1 Review of Literature

1.1 General description of Marek's disease

Marek's disease (MD) is a highly contagious neoplastic disease of poultry caused by a lymphotrophic alphaherpesvirus which is known as Marek's disease virus (MDV-1 /GaHV-2). MD is one of the major threats to the poultry industry, and the economic impact every year has been estimated to be US$ 1-2 billion (Morrow & Fehler, 2004).

MD was first described by Jozef Marek (Marek, 1907) as paralysis of the domestic fowl (polyneuritis), based on clinical diagnosis in 1907. In earlier days, MD was considered as a disease limited only to the nervous system (Marek, 1907; Van der Walle & Winkler-Junius, 1924). The association of MD with visceral tumours was described by Pappenheimer *et al*. (1929a), and they discovered the cytological composition of those tumours was similar to the lymphocytic infiltrations of the nerves. Up to the early 1960s MD diagnosis was limited by the inability to differentially diagnose it from avian lymphoid leucosis (LL) as both diseases generated visceral tumours, and there were no diagnostic tests to distinguish them. However, in the early 1960s MD was identified as a separate entity from LL, and, in 1961, it was suggested that it be named "Marek's disease" (Campbell & Biggs, 1961). The causative agent of MD was identified by Churchill and Biggs (1967) as a cell-associated herpesvirus. In later classifications, Marek's disease herpesvirus serotype 1, 2 and herpesvirus of turkeys (HVT) were identified as separate species in the genus *Mardivirus*.

MD became a severe threat to the poultry industry in the 1960s (Schat & Nair, 2008) when the industry was rapidly intensifying with a large increase of the poultry population. During this time, the lymphoproliferative form of the disease became more common causing infiltration of mononuclear cells into peripheral nerves and other visceral organs and also into skin and iris. Control of MD was achieved by live viral vaccination (Churchill *et al.*, 1969), a development which followed immediately after the discovery of the causative agent. Shortly after the initial serotype 1 vaccine was developed in the UK, the HVT vaccine was developed in the USA and made commercially available (Okazaki *et al.*, 1970). These vaccines were the first vaccines which conferred protection against tumours in any species (Davison & Nair, 2005), and MD was therefore considered as a model for herpesvirus oncology.

Vaccination successfully reduced the mortality and gross MD lesions but did not provide a sterile immunity, in that it did not prevent infection with, and shedding of, pathogenic MDV. These type of vaccines are known as imperfect vaccines. When the chickens were vaccinated with these MD vaccines, the vaccines prevented premature death of the host, even if they were infected with a highly virulent MD virus thus removing one of the major evolutionary constraints on increasing pathogen virulence. The higher virulence strains in vaccinated hosts also tend to have a higher replication rate than that of low virulence strains (Dunn *et al.*, 2014). These factors result in a reproductive advantage to the high virulence strains in vaccinated hosts. Thus, as for other imperfect vaccines, the vaccine itself is thought to be the reason for the evolution of the virulence of MD viruses, and there is evidence for the evolution of MD viruses towards greater virulence in the field (Witter, 1997; Gandon *et al.*, 2001). Therefore, the more virulent GaHV-2 s are clearly favoured over less virulent GaHV-2 s due to their higher total replication capability when the host is vaccinated with MD vaccine (Atkins *et al.*, 2013). More recently, Read *et al.* (2015) demonstrated that by prolonging infectious periods of GaHV-2 by vaccination, indeed can favour persistence of more pathogenic GaHV-2 strains. This resulted in vaccine breaks approximately ten years after their introduction (Pastoret, 2004). In order to control MD outbreaks in HVT-vaccinated flocks, new vaccines were introduced, to USA such as bivalent vaccines which are comprised of HVT and GaHV-3 viruses (Calnek *et al.*, 1983), and/or Rispens vaccine (Rispens *et al.*, 1972a, 1972b), which is an attenuated GaHV-2 vaccine.

MD is still economically important in the poultry industry, partly due to the cost of widespread use of vaccination in broiler chickens, as the vaccines used are cell associated and have high production and storage costs (Gimeno, 2004) prior to administration.

Moreover, the clinical manifestation of MD has changed over time, and outbreaks of the disease occur in different regions of the world at different times despite vaccination (Witter, 1997, 1998b). Sustainable vaccine strategies are therefore required to maintain the protection provided by vaccines. Furthermore, studies directed towards the understanding of the kinetics of the MD vaccines and pathogenic GaHV-2 s (Islam *et al.*, 2006a; Islam & Walkden-Brown, 2007; Islam *et al.*, 2008; Islam *et al.*, 2014) and monitoring the protection and efficacy of vaccines in the field levels (Purchase *et al.*, 1971a; Rispens *et al.*, 1972b; Witter *et al.*, 1984; Witter *et al.*, 1985) may also help prevent vaccine breaks and resultant MD outbreaks.

Proper vaccination and monitoring of vaccinal success are not sufficient to control MD. Biosecurity measures, such as providing a clean environment for the chickens, and vaccination and control of other immunosuppressive diseases, are also of utmost importance (Morrow & Fehler, 2004).

1.2 Brief history of Marek's disease

In 1907 Dr. Jozef Marek, an eminent Hungarian veterinarian, published a paper describing a disease of four cockerels which caused paralysis of their legs and wings (Marek, 1907). He examined one cockerel in detail and discovered thickening of sacral plexuses and spinal nerve roots, loss of nerve fibres, and infiltration of mononuclear cells into the affected nerves. Jozef Marek described this disease as "polyneuritis" or "neuritis interstitialis". This was supposedly the first published evidence of an economically important poultry disease, which later was given the name "Marek's disease".

Pappenheimer and colleagues (Pappenheimer *et al.*, 1926, 1929a; Pappenheimer *et al.*, 1929b), in their subsequent seminal studies, demonstrated that, apart from the nerve lesions, there were tumours in the visceral organs in some of the birds with this disease. Examining 60 birds between 3-18 month age exhibiting paralysis, they found 10% of birds had visceral lymphomas, mainly in ovaries and less frequently in liver, lungs, kidneys, adrenal glands, and muscle. They used the term visceral lymphomatosis to explain the tumours in the neurolymphomatosis gallinarum disease.

Ellermann *et al.* (1921), studied neoplastic conditions of the blood cells of chickens in parallel with the work of Marek, and they gave these conditions the term "leucoses". A more common form of leucoses was lymphoid leucosis (LL) under field conditions, and with the expansion of the poultry industry in the 1950s the incidence of LL increased. By pathological examination and visceral lymphomas of LL and MD, which was the only form of diagnosis at that time, scientists were unable to differentiate between the two diseases. Furthermore, the aetiological agents had not been identified, so the two diseases were considered to be a single entity. However, some scientists (Campbell, 1945, 1956) still considered fowl paralysis and leucosis to be two non-related diseases. In 1961, Campbell and Biggs (1961) presented papers to clarify the conflicting views about the two diseases and, afterwards, the two conditions were separated and fowl paralysis was termed Marek's disease.

Experimental transmission of MD was first successful in the early 1960s (Sevoian *et al.*, 1962; Biggs & Payne, 1963) despite the belief from the beginning that it was an infectious disease. Later, with the development of cell culture techniques, GaHV-2 was found to be an extremely cell-associated virus (Churchill & Biggs, 1967; Nazerian *et al.*, 1968; Solomon *et al.*, 1968), and the cytopathic effects of the virus resembled that of other herpesviruses. The detection of the enveloped herpesvirus particles in the feather follicular epithelium in chickens infected with GaHV-2, as well as demonstrating that cell-free viral particles were also infective, were important landmarks in MD research (Calnek & Hitchner, 1969; Calnek *et al.*, 1970). This finding also explained the extremely contagious nature of MD with the presence of GaHV-2, which can remain infective from 16 weeks (Witter *et al.*, 1968) to one year (Carrozza *et al.*, 1973; Hlozanek *et al.*, 1973), in infected desquamated epithelial cells in poultry house dust and litter (Beasley *et al.*, 1970). The aetiological agent of MD as a herpesvirus was confirmed after it was controlled efficiently with an attenuated GaHV-2 vaccine (Churchill *et al.*, 1969) and herpesvirus of turkeys (Okazaki *et al.*, 1970).

After the successful isolation and attenuation of the GaHV-2 strain HPRS16 (Churchill *et al.*, 1969), MD became the first naturally occurring animal cancer disease to be effectively controlled by a vaccine. Almost simultaneously, scientists from the USA isolated a herpesvirus from turkeys that was non-pathogenic to chickens and did not transmit between chickens, but gave good protection against MD (Okazaki *et al.*, 1970). In 1972, Rispens and colleagues from the Netherlands described the use of a mildly virulent attenuated strain of MDV which was known as CVI988 (Rispens *et al.*, 1972a, 1972b). This vaccine is still effective to date and is the most widely used vaccine in long-lived chickens such as layers and breeders; thus it is considered to be the gold standard of vaccination for MD (Davison & Nair, 2005).

The nature of MD has changed with time, especially after the widespread introduction of vaccines. In the era when the disease was discovered, the predominant nature of the disease was paralysis of legs and wings and was named “classical MD”. The more pathogenic “acute MD” with visceral lymphomas in younger birds appeared in the 1950s and was first reported in the USA (Benton & Cover, 1957). Acute MD or the lymphoproliferative form of MD predominated in the second half of the twentieth century and caused tumours in young layers from 6-16 weeks age with mortality as high as 60%. One of the reasons for this is thought to be the expansion and intensification of the poultry industry at that time. MD also took another

turn in the 1980s, producing a syndrome known as transient paralysis syndrome in maternal antibody (mab) negative chickens. In this syndrome, death occurred in young birds 9-12 days post-infection from vasculitis and vasogenic brain oedema without the development of tumours (Gimeno *et al.*, 1999).

Initially, MD was well under control with the HVT vaccine; however, a decade after introduction of the vaccine in the late 1960s, MD outbreaks were observed in vaccinated birds (Witter *et al.*, 1980). Bivalent vaccines consisting of HVT and GaHV-3 strains (SB-1) were introduced due to their greater protective efficacy than HVT alone. However, vaccine breaks in chickens vaccinated with bivalent vaccines continued to be reported in the late 1990s (Witter, 1997). Therefore, MDV isolates were subsequently divided into several pathotypes according to the protection levels provided by HVT and bivalent vaccines. The pathotypes were classified as mild (m), virulent (v), very virulent (vv), and very virulent plus (vv+) using a standardised animal vaccination-challenge model developed at the Avian Disease and Oncology Laboratory (ADOL) in the USA (Witter, 1997). The ADOL method was also expanded into tests using the Rispens vaccine. Identifying the pathotype of a field strain compared to a reference GaHV-2 strain is of immense value, as it can be used to predict possible vaccine breaks (Buscaglia *et al.*, 2004; Witter *et al.*, 2005).

The protective index (PI) provided by vaccination is calculated according to the following equation (Sharma & Burmester, 1982):

$$PI = \frac{\%\ \text{MD in unvaccinated chickens} - \%\ \text{MD in vaccinated chickens}}{\%\ \text{MD in unvaccinated chickens}}$$

Since their introduction, MD vaccines have controlled the disease very effectively, in that they reduced the mortality, clinical signs, and tumourigenesis. However, the MD vaccines do not prevent infection, replication, and shedding associated with the pathogenic field viruses, although they do reduce the rate of replication of pathogenic MD viruses. Thus, the MD vaccines fail to provide a sterile immunity in the chicken population. These types of vaccines which cannot confer a sterile immunity are generally known as “imperfect vaccines” and typically are an important factor which promotes the evolution of viruses with higher

virulence (Gandon et al., 2001). Indeed, the observation of the emergence of more virulent MD field viruses despite vaccination (Witter *et al.*, 1980; Witter, 1983, 1997, 1998b), led to the assumption that the vaccine itself was responsible for the evolution of GaHV-2 virulence. A recent modelling approach by Atkins *et al.* (Atkins *et al.*, 2013) using broiler experimental data confirmed that vaccination against MD with imperfect vaccines, is a likely driver for the evolution of virulence in GaHV-2. Furthermore, an experiment by Read *et al.* (2015) confirmed that enhancing the lifespan of chickens by vaccination can increase the shedding of pathogenic GaHV-2. Therefore, MD is likely to remain a significant problem for the poultry industry despite vaccination, due to the emergence of GaHV-2 strains with higher virulence and in turn, if these higher virulent strains cause vaccine failure.

Apprehending the connection between GaHV-2 virulence and vaccination, scientists started to study the kinetics of the vaccine viruses and their effects on wild-type GaHV-2, using viral loads and quantitative PCR techniques (qPCR) to measure viral load (Baigent *et al.*, 2011; Islam *et al.*, 2013a). Most importantly, the kinetics of vaccine virus and the pathogenic MD viruses in the same host (Islam & Walkden-Brown, 2007; Islam *et al.*, 2008; Islam *et al.*, 2014) was studied in order to understand the behaviour of the two viruses in the same host at a given time. Furthermore, the protection provided by the vaccines was tested against different GaHV-2 pathotypes to evaluate efficacy of the vaccine under experimental conditions and in the field.

Currently, as mentioned, the Rispens vaccine is considered the gold standard of MD vaccination (Davison & Nair, 2005). However, as Rispens vaccine is also a GaHV-2 virus, as are all pathogenic GaHV-2s, the difficulty of distinguishing the two viruses easily and rapidly was the main problem in studying the kinetics in Rispens-vaccinated hosts that were also infected with pathogenic GaHV-2. Some solutions to this were cloning of the Rispens vaccine virus prior to such studies (Baigent *et al.*, 2011) or measuring viral loads of both Rispens vaccine virus and the pathogenic viruses together (Tan *et al.*, 2007). However, more recently several scientists (Haq *et al.*, 2012; Baigent *et al.*, 2013; Renz *et al.*, 2013; Gimeno *et al.*, 2014) described qPCR tests that distinguish between the Rispens vaccine virus and the pathogenic GaHV-2 isolates, thus enabling the study of the two viruses in co-infected hosts.

1.3 Marek's disease in Australia

As in other parts of the world, MD caused significant losses in the poultry industry in Australia in the 1960s. However, the outbreaks were brought under control by introducing a locally produced cell-free HVT vaccine. With more genetically MD-resistant chicken strains and enhanced hygienic practices, Australia was able to delay, compared to the rest of the world, the emergence of vvMDV strains (Jackson, 2000). In 1978 another locally produced Australian cell-associated GaHV-3 vaccine known as Maravac® was developed and used alone or in combination with HVT (Jackson, 2000) to enhance vaccinal protection by HVT. The first isolation of vvMDV strains in Australia was in the late 1980s included isolates MPF6 and MPF23 (Mckimm-Breschkin *et al.*, 1990). This was followed by the isolate Woodlands 1 (Zerbes *et al.*, 1994).

Some evidence of vaccine-induced evolution of the virulence of MD, as described by Witter *et al.* (1980) could be observed in Australia after 1990. New high-performing poultry strains were imported to Australia in 1990, and they were not sufficiently protected by the conventional native vaccines. This led to massive outbreaks of MD in layers, breeders, and broilers between 1992 and 1997 with mortality reaching as high as 40% in layers (Cumming *et al.*, 1998). In this period, GaHV-2 strains were isolated from the chickens vaccinated with HVT and bivalent vaccines (De Laney *et al.*, 1995) and later they were named as MPF57 and Woodlands 1 strain (De Laney *et al.*, 1998). MPF57 was a vMDV, whereas the Woodlands strain was designated as a vvMDV.

To counteract the excessive losses by the MD outbreaks in both layers and broilers, the master seeds of Rispens and HVT vaccines were imported from France in 1997. New vaccination strategies were implemented including *in ovo* vaccination of broilers with cell-associated HVT vaccine replacing day-old vaccination with cell-free HVT. In addition, longer lived layers and breeders were vaccinated with Rispens vaccine, leading to successful control of MD throughout Australia (Jackson, 2000). An Australian attenuated serotype 1 vaccine was also produced using a local BH 16 strain, and shown to be as protective as the Rispens vaccine; however, it has not been commercialised (Karpathy *et al.*, 2002, 2003).

Subsequent formal pathotyping experiments using an adaptation of the ADOL method confirmed the presence of very virulent GaHV-2s in Australia (Renz *et al.*, 2012; Walkden-Brown *et al.*, 2013b), but no isolates of the vv+ pathotype have been reported. The v

pathotypes, although causing a high percentage of mortality and MD incidence in unvaccinated hosts (Renz *et al.*, 2012; Wajid *et al.*, 2015), were very well controlled by HVT and bivalent vaccines when compared with vv isolates (Renz *et al.*, 2012; Walkden-Brown *et al.*, 2013b). Despite less evidence of GaHV-2 evolving greater virulence in Australia compared with elsewhere in the world, the presence of vvMDV isolates remains a threat to the Australian poultry industry, especially with further intensification of poultry production and the more recent widespread introduction of *in ovo* vaccination in broilers.

How do we prevent MD outbreaks in Australia in the future? Better monitoring systems need to be developed for the early detection of vaccine efficacy, particularly for the Rispens vaccine and the presence of GaHV-2 in the field. This would assist with the early detection of future vaccine breakdowns associated with evolving virulence of GaHV-2. Further, measuring the protective efficacy of the Rispens vaccine against Australian GaHV-2 pathotypes (Islam *et al.*, 2013b) and performing kinetic studies of wild-type GaHV-2 and Rispens vaccine virus in co-infected chickens will give researchers an insight into the present situation of MD in Australia and the protection level provided by current vaccines.

1.4 Economic significance

One of the main obstacles in assessing the economic impact of the MD is the fact that MD is not a notifiable disease. In addition, low level MD losses are thought to be normal in vaccinated birds in the field and MD linked to low hygiene also goes unreported. The impact of MD for the world poultry industry is crudely estimated to be about US $ 1-2 billion per annum, which is roughly one percent of the total value of poultry products (Morrow & Fehler, 2004). This includes the costs of vaccinating birds against MD which are substantial.

After the 1950s, when acute MD became more prevalent, and vaccines were not available, there were significant losses in both layers and broilers due to high mortality and morbidity rates. Losses in layers were as high as 93% with some GAHV-2 strains (Eidson & Schmittle, 1968), but generally were around 30% (Purchase, 1985). In meat chickens in countries other than the USA, the mortality increased up to 30% (Purchase, 1985). In the USA, the main economic loss in this era for broilers was as a result of carcass condemnations. As a consequence of these massive losses, research efforts into control measures were increased dramatically, including the poultry industry being forced into the development of genetically resistant breeds for MD (Cole, 1968).

After MD vaccines were developed in the early 1970s and introduced worldwide, the incidence of the clinical disease declined dramatically (Purchase, 1985). Concurrently, the cost of mortality and carcass condemnation in broilers (Witter, 2001b) were also reduced substantially. In the USA, from 1970 to 1982, condemnations of young chickens declined from 1.57% to 0.08% which was a 94.9% reduction (Purchase, 1985). However, a new expense associated with MD was introduced, the cost of vaccination. In the USA, vaccination costs were estimated at 44.4 million USD in 1985 (Purchase, 1985), and 169 million USD in 2004 (Morrow & Fehler, 2004). MD vaccines are comparatively expensive. Firstly, most of the vaccines are cell-associated vaccines and therefore must be stored in liquid nitrogen, and maintenance of the cold chain at –196 ºC is a necessity for storage and transport. Secondly, the vaccines should be administered early in life via inoculation of the chicks before exposure to pathogenic GAHV-2. Unlike some poultry vaccines which can be administered using mass vaccination methods through water, feed or sprays, this is not possible for MD. Therefore the chicks are vaccinated at hatch or in some countries broilers are vaccinated on the 18th day of embryonic development using automatised equipment. Improper vaccination methods, storage or disruption of the cold chain has led to outbreaks in vaccinated chickens (Landman & Verschuren, 2003).

Undoubtedly the third economic impact of MD is the emergence of new virulent strains despite vaccination (Witter *et al.*, 1980; Witter, 1997, 1998b; Teng *et al.*, 2011). This has led to sporadic MD outbreaks in some countries. Also, sub-clinical disease due to MD infection can often go undiagnosed and results in poor performance in chickens. GAHV-2 is immunosuppressive and can result in a higher incidence of concurrent diseases. Countries where vaccination is not practised have experienced about 46% mortality due to MD outbreaks (Lobago & Woldemeskel, 2004).

Therefore, the economic impact of MD is difficult to estimate and also varies with each country, the vaccination schedules practiced, and the level of general hygiene and biosecurity.

1.5 Aetiology

1.5.1 Classification of MDVs

MD is caused by an alphaherpesvirus. One of the remarkable characteristics of herpesvirus pathogenesis is latency. With latency, hosts are infected lifelong with recurrent limited

replication of the virus. These recurrent replications of the virus lead to shedding of the virus and transmission of the disease. Latently infected hosts do not show any clinical signs and act as carriers of the disease (Maclachlan & Dubovi, 2010).

All herpesviruses share a common morphology with linear dsDNA; however, they are divided into three distinct families: *Herpesviridae*, *Alloherpesviridae*, and *Malacoherpesviridae*. There are three subfamilies in the *Herpesviridae* family, namely *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. The subfamilies are further divided into genera. The subfamily *Alphaherpesvirinae* consists of four major genera: *Simplexvirus, Varicellovirus, Mardivirus*, and *Iltovirus*. The common important viruses in these genera are: for genus *Simplexvirus*, herpes simplex virus 1; for genus *Varicellovirus*, varicella-zoster virus; for genus *Mardivirus*, *Gallid herpesvirus 2* (GaHV-2), and for *Iltovirus, Gallid herpesvirus 1* (Infectious laryngotracheitis virus).

The genus *Mardivirus* consists of five species including, *Gallid herpesvirus 2* (GaHV-2), *Gallid herpesvirus 3* (GaHV-3), and *Meleagrid Herpesvirus 1* (MeHV-1). The scientific nomenclature for MDV is GaHV-2, whereas herpesvirus for turkeys is MeHV-1. The species classification corresponds to earlier classification of MDVs into three serotypes on the basis of variation in antigenic determinants (von Bülow & Biggs, 1975a; 1975b). Serotype 1 corresponds to GaHV-2, serotype 2 to GaHV-3, and serotype 3 (HVT) to MeHV-1. Serotype 1 MDVs (GAHV-2) are pathogenic and cause tumours in chickens, while serotype 2 (MDV-2) of chickens and serotype 3 (HVT) of turkeys are non-oncogenic in chickens or turkeys (Kawamura *et al.*, 1969; Cho & Kenzy, 1972) and have been widely used as vaccine viruses. The classification of the MDV by serotype is still extensively used (Table 1.1).

Table 1.1 Serotypes of MDV

Serotype	Natural host	Mardivirus species	Tumours in chickens	Used as vaccine?
MDV-1	chicken	Gallid herpesvirus 2 (GaHV-2)	Yes	Yes
MDV-2	chicken	Gallid herpesvirus 3 (GaHV-3)	No	Yes
HVT	turkey	Meleagrid Herpesvirus 1 (MeHV-1)	No	Yes

GAHV-2 is a highly cell-associated alphaherpesvirus (Schat, 1985a) which behaves more like Gammaherpesviruses such as the Epstein-Barr virus in its tropism to lymphocytes. However,

its genomic and molecular structures indicate that GAHV-2 belongs to the alphaherpesvirus family and shares homology with herpes simplex virus (Buckmaster *et al.*, 1988; Lee *et al.*, 2000a; Tulman *et al.*, 2000).

Serotype 1 strains usually represent the pathogenic GAHV-2s and the avian diseases and oncology laboratory (ADOL) proposed a further classification into pathotypes after the discovery of GAHV-2s of varying virulence in the field (Witter, 1983). A description of the ADOL pathotype classification is given in Table 1.2

Table 1.2 USDA-ADOL classification of MDV pathotype, adapted from (Witter, 1997) by Walkden,-Brown *et. al.* (2007)

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 56 days post challenge (dpc), but induces lymphomas and nerve lesions in a high proportion of susceptible and vaccinated 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.
vvMDV (very virulent	Causes moderate levels of mortality by 56 dpc and induces lymphomas and nerve lesions in a high proportion of susceptible and vaccinated chickens. HVT/GAHV-3 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 level of mortality by 56 dpc and induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT and HVT/GAHV-3 are only partially protective. Classification based on significantly higher pathogenicity than MD5 in HVT/SB-1-vaccinated chickens.

This classification was later modified by Witter *et al.*, (2005) using Rispens vaccine and by using alternative poultry strains; Witter used the susceptible maternal antibody (mab) positive F1 progeny of ADOL line$15I_5$ male and line 7_1 female chickens in his pathotyping experiments. However use of Rispens vaccine did not work as it did not discriminate between protective indices of vv and vv+ MDV pathotypes.

1.5.2 Morphology

The morphology of MDV viral particles is typical and similar to other herpesviruses. The nucleocapsid alone is about 95-100 nm in diameter and consists of 162 hollow capsomeres (Nazerian & Burmester, 1968).

The virions are commonly observed in the nucleus and less frequently in the cytoplasm and extracellular spaces. Enveloped virus particles are 150-160 nm in diameter and can be observed in infected cell cultures. Non-enveloped virus particles are less commonly seen and appear as amorphous structures 273-400 nm in size seen in thin sections of negatively stained lysed feather follicular epithelium (FFE) cells (Calnek *et al.*, 1970).

The morphology of GAHV-3 and HVT is similar to that of GaHV-2, although HVT sometimes show a unique crossed appearance in thin sections (Nazerian *et al.*, 1971)

1.5.3 Genome structure of the virus

The genomes of the three important MDV species of *Mardivirus* appear to be very similar according to the complete genomic sequencing carried out to date (Lee *et al.*, 2000a; Afonso *et al.*, 2001; Izumiya *et al.*, 2001; Kingham *et al.*, 2001; Spatz *et al.*, 2007), with double-stranded DNA approximately of 160-180 kb. However, the guanine plus cytosine (G+C) ratio differs among the three serotypes. The ratio is 44% and 53.6% for GaHV-2 and GaHV-3, respectively, whereas it is 46.7% for HVT (Izumiya *et al.*, 2001; Silva *et al.*, 2001).

Similar to other alphaherpesviruses, the genomic structure of the three MDV serotypes contains a unique long sequence (U_L) and a unique short sequence (U_S) (Cebrian *et al.*, 1982). These unique sequences are flanked by several inverted repeat sequences, the terminal repeat long (TR_L), internal repeat long (IR_L), internal repeat short (IR_S), and terminal repeat short (TR_S). The schematic representation of the genomic organisation is given below in Figure 1.1. These repeat regions are unique among MDV species and disparate from other alphaherpesviruses. The genomic comparison between virulent GaHV-2 isolate GA and very virulent MDV isolate Md5 shows that the structural differences between them are very small. The U_L regions of these two strains are similar in length, whereas the U_S sequence of GA is longer than Md5 (Silva *et al.*, 2001).

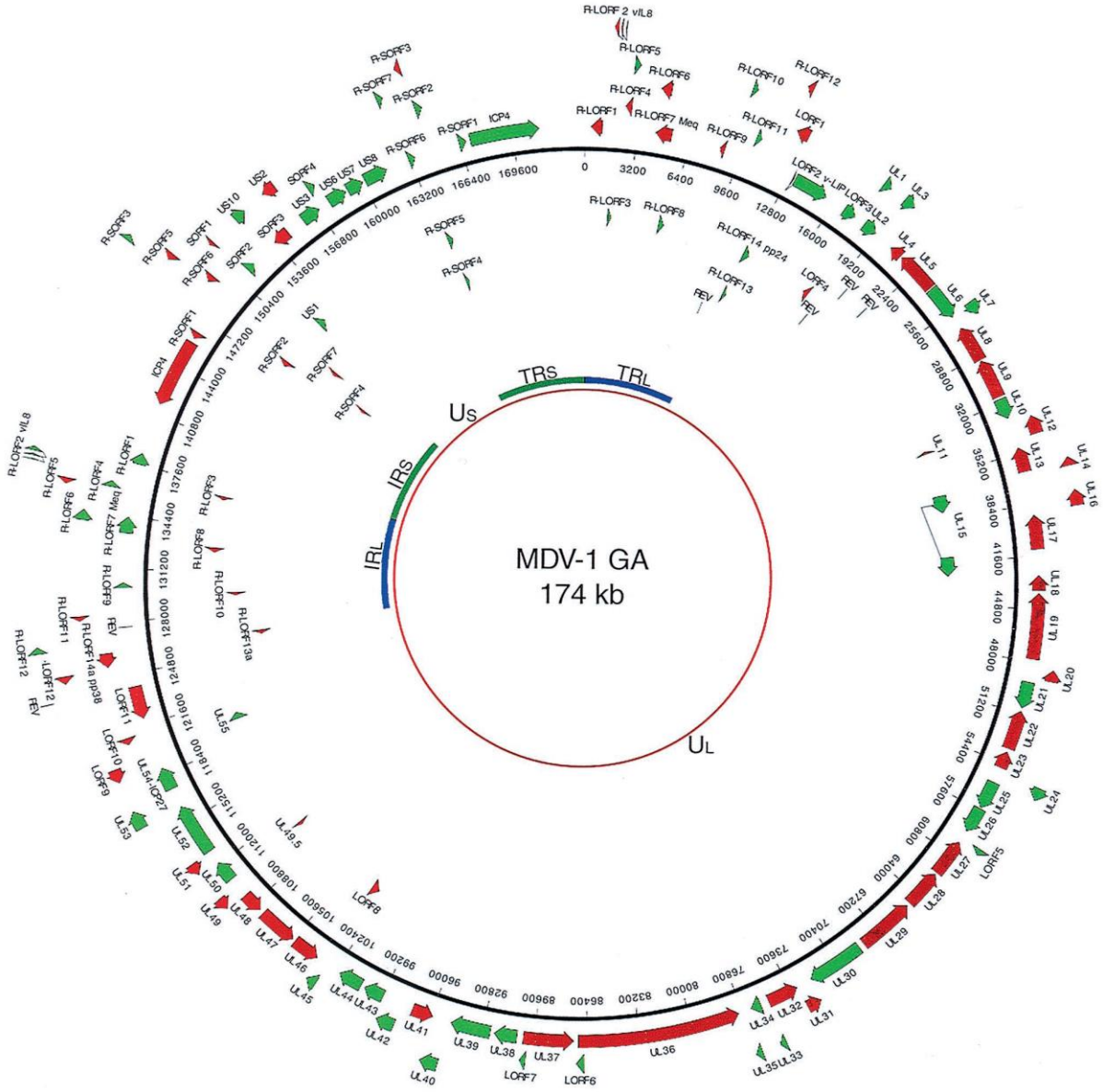

Figure 1.1 The replicative form of the GaHV-2 genome (Lee, *et. al.* (2000a))

The genes in MDVs can be divided into two broad categories: genes homologous with alphaherpesviruses and genes unique to MDV. There are several genes unique to each MDV. The repeats flanking the U_L regions, which are the R_L regions, and encoded genetic elements strongly associated with tumour formation. Some of the unique R_L encoded genes that are unique to GaHV-2 include *meq* (Jones *et al.*, 1992), *vIL-8* (Parcells *et al.*, 2001), and *pp38* (Cui *et al.*, 1990) (Figure 1.1).

Within TR_L and IR_L regions of the MDV genome, there are tandemly arranged 132 bp direct repeat sequences, which are used to distinguish GaHV-2 from other species as these are unique to GaHV-2. Furthermore, it has been found that there are 2-3 tandem repeats in pathogenic GaHV-2, whereas four or more tandem repeats are seen in attenuated GaHV-2, a

difference that was used to differentiate between pathogenic and attenuated GaHV-2 previously (Becker *et al.*, 1992; Silva, 1992; Zhu *et al.*, 1992).

The *meq* gene is considered the principal oncogene of GaHV-2 based on its transforming properties (Liu *et al.*, 1998) and results of gene deletion studies (Lupiani *et al.*, 2004; Li *et al.*, 2011). The *meq* gene is usually 1020 bp in length and encodes the protein known as Meq which consists of 339 amino acids (Jones *et al.*, 1992). Some cloned GaHV-2s with a deleted *meq* gene are able to confer protection against MD. (Lee *et al.*, 2008; Silva *et al.*, 2010; Lee *et al.*, 2013). Rispens vaccine virus, on the other hand, encodes a slightly longer Meq protein (398 amino acids (aa)) known as LMeq that consists of an additional 59 aa mainly in the form of proline-rich repeats in the transactivation domain (Chang *et al.*, 2002b). There are two to four aa differences between pathogenic GaHV-2 and Rispens vaccine virus in the transactivation and DNA-binding domains (Shamblin *et al.*, 2004). Later studies demonstrated that Meq proteins of Rispens vaccine are weak transactivators and hence non-oncogenic (Ajithdoss *et al.*, 2009).

Comparison of the *meq* gene of oncogenic and attenuated GaHV-2 isolates has revealed that attenuated GaHV-2 (Rispens vaccine) contains a 180 bp insertion (Lee *et al.*, 2000b; Chang *et al.*, 2002a; Spatz *et al.*, 2007). This insertion is thought to be responsible for the non-oncogenic characters of the Rispens vaccine (Lee *et al.*, 2000b; Chang *et al.*, 2002a; Spatz *et al.*, 2007). However, these studies demonstrated that there are also gene polymorphisms with point mutations, insertions, and deletions that are associated with the virulence of GaHV-2 when *meq* genes are aligned with each other (Lee *et al.*, 2000b; Chang *et al.*, 2002b; Shamblin *et al.*, 2004; Tian *et al.*, 2011). In a more recent study, Renz *et al.* (2012) showed that the Australian pathogenic GaHV-2 isolates possessed the 177 bp insertion which was previously thought to be associated with non-oncogenic characters of MDVs. The Australian isolate insertion was homologous with Rispens vaccine virus except at the first 3 bases and the single nucleotide polymorphism (SNP) at 646 position of field isolates (guanine) aligned to 649 position of the vaccine strain (cytosine). Furthermore, when the aa sequences were aligned, the virulence of Australian and international MDV isolates strongly correlated with the number of repeat sequences of prolines (PPPP) in the *meq* gene. There were generally two to eight PPPP repeats in the *meq* gene in the pathogenic MDV, and the fewer the proline repeats the higher was the virulence.

The overall length of the *meq* gene of Australian MDV isolates is 1197 bp compared with the length of Md5 being 1020 bp and Rispens vaccine virus 1200 bp. The *meq* gene of the Australian MDV isolates differs from other MDV isolates by having a 177 bp insert which is similar to the 180 bp insert linked with attenuation. Furthermore, the tested *meq* genes of the Australian isolates possessed a remarkably high homology. A phylogenetic tree analysis grouped the Australian isolates separately within the lower virulence GaHV-2 group of international GaHV-2 isolates (Renz *et al.*, 2012). However, the Australian isolates showed that they shared a more recent common ancestry with their Asian and USA GaHV-2 isolates and also possessed the similar amino acid substitutions in Meq protein reported by Shamblin *et al.* (2004).

The other genes unique to GaHV-2 are *v-IL8*, viral lipase, the 1.8 kb gene family, MDV-encoded telomerase RNA, and MDV-encoded microRNA.

1.5.4 New methods for manipulation of the MDV genome

As the MDVs comprise large genomes and are highly cell associated, studies investigating manipulation of viral genes have been difficult (Nair, 2013). A significant improvement was seen after MDV-infected bacterial artificial chromosomes (BAC) were produced (Schumacher *et al.*, 2000). There are several advantages of using BAC clones to study the MDV. Firstly, in BACs the MDV viral genomes can be propagated in the *E. coli* cells and do not need eukaryotic cell lines for maintenance. Secondly, mini F plasmids, which usually carry low copy numbers, can maintain genetic stability. Finally, the MDV clones are compliant with recombination of the *E. coli* host (Osterrieder & Vautherot, 2004). The BACs and overlapping cosmid clones containing MDV genomes were a significant breakthrough in manipulation of MDV genomes and studying gene functions in pathogenesis (Reddy *et al.*, 2002; Lupiani *et al.*, 2004; Jarosinski *et al.*, 2007; Kaufer *et al.*, 2011; Zhao *et al.*, 2011).

Furthermore, these novel strategies helped in the understanding of the evolution of virulence (Hunt & Dunn, 2015) and reduction of virulence (Hildebrandt *et al.*, 2014) in GaHV-2 strains *in vitro* and *in vivo*. In addition, the GaHV-2 BAC and cosmid clones as well as other vectors like poxviruses have been explored for production of recombinant vaccines to substitute or outperform the current "gold standard" vaccine Rispens vaccine (Lee *et al.*, 2003; Petherbridge *et al.*, 2003; Cui *et al.*, 2005; Baigent *et al.*, 2006a; Lee *et al.*, 2008; Singh *et al.*, 2010). The protection provided by these recombinant vaccines sometimes exceeded that of

the Rispens vaccine at the experimental level (Lee *et al.*, 2013; Su *et al.*, 2015) and thus show promising potential as future vaccine candidates for MD control.

1.6 Epidemiology

1.6.1 Incidence and distribution of disease

MD is a worldwide problem. In areas where there is a high poultry density, every chicken in a flock is susceptible to infection, and some economic losses may be incurred. It is difficult to measure the true incidence of disease as there is no effective reporting system and it is not considered a notifiable disease according to the World Organisation for Animal Health (OIE) (Morrow & Fehler, 2004). Furthermore, the disease may take several forms in susceptible chickens. Infected vaccinated and genetically resistant chickens may not show any clinical disease. Therefore incidence of infection may be higher than the incidence of the disease *per se* (Schat & Nair, 2008).

There are seasonal variations of MD incidence in broiler chicken populations in some countries (Kennedy *et al.*, 2015). In the USA the MD incidence has been measured using carcass condemnation data since 1961. The data show that incidence of the disease increased during the winter months compared to those during summer (Schat & Nair, 2008). Obviously the survival of GaHV-2 in the environment is favoured by the cooler weather, reduced ventilation in the winter months also contributed to the higher incidence of disease (Purchase, 1985). This seasonal variation was also confirmed in a recent study in Australia where higher GaHV-2 loads were detected in broiler house dust in the winter months (Walkden-Brown *et al.*, 2013a).

Furthermore, there are regional differences in MD incidence in some countries. In the USA, the 1960s MD outbreaks were initiated in the Delmarva and north-eastern regions. Throughout history, Delaware has consistently had the highest condemnation rates whereas Georgia had the lowest. However, after the HVT vaccination was introduced for broilers in the 1970s, carcass condemnation in the USA declined dramatically and between 1971 and 2000 it decreased 79-fold, 169-fold, and 958-fold in Delaware, the USA overall, and Georgia, respectively (Schat & Nair, 2008). Between 2001 and 2011 the condemnation rates of broilers in USA continued to reduce, with the lowest rate recorded in 2007 (Dunn & Gimeno, 2013). Similar findings for regional variation were reported from a recent study in Australia using broiler dust samples (Walkden-Brown *et al.*, 2013a).

However, the Australian data mentioned above is dependent on direct measurement of GaHV-2 from poultry house dust samples whereas the data from USA are dependent on post-mortem analysis and carcass condemnation data. Hence in the latter situation, the data is dependent on gross pathology and not on measuring the viral load.

1.6.2 Determinants of MD occurrence

1.6.2.1 Host factors

1.6.2.1.1 The lifespan of the bird

The longer birds live the greater the chance of their exposure to GaHV-2 and of infection to result in MD; hence, obligatory vaccination is practised for layers and breeders but not broilers. Once infected with GaHV-2, the infection will persist for life with recurrent replication and shedding (Witter et al., 1971) With extended grow-out periods of broilers to increase meat production, these birds now also potentially reach the age when they shed GaHV-2 into the environment, which subsequently acts as an infective source for the next batch (Groves, 1995). Therefore, the infected but not clinically ill broilers and layers serve as reservoirs of infection for the naïve birds in multi-age farming systems. Furthermore, as mortality in these GaHV-2-infected chickens is prevented and the lifespan is increased by the vaccines, they continue to shed pathogenic GaHV-2 to the environment throughout their lives.

1.6.2.1.2 Genetic resistance

Genetic selection for resistance was thought to be the answer to reducing MD incidence before vaccines were developed. Asmundson and Bailey (1932) observed that there was large variation in MD mortality between six poultry breeds even before the MD causative agent was identified. Significant differences in GaHV-2 infection rates were also observed in some breeds of meat chickens (Walkden-Brown *et al.*, 2008). Chicken lines which were resistant and susceptible to MD were subsequently developed at Cornell University (lines N and P) and at the USDA ADOL in East Lansing (Lines 6 and 7) (Stone, 1975). Genetic resistance was reported to have little correlation with production traits in many studies (Biggs *et al.*, 1968b; Ameli *et al.*, 1992). Later, genetic resistance was found to be associated with the B blood group locus (Hansen *et al.*, 1967). As the B blood group locus was a marker for the chicken genes of the major histocompatibility complex (MHC), genes of the MHC were identified as being associated with resistance (Longenecker *et al.*, 1976; Briles *et al.*, 1977;

Bacon *et al.*, 2001). The B^{21} haplotype of the MHC was shown to confer resistance in all chicken strains, whereas B^{1}, B4/B^{13}, B^{5}, B^{12}, B^{15}, and B^{19} haplotypes were associated with susceptibility to MD. The resistant haplotypes are usually dominant or co-dominant to the susceptible haplotype (Calnek, 1985); however, the exact mechanism of resistance is still unclear. In addition, there are also non-MHC-associated genes influencing MD resistance (Pazderka *et al.*, 1975).

Both genetically resistant and susceptible birds when challenged with a virulent GaHV-2 became infected with GaHV-2 (Cole, 1968). However, the virus neutralising antibody levels were higher in resistant chickens. Moreover, the development of clinical disease, mortality, and the lymphoproliferative lesions in organs were significantly lower in resistant birds (Sharma & Stone, 1972). The analysis of Cornell University's MD-resistant and susceptible chicken lines revealed that the susceptible line had a higher viral replication rate (Lee *et al.*, 1981; Bumstead *et al.*, 1997) from a very early stage to death.

The early genetic selections for MD resistance were based on the degree of susceptibility to MD after challenge (Cole, 1968; Stone, 1975; Morrisroe, 1976). Regarding selection methods, family selection (Cole, 1968) and mass selection (Maas *et al.*, 1981) for resistance have been practised. At first these studies were carried out on unvaccinated chickens, but Bacon *et al.* (1992) suggested that selection be made on vaccinated birds. Bacon *et al.* (2001) also suggested that mass selection may not be appropriate for commercial breeders due to loss of other favourable traits due to high mortality. Selection based on alleles of the MHC has been also studied (Gavora *et al.*, 1986; Lakshmanan *et al.*, 1997), specifically the B^{21} haplotype (Briles *et al.*, 1977), but negative associations were observed with some genetic traits for production (Gomez *et al.*, 1991) limiting the value of this approach. Selecting for non-MHC genes in commercial lines may be more important than selecting for MHC-associated genes. Bumstead *et al.* (1998) and Yonash *et al.* (1999), after mapping area-conferring genetic resistance, reported on 14 genes which confer resistance which are not associated with the MHC.

Furthermore, selection for multiple traits for immunity (Lamont *et al.*, 1996; Gao *et al.*, 2015), marker-assisted detection of quantitative trait loci (QTL) (Vallejo *et al.*, 1998; Liu *et al.*, 2001) and allele-specific genes associated with MD resistance (Perumbakkam *et al.*, 2013) have been conducted.

Although the genetic selection for MD resistance became less important after vaccines were introduced to control the disease, it has been found that vaccinated resistant chickens are more protected from MD challenge than their vaccinated susceptible counterparts (Spencer *et al.*, 1972; Zanella *et al.*, 1975; Chang *et al.*, 2014; Hunt & Dunn, 2015). Therefore, in addition to vaccination, genetic resistance still plays an important role in controlling MD, especially with emerging new pathogenic GaHV-2s. Walkden Brown *et al.* (2008) has demonstrated two commercial broiler strains were significantly different in MD resistance.

1.6.2.1.3 Active immunity (vaccination)

The introduction of MD vaccines controlled the disease successfully at a stage when acute MD outbreaks resulted in up to 30% mortality in poultry flocks (Churchill *et al.*, 1969; Okazaki *et al.*, 1970; Rispens *et al.*, 1972a; Schat & Calnek, 1978b). Some of these initial MDV vaccines, such as HVT and Rispens vaccine, are still widely used and are effective in the field.

Most MD vaccines are cell associated; however, HVT vaccines can be both cell free and cell associated. The cell-free HVT vaccine was as effective as the cell-associated HVT vaccine in chickens without maternal antibodies. However, for chickens with maternal antibodies, cell-free HVT provided lower protection due to interference from maternal antibodies (Witter & Burmester, 1979; Jackson, 2000). In comparison, GaHV-2 Rispens vaccines provide a better protection than GaHV-3 and HVT vaccines for more virulent MD strains (Witter, 1998b; Witter *et al.*, 2005).

The route of vaccine administration also has an effect on protection and subsequent MD occurrence. Generally, intraperitoneal and intramuscular routes give better protection than subcutaneous inoculation, while intranasal administration offers minimal protection (Rispens *et al.*, 1972a; Geerligs *et al.*, 2008). The intramuscular and subcutaneous vaccination routes are generally practised in the field and generally a dose of 2000 PFU is administered per chick (Schat & Nair, 2008). Intraocular and intranasal administration of MDV vaccines have been found to be ineffective (Purchase *et al.*, 1971b; Rispens *et al.*, 1972a). On the other hand, intra-embryonic or *in ovo* vaccination on the 18th day of incubation provide effective protection against early MD exposure (Sarma *et al.*, 1995; Reddy *et al.*, 1996; Ricks *et al.*, 1999) and is now widely used to vaccinate broilers with the HVT vaccine using a fully automatized process.

The vaccination challenge interval (VCI) has a major effect on subsequent MD occurrence for many MD vaccines. The ADOL pathotyping method proposed by Witter *et al.* (2005) uses a VCI of five days. When the VCI is less than five days, the protection provided by the vaccine may be limited due to insufficient time to mount an active immune response. This phenomenon was demonstrated clearly in various studies using HVT (Islam *et al.*, 2007) in broilers and Rispens vaccine in layers (Islam *et al.*, 2013b). When the VCI is increased to more than five days, both HVT (Islam *et al.*, 2008) and Rispens vaccine (Baigent *et al.*, 2007; Islam *et al.*, 2013b) vaccines were more successful in reducing the incidence of MD. Therefore, it is of utmost importance for the vaccination to be carried out before early GaHV-2 challenge in the field; as well as providing an additional advantage of the ease and labour saving achieved and *in ovo* vaccination.

The development of active immunity following vaccination is similar to that following natural infection with milder or low virulence strains of MDV (Biggs, *et al.* 1972; Rispens *et al.*, 1972a, 1972b).

1.6.2.1.4 Passive immunity (maternal antibodies)

Anti-MDV antibodies which are transferred from MDV vaccinated parent hens to chicks via the egg yolk are protective for the first three weeks of the chick's life. The effects of maternal antibodies (mab) for the development of MD are: reducing the levels of MDV viral antigens, lowering the frequency of tumours, delaying the onset of MD, reducing the destructive effect of MD on myeloid and lymphoid tissues, and reduction of MD mortality (Calnek, 1972; Payne & Rennie, 1973; Lee & Witter, 1991). Maternal antibodies for MD do not prevent infection with GaHV-2 (Calnek, 1972) but protect against the early mortality/paralysis syndrome associated with infection with GaHV-2 isolates of higher virulence (Walkden-Brown *et al.*, 2007; Read *et al*., 2015), when compared with chickens without mab (Gimeno *et al.*, 1999; Witter *et al.*, 1999).

One of the deleterious effects of the mab is their interaction with vaccines. When a chick is vaccinated against MD, the humoral antibody response is delayed by the existing maternal antibodies. The adverse effects of the mab for vaccines are higher for homologous antibodies than heterologous antibodies (King *et al.*, 1981). Therefore, this knowledge has prompted the chicken industry to vaccinate chickens of different generations with different serotype vaccines (Eidson *et al.*, 1978; Witter & Burmester, 1979). Furthermore, the cell-free MDV

vaccine HVT interact with mab more than cell-associated HVT vaccines (Calnek & Smith, 1972; Witter & Burmester, 1979; Jackson, 2000).

1.6.2.1.5 Host range

Chickens are the most important natural host of the GaHV-2, but the disease has been reported in other commercial poultry such as quails, turkeys, and pheasants. Other subspecies of fowl, including guinea fowl and jungle fowl are susceptible to GaHV-2 infection (Cho & Kenzy, 1975). The clinical signs of MD in quail are very similar to those seen in chickens and outbreaks have been reported in Japan (Kobayashi *et al.*, 1986; Imai *et al.*, 1991). Infected birds develop tumours in various organs, even though there are no pathological lesions in peripheral nerves (Kobayashi *et al.*, 1986). MD in turkeys is not a significant economic problem and vaccination is not generally used as a control measure. However, some acute MD outbreaks have been recorded in some countries, such as Scotland (Pennycott & Venugopal, 2002), Israel (Davidson *et al.*, 2002b) and Germany (Voelckel *et al.*, 1999). There is evidence on interspecies transmission of MD from quails to chicken (Imai et al., 1990) and turkey to chicken (Coudert et al., 1997) and vice versa.

1.6.2.1.6 Host sex

Various studies reported higher mortality in females following GaHV-2 challenge (Purchase & Biggs, 1967), with earlier deaths than males. However, in a recent study with Australian GaHV-2 isolates the males had a higher mortality rate than females in the early paralysis phase of the disease (11-16 dpc). The death rate of female birds at the lymphoma phase (34-55 dpc) was higher than that of males (Renz *et al.*, 2012).

1.6.2.1.7 Age at exposure

Susceptibility to infection with GaHV-2 is not dependent on age, with both older and newly hatched chicks being fully susceptible to cytolytic infection with GaHV-2 (Calnek, 2001). Furthermore a study by Witter & Gimeno (2006) confirmed that SPF chickens between 18-102 weeks are susceptible to infection with highly virulent MDV strains.

However, the cytolytic infection in older chickens is resolved more promptly (Buscaglia *et al.*, 1988), and the viral load also appears to be lower compared to day-old chicks. The occurrence of MD has been found to be lower in 4½-month-old chickens compared to day-old chickens when exposed to GaHV-2 (Calnek, 1973), and this is especially seen in genetically resistant lines. Furthermore, it has been observed that mortality and detection of

meq oncogenes after GaHV-2 challenge was lower in 96 week old mab-negative chickens when exposed to GaHV-2 compared to younger 14-week-old chickens (Ikezawa *et al.*, 2012).

the study confirmed that SPF chickens between 18-102 weeks are susceptible to infection with higly virulent MDV strains.

Sharma *et al.* (1973) concluded that the mechanism for age-related resistance to MD was lesion regression. In a study with neonatal thymectomised birds, the age-related resistance was attributed to an enhanced cell-mediated immunity rather than to an antibody-mediated response (Sharma *et al.*, 1973). Therefore the age related resistance to MD development is associated with maturation of host immune system and development of immunocompetence (Baigent & Davison, 2004).

1.6.2.2 Pathogen factors

1.6.2.2.1 Virulence of challenge MDV

The virulence of GaHV-2 strains varies widely (Witter, 1997). Variations in the virulence of GaHV-2s play an important role in the incidence of MD (Biggs, 1997). GaHV-2 strains may be classified according to their virulence into several pathotypes (refer to section 1.5.1). The more virulent strains cause higher MD incidence, mortality, and lymphoma in both susceptible chickens vaccinated with HVT and genetically resistant unvaccinated chickens.

In Australia, virulent and very virulent GaHV-2 pathotypes have been identified, however no vv+ viruses have been isolated so far. 04CRE and MPF57 have been classified as vMDV, whereas Woodlands FT158 and 02LAR have been classified as vvMDV according to pathotyping studies using an adaption of the ADOL method (Walkden-Brown *et al.*, 2007; Renz *et al.*, 2012; Walkden-Brown *et al.*, 2013b).

1.6.2.2.2 Dose/Level of exposure to challenge strain

Dose of the GaHV-2 virus strain may have an effect on the subsequent MD occurrence in natural situations. However a very low dose of some virulent GaHV-2 strains may instigate a severe MD response in highly susceptible chickens (Smith & Calnek, 1974).

1.6.2.3 Environmental factors

The seasonal variations for MD incidence are described in section 1.6.1. Other than these factors, broiler farms with more than four sheds and farms within 2 km of other farms were

found to be risk factors associated with the presence of GaHV-2 in shed dust. Biosecurity measures on farms, such as provision of protective clothing for visitors and using wood litter material, has been important for reducing GaHV-2 occurrence in dust (Groves *et al.*, 2008) and thereby reducing the spread of MD.

MD has an immunosuppressive effect on its host thus increasing susceptibility for subsequent secondary infections with bacterial, viral and other pathogens (Biggs *et al.*, 1968a; Abbassi *et al.*, 1999). Moreover, when the coexisting infection is also suppressing host immunity, both disease outcomes are aggravated. This has been observed in concurrent infections with reticuloendothelial virus (Witter *et al.*, 1979), chicken anaemia virus (Jeurissen & De Boer, 1993), and infectious bursal disease (von Bülow *et al.*, 1986). The presence of red mites and rats which may carry GaHV-2 infected dust could also predispose the occurrence of MD.

1.6.3 Transmission

GaHV-2 is transmitted by contaminated feather follicles either by direct or indirect contact with chickens (Biggs & Payne, 1967; Purchase, 1985; Abdul-Careem *et al.*, 2009). Fully infectious viral particles are shed only in keratinised epithelial cells of the feather follicles (Calnek *et al.*, 1970). These cells contaminate the environment and act as a source of infection to susceptible chickens. However, cell-free transmission of GaHV-2 is also possible (Nazerian & Witter, 1970). Feather dander is therefore the most important source of infection, (Beasley *et al.*, 1970) and it is an important component of poultry dust. GaHV-2 in poultry dust can remain infective for several months to years depending on the temperature of the environment (Carrozza *et al.*, 1973).

The young flock becomes infected from dust and dander remaining in sheds or pens or through contact with fomites, aerosols or people contaminated with them. Once GaHV-2 infects a chicken, even if it is vaccinated, the virus will replicate, shed into the environment and transmit horizontally. Infected chickens appear to shed the virus throughout their lifetime (Witter *et al.*, 1971).

MD does not transmit vertically (Solomon & Witter, 1973) and it is not transmitted to the progeny by egg contamination as the temperature and humidity conditions used during incubation do not favour MD virus survival (Calnek & Hitchner, 1973).

1.7 Pathogenesis

1.7.1 Entry of MDV into the host

Susceptible chickens are infected with GaHV-2 by inhalation of infected dander or dust (Figure 1.2). Scientists have tried to mimic these natural infections and, infection models for MD using aerosols containing GaHV-2 have been successfully developed by Abdul Careem *et al.* (2009). The portal of entry of MDV into the host is thought to be the lungs of the chicken. Probably, cell-free viruses can enter the circulation following binding to the lung epithelial cells with their receptors (Adldinger & Calnek, 1973). The viruses with keratinised epithelial cells may be phagocytised by macrophages and similarly enter the blood stream (Figure 1.2).

Calnek postulated that, after entry of the virus, the pathogenesis of MD can be divided into four phases. This is known as the Cornell model (Calnek & Witter, 1985; Calnek, 2001). These sequential events occurring in a bird infected with MDV are: 1) the early cytolytic phase, 2) the latent phase, 3) the late cytolytic phase, and 4) transformation. The demarcations of these phases are not very clear. At a given time, phases two to four can exist in the cells of the same host and sometimes when mab-negative chickens are infected with a vv+ GAHV-2 mortality may occur without the virus entering the latent phase. The presence of the late cytolytic phase is dependent on the genetic resistance of the birds. These events were mostly studied in mab-negative specific pathogen free (SPF) chickens so may differ somewhat from the field situation.

One of the important characteristics of pathogenesis of herpesviruses is the integration of the virus into the host genome in the latently infected cell; this can be also observed with GaHV-2 (Delecluse & Hammerschmidt, 1993; Robinson *et al.*, 2014). This latently infected GaHV-2 can be activated in the transformed lymphoma cells leading to the second wave of cytolysis (Delecluse *et al.*, 1993).

1.7.2 Early cytolytic phase

The main target cells of the early cytolytic phase are B lymphocytes (Shek *et al.*, 1983). However, a few T cells, found to be CD4+ and CD8+ T cells (Baigent *et al.*, 1996; Baigent *et al.*, 1998; Baigent & Davison, 1999), are also involved in this phase (Calnek *et al.*, 1984a; Calnek *et al.*, 1984b). As resting T cells are refractory to infection with MDV, these T cells are activated T cells expressing TCRαβ1 and TCRαβ2. As a result, transient atrophy of bursa

and thymus can be observed. As the thymus consists mostly of T lymphocytes; thymocytes can undergo massive apoptosis due to GaHV-2 infection (Baigent *et al.*, 1998) or cytokine reactions.

In the spleen, changes in the pro-inflammatory cytokine expression can be observed in splenocytes. Cytokines IFN-γ mRNA, IL-1β, IL-8, IL-6, IL18, and inducible NO synthase are upregulated (Xing & Schat, 2000; Djeraba *et al.*, 2002; Jarosinski *et al.*, 2002). The upregulation of these cytokines may explain enlargement of the spleen (Calnek *et al.*, 1979) as a consequence of hyperplasia of lymphoid and reticular cells. It has been discovered that the genetically resistant line 6 chickens have a significantly fewer infected B lymphocytes in the spleen than susceptible line 7 chickens (Lee *et al.*, 1981). Baigent *et al.* (1996; 1998) showed that there are more cells expressing pp38 in line 7 chickens than in line 6 chickens. Furthermore, in line 7 birds there were B cells expressing pp38+ surrounded by TCRαβ1+, CD4+, and CD8+ cells facilitating transfer of GaHV-2 from B cells to T cells. Therefore it appears that the B cells of line 7 birds were more susceptible to infection with GaHV-2 than the line 6 birds.

IFN-γ is activated 3-4 days post infection (dpi), and may upregulate the IL-8 receptor of T cells, then *vIL-8* can attract activated T cells to cytolytically infected B cells. This may enable transfer of the GaHV-2 from B cells to T cells (Schat & Xing, 2000; Parcells *et al.*, 2001). This process has been explained by using mutants lacking *vIL-8* wherein the early virus replication and later tumour formation was significantly reduced (Parcells *et al.*, 2001; Cortes & Cardona, 2004; Cui *et al.*, 2004). The *pp38* gene expression is also necessary to cytolytically infect B cells (Reddy *et al.*, 2002; Gimeno *et al.*, 2005b).

The early cytolytic infection is a semi-productive phase as no cell-free virus is produced and only non-enveloped intranuclear particles are produced (Baigent & Davison, 2004).

The early cytolytic infection can be reduced by vaccination and the presence of mab (Calnek, 1972; Smith & Calnek, 1974; Calnek *et al.*, 1980; Schat *et al.*, 1982), which also results in a reduction of the cells that get latently infected and transform into tumours. When one-day-old chicks are infected with GaHV-2, there is a prolonged early cytolytic phase, compared with older 2- to 7-week-old chicks (Buscaglia *et al.*, 1988). In addition, infection with vv and vv+ strains can cause severe lymphoid atrophy and early death in the absence of lymphomas (Witter *et al.*, 1980; Calnek *et al.*, 1998).

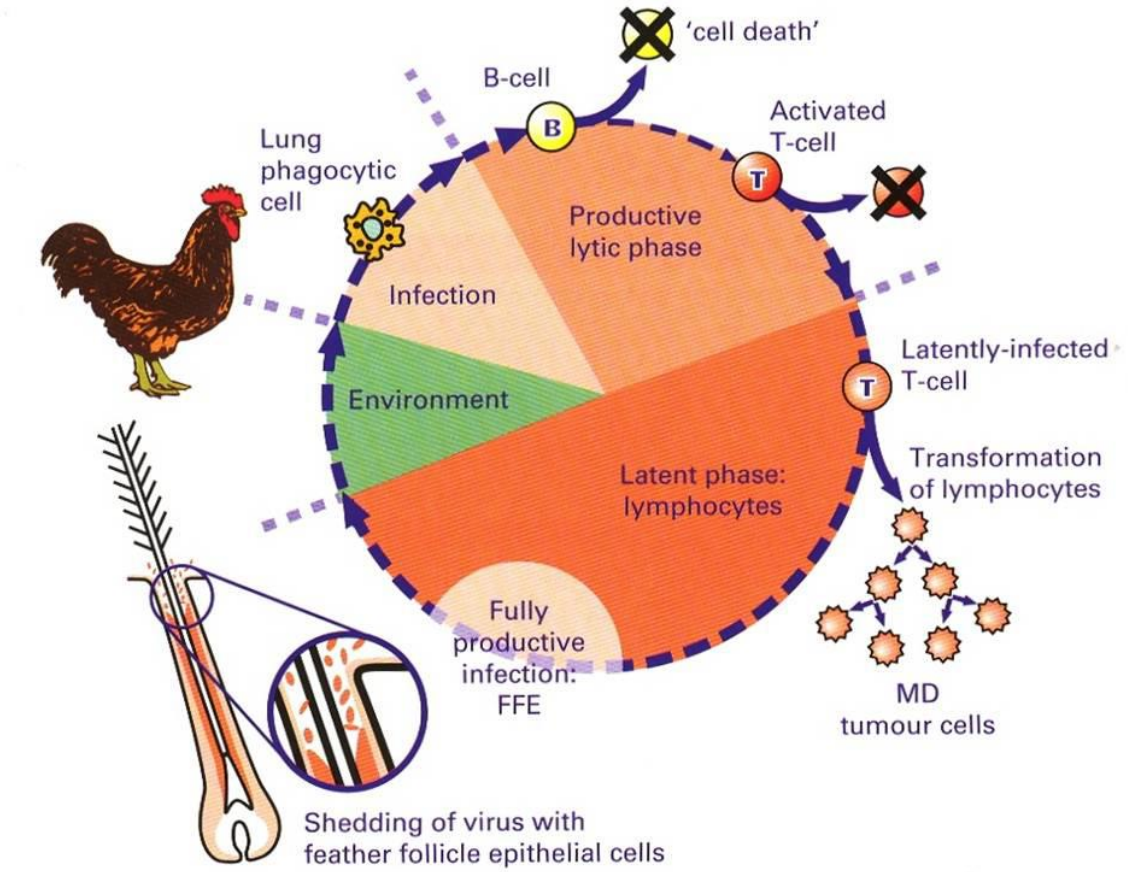

Figure 1.2 Stages in the cycle of MD pathogenesis.

Birds acquire infection by inhalation of infectious virus shed from the feather follicle epithelium (FFE). Following early replication in the lungs, the virus replicates in the lymphoid organs. B-cells go through a lytic infection, resulting in the activation of T cells, which are target cells of the virus. Throughout the lytic infection, noticeable cytolysis in both B and T cells (cells with cross mark) occur. T cells are transformed by the virus to produce tumours in different organs. Infected T cells convey the infection to the FFE. Adapted from Baigent & Davison (2004).

1.7.3 Latent phase

Six to seven days after infection with GaHV-2, the expression of GaHV-2 antigens cannot be observed as cytolytic damage begins to resolve and the infection becomes latent. Being a herpesvirus, MDV infection of a chicken will be lifelong with recurrent limited replications of the virus (refer section 1.5.1), especially in genetically resistant birds (Witter *et al.*, 1971).

The T lymphocytes affected are mainly CD4+ T cells; these are the target cells for the latent phase. However, CD8+ cells and B cells can be involved (Shek *et al.*, 1983; Calnek *et al.*, 1984b; Ross *et al.*, 1997). The importance of T cells in latency was demonstrated by Schat *et*

al. (1981) in embryonically bursectomised chickens challenged with GaHV-2. Furthermore, soluble factors like IFN-α, IFN-γ, latency maintenance factor, and NO may have a role in initiating latency (Buscaglia *et al.*, 1988; Xing & Schat, 2000) and the *meq* gene has a role in maintaining the latency (Parcells *et al.*, 2003). In the latent phase, viral transcription is only limited to the production of latency-associated transcripts (LATs) (Cantello *et al.*, 1994; Cantello *et al.*, 1997).

The host immune responses appear to be crucial in induction and maintenance of the latency period (Buscaglia *et al.*, 1988) as younger day-old immunologically immature birds fail to enter latency when challenged compared to more immunocompetent older birds. In general, chickens which are genetically susceptible and infected with vvMDV enter a second pathological cycle two to three weeks after infection (Calnek & Witter, 1985). The latently infected lymphocytes will transform into lymphoblastoid cells in the biological cycle of GaHV-2 (Baigent & Davison, 2004).

1.7.4 Late cytolytic phase

In MD-susceptible birds there can be a wave of a second semi-productive, cytolytic infection 2-3 weeks after the initial infection. In addition to the birds being permanently immunosuppressed, this phase is associated with lymphoma formation. The lymphoid organs and epithelial tissues of adrenal glands, proventriculus, and kidney are often affected during this phase. The bursa and thymus can be atrophied and a massive influx of mononuclear cells can be observed in epithelial tissues after the necrosis of lymphocytes and epithelial cells (Adldinger & Calnek, 1973; Calnek, 2001).

This second phase of cytolytic infection is also dependent on the virulence of the challenge GaHV-2 and can be seen in birds infected with vv and vv+ MDV (Schat & Nair, 2008).

1.7.5 Fully productive infection in the feather follicular epithelium

The virus is probably carried to the skin epithelium by latently infected peripheral blood lymphocytes and can be detected 10-12 dpi. After 13 dpi, virus replication in the skin becomes fully productive, and enveloped cell-free virus particles are released (Calnek *et al.*, 1970). Infection of FFE and shedding of free virus is seen regardless of whether the bird is genetically susceptible or resistant and whether the virus is mild or virulent. The shedding of FFE infected with fully infectious MD viral particles is the main method of entry of MDV

into the environment. Lymphoid aggregates can be seen in perifollicular dermis 7 days after infection containing small lymphocytes with inclusions, presumably GaHV-2 (Cho *et al.*, 1996). Viral DNA has been detected on 7 dpi in feathers for Rispens vaccine (Baigent *et al.*, 2005a). These lymphoid aggregates can either become necrotic areas or skin tumours. The skin tumours are associated with a limited number of pp38+ cells. Skin tumours in broilers are a major reason for carcass condemnations in the USA (Schat & Nair, 2008).

1.7.6 Lymphoma phase

The lymphoproliferative changes of the GaHV-2 life cycle may progress to tumour development, although in some cases regression of lesions has been reported depending on genetic resistance and age at infection (Sharma *et al.*, 1973). Mortality due to lymphoma can occur at any time after 3 weeks depending on host resistance, immunity, and virulence of the infecting MDV.

MD lymphomas comprise several types of lymphocytes including tumour, inflammatory and immunologically committed and non-committed cells (Payne & Roszkowski, 1972). Although both T and B cells are present, there are more T cells than B cells (Payne & Rennie, 1976; Baigent, 1995). The target cells for transformation are CD4+ cells and they express TCRαβ1, TCRαβ2, and also MHC class II receptors (Schat *et al.*, 1991). The lymphomas in visceral organs are usually proliferative in character, whereas in skin, eye and nerves, inflammatory lesions may predominate. A phenotype analysis of tumour cells revealed that, double negative T cells ($CD3^+$, $CD4^-$, $CD8^-$ and $CD3^-$, $CD4^-$, $CD8^-$) are also transformed (Schat *et al.*, 1991; Okada *et al.*, 1997). MD tumours and cell lines derived from tumours, express additional antigens such as MATSA (Marek's disease Tumour-Associated Surface Antigen) and AV37. Furthermore some MD lymphomas contain cells that express chicken foetal antigens at varying degrees, which indicates that tumour cell dedifferentiation varies among strains of chicken (Powell *et al.*, 1983). MicroRNA (miRNA) has also been found to have a role in oncogenesis (Zhao *et al.*, 2011; Hicks & Liu, 2013) and regulation of lymphoma formation (Yu *et al.*, 2014). It has been discovered that the GaHV-2 miRNA miR-M4 has a host cellular ortholog miR-155 and expression of these were responsible in induction of tumour formation (Zhao *et al.*, 2011).

Therefore, it has been hypothesised that activation of T cells in response to cytolytically infected B cells could be a major event of pathogenesis by providing a supply of T cells as the targets of transformation. This hypothesis was supported in studies by Calnek (1989a;

1989b), showing that tumour induction was heightened by enhancing a cell-mediated immune response against allogenic cells (antigenically different cells from the same species) at the site.

1.8 Clinical signs and pathobiology

The clinical signs of MD can vary according to the type of the syndrome; the main syndromes being the lymphoproliferative (lymphomas, nerve lesions, fowl paralysis, skin leucosis and ocular lesions), the lymphodegenerative (early mortality syndrome), the central nervous system (transient paralysis and persistent neurological disease), and the vascular syndromes (atherosclerosis) (Schat & Nair, 2008).

1.8.1 Classical MD/ fowl paralysis

Fowl paralysis is generally observed in older birds from 8-20 weeks of age. The signs are mainly asymmetric paresis, which leads to complete paralysis of one or more wings and legs. If the vagus nerve is involved, there can be paralysis and distension of the crop. In general, incoordination and stilted gait may be the first observed clinical signs. Drooping of one or both wings also can be observed (Schat & Nair, 2008). A classical clinical presentation is a bird with one leg stretched forward and one leg backward as a result of paralysis or paresis of a leg on one side (Pappenheimer *et al.*, 1929a). The chickens usually do not recover from the disease and die. Currently, this syndrome is rarely observed in commercial chickens.

1.8.2 Acute MD/lymphoma syndrome

Acute MD is mainly characterised by lymphomas in visceral organs of the chickens, and the onset of the disease can be observed in younger birds after 2-8 weeks post infection. The lymphomatous infiltrations are mainly seen in gonads, lung, heart, mesentery, kidney, liver, spleen, bursa, thymus, adrenal gland, pancreas, proventriculus, intestine, iris, skeletal muscle, and skin. This is the most common form of disease observed in the field and can result in up to 60% mortality in layers, unless prevented by vaccination. In acute MD, nervous lesions and signs are also common (Schat & Nair, 2008).

Chickens with visceral tumours may show fewer specific clinical signs and simply become depressed, then recumbent and comatose before death. Sudden death is also possible, particularly with involvement of the heart. In commercial poultry the onset of death may be accelerated by inability to reach food and water and sometimes due to trampling by other chickens. Nervous signs also can be seen in these syndromes.

1.8.3 Transient paralysis (TP) and persistent neurological diseases

There are two types of TP, classical and acute. In the classical form, birds recover some time after paralytic signs (Kenzy *et al.*, 1973) but die due to lymphoma development at a later stage of life, whereas in the acute form, death occurs very rapidly within 24-72 hours after the onset of paralytic signs (Witter *et al.*, 1999; Renz *et al.*, 2012). TP is found generally in 8- to 12-day-old experimental chickens and usually resolves within one to two days (Calnek, 2001). The syndrome is mainly due to vasogenic brain oedema (Swayne *et al.*, 1989a).

The acute paralysis (APS)/early mortality syndrome (EMS) has mostly been observed under experimental conditions with some strains of GaHV-2 (Payne & Rennie, 1976; Witter *et al.*, 1980; Witter, 1983; Nair *et al.*, 1996). There is only very limited data available reporting this syndrome in the field (Carvallo *et al.*, 2011), and this warrants further investigation. EMS is characterised mainly by depression, paralysis and sudden early death in 5- to 10-day-old chickens. In this syndrome there is no lymphoma formation. High mortality can be observed in EMS in young stock 8-16 days after infection with virulent MD viruses (Witter *et al.*, 1980; Nair *et al.*, 1996).

Gimeno *et al.* (1999) described another two neurological syndromes as late paralysis (late onset of neurological signs at 20 dpi) and a persistent neurological syndrome (persistence of neurological signs after recovery). The persistent neurological disease is seen in birds that have recovered from classical TP, but the birds continue to display nervous signs such as ataxia, tics or torticollis. All these neurological syndromes are mainly seen in mab –ve birds under experimental conditions and not in the field.

1.8.4 Lymphoma and other lesions of the eye

In some instances the GaHV-2-infected birds exhibit lesions of the eyes and this may lead to blindness (Ficken *et al.*, 1991). When the tumour involves the iris (iridocyclitis) its colour turns to grey, and this is commonly known as “grey eye”.

The other lesions of the eye include conjunctivitis (Ficken *et al.*, 1991), uveitis, keratitis, retinitis and even necrosis of the retina (Pandiri *et al.*, 2008).

1.8.5 Skin leucosis

Mortality from this syndrome is rare; however, economic loss occurs due to carcass condemnations of broilers. This condition is characterised by swollen feather follicles and the condition known as "red leg" in broilers.

1.8.6 Atherosclerosis

This syndrome is mainly observed in adult mab-negative birds under experimental conditions. After being challenged with GaHV-2, chickens develop a deposition of cholesterol in the arteries (Fabricant *et al.*, 1983; Fabricant & Fabricant, 1999). Clinical signs have not been observed with this syndrome.

1.8.7 Incubation Period

The incubation period in experimental birds after being inoculated with the virus on day 1 is about 3-4 weeks (Purchase, 1985). Infiltration of mononuclear cells into organs of experimental birds was found to be 5-7 days (Payne & Rennie, 1973) after infection and the clinical signs could be observed in the third or fourth week (Payne & Biggs, 1967).

Death of chickens occurs as early as 8-14 days post infection with EMS (Witter *et al.*, 1980). The virus causes cytolytic reactions in primary immune organs such as the bursa and thymus 3-6 days post infection and degenerative lesions can be observed after 6-8 days (Calnek, 2001). The clinical manifestations of TP in experimental conditions usually occur 8-18 days after infection (Kenzy *et al.*, 1973; Witter *et al.*, 1999).

MD can be first observed in 3-4 week old unvaccinated layers in field situations. When the chickens are vaccinated in commercial situations, MD outbreaks are known as "early" or "late" breaks (Witter, 2001b). These breaks are mainly due to the inability of the vaccine to provide adequate protection against disease, and late breaks are found to be more problematic.

1.8.8 Immunosuppression caused by MD

Immunosuppression induced by MD can be the result of loss of lymphocytes due to cytolytic replication of GaHV-2, virus induced changes in the regulation of immune responses and immunosuppression due to tumours. The first phase of impairment of immunity is seen in early cytolytic infection of lymphocytes with GaHV-2 which will die eventually. The cells involved are CD4+ T cells expressing $(TCR)_{\alpha\beta,}$ $TCR_{\gamma\delta}$ and CD8+ cells (Burgess & Davison,

2002; Barrow *et al.,* 2003). The GaHV-2 induced cell death is most likely due to apoptosis as in the case with other viral infections (O'Brien, 1988). CD4+ and CD8+ thymocytes can undergo massive apoptosis during the cytolytic period (Morimura *et al.,* 1996). The reduction of lymphocytes in the early cytolytic phase eventually will lead to enhanced tumour formation. Furthermore genes involved in immune mechanisms are upregulated or downregulated by GaHV-2 infections. The production of specific cytokines are upregulated, however upregulation of proinflammatory cytokines IL-6 and IL-18 in susceptible birds may enhance lesions and immunosuppression. The upregulation and down regulation also varies with the time after infection with GaHV-2 (Heidari *et al.,* 2010). The second phase and permanent immunosuppression can be observed in the second cytolytic infection which correlates with subsequent tumour formation. Immunosuppression when associated with transformed lymphoblasts may trigger another lytic cycle which may lead to loss of more B and T lymphocytes and results in bursal and thymic atrophy (Schat & Nair, 2008).

1.9 Pathology

1.9.1 Gross pathology

The most common pathological lesions in MD can be observed in visceral organs and nerves.

Lymphoma lesions can be observed in visceral organs including the ovary, liver, spleen, proventriculus, lungs, adrenal glands, kidneys, intestines, mesenteries, and heart (Fujimoto *et al.*, 1971), However, thymus, skeletal muscle, iris and skin may also be involved. The organ distribution is influenced by host genetics and virus strain. Visceral tumours are more common in infection with highly virulent GaHV-2 strains (Witter, 1997). These tumours may appear as either diffuse enlargement of the organs or may occur as focal nodular growths of different sizes.

Lesions of the skin are one of the important causes of condemnation of carcasses of broilers. Involvement of skin is associated with feather follicles which can be clearly seen as white coloured nodules in carcasses. Erythema is seen in shanks of broilers in infection with virulent strains, and this is commonly known in the USA as "Alabama redleg". Tumorous growth in comb and wattle can also occur, which leads to swelling (Ekperigin *et al.*, 1983).

Loss of pigmentation of the iris and irregularity of the pupils are gross lesions observed in eyes. In some studies lesions of the eye have been observed with conjunctivitis (Ficken *et al.*, 1991). Nevertheless, Witter (1997) reported that ocular lesions can be induced by field isolates of GaHV-2 both in unvaccinated and HVT-vaccinated chickens. Pandiri *et al.,* (2008) demonstrated early and late ocular lesions after GaHV-2 infection. The early lesions included mild to moderate lymphohistiocytic uveitis, whereas the late lesions consisted of severe uveitis, keratitis, pectenitis, viteitis, retinitis and retinal necrosis.

Enlargement of most nerves and nerve plexi are observed in MD; however, sciatic and brachial plexuses are more enlarged. The peripheral nerves in the acute form of the disease show enlargement and may exhibit yellow discolouration, loss of striations, and oedema. Usually these nerve lesions are unilateral and small, which necessitates comparison with the opposite nerves and in experiments, control birds of the same age (Schat & Nair, 2008).

In birds exhibiting EMS, there is severe atrophy of bursa and thymus present, whereas in birds with atherosclerosis due to MD, the coronary artery, aorta, and other major arteries are occluded by fatty depositions (Schat & Nair, 2008).

1.9.2 Microscopic Pathology

Two lymphoproliferative lesions can be identified in nerves. The first is tumorous and comprises infiltration of pleiomorphic lymphocytes. The second type is associated with inflammatory reaction and is characterised by infiltration of small lymphocytes with oedema and also demyelination of nerves. These changes were first described by Payne and Biggs (1967) as type A and type B lesions. Later studies (Lawn & Payne, 1979) revealed that cellular infiltration can occur in nerves as early as 5 days post infection with GaHV-2 in day-old chicks. Subsequently, type B lesions could be seen with destruction of the myelin sheath of nerves at 4 weeks.

The TP syndrome is mainly associated with vasogenic brain oedema and perivascular cuffing which can be most consistently observed in the cerebellum (Swayne *et al.*, 1989a, 1989b). In the EMS there are a large number of intranuclear inclusions in the thymus and bursa (Calnek, 1972; Nair *et al.*, 1996; Schat & Nair, 2008).

The lesions in visceral organs are more homogeneously proliferative than in nerves, and cellular composition consists of small to medium lymphocytes, lymphoblasts, and primitive

reticulum cells (Purchase and Biggs, 1967). As host immunity develops, macrophages can be seen in some tumours, but the cellular composition of the tumours does not differ from organ to organ.

The lesions of the skin are mostly inflammatory, but there are tumours as well associated with feather follicles. Aggregates of proliferating cells can be seen around dermal capillaries and histiocytes, and plasma cells can be seen in the dermis (Helmboldt *et al.*, 1963). Lymphoproliferative lesions can be seen in feather pulp and can be used as a tool for ante-mortem MD diagnosis (Cho *et al.*, 1998). GaHV-2 antigens and intranuclear inclusions are present in the tumorous nodules surrounding the feather follicular epithelium (Calnek *et al.*, 1970).

A commonly observed change is mononuclear infiltration of the iris. Ocular lesions were experimentally induced by Smith *et al.* (1974) who reported that cell infiltration also occurs in the optic and ciliary nerves. Pandiri *et al.*, (2008) demonstrated that GaHV-2 induced eye lesions could be observed at 6dpi, and consist of hypertrophy of endothelial cells, CD8+ lymphocytes, CD4+ lymphocytes and macrophages. Plasma cells and granulocytes were also observed by these scientists in the ocular lesions.

1.10 Control

1.10.1 Vaccination

The development of vaccines revolutionised the control of Marek's disease in the field. The first attempt at developing a vaccine that could be adapted for commercial use was in the UK (Churchill *et al.*, 1969). This vaccine was developed from a GaHV-2 strain known as HPRS-16 and was attenuated by passaging through chicken kidney cell cultures. However, the HPRS-16 vaccine was replaced by another vaccine developed in the USA based on HVT (Okazaki *et al.*, 1970; Witter *et al.*, 1970). The FC126 strain of HVT, which was first successfully used in the USA (Purchase & Okazaki, 1971), was adopted as a vaccine by many other countries, and is still widely used either alone or in combination with other vaccines. Both cell-free and cell-associated vaccines are available for HVT, but cell-associated vaccines are mostly used as they are less affected by the interference of mab (Witter & Burmester, 1979).

In 1972, a low pathogenic GaHV-2 field isolate in the Netherlands was developed into a vaccine named CVI988 (Rispens vaccine) after its accession number at the Central Veterinary Institute at Lelystad in the Netherlands and the developer of the vaccine Dr. Bart Rispens (Rispens *et al.*, 1972a, 1972b). This vaccine was found to provide good protection in both laboratory and field trials. Although the Rispens and HVT vaccines induced similar protection levels in early studies (Vielitz & Landgraf, 1971), later studies reported that Rispens vaccine provided greater protection than HVT in birds challenged with highly virulent GaHV-2s (Witter *et al.*, 1995; Buscaglia *et al.*, 2004; Witter *et al.*, 2005). It has since become the gold standard MD vaccine and is used worldwide, primarily to protect long-lived layer and breeder chickens, while broilers tend to be vaccinated mainly with HVT.

Vaccines were also developed from GaHV-3 isolates (Schat & Calnek, 1978a). The GaHV-3 vaccines, especially the SB-1 strain were combined with HVT (Schat *et al.*, 1982; Witter, 1982) to provide superior immunity compared to when either of them was administered alone.

More vaccines have been developed using the GaHV-2 strains, such as the US Md11/75C/R2/23 strain (Witter *et al.*, 1995) and Australian BH16 vaccine (Karpathy *et al.*, 2002, 2003). The latter vaccine provides a similar level of protection as the Rispens vaccine (Karpathy *et al.*, 2002, 2003) but has not been commercialised, whereas the former vaccine was not as protective as Rispens vaccine. Experimental recombinant vaccines have also been developed from all MDV serotypes (Ross *et al.*, 1993; Petherbridge *et al.*, 2003; Baigent *et al.*, 2006a; Singh *et al.*, 2010) as well as a recombinant fowl pox (Nazerian *et al.*, 1992) vaccine expressing MDV genes. A recently developed *meq* null (*meq* gene deleted) GaHV-2 vaccine (Lee *et al.*, 2010; Lee *et al.*, 2013) was found to offer more protection than Rispens vaccine for vv+ strains, both in the laboratory and in the field but has yet to be released for commercial use.

As early development of immunity is desirable, chicks are usually vaccinated at or before hatch. Administration of both cell-associated vaccines and cell-free vaccines may be by intramuscular or subcutaneous routes. The development of *in ovo* vaccination at the 18th day of incubation (Sharma & Burmester, 1982) with subsequent mechanisation and automation of this process (Johnston *et al.*, 1997) has led to widespread adoption of MD vaccination in the broiler industry. MDV vaccines of all serotypes have successfully conferred protection with *in ovo* vaccination. As most of the vaccines are cell associated, it is necessary to ensure

proper handling of vaccine in liquid nitrogen, proper reconstitution and completion of vaccination within one hour to ensure successful vaccination responses (Halvorson & Mitchell, 1979; Jackson, 1999). The cell-free lyophilised vaccines, unlike the cell-associated vaccines, can be stored at 4 ºC (Baigent *et al.*, 2006b).

1.10.1.1 Response to vaccination

MDV vaccines may protect chickens both through anti-tumour effects (Purchase *et al.*, 1971a; Hiramoto *et al.*, 1996), and anti-viral effects (Islam *et al.*, 2006a; Baigent *et al.*, 2011). Most commercially used MDV vaccines do not have adverse effects on chickens (Witter *et al.*, 1970; Rispens *et al.*, 1972a; Schat & Calnek, 1978a; Witter & Lee, 1984). However, it has been reported that HVT causes mild reduction of B and T lymphocytes (Islam *et al.*, 2002). Both antibody-mediated and cell-mediated immune responses (CMI) can be seen in MDV vaccination similar to virulent GaHV-2 strains. Anti-viral antibodies can be observed within 1-2 weeks post vaccination (Melchior *et al.*, 1973) and are present for the lifetime of the chicken (Rispens *et al.*, 1972a; Witter, 1982).

The antibody-mediated immune mechanisms against MD are not very clear. However, when CMI mechanisms are taken into consideration in highly protective (HP) MDV vaccines, these vaccines induce more T cells expressing MHC I and MHC II antigens than lower protecting (LP) MDV vaccines (Gimeno *et al.*, 2004). Furthermore, the HP vaccines replicate better *in vivo*, and induce higher expansion of total T cells including helper and cytotoxic T cells than LP vaccines. In feather pulp, MDV vaccines enhance expression of the IFN-γ and infiltration of CD8+ T cells (Abdul-Careem *et al.*, 2008b). One of the models on anti-tumour effects of MDV vaccine proposes that GaHV-2-infected cells are killed by CD8+ T cells and the remaining tumour cells are destroyed by natural killer cells (Morimura *et al.*, 1998). According to this model, infected CD4+ T cells are destroyed by apoptosis reducing targets for transformation and lymphoma development.

The vaccine viruses replicate systemically in the lungs and lymphoid tissues such as spleen, bursa and thymus similar to virulent GaHV-2 viruses. However the HP MDV vaccines have a higher replication rate in the lymphoid tissues when compared with LP vaccines (Gimeno *et al.*, 2004). The highest viral load of Rispens vaccine was demonstrated during the cytolytic phase (1-7 days) in spleen, bursa and thymus by Baigent *et al.*, (2005b). These scientists suggested that higher the replication of Rispens virus in spleen in cytolytic phase the higher

will be the stimulation of immune responses as main immune responses could be observed in the spleen.

The vaccine virus can be detected in peripheral blood leucocytes (PBL) as early as 4 dpv with the Rispens vaccine (Baigent *et al.*, 2005a) with a peak viral load at 7 dpv (Islam *et al.*, 2013a). GaHV-3 vaccines have been detected at 5-6 dpv peaking at 17 to 21 dpv (Calnek *et al.*, 1979; Witter & Lee, 1984), whereas the HVT vaccines have been detected 2 dpv and vaccine viral loads increased up to 61 dpv (Islam *et al.*, 2006a).

Shedding of vaccinal virus through the FFE has been reported with GaHV-2 Rispens vaccine and GaHV-3 and HVT vaccines (Witter *et al.*, 1970; Rispens *et al.*, 1972a; Islam & Walkden-Brown, 2007). The Rispens vaccine is effectively transmitted horizontally between chickens (Rispens *et al.*, 1972a; Islam *et al.*, 2013a). Furthermore, it has been observed that subsequent GaHV-2 challenge increased shedding of MDV vaccine viruses HVT and GaHV-3 (Islam & Walkden-Brown, 2007).

1.10.1.1.1 Factors affecting efficacy of vaccines

Dose

In order to achieve vaccinal protection against MD, the vaccine doses should be adequate to create infection in the chicken (Patrascu *et al.*, 1972; Purchase *et al.*, 1972; De Boer *et al.*, 1986). The general vaccine dose used commercially is 2000-6000 plaque forming units (pfu) per chick; however, for broilers this dose may be considerably reduced (Schat & Nair, 2008).

Increasing the dose of HVT vaccines against very virulent GaHV-2 strains has been found not to provide enhanced protection for chickens (Eidson *et al.*, 1978; Witter *et al.*, 1980). HVT doses between 125 and 64000 pfu provided protective indices of between 66 to 100% (Islam *et al.*, 2007), and had a significant effect on the PI. Gimeno et al., (2011) carried out an experiment in which the MD vaccines HVT and HVT/SB1 bivalent vaccine were diluted and administered to day old chickens and subsequently challenged with pathogenic GaHV-2. These scientists found that that dilution of these vaccines led to reduced MD protection.

Maternal antibodies

Refer to section 1.6.2.1.4

Interval between vaccination and challenge

Refer to section 1.6.2.1.3

Stress and immunosuppression

It has been reported that some infectious diseases of poultry hinder the initiation of vaccinal immunity due to immunosuppressive effects of those diseases. These are infectious bursal disease (Sharma, 1984), reovirus (Rosenberger, 1983), reticuloendothelial virus (REV) (Witter *et al.,* 1979) and chicken anaemia virus (Otaki *et al.,* 1988a; b). MD can be induced in vaccinated chickens that have been subjected to immunosuppressive treatment with betamethasone and corticosterone (Powell & Davison, 1986). The stress associated with high productivity and high stocking densities have made chickens susceptible to infection with GaHV-2 at a very early age (Witter, 2001a).

Route of administration

Refer to section 1.6.2.1.3

1.10.2 Management Procedures

In order to control MD, even when good vaccination practices are in use, strict biosecurity measures are necessary to prevent the early exposure of chicks to GaHV-2. Unfortunately most commercial layer farms do not practise all-in-all-out systems and have multiple age groups placed very close to each other. While broiler farms mostly use all-in-all out systems, in some countries litter is reused from batch to batch and this provides a good avenue for transmission of GaHV-2 as it is known to be a litter-transmitted disease and very persistent in the environment (Witter *et al.,* 1968; Carrozza *et al.,* 1973).

It has been shown in SPF flocks that MD can be controlled successfully by using strict biosecurity measures and using positive pressure poultry houses with filtered air (Drury *et al.*, 1969; Anderson *et al.*, 1972). However, these methods are too expensive to be practised on a commercial basis; therefore, adoption of common general hygienic measures is of more importance. It is essential to clean poultry houses for broilers as well as for layers and breeders (Pattison, 1985) and basic recommendations include:

- The litter should be removed and deposited as far as possible away from poultry sheds and the inside should be washed with a disinfectant using high-pressure cleaning devices.
- All dust and organic matter should be removed from the building.
- Disinfectants and insecticides should be used to clean surfaces of building
- After cleaning the building can be fogged with formaldehyde
- Poultry houses should be rested as long as possible before introduction of new litter and clean equipment. Generally four weeks of rest of poultry houses is recommended for breeder flocks (Pattison, 1985).

1.10.3 Genetic resistance

The vaccination responses are found to be better in MD resistant chickens than the susceptible chickens (Spencer *et al.,* 1974). Refer to section 1.6.2.1.2.

1.11 Diagnosis

1.11.1 Diagnosis of clinical disease

Diagnosis of MD in the field is still difficult as there are no pathognomonic lesions for MD even though clinical diagnosis has been documented since the 1970s (Siccardi & Burmester, 1970). Also, poultry can be simultaneously infected with other viral diseases such as LL virus and REV which can cause tumours and also gross enlargement of nerves (Davidson & Borenstein, 1999).

Diagnostic accuracy can be improved by considering the following steps:

- Clinical data and gross pathology – The flock history and post-mortem lesions, mainly a) leucotic enlargement of peripheral nerves, b) lymphoid tumours in visceral organs (heart, liver, gonads, skin, and muscle, and c) tumorous infiltrations in eye causing discolouration of the eye and irregularities of the pupil
- Histology, cytology, and histochemistry of frozen cells – Pathological tissue can be fixed or fresh frozen tissue can be used to prepare sections needed for these methods. The visceral tumours and nerve lesions of MD consist of lymphocytes, lymphoblasts, plasma cells and macrophages. Expression of the *meq* gene detected by immunohistochemistry is also a useful criterion for diagnosing MD lymphomas (Gimeno *et al.,* 2005a).

- Virological methods – Once the above two criteria have been satisfied more specific tests such as immunohistochemistry, fluorescent antibody test, and PCR can be used to confirm the diagnosis (Schat & Nair, 2008).

1.11.2 *In vitro* diagnosis

The principal methods to identify GaHV-2 infection in the laboratory are isolation of the virus, demonstration of viral DNA or antigens in tissue, and detection of GaHV-2-specific antibodies. Confirmation of diagnosis using two different tests such as virus isolation and the more sensitive polymerase chain reaction (PCR) test is ideal but may be costly. MDV vaccines and vaccination responses can interfere with some tests and diagnosis (Zelnik, 2004); however, tests are available which can distinguish between all three serotypes as well as wild-type GaHV-2 and vaccinal GaHV-2 (Becker *et al.*, 1992; Handberg *et al.*, 2001; Islam *et al.*, 2004; Renz *et al.*, 2013).

1.11.2.1 Virus isolation and identification

Earlier, electron microscopy was used to identify the morphology and structure of GaHV-2 (Nazerian & Burmester, 1968; Nazerian *et al.*, 1971). This method can be used as a diagnostic tool to identify viral particles in the FFE (Calnek *et al.*, 1970). However, it is not feasible to use electron microscopy in routine diagnosis due to the need for expensive and specialised equipment (Zelnik, 2004).

Ideally, virus isolation and propagation is necessary to identify the infectious agent and to isolate virus for further studies and characterisation. As the virus is cell associated, the sample material should ideally contain intact viable cells, but the virus can be present in cell-free preparations from skin, dander, feather or infected feather follicles (Calnek *et al.*, 1970). The samples generally used are blood lymphocytes, heparinised blood, splenocytes and tumour cells (Schat & Nair, 2008). The most widely used technique for primary isolation is inoculation of susceptible cell cultures with blood lymphocytes or single-cell suspensions from affected organs (Schat & Nair, 2008).

Chicken kidney cell cultures (CKC) and duck embryo fibroblast cell cultures (DEF) are generally suitable for primary isolation of GaHV-2 (Churchill & Biggs, 1967; Nazerian *et al.*, 1968; Solomon *et al.*, 1968). Chicken embryo fibroblast (CEF) can be used for GAHV-3 and MeHV-1, and also to isolate vaccine strains. However, some serotype 1 strains also grow well in CEF even in primary isolation (Tan *et al.*, 2008). The three MDV species can be

identified tentatively by their plaque size and morphology (Witter, 1983; Schat, 1985). MDV plaques are grossly visible signs of cytopathic damage in cell cultures with rounded refractile cells known as syncytia. The virulent GaHV-2 propagates in cell culture slowly with small to medium sized plaques appearing after 5-7 days post inoculation. However, the attenuated GaHV-2 plaques appear as early as 2-4 days post inoculation and produce larger plaques. Even though a tentative identification about the isolate can be made depending on plaque size and morphology (Zelnik, 2004), more specific techniques like immunohistochemistry using monoclonal antibodies for MDV may be needed for confirmation of diagnosis.

1.11.2.2 Detection and characterisation of MDV antigens and antibodies

1.11.2.2.1 Antigen detection

Several methods have been developed to detect viral antigens in infected tissue and cell cultures, after the MD virus has been successfully isolated in cell cultures. Development of monoclonal antibodies to type-specific epitopes of all three MDV species (Lee *et al.*, 1983) has been a great advancement in differentiation between MDV species. Fluorescent antibody techniques have been developed (Spencer & Calnek, 1970) to detect viral antigens in FFE, feather tips, brain or infected cell cultures. Other methods, such as agar gel precipitation tests (AGPT) (Haider *et al.*, 1970; Lesník *et al.*, 1981) and enzyme-linked immunosorbant assays (ELISA) (Scholten *et al.*, 1990), are also available to detect viral antigens.

AGPT used to be a common test and it can be still used to screen for MD. This test can be performed to identify antigen from infected cells, feather pulp or skin extracts (Haider *et al.*, 1970). In this test, serum containing specific antibodies for GaHV-2 reacts with the antigen in the wells of an agar gel plate (Chubb & Churchill, 1968). The formation of a precipitation line between the antigen and antibody in serum is regarded as a positive reaction.

Immunoflourecent (IF) assays to identify MDV antigens can also be practised, and specific MDV serotypes can be detected if monoclonal antibodies and monospecific antisera are used. These specific monoclonal antibodies are very useful in distinguishing GaHV-2 and HVT antigens (Ikuta *et al.*, 1982; Lee *et al.*, 1983; Dorange *et al.*, 2000). The most useful diagnostic approach, however, is to grow the virus in cell culture media, and identify it by immunofluorescence staining (Ikuta *et al.*, 1982; Lee *et al.*, 1983) or immunoperoxidase (Silva *et al.*, 1997).

Scholten *et al.* (1990) developed an ELISA technique to detect the GaHV-2 antigen in feather tips of infected birds using a monoclonal antibody. This technique was found to be more sensitive than AGPT.

1.11.2.2.2 Antibody detection

GaHV-2-infected birds have antibodies throughout their lifetime because once infected with GaHV-2, the infection is lifelong. Rather than laboratory diagnosis of field cases, specific antibody detecting tests have been useful in studies of the MD pathogenesis and monitoring of specific pathogen free (SPF) flocks. These tests include AGPT, ELISA, IF and virus neutralisation (VN) tests. All these tests are non-specific and do not differentiate between the three MDV serotypes (Schat & Nair, 2008).

If tests to detect antibodies in serum utilise fixed GaHV-2-infected tissue, culture monolayers and secondary labelled anti-chicken antibodies are required in order to elicit positive fluorescence (Zelnik, 2004).

ELISA for antibody detection

Cheng *et al.* (1984) developed an ELISA technique to detect MDV antibodies in serum. The basis of any ELISA test is the specificity of an antigen-antibody reaction. The ELISA test by Cheng *et al.* utilised CEF inoculated with MDV and HVT as the antigen.

This ELISA method was not adopted even though it was more sensitive than the indirect IF test. Zelnik (2004) suggested that when the antigen that is used to coat the ELISA plates is prepared using CEF it gives a higher background reading in the results. This is because the chickens have already developed antibodies against CEF-cultured MD vaccines.

This problem of higher background reading has been overcome by sourcing MDV antigen from CKC cultures, a different cell culture (Zelnik *et al.*, 2004) for the coating antigen of the ELISA plates. These assays can also be used to distinguish between infected and vaccinated chickens.

1.11.2.3 Detection of viral DNA

1.11.2.3.1 Polymerase chain reaction assays

Conventional PCR in MD diagnosis

The use of conventional PCR in MD diagnosis was first attempted by Silva *et al.* (1992) using a method that could differentiate pathogenic and non-pathogenic GaHV-2 based on the 132 base pair (bp) tandem repeat. Furthermore, this test can be used to differentially diagnose MD from LL and REV. In the early stages, PCR tests were developed with the aim to differentiate the serotypes of MDV (Becker *et al.*, 1992; Handberg *et al.*, 2001) and to diagnose natural GaHV-2 infections (Davidson *et al.*, 1995).

The advantages of PCR over the virus isolation methods are undoubtedly its superior sensitivity, less time consumption, and the fact that DNA isolated from crude biological material can be used for analysis. More expensive equipment and reagents when compared to serological methods are a disadvantage of this method. The inability of PCR to differentiate live intact virus from the inactivated virus particles is also a problem.

Real-time PCR

Real-time PCR is the collection of fluorescent signals from one or more polymerase chain reactions continuously over a range of cycles. Quantitative PCR (qPCR) is the conversion of fluorescent signals emitting from each reaction to a numerical value for every sample (Shipley, 2006). Real-time PCR is based on the same principle as conventional PCR; however, the detection of the desired amplified DNA can be visualised as the reaction is occurring (Lomeli *et al.*, 1989). Real-time PCR therefore requires a fluorescent molecule to associate with the target PCR product and report its presence (Kubista *et al.*, 2006). Typically this takes the form of a fluorescent probe that binds to the amplicon or non-specific fluorescent dyes such as SYBR-green that intercalate with DNA thus detecting the two stranded amplicon and any other double stranded DNA in the reaction at the point of reading.

The major importance of real time qPCR assay is its ability to quantify the genomic copy numbers in particular tissue (Bumstead *et al.*, 1997; Burgess & Davison, 1999; Islam *et al.*, 2004; Baigent *et al.*, 2005a) or an environmental sample such as shed dust (Islam *et al.*, 2006a; Renz *et al.*, 2006; Walkden-Brown *et al.*, 2013b). Furthermore, this method enables the study of the behaviour of pathogenic GaHV-2 (Baigent *et al.*, 2011; Cortes *et al.*, 2011;

Baigent *et al.*, 2013) and vaccinal GaHV-2 (Baigent *et al.*, 2005a; Baigent *et al.*, 2005b, 2007) over a given time period *in vivo* and provides additional insight into the pathogenesis of GaHV-2s. The kinetics of pathogenic and vaccinal viruses in single and mixed infections was extensively studied after qPCR assays were developed for those viruses (Islam & Walkden-Brown, 2007; Islam *et al.*, 2008; Islam *et al.*, 2014). The MDV qPCR assays are at least tenfold more sensitive than the conventional PCR (Islam *et al.*, 2004). As there is no handling of the PCR products after the procedure, the risk of carry-over contamination, are much lower than with conventional PCR.

Loop mediated isothermal amplification of DNA (LAMP)

LAMP is a more recent technique which does not require elaborate instruments such as thermocyclers, as the DNA amplification can be performed under isothermal conditions (50 – 60 ºC). This method uses a DNA polymerase and four to six primers, and the final result is stem-loop DNAs and this product can be measured by turbidity, fluorescence or colour change by using intercalating dyes such as SYBR-Green. This PCR amplifies DNA with high specificity, efficiency, and rapidity similar to PCR and real-time PCR.

LAMP has become very popular due to its relatively low cost and simplicity and many protocols have been developed to diagnose animal diseases using this test (Dhama *et al.*, 2014).

LAMP assays have been developed targeting the *meq* gene (Wei *et al.*, 2012) of GaHV-2 and to rapidly detect GaHV-2 in feathers (Wozniakowski *et al.*, 2011; Angamuthu *et al.*, 2012) and dust (Woźniakowski & Samorek-Salamonowicz, 2014). Furthermore, assays have been developed which distinguish the three MDV serotypes (Wozniakowski *et al.*, 2013).

One of the advantages of this assay is the comparatively low cost of the equipment and reagents. Moreover, the test is highly specific and rapid and can be developed into a quantitative test by the use dyes that intercalate with DNA.

1.11.2.3.2 Importance of PCR methods for MD diagnosis and research

The complete genome sequencing of all three serotypes of MDV (Tulman *et al.*, 2000; Afonso *et al.*, 2001; Izumiya *et al.*, 2001; Kingham *et al.*, 2001) has allowed the development

of PCR tests that distinguish them from each other (Davidson *et al.*, 1995). Many conventional and qPCR tests have been developed since the complete genomes were sequenced (Handberg *et al.*, 2001; Bumstead *et al.*, 1997; Reddy *et al.*, 2000).

In the first method that describes diagnosis of MD using qPCR, primers tagged with fluorescent dyes were used. This qPCR was similar to a conventional PCR and was run over 21 cycles and processed in an acrylamide gel, and the fluorescent bands were quantified using specialised software. The results of this qPCR method had a significant correlation with the development of disease in chickens (Bumstead *et al.*, 1997). This method was further developed to enable the quantification of the number of GaHV-2 genomes in two different lymphoma cultures (Burgess & Davison, 1999). Reddy *et al.* (2000) included a competitor DNA in addition to the GaHV-2 DNA and also confirmed that the more virulent GaHV-2 replicates better than the less virulent GaHV-2s. Islam *et al.* (2004) developed a Taqman® qPCR that could distinguish the three MDV serotypes using spleen samples of Australian field and vaccinal MDV strains.

Soon after the introduction of qPCR for MD diagnosis, quantitation of MDV from feather samples (Baigent *et al.*, 2005a; Cortes *et al.*, 2011; Haq *et al.*, 2012) was reported. The automation and use of qPCR methods enabled the study of the kinetics of GaHV-2 infection (Baigent *et al.*, 2005b; Islam *et al.*, 2006a) and its effect on viral loads during MD pathogenesis (Islam *et al.*, 2008). MDV vaccine strains have also been studied for quantitation (Renz *et al.*, 2006) and its kinetics either in single or mixed infections with pathogenic GaHV-2 (Islam *et al.*, 2006a; Islam & Walkden-Brown, 2007; Islam *et al.*, 2014). The co-infection kinetic studies of vaccine and pathogenic GaHV-2 led to an understanding of kinetics of the two viruses at a given time or over the first two months after infection (Islam & Walkden-Brown, 2007; Islam *et al.*, 2008). Quantification of viral genomes in dust rather than the tissue samples was also introduced (Islam *et al.*, 2006a; Renz *et al.*, 2006; Baigent *et al.*, 2013), which helped in monitoring vaccinal and pathogenic GaHV-2 in the field (Walkden-Brown *et al.*, 2013a).

Protection studies incorporated the measurement of pathogenic GaHV-2 and vaccine viral loads as an early marker of subsequent MD incidence (Islam *et al.*, 2008; Renz *et al.*, 2012; Islam *et al.*, 2013b; Walkden-Brown *et al.*, 2013b). Furthermore, the kinetics of MDV replication in resistant and susceptible chicken breeds has been studied using qPCR (Yunis *et al.*, 2004).

Initially, a standard PCR was developed to differentiate the GaHV-2 Rispens vaccine and pathogenic GaHV-2 by amplifying the 132 bp region, where the pathogenic GaHV-2 was thought to have only two tandem copies, whereas CVI998 has more than two tandem copies (Davidson *et al.*, 2002a). This test subsequently proved to return misleading results. More recent tests were developed that distinguished Rispens and pathogenic GaHV-2 successfully and were mainly based on sequence differences between Rispens and pathogenic GaHV-2s (Haq *et al.*, 2012; Renz *et al.*, 2013; Gimeno *et al.*, 2014). The assays developed by Renz *et al.* (2013), however, could only distinguish Australian GaHV-2 isolates from Rispens virus. As the Rispens vaccine is mostly used to vaccinate long-lived breeders and layers these qPCR methods are of immense use in the development of a field-monitoring test of the Rispens vaccine efficacy. The most recent assay by Gimeno *et al.* (2014) is a highly specific mismatch mutation assay (MAMA) that involves inserting an intentional mismatch base at the penultimate position of the primer (Cha *et al.*, 1992).

1.12 Rispens CVI 988 virus history and characteristics

1.12.1 History of Rispens virus

Rispens *et al.* (1972a) identified an apparently avirulent GaHV-2 from a flock of layers with a high frequency of virus isolation at 11 weeks of age but with no MD mortality or MD diagnosis in *postmortem* examinations. The virus did not cause MD on its 26th DEF passage when injected into chickens. Furthermore, it provided very good protection when these birds were exposed to chickens previously exposed to a virulent GaHV-2, on the same day of the vaccination. However, the virus caused minor microscopic lesions in mab-free chickens at 4th DEF passage level, but further passaging through DEF cell culture up to 26th level resulted in a completely avirulent GaHV-2 isolate. The vaccinated birds maintained high antibody levels for 2 years and virus shedding persisted throughout. Further clinical trials (Rispens *et al.*, 1972b) in the field suggested that inoculation of 3000 pfu of this virus into day-old chicks reduced the MD mortality in vaccinated broiler breeder chickens (0.15%) when compared with unvaccinated birds (14.3%) reared under similar conditions. Rispens vaccine virus was also found to successfully laterally transmit from unvaccinated to vaccinated chickens, but the time period between exposure to vaccinated birds and acquisition of complete protection was about 6 weeks.

The Rispens vaccine was licensed in the Netherlands and initially used in European and Asian countries. It was introduced into the USA much later, in 1994 (Bublot & Sharma, 2004), after it was shown to protect against vvMDV isolates (Witter, 1992; Witter, 1998a; Witter *et al.*, 2005). Witter (1996) also showed that the Rispens vaccine provides protection against the most virulent vv+MDV pathotypes (Witter *et al.*, 1995; Baigent *et al.*, 2006b). The Rispens vaccine was introduced into Australia in 1997 to help bring a major outbreak of MD under control (Jackson, 1998).

The Rispens vaccine is considered to be the gold standard of MDV vaccines at present and virtually all commercial long-lived chickens (breeders and layers) are vaccinated at hatch using Rispens vaccine (Bublot & Sharma, 2004).

1.12.2 Characteristics of the Rispens vaccine virus

Complete genome sequencing of the three MDV serotypes has revealed that the GaHV-2 genome encodes some unique genes including *meq* (Jones *et al.*, 1992), and *pp38* (Cui *et al.*, 1991; Chen *et al.*, 1992). Lee *et al.* (1983) developed monoclonal antibodies for the three MDV serotypes and Ikuta *et al.* (1985) performed further studies in this area. All pathogenic MDVs reacted with mab H19 except for Rispens virus. The difference was found to be a single nucleotide polymorphism of the *pp38* ORF at the 320th bp position. In pathogenic GaHV-2 viruses, the base was adenine coding for glutamine amino acid at 107th aa of the pp38 protein, whereas in Rispens virus it was guanine resulting in arginine (Cui *et al.*, 1998; Cui *et al.*, 2004). Haq *et al.* (2012) developed a real time PCR based on an SNP within *pp38* to differentiate virulent GaHV-2 and Rispens virus.

The attenuated GaHV-2 vaccine strain Rispens virus also encodes the *meq* gene, but differs from its oncogenic counterparts by having a 177 to180 bp insertion in the gene, and this is thought to be responsible for the non-oncogenic character of the vaccine strain (Lee *et al.*, 2000a; Chang *et al.*, 2002a; Spatz *et al.*, 2007). The LMeq protein encoded by *meq* gene of Rispens virus has been found to be a weak transactivator, which therefore further explains its non-oncogenic character (Ajithdoss *et al.*, 2009)

However, this was not found to be true for Australian GaHV-2s. The *meq* sequence of five Australian MD (O2LAR, FT158, Woodlands, MPF132/5, O4CRE, and MPF57) isolates and Rispens virus was reported by Renz *et al.* (2012). This study showed that the *meq* gene of all Australian MD isolates had the 177 bp insertion, yet were pathogenic, whereas Rispens

CVI988 had a 180 bp insertion. Based on this sequence variation, Renz et al. (2013) developed a specific qPCR test that can distinguish the Australian field isolates and Rispens vaccine. The PCR that differentiates a single nucleotide polymorphism (SNP) is not very efficient. Recently, Gimeno *et al.* (2014) developed a highly specific mismatch mutation assay (MAMA) to differentiate pathogenic GaHV-2 from Rispens virus modifying the Real-time PCR assay used by Haq *et al.* (2012).

Another important characteristic of Rispens virus is lateral transmission. The first authors who described the Rispens virus found that it can be effectively transmitted between chickens at its 35th passage level (Rispens *et al.*, 1972a). However, in subsequent studies a plaque purified clone of Rispens virus at the 65th passage level (Witter *et al.*, 1995) and Rispens CVI988 at the 42nd passage level (Witter, 1987) exhibited only limited transmission between chickens. The Netherlands Central Veterinary Laboratory provided the 33rd passage level of Rispens vaccine to the commercial vaccine manufacturers in the 1980s (Schumacher *et al.*, 2000). However, the passage level of the currently available commercial vaccines is thought to be at a higher passage level (40-45) and has been found to be efficiently transmitted between chickens (Islam *et al.*, 2013a). Furthermore, as commercial Rispens vaccines are shed in high amounts by vaccinated chickens (Islam *et al.*, 2013a) led to investigate in this thesis whether the Rispens vaccine has actually "escaped" from the vaccinated flocks and established itself in the free living MDV population in this thesis.

The protective efficacy of the Rispens vaccine against vv+MDV is superior to HVT and bivalent vaccines (Buscaglia *et al.*, 2004; Witter *et al.*, 2005). Perhaps one of the reasons may be Rispens and pathogenic GaHV-2 belong to the same species of MDV, hence immune responses provided Rispens vaccine may be more appropriate (homologous) than the response to other MDV species which is heterologous to GaHV-2. However, the protection provided by Rispens vaccine has, unlike the HVT vaccine, not been widely studied under commercial conditions with mab-positive chickens, despite its widespread use worldwide. Although Rispens vaccine provides superior protection overall, some studies have reported inadequate protection for some vv and vv+MDV strains (Witter *et al.*, 2005; Gong *et al.*, 2014). Protection for vMDV strains by Rispens vaccine has hardly been studied at all. Furthermore, the viral loads of Rispens and pathogenic virus have not been compared in most studies with regards to the vaccinal protection. When the vaccination challenge interval increases, Rispens vaccine provides superior protection (Islam *et al.*, 2014). It has been also

observed that the protective immunity of Rispens vaccine is generated late, and therefore maximum protection is not provided in early stages (Geerligs *et al.*, 1999). This could be overcome by combining Rispens vaccine with HVT (Geerligs *et al.*, 2008; Gong *et al.*, 2014).

Different Rispens vaccine strains at different passage levels have been studied to assess their protective efficacy. Witter *et al.,* (1995) demonstrated that protection provided by Rispens CVI988 (passage 40) was superior to that of CVI988/C (clone C). The first Rispens vaccine introduced to Australia was CV1988/C/R6 which was thought to be a good vaccine candidate in the preliminary trials. However it failed to provide sufficient protection for mab positive commercial chickens in subsequent field trials (Jackson, 1996). Furthermore, in recent protection experiments it has been found that different commercial preparations of Rispens CVI988 provide diverse protection levels for pathogenic GaHV-2 challenge (Lee *et al.*, 2010; Lee *et al.*, 2013; Chang *et al.*, 2014).

The kinetics of Rispens CVI988 virus has been studied using qPCR methods. The Rispens viral load once administered at hatch reaches its peak level in the spleen, bursa, and thymus at 7 dpv in the early cytolytic infection phase (Baigent *et al.*, 2005b). The Rispens virus can be detected after 7 dpv in feathers (Baigent *et al.*, 2005b; Haq *et al.*, 2012; Islam *et al.*, 2013a; Islam *et al.*, 2013b) and the viral load has been found to increase in feathers about 1000 times more than in the lymphoid organs. In PBL the viral load increases with time but remains at lower levels. The higher immunity induced by the Rispens vaccine is partly due to higher replication of the virus in the spleen during the cytolytic phase (Baigent *et al.*, 2005b).

The Rispens virus, when co-infected with a pathogenic GaHV-2 in a host, has a lower replication rate than the pathogenic virus. Furthermore, when vaccinated with Rispens vaccine prior to challenge with pathogenic GaHV-2, it reduces replication of the pathogenic GaHV-2 in co-infected hosts, which has been observed in viral loads in feather (Haq *et al.*, 2012; Islam *et al.*, 2014). However, in co-infected hosts the viral load of Rispens virus has a limited value in predicting the subsequent MD incidence (Islam *et al.*, 2014).

After the initial long-term studies of Rispens CVI988 (Rispens *et al.*, 1972a), limited studies have been carried out to monitor the long term kinetics, immunity, and protection level offered in the field by current commercial Rispens vaccines. As Rispens vaccine is an imperfect vaccine, the present levels of co-infection with pathogenic GaHV-2 with

commercial Rispens vaccines in the field also need to be investigated in order to avoid future vaccine breaks.

Finally, vaccines providing better protection than Rispens vaccine have been produced, but are yet to be employed for field use (Lee *et al.*, 2012; Lee *et al.*, 2013). Thus, Rispens vaccine is still the most widely used vaccine and it is of utmost importance that more protection and kinetic studies both under experimental and field conditions are conducted in order to develop and maintain useful vaccination and monitoring strategies in the field.

1.13 Conclusions

More than a century has elapsed since Jozef Marek, in 1907, gave the first description of Marek's disease as a paralytic disease in four cockerels. The disease became a major problem in the 1960s when the poultry industry became more intensified throughout the world. The causative agent of MD was only identified in 1968 (Churchill, 1968) and effective vaccines were discovered soon thereafter (Churchill *et al.*, 1969; Okazaki *et al.*, 1970; Rispens *et al.*, 1972a).

The vaccines successfully controlled MD in the field, but outbreaks were found despite vaccination, and GaHV-2 isolates were found to be increasing in virulence (Witter, 1998b). It is now postulated that the increase of virulence of MD may be driven by the vaccination itself (Witter, 1998b; Witter *et al.*, 2005; Atkins *et al.*, 2013). No MD vaccines, including Rispens CVI988, provide sterile immunity; they protect against clinical disease but not infection replication and shedding of wild-type virus (Gandon *et al.*, 2001; Atkins *et al.*, 2013). However, Rispens vaccine has been a highly effective vaccine even it was introduced in 1970s unlike HVT and HVT/ GaHV-3 bivalent vaccines. The reason for this could be the Rispens vaccine is only used in layers and breeders but not in broilers which is the largest poultry population. Therefore GaHV-2s in the meat chickens the population do not face selection for improved fitness in Rispens vaccinated hosts.

Rispens CVI988 vaccine is considered the gold standard of MD vaccines, but how long it will protect against the disease? With all the new tools and understanding of GaHV-2 that we have, how can we deploy these methods and knowledge to better understand and control the disease? From the currently available tools it is possible to compare the kinetics of the two viruses in the same host and factors influencing the kinetics. This has been studied mainly with vv+ and vv (mainly RB1B) viruses and generally with mab-negative chickens.

Therefore, it will be useful to study the protection provided by the vaccine for other GaHV-2 isolates of varying virulence with different genotypes of mab-positive birds and the associations of viral loads in tissue samples and dust with MD incidence.

Competitive kinetic studies have been used to investigate the difference in behaviour between Rispens and other wild-type viruses (Baigent *et al.*, 2011; Haq *et al.*, 2012; Islam *et al.*, 2014). However, viral loads in co-infected hosts in PBL and dust have been studied up to 56 dpi, but data on viral kinetics in feathers is sparse. The success of Rispens vaccination can be measured using feather, PBL and/or chicken dander. Feather and chicken dander are preferred samples in the field being relatively non-invasive and less expensive. However, limited long-term field studies have been carried out to determine which sample to collect and at what time to measure the vaccinal success (Rispens *et al.*, 1972a). The presence of co-infection of pathogenic GaHV-2 and Rispens viruses has also not been extensively studied in the field. Furthermore, studies have not been carried out to observe whether Rispens virus is transmitted and replicated in birds that have not been vaccinated with Rispens vaccine.

In this thesis I will investigate the effects of co-infection with Rispens vaccine with two virulent Australian field isolates. Consequently, I will study the type of samples to be collected and the time of sampling to measure the vaccine take. I will make long-term observations of the viral kinetics of the vaccine in the field, extent of birds infected with wild-type virus despite vaccination. I will also examine whether the vaccine virus has actually spread to unvaccinated flocks nearby.

2 General Materials and Methods

2.1 The experiments

Two major experiments were conducted during this PhD thesis. The first experiment was a protection and viral kinetic study in which chickens with maternal antibodies were vaccinated with Rispens CVI988 vaccine and challenged with a virulent GaHV-2 or a very virulent GaHV-2 isolate. The second experiment was a field experiment to study the efficacy of Rispens CVI988 vaccine under commercial conditions.

The title, experimental name, timeframe, location of experiment and UNE animal ethics approval number are given in Table 2.1.

Table 2.1 Experiment details

Code	Name of the experiment	Timing	Location	AEC approval
RISP12-AE-KIN3	Protection and kinetics of Rispens CVI988 against two Australian pathogenic GaHV-2 viruses in co-infected chickens	12th April 2012 to 12th June 2012	PC2 Isolator facility, UNE	AEC12-020
RISP12-SR-FLD	Evaluating the field efficacy of the Rispens CVI988 vaccine	15th June 2012 to 27th February 2013	Tamworth and Port Macquarie	AEC12-021

2.2 Handling of experimental animals

All chickens in 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 2005. The Animal Ethics Committee of the University of New England approved all experimental protocols.

2.3 Source of experimental animals

Commercial ISA Brown layer chickens were used for the isolator experiments and were sourced from the Country road hatchery, operated by the Baiada Poultry Pty Ltd., Tamworth.

The three farms which participated in the field experiment also obtained their commercial layers from Baiada Poultry. The three farms are referred to as farm A, B, and C. Farm A had

Hy-line Brown chickens, whereas farm B had ISA Browns, and farm C had ISA Browns with a small number of English Leghorns.

Figure 2.1 (A) English Leghorns and (B) ISA Brown pullets on farm C

2.4 Animal rearing

2.4.1 UNE isolator facility

The first experiment was conducted in the UNE isolator facility, which is an approved physical containment level 2 (PC2) facility. There are 24 isolators in this facility and the incoming air is filtered through high efficiency particulate air (HEPA) filters. The central air supply system provides temperature-controlled filtered air to the isolators (Figure 2.2) and each isolator scavenges its air supply through exhaust air ducts. The exhaust air of the isolators is also HEPA filtered. The soft-bodied isolators are kept under positive pressure, whereas the isolator building itself, is under negative pressure. The air flow and isolator pressures can be controlled manually. There were 12 to 23 changes of air per hour in the isolator units depending on the settings, which varied according to the age of the chickens. The body of each isolator was made of disposable plastic with a stainless steel frame. The floor was made of 2.5 mm thick perforated stainless steel with 12.7 mm holes on 17.45 mm staggered centres in order to prevent accumulation of faeces during the experimental period.

The faeces of the animals accumulated beneath the floor of each isolator over the experimental period. With the aim of improving their welfare, the chickens reared in these isolators had access to two trays of dry litter and nylon strings in order to minimise stress and

to reduce cannibalism (Figure 3). This environmental enrichment was very successful and there were no records of cannibalism in these two experiments.

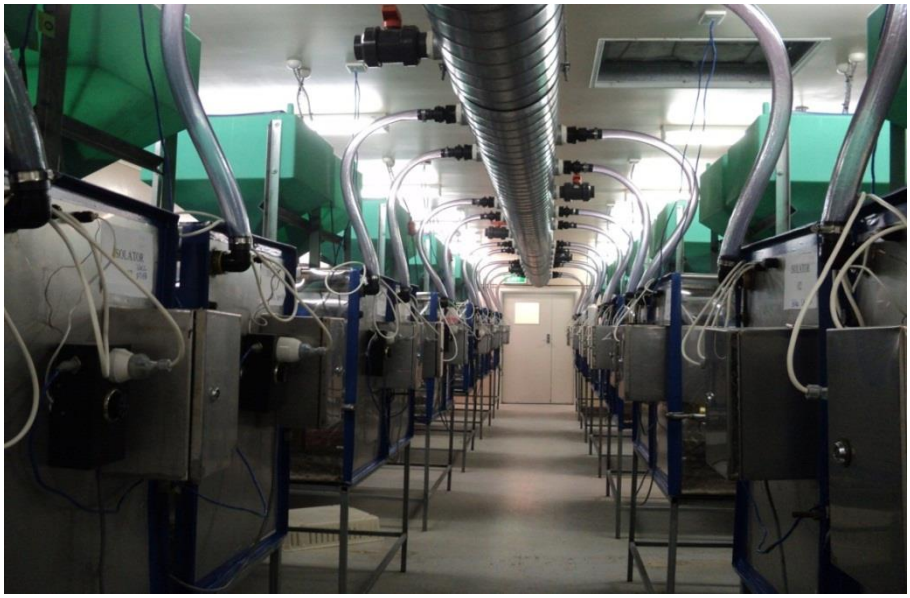

Figure 2.2 Interior of the UNE isolator facility containing 24 isolators

On the day the chickens were introduced, the isolator temperature was set to 34 ºC which was maintained for 24 hours, after which the temperature was manually decreased by 1 ºC every other day. The temperature was reduced to 21 ºC and maintained throughout the rest of the experiment.

Figure 2.3 Birds supplied with litter trays and nylon strings to improve welfare

2.4.1.1 Cleaning and disinfection in the isolator facility

The whole isolator unit and its appliances were cleaned between experiments. The unit and the parts used to build the isolators were thoroughly cleaned with a virucidal agent (0.5 - 1% Virkon S, Antec International Ltd., UK) followed by high pressure steam cleaning. A second cleaning was also carried out one week before the commencement of each experiment followed by steam cleaning and complete treatment with Virkon S. The isolator unit was fumigated using formaldehyde before each experiment. All experimental equipment was kept in the access box of each isolator and sprayed with Virkon S before introduction to the isolator. All the personnel working in the isolator were PC2 trained and they changed into protective overalls, footwear, and hair nets before entering the isolator units in the anteroom of the isolator facility.

2.4.1.2 Feed and water supply in the isolator facility

The feed for the whole experiment was calculated beforehand and was loaded into the large feed hoppers located above each isolator and these were sealed to prevent the entry of pathogens. Automatic nipple drinkers were used to supply water *ad libitum* (Figure 2.3). The chickens also had access to ad libitum feed which was a commercial layer starter followed by layer finisher (Ridley Agriproducts, Tamworth).

2.4.2 Commercial farms

Farm A had Hy-Line Brown chickens in 4-5 age groups about 19 weeks apart in age. There was one group of young pullets on the floor and four age groups of layers in two caged layer sheds (two age groups per shed). These sheds were mechanically ventilated. Replacement pullets were reared to 15 weeks of age in four barns separated from the older birds by approx. 0.5 km.

Farm B housed ISA Brown chickens purchased as started pullets around 15 weeks of age, run as layers to 65 weeks, then moulted and taken through a second lay to 90-92 weeks of age. The 50-week-old group were in the new climate controlled shed with the other groups in the older conventional shed with open sided ventilation. There were 9000 birds in each from the older groups and 7000 birds from the youngest age group.

Farm C housed pre-lay pullets, mostly ISA Brown with one group of English Leghorns. At the time of the first sampling there were 5 age groups of 5600-6000 birds per age group in

single age sheds. The English leghorns were 2.5 weeks old and there were 3000 birds in one shed together with the same aged ISA Browns. The birds were reared on the floor.

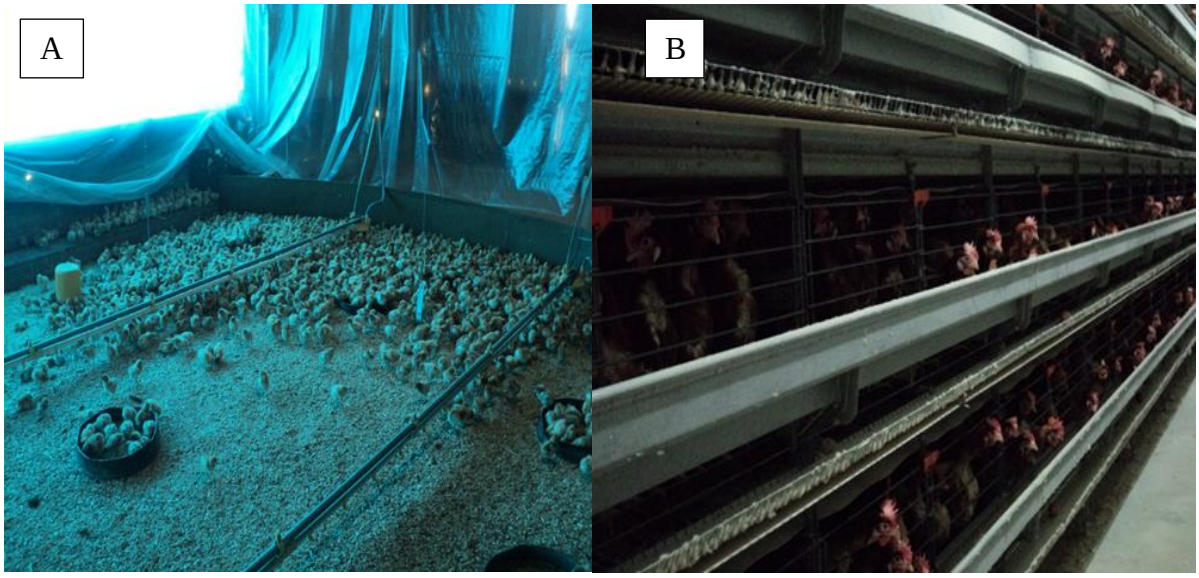

Figure 2.4 (A) Chicks on farm A being brooded (B) Laying hens on farm A under a battery cage systems

All the chickens in these farms were vaccinated for Marek's disease with Rispens CVI988 Bioproperties vaccine at hatch. Feed and water was given to these birds by automatic feeders and waterers typical for commercial barn/battery systems.

2.5 Vaccination

The chickens in the isolator experiment were vaccinated at hatch with Rispens CVI988 vaccine (RIS8111, expiry: 2012/11, Bioproperties Ltd., Australia). The vaccination was performed manually at UNE using the diluent supplied by the manufacturer. The vaccine was thawed at 36 ºC using a water bath and administered within 30 minutes to prevent the loss of viability as it is a cell-associated vaccine. To avoid vaccinator error the same vaccinator administered the vaccine using 23 G needles throughout, and the vaccine was administered subcutaneously to the loose skin of the neck using the recommended vaccine dose (4000 µl) in a volume of 0.2 ul.

2.6 Challenge of birds with GaHV-2

In experiment 1 the chickens were challenged with two GaHV-2 isolates – MPF57 which is a virulent (v) GaHV-2 pathotype or FT158 which is very virulent (vv) GaHV-2 pathotype. Details are as follows (Renz *et al*., 2012)

1. MPF57 Batch No. P 4181109, initial titre-60,000 plaque forming units/ml pfu/ml
2. FT158 Batch No. P40211209 initial titre-165,000 pfu/ml -very virulent (vv)

The challenge dose used was 500 pfu. These virus isolates were grown and titrated previously in chicken embryo fibroblast (CEF) cell cultures at UNE.

They were stored in liquid nitrogen and prior to use thawed in a water bath at 37 ºC. the viruses were diluted with M199 media (Invitrogen, Australia) which contained 10% foetal calf serum with antibiotics and antimycotic (Invitrogen, Australia). The chickens were injected with given dose in the subcutaneous route on day 5 post vaccination.

2.7 Euthanasia of chickens

The chickens were euthanised using the method described by Andrews *et al.*, (1993) by cervical dislocation. The chickens were held in a fixed position with legs flexed with one hand. The thumb and the index finger circled the base of the skull, and the middle and ring fingers were held under the beak. The cervical dislocation was achieved by rapid extension of the arm holding the head, with dorsal flexion of the head at the same time.

2.8 MD lesion detection and scoring immune organs

A standard necropsy examination (Bermudez & Stewart-Brown, 2008) of all birds that were euthanised or died throughout the experiment was carried out. The carcasses were dampened with warm water containing detergent and examined for tumorous lesions in the skin. After opening the skin the breast and thigh muscles were checked for tumour infiltrations and nodules. The skin was exposed in the neck region and the thymus was scored for atrophy from 0-3 in ascending severity as follows; 0 = normal, 3 = complete or almost complete atrophy. The sciatic nerve plexus was examined for gross enlargement and inflammation. After opening the carcass the visceral organs such as heart, liver, spleen, proventriculus, mesenteries, gonads, lungs, and gastrointestinal tract were observed for gross enlargements and diffuse or discrete tumour infiltrations. The bursa of Fabricius was examined and scored

for atrophy similar to that of the thymus. Some of the tumour lesions found in the experiment is shown in Figure 2.6.

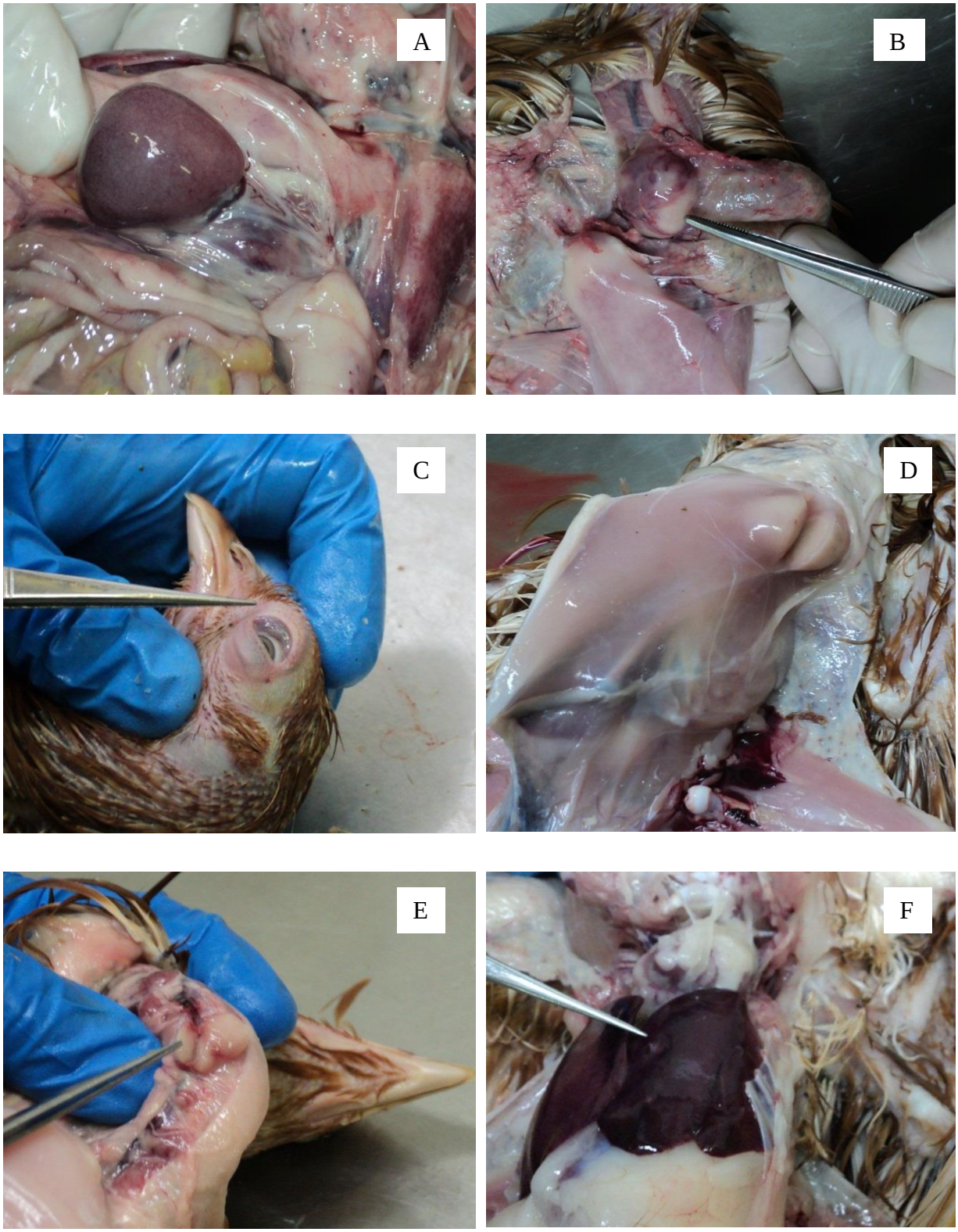

Figure 2.5 MD lymphomas in A) Spleen B) Heart C) Conjunctiva of eye D) Breast muscle E) Thymus and F) Liver

2.9 Sample collection

Blood samples were collected from puncture of the brachial vein with a sterile 21 gauge needle followed by aspiration of blood with a sterile Pasteur pipette and transfer into a 1.5 ml microfuge tube containing 150 µl of 3% sodium citrate. The samples were mixed gently and kept on ice until further processing. In the field experiment the blood samples were collected from the brachial vein into sterile 1.5 ml microfuge tubes and chilled until further processing at UNE laboratories.

Spleen samples were washed in sterile PBS, and put into sterile 5 ml tubes, weighed and stored at -20 ºC until further processing.

Feather sampling - Three to five feathers were collected from the axial tract of the birds and placed in a sterile container. In younger birds (7 to 14 days of age) feathers were collected from the wing. Samples were stored at -20 ºC until further processing.

Dust samples from isolators were collected from exhaust air outlets at the point where the dust naturally accumulates. The exhaust air valve was closed temporarily and dust was collected using a disposable wooden spatula into a 1.5 ml microfuge tube. The outlet was cleaned so that the next dust collection represented the collection of each subsequent week. The dust samples were stored at -20 ºC until further processing. Dust samples from the field experiment were collected from any accessible surface within the barn or battery cages with obvious dust accumulation and placed into 1.5 ml microfuge tubes and stored at -20 ºC until further processing.

2.10 Sample preparation and processing

2.10.1 Separation of peripheral blood lymphocytes

1. 300 µl of citrated blood sample was transferred slowly onto 300 µl of Ficoll-Paque™ PREMIUM (Amersham Biosciences, Sweden) in a 1.5 ml microfuge tube.
2. The microfuge tube was centrifuged at 900 g for 20 minutes at approximately 8 °C.

3. Lymphocytes were then carefully aspirated from the Ficoll-Paque™ interface with a sterile pipette and transferred to another 1.5 ml microfuge tube containing 500 µl PBS.
4. The samples were then centrifuged at 3500 g for 5 minutes at approximately 8 °C. The supernatant was removed using a sterile pipette and the PBL pellet was stored at -20 °C until further processing.

2.10.2 DNA extraction from PBL

DNA was extracted from PBL using the automated DNA X-tractor Gene (Corbett Robotics, Australia). Prior to loading the samples on the robot, the PBL pellet was re-suspended in 100 µl PBS and then diluted 1:5 in PBS. The diluted samples were loaded manually into the 96-well lysis block, 200 µl per well. The lysis block was transferred to the X-tractor gene. The extraction protocol was as per the manufacturer's instructions below:

1. 100 µl of DX Liquid Digest buffer (DXL) with 10% DX digest enzyme was added per well to the lysis plate, mixed and incubated for 20 minutes
2. 400 µl of DX Binding buffer (DXB) with DX binding additive was added per well to the lysis plate and mixed and incubated for 5 minutes
3. 600 µl from each well of the lysate was transferred from the lysis plate to the capture plate and vacuumed at 30 kPa for 3 minutes
4. 200 µl of DX Binding buffer (DXB) with DX binding additive was added per well to the capture plate and again vacuumed at 30 kPa for 3 minutes
5. 600 µl of DX wash buffer (DXW) per well was loaded into the capture plate and vacuumed at 25 kPa for 1 minute
6. The above step was repeated, and 600 µl of DX final wash buffer (DXF) per well loaded to the capture plate and vacuumed at 35 kPa for 5 minutes to dry the plate
7. The carriage was moved to the elution chamber and 100 µl of elution buffer (E) per well was loaded to the capture plate, incubated for 5 minutes and again vacuumed at 30 kPa for 1.5 minutes. After this step the elution plate was removed from the robot and stored at -20 °C.

2.10.3 DNA extraction from feathers using the DNA extraction robot

Before the DNA extraction from feathers, 3-5 feathers with plenty of pulp were selected. They were cut about 3-5 mm from the proximal shaft using a sterile scalpel blade and transferred into a labelled sterile microfuge tube. Prior to loading the feather samples on the lysis block, they were digested manually with 200 µl of X_1 buffer (100 mM, Tris HCl pH8, 1 ml; 0.5M EDTA pH8, 200 µl; 1M NaCl, 1 ml; 10% SDS, 2 ml; 1M DTT 400 µl; 250 ug/ml Proteinase K, 125 µl; Milli-Q water, 5.275 ml)

The feather tips containing the X1 buffer were kept in a water bath at 55 ^{0}C for 3 hours. This manual digest was necessary to prevent blockage of the filter block of the Xtractor Gene. The digested samples were loaded into the lysis block and the original lysis step on the Xtractor Gene was skipped. All remaining steps were performed identically on the Xtractor Gene as outlined above for the PBL extraction.

2.10.4 DNA extraction from feathers, dust and spleen using the ISOLATE Genomic DNA mini kit

In the field experiment, DNA was also extracted manually from feathers, and from 5 mg of dust and 5 mg of spleen using the ISOLATE Genomic DNA Mini Kit (Bioline, Australia).

5 mg of dust or spleen or 3-5 feather sections were placed in a 1.5 ml microcentrifuge tube.

Then 400 µl Lysis Buffer D and 25 µl Proteinase K was added, vortexed, and incubated at 50 ºC with mixing every 15 minutes until the sample was completely lysed, this was usually within 1 - 2 hours. If there was a large amount of sediment present after incubation, the sample was centrifuged at 12,000 rpm (10,000 g) for 1 minute in a benchtop centrifuge, and then the supernatant was taken off and used for further extraction.

Next, 200 µl binding buffer D was added to the sample and mixed by vortexing for 15 seconds.

Then the mixture was pipetted into spin column D and centrifuged at 12,000 rpm (10,000 g) for 2 minutes. Then the collection tube was discarded and the column was placed into a new collection tube. Next, 700 µl of wash buffer was added and the column centrifuged at 12,000 rpm (10,000 g) for 1 minute. The filtrate was discarded and the washing procedure was

repeated. After this, the column was centrifuged for 2 minutes at 13,000 rpm (12,470 g) to remove all traces of ethanol.

Then the column was placed into a 1.5 ml microfuge tube and the DNA eluted with 100 µl elution buffer and incubated at room temperature for 1 minute. Finally, the tube was centrifuged at 8000 rpm (4722 g) for 1 minute and the supernatant containing the extracted DNA was stored at −20 ºC.

2.10.5 DNA quantification and storage

All DNA was quantified using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop® Technologies Wilmington, USA). The absorbance ratio of the sample at 260 and 280 nm was assessed as a measurement of DNA quality. All DNA was stored at −20 °C until further analysis (qPCR).

2.10.6 Quantitative real-time polymerase chain reaction (qPCR)

All extracted DNA samples of the isolator experiment and were subject to two TaqMan® qPCR assays, one of which only detects Rispens vaccine GaHV-2, while the complementary test only detects wild type pathogenic GaHV-2 (Renz, *et al.,* 2013). The target gene for both of these tests was the *meq* gene. The primers and probes for the two tests are given in Table 2.1. The GaHV-2 genome copy number was determined by absolute quantification as reported by (Renz *et al.,* 2013).

Table 2.2 Sequences of meq-gene primers and probes used for qPCR differentiation between Rispens/CVI988 and pathogenic assays (Renz *et al.,* 2013)

Target gene	Primer/probe sequence
meq (Rispens serotype 1)	
Probe	5'-(FAM)TGCAGAGGGCCAATGAACACCTACG (BHQ1)-3'
Primer BCH402	5'-TCGGAGAAGACGCAGGAA-3'
Primer BCH403	5'-GCTCATGACAAGCCAACTGTA-3'
meq (Non-Rispens serotype 1)	
Probe	5'-(FAM)-TGCAGAGGGCCAATGAACACCTACG (BHQ1)-3'
Primer BCH406	5'-TCGGAGAAGACGCAGGTC-3'
Primer BCH445	5'-GTAAGCAGTCCAAGGGTCACC-3'

The respective TaqMan® real-time qPCR assay was performed using a RotorGene-Q realtime PCR instrument (Qiagen, Australia). The qPCR cycling parameters consisted of: 50 °C for 2 minutes, 95 °C for 2 minutes, followed by 45 cycles consisting of denaturation at 94 °C for 15 seconds and annealing/extension at 60 °C for 60 seconds for the Rispens vaccine specific and pathogenic assays. Each reaction tube contained 0.3 µM of each primer, 0.2 µM of the probe, 12.5 µl of KAPA probe fast qPCR kit master mix (2x) universal (KAPABiosystems, USA), 5 µl of DNA template (25 ng of DNA) in a total reaction volume of 25 µl. A Corbett CAS1200 liquid handling station (Corbett Research, Sydney, Australia) was used to prepare the reaction tubes for all qPCR assays.

A standard curve for each primer set was generated in each assay and used to derive the copy number of target sequences in unknown samples. For each assay run, individual standard curves were generated using 10-fold serial dilutions of GaHV-2 standards of known concentration of GaHV-2 DNA. All samples were randomised across assays for the first experiment, and for the second experiment individual bird samples were grouped together before randomizing on an individual bird basis to minimise individual assay effects. A single reaction per sample was performed for PBL DNA samples and duplicate reactions for feather, dust, and spleen DNA samples. Standards and quality controls (QCs) were also assayed in duplicate for all assays.

2.10.7 Enzyme Linked Immunosorbent Assay (ELISA)

The serum samples were subjected to analysis using a Marek's disease-specific ELISA adapted from Zelnik *et al.* (2004). The full ELISA procedure is given below.

The test serum samples were diluted 1:100 with PBST (0.5 ml/litre Tween 20 added for 1 litre of PBS). The samples were stored at 4 $^{\circ}$C until required. The ELISA plates (Immulon ® 2 flat-bottom microtitre plates, Cat. No. 011-010-3455) were coated with GaHV-2 antigen (the dilution was determined in the previous stage) diluted with carbonate buffer (0.05M, pH 9.6). One hundred microlitres of the diluted antigen was added to each well and incubated at 4 $^{\circ}$C for 16 hours. After the incubation period, the plates were washed with PBST twice. One hundred microlitres of PBST containing 1% skim milk (1 g of skim milk powder in 100 ml of PBST) was added to each well to block the plates. The plates were covered and left for 1 hour at room temperature. The plates were flicked at the end of incubation period and the skim milk PBST was flicked out. One hundred microlitres of the diluted samples, standards, negative control samples and blanks (PBST+1% skim milk) were added to the plate and

incubated for one hour at 37 °C. The positive control samples were obtained from experiments which have utilised specific pathogen-free chickens challenged with Marek's disease virus and with known antibody titres. The negative control samples were taken from unchallenged control birds from the same experiments. The plates were washed with PBST twice. One hundred microlitres of anti-chicken rabbit antibody whole molecule conjugated with peroxidase enzyme (Sigma cat no. A9046, diluted 1:5000 with PBST) was added to each well of the ELISA plate. The plate was covered and incubated for 1 hour at 37 °C. The plates were washed with PBST three times and 100 µl of substrate (34 mg of o-Phenylenediamine and hydrogen peroxide 30% w/v [Univar/Chem-Supply] with 100 ml of citrate phosphate buffer [pH 5.0]) was added to all wells. The plates were covered with aluminium foil and incubated for 10 minutes at room temperature. The chemical reaction was stopped by addition of 50 µl of 98% sulphuric acid per well. The plate was mixed for 5 seconds and read by microplate reader (Bio-Rad, Benchmark) at 490 nm, and the optical density values were obtained, averaged over duplicate samples. The antibody titre was determined by comparing the average optical density value with the standard curve generated by the samples.

Preparation of antigen: Rispens vaccine (Bioproperties Vaxsafe RIS) was used to prepare the antigen for ELISA. A live vaccine vial was thawed by placing in warm water (27-29 °C) and contents were transferred into a sterile tube using a syringe with an 18 gauge needle attached to it. About 2-3 ml vaccine diluent was also added to the sterile tube and was mixed well. Then it was centrifuged at 2500 rpm (748 g) for 10 minutes at 4 °C (Allegra X-15R ® centrifuge, Beckman Coulter). The supernatant was discarded and the pellet was retained. The remaining pellet was frozen at -20 ^{0}C and about four freeze-thaw cycles were carried out. After the last thawing the pellet was broken up and dispersed using a homogenizer (MSE Soniprep 150) by addition of phosphate buffered saline (PBS, Sigma P-5368, pH 7.4). Sonication was carried out for 1-2 minutes at 12 Amperes. The homogenised antigen was centrifuged at 4 °C at 3000 rpm (1077 g) for 10 minutes and the supernatant was retained. The concentration of the antigen was determined by a spectrophotometer (M7 Bio-Rad SmartSpec^{TM} 3000) using bovine serum albumin standards (Sigma, A-3803) and Bradford reagent (Sigma-Aldrich). Antigen was stored at -20 ^{0}C until required. Optimum antigen concentration to coat the plates was determined by serial dilution of the antigen against known standards, PBS, and negative samples.

Validation of assays: A standard curve was generated in each assay. All samples from an individual bird were performed in duplicate on one plate to minimise between-plate effects. The sensitivity of the assays for GaHV-2 was determined by running 2-fold serial dilutions of the standards with known titres. Standards and quality control (QC) were also assayed in duplicate for all assays. Standards were made up from pooled sera from breeder broiler chickens vaccinated against GaHV-2 with Rispens vaccine. The titre cut-off value was determined as 500 based on the results of previous ELISA tests. The inter-assay CV was determined from the negative control samples from the each run, whereas the intra-assay CV was determined by duplicates of each sample. The mean intra-assay CV was 14.8%, whereas the inter-assay CV was 7.8%.

Chapter 3

Protection provided by Rispens CVI988 vaccine against Marek's disease virus isolates of different pathotype and early prediction of vaccine take and MD outcome

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Chapter 4

Effects of Rispens CVI988 vaccination followed by challenge with Marek's disease viruses of differing virulence on the replication kinetics and shedding of the vaccine and challenge viruses.

Manuscript submitted to

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Chapter 5

Field studies on the detection, persistence and spread of the Rispens CVI988 vaccine virus and the extent of co-infection with Marek's disease virus

Manuscript submitted to

The Australian Veterinary Journal

6 General discussion and conclusions

The focus of the research undertaken for this thesis was to utilise qPCR tests which allow differentiation between Rispens CVI988 and pathogenic MDV-1 to investigate important issues relating to vaccination with Rispens. The kinetics of two GaHV-2 pathotypes of different virulence in Rispens-vaccinated birds was first studied in a controlled environment (isolators) to determine influences on each other and comparative fitness. This isolator experiment also aimed to determine early predictors of MD incidence and vaccinal protection in Rispens-vaccinated chickens. Early indicators of vaccine take were also investigated. Subsequently, field studies were carried out on commercial farms to measure the Rispens vaccine take, level of co-infection with pathogenic GaHV-2, the long-term viral kinetics of Rispens vaccine, and the possibility and extent of Rispens virus spread to unvaccinated broiler flocks.

6.1 Protection provided by Rispens CVI988 against two different GaHV-2 pathotypes

The protection provided by Rispens CVI988 vaccine against several non-Australian vv and vv+ GaHV-2 isolates has been explored in previous formal protection studies and has been found to be variable (Witter *et al.*, 2005; Zhang *et al.*, 2015). However, amongst the vv and vv+ pathotypes examined, Rispens vaccine protection did not appear to be affected by GaHV-2 pathotype (Witter *et al.*, 2005). There are no studies reporting the protective effect of Rispens vaccine against vMDV pathotypes, as these pathotypes are generally adequately protected against by HVT and HVT/GaHV-3 bivalent vaccines. Therefore, the first experiment aimed to compare the level of protection offered by the Rispens CVI988 vaccine against Australian v and vvMDV isolates to determine whether the lack of association between protection and challenge pathotype extends to vMDV pathotypes.

In this experiment, the protection provided by Rispens vaccine did not differ between MPF57 (vMDV) and FT158 (vvMDV). The protection indices provided by Rispens vaccine for these pathotypes were 66% and 61%, respectively. As predicted, the GaHV-2 pathotype did not affect the protective index, thus supporting the idea that protection provided by the Rispens vaccine is unrelated to pathotype. This finding needs to be confirmed using a wider range of

isolates of these pathotypes. Despite the similar protective indices, the FT158 pathotype was clearly more virulent than MPF57 inducing higher MD incidence and MD mortality across both unvaccinated and vaccinated chickens (Chapter 3). There was evidence that virulence and the vaccine resistance are two different traits of a virus (Renz *et al.,* 2012, Walkden-Brown *et al.,* 2013b). The finding of a lack of association between pathotype and protection provided by Rispens vaccine is supportive of the idea that the "vaccine resistance" component of GaHV-2 pathotype classification (which is based on the response to vaccination by HVT and HVT/bivalent vaccines) does not correlate with vaccine resistance to the Rispens vaccine. This suggests that different mechanisms of vaccine resistance are involved for Rispens vaccine than those causing resistance to the HVT vaccine.

This experiment has provided additional insight into the multiple factors that affect the PI provided by the Rispens vaccine. One of the factors which influences PI is the host genotype. In previous experiments with ISA Brown chickens as the host, HVT or HVT/Bivalent vaccines against challenge with MPF57 conferred a lower protection (38.3 and 58.9%, respectively; Renz, 2008) than Rispens vaccine, supporting its superiority. However, in commercial broilers the protection against challenge with FT158 provided by HVT or HVT/Bivalent vaccine (61.4 and 57.7%, respectively [Walkden-Brown *et al.*, 2013b]) was similar to that provided by Rispens vaccine in this experiment. In mab −ve SPF chickens challenged with FT158 and MPF57 the HVT vaccine provided 100% protection against MPF57 and 61% against FT158 (Renz *et al.*, 2012).

The lack of association between protection indices for vv and vv+ pathotypes provided by Rispens vaccine and that provided by bivalent vaccine (HVT/SB1) in the study of Witter *et al.* (2005) is shown in Figure 6.1. There is clearly no association between the PIs provided by these two vaccines. The comparison of the average protection provided by the Rispens vaccine for different pathotypes of GaHV-2 throughout the world is given in Figure 6.2. Again, protection is unaffected by pathotype. As pathotype is determined in large part by the PI provided by HVT and bivalent vaccines, the lack of association suggests that there is no "cross resistance" between Rispens vaccine with other *Mardivirus* vaccines.

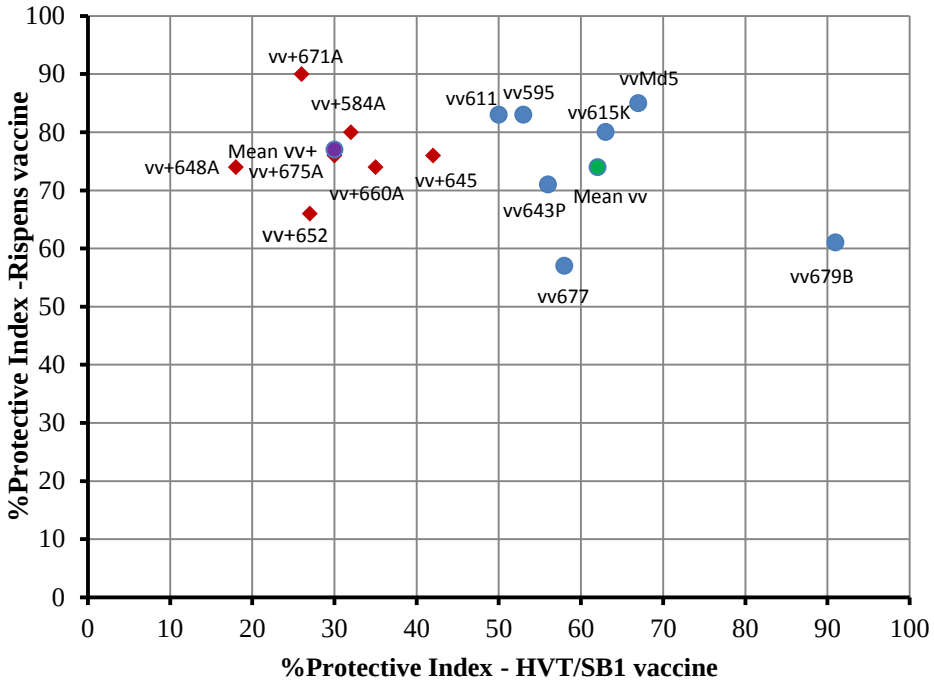

Figure 6.1 Scatterplot showing the association between protective indices provided by the Rispens and bivalent (HVT/SB1) vaccines against vv and vv+MDV pathotypes using SPF chickens

Redrawn from the data of Witter *et al.* (2005)

Legend: ●, vv pathotypes; ♦, vv+ pathotypes; ●, mean PI of vv pathotypes; ●, mean PI of vv+ pathotypes

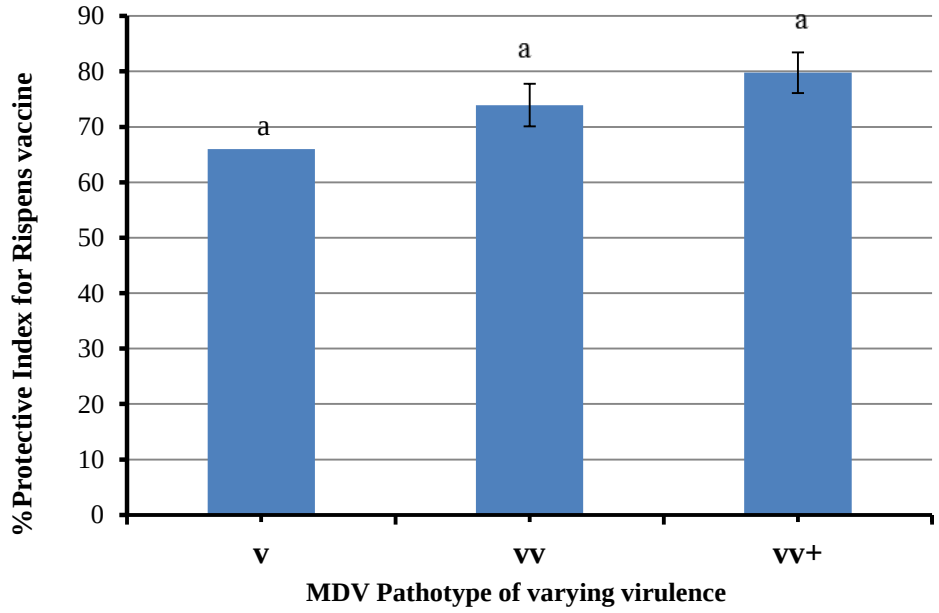

Figure 6.2 The mean protective indices of Rispens for v (1 isolate), vv (11 isolates) and vv+MDV (9 isolates) and data from this thesis (one v and one vv isolate).

Compiled from the reports of Witter *et al.* (2005); Lee *et al.* (2010); Gong *et al.* (2014); Zhang *et al.* (2015),

Means sharing a common letter do not differ significantly (P>0.05).

In general, Rispens vaccine provides higher protection for vv+ pathotypes than HVT or bivalent vaccines. However, for some vv pathotypes the Rispens vaccine protection appears to be inferior, and some researchers found that combining Rispens vaccine with HVT enhances the vaccinal protection (Geerligs *et al.*, 1999; Gong *et al.*, 2014). The inferior protection was also observed in the study reported in this thesis and investigation should be conducted into whether combining Rispens vaccine with HVT enhances protection against challenge with v and vv Australian isolates as is the case in other parts of the world.

Another explanation for the low PI in the experiment is the vaccination to challenge interval. In a recent experiment using ISA Brown chickens and a vvMDV, Islam *et al.* (2013b) showed that when the vaccination challenge interval (VCI) is increased from 5 to 10 days the PI increased from 85% to 100%. Some scientists have observed that early protective immunity of Rispens is delayed (Geerligs *et al.*, 1999). Therefore, the low PI in this study may be also attributed to the low VCI of 5 days used in USDA-ADOL-type challenge studies.

Previous studies have found that Rispens administration via intra-abdominal and intramuscular routes provides better protection levels than the subcutaneous route (Rispens *et al.*, 1972a; Geerligs *et al.*, 2008). However, in the present study, the Rispens vaccine was administered subcutaneously following the manufacturer's instructions. Given that this is the route by which chickens are vaccinated with Rispens vaccine worldwide, its use in the present experiment is sensible.

One of the limitations of this study was that it tested only a single isolate of each pathotype limiting the strength of the conclusions that could be drawn. If more isolates had been included and the same result obtained, the conclusion that lack of association between pathotype and Rispens vaccine protection level extends to vMDV isolates would have been stronger.

6.2 Early predictors of MD incidence

In the isolator experiment, the viral loads of pathogenic GaHV-2 in the first 3 weeks in different samples proved to be better predictors of subsequent MD incidence than viral loads of Rispens in the vaccinated and challenged chickens. Previous studies using other vaccines reported that GaHV-2 viral load from 7-21 days provides a good predictive measurement for

subsequent MD outcome (Islam *et al.*, 2006b; Islam *et al.*, 2007; Gimeno *et al.*, 2008; Islam *et al.*, 2008; Islam *et al.*, 2013b). Immune organ atrophy measures are also a good measure for mab –ve SPF chickens with other GaHV-2 vaccines but not for mab +ve chickens (Islam *et al.*, 2006; Gimeno *et al.*, 2008; Walkden-Brown *et al.*, 2013b; Dunn *et al.*, 2014).

In this experiment, GaHV-2 load of PBL at 7 dpc was the earliest most significant predictor of MD followed by GaHV-2 load of spleen at 14 dpc, feather at 14 dpc, and dust at 21 dpc. This study confirmed the finding of PBL as an early indicator of MD at 7 dpc from the previous kinetic studies (Islam *et al.*, 2006; Gimeno *et al.*, 2008). This experiment also confirmed the importance of other samples such as spleen (Islam *et al.*, 2007; Walkden-Brown *et al.*, 2013b), feather (Islam *et al.*, 2013b), and dust (Walkden-Brown *et al.*, 2013b) on 14 and 21 dpc as good early MD indicators, as ascertained in previous experiments. Thus, non-invasive samples such as feathers and dust could be collected as early as 2 and 3 weeks, respectively, following challenge to predict the MD outcome in experimental situations where full expression of MD is precluded on ethical or economical grounds. Feather samples would provide data of individual birds, whereas, due to lower cost, dust could be potentially suitable for analysing the flock situation and monitoring MD in the field. Nevertheless, these correlations are not necessarily easily extrapolated to the field where the time of challenge and dose of challenge is not known. Furthermore, the Rispens viral loads in dust did not provide an equally strong prediction of subsequent development of MD. This may largely be due to the absence of sufficient data, as there were only four isolators with vaccinated and challenged birds as opposed to the eight isolators with challenged birds. Baigent *et al.* (2007), in a more powerful design, showed a strong a relationship between protection and Rispens viral load. The reason for the stronger relationship in that study may be due to their use of different vaccine doses (0.01, 0.1, and 1.0 commercial dose of Rispens CVI988) and, more importantly, having longer VCIs ranging from 14 to 28 days. In the present experiment the challenge was much earlier, being five days after vaccination, and the single VCI and dose rate resulted in a much lower range of Rispens viral loads and MD outcomes on which to measure association. However, the directions of the association between Rispens viral load and MD incidence was consistently negative, suggestive of some association, as was also observed in the recent study of Islam *et al.* (2014). Therefore, if the experimental design had more isolators with vaccinated and challenged birds, we may have detected significant negative correlations between Rispens viral loads and MD incidence.

The Rispens viral loads provided more prediction on the vaccine take. This will be discussed in Section 6.3

6.3 Sampling to assess vaccine take under experimental and field conditions

Another investigation undertaken in both the isolator and field experiments was to explore what samples and at what time the samples should be collected to best measure vaccination success (vaccine take).

In the isolator experiment, the percentages of positive Rispens virus samples from PBL during the second and third weeks post vaccination were 72 and 90%, respectively. The percentage of positive samples for spleen, which was only sampled on 14 dpc, was 53%. Non-invasive feather samples were 80 and 93% positive, respectively, during weeks 2 and 3 post vaccination, whereas dust was 100% on 21 dpv. Therefore, in this experiment both invasive and non-invasive samples produced comparable results for prediction of vaccine take (Table 6.1).

Table 6.1 Comparison of the number and percentage of different sample types positive for the Rispens virus in all vaccinated chickens from between 7 and 24 days post vaccination (dpv) in the isolator and field experiments

Sample type	Percentage samples qPCR positive for Rispens					
	Experiment	7 dpv	14 dpv	17 dpv	21 dpv	24 dpv
Spleen	Isolator	-	53%	-	-	-
	Field	-	-	-	-	-
PBL	Isolator	17.8%	72.4%	-	90%	-
	Field	-	-	-	-	-
Feather	Isolator	82.7%	80%	-	93%	-
	Field	15%	45%	80%	55%	80%
Dust	Isolator	66.6%	50%	-	100%	-
	Field	100%	50%	100%	100%	100%

Similarly, in the field experiment, results suggest that the vaccine success can be measured earliest with high accuracy by using feathers at 17 dpv and poultry house dust collected at 24 dpv. At 17 dpv, the feather samples were 80% positive with a high mean Rispens viral load of 2.5×10^5 VCN per 10^6 feather cells. The dust samples were 100% positive at 24 dpv and contained the highest viral load of 10^6 VCN/mg of dust. The feather test provides an earlier measure of an individual animal vaccine take, whereas the dust test provides a more convenient and inexpensive flock test of vaccine take. These results agree with the studies of

Islam *et al.* (2013a) in which the Rispens viral load peaked for feather at 14 dpv and dust at 21 dpv. Feather sampling may not provide definitive evidence of vaccination success if some birds have acquired the vaccine by natural transmission. However if the birds are sampled within the first 10 days of their lives the Rispens vaccine is mainly due to vaccination and not contact transmission. Nonetheless, if sampling for vaccine take was carried out at 3 weeks of age, the vaccine can be horizontally transmitted as well as Rispens vaccine may replicate in the birds which received a low level reach a plateau. The correlation between Rispens virus VCN and protection is also not well defined and may vary between different breeds of chicken and mab status. Hence a positive low level of Rispens VCN may not confer sufficient protection. On the other hand the as GaHV-2 infection is lifelong (Witter, 1971) the persistent infection, as demonstrated in this study may be the reason for the immunity provided by Rispens vaccine.

There were more samples positive for MDV ELISA than Rispens virus in feathers in the field experiment. The reasons for this may include 1) In samples from young birds, Rispens vaccine has not yet replicated to significant levels, but mab gives a positive ELISA result; (2) In older birds, Rispens virus is at low latent levels, but antibody persists and (3) the VCN of birds that receive very low dose of the vaccine may be undetectable by the technique at the time.

6.4 Viral loads of Rispens and two pathogenic GaHV-2s of different virulence in co-infected hosts

In the chickens vaccinated with Rispens CVI988 and challenged with two different GaHV-2s (vMPF57 and vvFT158), the overall genomic copy number ranking was FT158 > MPF57 > Rispens in PBL, feathers, and dust, but the differences only achieved statistical significance in PBL. This may be due to lymphocytes being the primary target cells of MDV and having the main role in disease pathogenesis, while feathers and dust involved only in virus transmission. Immune responses might restrict levels of systemic vaccine and challenge virus in PBL, but perhaps virus levels in the skin are not subjected to such immune control. This is consistent with the findings Yunis *et al.*, (2004) and Dunn *et al.*, (2014) who reported differences in viral loads between GaHV-2s differing in virulence, especially between v and vv+MDV isolates or the studies showing that viral load of vaccinal viruses is generally lower than pathogenic viruses (Islam & Walkden-Brown, 2007; Islam *et al.*, 2008; Walkden-Brown *et al.*, 2013b; Islam *et al.*, 2014).

6.5 Effect of Rispens vaccination on pathogenic viral load and implications for vaccination-driven evolution of virulence

Vaccination induced a highly significant reduction in pathogenic GaHV-2 load in PBL and feathers, but in dust the reduction was much smaller and was only a non-significant trend. In feathers, vaccination had a greater suppressive effect on viral load of the less virulent vMPF57 than the more virulent vvFT158. When the difference in GaHV-2 viral load between unvaccinated and vaccinated chickens using paring based on viral load rank, the difference (reduction) was higher for MPF57 than FT158 in all three sample types, suggesting a greater inhibitory effect of vaccination on GaHV-2 virus of lower virulence. In both PBL and feathers, the pathogenic GaHV-2 viral load was reduced by Rispens vaccination consistently from 7dpc. Therefore it is clear that Rispens vaccine has an antiviral effect in addition to any anti-tumour effect it may have. When the total genome copy numbers shed over the 56 days of the experiment were calculated (taking into account mortality) there was again some evidence that fewer MPF57 copy numbers were released by vaccinated hosts, whereas the FT158 copy numbers were not affected; however, this effect was not statistically significant. The mixture of significant differences and non-significant trends observed in this regard require confirmation in a larger experiment with higher levels of replication at the chicken and isolator level, and possibly also a wider range of virulence in the GaHV-2 challenge viruses.

Success of the Rispens vaccine may be due to its ability to reduce the pathogenic GaHV-2 load than HVT and HVT/bivalent vaccines (Baigent *et al.,* 2013). However, in this experiment high level of reduction of pathogenic GaHV-2 by Rispens vaccination could not be observed presumably due to differences in breeds of chickens, dose of vaccine, vaccination challenge interval and strain of challenge viruses. The presence of anti-MDV antibodies also may have interfered the early replication and immune responses to Rispens vaccine in the current experiment.

In a recent experiment, Read *et al.* (2015), using HVT vaccination and challenge with several GaHV-2s of varying virulence, reported that vaccination enhanced the lifespan of chickens challenged with virulent GaHV-2s and thus prolonged the period of shedding and transmission of virulent GaHV-2s, thereby providing an environment that favoured the more

pathogenic GaHV-2 isolates which have higher replication and shedding rates than low virulence GaHV-2s. Examination of the data of these authors also revealed that vaccination with HVT depressed viral shedding in dust to a greater extent in low virulence than high virulence pathotypes, thus also contributing to the more favourable environment for higher virulence GaHV-2s in vaccinated hosts. The results of the isolator experiment similarly suggest that the Rispens vaccine has a greater inhibitory effect on lower virulence viruses. In this respect, it appears to be no different to other imperfect HVT and bivalent MDV vaccines. Rispens vaccine appears to favour FT158 over MPF57 by both enhancing the lifespan of the hosts challenged with FT158 and by exerting lesser inhibition of replication for FT158 than for MPF57.

If the Rispens vaccine's effects in favouring higher virulence GaHV-2s are no different from HVT and bivalent vaccines, why is it still effective rather than succumbing to higher virulence GaHV-2s as has occurred for HVT and HVT/GaHV-3 vaccines? One of the reasons may be that the Rispens vaccine is mostly used in long-lived layers and breeder populations and not in broilers (Dunn & Gimeno, 2013). Thus, the GaHV-2s circulating in the much larger broiler population do not encounter selection for improved fitness in Rispens-vaccinated birds. Therefore they are not exposed, or "in refugia" to selection for resistance to the effects of Rispens vaccine. Furthermore, it has been demonstrated by mathematical modelling studies that reduced the lifespan of broilers could lead to increase of GaHV-2 virulence as increases in viral fitness are not constrained by host death (Atkins *et al.,* 2013). The Rispens vaccine has not been exposed to this effect, due to its use in breeders and layers which have not had an equivalent reduction in lifespan as broilers. While it may be argued that the long lifespan of layers and breeders may counteract their smaller population size, the population of broilers is always higher in a given point of time. For example based on Australian data for 2013/2014 (ACMF, 2014; AECL, 2014) the number of "chicken days" for broilers in in Australia was 28 billion whereas for layers it was 7 billion. Witter *et. al.,* (1997) has found that isolates of vv+ MDV originated both from layers and broilers suggesting that selection for virulence is active in both populations.

6.6 Effect of GaHV-2 challenge viruses on Rispens viral load

Challenge with pathogenic virus 5 dpv reduced the Rispens viral load significantly in PBL, but not in feather and dust. The reason behind this could be that the cytolytic infection of FT158 destroying a higher proportion of lymphocytes in which Rispens vaccine replicates. Furthermore, in PBL, the reduction of Rispens viral load was greater following challenge with vvFT158 than vMPF57. In a previous study, Haq *et al.* (2012) showed that when chicks are vaccinated with Rispens vaccine via the intraembryonic route on the 18th day of incubation and challenged on day 5 of age, challenge with pathogenic GaHV-2 (RB-1B) has little effect on Rispens viral load. Islam *et al.* (2014) investigated GaHV-2 and Rispens kinetics with a range of intervals between vaccination and challenge and found that when challenge with GaHV-2 was carried out before Rispens vaccination, there was a significant suppression of Rispens viral load, but not when challenge followed vaccination. The first GaHV-2 which enters the host has a suppressive effect on the second virus, and this effect is enhanced by the length of the time period between the two infections as well as the virulence of the viruses (Dunn *et al.*, 2010; Dunn *et al.*, 2012; Islam *et al.*, 2014). Dunn *et al.* (2010; 2012) demonstrated, using virulent GaHV-2s, that the more virulent virus had the competitive advantage over the less virulent virus in mixed infections; however, the less virulent virus was never eliminated. The finding in this thesis is the first report that GaHV-2 challenge at 5 dpv has caused suppression of the Rispens viral load. Possibly the higher GaHV-2 challenge dose used in this thesis (4000 PFU) and the virulence of FT158 contributed to the difference in findings to those of Islam *et al.* (2014).

In contrast, Islam and Walkden-Brown (2007), in a study in which chickens were vaccinated with HVT and GaHV-3 vaccines then challenged with pathogenic GaHV-2 isolates 5 days later, found that the challenge with pathogenic GaHV-2 markedly increased the shedding of the vaccinal viruses HVT and GaHV-3. This could possibly be due to the immunosuppressive effects of the challenge GaHV-2. There was no evidence of a similar phenomenon for the Rispens vaccine in the isolator experiment. In fact, as noted above, there was evidence of a reduction in Rispens viral load in chickens challenged with the more virulent FT158.

6.7 Viral loads of different tissues

In this experiment, it was clear that the effects of vaccination and challenge, which were very clearly observed in feather and PBL, were not seen or were greatly reduced in magnitude in dust. These treatment effects for feather and PBL appear to be very similar in the present study as well as in previous studies (Islam *et al.*, 2014); however, dust is different. The viral load of feather was always higher than the load in PBL. The reason may be that in PBL we assess the viral load in the lymphocytes, whereas in feather or the feather pulp we assess a collection of epithelial cells with infiltrated lymphocytes and lymphoid cell aggregations. Also the higher load may reflect a higher proportion of infected cells rather than a higher viral load in infected cells. Dust, on the other hand, is the only sample that only contains virus shed from the FFE of the bird. Therefore, dust, and not the other two tissues, is the only sample we can use to make inferences about viral fitness, shedding or transmission. However, in the present experiment there was no treatment effect on the dust as there was a very small difference between the continuous shedding of pathogenic GaHV-2 and Rispens virus without any significant effect from the vaccination and challenge. The lack of clear treatment effects on MDV shedding in dust have also been observed in a previous study (Islam *et al.*, 2014). Therefore, the conclusions regarding viral transmission deriving from inferences on viral loads of tissues other than dust or dander should be done cautiously.

On the other hand, a high correlation between Rispens viral load in feather and dust was observed in a recent study by Baigent *et al.* (2013) where vaccination was carried out on day 1 and challenge on day 7. Those scientists used different levels of vaccine (1000 PFU) doses, different dust collection methods, and a different experimental duration (31 dpc), all of which may have contributed to the high correlation level observed.

6.8 The patterns of pathogenic GaHV-2 and Rispens vaccine viral load over time

The pattern of Rispens viral load over time differed from pathogenic GaHV-2 viral load in PBL and feather but not in dust. A peak viral load was observed at 21-28 dpv in Rispens virus, before declining to the end of the experiment although the peaks were later than those seen in previous studies (Abdul-Careem *et al.*, 2008; Baigent et al., 2005b; Islam *et al.*, 2013; Islam *et al.*, 2014). This may be mainly due to the chickens in the current experiment were positive for mab, which could delay the Rispens virus replication. On the other hand the

pathogenic GaHV-2 viral load continued to increase or plateaued after 21 dpv. This suggests that the attenuation of Rispens virus has had a greater effect on viral load in lymphoid tissues rather than on viral shedding in dander.

Yunis *et al.* (2004) demonstrated that using viral loads of lower virulent virus JM-16 and a high virulent virus RK-1 that JM-16 went into latency after 6 dpi but vv+ RK-1 did not go into latency during the entire experimental period of 10 days. These scientists and others (Dunn *et al.*, 2014) also described the higher virulent GaHV-2 having a higher replication rate continuing to increase viral load with time. In the present experiment, probably the Rispens virus may have gone into latency after 21 days in PBL and feathers which explains why the Rispens viral load declined after 21 dpv. Furthermore, the two pathogenic GaHV-2 s did not go into latency at least within this experimental period.

6.9 Kinetics of Rispens CVI988 in long lived chickens

One of the objectives of the field study was to determine whether the infection with current commercial vaccine strains of Rispens CVI988 virus is lifelong. The results of this experiment were consistent with those of Rispens *et al.* (1972a) who found the Rispens virus (26^{th} passage duck embryo fibroblast culture) could be isolated in feathers of the birds for up to 2 years; however, the detection frequency varied between 30 and 70% (Rispens *et al.*, 1972a). The results of our study are consistent with those initial findings of Rispens. The virus was readily detected in feathers and dust in chickens aged between 3 days and 91 weeks with no obvious decline in viral load with age. It can be postulated that the long-lived birds are constantly exposed and may get re-infected with Rispens virus-contaminated poultry house dander, thereby maintaining the lifelong Rispens virus infection. In a recent experiment (Islam *et al.*, 2013a), current commercial Rispens vaccines available in the market were found to transmit effectively between chickens. We were unable to determine whether the birds were infected throughout life or whether their infection was due to recurrent exposure. However, it is more likely that the birds are infected lifelong as this is also a characteristic of herpesviruses in general (Maclachlan & Dubovi, 2010). Lifelong infection has also been reported for pathogenic MDVs (Witter *et al.*, 1971) and for vaccines of infectious laryngotracheitis (ILT), which is also a *Gallid herpesvirus* (Hughes *et al.*, 1991).

6.10 Co-infection of Rispens and GaHV-2 in the field

Until recently, due to lack of rapid molecular tests enabling differentiation between Rispens virus and pathogenic GaHV-2, it was not possible to study the level of co-infection of Rispens virus and pathogenic GaHV-2 in the field. Like all GaHV-2 vaccines, Rispens vaccine is an imperfect vaccine, as it reduces mortality and tumour formation caused by GaHV-2 but does not prevent infection, replication, and shedding of GaHV-2. In the field study, 120 feather DNA samples and 42 dust samples from layer chickens vaccinated with Rispens vaccine were subjected to a pathogenic GaHV-2-specific qPCR. The level of co-infection of Rispens and wild-type viruses in the field was at a very low level, being 7% in feather samples and 5% in dust samples. This could be due to a low level of challenge with GaHV-2, effective blockade of natural infection by the vaccinated chickens, or failure to detect low levels of pathogenic GaHV-2 in co-infected chickens. All three may be involved, particularly when the interval between vaccination and exposure to irregular challenge may be prolonged in the field. Islam *et al.,* (2014), using challenge with vvMDV pathotype 02LAR at various times relative to vaccination with Rispens (-10, -5, 0, 5, 10 days), showed that as the VCI increases detection of GaHV-2 become more difficult. At the maximum VCI of 10 days only 15% of PBL and less than 40% of feather samples were positive for GaHV-2, showing the inhibitory effect of Rispens vaccine. The farms in this experiment were in country areas with perhaps lower challenge than in dense peri-urban areas with high chicken densities.

6.11 Presence of Rispens virus in unvaccinated broiler flocks

Rispens *et al.* (1972a) reported that the virus readily transmitted between chickens at passage level 35. At very high passage levels transmission ceases (Witter, 1987; Witter *et al.*, 1995) and at the passage level used in today's commercial vaccines (likely to be between 40-45) a high level of virus shedding in dust and effective transmission to in-contact flock mates again occurs (Islam *et al.*, 2013a). In light of these findings the presence of Rispens CVI988 in unvaccinated chicken populations was investigated as part of this thesis. Dust DNA from 100 unvaccinated broiler flocks was analysed for the presence of Rispens vaccine. These 100 samples had previously returned positive results from a generic GaHV-2 qPCR assay. Of the samples, only 7% were found to be positive for the Rispens virus. This result provided preliminary evidence that the Rispens virus can escape to unvaccinated flocks and replicate

within them. It has been suggested by Jackson *et al.* (1976) that infection with apathogenic viruses circulating in the field occurs and protects against subsequent virulent GaHV-2 infection. However, can Rispens virus be successfully maintained in a chicken population in competition with other GaHV-2 s? In the results reported in Chapter 5, there was no difference in the viral copy numbers being shed in dust between pathogenic GaHV-2 and Rispens virus, lending support to the idea that it is possible for Rispens virus to become established in poultry populations naturally. One of the limitations of this study was the inability to obtain the precise location of these positive farms in relation to other farms where Rispens vaccination had been performed. Without this data, it can only be assumed that the farms that returned a positive result for Rispens virus from dust samples were in close proximity to breeder or layer farms where Rispens vaccination was a routine management measure. Occasionally, broiler farms are placed with surplus chicks for breeder farms and while they are not usually vaccinated with Rispens vaccine in this case it is possible that this may occasionally occur. There was, however, no evidence of such placements in the broiler flocks from which the dust samples were drawn. These findings of "escaped" Rispens virus warrant a more detailed and focussed study.

6.12 Conclusions

1. The protection provided by Rispens CVI988 vaccine is highly variable and appears to be independent of pathotype. This suggests that there is no "cross resistance" between the resistance to the effects of HVT vaccination (on which pathotype is largely based) and Rispens vaccination.
2. Rispens vaccine take can be successfully measured using Rispens qPCR assay of PBL and feathers at 14 dpv and 21 dpv, and dust at 21dpv under experimental conditions. Similarly, in the field, the vaccinal success can be measured effectively in feather tips at around 2 weeks and dust at around 3 weeks of age.
3. Early prediction of MD can be more reliably assessed by pathogenic GaHV-2 load in various tissues and environmental samples. GaHV-2 load in PBL provides the earliest prediction of subsequent MD incidence at 7 dpc, followed by feather at 14 dpc, and dust at 21 dpc. Viral loads of Rispens virus did not provide an equally predictive power for the subsequent MD outcome. However, this may be partly due to the low power to detect associations in this experiment as there were only four isolators with vaccinated and challenged birds.

4. The inhibitory effects of vaccination with Rispens vaccine on GaHV-2 viral load were greatest for the lower virulence challenge virus (MPF57), thus favouring the higher virulence FT158. Taken as a whole, the data are supportive of the proposition that the Rispens vaccine is similar to HVT in providing a host environment that favours more virulent GAHV-2 s. The ongoing efficacy of the vaccine has other plausible explanations that do not involve effects on selection for more virulent GAHV-2s.
5. Virulence of the three MDVs in the current experiment was positively correlated with replication of the virus in PBL and feathers, and to a lesser extent in shedding of the virus.
6. There is a very low level of co-infection of pathogenic GAHV-2 in Rispens-vaccinated chickens in the field, in the areas investigated.
7. There is preliminary of evidence of escape of the Rispens CVI988 vaccine to unvaccinated chicken populations under field conditions. Further studies should be carried out to confirm this finding.

6.13 Future work

1. The viral kinetics of the Australian commercial Rispens vaccines has been studied in an earlier experiment by Islam *et al.* (2013a). However, the protection provided by these vaccines has not been compared. As there is a variability of the PI between Rispens vaccines from different origins and having different passage histories, an experiment which compares the protective abilities would be of use for the Australian poultry.
2. In order to prove Rispens vaccines actually favours transmission of highly virulent GaHV-2 over low virulent viruses, it would be useful to perform transmission studies using sentinel birds. Read *et al.* (2015) recently examined this for HVT showing that HVT vaccination did favour transmission of highly virulent GaHV-2.
3. The pathotype of the co-infecting GaHV-2 viruses with Rispens vaccine from the field experiment were not identified. It would be beneficial to identify these viruses for the industry and determine if they represent a particularly virulent pathotype.
4. We have selected 100 dust DNA samples from unvaccinated broiler farms to identfy whether the Rispens vaccine has “escaped” to unvaccinated broiler populations. It would be useful to confirm this in a follow up study and also to determine whether the

Rispens vaccine is found in HVT vaccinated broiler populations as well as in unvaccinated backyard chickens.

5. One of the limitations of the isolator experiment was comparing the protection provided by Rispens vaccine for only two GaHV-2 isolates. If we could have compared more isolates with perhaps a wider range of pathotypes we could have arrived at stronger conclusions.
6. The PIs obtained in the isolator experiment were moderate for the MPF57 and FT158 isolates. It is possible that a Rispens+HVT bivalent vaccine may provide superior protection against Australian GaHV-2 strains and this should be investigated.
7. In the current work, the ad hoc method of field dust sample collection probably led to unwanted additional variation in viral load values. For future routine surveillance or diagnostic work a standardized collection method such as settle plates or a fixed sampling site within sheds should be tested, validated and introduced.
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9. Feather pulp samples can be collected in FTA ®□filter cards and could be transported and stored without a cold chain (Cortes et al., 2009). Future studies should examine the robustness and practicality of this method. Contamination of samples during collection from airborne GaHV-2 would be a significant risk to manage.

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