, a WLH type considered to exhibit significant resistance to MD. Thus, the SPF birds used in experiment 4 were likely more resistant to MD than birds used in previous experiments. However this was the only strain of SPF chicken available commercially in Australia at the time.

SPF chickens were randomly sorted to provide 16 chickens per isolator including the two external negative control groups (Mat-Ab negative). SPF chickens in each isolator were individually wing tagged and toe web marked to identify 3 subgroups of 5 to 6 birds each indicating IgY doses (0, 1 or 4 egg equivalents).

A single external positive control group (Mat-Ab positive) comprised 20 ISA Brown chickens. Ten chickens were used terminally for serum collection at 0 day while other 10 were retained and used for collection of sera until 6 weeks of age. The duration of the experiment was 62 days (56 dpc).

6.1.5.2 Administration of treatments – Expt. 4

The antibody material in the experiment comprised pooled material from 10 of the batches extracted and tested in experiment 3 (Batches 1, 3, 6, 7, 8, 10, 12, 13, 14 and 15) diluted to the same concentration in terms of eggs extracted/given volume. Antibody treatments were administered to SPF chickens from 4 days of age as follows. The 0 egg IgY group received only sterile PBS on day 4. The 1 and 4 egg IgY groups received IgY equivalent to one egg, which was 0.634 ml of the extracted IgY, on day 4. Chickens receiving 4 eggs per treatment received their 2^{nd}, 3^{rd} and 4^{th} egg equivalents on days 6, 7, and 8 of age respectively. The IgY was administered s.c while the doses of MDV (02LAR) were administered i.a. At 6 days of age, chickens in the 12 non-control isolators were challenged with 500 or 2000 PFU of MDV s.c. in 200 µl of diluent. The isolate used was 02LAR (Batch P4 No.181109), grown and titrated at UNE in chick embryo

fibroblasts. 02LAR is a very virulent MDV isolated from an unvaccinated broiler flock on the Mornington Penninsula of Victoria in 2002 (Renz *et al.* 2012; Walkden-Brown *et al.* 2013b).

6.1.5.3 Measurements and laboratory methods - Exp. 4

Chickens were monitored daily for the presence of clinical signs using a scoring system based on the classification of Gimeno *et al.* (1999) and described in detail in Chapter 5. Blood was sampled individually weekly between 7 and 49 dpc for both sera and extraction of PBL. All chickens that died were examined post mortem for gross MD lesions as described in Chapter 3. Surviving chickens were euthanased at 56 dpc and examined post mortem for MD lesions and had the weight of body, bursa and spleen recorded.

MDV antibody titre in weekly sera was determined using the MDV-specific ELISA described in section 3.9 3.1 in Chapter 3.

6.1.6 Statistical analysis

Treatment effects on discrete variables such as the incidence of clinical signs, MD and mortality were analysed using contingency table analysis with likelihood ratio test. Mortality patterns were also investigated using survival analysis (Kaplan-Meier product-limit method). Treatment effects on continuous variables such as live weight, relative immune organ weights, antibody titres and qPCR data were investigated by fitting least squares models incorporating relevant fixed effects such as of sex, antibody status, MDV challenge isolate, challenge dose and two-way interactions between the main effects. Where repeated samples were collected from the same chicken or isolator, a repeated measures analysis was used by using a mixed REML model fitting the chicken or isolator as a random effect and the other treatment effects and day of sampling as fixed effects. Following an overall significant effect in either model, the significance of differences between means within the effect was tested using Student's T test. Where necessary, MDV viral load and antibody titre data were transformed using a Log^{10} (y+x) transformation to stabilise the variance. Association between variables was analysed using correlation and linear regression analysis. Analyses were performed with JMP10 (SAS Institute, Cary NC, USA). Data for continuous variables are generally reported as

least squares means and standard errors. A statistical significance level of $P<0.05$ is used throughout.

6.2 Results

6.2.1 Results of Experiment 1. Effect of Mat-Ab on ATP in white leghorn chickens

6.2.1.1 Preliminary experiment. Breeding of chicks of different Mat-Ab status.

MDV antibody titres measured on 120 day post vaccination revealed significantly higher $\log^{10}$ anti-MDV antibody titre of vaccinated (3.38 ± 0.09) than unvaccinated (2.86 ± 0.08) chickens ($P=0.0003$). The fact that not all unvaccinated chickens were negative suggested that Rispens CVI988 vaccine virus had been transmitted from the vaccinated sheds to the unvaccinated sheds or it could also be the results of infection with serotype 1 field virus and thus, chicks hatched from unvaccinated hens could not be expected to be free of maternal antibody.

6.2.1.2 Expt. 1. MDV serology to confirm Mat-Ab status.

The titres of maternal MDV-Ab in uninfected control sera between days 0 and 28 differed between UNE-hatched chickens from Rispens vaccinated parents and SPAFAS SPF chickens from unvaccinated parents (Figure 6.1). Titres in in the UNE-hatched chickens titres showed a rapid decline from 470 on hatch day to negligible levels at day 21 and undetectable levels at day 28 of age. The majority of SPF chicks had undetectable MDV titres throughout but on days 0 and 21 there was some absorbance above the blank, suggestive of non-specific reaction in the assay. Out of 10 chicks, four chicks had anti-MDV Ab titre of 150 at hatch or higher.

Sera were tested for the presence of CAV-specific antibodies using a commercially competitive ELISA kit. The serological results showed only 3/20 chicks were positive at hatching (d 0).

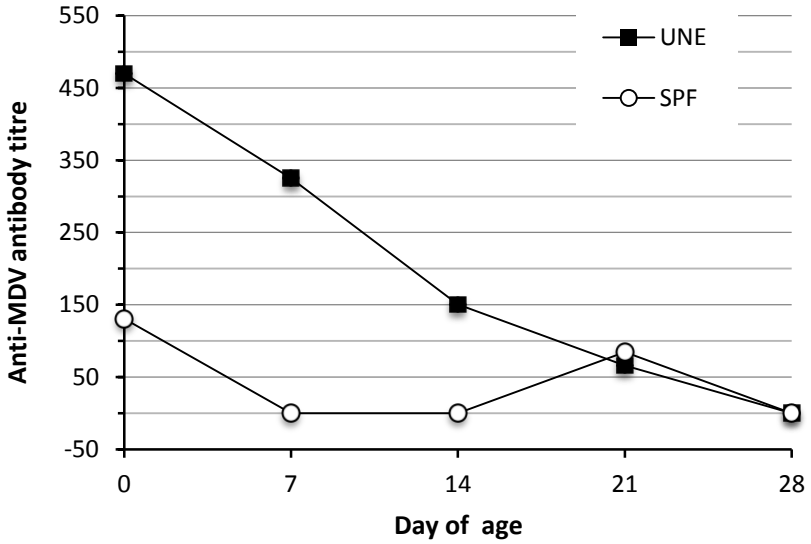

Figure 6.1: Experiment 1. Anti-MDV titres from hatching (day 0) to 28 d old in serum from SPF white leghorn chickens and UNE-hatched white leghorn chickens from parents vaccinated with the Rispens CVI988 vaccine.

6.2.1.3 Expt. 1. Viral load in PBL

PBL samples from 10 unchallenged control chickens collected at 16 days of age were negative for MDV. When the MDV-1 load in PBL in samples collected from isolators 1 (02 LAR day 7 challenge) and 2 (MPF57 day 7 challenge) at 16 days of age (9 dpc) was analysed, there was a significant effect of challenge virus (P = 0.027), but not Mat-Ab status (P = 0.99), challenge dose (P = 0.33) sex (P = 0.61) or two way interactions between these factors. The load of MDV in PBL was higher for the vvMDV isolate 02LAR ($8.3 \times 10^5 \pm 1.0 \times 10^5$ copies per 10^6 cells) than for the vMDV isolate MPF57 ($5.3 \times 10^5 \pm 8.6 \times 10^4$ copies per 10^6 cells).

When the MDV-1 load in PBL in samples collected from isolators 4 (02LAR day 0 challenge) and 1 (02 LAR day 7 challenge) at 16 days of age (16 or 9 dpc) was analysed there was no significant overall effect Mat-Ab status (P = 0.84), challenge age (P = 0.09), challenge dose (P = 0.88) or sex (P = 0.66) or two way interactions between these factors. There was a trend (P=0.09) towards higher viral load in birds challenged at day 7 ($8.5 \times 10^5 \pm 1.2 \times 10^5$ copies per 10^6 cells) than those challenged at day 0 ($5.9 \times 10^5 \pm 1.0 \times 10^5$ copies per 10^6 cells). Looking at two way interactions there was significant interaction between the effects of challenge age and challenge dose (P = 0.04) such that birds challenged at day 7 had significantly higher MDV load than those challenged at day 0 when the challenge dose was 500 PFU but not 1000 or 2000 PFU.

Amongst PBL samples collected on day 16, samples from the 18 chickens that had ATP had significantly higher (P = 0.01) MDV load ($8.4 \times 10^5 \pm 1.0 \times 10^5$ copies per 10^6 cells) than the 69 chickens that exhibited no ATP ($5.5 \times 10^5 \pm 5.5 \times 10^4$ copies per 10^6 cells).

6.2.1.4 Expt. 1. Clinical signs and mortality

During the first five days of the experiment, three chickens died due to omphalitis and peritonitis and they were excluded from data analysis.

Chickens were examined for clinical signs during the experiment with signs scored mild, moderate or severe. For the final analysis, birds with moderate or severe signs were scored as ATP and those with no or mild signs were scored as no ATP. Complete paralysis and prostration culminating in euthanasia was observed in 3/117 chickens (2.5%) and they were sorted as EMS. These were Mat-Ab negative and challenged with 2000 PFU of 02LAR (one on d7 and two on d0). ATP was observed in 19/89 chickens (21.3%) challenged with MDV. The frequency of ATP by treatment and challenge dose is shown in Table 6.4.

Comparison of the incidence of ATP in isolators 1 (02 LAR day 7 challenge) and 2 (MPF57 day 7 challenge) showed no significant effect of maternal antibody on the incidence of ATP (33.3 and 27.1% in Mat-Ab negative and positive chickens respectively, P = 0.22) or challenge dose (19.1, 21.1 and 42.2 % respectively for 500, 1000 and 2000 PFU doses respectively, P = 0.21). On the other hand the effect of challenge virus approached statistical significance (16.7 and 37.9 % respectively for 02LAR and MPF57, P=0.06). However, when mild clinical signs were included in the definition of ATP, the effect of challenge virus became highly significant (86.2 and 43.3 % respectively for 02LAR and MPF57 respectively P <0.001).

Comparison of the incidence of ATP in isolators 4 (02LAR day 0 challenge) and 1 (02 LAR day 7 challenge) showed a significant effect of age at challenge (10.0 and 37.9% in chickens challenged at days 0 and 7 respectively, P < 0.001). The proportion of ATP, cases in Mat-Ab negative chickens (29.0%) was not significantly higher than that in Mat-Ab positive chickens (17.9%) (P = 0.31). The same was observed for challenge dose (15.0, 21.1 and 35.0 % respectively for 500, 1000 and 2000 PFU doses respectively, P = 0.32).

Table 6.4: Experiment 1. Number of chickens exhibiting acute transient paralysis by challenge treatment and challenge dose.

Isolator	Treatment	Chall dose (PFU)	Immune status	Number of birds			% ATP
				No ATP	ATP	Total	
3	Control			28	0	28	0.0
2	MPF57 d7	500	Ab+	6	0	6	0.0
			Ab-	4	1	5	20.0
		1000	Ab+	3	0	3	0.0
			Ab-	6	1	7	14.2
		2000	Ab+	3	2	5	40.0
			Ab-	3	1	4	25.0
1	02LAR d7	500	Ab+	3	1	4	25.0
			Ab-	4	2	6	33.3
		1000	Ab+	3	2	5	40.0
			Ab-	3	1	4	25.0
		2000	Ab+	3	1	4	25.0
			Ab-	2	4	6	66.6
4	02LAR d0	500	Ab+	5	0	0	0.0
			Ab-	5	0	0	0.0
		1000	Ab+	4	1	5	20.0
			Ab-	5	0	0	0.0
		2000	Ab+	5	0	0	0.0
			Ab-	3	2	5	40.0

6.2.1.5 Expt. 1. Gross lesions, body and relative immune organ weights

Severe atrophy of the bursa and thymus without MD nerve enlargement were the predominant findings in birds with severe ATP, with less severe effects in surviving birds. Indication of early visible lymphomas was seen at the termination of the experiment with gross enlargement of the ovary and liver and spleen observed in 2 cases.

Final body weight at 28 days of age in chickens challenged at day 7 with 02LAR or MPF57 was significantly reduced by MDV challenge (P = 0.001) relative to controls with no difference between the two MDV isolates (Figure 6.2A). The effect of sex was also significant (P = 0.03) but there was no significant effect of Mat-Ab status (P = 0.84) or interaction between these effects. In the challenged treatments, there was no significant

effect of challenge dose (P = 0.67). In chickens challenged at day 0 with 02LAR, final body weight at day 21 was also reduced by challenge (P = 0.05, Figure 6.2A) with no effect of Mat-Ab status (P = 0.32), sex (P = 0.31) or interactions between the main effects.

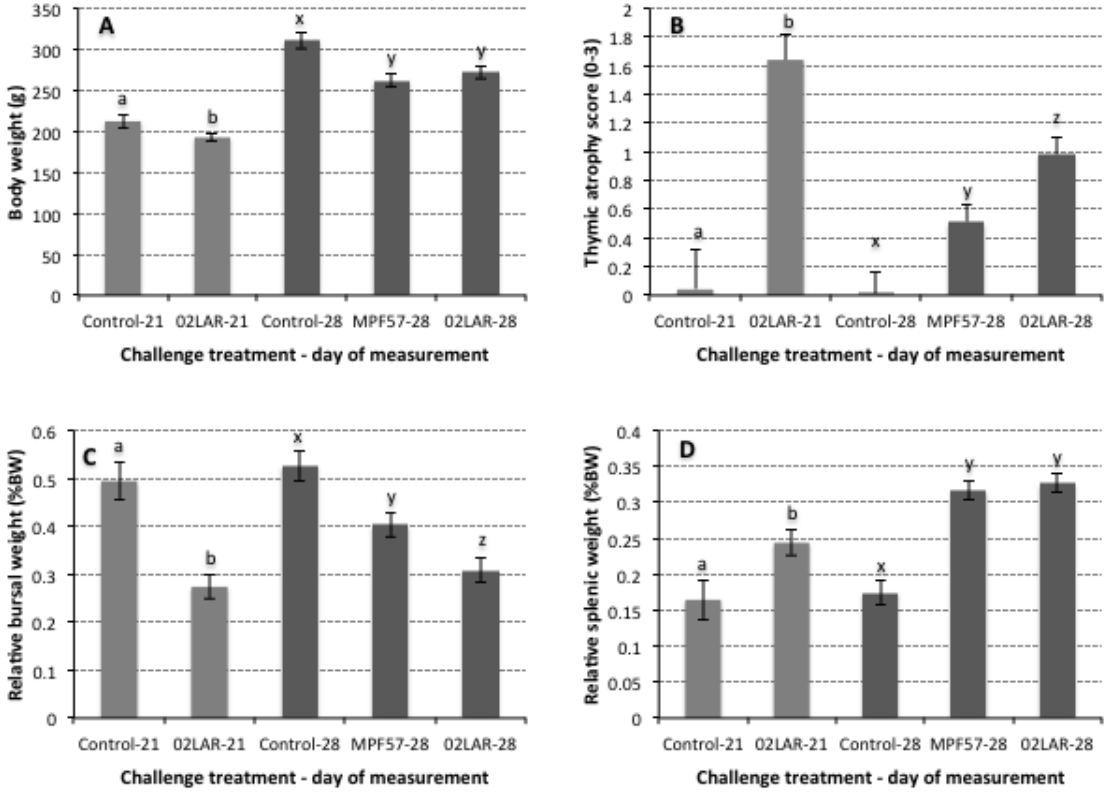

Figure 6.2: Experiment 1. Least squares means (±SEM) for body weight (A) thymic atrophy score (B), relative bursal weight (C) and relative splenic weight C) showing the effects of virus challenge treatment in surviving chickens at 21 and 28 dpc. abc,xyz Means within day of measurement differ significantly if they do not share a common letter.

Thymic atrophy score (0 = nil, 3 = severe) at 28 days of age in chickens challenged at day 7 with 02LAR or MPF57 was significantly increased by MDV challenge (P = 0.001) relative to controls with significantly higher scores for 02LAR than MPF57 (Figure 6.2B). There was a significant interaction between the effects of challenge treatment and Mat-Ab status (P = 0.025) with no effect of Mat-Ab status in control chickens or those challenged with MPF57, but a significant effect of Mat-Ab status in chickens challenged with 02LAR (1.36 ± 0.14 in Mat-Ab negative, 0.60 ± 0.19 in Mat-Ab positive). There was no significant effect of sex overall (P = 0.85) but significant interaction between the effects of sex and challenge treatment (P= 0.04) with higher scores in male chickens challenged with 02LAR, but female chickens challenged with MP57. In the challenged

treatments there was no significant effect of challenge dose (P = 0.61). In chickens challenged at day 0 with 02LAR, Thymic atrophy score at day 21 was increased by challenge (P < 0.0001, Figure 6.2B) but there was no significant effect of Mat-Ab status (P = 0.66), sex (P = 0.97), or interactions between the main effects.

Relative bursal weight (% of bodyweight) at 28 days of age in chickens challenged at day 7 with 02LAR or MPF57 was significantly reduced by MDV challenge (P = 0.001) relative to controls with significantly lower values for 02LAR than MPF57 (Figure 6.2C). There was a significant interaction between the effects of challenge treatment and Mat-Ab status (P = 0.05) with no effect of Mat-ab status in control chickens but in chickens challenged with either virus there was a significantly lower relative bursal weight in Mat-Ab negative (0.30) than Mat-Ab positive (0.42) chickens. There was no significant effect of sex (P = 0.56) other interactions between the main effects. In the challenged treatments, there was no significant effect of challenge dose (P = 0.81). In chickens challenged at day 0 with 02LAR, relative bursal weight at day 21 was reduced by challenge (P < 0.0001, Figure 6.2C) but there was no significant effect of Mat-Ab status (P = 0.44), sex (P = 0.51), or interactions between the main effects.

Relative splenic weight (% of bodyweight) at 28 days of age in chickens challenged at day 7 with 02LAR or MPF57 was significantly increased by MDV challenge (P = 0.001) relative to controls with no difference between the two viruses (Figure 6.2D). There was no significant effect of Mat-Ab status (P = 0.18), sex (P = 0.98) or interactions between the main effects apart from a significant interaction between the effects of challenge treatment and sex (P = 0.04) due to higher relative splenic weights in males in the control treatment, but in females in the challenge treatments. In the challenged treatments there was no significant effect of challenge dose (P = 0.31). In chickens challenged at day 0 with 02LAR, relative splenic weight at day 21 was increased by challenge (P < 0.0001, Figure 6.2D) but there was no significant effect of Mat-Ab status (P = 0.32), sex (P = 0.31), or interactions between the main effects.

6.2.2 Results of experiment 2. Extraction of IgY from eggs and effect on anti-MDV titres in chicks following inoculation at different doses and by different routes.

6.2.2.1 ELISA test to assay anti-MDV Ab (Serology)

High titres of MD-Ab were detected in the extracted IgY from egg yolks obtained from hens immunized with Rispens CVI988. As a consequence, SPF chickens were inoculated with various doses (2, 4 and 6 egg equivalents) of the extracted IgY on the day of hatch. Untransformed mean anti-MDV titres of chickens with natural and injected antibody are presented in Figure 6.3. ISA Brown birds showed a decline in maternal antibody to day 14 with no further decline at day 21. Inoculation with extracted IgY increased anti-MDV titres in SPF chicks in a dose related manner, but even the IgY extracted from 6 eggs failed to raise titres to those observed in the ISA Brown birds. The decline in titre in inoculated birds mimicked that seen in ISA Brown chickens, with a rapid decline between days 7 and 14 but no further decline to day 21.

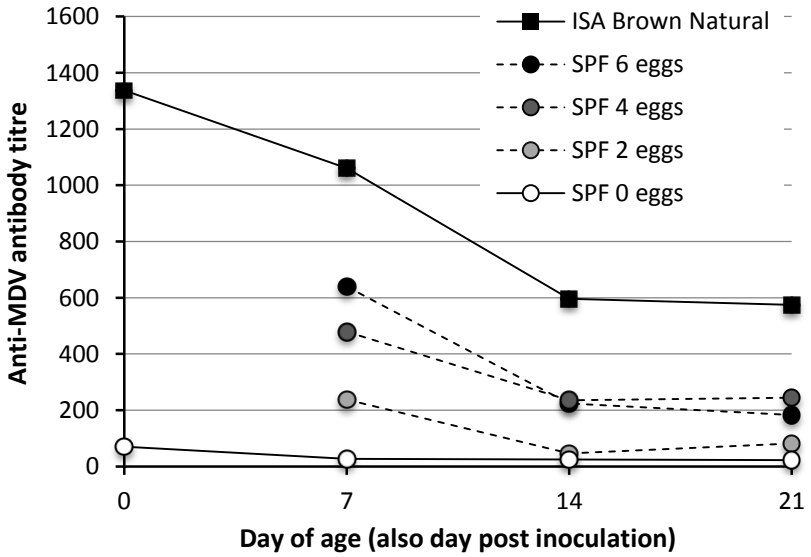

Figure 6.3: Experiment 2. Mean untransformed anti-MDV titres of ISA Brown chickens with natural maternal immunity against MDV and white leghorn SPF chicks inoculated with 0, 2, 4 or 6 egg equivalents of extracted Ig Y from eggs from the same parent flock from which the ISA Brown chickens were bred.

Analysis of Log^{10} (y + 80) anti-MDV antibody titre from days 7 to 21 in all treatments showed a significant effect of dose of antibody ($P < 0.001$) and day post inoculation ($P < 0.001$) with no significant interaction between them ($P = 0.12$). Overall mean titres were significantly higher in chicks with natural maternal immunity (ISA Brown, 2.86 ± 0.05), than those injected with 4 (2.54 ± 0.05) or 6 (2.52 ± 0.05) egg equivalents, which in turn

were significantly higher than those injected with 2 eggs (2.17 ± 0.05) which were higher than those given no injection of IgY (1.95 ± 0.05). Overall titres were higher at day 7 (2.59 ± 0.04) than at days 14 (2.32 ± 0.04) or 21 (2.33 ± 0.04).
nalysis of Log^{10} (y + 80) anti-MDV antibody titre in SPF chicks alone revealed significant effects of dose of antibody ($P < 0.001$) and day post inoculation ($P < 0.001$) but not route of administration (i.a. 2.29 ± 0.03, s.c. 2.31 ± 0.03, $P = 0.76$) with no significant two-way interaction between these effects. However the dose x day interaction approached significant ($P = 0.08$) as the effect of day was only observed in chicks injected with IgY, not in the control treatment given no IgY.

6.2.3 Results of experiment 3. Extraction of IgY from commercial layer eggs and effects on anti-MDV titres in commercial ISA Brown chicks.

ELISA was used to identify antibody titre in the different 17 batches (B) of the extracted IgY by selecting sample dilutions of 1:100. The titre in different batches from (B 1 to B 17) were as follows; B1 154, B2 424, B3 745, B4 906, B5 493, B6 267, B7 622, B8 985, B9 790, B10 670, B11 434, B12 242, B13 2, B14 494, B15 196, B16 416, B17 26.

Untransformed mean anti-MDV titres of chickens with natural and injected antibody are presented in Figure 6.4. Control birds showed a decline in maternal antibody to day 14 with a slight further decline to day 28. Inoculation with extracted IgY increased anti-MDV titres at day 5 and to some extent day 14 post inoculation, but not thereafter, in a dose-dependant manner. At day 5 post injection with either 1 or 2 ml of extracted IgY antibody titres were more than doubled with injection of 2 ml appearing to provide some elevation over control chickens at day 14.

Analysis of Log^{10} (y + 80) anti-MDV antibody titre revealed significant effects of treatment ($P = 0.002$) and day post injection ($P < 0.0001$) with significant interaction between these effects ($P = 0.001$). The significant interaction was because the effect of treatment was only evident on days 5, 14 and 28 post inoculation (Figure 6.4, Table 6.5).

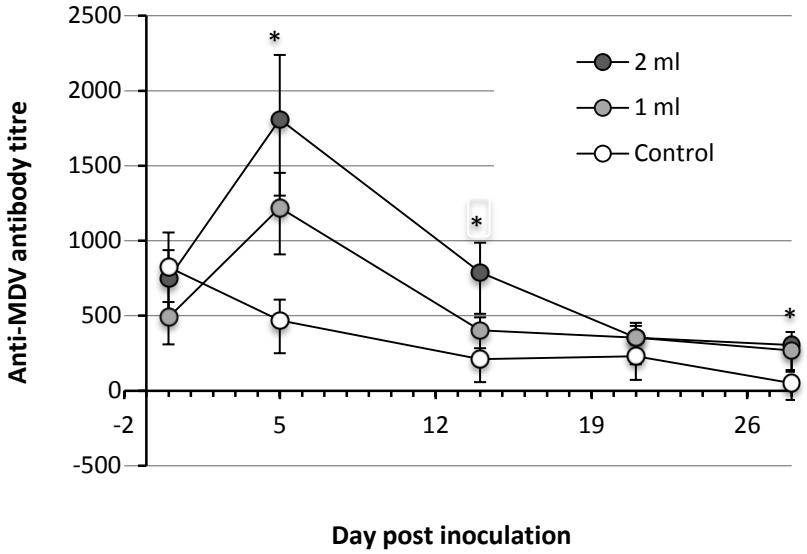

Figure 6.4: Experiment 3. Back transformed LS mean anti-MDV titres (± SEM asymmetric on back transformation) of ISA Brown chickens with natural maternal immunity against MDV (Control) or injected subcutaneously and intra-abdominally with 1ml or 2ml of pooled reconstituted IgY extracted from commercial layer eggs. Chicks were injected with extract from different pools having a mean concentration of 1.64 eggs equivalent / ml of IgY (range 1.1-2.8 eggs/ml). The means are from 9, 16 and 10 birds sampled longitudinally in control, 1 ml and 2 ml treatments respectively. Asterisks indicate weeks in which treatments differed significantly ($P < 0.05$).

Table 6.5: Experiment 3. Least squares means (± SEM) of Log^{10} (Y + 80) anti-MDV titre of ISA Brown chickens with natural maternal immunity against MDV (Control) or injected subcutaneously and intra-abdominally with 1ml or 2ml of pooled reconstituted IgY extracted from commercial layer eggs.

Treatment	Day post inoculation with extracted IgY				
	0	5	14	21	28
Control	2.96 ± 0.10^{a}	2.74 ± 0.10^{a}	2.46 ± 0.10^{a}	2.49 ± 0.10^{a}	2.11 ± 0.10^{a}
1ml	2.76 ± 0.07^{a}	3.11 ± 0.07^{ab}	2.68 ± 0.07^{a}	2.64 ± 0.07^{a}	2.54 ± 0.07^{b}
2ml	2.92 ± 0.09^{a}	3.28 ± 0.09^{c}	2.94 ± 0.09^{b}	2.64 ± 0.09^{a}	2.58 ± 0.09^{b}

^{abc}Means within columns not sharing a common letter are significantly different ($P < 0.05$)

6.2.4 Results of experiment 4. Effect of administered anti-MDV IgY on the pathogenesis of MD.

6.2.4.1 MDV-specific Serology

Data for the ELISA test results for this experiment were fully analysed some time after the assays were performed and in the intervening period the samples were lost due to inadvertent discarding by someone not associated with the research. Samples from different treatments and times were distributed across all plates and analysis of these and quality controls revealed that results from plates 2 and 7 of 10 plates were unusable, so they have been excluded from the analysis presented below.

Analysis of the remaining results produced some unexpected findings. While inoculation with extracted IgY clearly increased anti-MDV titres in challenged chickens (Figure 6.5), the differences did not diminish over the course of the experiment as may be expected with an active MDV infection. Nor was there any major increase in overall titre over the 49 days post challenge, and there was no effect of MDV challenge dose (Figure 6.5). In unchallenged chickens there was a steady decline in titre after day 21 but the negative control birds did not differ in titre from treated birds at any of the time measured (Figure 6.6).

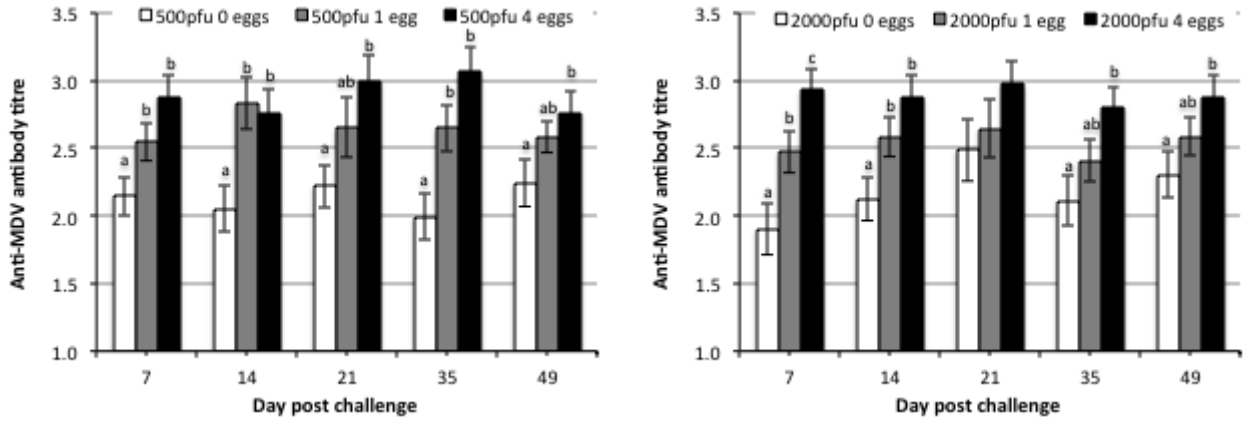

Figure 6.5: Experiment 4. Anti-MDV titres [Log^{10} (y+80)] (LSM ± SEM) of SPF chickens challenged with 500 pfu (left) or 2000 pfu (right) of vvMDV 02LAR at 6 days of age. Chicks had been inoculated with 0 (diluent only), 1 or 4 egg equivalents of extracted IgY from commercial layer chickens vaccinated against MD with the Rispens CVI988 vaccine. Columns within time periods not sharing a common letter differ significantly ($P < 0.05$). See the text for the explanation of the incomplete treatment combinations.

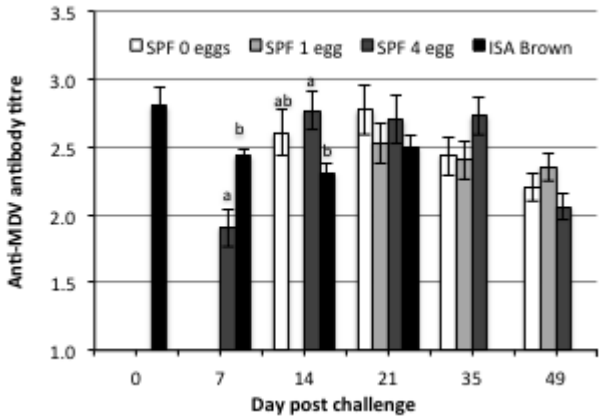

Figure 6.6: Experiment 4. Anti-MDV titres [Log^{10} (y+80)] (LSM ± SEM) of control SPF chickens injected 0, 1 or 4 egg equivalents of extracted IgY and ISA Brown chickens hatched from parents vaccinated against MD with the Rispens CVI988 vaccine. Chicks were not challenged with MDV but were administered diluent only at 6 days of age. Days post challenge refers to this, and enables direct comparison with Figure 6.5. Columns within time periods not sharing a common letter differ significantly ($P < 0.05$). See the text for the explanation of the incomplete treatment combinations.

6.2.4.2 Clinical signs

There was mortality due to bacterial infection during the early stages of the experiment. Antibiotic treatment largely stopped mortality by 11 days of age (6 dpc) apart from a small number of apparent bacterial infections after this. Thus the data on clinical signs below refers only to birds after 14 dpc when bacterial infection subsided.

The unchallenged control chickens did not show neurological signs while the challenged groups began to exhibit signs of clinical MD from 10 dpc. The clinical signs began with depression or unwillingness to move followed by paresis and dragging of the leg, paresis and drooping of wings, and then paralysis in some cases. The challenge dose of MDV influenced the onset and incidence of clinical signs (Figure 6.7), and appeared to influence their severity (Figure 6.8). The mean time of clinical signs post challenge was earlier in birds challenged with 2000 pfu of MDV (39.2 ± 1.7 days) than those challenged with 500 pfu (44.1 ± 1.9 days) ($P = 0.05$). The severity score for clinical signs in sick birds did not differ significantly between the two doses but there was a trend towards higher scores in birds challenged with 500 pfu of MDV (4.06 ± 0.26) than those challenged with 2000 pfu (3.47 ± 0.25) ($P = 0.09$).

The dose of IgY administered to the chicks also had a marked effect on the onset and incidence of clinical signs (Figure 6.9), but not their severity (Figure 6.10). The mean time of clinical signs post challenge was greater in birds administered 4 egg equivalents of IgY (50.3 ± 3.0 days) than those administered either 1 egg equivalent (38.4 ± 31.8) or none (36.3 ± 1.9 days) ($P < 0.001$). On the other hand, the severity score for clinical signs in sick birds did not differ significantly between birds given the different IgY doses ($P = 0.61$).

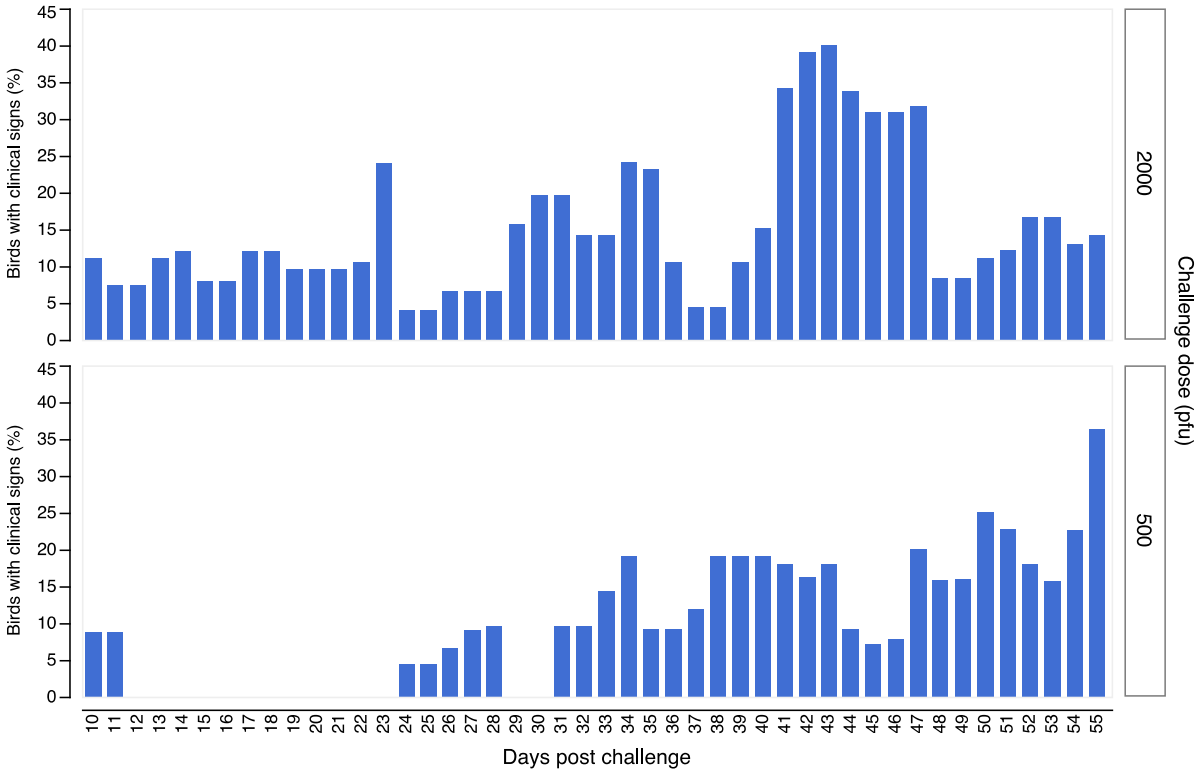

Figure 6.7: Experiment 4. Percentage of chickens showing clinical signs between days 10 and 55 following challenge with 500 or 2000 pfu of vvMDV isolate 02LAR. All IgY treatments are pooled.

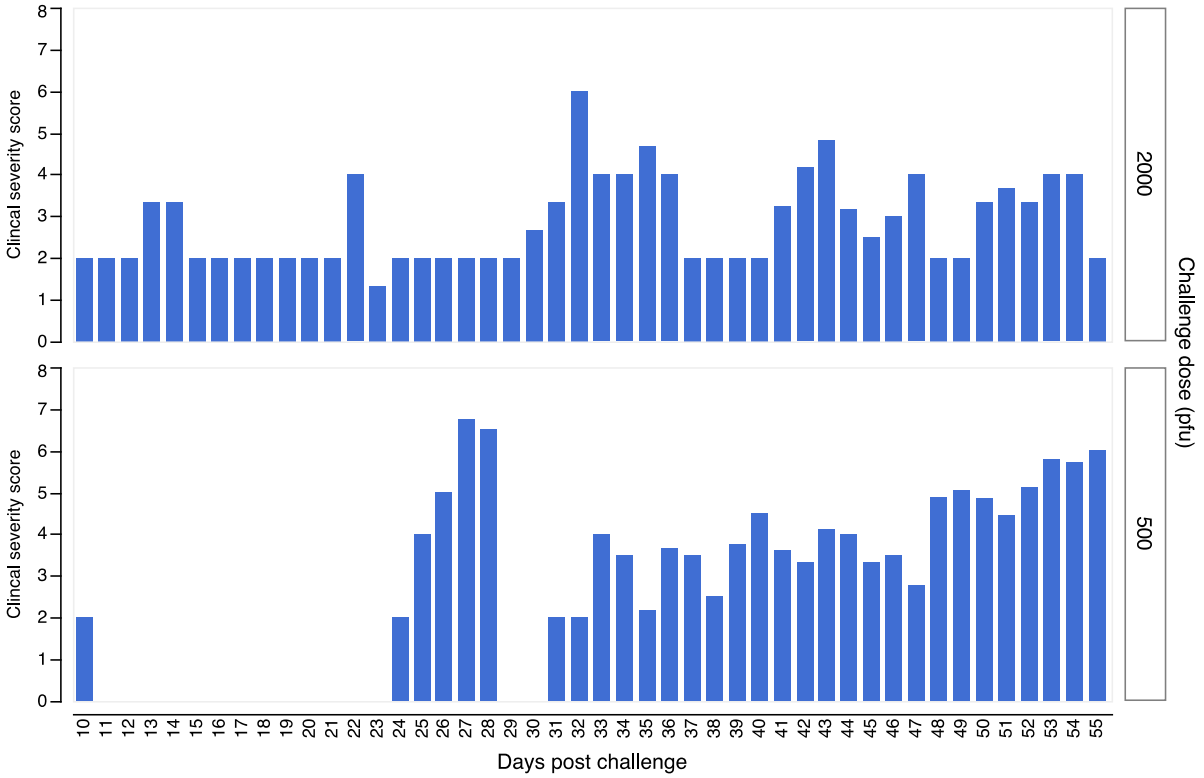

Figure 6.8: Experiment 4. Severity of clinical signs in chickens following challenge with 500 or 2000 pfu of vvMDV isolate 02LAR. All IgY treatments are pooled.

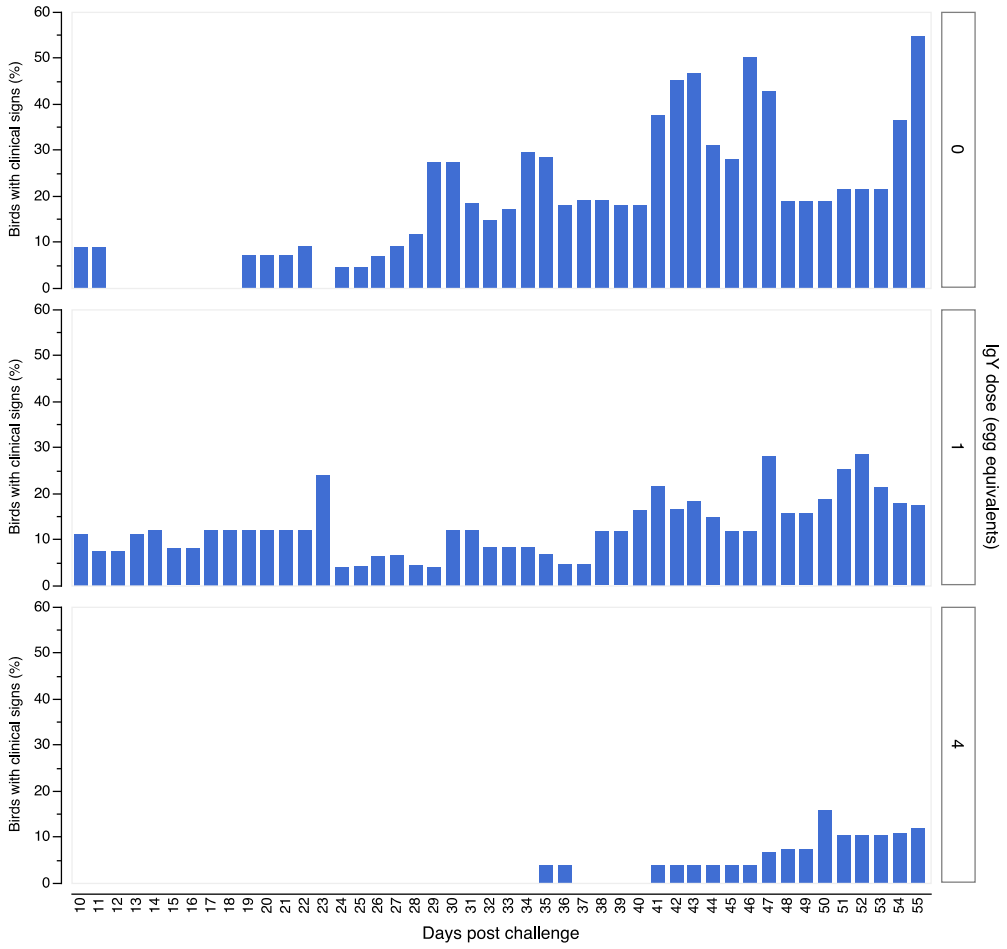

Figure 6.9: Experiment 4. Percentage of chickens given different doses of egg-extracted IgY showing clinical signs between days 10 and 55 following challenge with isolate 02LAR. Challenge treatments of 500 and 2000 pfu are pooled.

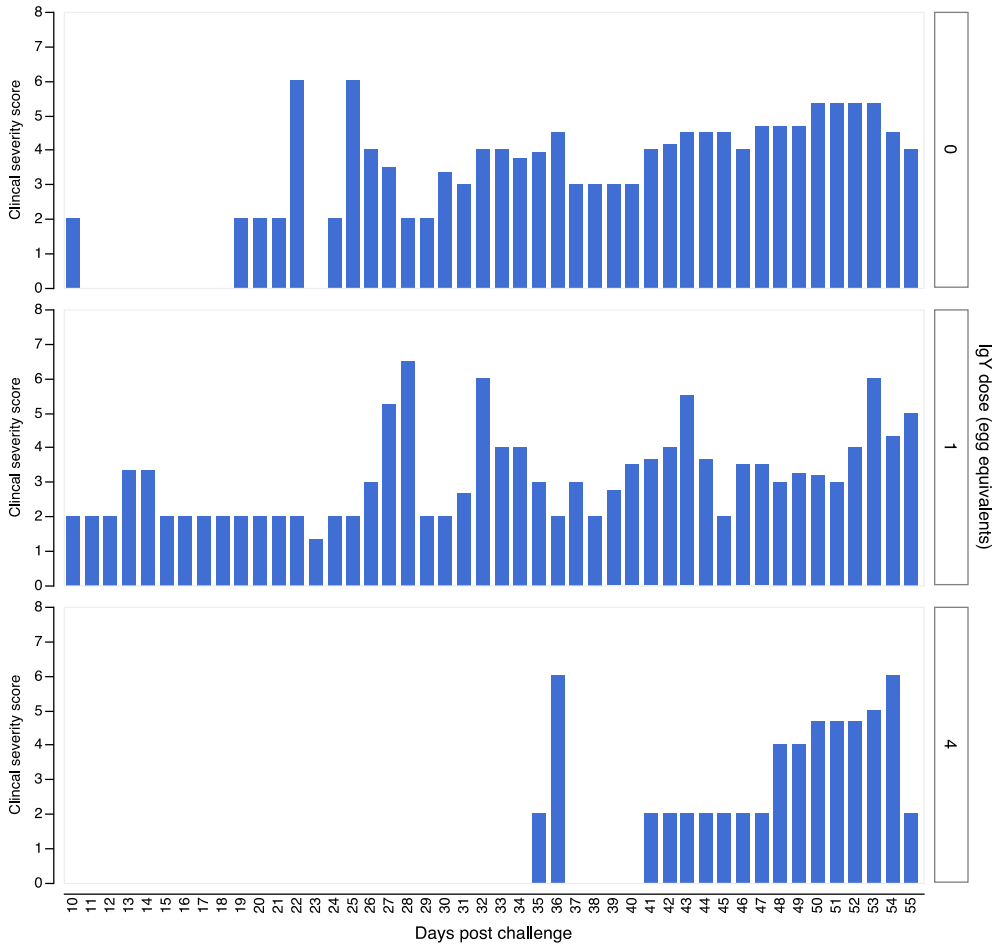

Figure 6.10: Experiment 4. Severity of clinical signs between days 10 and 55 following challenge with isolate 02LAR in chickens given 0, 1 or 4 egg equivalents of extracted IgY. Challenge treatments of 500 and 2000 pfu are pooled.

6.2.4.3 Mortality and MD incidence

During the early stages of the experiment there was extensive mortality due to bacterial infections, requiring antibiotic treatment in water. Mortality was largely complete by 11 days of age (6 dpc) but there were a small number of additional cases up to 19 days of (14 dpc). While there was some evidence of treatment effects on this early mortality it was observed in all SPF chick treatments including controls and for this reason the mortality analysis was restricted to chicks alive from 14 dpc (232 chickens, population at risk).

Survival analysis, which was restricted to chicks alive from 14 dpc (232 chickens) of the population at risk revealed a highly significant effect of treatment ($P < 0.001$ Figure 6.11). This was due to significant effects of both IgY dose ($P < 0.001$, Figure 6.12 Left) and

MDV challenge dose (P = 0.006, Figure 6.12 Right). There was no mortality in unchallenged control chickens. In challenged chickens without IgY administered, mortality accelerated between 28 and 32 dpc with a further increase between between 42 and 56 dpc. In those that received 1 egg equivalent of IgY, mortality accelerated from day 40 and continued to the end of the experiment. In chickens that received 4 egg equivalents of IgY, mortality was delayed markedly, accelerating from day 48 and contining to the end of the experiment. The effect of challenge dose was due to the effect of the (zero IgY) dose group, with no difference in the mortality pattern between chickens challenged with 500 or 2000 pfu of vvMDV.

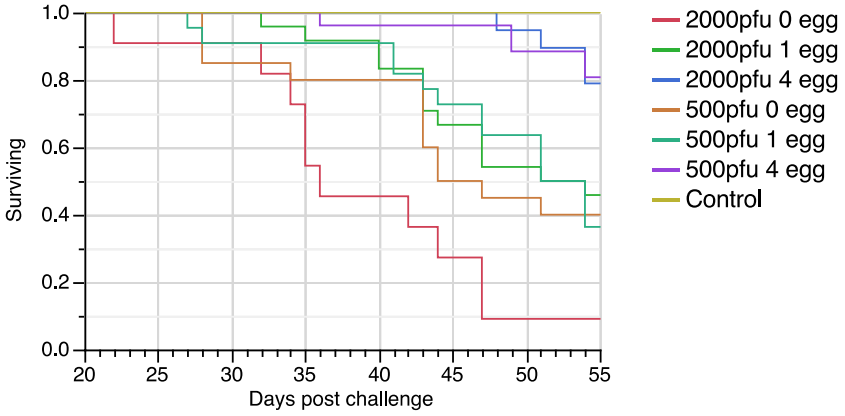

Figure 6.11: Experiment 4. Survival pattern showing the effect of treatment (P < 0.0001).

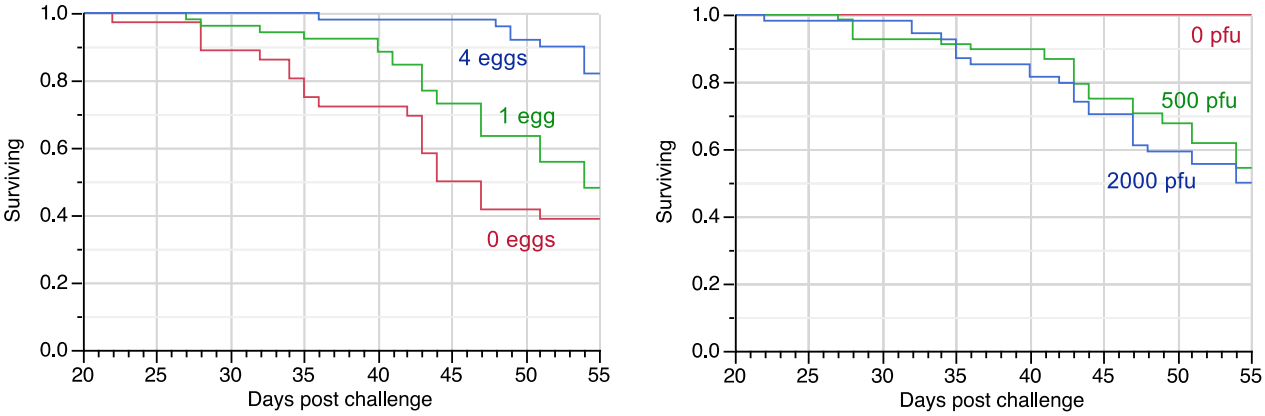

Figure 6.12: Experiment 4. Survival patterns in chickens challenged with 0, 500 or 2000 pfu of vvMDV (02LAR) with prior inoculation with 0, 1 or 4 egg equivalents of IgY extracted from commercial layer eggs showing the overall effects of IgY treatment (Left, P < 0.0001) and challenge dose (Right, P = 0.006).

The overall incidence of mortality and MD lesions is detailed in Table 6.6. Amongst all chickens challenged with vvMDV 59/122 (48.4%) died or were euthanased and 76/122

(62.3%) exhibited gross MD lesions up to the end of the experiment on 56 dpc. No unchallenged control chickens died or exhibited MD lesions. As may be predicted from the survival analysis, the level of administered IgY had a highly significant effect on both mortality ($P < 0.0001$) and incidence of MD ($P = 0.0002$) with administration of 4 eggs but not 1 egg equivalents significantly reducing mortality and MD relative to chicks not provided with any (Table 6.6). The dose of vvMDV used at challenge on the other hand, did not affect either mortality ($P = 0.96$) or MD incidence ($P = 0.2$) significantly.

Table 6.6: Mortality rate and incidence of MD to day 56 dpc by challenge treatment and IgY dose. The table shows information on only birds after 14 dpc.

IgY Treatment	Overall IgY treatment effect*		Challenge dose (pfu)	Challenge treatment effect*	
	Mortality	MD		Mortality	MD
0	23/31 (74.2%)[a]	26/31 (83.9%)[a]	500	13/20 (65.0%)	15/20 (75.0%)
0			2000	10/11 (90.1%)	11/11 (100%)
1	26/46 (56.5%)[a]	32/46 (69.6%)[a]	500	14/22 (63.6%)	14/22 (63.6%)
1			2000	12/24 (50.0%)	18/24 (75.0%)
4	10/45 (22.2%)[b]	18/45 (40.0%)[b]	500	6/26 (23.1%)	10/26 (52.4%)
4			2000	4/19 (21.1%)	8/19 (42.1%)
All	59/122 (48.4%)	76/122 (62.3%)	500	33/68 (48.5%)	39/68 (57.3%)
			2000	26/54 (48.2%)	37/54 (68.5%)
Control	0/22 (0%)	0/22 (0%)	0		

* Overall effect of IgY was significant for mortality ($P < 0.0001$) and MD incidence ($P = 0.0002$). Overall effect of challenge dose was not significant for mortality ($P = 0.96$) or MD incidence ($P = 0.20$)

[ab] Means not sharing a common letter in the superscript differ significantly ($P<0.05$)

6.2.4.4 Bodyweight and relative immune organ weights

Mean bodyweight (at 22 and 56 dpc) and thymic atrophy score, relative bursal weight and relative splenic weights of surviving birds at 56 dpc are shown by treatment in Table

6.7. Concerning body weight, inoculation with IgY had no adverse effect in unchallenged control chickens when measured at 22 and 56 dpc, thus the control treatment could be grouped irrespective of IgY administration.

Bodyweight at 22 dpc was significantly depressed by challenge with 2000, but not 500 pfu of vvMDV (P = 0.01) (Table 6.7). At 56 dpc, bodyweight was uniformly lower in challenged than control chickens (P = 0.004) with no significant difference in challenged birds due to either challenge dose or IgY dose, although the effect of the latter in a specifc contrast approached significance (P=0.065).

Table 6.7: Experiment 4. Bodyweight (at 22 n=4-6 birds/group, at 56 dpc n= 5-20 birds/group), thymic atrophy score, relative bursal weight and relative splenic weights of surviving birds at 56 dpc (LSM ± SEM) showing the effects of MDV challenge dose and IgY dose. Sex was taken into account in the model.

MDV challenge (pfu)	IgY dose (eggs)	Body weight at 22 dpc (g)	Body weight at 56 dpc (g)	Thymic atrophy score (0-3)	Relative bursal wt (%BW)	Relative splenic wt (%BW)
2000	0	167 ± 21^{b}	IC	IC	IC	IC
2000	1	NM	729 ± 29^{b}	0.46 ± 0.25^{bc}	0.30 ± 0.03^{ab}	0.31 ± 0.02^{a}
2000	4	NM	781 ± 24^{b}	0.71 ± 0.21^{bc}	0.35 ± 0.02^{a}	0.29 ± 0.02^{a}
500	0	233 ± 15^{a}	NM	2.04 ± 0.30^{a}	NM	NM
500	1	NM	728 ± 33^{b}	1.16 ± 0.28^{b}	0.23 ± 0.03^{b}	0.28 ± 0.02^{a}
500	4	NM	777 ± 21^{b}	0.49 ± 0.18^{c}	0.31 ± 0.02^{a}	0.30 ± 0.02^{a}
0 (Control)	All	239 ± 10^{a}	845 ± 19^{a}	0.19 ± 0.17^{c}	0.34 ± 0.02^{a}	0.19 ± 0.01^{b}
0 (Control)	0	254 ± 35^{x}	862 ± 31^{x}	0.00 ± 0.00^{x}	0.33 ± 0.03^{x}	0.20 ± 0.01^{xy}
	1	218 ± 33^{x}	841 ± 30^{x}	0.25 ± 0.18^{x}	0.33 ± 0.03^{x}	0.16 ± 0.01^{y}
	4	246 ± 35^{x}	843 ± 31^{x}	0.17 ± 0.19^{x}	0.37 ± 0.03^{x}	0.20 ± 0.01^{x}

^{abc}Overall treatment means not sharing a common letter in the superscript differ significantly (P<0.05)

^{xyz} Control means not sharing a common letter in the superscript differ significantly (P<0.05)

NM – No Measurement, IC – Insufficient chickens (1) to include in model

Thymic atrophy score (0-3, maximum atrophy score = 3) at 56 dpc was significantly lower in control chickens than those in all challenge treatments except 500pfu and 1 egg (P < 0.0001) (Table 6.7). There were no significant trends towards greater atrophy in chicks

receiving 1 rather than 4 egg equivalents of IgY (P=0.37) and in those challenged with 2000 rather than 500 pfu of vv MDV (P = 0.31).

Relative bursal weight at 56 dpc was significantly lower in birds in the 500 pfu and 1 egg treatment than control chickens (P = 0.03) with the other treatments having intermediate values (Table 6.6). There was also a significant sex effect (P = 0.03) with females having significantly higher values (0.33 ± 0.01) than males (0.28 ± 0.02).

Relative splenic weight at 56 dpc was uniformly higher in chickens challenged with MDV compared to control chickens (P < 0.0001) (Table 6.6). Interestingly there was a slight but significantly lower relative spleen weight in control birds given 1 egg equivalent of IgY relative to those given no IgY or 4 egg equivalents (Table 6.7).

6.3 Discussion

The main purpose of this study was to assess the restrictive effect on the pathogenesis of MDV of passively transferred anti-MDV antibody derived from egg yolks of commercial chickens vaccinated with Rispens against vvMDV. To test this, a series of experiments was conducted, some to develop and test the means of generating populations of chickens differing in anti-MDV ab status (Expts 1, 2 and 3), and others to test the effects of differing ab status on the pathogenesis of MD (Expts 1 and 4. In experiment 1, differences in anti-MDV status had no significant effects on the development of EMS, ATP or MDV viral load in PBL whereas MDV challenge dose and pathotype did influence this. The main effect of presence of anti-MDV ab was to reduce immune organ damage and this finding is in agreement with (Powell *et al.* 1980; Islam *et al.* 2007).

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The main effect of presence of anti-MDV ab was to reduce immune organ damage. Experiments 2 and 3 confirmed that inoculation of IgY extracted from eggs from Rispens vaccinated parents significantly increased anti-MDV titres in chicks and this was implemented in a challenge experiment in experiment 4. In this experiment inoculation with extracted antibody conferred significant protection against MD, and MD induced mortality and changed the pattern of clinical signs observed. Unfortunately the incidence of ATP could not be ascertained with accuracy in this experiment due to confounding bacterial infection.

In the preliminary experiment component of experiment 1, a moderate level of antibody was found in unvaccinated hens suggestive of the spread of CVI988 between isolation sheds during the experiment. Effective shedding and spread of the current commercial vaccine strains of Rispens CVI988 has subsequently been demonstrated (Islam *et al.* 2013), in support of this. As a consequence, SPF chickens free from Mat-Ab were purchased from the SPAFAS Company. These SPF chickens were from the same genetic back ground to those chickens with anti-MDV Mat-Ab generated at UNE. The chickens are derived from the CSIRO HWL line which has been shown to be comparatively resistant to MD (McKimm-Breschkin *et al.* 1990), but in which significant ATP has been observed (Renz et al., 2012).

The ELISA test to detect antibody directed against MDV confirmed that the UNE chickens had anti-MDV maternal Ab towards MD with titres declining in a classical fashion to very low levels at 21 days of age. This result was in agreement with previous studies that used the ELISA test to confirm antibody transmission from hens to the sera of their offspring (Davison *et al.* 2008; Grindstaff 2010; Kowalczyk *et al.* 1985; Patterson *et al.* 1962).

The main part of experiment 1 involved experimental challenge with MDV-1 strains 02LAR (vvMDV) or MPF57 (vMDV) on days 0 and 7 of age. Chickens challenged on d 0 were protected against ATP mortality but not against MDV infection relative to those challenged on day 7. Although the effect of Mat-Ab treatment was not significant, there was a trend towards lower ATP in the group with Mat-Ab (17.9 vs. 29.0%). In the group challenged on d 7 more ATP and some mortality (only in Mat-Ab negative chickens) was observed. Once again there was a non-significant trend for the incidence of ATP to be lower in the group with Mat-Ab (27.1 vs. 33.3%) These findings only partially support hypothesis 2 that Mat-Ab would be protective against ATP, particularly when challenge was at day 0. On the other hand, there was strong support for hypothesis 3 (more ATP induced by vvMDV than vMDV) with chickens challenged with MPF57 (vMDV) on d 7 inducing less ATP and other clinical signs than 02LAR (vvMDV). Hypothesis 4 that ATP incidence would be positively correlated with challenge dose was only partially supported with a clear trend towards increased incidence with increasing dose in each of the challenge isolators, but this did not achieve statistical significance. The results of the experiment indicate that Mat-Ab may confer immunologic protection against ATP as previously hypothesised on the basis of observed incidence only in maternal antibody negative chicks under experimental conditions (Renz et al., 2012) and not in commercial maternal antibody positive chicks under identical conditions in separate experiments

(Walkden-Brown et al., 2013). However, the Mat-Ab levels in Experiment 1 did not confer the complete protection that was hypothesised. Such protection is in accordance with early research on the role of immune-mediated maternal effects showing that Mat-Ab has a restrictive effect on the early pathogenesis of MDV (Burgoyne and Witter 1973; Calnek 1972b; Kermani-Arab *et al.* 1976).

This restrictive effect was not clearly evident in the MDV viral load in PBL in which clear effects of MDV pathotype but not Mat-Ab status were observed. This finding was in disagreement with an early report that Mat-Ab lowers the amount of MDV antigen in positive tissues based on fluorescent antibody test (Calnek 1972b). Some indirect support for an effect of Mat-Ab could be found in the higher viral loads seen in chickens challenged at day 7 than day 0 in some treatments, with a strong trend overall. Hypothesis 5, that there would be a positive association between ATP and MDV load in PBL was supported with significantly higher MDV load observed in chicks affected by ATP than those not affected.

Hypothesis 6 that Mat-Ab would be protective against bodyweight loss and immunosuppression induced by MDV challenge was partially supported. There was no effect on bodyweight, but in chickens challenged with MDV Mat-Ab offered significant protection against both thymic and bursal atrophy. The hisopathology of chickens with ATP showed nerve lesions type (A and B), various degrees of vasculitis, perivascular mononuclear cell cuffs of the brain stem and vacuolation of cerebrum. Only one chicken with EMS was submitted to hisopathology and it exhibited perivascular mononuclear cell cuffs of the brain stem and vacuolation of cerebrum This histopathological finding for EMS is in agreement with previous studies (Swayne *et al.* 1989a; Swayne *et al.* 1989b; Witter *et al.* 1999).

Experiments 2 and 3 were preparatory trials. Experiment 2 showed dose-dependant increases in anti-MDV titres in SPF chickens inoculated with 2, 4 or 6 egg equivalents of Mat-Ab in support of hypotheses 1 and 2. However, even inoculation of 6 egg equivalents failed to induce titres equivalent to those obtained from chicks hatched from eggs from the same parent flock from which the egg-extracted IgY was derived, leading to rejection of hypothesis 3. Titres in these commercial layer chicks were considerably higher than those seen in the UNE-hatched chicks in experiment 1, and persisted for longer. As hypothesised (hypothesis 4) there was no difference in titre following IgY administration via the i.a. or the s.c. route so a combination of the two routes was used in subsequent experiments. Inspection of the antibody titre profile demonstrated a similar decay curve of injected anti-MDV Ab in SPF chickens and naturally acquired antibody in

commercial chickens, in accord with hypothesis 5. The natural profile in the commercial layers suggests that under field conditions significant titres may persist at least for 3 weeks and these could protect the chickens during the critical period between *in ovo* vaccination and development of solid vaccinal immunity. In experiment 2 the decline of MDV-Ab did not continue after 14 days as in the experiment 1. The real cause is not clear but possibly it was due to slow release of the injected antibody in to the chickens' circulation.

Also this study concluded that anti-MDV IgY extraction by the ammonium sulphate-caprylic acid method (Akita and Nakai 1992; McLaren *et al.* 1994) was satisfactory. Thus, the result provided an evidence to go a head to design Experiment 3 to extract IgY from 2000 eggs and test them for use in subsequent challenge experiments. The large number of eggs targeted was a result of the comparatively inefficient translation of extracted and inoculated IgY to serum titres, relative to natural maternal transfer.

Due to the failure of a hatch of SPF chickens at UNE, Experiment 3 utilised only commercial layer chickens with anti MDV ab. The experiment was very successful with injection of 1 or 2 ml (1.1-2.8 egg equivalents/ml) of extracted IgY from 16 batches producing marked and sustained elevations of anti-MDV titre (Figure 6.4, Table 6.5) in agreement with all of the hypotheses proposed. From this result, it was apparent that the IgY extractions from these commercial layer eggs had produced a more potent extract than that used in experiment 2, with a doubling or trebling of the natural titres attained following injection. It is not clear whether this difference was due to improvements in extraction efficiency or superior anti-MDV titres in the commercial layers producing the eggs for Experiment 3 than those in the layer breeder hens producing the eggs used in experiment 2. The former explanation is probably more likely.

The success of experiment 3 enabled Experiment 4 which, like experiment 1, was designed to study the protective effect of anti-MD IgY against Australian vv and v strains of MDV strains of the Australian isolates. However, in the interim, the genotype of the SPF chickens available in Australia changed from the ex CSIRO HWL line used in previous experiments to the Lohmann LSL classic, a white leghorn type bird bred in Germany with some genetic selection for resistance to MD.

Unfortunately the SPF chicks acquired bacterial infections during hatching and/or transfer to isolators leading to considerable illness (navel ill, yolk sac infection, peritonitis, septicaemia) and mortality during the early stages of the experiment, confounding or

obscuring any ATP which was induced. Issues with sample identification and analysis followed by premature loss of the serum samples also adversely affected the clarity of the serological results. Nevertheless, the antibody and challenge treatments were successfully implemented and some very clear results were obtained.

Hypothesis 1 of Experiment 4 was that MDV challenge would induce dose-dependent increases in ATP and MD in SPF chicks lacking maternal antibody. While MDV challenge certainly induced significant MD and mortality, induction of ATP was obscured by a concurrent bacterial infection. Furthermore there were no significant effects of viral dose on clinical signs, mortality and MD incidence, although trends were in the expected direction. However, direct comparison of the survival curves of the 2000 and 500 pfu challenge treatments given no maternal antibody revealed a significantly faster and greater rate of mortality in the 2000 pfu treatment ($P = 0.04$).

Hypothesis 2, that inoculation of SPF chicks with egg-extracted IgY would delay and reduce ATP could not be effectively tested given the issue with early bacterial infection. This was brought under control by water-administered antibiotics. However, IgY administration profoundly influenced the pattern of clinical signs observed, with later onset of signs and reduced incidence with increasing dose of IgY. Fewer chickens in the 4 egg injected groups showed neurological signs or ataxia symptoms. This suggests that administration of IgY leads to enhancement of protection during the early period of MDV replication. Similarly the protective effects of IgY administration against bodyweight loss and immunosuppression (Hypothesis 3) were compromised by the lack of birds available in the zero IgY but MDV challenged treatments at the end of the experiment. Where data were available generally supported the hypothesis, though only achieving statistical significance rarely (eg for thymic atrophy).

In contrast to the earlier hypotheses, the experiment provided very strong support for hypothesis 4 that inoculation with IgY would reduce the incidence and delay the onset of MD in a dose dependent manner. Survival analysis revealed highly a significant ($P<0.001$) effect of IgY dose with higher doses delaying the onset of mortality primarily (Figure 6.12). Analysis of overall rates of mortality and MD also revealed highly significant effects of IgY dose with the major effect being due to a marked reduction in the 4 egg equivalent group (Table 6.6). This result in aggrement with earlier studies showing restrictive effects of anti-MDV Ab to the development of MD (Chubb and Churchill 1969; Kermani-Arab *et al.* 1976).

The differences in MD observed in challenged groups with differing IgY administration were supported by evaluation of anti-MDV titres using ELISA with sustained dose dependent differences in antibody titre between the three IgY treatments (0, 1 or 4 eggs) (Figure 6.6). Surprisingly though, the differences were maintained for the duration of the experiment, with little change in overall titre as the experiment progressed. This is difficult to explain as it would be expected that the chickens administered no antibody would show a late rise in anti-MDV titre as active immunity develops. Chickens administered exogenous IgY could be expected to see a decline in the short term as passive immunity declined, followed by an increase as active immunity developed. The IgY preparation used in this experiment had a thick viscous consistency with some salts still present, and it is possible that this formed a slow release depot for the IgY. This was supported by examination of s.c. injection sites at various times after inoculation. However, this would not explain the lack of a rise in titre in chicks not receiving any antibody. Even more baffling are the ELISA results for the unchallenged control chickens in which control chicks with no administered IgY had similar (low) titres as birds administered IgY or having natural maternal anti-MDV antibody. The reasons for this are not clear, but at least in the control birds there was a gradual decline in titre over time, whereas there was an overall gradual increase in the challenged birds.

Overall this series of experiments has thrown up an interesting mixture of expected an unexpected results. Extraction of IgY from egg yolk and inoculation into chickens successfully raised anti-MDV titres and provided important protective effects against MD infection, mostly, but not always, consistent with inhibition of early viral replication and pathogenesis (Experiment 4). On the other hand, the presence or absence of natural anti-MDV antibodies in Experiment 1 induced surprisingly few effects on MD or MDV with the main effect of anti-MDV antibody being to reduce immune organ damage. Frustratingly, the original objective of determining the role of anti-MDV antibodies in the development of ATP has not been able to be fully met due to a combination of an apparent lack of IgY effect in Experiment 1 and inability to detect an effect in Experiment 4 due to concurrent bacterial infection. Changes in chicken genotype and MDV titration method to determine pfu may also have contributed to a lower incidence of ATP than originally reported in Australian SPF chickens by Renz et al (2012). Also the chicken genotype used in this experiment 4 (Lohman LSL CLassic) appeared to show more tendencies to neurological signs with nerve swelling than tumour development than the SPF strain used in Experiment 1 (SPAFAS Australia bird).

Chapter 7 Investigation of a field outbreak of apparently atypical Marek's disease

7.1 Introduction

Marek's disease (MD) is a widespread contagious lymphoproliferative disease of chickens caused by the MD virus (MDV) serotype 1 (MDV-1). Vaccination with herpesvirus of turkeys (HVT), used successfully since 1970, has dramatically decreased the incidence of this disease (Calnek and Smith 1972; Purchase and Okazaki 1971).

Following a major outbreak of MD in Australia in the 1990s and resultant introduction of *in ovo* vaccination in 1996 (Islam *et al.* 2001; Jackson 1999), vaccination of broiler chickens against MD has become a widespread practice, as it is in many other countries, with approximately 50% of broilers in the country vaccinated *in ovo* with HVT. Prior to this broiler chickens were rarely vaccinated and long lived layer and breeder birds were protected against MD through vaccination with HVT alone or in combination with serotype 2 virus (Karpathy *et al.* 2002). Broilers received some protection in the form of passively acquired maternal immunity. As existing vaccines failed to control MDV in the 1990s outbreak of MD the serotype 1 vaccine, CVI988 (Rispens), was also approved for use in Australia in 1997 and it is now universally used in layer and breeder hens there, sometimes in combination with HVT (Karpathy *et al.* 2002). Thus, broiler chickens in Australia receive passive maternal immunity against MDV from their vaccinated parents. However, such chickens may receive an MDV challenge of variable magnitude at any time after placement if the exposure is to virulent strains of MDV-1 (Witter 1997) or after the decay of maternal antibodies Mat-Ab (Calnek 1972b).

The main infective source of MDV-1 is poultry dust that contains feather dander (Calnek *et al.* 1970; Carrozza *et al.* 1973). MDV-1 can survive in the dust and litter for long periods (Baigent and Davison 2004; Carrozza *et al.* 1973; Witter *et al.* 1968). Recent Australian studies demonstrate that the use of real-time PCR is a highly sensitive method of detecting MDV-1 in dust of commercial poultry farms across Australia (Islam *et al.* 2004; Renz *et al.* 2006; Walkden-Brown *et al.* 2013a; Walkden-Brown *et al.* 2004).

In late May 2010, a syndrome of late paralysis with high mortality occurred in broiler chickens in one broiler growing region of NSW. The syndrome started in two broiler

farms over two weeks (wk) and in the following two wk, three more farms of the same company and within 1 km reported illness. The syndrome occurred in chickens at 27, 32 and 42 days (d) old. According to the history provided by the company's Manager and the veterinarians who were responsible for entire poultry operations, the mortality in chickens from the affected farms could reach 25% in some sheds (8000-10000) birds. The affected birds became prostrate and died within a day or so of developing clinical signs. The affected birds had been in good condition and ate well prior to developing clinical signs. Chickens were vaccinated against infectious bronchitis at the hatchery, but were not vaccinated against Marek's disease.

A trial was conducted to treat the flocks with Amoxicillin in the drinking water at the recommended dose rate for 3-4 d. There was good response at the beginning, but later the pattern of morbidity and mortality remained unchanged. Dead and moribund birds from affected flocks were submitted for necropsy by veterinarians for the farms. No nerve enlargement or visceral tumours were detected.

The broiler chickens originated from the company's hatchery. They were housed in large sheds typically containing about forty-four thousand chickens per shed. The shed floors were mostly concrete and had a fresh deep litter of wood shavings. The sheds had a controlled environment in relation to ventilation, lighting and temperature, and programs had not been changed since past successful production. Feed was supplied by the same company, using previously used formulations. Chickens had been vaccinated after hatching against Newcastle disease and infectious bronchitis according to the manufacturer's direction. Flocks were housed in standard buildings, which were totally cleaned with high pressure water after the litter had been cleaned out. Afterwards the floors were left to dry, and then subjected to "bomb fumigation" with formaldehyde before the chickens were housed. No MD vaccine had been used in the chickens, but birds were generated from parents vaccinated with HVT and Rispens. In this outbreak natural MD Mat-Ab did not protect the chickens against the syndrome, which was diagnosed as MD on the basis of the histopathological lesions by the NSW Department of Primary Industries diagnostic service at the Elizabeth McArthur Agricultural Institute. However, MDV Mat-Ab, like any Mat-Ab has a decay curve. Mat-Ab of MDV have been shown to fall to as low as 2.1% of the original level at hatching in chicks by 21 d of age (Biggs *et al.* 1972).

The chickens in this study exhibited signs characterised by ataxia, paresis. Classical MD occurs as a nervous form (Cho *et al.* 1970; Cho *et al.* 1998; Gimeno *et al.* 1999;

Pappenheimer *et al.* 1926), appearing as a gradual advancing paralysis of neck, wings and one or both limbs, generally in chickens of older age than those in the present outbreak. Acute MD which is the dominant form of MD observed in Australia in recent years, has been characterised more by lymphoma formation than neurological signs (McKimm-Breschkin et al., 1990; Renz et al., 2012; Walkden-Brown et al., 2013) although Renz et al (2012) reported the occurrence of an early paralysis and mortality syndrome between days 9-15 in challenged chicks. The latter was very similar to the acute transient paralysis reported in the USA (Witter et al., 1999). However, this ATP was seen much earlier in life than in the current outbreak, and has only been seen in chickens not having maternal antibody against MD. It is thus not a syndrome that has been observed in the field.

The microscopic lesions of MD in the chicken have been well described (Fujimoto *et al.* 1971; Helmboldt 1972; Pappenheimer *et al.* 1926; Wight 1962a). The classic microscopic lesions that involve the CNS are infrequent and include lymphocytic perivascular cuffing, occasional focal demyelinating plaques and gliosis and on rare occasions lymphomatous lesions (Cho *et al.* 1970). The more severe transient and acute transient paralysis associated with very virulent MDV administration to maternal antibody negative chicks are associated with oedema due to vasculitis and perivascular mononuclear cell cuffs, leakage of albumin, and vacuolation. The development of clinical signs probably results from the development of vasogenic brain oedema. Vasculitis is caused by the indirect effect of vv MDV, resulting in ischaemic malacia (Swayne *et al.* 1989a; Swayne *et al.* 1989b; Witter *et al.* 1999).

In MDV-1 infection, lymphocytes are the main target cells; hence early 3-7 days post-infection (dpi) cytolytic infection of B lymphocytes causes transient immunosuppression, while late cytolytic infection of T lymphocytes leads to immunosuppression. Malignant transformation of T cells is the cause of lymphoid tumour formation and death (Calnek 2001a; Calnek *et al.* 1998; Venugopal and Payne 1995). However, infection with virulent MDV induces severe neurological clinical signs of MD, including ATP, early mortality syndrome (EMS) may build up before lymphoma shaping (Silva and Witter 1996; Smith and Calnek 1974; Witter *et al.* 1980). In the present study, tumour formation or enlargement of nerves was not encountered in this syndrome which may occur before the onset of lymphomas. ATP or the Australian EMS is associated with immunosuppression, which is manifest as severe atrophy of thymus and bursa (Walkden-Brown *et al.* 2007a; Witter *et al.* 1999).

Diagnosis of MD in the field is usually dependent on clinical signs, gross examination at necropsy and confirmation by histopathological findings together with the isolation and identification of MDV-1 from infected flocks, and presence of specific antibodies especially when MD infection has not induced obvious pathological changes (Davison and Nair 2004; Sharma 1985). Virus propagation in cell culture is the commonly used method for virus isolation and quantification of the virus by plaque assays (De Laney *et al.* 1995). Routine monitoring of MDV viral copy number in poultry dust from the region indicated that MDV levels were approximately 3 logs higher than in other parts of Australia (Region D, Walkden-Brown et al., 2013) and this, and also because the affected broiler chicks had not been vaccinated against MD and showed nervous signs including leg and wing paralysis led to a suggestion that the syndrome could be an atypical form of MD.

7.2 Materials and Methods

7.2.1 Data collection

The UNE research group was invited to visit the outbreak and collect samples for isolation of MDV and other diagnostic purposes. A visit to the farm by Salih Wajid, Katrin Renz and Sue Burgess was made on 6-7June 2010, and on that day, a farm (67-shed 2) was reported to be newly infected and was included in the survey. The total number of meat broilers in the five affected farms was about 1.6 million. Flocks sizes were 44,000 chickens per shed on average.

Before a visit was arranged, a three-page survey was designed by Prof. Steve Walkden-Brown to gather information such as farm identification, farming system, age of affected flocks, immunization status, total number of affected chickens, number of affected poultry sheds on each farm, morbidity and mortality rate, history of other disease problems, security measures, data on vaccination and treatment used. The farm manager (Mr. Graham Kirby) and two of the company veterinarians, who looked after the broilers on the affected farms, kindly, answered the questionnaires and took the group around the five affected farms.

On the visit of June 6, 2010, close observations of the chickens was carried out. In total, eight sheds on eight farms, with a total of 352,000 birds, which were 21, 27, 32, 35, 42, and 49 d old, were visited. Sick chickens showed clinical signs of ataxia, paresis and

prostration, apparently due to paralysis. Other chickens were in good body condition. Photographs and movies were taken of affected birds. Mortality figures were documented in the company's daily record, and the mortality rate was 25%. The strain of chicken was Ross broiler. The clinical signs occurred at 32-42 d of age.

7.2.2 On-farm measurements and sampling

7.2.2.1 Blood samples

As the chickens of this study were not vaccinated against MDV-1, but were generated from dams vaccinated against MDV-1, sampling for detection of anti-MDV antibody by ELISA was designed to differentiate passively acquired maternal antibody from that arising from infection with MDV on the basis of change in antibody titre over time. Blood samples from both affected (n = 41) and unaffected chickens (n = 30) were collected as shown in Table 7.1. Approximately 3 ml of blood was collected from each chicken from the wing vein. Blood samples were collected into disposable plastic tubes free from anticoagulant.

Table 7.1. Details of blood samples collected for anti-MDV ELISA test.

Farm	Shed No.	Shed status	Bird age (d)	Blood samples collected	
				Unafffected birds	Affected birds
F61	16	Unaffected	21	11	
F60	2	Affected	27	10*	
F60	24	Unaffected	35	10	
F68	3	Unaffected	42	5	
F67	2	Affected	49	5*	10
F60	15	Affected	32		10
F68	1	Affected	42		10
Total			41		30

*The sampled chickens were healthy apparently.the clinical signs of sickness started on sampling day.

7.2.2.2 Spleen collection for qPCR detection of MDV and virus isolation

Three spleens were removed from chickens with clinical signs. After displacement of the liver to the left side, the spleen was removed using sterile forceps, washed in Phosphate

Buffered Saline (PBS), placed into a sterile plastic sample jar and shipped on ice to UNE for virus isolation in tissue culture and detection of MDV using qPCR and standard PCR.

7.2.2.3 Dust collection for qPCR detection of MDV

Dust samples were collected from four affected sheds and four unaffected sheds as shown in Table 7.2. Using a disposable wooden spatula, approximately 1 g of dust was scraped from the surfaces of the fan housing of each shed into an Eppendorf tube, and used for DNA extraction.

Table 7.2. Summary of dust samples collected

Farm	Shed No.	Shed status	Bird age (d)
F67	2	Affected	49
F67	3	Affected	47
F61	16	Not affected	21
F60	15	Affected	32
F60	24	Not affected	35
F60	2	Not affected	27
F68	1	Affected	42
F68	3	Not affected	42

7.2.2.4 Gross necropsy

For post mortem examination, 30 chickens were selected randomly from different infected or uninfected farm sheds as summarized in Table 7.3. Twenty of the chickens exhibited clinical signs of ataxia and paresis. The remaining 10 chickens showed no clinical signs. However, five of them were from sheds where clinical signs were seen. Birds were euthanized humanely by dislocation of the cervical vertebrae. Gross necropsies (as described in Chapter 3 section 3.2) were performed immediately, lesions scored for severity 0-4 depending on the absence (0) or presence of mild (1), moderate (2), severe (3) and very severe (4) lesions. In order to prevent the spread of infection and to reduce the environmental contamination, disposable suits, boots, head caps and gloves were used. Care was taken to decontaminate the necropsy instruments, and the vehicle used to visit the farms.

Table 7.3. Details of birds euthanized for post-mortem examination and histopathology sampling

Farm	Shed	Shed status	Bird status	Age	No. of birds
68	1	Affected	Affected	42	10
68	3	Not affected	Not affected	42	5
67	2	Affected	Not affected	49	5
67	2	Affected	Affected	49	5
60	15	Affected	Affected	32	5

7.2.2.5 Tissue samples for histopathological evaluation

Fresh tissues samples of brain, sciatic nerve, trachea, lung, heart, spleen, proventriculus, kidney, liver, thymus, and bursa of Fabricius were collected from necropsied chickens and placed in 10% neutral buffered formalin. Fixed tissues were then trimmed, embedded in paraffin, sectioned in 4-5 µm slices and processed for staining with haematoxylin and eosin (H & E) as described in Chapter 3 section 3.9.4.

7.2.2.6 Scoring of the histopathological lesions

In a similar way to that done in scoring gross pathological lesions, histopathological lesions of the processed tissues were scored during microscopic examination. The severity of lesions was graded on a scale of increasing severity of the lesion from 0 to 4. A score of 1 was given to those specimens with minimal changes. A score of 2 was for tissue with mild changes, 3 for moderate changes, and score 4 for severely damaged tissue. The severity of haemorrhage, inflammatory response, degree of epithelial necrosis in different tissues was considered in the grading. In the thymus, the grading was measured on the severity of lymphocyte lysis. In the bursa, grading was dependent on the degree of lymphocyte lysis, cyst formation in the depleted follicles and epithelialisation of atrophied follicles, repopulation of lymphocytes and intra-follicular connective tissue proliferation. In the spleen, grading was measured on depletion of lymphocytes and proliferation of epithelioid tissue and macrophages in periarterial lymphocytic sheaths and intensity of engorgement of venous sinuses. In the kidney, measurement was dependent on the extent of nephrosis or nephritis and the degree of congestion and haemorrhage. Finally, the CNS grading was measured on congestion,

haemorrhage, with or without perivascular cuffing (PVC) of lymphocytes, and presence or absences of encephalomalacia.

7.2.2.7 Cell culture and MDV isolation

Chicken embryo fibroblast (CEF) cultures were prepared from 9–11 d old chicken embryos (SXPF) according to a standard method (De Laney *et al.* 1995; McKimm-Breschkin *et al.* 1990). To isolate MDV-1, two cell culture flasks of 25 cm^2 surface area (Greiner Cat.-No.: 658175) of secondary CEF were inoculated with 500 µL of cryopreserved splenocytes (1x10^7 cells/ml) prepared from spleens collected from chickens on farm 68 sheds 1 and 2. The culture medium was M199 (Invitrogen cat number 31100-035) with 20% (growth) and 10% (maintenance) foetal calf serum (Invitrogen cat number 10100-139. Both media contained antimycotics and antibiotics (Antibiotic-Antimycotic (100X), liquid Product Invitrogen cat number 15240-062). The cells were incubated at 37.5°C in 4-5% CO_2. The culture media were changed about 24 h after infection with splenocytes. Media was replaced with maintenance media as soon as cells were 90-100% confluent and subsequent media changes were performed every second day until day 6 or 7. The cells were then trypsinised with 2-3 ml pre-warmed solution (Invitrogen cat number 15050-065) and passaged onto new secondary CEFs which were about 70-90% confluent.

7.2.2.8 ELISA to detect anti-MDV antibodies

Samples of serum were tested for the presence of MDV antibodies, using an indirect ELISA procedure adapted from that described by (Zelnik *et al.* 2004) and the details is described in Chapter 3 section 3.9.3.ELISA for MDV Abs.

7.2.2.9 Extraction of DNA

DNA was extracted from 5 mg of spleen tissue samples and 10 mg of dust samples, using the Bioline Isolate DNA Kits. All DNA was stored at –20^o C. and then quantified using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop® Technologies Wilmington, USA) to assess DNA quality and quantity as described in Chapter 3 section 3.9.2.

7.2.2.10 Assay of MDV-1 genome copy number by qPCR

MDV-1 genome copy in spleen and dust samples was determined in duplicate using the qPCR method of (Islam *et al.* 2006; Islam and Walkden-Brown 2007; Renz *et al.* 2006)as detailed in Chapter 3 section 3.9.3.

7.2.2.11 Amplification and sequencing of the meq gene

Extracted DNA was also used as template for a standard PCR to amplify the *meq* gene from MDV-positive samples using the *meq*-specific primers BCH342, SJW1, BCMD07,and BCH343 (Table 2.6) Standard PCR was performed in a 25 µL reaction mixture reaction buffer (Fisher Biotec, Perth, Australia). The amplified fragments were separated on an agarose gel (1%) and visualized under ultraviolet light by staining with ethidium bromide. The PCR products were then purified using the Wizard DNA purification Kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The *meq* gene, amplified from one dust sample, was used for DNA sequencing. DNA sequencing and analysis was conducted by Macquarie University (Sydney, Australia) using an ABI 377 sequencer (Applied Biosystems Inc.). All primers used for sequencing are shown in Table 3.7. The DNA sequence data were analysed using Chromas 1.43 for analysis of chromatograms and SEQAID IITM 3.70 for sequence alignment (Rhodas and Roufa 1989).

7.2.3 Statistics

Most of the results in this chapter are descriptive. Anti MDV antibody titres were $\log^{10}$ transformed to better meet the assumptions of analysis of variance and the effects of age, and affected status nested within age were fitted in a least squares linear model. Both untransformed means and least squares means and standard errors of the mean are reported. Data were analysed using JMP 10 (SAS Institute Inc., NC, USA). A significance level of $P<0.05$ is used.

7.3 Results

The visited company is one of major commercial poultry companies in Australia, where intensive poultry farming is used for the production. The floors of the visited sheds were concrete and had a fresh deep litter of wood shavings. Environmental factors (ventilation,

lighting and temperature) in the sheds were under automatic control and the visited affected farms (sheds) were used for commercial Ross broiler chickens (meat production). Chickens were offered feed and water *ad libitum*. The affected chickens were clustered in separated groups away from the feed source. They were inactivity, standing or sitting with feathers ruffled. The chickens were of a uniform size and the breast muscles covered the breastbone in the sick chickens, indicating that the affected chickens had been eating well prior to sickness as in the history. Only one chicken was smaller than other birds and was sitting on the hock joints. The feathers of some affected chickens were rough in comparison to the active mate chickens. Some chickens in shed (2) in the farm (67) started to exhibit the clinical signs of paresis on the day of the visit.

7.3.1 Clinical signs

The disease was reported by farm staff to affect chickens between the ages of 27 and 42 d old. The disease had occurred on farms with chickens of 32, 42 d old prior to our visit, and was observed in chickens of 27 d old during the visit. The clinical signs were characterized by weakness/paresis, depression, or complete flaccid paralysis with chickens in sternal recumbency with neck and wings outstretched (Figure 7.1). Some of these chickens appeared to be dying (Figure 7.2). The majority of the examined chickens were in good body condition. Some chickens showed signs of gasping. According to the case history, the course of the disease was not more than 48 h from the beginning of the clinical signs until death or euthanasia.

Figure 7.1 A terminally comatose chicken (42 days old) from an affected shed.

Figure 7.2. Dead 42 day old chickens that were in good body condition. They were head down in the litter.

7.3.2 Anti-MDV antibody titres

Untransformed anti-MDV antibody titres increased between days 21 and 42 of age, with little difference between affected and unaffected birds (Figure 7.3). Analysis of log^{10} transformed titres fitting the effects of bird age and chicken disease status (affected/unaffected) nested within bird age, revealed a highly significant effect of bird age (P<0.0001, Figure 7.4) but not disease status (P = 0.80).

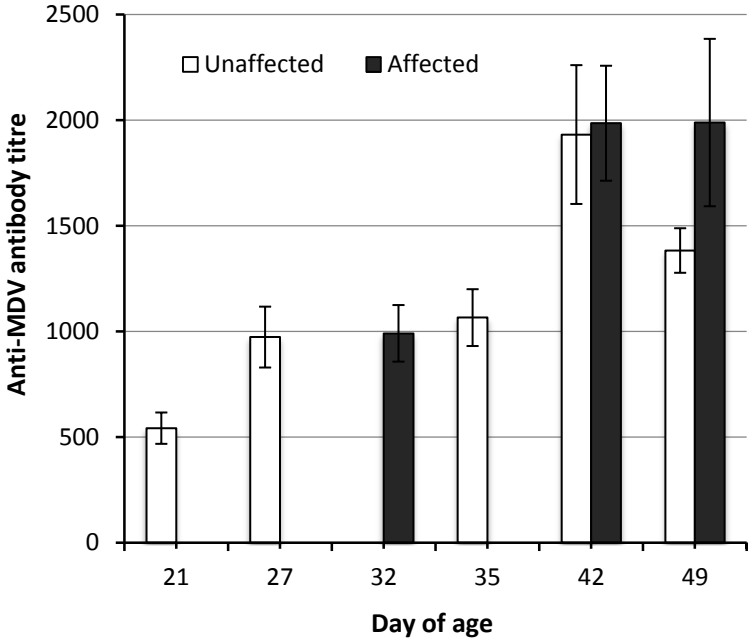

Figure 7.3: Untransformed mean anti-MDV titre (± SEM) in sera of chickens of different ages, affected and unaffected by the paralysis syndrome. Chickens were not vaccinated against MD.

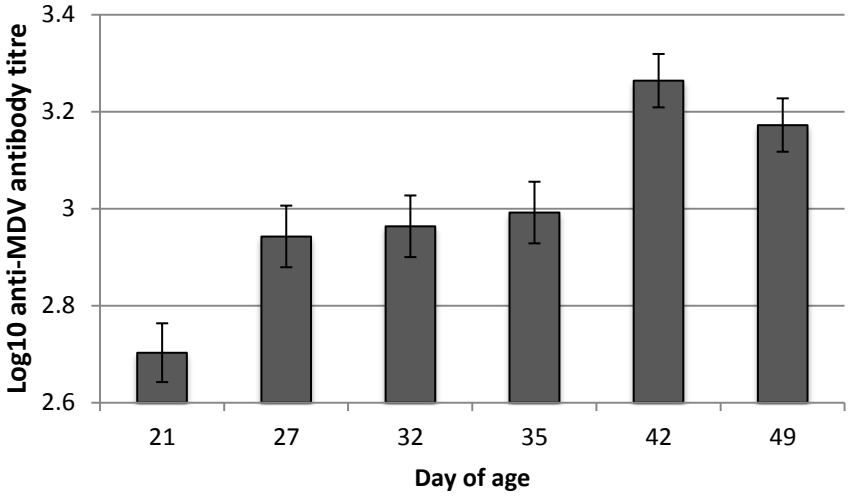

Figure 7.4: Least squares means (± SEM) of log^{10} anti-MDV titre in sera of chickens showing the overall effect of age (P<0.0001). Chickens were not vaccinated against MD.

7.3.3 Necropsy findings

Thirty dead and euthanized chickens were subjected to post-mortem examination, 20 clinically affected and 10 unaffected. Main findings for each chicken are detailed in Table 7.4. Most chickens were in good body condition, even those that were found dead in the sheds. Gross pathological lesions observed in different flocks were similar, but varied in severity. There were no statistically significant differences in lesion incidence between clinically affected and unaffected chickens, but there were trends towards increased incidence of congested trachea, breast muscle and intestines in clinically affected birds. On the other hand this trend was reversed for lung and kidney congestion. There were also numerically, but not statistically higher bursal (P = 0.43) and thymic atrophy scores (P = 0.41) in clinically affected chickens.

All birds examined had moderately ruffled feathers. 5 of 30 (16. 6%) and necropsied chickens showed green pasty to watery faeces soiling the vent region. Petechial or extensive haemorrhages in the breast muscle were observed in 5 (16. 6%) chickens. There was some feed in the chickens' crops and the intestinal tracts of some affected chickens were semi-empty. Gall bladders were distended in some birds. The intestinal tracts of some affected chickens were distended with gas. Livers were swollen, congested and friable and they were either dark red or pale to yellow. They had red pin-point petechial or ecchymotic subcapsular haemorrhage in 18 of the 30 (60%) examined birds.

Congested and consolidated lungs were noticed in 6/30 chickens (20%) birds. The trachea mucosa showed slight congestion in two cases (6.6%). Kidneys were moderately enlarged and usually congested and 5/30 (16. 6%) had patchy subcapsular haemorrhage. The proventriculi were either semi-filled or contained gas. One case showed moderate thickening of the proventricular wall. The majority of spleens examined showed no abnormalities, but in one case, the spleen was one and a half times the normal size, and in another case it was reduced to half size. Both of these spleens were soft and dark red in colour.

Complete atrophy of the thymus was detected in one chicken, while six chickens had moderate reduction in the size of the thymus. In the remaining 15 cases, the thymus showed no observable change. Severe atrophy of the bursa of Fabricius was detected in 8/30 (26.6%) chickens while 11 (36.3%) chickens' bursa were not affected (Table 7.4).

Table 7.4: Summary of main gross findings at necropsy by individual bird (cong = reddened/congested, haem = haemorrhage/petechiae/ echymoses, v = very, enl = enlarged, subcaps= subcapsular, dist = distended).

Chicken No	Bird ID	Farm	Shed	Shed affected?	Sex	Bird affected?	Age (d)	Breast Muscle	Cloaca	Heart	Trachea	Lung	Liver	Gall bladder	Spleen	Kidney	Proventriculus	Thymic atrophy score (0-3)	bursal atrophy score (0-3)	Intestines
1	60/15-1	60	15	Yes	F	Yes	32	cong					haem fragile	dist				0	0	
2	60/15-2	60	15	Yes	M	Yes	32						haem fragile					0	0	
3	60/15-3	60	15	Yes	M	Yes	32						v enl		enl			0	0	
4	60/15-4	60	15	Yes	M	Yes	32						enl haem	dist				0	0	cong serosa
5	60/15-5	60	15	Yes	M	Yes	32				cong		enl haem subcaps					0	0	cong serosa
42	67/2-1	67	2	Yes	M	No	42					cong haem cons	enl haem subcaps		enl			2	2	
43	67/2-2	67	2	Yes	M	No	42					cong haem				cong		1	2	
44	67/2-3	67	2	Yes	F	No	42					cong	haem subcaps					0	2	
45	67/2-4	67	2	Yes	M	No	42		dirty			v cong	haem					2	2	
46	67/2-5	67	2	Yes	M	No	42						enl	dist		cong		2	2	
48	67/2-6	67	2	Yes	M	Yes	42		dirty			cong	enl haem					3	3	
49	67/2-7	67	2	Yes	M	Yes	42						enl haem			cong		3	2	
50	67/2-8	67	2	Yes	M	Yes	42						enl haem					2	3	
51	67/2-9	67	2	Yes	F	Yes	42		dirty				enl sev haem					3	3	
52	67/2-10	67	2	Yes	M	Yes	42		dirty				enl haem			cong		3	3	
57	68/1-1	68	1	Yes	M	Yes	42	cong			cong		enl haem		enl spotty			0	0	
58	68/1-2	68	1	Yes	M	Yes	42				cong		enl haem					0	0	cong gas caecum
59	68/1-3	68	1	Yes	M	Yes	42			pericarditis	cong		enl haem subcaps		small			1	0	
60	68/1-4	68	1	Yes	M	Yes	42				cong			dist				3	3	
61	68/1-5	68	1	Yes	M	Yes	42				cong		enl cong					3	3	cong serosa
62	68/1-6	68	1	Yes	M	Yes	42	cong					haem	dist				3	3	cong serosa
63	68/1-7	68	1	Yes	M	Yes	42						haem	dist				4	1	cong serosa
64	68/1-8	68	1	Yes	M	Yes	42								small	enl	cong	3	3	cong serosa
65	68/1-9	68	1	Yes	F	Yes	42						enl haem	dist				2	2	
66	68/1-10	68	1	Yes	M	Yes	42	haem	dirty				enl cong	dist				2	3	
67	68/3-1	68	3	No	F	No	42						pale					3	0	
68	68/3-2	68	3	No	M	No	42	haem					enl haem subcaps					0	0	
69	68/3-3	68	3	No	M	No	42						enl haem subcaps	dist				3	0	
70	68/3-4	68	3	No	M	No	42					v cong	enl haem subcaps			enl	Dist gas cong	0	1	
71	68/3-5	68	3	No	M	No	42						enl haem subcaps	dist		cong		0	1	

Table 7.5: Summary of histopathological lesion severity score by individual bird. Empty cells indicate that no evaluation was done on that sample. The severity of lesions was graded on a scale of increasing severity of the lesion from 0 to 4. A score of 1 was given to those specimens with minimal changes. A score of 2 was for tissue with mild changes, 3 for moderate changes, and score 4 for severely damaged tissue.

Chicken No	Sample ID	Farm	Shed	Shed status	Bird status	Sex	Cerebellum	Cerebrum	Meninges	Choroid plexus	Sciatic nerve	Breast muscle	Trachea	Lung	Liiver	Spleen	Kidney	Proven-triculs	Thymic atrophy score (0-4)	bursal atrophy score (0-4)
1	60/15-1	60	15	Affected	Affected	F	4	3	2	2	0	1	0	2	4	0	1	0	0	
2	60/15-2	60	15	Affected	Affected	M	3	4	0	2	0	0	0	1	3	1	1	0	0	1
3	60/15-3	60	15	Affected	Affected	M	2	3	1	2	0	0	2	2	2	2	2	0	0	2
4	60/15-4	60	15	Affected	Affected	M	2	2	2	2	0	0	0	4	2	1	2	1	1	1
5	60/15-5	60	15	Affected	Affected	M	0	2	0	0		0	3	2	2	1	3	0	1	4
42	67/2-1	67	2	Affected	Unaffected	M	2	4	2	0	1	0	0	3	4	3	3	2	0	3
43	67/2-2	67	2	Affected	Unaffected	M	2	2	1	0	0	0	2	4	2	0	2		1	2
44	67/2-3	67	2	Affected	Unaffected	F	1	1	1	0	0	0	0	4	2	0	2	0	0	1
45	67/2-4	67	2	Affected	Unaffected	M	2	4	0	2	0	0	3	4	3	0	1	0	1	3
46	67/2-5	67	2	Affected	Unaffected	M	3	2	2	0	0	0		4	3	0		0		2
48	67/2-6	67	2	Affected	Affected	M	4	4	0	2	0	0		4	1	0	3	0		3
49	67/2-7	67	2	Affected	Affected	M	2	3	2	0	0	0		4	3		1	3		4
50	67/2-8	67	2	Affected	Affected	M	1	4	0	3	0	0			1			2	1	3
51	67/2-9	67	2	Affected	Affected	F	3	3	2	2	0	0	1		1		1	0		2
52	67/2-10	67	2	Affected	Affected	M	2	4	0	0	0	0			3		3	1		
57	68/1-1	68	1	Affected	Affected	M	2	2	2	0	0	1	1	3	1	2	1	2		0
58	68/1-2	68	1	Affected	Affected	M	3	3	2	2	0	0	0	2	2			2		0
59	68/1-3	68	1	Affected	Affected	M	3	3	2	0	0	0		2	3	0	1	2	0	3
60	68/1-4	68	1	Affected	Affected	M	3	3	2	2	0	0	1	3	3	1	1	3	0	3
61	68/1-5	68	1	Affected	Affected	M	3	3	2	0	0	0	0	2	2	0	2	1		3
62	68/1-6	68	1	Affected	Affected	M	4	4	2	2	2	1		4	2	0	2	0		3
63	68/1-7	68	1	Affected	Affected	M	3	3	1	2		0	2		3		2		0	4
64	68/1-8	68	1	Affected	Affected	M	3	2	0	2	0	0		1	3	2	3	2	2	4
65	68/1-9	68	1	Affected	Affected	F	3	2	2	3	0	0	1	2	2	1		0		
66	68/1-10	68	1	Affected	Affected	M	2	4	2	3	0	2	1	4	2	0		1		
67	68/3-1	68	3	Unaffected	Unaffected	F	2	3	2	3	0	0	0		3	0	1	0		2
68	68/3-2	68	3	Unaffected	Unaffected	M	0	0	0	2	0	2	0	2	2	0	1	2	0	3
69	68/3-3	68	3	Unaffected	Unaffected	M	2	2	2	0	0	0	0	2	1	0	2	0		2
70	68/3-4	68	3	Unaffected	Unaffected	M	1	1	2	2	0	0	1	1	2	0	3	0	0	3
71	68/3-5	68	3	Unaffected	Unaffected	M	1	3	0	0	0	0	1	3	2	0	2	0	0	4

7.3.4 Main histological findings and differences between clinically affected and unaffected chickens

The major histopathological lesions in both affected and apparently normal chickens were in the CNS and peripheral nerves (brain, sciatic nerve) liver and lungs, and kidneys in addition to lymphoid atrophy of the bursa. The principal histopathological lesions were early lymphomatous foci in the liver and lungs. There were also variable degrees of haemorrhage. The hemorrhagic lesions occurred in the central nervous system and in the visceral organs, mainly lungs, liver and kidneys. Haemorrhage was associated with pneumonia, and interstitial glomerulonephritis. In the CNS, haemorrhage was frequently associated with perivascular cuffing, exudation of proteinaceous fluid in the Virchow spaces, and encephalomalacia. Of all of the severity scores shown in Table 7.5 the only organ for which there was a significant difference in severity score between affected and unaffected chickens was the cerebellum (affected 2.90 ± 0.3, unaffected 1.56 ± 0.04 P = 0.009)

7.3.4.1 Trachea

Histopathological examination on 22 tracheas of 30 chickens of affected and unaffected chickens revealed that 12 of them showed pathological changes ranging from mild mucosal congestion in three cases with engorged blood vessels of the tunica propria. The tunica propria was hypaeremic, oedematous, and showed diapedisis of few erythrocytes (Figure 7.5). In four cases, the trachea had a saw-tooth appearance from desquamation and ulceration of the epithelium, three cases showed squamous metaplasia of the lining epithelium which is normally a pseudostratified ciliated epithelium being replaced by a simple squamous to low cuboidal epithelium, and three cases also exhibited, desquamation of the ciliated lining, hypertrophy of the mucous glands and degeneration of the glandular cells, with only one of them showing infiltration of the glandular lumen with granulocytes and mononuclear cells, but without disarrangement of the epithelium. The remaining tracheas had non-ciliated pseudostratified columnar epithelium. The underlying tunica propria was hyperaemic and slightly infiltrated with lymphocytes.

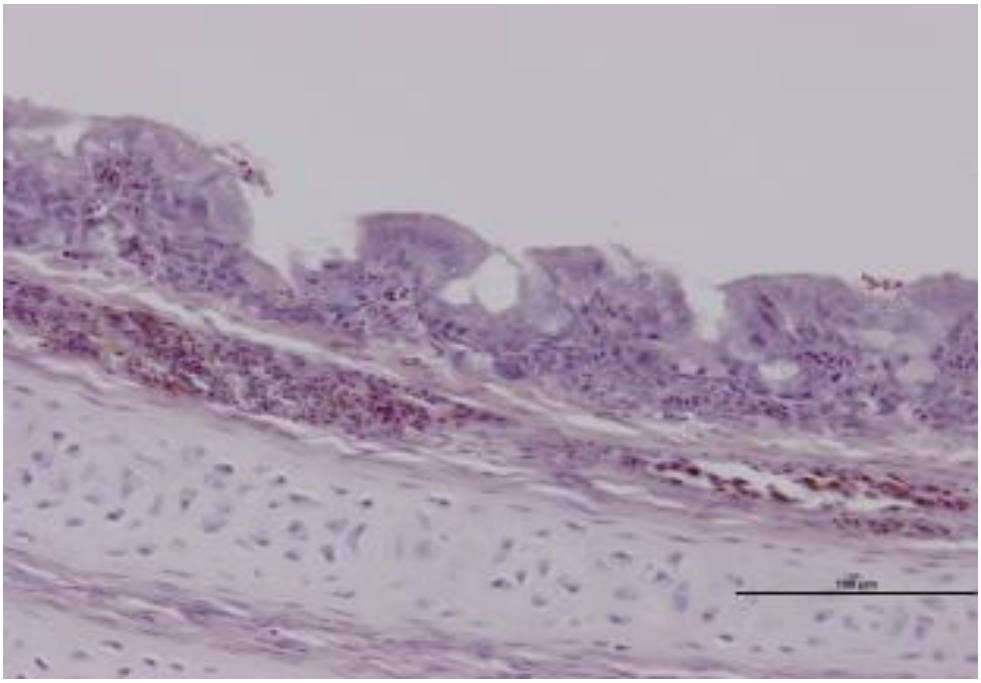

Figure 7.5. Tracheitis in an affected bird (49 days old. Note the saw-tooth appearance of the epithelium. The normal pseudostratified ciliated columnar epithelium was partially lost. The remaining fronds were non-ciliated. The tunica propria was hyperaemic with some diapedesis of erythrocytes and infiltration of lymphocytes. Haematoxylin and eosin staining (H & E), 200X. Bar=100 µm.

7.3.4.2 Lung

The microscopic findings of the lungs are based on examination of sections of 25 of the 30 lungs collected. Inflammation occurred in the majority of lung sections examined with lesions ranging from congestion to exudation of proteinaceous materials and inflammatory cells such as plasma cells, granulocytes and macrophages.

Lungs with mild to severe congestion with diapedesis of blood cells or oedema were noticed in 10 chickens, which were six chickens from ill birds and four from non affected sheds. The dilated blood capillaries allowed the passage of two or three erythrocytes at once. There were variable degrees of proliferation of macrophages and lymphoid cells, which were limited in the epithelial linings of the alveoli or were more intense and filled some alveoli (Figure 7.6).

During the microscopic examination of the lungs, hyaline cartilage nodules were found. More than one nodule was found in the same section. They may responsible for the grittiness found during the sectioning. These nodules were frequently adjacent to an

interlobular septum and faraway from large airways and blood vessels. A mass of ossification was also noticed. The cartilage nodules were either associated with oedema, lung congestion (Figure 7.7), or pneumonitis, where lung tissues were infiltrated by mononuclear cells, consisting of lymphocytes, plasma cells, and granulocytes.

Perivascular oedema was identified in the parabronchial lumen and in the interlobular interstitial septae. Proteinaceous fluid often contained fibrin threads with or without an inflammatory response, consisting of macrophages, plasma cells and granulocytes with or without mild haemorrhage were presented in the air capillaries and interlobular septae (Figure 7.8 and Figure 7.9). In general, haemorrhage occurred in the lungs and it was mainly prominent in the mesobronchi, parabronchial lumen and within the air capillaries and interstitial septae (Figure 7.9).

The most significant lesions in the lungs were the infiltration of lymphoid cells. Two cases showed extensive microscopic foci of pleomorphic lymphoid cells filling some of the alveoli (Figure 7.10). Degenerative lymphoblast cells recognized as large dark cells comparable to what are referred to as MD cells were identified. Another case showed moderate multifocal lymphoid cell infiltration in the bronchial wall, in the capillaries and interstitial septae. Contraction of the smooth muscle of the parabronchi resulted in considerable atresia of the parabronchial lumen. Atria and septum was also lightly infiltrated with the pleomorphic lymphoid cells.

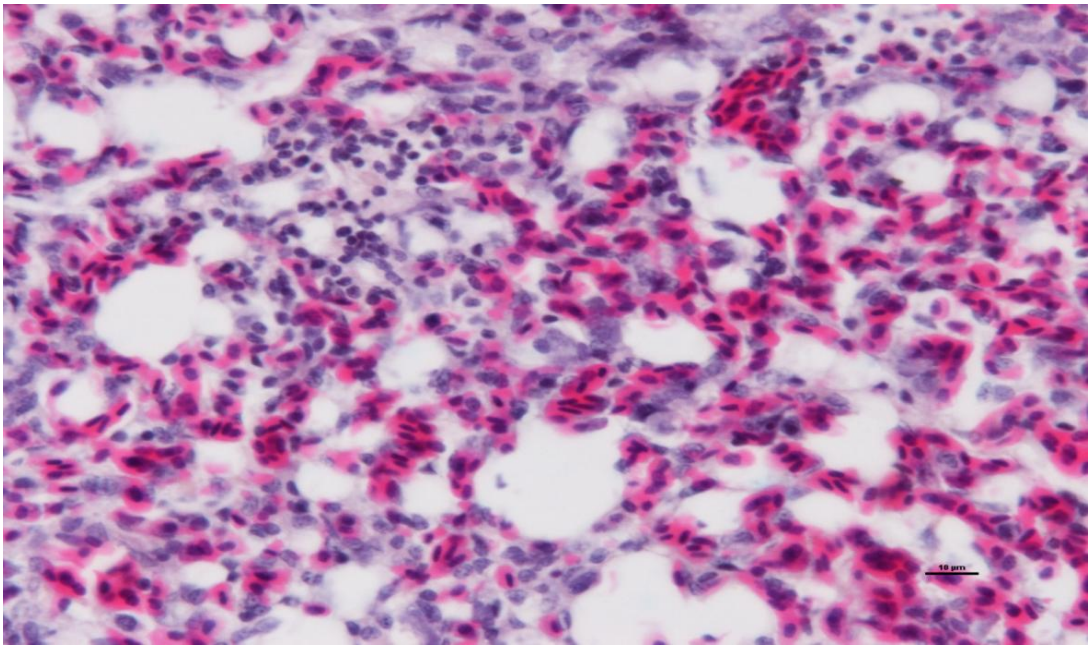

Figure 7.6. Congested lung showing dilated blood capillaries more than twice normal size. Note lymphocyte infiltration into the air capillaries. H & E, 400X. Bar=10 µm.

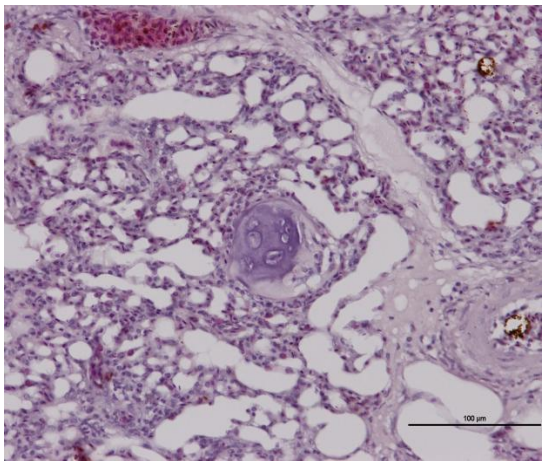

Figure 7.7: Example of a mineralised cartilage nodule in pulmonary tissue. The section also shows inflammatory oedema (pale pink staining fluid) in the septum causing distension of the interstitial space. H & E, 200X. Bar=100 µm.

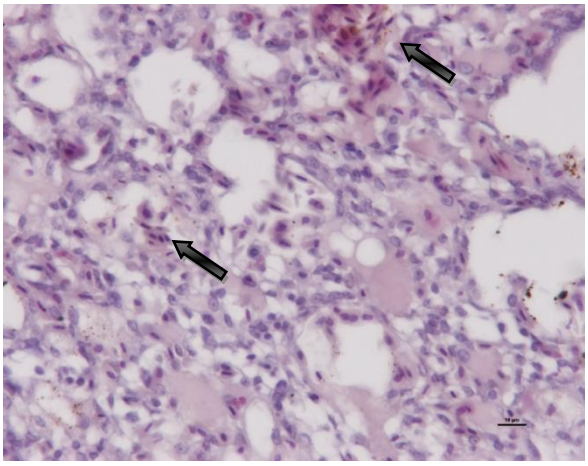

Figure 7.8: Lung with interstitial oedema diapedesis of erythrocytes into the air capillaries (arrows) and proliferation of mononuclear cells. H & E, 400X. Bar=10 µm.

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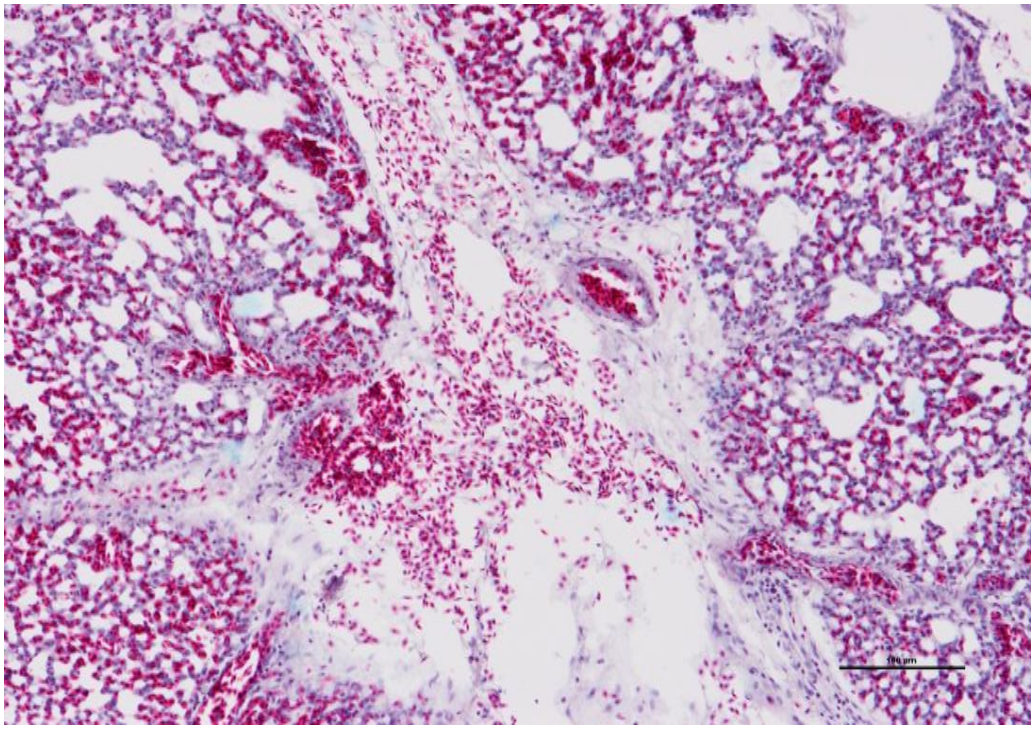

Figure 7.9: Lung from a 32 days old ill chicken showing haemorrhage in the respiratory atria and air capillaries in the adjacent parabrochial lobules which are separated by greatly distended connective tissue of the interlobular interstice septum by inflammatory oedema and extensive haemorrhage. H & E, 200X. Bar=100 µm.

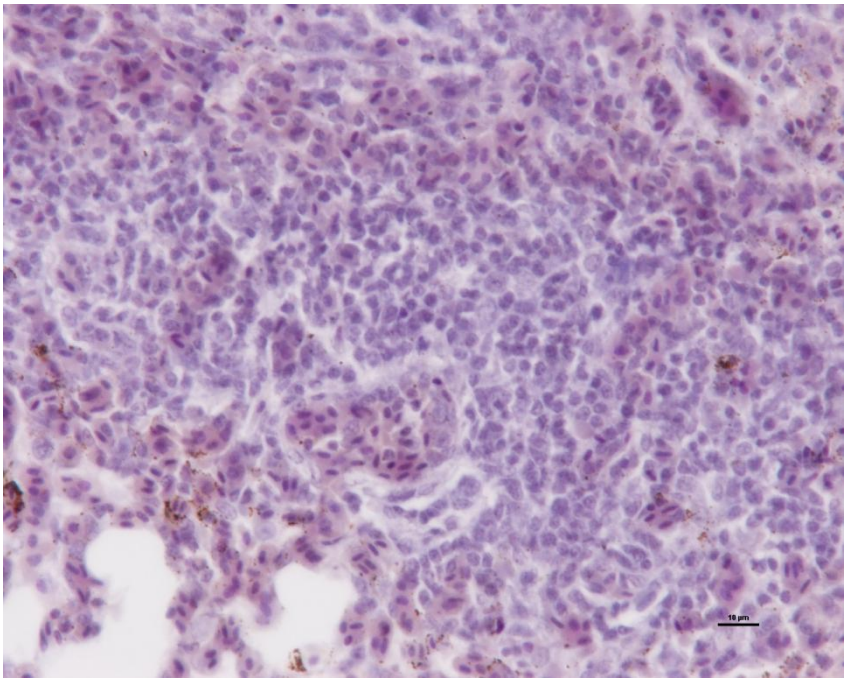

Figure 7.10: Lung from a 49 days old ill chicken showing an early lymphomatous aggregation typical of MD. Pleomorphic lymphoid cells including small, medium and large lymphocytes, lymphoblasts and reticulum cells fill the air capillaries. H & E, 400X. Bar=10 µm.

7.3.4.3 Liver

Liver sections from 30 chickens were examined microscopically. Early lymphomoid nodules were identified in sections of livers taken from six chickens. Two of them were apparently normal. Only one chicken was from an unaffected shed. The lymphomatous nodules consisted of a polymorphic population of atypical lymphoid cells including small medium and large lymphocytes, lymphoblasts and epithelioid cells. The lymphomatous nodules were found in the triad region or in the liver parenchyma, but were more frequent in the perivascular regions. The central vein and blood sinusoids were dilated due to engorgement with blood in 12 livers (Figure 7.11 and Figure 7.12), and two of them had accumulations of blood in the subcapsular region and appeared as "blood lakes." in the liver parenchyma. These blood “lakes” could be few mm in diameters and were easily detected during tissue trimming. These areas remained uncapsulated.

Two types of vacuolation of hepatocytes were detected microscopically. The first type of vacuolation was hydropic swelling or probably due to glycogen accumulation, and was a prominent feature in livers of two chickens. The hepatocyte cells seemed swollen and had small enclosed spaces with unclear outline within the cytoplasm of the cells. The hepatocyte nuclei were centrally located. In contrast, in the second type, the vacuoles

appeared like fat droplets and were detected in six livers. These vacuoles had a distinct border and gave the cytoplasm a foamy appearance with nuclear displacement. The nuclei of the hepatocytes were pushed away to the periphery by coalescent vacuoles, showing a signet cell appearance which is consistent with fat vacuoles. This alteration was diffuse, and not limited to areas around the central vein. Focal accumulation or scattering of granulocytes and plasma cells was present in most of examined sections.

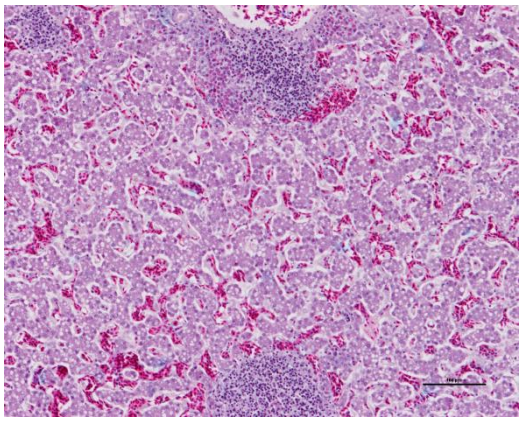

Figure 7.11: Early lymphoid nodules in liver consisting of a polymorphic population of atypical lymphoid cells including small, medium lymphocytes in the perivascular regions. The sinusoids are engorged with blood. The hepatocytes contain fat droplets. H & E, 100X. Bar=100 µm.

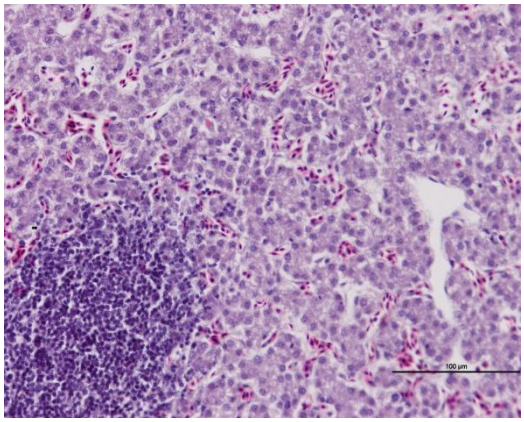

Figure 7.12: A liver section showing a polymorphic population of atypical lymphoid cells (not seen in this magnification) in a lymphoid micronodule. The hepatocytes do not contain fat droplets H & E, 200X. Bar=100 µm.

7.3.4.4 Spleen

A total of 24 spleens was submitted for histopathological examination, and neither gross nor microscopic changes were detected in 15 chickens. Only one spleen exhibited moderate enlargement due to reticuloendothelial cell hyperplasia in the periarterial lymphocytic sheaths while other cases showed milder proliferation and lymphoid depletion.

7.3.4.5 Proventriculus

The proventriculi from 28 chickens were examined histopathologically. Mild to extensive diffuse lymphoid cell infiltration was encountered in 13 cases. Extensive lymphoid cells were scattered in the lamina propria, with or without plasma cells, and granulocytic cells of four cases. A focal aggregation of lymphoid cells in the tunica muscular was noticed in

one chicken. Other case showed heavy infiltration of lymphoid cells in the lamina propria and the glandular epithelium.

7.3.4.6 Bursa of Fabricius

The dominant pathological change observed in the bursa of Fabricius was atrophy of the lymphoid follicles. Out of the 28 bursae, 14 bursae displayed normal histopathological structures, where the plicas were completely filled with follicles and separated by a thin sheet of connective tissue. In the affected bursae, the form and size of follicles varied. Bursae underwent cytolytic changes and ranged from mild (three cases), moderate (six cases) to severe cytolysis of the lymphocytes in the cortex and medulla of the follicles (five cases). Three of the five cases showed depletion of the follicles with hyperplasia of epithelium instead of reduction of plical size. In three cases, the severely atrophied bursae showed infiltration of the interfollicular connective tissue with pleomorphic lymphoid cells. In two cases there was diapedesis of erythrocytes (haemorrhage) in the interfollicular tissue, and one of them had erythrocytes in the lumen. Epithelialisation of the follicles was observed. Bursitis was associated with infiltration of granulocytes and plasma (Figure 7.13 and Figure 7.14).

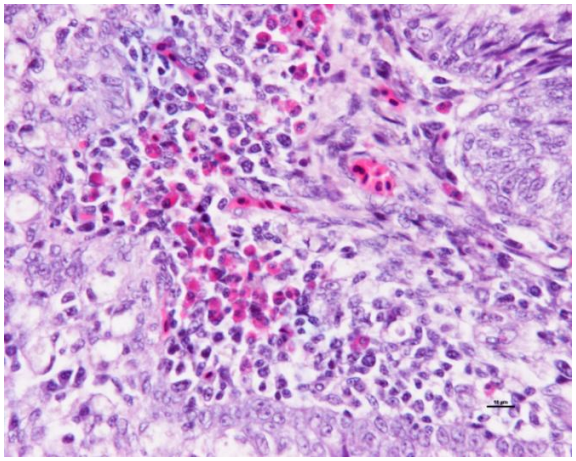

Figure 7.13: Bursitis in a 42-day-old chicken. Note pleomorphic lymphoid cells as well as plasma cells, granulocytes, oedema and diapedesis of a few erythrocytes. H & E stain. X600. Bar=10 µm.

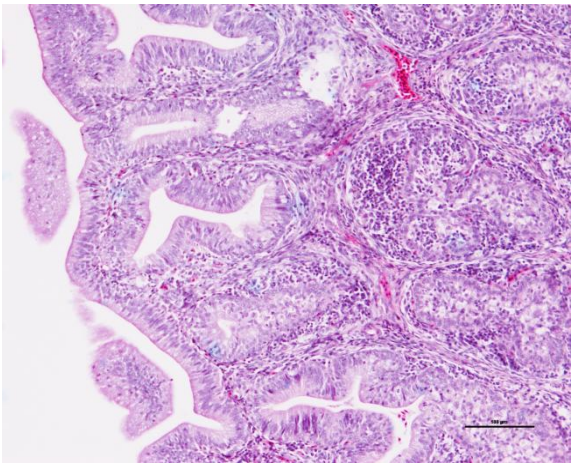

Figure 7.14: Bursa from broiler chicken showing depletion of lymphoid follicles leading to reduction of the cortex to very narrow rim of lymphocytes. Note the intraepithelial cyst formation and infiltration of inflammatory cells in interfollicular stroma, marked epithelialisation of the follicular structure and papillary folding of surface epithelium. H & E stain. X200. Bar=100 µm.

7.3.4.7 Thymus

Gross atrophy of all the lobes of the thymus was observed in four birds at necropsy, whereas in the other birds the thymus was either normal or showed slight atrophy. On histopathological examination, they showed slight lymphoid depletion but they had not lost the demarcation between cortex and medulla.

7.3.4.8 Kidney

A total of 25 kidneys were processed and examined histopathologically. Vascular congestion was encountered in 14 cases while congestion plus haemorrhage was noticed in another four cases while focal interstitial nephritis with granulocytic infiltration was observed in other four cases. In general, kidney lesions consisted of diffuse areas of congestion and haemorrhage was most obvious in the branches of the ureters and in the intratubular spaces (Figure 7.15). Focal or multifocal accumulations of lymphoid cells in the interstitial tissue were detected in three cases (Figure 7.16). Bowman spaces of glomeruli were clear in some instances while in other regions of the same section, they were absent. Observed differences in size of some glomeruli were due to hyperplasia and hypertrophy of the cells of the visceral layer of Bowman's capsule.

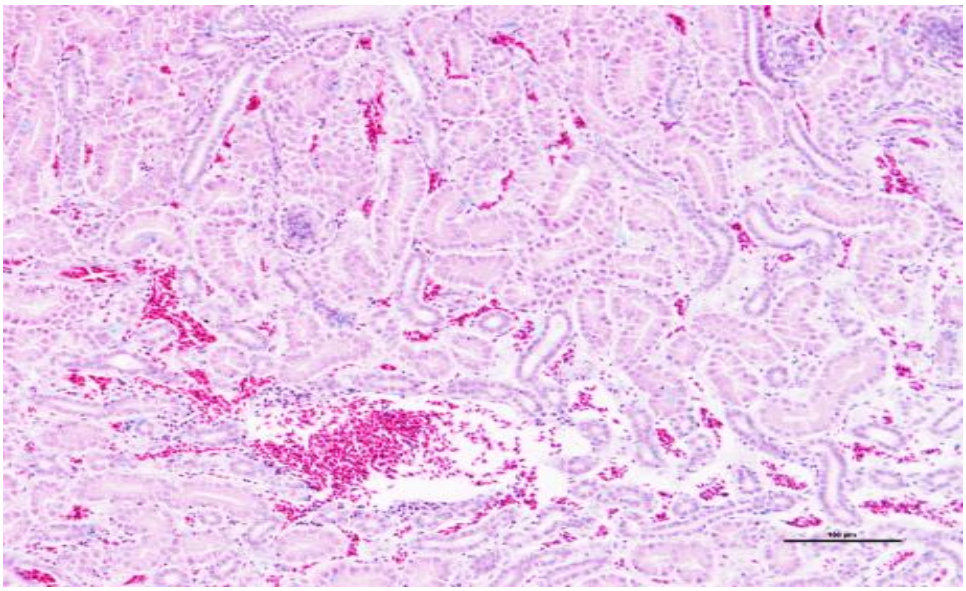

Figure 7.15. Kidney section from an affected chicken showing congested blood vessels and haemorrhage in the intertubular area. H&E, 200X. Bar=100 µm.

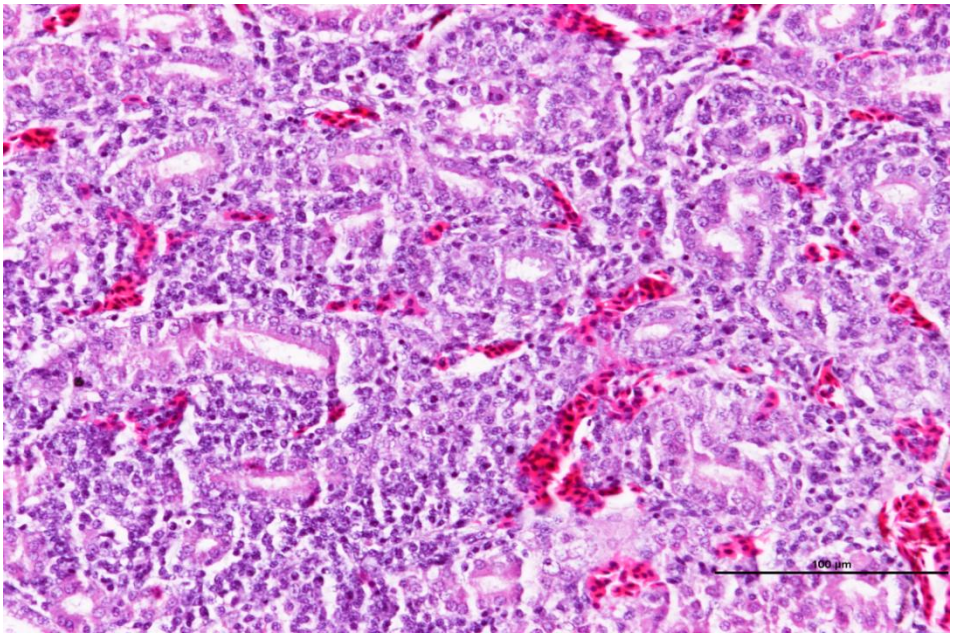

Figure 7.16. Kidney section of an affected chicken showing infiltration of lymphoid cells in the congested interstitial tissue. H&E, 100X. Bar=100 µm.

7.3.4.9 Sciatic Nerve

From the microscopic examination of the sciatic nerve obtained from 30 chickens, two nerves had haemorrhage in the perineurium, while a third sciatic nerve showed accumulation of lymphoid cells in the perineurium. Only two sciatic nerves showed type C lesions, consisting of simple mononuclear cell infiltration without oedema (Figure 7.17).

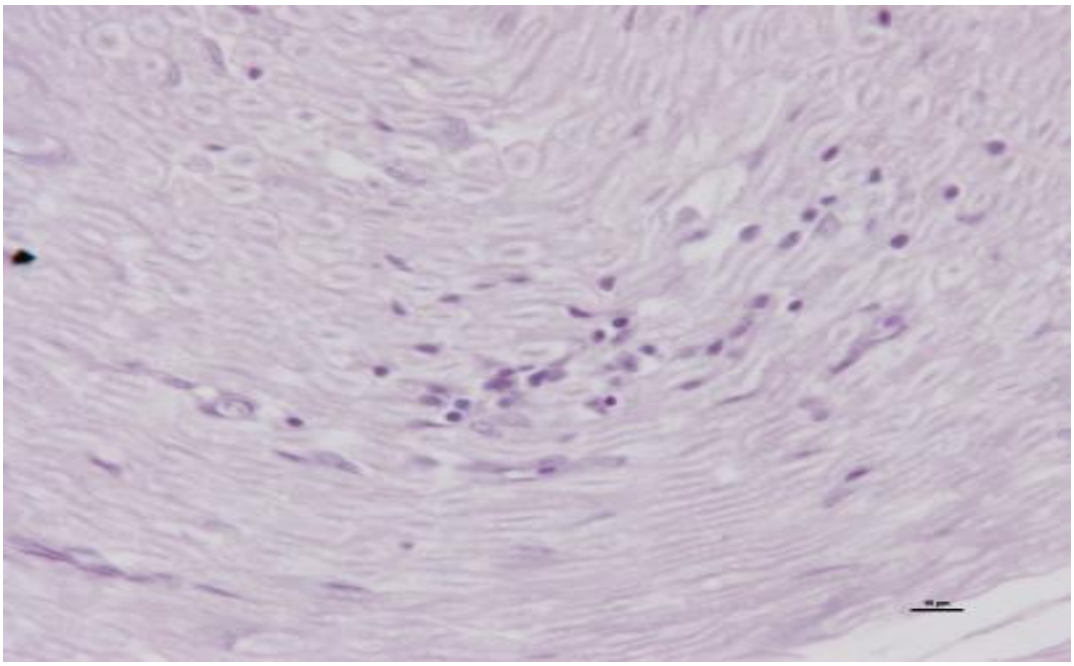

Figure 7.17. Sciatic nerve from an affected chicken. This is an MD type C lesion consisting of scattered lymphoid cells in the sciatic nerve. H & E, 400X. Bar=10 µm.

7.3.4.10 C.N.S

The most significant finding was frequent haemorrhage and malacia. In addition, there was lymphoid perivascular cuffing in the Virchow spaces associated with haemorrhage and exudation of proteinaceous oedema fluid in the Virchow space and brain parenchyma. These changes were noticed in the cerebrum, cerebellum, choroid plexus, and the meninges. The vessels of the meninges, cerebrum and cerebellum were usually distended with blood, and haemorrhages of variable degree were found in the subarachnoid space and in the pia mater. The frequency of haemorrhage was similar in the meninges of both the cerebellum (Figure 7.18) and cerebrum. The incidence of meningeal haemorrhage was nine of the 30 chickens examined. Haemorrhage was found in 11 chickens, eight of them with perivascular haemorrhages in the parenchyma and they were sick. The remaining cases were three chickens had submeningeal haemorrhages from affected sheds but the chickens were apparently healthy. Haemorrhages were commonly seen in the cerebral and cerebellar white matter and it was rarely in the ventricles, medulla oblongata and choroid plexus. The haemorrhages were accompanied by oedema and vacuolization of the white matter. Exudation of proteinaceous fluid was most visible in some Virchow spaces and was usually associated with diapedesis of a high number of erythrocytes Figure 7.19). The incidence of cerebral haemorrhage was more severe than that in the cerebellum. Thromboses were encountered in the cerebrum infrequently. Malacia was observed in 6/30 cases in the cerebrum and 4/30 in the cerebellum.

The areas of malacia stained poorly and their boundaries were more easily detected at low power magnification. The encephalomalacia observed in the cerebellum occurred with or without perivascular bleeding in the white matter. Vacuolation in and around the white matter of the folia was also infrequently encountered. A few Purkinje cells were undergoing neuronal degenerative changes including loss of Nissl granules, being smaller and darker than normal adjacent Purkinje cells, and in some parts Pukinje cells were missing.

PVCs were observed with mononuclear and lymphoid cells (Figure 7.20). PVCs were observed in 8/30 cerebrums whereas PVCs were observed in 4/30 cerebellums. The blood vessels had either 3-4 cell layers or had a compact cuff of mononuclear cells, mainly lymphocytes. Some cases showed PVCs and vascular haemorrhage in the same section. Choroiditis was also encountered with granulocytic and lymphocytic infiltration in one case.

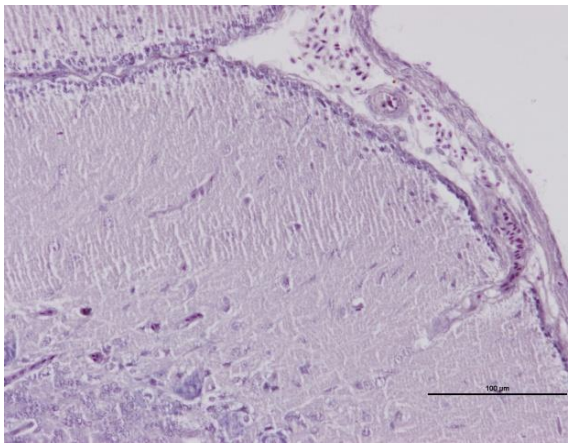

Figure 7.18. Section of cerebellum of an affected chicken showing haemorrhage in the meninges. H & E. 200X. Bar=100 µm.

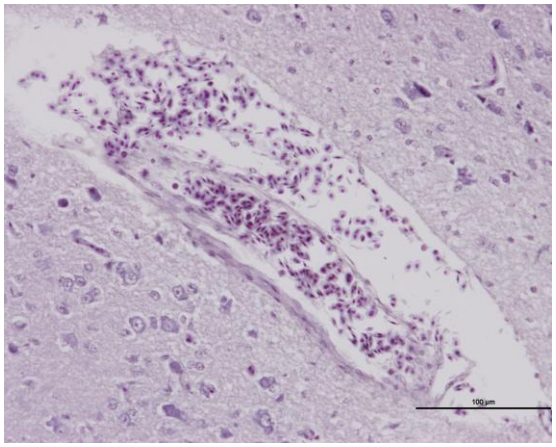

Figure 7.19. Figure 6.17. Section of cerebrum from an affected bird showing erythrocytes in the Virchow-Robin space surrounding blood vessels.

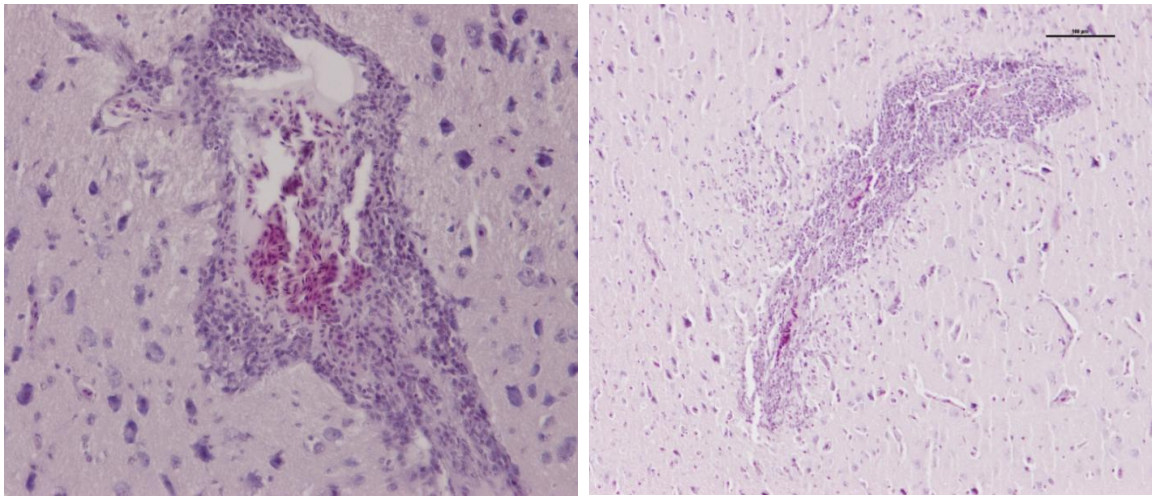

Figure 7.20. Section of cerebrum from an affected bird showing encephalitis. Note the perivascular cell proliferation in the pons region (left) and in a cerebral blood vessel (right). Perivascular accumulations of lymphoid cells fill the Virchow-Robin space. Some lymphoid cells have crossed the glial barrier and extended into the surrounding cerebral parenchyma. H&E 200 X. Bar=100µm.

7.3.5 Virus Detection

7.3.5.1 MDV isolation in cell culture

No distinct typical MDV-1 plaques were visible within the CEF tissue cultures after three passages. Culture material was assayed for MDV-1 using qPCR and found to be negative.

7.3.5.2 Virus detection by qPCR

After DNA extraction and quantification with the use of Bioline Isolate DNA Kits and NanoDrop spectrophotometer, DNA was diluted to a fixed concentration of 5 ng /µl before use as a template for qPCR. Average DNA yields of spleens were between 752.5 and 116.4 ng/µl while average yields of dust samples were between 29.8 and 59.0 ng/µl.

The Log^{10} of viral copy number (VCN)/mg dust detected in DNA extracted from the dust of eight sheds (four farms plus one extra shed from farm 67 (affected 49 days) is shown in Figure 7.22. MDV load in dust increased between days 21 and 49 to high levels (>10^6 VCN per mg dust) (Figure 7.23).

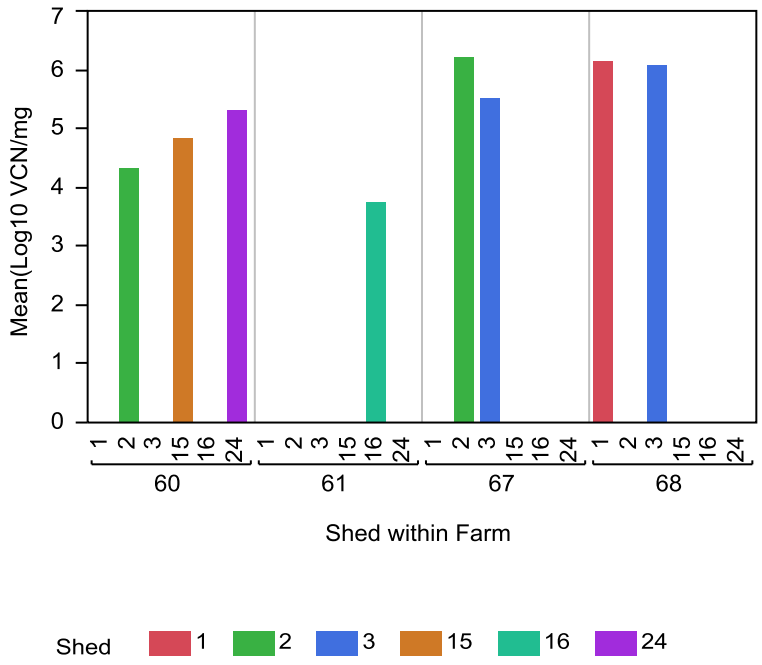

Figure 7.21: Log^{10} MDV viral copy number per mg of shed dust from sheds within farms.

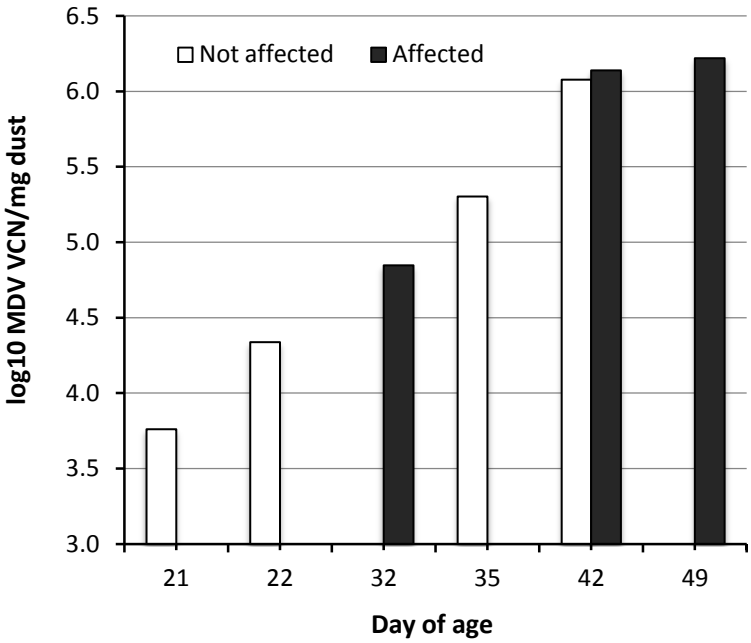

Figure 7.22: Log10 MDV viral copy number per mg of shed dust from sheds containing chickens of different ages, affected and unaffected by the paralysis syndrome.

Two spleen samples were tested for MDV by qPCR. MDV-1 load in spleen [Log^{10} (VCN/$10^{\wedge 6}$ cells)] was 4.65 from Farm 68 (affected 42 days of age) and 5.16 from Farm 67 (affected 49 days of age).

1.1.1 *meq* gene sequencing and alignment

The MDV-1 *meq* gene was successfully amplified from a dust sample in this study. It was from farm 67 (shed 3) and was named 10GRF. It was sequenced and the sequence aligned with the *meq* gene sequence of the MDV-1 isolate Md5 (Tulman *et al.* 2000) (GenBank accession number AF243438). This MDV-1 isolate is classified as vv according to the classification system (Witter 1997). The sequence was also compared with the *meq* gene of the vaccinal MDV-1 isolate Rispens/CVI988 vaccine strain (Spatz *et al.* 2007) and the *meq* gene sequence of two Australian vv MDVs isolates, 02LAR and the Australian reference strain MPF57 (Renz *et al.* 2012). The results showed that the overall length of the 10GRF *meq* gene coding region from the Australian outbreak was 1197 bp, exactly the same as the length of the *meq* gene sequence of MPF57 and

02LAR (Australian vv MDVs). The length of the *meq* gene sequences from Md5 and CVI988 reference isolates are 1020 bp and 1200 bp, respectively.

The 10GRF *meq* gene had an insertion of 177 bp in the proline-rich region relative to Md5 (Figure 7.24), and this was exactly the same as that of the Australian reference strain of MDV-1 (MPF57).

In the 10GRF *meq* gene sequence, there were specific single nucleotide polymorphisms (SNPs). The number of polymorphisms present relative to Md5 was four and relative to others Australian isolate was three. At positions 229 and 230 the sequence is GG whereas it is AA, GA and GC in the sequences of Md5, CVI988 and the previously reported Australian isolates respectively. At position 521 there is an A in the 10GRF *meq* gene sequence, whereas in the other isolates there is a C. At position of 938, the 10GRF *meq* gene has C while the other isolates have T (Figure 7.24).

The amino acid alignment of 10GRF *meq* gene of this study (Figure 7.25) showed that it encodes 398 aa similar to the Australian isolates whereas the Md5 *meq* gene and CVI988 encode 339 and 399 aa respectively. The insertion of 59 aa is identical with the insertion that is present in the Australian isolates while CVI988 has an additional proline residue. The overall proline content in 10GRF Meq was (22.6%) more than Md5 (21.3%), less than Rispens CVI988 (23.3%) and equal to MPF57 and 02LAR (22.6%). The number of repeat sequences of four prolines (PPPP repeats) in the *meq* gene was five as is the case with MPF57 and 02LAR while Md5 and Rispens/CVI988 contain four and eight PPPP repeats respectively. The 10GRF Meq has asparagine instead of the threonine at aa 174 in the other isolates. Like MPF57, 10GRF Meq has alanine at position aa 80 and glutamine as aa 186, while 02LAR has aspartic acid and histidine at those positions. The 10GRF Meq differs from the others in being the only Meq that has threonine at aa 313, whereas other isolates have isoleucine.

```
10GRF           ATGTCTCAGGAGCCAGAGCCGGGCGCTATGCCCTACAGTCCCGCTGACGATCCGTCCCCC 60
MPF57           ATGTCTCAGGAGCCAGAGCCGGGCGCTATGCCCTACAGTCCCGCTGACGATCCGTCCCCC 60
02LAR           ATGTCTCAGGAGCCAGAGCCGGGCGCTATGCCCTACAGTCCCGCTGACGATCCGTCCCCC 60
RISPENS         ATGTCTCAGGAGCCAGAGCCGGGCGCTATGCCCTACAGTCCCGCTGACGATCCGTCCCCC 60
Md5             ATGTCTCAGGAGCCAGAGCCGGGCGCTATGCCCTACAGTCCCGCTGACGATCCGTCCCCC 60
                ************************************************************

10GRF           CTCGATCTTTCTCTCGGGTCGACTTCGAGACGGAAAAAAAGGAAAAGTCACGACATCCCC 120
MPF57           CTCGATCTTTCTCTCGGGTCGACTTCGAGACGGAAAAAAAGGAAAAGTCACGACATCCCC 120
02LAR           CTCGATCTTTCTCTCGGGTCGACTTCGAGACGGAAAAAAAGGAAAAGTCACGACATCCCC 120
RISPENS         CTCGATCTTTCTCTCGGGTCGACTTCGAGACGGAAAAAAAGGAAAAGTCACGACATCCCC 120
Md5             CTCGATCTTTCTCTCGGGTCGACTTCGAGACGGAAAAAAAGGAAAAGTCACGACATCCCC 120
                ************************************************************

10GRF           AACAGCCCCTCCAAACACCCCTTCCCTGACGGCCTATCTGAGGAGGAGAAACAGAAGCTG 180
MPF57           AACAGCCCCTCCAAACACCCCTTCCCTGACGGCCTATCTGAGGAGGAGAAACAGAAGCTG 180
02LAR           AACAGCCCCTCCAAACACCCCTTCCCTGACGGCCTATCTGAGGAGGAGAAACAGAAGCTG 180
RISPENS         AACAGCCCCTCCAAACACCCCTTCCCTGACGGCCTATCTGAGGAGGAGAAACAGAAGCTG 180
Md5             AACAGCCCCTCCAAACACCCCTTCCCTGACGGCCTATCTGAGGAGGAGAAACAGAAGCTG 180
                ************************************************************

10GRF           GAAAGGAGGAGAAAAAGGAATCGTGACGCCTCTCGGAGAAGACGCAGGGGGCAGACGGCC 240
MPF57           GAAAGGAGGAGAAAAAGGAATCGTGACGCCTCTCGGAGAAGACGCAGGGGGCAGACGGCC 240
02LAR           GAAAGGAGGAGAAAAAGGAATCGTGACGCCTCTCGGAGAAGACGCAGGGGGCAGACGGAC 240
RISPENS         GAAAGGAGGAGAAAAAGGAATCGTGACGCCTCTCGGAGAAGACGCAGGGGGCAGACGGAC 240
Md5             GAAAGGAGGAGAAAAAGGAATCGTGACGCCGCTCGGAGAAGACGCAGGGAAGCAGACGGAC 240
                *************** ************** ***************** ******** *

10GRF           TATGTAGACAAACTCCATGAAGCATGTGAAGAGCTGCAGAGGGCCAATGAACACCTACGT 300
MPF57           TATGTAGACAAACTCCATGAAGCATGTGAAGAGCTGCAGAGGGCCAATGAACACCTACGT 300
02LAR           TATGTAGACAAACTCCATGAAGCATGTGAAGAGCTGCAGAGGGCCAATGAACACCTACGT 300
RISPENS         TATGTAGACAAACTCCATGAAGCATGTGAAGAGCTGCAGAGGGCCAATGAACACCTACGT 300
Md5             TATGTAGACAAACTCCATGAAGCATGTGAAGAGCTGCAGAGGGCCAATGAACACCTACGT 300
                ************************************************************

10GRF           AAGGAAATTCGAGATCTAAGGACTGAGTGCACGTCCCTGCGTGCACAGTTGGCTTGTCAT 360
MPF57           AAGGAAATTCGAGATCTAAGGACTGAGTGCACGTCCCTGCGTGCACAGTTGGCTTGTCAT 360
02LAR           AAGGAAATTCGAGATCTAAGGACTGAGTGCACGTCCCTGCGTGCACAGTTGGCTTGTCAT 360
RISPENS         AAGGAAATTCGAGATCTAAGGACTGAGTGCACGTCCCTGCGTGTACAGTTGGCTTGTCAT 360
Md5             AAGGAAATTCGAGATCTAAGGACTGAGTGCACGTCCCTGCGTGTACAGTTGGCTTGTCAT 360
                ****************************************** ***************

10GRF           GAGCCAGTTTGCCCTATGGCGGTACCCCTAACGGTGACCCTTGGACTGCTTACCACCCCG 420
MPF57           GAGCCAGTTTGCCCTATGGCGGTACCCCTAACGGTGACCCTTGGACTGCTTACCACCCCG 420
02LAR           GAGCCAGTTTGCCCTATGGCGGTACCCCTAACGGTGACCCTTGGACTGCTTACCACCCCG 420
RISPENS         GAGCCAGTTTGCCCTATGGCGGTACCCCTAACGGTGACCCTTGGACTGCTTACCACCCCG 420
Md5             GAGCCAGTTTGCCCTATGGCGGTACCCCTAACGGTGACCCTTGGACTGCTTACCACCCCG 420
                ************************************************************

10GRF           CACGATCCCGTTCCTGAACCTCCCATTTGCACTCCTCCACCTCCCTCACCGGATGAACCT 480
MPF57           CACGATCCCGTTCCTGAACCTCCCATTTGCACTCCTCCACCTCCCTCACCGGATGAACCT 480
02LAR           CACGATCCCGTTCCTGAACCTCCCATTTGCACTCCTCCACCTCCCTCACCGGATGAACCT 480
RISPENS         CACGATCCCGTTCCTGAACCTCCCATTTGCACTCCTCCACCTCCCTCACCGGATGAACCT 480
Md5             CACGATCCCGTTCCTGAACCTCCCATTTGCACTCCTCCACCTCCCTCACCGGATGAACCT 480
                ************************************************************

10GRF           AACGCTCCACATTGCTCCGGTTCCCAACCTCCTATCTGTAACCCCCCCTCCTCCCGATACG 540
MPF57           AACGCTCCACATTGCTCCGGTTCCCAACCTCCTATCTGTACCCCCCCTCCTCCCGATACG 540
02LAR           AACGCTCCACATTGCTCCGGTTCCCAACCTCCTATCTGTACCCCCCCTCCTCCCGATACG 540
RISPENS         AACGCTCCACATTGCTCCGGTTCCCAACCTCCTATCTGTACCCCCCCTCCTCCCGATACG 540
Md5             AACGCTCCACATTGCTCCGGTTCCCAACCTCCTATCTGTACCCCCCCTCCTCCCGATACG 540
                ****************************************** ******************

10GRF           GAGGAACTTTGCGCCCAGCTCTGCTCGACCCCACC---TCCCATCTCTACTCCCCATATT 597
MPF57           GAGGAACTTTGCGCCCAGCTCTGCTCGACCCCACC---TCCCATCTCTACTCCCCATATT 597
02LAR           GAGGAACTTTGCGCCCATCTCTGCTCGACCCCACC---TCCCATCTCTACTCCCCATATT 597
RISPENS         GAGGAACTTTGCGCCCAGCTCTGCTCGACCCCACCACCTCCCATCTCTACTCCCCATATT 600
Md5             GAGGAACTTTGCGCCCAGCTCTGCTCGACCCCACC------------------------- 575
                ***************** *****************

10GRF           ATCTACGCTCCGGGGGCCTTCCCCCCTCCAACCTCCTATCTGTACCCCCGCTCCTCCCGAT 657
MPF57           ATCTACGCTCCGGGGGCCTTCCCCCCTCCAACCTCCTATCTGTACCCCCGCTCCTCCCGAT 657
02LAR           ATCTACGCTCCGGGGGCCTTCCCCCCTCCAACCTCCTATCTGTACCCCCGCTCCTCCCGAT 657
RISPENS         ATCTACGCTCCGGGGGCCTTCCCCCCTCCAACCTCCTATCTGTACCCCCTCTCCTCCCGAT 660
Md5             ------------------------------------------------------------
```

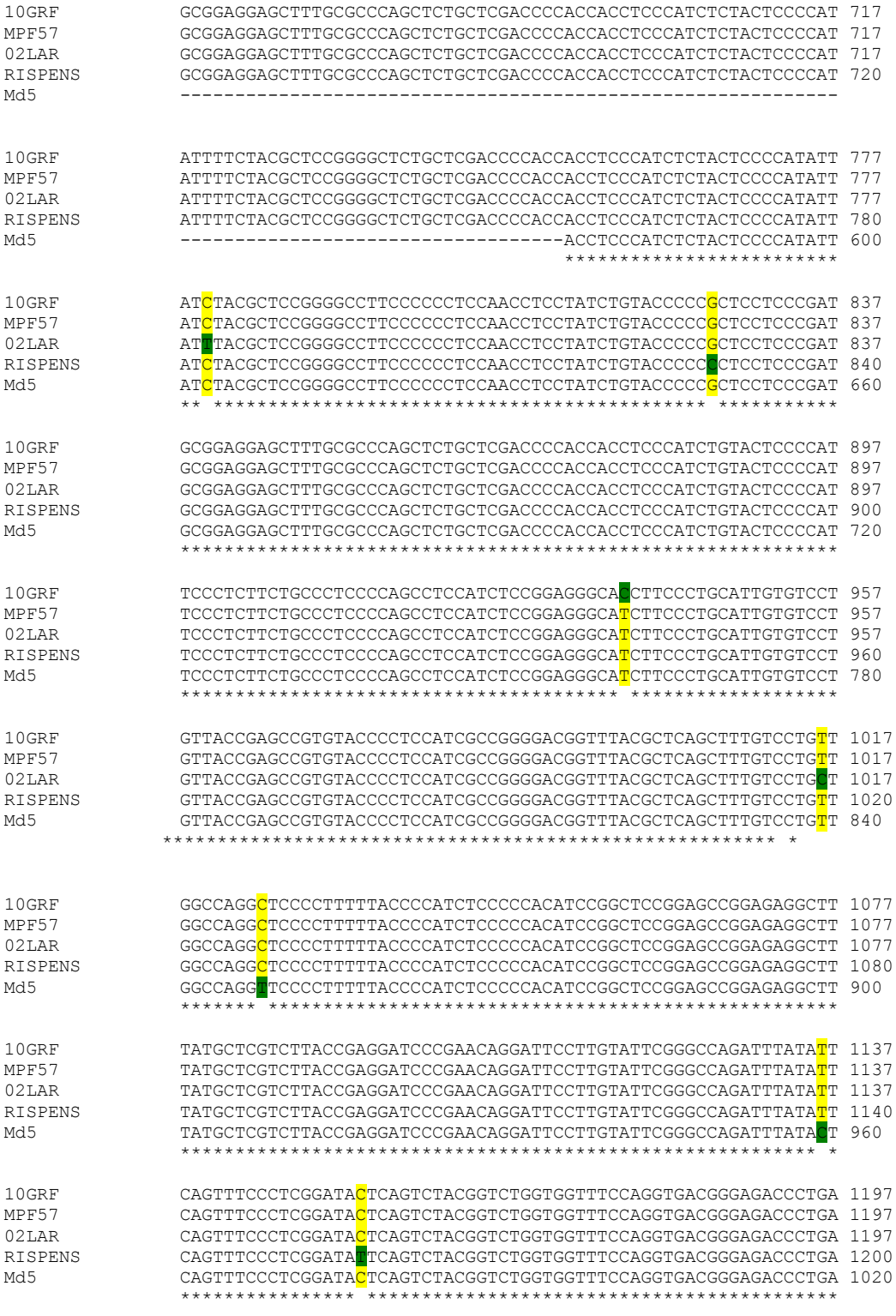

Figure 7.23: Alignment of the sequence of the 10GRF *meq* gene coding region with *meq* genes of the USA MDV isolate Md5, Netherlands vaccine strain Rispens CVI988 and Australian isolates MPF57 and 02LAR. The 177 bp insertion in the 10GRF *meq* gene is identical to the insertion in MPF57 and 02LAR. Missing nucleotides are marked by dashes, SNP's are indicated by shading and asterisks indicate identical nucleotides.

```
10GRF           MSQEPEPGAMPYSPADDPSPLDLSLGSTSRRKKRKSHDIPNSPSKHPFPDGLSEEEKQKL 60
MPF57           MSQEPEPGAMPYSPADDPSPLDLSLGSTSRRKKRKSHDIPNSPSKHPFPDGLSEEEKQKL 60
02LAR           MSQEPEPGAMPYSPADDPSPLDLSLGSTSRRKKRKSHDIPNSPSKHPFPDGLSEEEKQKL 60
Rispens         MSQEPEPGAMPYSPADDPSPLDLSLGSTSRRKKRKSHDIPNSPSKHPFPDGLSEEEKQKL 60
Md5             MSQEPEPGAMPYSPADDPSPLDLSLGSTSRRKKRKSHDIPNSPSKHPFPDGLSEEEKQKL 60
                ************************************************************

10GRF           ERRRKRNRDASRRRRRGQTAYVDKLHEACEELQRANEHLRKEIRDLRTECTSLRAQLACH 120
MPF57           ERRRKRNRDASRRRRRAQTAYVDKLHEACEELQRANEHLRKEIRDLRTECTSLRAQLACH 120
02LAR           ERRRKRNRDASRRRRRAQTDYVDKLHEACEELQRANEHLRKEIRDLRTECTSLRAQLACH 120
Rispens         ERRRKRNRDASRRRRRPQTDYVDKLHEACEELQRANEHLRKEIRDLRTECTSLRVQLACH 120
Md5             ERRRKRNRDAARRRRRKQTDYVDKLHEACEELQRANEHLRKEIRDLRTECTSLRVQLACH 120
                ***** *****:***** ** **********************************.*****

10GRF           EPVCPMAVPLTVTLGLLTTPHDPVPEPPICTPPPPSPDEPNAPHCSGSQPPICNPPPPDT 180
MPF57           EPVCPMAVPLTVTLGLLTTPHDPVPEPPICTPPPPSPDEPNAPHCSGSQPPICTPPPPDT 180
02LAR           EPVCPMAVPLTVTLGLLTTPHDPVPEPPICTPPPPSPDEPNAPHCSGSQPPICTPPPPDT 180
Rispens         EPVCPMAVPLTVTLGLLTTPHDPVPEPPICTPPPPSPDEPNAPHCSGSQPPICTPPPPDT 180
Md5             EPVCPMAVPLTVTLGLLTTPHDPVPEPPICTPPPPSPDEPNAPHCSGSQPPICTPPPPDT 180
                ************************************************.******

10GRF           EELCAQLCSTPPP-ISTPHIIYAPGPSPLQPPICTPAPPDAEELCAQLCSTPPPPISTPH 239
MPF57           EELCAQLCSTPPP-ISTPHIIYAPGPSPLQPPICTPAPPDAEELCAQLCSTPPPPISTPH 239
02LAR           EELCAMLCSTPPP-ISTPHIIYAPGPSPLQPPICTPAPPDAEELCAQLCSTPPPPISTPH 239
Rispens         EELCAQLCSTPPPPISTPHIIYAPGPSPLQPPICTPFPPDAEELCAQLCSTPPPPISTPH 240
Md5             EELCAQLCSTPPPP------------------------------------------------ 194
                *****:*******

10GRF           IFYAPGLCSTPPPPISTPHIIYAPGPSPLQPPICTPAPPDAEELCAQLCSTPPPPICTPH 299
MPF57           IFYAPGLCSTPPPPISTPHIIYAPGPSPLQPPICTPAPPDAEELCAQLCSTPPPPICTPH 299
02LAR           IFYAPGLCSTPPPPISTPHIIYAPGPSPLQPPICTPAPPDAEELCAQLCSTPPPPICTPH 299
Rispens         IFYAPGLCSTPPPPISTPHIIYAPGPSPLQPPICTPPPPDAEELCAQLCSTPPPPICTPH 300
Md5             --------------ISTPHIIYAPGPSPLQPPICTPAPPDAEELCAQLCSTPPPPICTPH 240
                              **********************.**********************

10GRF           SLFCPPQPPSPEGTFPALCPVTEPCTPPSPGTVYAQLCPVGQAPLFTPSPPHPAPEPERL 359
MPF57           SLFCPPQPPSPEGIFPALCPVTEPCTPPSPGTVYAQLCPVGQAPLFTPSPPHPAPEPERL 359
02LAR           SLFCPPQPPSPEGIFPALCPVTEPCTPPSPGTVYAQLCPAGQAPLFTPSPPHPAPEPERL 359
Rispens         SLFCPPQPPSPEGIFPALCPVTEPCTPPSPGTVYAQLCPVGQAPLFTPSPPHPAPEPERL 360
Md5             SLFCPPQPPSPEGIFPALCPVTEPCTPPSPGTVYAQLCPVGQVPLFTPSPPHPAPEPERL 300
                ************* ************************.**.*****************

10GRF           YARLTEDPEQDSLYSGQIYIQFPSDTQSTVWWFPGDGRP 398
MPF57           YARLTEDPEQDSLYSGQIYIQFPSDTQSTVWWFPGDGRP 398
02LAR           YARLTEDPEQDSLYSGQIYIQFPSDTQSTVWWFPGDGRP 398
Rispens         YARLTEDPEQDSLYSGQIYIQFPSDKQSTVWWFPGDGRP 399
Md5             YARLTEDPEQDSLYSGQIYTQFPSDTQSTVWWFPGDGRP 339
                ****************** ***** *************
```

Figure 7.24. Deduced aa sequences of the Meq protein encoded by the *meq* gene of the same isolates as shown in Figure 6.21. Identical aa are denoted by an asterisk, dashes indicate missing aa, green or yellow shading indicates a polymorphic aa and grey shading indicates 4-proline repeats.

7.4 Discussion

A field study was conducted into a natural outbreak of suspected Marek's disease in a commercial poultry complex containing about 1.6 million Ross broiler chickens in NSW, Australia. The existing information on epidemiological, clinical and pathological features in chickens contained features both consistent and not consistent with Marek's disease as typically observed in Australia. The field study did not resolve the issue of causation of

the paralysis syndrome as it also produced findings supportive and non-supportive of MDV as the main cause of the syndrome. These are summarised in Table 7.6 and discussed below in more detail.

Table 7.6: Summary of outbreak features supportive and not supportive of MDV as the necessary causal factor.

Area	Supportive of MDV causation	Not supportive of MDV causation
Epidemiology	No MD vaccination	No history of problems with MD - hence no MD vaccination
Clinical signs	Paralysis is typical of classical MD Lack of long term response to antibiotic treatment	Classical MD rarely seen in Australia and no history of occurrence in broilers. Very acute course. Clinical syndrome is unique – not reported for MD previously anywhere in the world.
Gross pathology	Nil	Lack of lymphomas (Acute MD). Lack of marked bursal and thymic atrophy Presence of haemorrhage
Histopathology	CNS, PNS, liver, lung kidney and bursal lesions consistent with MD	Lack of lymphomas Lack of clear differences in pathology between affected and unaffected chickens
Serology	Evidence of active MDV infection (titres rising with time)	No difference in MDV titre between affected and unaffected chickens or sheds.
Microbiology	High levels of MDV in routine dust samples collected for monitoring, with load increasing with bird age	No difference in MDV load in dust between affected and unaffected sheds.

The clinical signs observed in this study included neurological signs; paresis progressing to paralysis and recumbency, coma and death within 24 h. The morbidity rate was around 25% in affected sheds and the case fatality rate approached 100%. The disease started in birds of 32 and 42 d old, but later it was observed in younger birds of 27 d old. The patterns of morbidity and mortality remained unchanged in spite of the use of antibiotics by company veterinarians. The clinical signs were not consistent with typical

Marek's disease as reported in Australia (Cumming *et al.* 1998; Groves 1995; Walkden-Brown *et al.* 2005). Since the mid 1980s the predominant form of MD in Australia has been acute MD with lymphoma formation. In broiler chickens MD generally manifests either as immunosuppression with higher levels of intercurrent disease, or as acute MD with lymphoma appearance late in the batch days. Purely paralytic syndromes without lymphomas have been reported (Renz *et al.* 2012; Walkden-Brown *et al.* 2005) but these occur during the first 20 days of life, unlike the present field situation, and have only been reported in SPF chickens free of maternal antibody against MDV. The clinical picture of late paralysis without MD tumours in the present outbreak would therefore be a unique form of MD if the causative agent was MDV.

The anti-MDV titres in unvaccined chickens clearly revealed the presence of an active infection process. Chickens at 21 d old had baseline levels of anti-MDV antibody consistent with decline of maternally transferred antibody (Chapter 5). However there was a marked increase in titre between days 22 and 49 indicating the presence of an active immune response to infection. As the chickens were not vaccinated against MD, this is presumably due to field challenge. However there were no differences in titre between affected and unaffected chickens or sheds of the same age (limited data) which tends not to support the proposition that MDV is the causative agent of the paralytic syndrome.

The serological findings were supported by the measurement of MDV genome copy number in dust samples. QPCR of dust samples collected revealed high levels of serotype-1 MDV particularly during the later stages of the broiler lifespan. Viral load on a shed basis increased by 2.5 logs (from 3.76 to 6.22) between days 21 and 49, again indicating active replication and shedding of MDV. However, as for the serological results, there was no difference between the MDV load in dust between sheds affected and unaffected by the syndrome, casting doubt on the role of MDV in its causation. The late batch $\log_{10}$ (<6) values are higher than the values of 4-5 for end of batch dust reported from detailed studies on two unvaccinated broiler farms with no clinical or pathological signs of MD (Walkden-Brown *et al.* 2013a), but are about one log lower than late batch levels (>7) reported in isolator dust from challenge experiments in which MD was induced (Islam and Walkden-Brown 2007; Walkden-Brown *et al.* 2013b).

Serotype-1 MDV was not able to be cultured despite spleen samples being positive for MDV by qPCR, This is suggestive of a failure of isolation and growth in cultured cells rather than an absence of MDV. Isolation of field virus on CEF is has been reported to be

less sensitive than on CKC (Schat, 2005), and this could be one of the possible causes of the negative result. However many Australian MDVs have been isolated and amplified in CEK (Zerbes *et al.* 1994 Delaney *et al.*, 1995) or CEF (Renz *et al.* 2012, Walkden-Brown et al. 2013) when they could not be grown in CKC previously.

Gross and microscopic lesions in the CNS, sciatic nerve, visceral organs and primary lymphoid organs would support a diagnosis of MD. The pathological findings did not include gross visceral lymphomas or enlargement of the peripheral nerves. However, histopathological examinations provided confirmative diagnostic lesions of MD in some chickens. These included gross and microscopic lesions indicative of early stages of MD in visceral organs, sciatic nerves and the bursa of Fabricius. Early micro-foci of lymphomas were encountered in lungs, kidney and livers. The pleomorphic quality of the lymphomatous lesions and lymphoid cell infiltration of visceral organs is in agreement with previous studies on MD (Akiyama and Kato 1974; Powell *et al.* 1975; Schat *et al.* 1991). In the peripheral nervous system infiltration of the sciatic nerve in three chickens with small numbers of lymphoid cells (type C lesion) is also strongly supportive of MD. With MD, the type C lesion in the peripheral nerves consists of a very light cellular infiltration in the peripheral nerves and the lesion has a more inflammatory appearance of polyneuritis (Marek 1907; Payne and Biggs 1967). The lesions in the bursa were also indicative of early MD. The principal lesion was severe atrophy of the bursa due to necrosis of lymphoid cells. Complete microscopic absence of lymphoid follicles and aplasia of epithelial tissue of the plica was detected in many instances. Eight chickens had necrosis of the lymphoid follicles with cyst formation. The cysts either contained cellular debris or oedema fluid. Cysts resulting from the degenerative change also underwent epithelialisation in three cases. The lining epithelium showed corrugation and metaplasia. The bursal damage induced by MD in this study is similar to that reported by others as typical of MD infection infection (Jakowski *et al.* 1969; Purchase 1970). Bursal haemorrhage in two cases in the existing study also provides more evidence of the severity of MDV-1 caused in this spontaneous outbreak. Randall and Reece (1996) postulated that the bursal bleeding is an indication of the virus's virulence and it is not a specific lesion. Gumboro or infectious bursal disease virus (IBDV) is also capable of causing atrophy of the bursa. However, there are differences in the histolopathological lesions of IBDV and MDV-1. Invagination of the bursa's columnar epithelium lining the interior surface and reticulum cell proliferation in the interfollicular spaces are characteristic of the histological response to MDV-1 infection. These changes were in agreement with that reported by Jakowski *et al*. (1969).

Taken together the histopathological lesions in the visceral organs, sciatic nerve and bursa are indicative of early MD but are not consistent with the severity of clinical syndrome observed. The degeneration of the bursa and thymus is likely to lead to an immunosuppression (Islam *et al.* 2002) which may explain the occurrence of secondary infections that may be responsible for extensive haemorrhagic lesions observed in the internal organs and the severity of the clinical signs.

Lesions in other tissues were less clearly diagnostic for MD. In the CNS PVC, oedema, haemorrhage and ischemic malacia of the brain were observed, suggestive of non-suppurative encephalitis. Areas of malacia were noted in 4/30 sections of cerebellum, and 6/30 sections of cerebrum. Lymphocytic PVC, thrombi or haemorrhage were obvious in brains with malacia. According to Jubb and Huxtable (1993), infections of the CNS with viruses could be associated with malacic lesions due to indirect effects such as ischaemia or anoxia. The vv strains of MDV have been found to induce vasculitis and oedema of the brain (Gimeno *et al.* 1999; Swayne *et al.* 1988; 1989b; Swayne *et al.* 1989d; Witter *et al.* 1999), and marked malacic foci in the cerebellum and cerebrum (Cho *et al.* 1998). However these lesions have been reported only under experimental conditions in SPF chickens free of maternal antibody directed against MDV, and tend to be observed at younger ages (<20 days) than chickens affected in the present outbreak. Induction of malacic foci in the CNS in MDV-1 infection may be attributed to haemorrhage in the brain and visceral organs, particularly the lung, leading to ischaemia and hypoxia and in turn malacia in the brain, as the brain is the most sensitive organ to ischaemia and hypoxia. The choroid plexus was also infiltrated with lymphocytes. Perivascular cuffing in the CNS has been reported previously with lesions due to MDV-1 (Gimeno *et al.* 1999; Jakowski *et al.* 1969; Pappenheimer *et al.* 1926). Excessive numbers of mononuclear cells in the choroid plexus following MDV infection were seen by Sevoian and Chamberlain (1964). The Virchow space and choroid plexus are considered the entry sites for circulating inflammatory cells into the CNS under pathological conditions (Engelhardt 2006; 2006.; Engelhardt and Ransohoff 2005; Stolp and Dziegielewska 2009). The findings of haemorrhage and ischaemic necrosis (malacia) of the CNS with or without PCVs and lesions in peripheral nerves, can explain the clinical nervous signs of the affected broilers, which included incoordination, paresis and tremor, and complete prostration with paralysis or coma.

Cartilage nodules were seen in many of the lung sections examined but these may or may not be pathological. Cartilage nodules observed in the existing study are similar to the findings reported previously (Julian 1992; Sarango and Riddell 1985; Wight and Duff

1985). Their significance and origin remains unknown, but their numbers may increase in certain disease states.

Sequencing of *meq* gene from the PCR product of one dust sample from farm 68 (affected chickens), revealed that it had an insertion of 177 bp relative to Md5 that is identical in length to the insertion seen in MPF57, and other Australian isolates of vv MDV (Renz et al., 2012). The *meq* gene of Rispens/CVI988 has a longer insertion (180 bp). The 10 GRA *meq* gene also has three points of SNPs (Figure 7.25) and amino acid substitutions that enable it to be differentiated from the other Australian isolates. Importantly it has a SNP at the binding site of one of the primers for the pathogenic MDV1-specific qPCR test of Renz et al., (Renz *et al.* 2013) meaning that this MDV is not detected by this test. However an alternative primer (BCH446) was shown to work.

The *meq* gene is a key gene involved in the ability of MDV-1 to induce lymphoid tumours. Differences in the *meq* gene between oncogenic and attenuated isolates of MDV-1 have been reported, with a 177 to 180 bp insertion in the *meq* gene of the attenuated vaccinal isolate CVI988 being possibly responsible for its reported non-oncogenicity (Chang *et al.* 2002a; Lee *et al.* 2000; Spatz and Silva 2007). A new dogma was introduced by the finding of Renz *et al.* (2012) that the Australian isolates have 177 base-pair insertion but, rather than being attenuated, they belong to the category of virulent or vv MDV based on the results of the pathotyping experiments, according to the classification system suggested by (Witter 1997). Consequently, the insertion alone is not an indicator of attenuation.

The final hypothesis (hypothesis 5) was that the combination of epidemiological, clinical, and diagnostic features of the syndrome would support a diagnosis of MD. From the results it is clear that the chickens were infected with MDV and the virus was replicating and spreading within the flocks. Early lesions of MD were also observed, and it is possible or even likely that, had the chickens survived to ages beyond the normal killing age for broilers, gross lymphomas would have been observed. However, it is far from clear that MDV and the MD lesions observed are responsible for the severe clinical and pathological syndrome observed. As is shown in Table 7.4, there are many epidemiological, clinical and pathological findings from this syndrome that are not consistent with MD. It is possible, but unlikely, that the few mutations observed in the *meq* gene of the sequenced MDV from the outbreak are responsible for an entirely new form of MD. It is more likely that, while MDV is clearly present and inducing early lesions of MD, some other factor or agent is responsible for the severity of the observed clinical

syndrome. Prior infection with MDV leading to immunosuppression may form part of the causation for the syndrome, but on balance it would appear that MDV alone does not fully account for the observed syndrome.

Chapter 8 General discussion

8.1 Review

MDV-1 is a highly contagious *alphaherpesvirus*. Virulent strains of MDV-1 have the capacity to transform T-lymphocytes within weeks, resulting in the formation of tumours at many locations including viscera and skin. In addition, MDV-1 causes immunosuppression, neurological symptoms and ocular lesions. The chicken is the natural host of MDV-1 but it has also been reported to infect quail (Kenzy and Cho 1969), ducks (Cottral and Winton 1953), In Japan, MDV was detected in about 30% of of wild geese migrating to Japan (Murata *et al.* 2007) and lesions similar to those of MD have also been reported in owls (Halliwell 1971).

The virulence of MDV-1 has evolved over a period of more than sixty years. The host range of MDV-1 has expanded because of its higher virulence (Fadly *et al.* 2004), and MD outbreaks in commercial turkey flocks (Fadly *et al*. 2004; Malkinson *et al.* 1996; Voelckel *et al.* 1999) have led to the suggestion that the outbreaks are one aspect of the evolutionary trend of MDV towards greater virulence. Another phenomenon linked with evolution of MDV virulence is a change in the tissue tropism with increases in occular lesions and CNS involvement. Highly virulent MDVs induce a range of neurologic syndromes, including classical transient paralysis (TP), which is characterized by a rapid onset of flaccid paralysis at about 8–10 dpi with MDV. Recovery from paralysis usually occurs between 24-48 h (Zander 1959), but the birds later succumb to lymphoma of the internal organs (Swayne *et al.* 1989d). A more severe form of TP termed “acute TP”, in which the onset of paralysis is followed by death within 24–72 h (Witter *et al.* 1999), and persistent neurologic disease (PND) can occur after recovery from paralysis (Gimeno *et al.* 1999). However these phenomena have never been reported in the field and are only observed in maternal antibody negative chickens under experimental conditions. Under these conditions, highly virulent strains of MDV have the ability to kill their host without lymphoma or nerve enlargement. The onset of paralysis results from the development of vasogenic brain oedema secondary to vasculitis (Swayne *et al*. 1989a). However, neurological signs in chickens also can be induced by a variety of infectious agents other than herpesviruses such as picornaviruses, myxoviruses, retroviruses and arboviruses or bacteria such as *Pasteurella spp*., *Clostridium botulinum* or fungi. Neurological symptoms can also be due to non-infectious causes such as nutritional disease or exposure to toxins (Helmboldt 1972). Moreover, MDV-1 induces nerve lesions and lymphomas that

closely resemble, both grossly and microscopically, tumours induced by Lymphoid Leukosis (LL) and Reticuloendotheliosis Virus (REV) (Barth *et al.* 1990 ; Witter *et al.* 1970b). Thus, differential diagnosis of MD from other nervous diseases of fowls relies on the isolation or molecular detection of the infectious agent and antigenic structures of avian tumor viruses or detection the non-infectious causes to confirm the histopathological findings.

8.2 Thesis goal

The broad aims of this doctoral thesis were determine the protective effect of anti-MDV Ab on the pathogenesis of MDV in SPF chickens in addition to using molecular methods for quantification of MDV-1 for diagnosis and epidemiology of MD in Iraqi and Australian broiler chickens industry under natural and outbreak conditions. This involved detection and quantification of MDV in chicken tissues and environmental samples by qPCR and sequencing of *meq* gene of MDV, together with serological screening for MDV-Abs using the ELISA test and microscopical examination of the affected tissues of chickens.

Quantitative polymerase chain reaction (qPCR), using specific primers and fluorescent labeled probes to identify MDV, has increasingly been used to detect MDV and has advantages in sensitivity and specificity over the traditional MDV detection via viral isolation. QPCR can provide results in hours after receiving samples. In contrast, standard PCR methods are labour intensive and require post-PCR electrophoresis or other handling steps to quantify the amplicon. Moreover, MDV can be detected by qPCR as early as 5 dpi.

8.2.1 Prevalence of MDV in commercial and village chickens in Iraq and *meq* gene sequence variation

In a survey of six provinces in the south of Iraq, the prevalence of MDV-1 was examined in suspected samples (dust and spleens) that were collected from non-vaccinated and apparently healthy chickens of both commercial Ross breed broilers and local indigenous layer chickens (village chickens). The objectives of the study were to determine the prevalence of MD in the Iraqi chickens and to predict MDV pathotype based on *meq* sequence variation.

The investigation was the first of its kind in Iraq and indicated the suitability of qPCR to detect the presence and quantity of the virus in DNA extracted from the different sample types. The survey involved 109 samples (broiler dust and spleens of broilers and local layer chickens). Of 109 samples tested, 54 were positive, giving an overall prevalence of MDV1 of 49.5%. The distribution of positive samples were 27/52 (51.9%) broiler dust, 14/30 (46.7%) broiler spleen and 13/27 (48.1%) layer spleens. Comparing the distribution of positive/negatives by provinces showed the prevalence in different provinces ranged from 36.8% in Karbala and Nassiriya to 65% in Amarah.

The survey indicated that MDV-1 is circulating among the free living local breeds of layers of the Iraqi villages in addition to commercial broiler farms. The study detected the early presence of MDV-1 in the environment and tissues of healthy chickens either resistant to MD or at a stage of pathogenesis before the appearance of clinical signs. In the case of the village layers the former is more likely as the birds were old enough to display clinical MD. However commercial broilers are usually killed before 40 days of age and so may be susceptible but still not clinically affected. There are another causes which delay the appearance of clinical signs of infection in unvaccinated chickens such as the presence of specific maternal antibody at hatch, which is acquired naturally from vaccinated commercial breeders. In previous studies, shedding of MDV1 was reported to commence at 7 dpi (Baigent *et al.* 2005a; Islam *et al.* 2005b), before the appearance of clinical signs (Walkden-Brown *et al.* 2013a), and may continue throughout the life of the chicken (Carrozza *et al*. 1973; Witter *et al.* 1971).

This survey is the first epidemiological study in Iraq to use molecular approaches to identify MDV. The study provides information which can be used to design control strategies in Iraq including the vaccination and limitation of movement of fowls among different locations. Because MDV is usually transmitted by inhalation of contaminated chicken dust (containing feather dander), rigid biosecurity and high levels of hygiene can aid in MD control. Application of vaccination in the hatchery by *in ovo* or at day-old will ensure protection from MD. Routine monitoring of MDV in broiler dust samples as a disease management strategy has been been practiced successfully in Australia (Walkden-Brown et al., 2013c).

The survey in Iraq also included sequence analysis of the meq gene of MDV-DNA from the Iraqi samples in order to detect molecular markers for putative virulence of MDV-1. The sequences of *meq* gene of Iraqi samples demonstrated a high level of similarity, indicating that similar strains of MDV-1 are distributed in both commercial broiler and

local layer chickens in the surveyed six provinces in the south of Iraq, or that sequence variation may be found in other parts of the MDV genome. Widespread distribution of similar strains may be due to relaxation in biosecurity due to the Iraq war and disruptions arising from this. So, this survey affords an epidemiological perspective of MDV at the flock level and can facilitate and clarify means for MDV control and surveillance in Iraq.

The result of comparisons of nine Iraqi *meq* gene sequences with the very virulent US isolate Md5 showed that there was deletion of 123 bp relative to the reference strain Md5. The Iraqi *meq* sequences also contained single nucleotide polymorphisms resulting in differences in the amino acid sequence.

Amino acid alignment of the *meq* gene from the nine Iraqi samples and international isolates revealed that all the Iraqi Meq sequences had two four-proline repeat sequences, and there was limited sequence variation between them. All contained a deletion of 41 aa relative to Md5 (Tulman *et al.*, 2000), and larger deletions relative to Australian MDVs (Renz *et al.*, 2012) or Rispens CVI988 (Spatz and Silva, 2007). Their proline content of 19.6% is lower than the content reported for most other isolates. Shamblin et al. (2004) found that higher virulence MDVs had point mutations in the proline-rich repeats that interrupted stretches of four prolines, and the most virulent MDVs had the greatest number of such interruptions. This was confirmed and expanded upon by the finding of Renz et al (2012) that deletion in the section of the gene encoding consecutive proline repeats (PPPP) found in the proline-rich region was also associated with increased virulence. They reported that the number of PPPP sequences was statistically associated with pathotype of pathotyped MDVs with fewer PPPPs associated with the greatest virulence. Thus, polymorphisms in the sequence of meq genes have been linked with virulence. MDV1 strains with a low copy number of the proline-rich repeat region of *meq* tended to be highly virulent strains and statistically, there was strong association between the number of 4-proline repeats in the sequence and pathotype. More pathogenic MDV was associated with a lower overall proline content and fewer 4-proline repeat sequences. The negative association between the number of 4-proline repeats in Meq and MDV virulence suggests that the Iraqi MDVs are putatively vv+, but this needs to be confirmed by formal pathotyping *in vivo*.

8.2.2 Neuropathotyping Australian isolates of MDV1 in SPF chickens

Another hypothesis tested in this thesis was that the shorter neuropathotyping method proposed by Gimeno *et al.* (2002) to test for virulence in MDV will be applicable in Australia and correlate with formal pathotyping results based on lymphoma-based outcomes in experiments of longer duration. The neuropathotyping method was used to test the virulence of the Australian isolate of MPF23 against that of the reference strain of the Australian MDV-1, MPF57, as a complement to previous Australian pathotyping studies (McKimm-Breschkin *et al.* 1990; Renz *et al.* 2012; Walkden-Brown *et al.* 2013b) more latterly using the ADOL method (Witter 1997; Witter *et al.* 2005) based on induction of gross MD pathology in vaccinated and control maternal antibody positive chickens relative to that of prototype viruses of known pathotype. MPF23 was isolated in Australia in the mid 1980s and was the most pathogenic isolate identified at the time (McKimm-Breschkin et al, 1990), whereas MPF57 was a pathogenic isolate from the early 1990s (De Laney et al., 1995; 1998).

This is the first time that neuropathotyoing of MDV has been attempted in Australia to examine the virulence of MDV isolates. The experiment was successfully implemented in specific pathogen free (SPF) chicks over a 56 dpi period and the two challenges induced a wide range of neurological signs with agreement between virus ranking on the basis of neurological signs over the entire experimental period and incidence of tumours induced. However, the neuropathotyping model constructed by Gimeno *et al.* (2002) to classify MDV isolates did not clearly differentiate between the two Australian isolates (MPF23 and MPF57) tested in this thesis. Only with extension of the period of assessment of clinical signs beyond 26 dpc were clear differences observed. During the experiment chickens expressed a biphasic pattern of neurological signs including both TP and PND; an initial period of neurological signs between 5 and 17 dpc followed by a period of no clinical signs, then a second period of clinical signs starting on 25 dpc for MPF23 and 41 dpc for MPF57. The neurological signs between 5 and 17 dpc ranged between 81 and 71 % for MPF23 and MPF57 respectively; and 79, 71 and 79 % respectively for 500, 2000 and 8000 pfu) There was no significant effect of challenge virus ($P = 0.47$) or dose ($P = 0.88$) on the percentage of chicks exhibiting early neurological signs. However, between 21 and 56 dpc, there was a highly significant effect of challenge virus ($P = 0.006$) but not dose ($P = 0.70$) The percentage of chicks exhibiting late neurological signs ranged between 90 and 52 % for MPF23 and MPF57 respectively; and 64, 79 and 71 % respectively for 500, 2000 and 8000 pfu). Only one chick (2.4%) showed acute transient paralysis (ATP) leading to death, 45% showed classical transient paralysis (CTP) with

signs of 4 days duration or less, 29% showed neurological signs over a span of more than 4 days (PND). Also, during the experiment, chickens exhibited late neurological signs with typical leg paralysis with one leg extended backward and one leg extended forward with swelling of the sciatic nerves and loss of the normal shiny appearance and cross-striation.

The overall mortality rate was higher in the MPF23 group (81%) than the MPF57 group (62%) ($P<0.05$). The first MD lymphoma was observed at 29 dpc. MD was diagnosed in 100% of birds in the MPF23 group compared with 76% in the MPF57 group ($P = 0.01$), with no significant effect of dose ($P = 0.2$).

Microscopic pathology of peripheral nerves (sciatic) revealed three types of nerve lesions as described by Payne and Biggs (1967); A-type with the nerve showing infiltration and proliferation of lymphoid cells with presence of Marek's disease cells, which are degenerative lymphoblasts. B-type, characterized by diffuse infiltration by mainly small lymphocytes and plasma cells plus interneuritic edema; and C-type, characterized by light infiltration by plasma cells and small lymphocytes.

The two virus strains induced a similar histopathological pattern in chicken brains characterised by perivascular infiltrates of the mononuclear cell or cuffing (PVC) with variable degrees of vacuolation comparable with finding of previous studies (Kornegay *et al.* 1983; Swayne *et al.* 1989d) .

Neoplastic proliferation of lymphoid cells in other organs, including the skin was prominent during this study. Grossly variable sized, firm, white to grey tumours were observed in the ovary, lungs, kidneys, heart, liver, face and skin. Occasionally, bursal swelling was encountered due to diffuse interfollicular lymphoproliferative response. Thymic tumors also occurred but were infrequent. Atrophy was the predominant change in thymus and bursa.

The results of this experiment and others (Renz *et al.* 2012) provide no evidence of a change in the virulence of MDV-1 in Australia from vvMDV to vv+ pathotypes since the 1980s. Thus, the situation of the Australian poultry industry should be monitored with occasional pathotyping of new isolates to develop a profile of virulence over time. There is less evidence of virulence evolution in Australia than in the USA. Although HVT provides only limited protection against some isolates MDV, vaccination programs in Australia are still providing relatively good control of MD in Australia. This may be due to

less universal vaccination of broilers (Atkins *et al.* 2012). In contrast, in the USA there has been a regular change resulting in an increase in the virulence of MDV over time, due to selection pressure for MDV-1 in the face of widespread MD routine vaccination of broilers and extensive re-use of litter. This has caused vaccines such as HVT to become ineffective in some regions as HVT and bivalent vaccines were unable to control the most virulent MDV strains in the USA (Witter 2001b). Furthermore, the density of the chicken population is lower in Australia than some parts of the USA and MDV may not be as widespread as it is in the USA. Australian studies using molecular methods for differentiation and quantification of MDV in broiler shed dust samples showed only 23% of flocks have detectable MDV in dust (Walkden-Brown *et al.* 2013a).

8.2.3 Protective effect of maternal and administered egg yolk antibody directed against MDV

The original main goal of this thesis was to elucidate the role of anti-MDV antibody in protecting chickens against acute transient paralysis. Four experiments were undertaken in chickens with and without passive antibody, comprised of two challenge experiments with both types of chickens and two experiments to evaluate the ability of extracted IgY from eggs of chickens vaccinated with Rispens, to increase the serum anti-MDV titre of SPF and commercial chickens.

Experiment 1 involved experimental challenge with MDV-1 strains 02LAR (vvMDV) or MPF57 (vMDV) on days 0 and 7 of age. Chickens differing in anti-MDV Ab status used for this purpose. The presence of parental anti-MDV in serum was examined ELISA. The ELISA confirmed that the UNE chickens had anti-MDV with titres declining in a classical fashion to very low levels at 21 days of age. Presence of Mat-Ab protected against ATP mortality in chickens challenged on d 0 by comparison to those challenged on day 7. ATP in the chickens with Mat-Ab was lower than the chickens free from Mat-Ab (17.9 vs. 29.0%) but the effect of Mat-Ab treatment was not significant. In the group challenged on d 7 more ATP and some mortality was observed in chickens free from Mat-Ab. The incidence of ATP was again numerically lower in the group with Mat-Ab (27.1 vs. 33.3%) but the effect was not significant. However, the presence of parental anti-MDV Ab significantly reduced immune organ damage. Chickens with MDV Mat-Ab offered significant protection against both thymic and bursal atrophy due to MDV challenge.

There was an effect of MDV strain on induction of clinical signs and mortality. Chickens challenged with 02LAR (vvMDV) on d 7 showed more clinical signs (ATP and PN) than with MPF57 (vMDV). Also the clinical signs were positively correlated with challenge dose but this did not reach statistical significance.

MDV viral load in PBL was affected by MDV pathotype, being higher with 02LAR (vvMDV) than with MPF57 (vMDV). Also there was a positive association between ATP and MDV load in PBL with significantly higher MDV load observed in chicks affected by ATP than those not affected. However, there was no effect of Mat-Ab status on viral loads. This finding was in disagreement with an early report that Mat-Ab lowers the amount of MDV antigen in positive tissues based on fluorescent antibody test (Calnek 1972b). On the other hand, higher viral loads were seen in chickens challenged at day 7 than day 0 which could be attributed to effect of decrease of Mat-Ab with age as evidenced in ELISA test results.

The results of the experiment indicate that Mat-Ab may confer immunological protection against ATP as previously hypothesised on the basis of observed incidence only in maternal antibody negative chicks under experimental conditions (Renz *et al.* 2012) and not in commercial maternal antibody positive chicks under identical conditions in separate experiments (Walkden-Brown *et al.* 2013b). However the Mat-Ab levels in Experiment 1 did not confer the complete protection that was hypothesised. Such protection is in accordance with early research on the role of immune-mediated maternal effects showing that Mat-Ab has a restrictive effect on the early pathogenesis of MDV (Burgoyne and Witter 1973; Calnek 1972b; Kermani-Arab *et al.* 1976).

Experiments 2 and 3 were preceding and preparing for experiment 4 to evaluate the efficacy of injected immune globulin of egg yolks extracted from eggs from Rispens vaccinated parents to increase anti-MDV titres in chicks. Experiment 2 confirmed that inoculation of IgY with 2, 4 or 6 egg successfully increased anti-MDV titres in SPF chicks but not to titres equivalent to those in chicks containing natural Mat-Ab hatched from the same breeder flock that provided the eggs from which Ig-Y was extracted. There was no difference in titre following IgY administration via the i.a. or the s.c. route so a combination of the two routes was used in subsequent experiments. Inspection of the antibody titre profile demonstrated a similar decay profile of injected anti-MDV Ab (but with lower titres) in SPF chickens and naturally acquired antibody in commercial chickens. The natural profile in the commercial layers suggests that under field conditions significant titres may persist at least for 3 weeks and these could protect the

chickens during the critical period between *in ovo* vaccination and development of solid vaccinal immunity.

In experiment 3, inoculation of IgY extracted from 2000 commercial layer eggs significantly increased anti-MDV antibody titre in commercial layer chicks in a dose dependent manner, ensuring that this batch of IgY could be used in a major challenge experiment (Experiment 4).

Experiment 4 was a challenge experiment in which inoculation with extracted antibody conferred significant dose-dependent protection against MD, MD induced mortality and changed the pattern of clinical signs observed. The percentage of chickens with MD at 56 dpc was 84, 70 and 40% respectively for treatments receiving 0, 1 or 4 egg equivalents of extracted IgY. There were no significant effects of viral challenge dose on clinical signs and mortality. While MDV challenge certainly induced significant MD and mortality, induction of ATP was obscured by a concurrent bacterial infection that was initiated as at some point between setting of eggs and placement of chicks in the isolators. This was brought under control by water-administered antibiotics. However, IgY administration profoundly influenced the pattern of clinical signs observed, with later onset of signs and reduced incidence with increasing dose of IgY. Fewer chickens in the 4 egg injected groups showed neurological signs or ataxia symptoms. This suggests that administration of IgY leads to enhancement of protection during the early period of MDV replication which delays the onset of MD in a dose dependent manner. Survival analysis revealed highly significant ($P<0.001$) effect of IgY dose with higher doses delaying the onset of mortality primarily (Figure 6.12). Analysis of overall rates of mortality and MD also revealed highly significant effects of IgY dose with the major effect being due to a marked reduction in the 4 egg equivalent group (Table 6.5). This result is in agreement with earlier studies showing restrictive effects of anti-MDV Ab to the development of MD (Chubb and Churchill 1969; Kermani-Arab *et al.* 1976).

The precise mechanism by which antibody provides protection against an avidly cell-associated virus such as MDV remains unknown. Recent work in human cells has shown that antibody bound to virus enters the cell with the virus and mediates an intracellular immune response that disables virions or degradation of virions in the cytosol before translation of virally encoded gene (Mallery et al. 2010). A similar mechanism may explain the protective effects of maternal antibody against MDV.

8.2.4 Outbreak in an Australian commercial poultry farm

A field study in NSW, Australia was conducted on a natural outbreak of late paralysis in a commercial poultry complex containing about 1.6 million Ross broiler chickens that were not vaccinated against MDV but were generated from hens vaccinated with Rispens. The disease started in birds of 32 and 42 d old, but later it was observed in younger birds of 27 d old. The affected birds had been in good condition and ate well prior to developing clinical signs of paralysis and recumbency, coma and death within 24 h. The morbidity rate was around 25% in affected sheds and the case fatality rate approached 100%. In spite of treatment with Amoxicillin, the pattern of morbidity and mortality of the flocks remained unchanged and the course of the syndrome was very acute.

While the clinical signs had some features of classical MD, mainly paralysis, they were not consistent with typical Marek's disease as reported in Australia (Cumming *et al.* 1998; Groves 1995; Walkden-Brown *et al.* 2005). Since the mid 1980s, the predominant form of MD in Australia has been acute MD with lymphoma formation. In broiler chickens, MD generally manifests either as immunosuppression with higher levels of intercurrent disease, or as acute MD with lymphoma appearance late in the batch days. No lymphomas were observed in the outbreak investigated. Purely paralytic syndromes without lymphomas have been reported (Renz *et al.* 2012; Walkden-Brown *et al.* 2005) but these occur during the first 20 days of life, unlike the present field situation, and have only been reported in SPF chickens free of maternal antibody against MDV. The clinical picture of late paralysis in broilers without MD tumours in the present outbreak would therefore be a unique form of MD if the causative agent was MDV.

For post mortem examination, 30 chickens (20 sick birds and 10 apparently healthy chickens of 27, 32 and 42 d old) were selected randomly. None of the autopsied chickens exhibited gross visceral lymphomas or peripheral nerve enlargement. Gross peticheal haemorrhagic lesions were observed in the liver, kidney and lungs of most chickens.

There was a lack of clear difference in pathology between affected and unaffected chickens, but histopathological examination provided confirmative diagnostic lesions of MD in some chickens. These were indicative of early stages of MD in visceral organs, sciatic nerves and the bursa of Fabricius. Early micro-foci of lymphomas were encountered in lungs, kidney and livers. The pleomorphic quality of the lymphomatous lesions and lymphoid cell infiltration of visceral organs is in agreement with previous

studies on MD (Akiyama and Kato 1974; Powell *et al.* 1975; Schat *et al.* 1991). In the peripheral nervous system infiltration of the sciatic nerve in three chickens with small numbers of lymphoid cells (type C lesion) is also strongly supportive of MD and the lesion is in accord with the polyneuritis (Marek 1907; Payne and Biggs 1967). The lesions in the bursa were also indicative of early MD. The principal lesion was severe atrophy of the bursa due to necrosis of lymphoid cells.

Lesions in other tissues were less clearly diagnostic for MD. In the CNS PVC, oedema, haemorrhage and ischemic malacia of the brain were observed, suggestive of non-suppurative encephalitis. The CNS PVC has been reported previously with lesions due to MDV-1 (Gimeno *et al.* 1999; Jakowski *et al.* 1969; Pappenheimer *et al.* 1926). Malacia of CNS was noted in 4/30 sections of cerebellum, and 6/30 sections of cerebrum. Lymphocytic PVC, thrombi or haemorrhage were obvious in brains with malacia. According to (Jubb and Huxtable 1993), infections of the CNS with viruses could be associated with malacic lesions due to indirect effects such as ischaemia or anoxia. Other studies also reported that vv strains of MDV have been found to induce vasculitis and oedema of the brain (Gimeno *et al.* 1999; Swayne *et al.* 1988; 1989b; Swayne *et al.* 1989d; Witter *et al.* 1999), and marked malacic foci in the cerebellum and cerebrum (Cho *et al.* 1998) when maternal antibody is not present.

Serological testing for the presence of MDV antibodies provided evidence of active MDV infection, with sera MDV titres rising with time. Sera MDV titres increased between days 21 and 42 of age, with little difference between affected and unaffected birds revealed a highly significant effect of bird age ($P<0.0001$) but not disease status ($P = 0.80$). Chickens at 21 d old had baseline levels of anti-MDV antibody consistent with decline of maternally transferred antibody (Chapter 5). However there was a marked increase in titre between days 22 and 49 indicating the presence of an active immune response to infection. As the chickens were not vaccinated against MD, this is presumably due to field challenge. Measurable levels of maternal Mat-Ab against MDV persist for about 3 weeks in chickens (Calnek 1972b) and so higher levels of antibody in chickens older than 21 days is likely to be evidence of active infection (Calnek 1972a; Kottaridis and Luginbuhl 1972).

The serological evidence of active MDV infection was supported by the measurement of MDV genome copy number in dust samples. Dust samples collected from sheds for MDV specific qPCR revealed high levels of serotype-1 MDV with load increasing with bird age. The Log^{10} of viral copy number (VCN)/mg increased by 2.5 logs (from 3.76 to 6.22)

between days 21 and 49, again indicating active replication and shedding of MDV. Three spleen samples were tested for MDV by qPCR and 2/3 spleen samples were positive. MDV-1 load in spleen [Log^{10} (VCN/$10^{\wedge 6}$ cells)] was 4.65 from Farm 68 (affected 42 days of age) and 5.16 from Farm 67 (affected 49 days of age). However, MDV was not able to be cultured from spleen samples and there was no distinct typical MDV-1 plaques were visible in the CEF tissue cultures after three passages. Culture material was assayed for MDV-1 using qPCR and found to be negative. This is suggestive of a failure of isolation and growth in cultured cells rather than an absence of MDV. This suggests that qPCR was more sensitive than virus isolation and that qPCR of dust was more effective at detecting flock infection, than individual spleens. There was no difference between the MDV load in dust between sheds affected and unaffected by the syndrome, casting doubt on the role of MDV causing the paralytic syndrome. The late batch log^{10} values for MDV load (<6) are higher than the values of 4-5 for end of batch dust reported from detailed studies on two unvaccinated broiler farms with no clinical or pathological signs of MD (Walkden-Brown *et al.* 2013a), but are about one log lower than late batch levels (>7) reported in isolator dust from challenge experiments in which MD was induced (Islam and Walkden-Brown 2007; Walkden-Brown *et al.* 2013b).

Purified *meq* PCR products, obtained from a dust sample of an infected shed, were sequenced. The length of the *meq* ORF (1179 bp) was identical to the *meq* gene of previously characterised Australian MDV isolates (Renz et al., 2012). Sequencing of *meq* gene revealed that it has an insertion of 177 bp relative to Md5, and this was a common feature with MPF57, and other Australian isolates of MDV. The *meq* gene of Rispens/CVI988 has a longer insertion (180 bp). The analysed *meq* gene also had point mutations and changes in the amino acid sequence that enable it to be differentiated from the other Australian isolates.

From the results of this investigation, it is clear that the chickens were infected with MDV and the virus was replicating and spreading within the flocks. However, it is unlikely that MDV and the MD lesions observed were responsible for the severe clinical syndrome observed. There were many epidemiological, clinical and pathological findings from this syndrome that were not consistent with MD. It is possible, but unlikely, that the few mutations observed in the *meq* gene of the sequenced MDV from the outbreak are responsible for an entirely new form of MD. However, it is more probable that some other factor or agent is responsible for the severity of the observed clinical syndrome and this is deserving of further investigation. Prior infection with MDV leading to

immunosuppression may form part of the causation for the syndrome, but the evidence indicates that MDV alone is likely insufficient to induce the observed syndrome.

Taken together the main findings and outcomes of this thesis are:

a) Demonstration of the practical utility of surveying the presence of MDV and putative pathotype using qPCR of tissue and dust samples.

b) High prevalence of MDV in Iraqi, with little *meq* sequence variation and of putatively vv+ pathotype.

c) Failure of the neuropathotyping method of Gimeno *et al.* (2000) to differentiate between Australian MDVs of significantly different virulence.

d) Confirmation that MPF23, isolated in 1985 is a highly virulent MDV and that there appears to be no clear increase in the virulence of MDVs isolated in Australia between 1985 and the early 2000s.

e) ATP is difficult to induce in current SPF chickens in Australia, despite using the same challenge viruses and doses that reliably induced it in the past. Nevertheless, some ATP and mild clinical signs were induced in several experiments and the histopathology of such cases was consistent with that reported elsewhere.

f) Extraction of IgY from commercial layer eggs provides a good source of anti-MDV antibody and inoculation of chicks with this material significantly delays the pathogenesis of MD. However, based on egg equivalents, higher doses of extracted IgY need to be administered to obtain effects comparable with those of naturally transmitted IgY.

g) A serious outbreak of late paralysis in broiler chickens in a major poultry producing region of NSW was associated with active MDV infection, replication and early lesions, but MDV appears unlikely to be the sole cause of the observed syndrome.

Future research which would flow naturally from the findings of this PhD project, includes:

a) Formal pathotyping of Iraqi MDV isolates, and further confirmation of the association between the number of PPPP repeats in the *meq* gene and pathotype.

b) Formal pathotyping of MPF23 and comparison with isolates from the 1990s, 2000's and early 2010s. This will provide an indication of virulence change in Australia over a time period of nearly 30 years

c) A reliable experimental model for induction of ATP in current SPF chickens needs to be developed before further research on this topic should occur.

d) Detailed studies into the immunological basis of antibody protection against MD at a cellular and subcellular level.

e) Experimental reproduction of the late paralysis in broilers syndrome, followed by detailed studies into the causation of the syndrome.

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