1 Review of Literature

1.1 Introduction to Marek's disease

Marek's disease (MD) was first described by Jozsef Marek as fowl paralysis in 1907 (Marek, 1907b). He described a paralytic disease affecting the legs and wings of cocks and characterized by thickening of the sacral plexus and spinal nerve roots, loss of nerve fibres and infiltration of affected nerves by mononuclear cells; however he could not determine the causative agent. Subsequently many researchers worldwide tried to identify the cause of the disease as it became increasingly prevalent. At last in 1967, Churchill & Biggs (1967) identified the causative agent of MD was a cell associated herpes virus. Later Marek's disease virus (MDV) classified as an alphaherpesvirus of the genus *Mardivirus* that has 3 species namely gallid herpesvirus 2 (GaHV-2), gallid herpesvirus type 3 (GaHV-3) and meleagrid herpesvirus type 1, (MeHV-1) which are commonly referred to as MDV serotype 1 (MDV-1), MDV serotype 2 (MDV-2) and turkey herpesvirus (HVT) respectively (King*, et al.*, 2012). MD is a lymphoproliferative disease that can involve most organs and tissues including peripheral nerves. The most commonly affected organs and tissues are liver, spleen, gonads, kidney, proventriculus, mesenteries, gastro-intestinal tract, heart and lungs (Biggs, 2001). MDV is a host specific virus that affects various birds and other animals including humans are not at risk of infection (Tai, 2006).

MD was a severe economic threat to the poultry industry until 1969 when live virus vaccines against MD were developed and made commercially available (Churchill*, et al.*, 1969a; Islam*, et al.*, 2006b; Okazaki*, et al.*, 1970). The introduction of vaccination in the early 1970s was a major advancement in the science of vaccinology, this being the first demonstration of effective and widespread use of vaccination to prevent a cancer in any species. The magnitude of MD morbidity and mortality was significantly reduced, but vaccine breaks were reported some time later and increased virulence of challenge viruses occurred within a decade or so (Pastoret, 2004). To combat the increasing level of virulence of MDV new vaccines were introduced such as bivalent (HVT + MDV-2) vaccine, Rispens/CVI988 (attenuated MDV-1) vaccine (Schat and Calnek, 1978).

The clinical manifestation of MD continued to change and MDV continues to be a threat to poultry production, despite the widespread and intensive use of vaccination, including vaccination of the late embryo (Pastoret, 2004). The disease can be successfully controlled by vaccination with attenuated or non-pathogenic MDV strains; however, none of the available vaccines produces sterile immunity. Vaccine failures do occur as field strains continue to evolve towards greater virulence, and this evolution is likely to be driven by the vaccines themselves (Gandon*, et al.*, 2001; Witter, 1997). Two general strategies can be considered to improve protection by vaccination. Firstly by the development of novel vaccines, particularly those that can prevent replication and shedding of pathogenic virus by the vaccinated host or secondly by maximizing the potential of existing vaccines. The second goal requires investigation of optimal timing and vaccine delivery route, and optimal vaccination regimes for different chicken breeds.

1.2 Economic Significance of Marek's Disease

MD is an economically important lymphoid neoplasm of chickens. The resulting morbidity and mortality is responsible for decreased productivity and major economic losses (Morrow and Fehler, 2004). Before the introduction of vaccines, MD represented a serious economic threat to the poultry industry causing up to 60 % mortality in layer flocks and up to 10 % condemnations in broiler flocks (Schat and Nair, 2008). Vaccination against MD was introduced in 1970 and effectively protected against disease and mortality. It is intensively used worldwide with about 50 billion doses administered per year (Morrow and Fehler, 2004). Because vaccines are not 100 % effective, periodic losses still take place, however comprehensive reports on the worldwide MD situation are difficult to obtain. There are different reasons for this (Morrow and Fehler, 2004):

- MD is an endemic disease and is not considered a notifiable disease;
- Low-level losses after MD vaccination are normally accepted and treated as vaccination failures rather than vaccine breaks;
- The incidence of MD is often linked to financial claims between rearing companies and hatcheries, or hatcheries and vaccine manufacturers. Frequently such cases are not made public;

- As prevention of the disease involves optimal hygiene and biosecurity measures, as well a number of other management procedures, many MD cases are not reported due to avoid damaging the status of the company; and
- Diagnosis was not easy until the recent development of molecular techniques.

Moreover, the number of unreported cases of MD is probably very high (Morrow and Fehler, 2004). These issues seriously weaken the assessment of worldwide economic losses from MD. The economic impact of MD on the world poultry industry is thought to be in the range of roughly US$ 1 - 2 billion yearly (Morrow and Fehler, 2004). The Food and Agriculture Organization (FAO) estimates a total number of 99 million tonnes broiler meat and 69 million tonnes of eggs produced in the year 2010, with a total value of about US$ 100 - 200 billion. This means that the damage estimate given above corresponds to an MD-induced loss of about one per cent of the value.

1.3 Public Health significance of Marek's Disease

The public health significance of MD has been of little concern. It was suggested that MDV may have had a role of causing multiple sclerosis of humans (Macgregor and Latiwonk, 1993) but this was proven to be a misconception later on (Hennig, *et al.*, 1998). These authors investigated 107 well defined multiple sclerosis patients with regard to latent MDV infection and did not find MDV-related sequences in leukocyte DNA of any of the patients. More recently, it has been argued that HVT or HVT-like viruses are responsible for multiple sclerosis of humans as they found high incidence of multiple sclerosis clusters within specific geographical distributions where there is high density of wild birds so they attempted to draw a link between HVT or HVT-like viruses, probably spread with the global migration wild bird flyways, and its possible involvement in multiple sclerosis aetiology, as an initiating or causative agent (Bougiouklis, 2006). However there is no supportive proof for their claim. The DNA sequence of the gD gene of MDV has been found in human sera from persons with and without contact with poultry (Laurent, *et al.*, 2001), though MDV unique genes were not analyzed in these studies. However, MDV sequences could not be detected by quantitative real time PCR in 300 human plasma samples in another study (Hennig, *et al.*, 2003). Currently, there is no convincing evidence that suggests that MDV infects humans or negatively affect human health (Schat and Nair, 2008).

1.4 Scientific Significance of Marek's Disease

MD has a great influence in the fields of veterinary medicine, basic science, and comparative oncology. The disease is especially multifaceted involving interaction between neoplasia and inflammation resulting in a wide range of pathology and clinical signs which are modified in significant ways by host genetic influences. All these features give this disease an exceptionally multifarious pattern. MDV, an alphaherpesvirus with lymphotropic properties more commonly observed in gammaherpesviruses, is highly cell associated, establishes latency in lymphocytes, and contains an oncogene (*meq*) in its genome so is capable of inducing lymphomas. However, MDV is directly transmitted by inhalation of contaminated feather dander released by infected chickens (Beasley, *et al.*, 1970) and its virulence is increasing over time (Witter, 1997). It has two distinctive non-oncogenic closely related viruses which naturally infect chickens and turkeys (MDV serotype 2 and HVT respectively). Infection stimulates complex immune responses that frequently result in a high level of protection. Vaccination for MD represents a magnificent example of successful disease control in veterinary medicine and this was the first effective vaccine against cancer in any species. However, vaccination is imperfect and thus vaccinal and pathogenic virus can co-exist in the host. MD vaccines prevent tumor formation in susceptible chickens but do not prevent MDV infection and transmission and may escalate continuous MDV evolution towards higher virulence (Atkins, *et al.*, 2012).

1.5 Aetiology of Marek's Disease

The initial steps towards identifying the cause of MD were to determine and isolate the infectious agents or material by which the disease transmits and the development of a routine assay system (Biggs and Payne, 1967; Biggs and Payne, 1963; Sevoian, *et al.*, 1962). Subsequently, it was shown in a series of experiments that infectious material in both blood and tumour cells that induced MD was highly cell associated and it was concluded from these studies that the infectious element was an avian cell (Biggs and Payne, 1967; Biggs, *et al.*, 1968; Biggs, 2004). It was then correctly assumed that the infection could be due to a cell-associated virus that requires viable cells for the successful transmission of the infection. On the basis of this, efforts were made to isolate the virus and develop an assay system in cell culture using tumour cells or whole blood as the inoculum. This was eventually successful and a cytopathic effect was observed in the cell cultures, which was typical for herpesviruses (Churchill and Biggs, 1967, 1968; Nazerian and Burmester, 1968).

Marek's disease virus (MDV) is a DNA virus belonging to the family Herpesviridae, sub-family Alphaherpesvirinae and genus Mardivirus (Fauquet, *et al.*, 2005; King, *et al.*, 2012). The herpesvirus genomes are molecules that range from 108 to 230 kbp in size (Davidson, *et al.*, 2002; Maotani, *et al.*, 1986; Nazerian and Burmester, 1968). Six different universal genome organizations are notable in the herpesviruses known as classes A to F, where only class D and E genomes are established in the Alphaherpesvirinae and the MDVs represent class E genomes, which have an organization that is identical to herpes simplex virus type 1 (HSV-1) (Roizman and Pellett, 2001). On the other hand, based on pathological characteristics MDVs were primarily classified as gammaherpesviruses, similar to the Epstein-Barr virus (Witter, *et al.*, 1969).

1.6 Biology of Marek's Disease virus

1.6.1 Classification

MDV can be classified based on serotype, pathotype and neuropathotype. A brief description of each is given below:

1.6.1.1 Serotype or species

There are three closely related but distinct species within the genus Mardivirus. Prior to phylogenetic and taxonomic studies enabling clear species distinctions the three species were serologically typed and classified as serotypes of MDV. Serotype 1 MDV (MDV-1) contains all pathogenic MDVs whereas serotype 2 MDV (MDV-2) and serotype 3 which is also referred to as turkey herpesvirus (HVT), are apathogenic or only very weakly pathogenic isolates. However, the official nomenclature of these serotypes according to the International Classification Taxonomy of viruses (ICTV) are as follows:

- gallid herpesvirus type 2, (GaHV-2) referred to as MDV-1
- gallid herpesvirus type 3, (GaHV-3) referred to as MDV-2 and
- meleagrid herpesvirus type 1, (MeHV-1) referred to as turkey herpesvirus (HVT)

Because the serotype classification is still widely used and convenient, this classification will be used throughout the thesis.

The sequence similarity of virus proteins between the three serotypes ranges from 50 % to 80 %. Only MDV-1 causes clinical disease in chickens, the other two species are non-pathogenic

and used as vaccine. (Afonso, *et al.*, 2001; Schat and Calnek, 1978; Witter, 1982). An overview of these serotype are described in Table 1.1.

1.6.1.2 Pathotype

The term "pathotype", although properly used to designate classes of organisms that induce different types of pathology, has in the case of Marek's disease (MD) been widely applied to designate differences in the virulence of isolates as measured by the frequency and severity of disease induced (Witter, *et al.*, 2005). On the basis of these studies, four pathotypes of MDV are recognized - mild (mMDV), virulent (vMDV), very virulent (vvMDV) and very virulent plus (vv+MDV) (Witter, 1997). An overview of this classification is described in Table 1.1. This method has been used to pathotype more than 45 isolates and is claimed as the 'gold standard' for pathotype classification of MDV-1 strains (Witter, *et al.*, 2005). However, the requirement for a specific type of chicken (15 x 7 ab+) with the high cost of keeping large numbers of those chickens through a 9-week experimental period, and a statistical method to compare the lesion responses to those of the isolates JM/102W (prototype vMDV-1) and Md5 (prototype vvMDV-1) have prevented other laboratories using this method.

Table 1.1 Serotypes and pathotypes of MDV

Serotype	Pathotype	ICTV taxonomy	Origin	Disease in chickens	Used as vaccine
MDV-1	mild (mMDV)	Gallid herpesvirus type 2, (GaHV-2)		Yes	No
	Virulent (vMDV)			Yes	No
	very virulent (vvMDV)			Yes	No
	very virulent plus (vv+MDV)			Yes	No
MDV-2	non - oncogenic	Gallid herpesvirus type 3 (GaHV-3)	chicken	No	Yes
HVT	non - oncogenic	Meleagrid herpesvirus type 1, (MeHV-1)	turkey	No	Yes

1.6.1.3 Neuropathotype

Gimeno *et al*. (2002) suggested a new classification system based on neurological responses which was complementary to ADOL pathotyping. In this system, an MDV isolate may be classified in one of three groups, designated neuropathotype A, B and C, which roughly correspond to the virulent, very virulent and very virulent plus pathotypes (Gimeno*, et al.*, 2002). An additional advantage of this system was it included a combination of variables that involve two measures of the early cytolytic events (transient paralysis and mortality before 15 days post inoculation) and one of the late lymphoproliferative lesions (Persistent Neurologic Disease). Another advantage of this system was that it required fewer birds and shorter time compared with the pathotyping system. However this system can be used only in maternal antibody negative (Mab-), unvaccinated chickens (Gimeno*, et al.*, 2002). Details of the classification are given in Table 1.2. However, recent studies showed that if the clinical observation period was extended beyond 0 - 23 dpc which Gimeno *et al*. (2002) used, a more sensitive measure of virulence may be obtained (Wajid*, et al.*, 2013).

Table 1.2 Classification of MDV on the basis of neuropathotype (Gimeno, *et al.*, 2002)

Classification	Description
Neuropathotype A	Produced transient paralysis (TP) in a low to medium percentage of chickens (less than 50 %). Very low mortality 9 - 15 day post-inoculation (DPI) (less than 10 %). Produced Persistent Neurologic Disease (PND) in a proportion of chickens between 21 to 23 DPI (less than 15 %).
Neuropathotype B	Both TP and PND have higher frequencies than in Neuropathotype A (paralysis ranged between 33 % and 96 %and PND ranged between 18 % and 58 %). Low mortality rate (less than 25 %).
Neuropathotype C	Induced most severe neurologic responses. The frequencies of TP and PND are very high (up to 100 %). High rate of mortality (ranging between 17 % to 100 %).

1.6.2 Morphology

The morphology of MDV is similar to other herpesviruses. The shape of the virus is hexagonal and the mature virion is about 180 nm in diameter, enclosed in a complex envelope. The envelope surrounds a capsid of 100 nm in diameter consisting of 162 capsomeres. The electron-dense inner zone is constituted of a double stranded DNA. The external membrane of the viral envelope is irregular and 20 nm thick (Hamdy, *et al.*, 1974; Morgan, *et al.*, 1959; Nazerian and Burmester, 1968). Figure 1.1 shows electron micrographs of MDV.

Viruses of all 3 serotypes of MDV have a general genome structure similar to herpes simplex virus genome with a unique long (U_L) sequence and unique short (U_S) sequence which are flanked by sets of inverted repeat sequences: terminal repeat long (T_{RL}), internal repeat long (I_{RL}), internal repeat short (I_{RS}), terminal repeat short (T_{RS}) . Alpha (α) like sequences typical for all alphaherpesviruses are located at the terminal ends of the T_{RL} and T_{RS} and between the I_{RL}, and I_{RS} regions (Afonso, *et al.*, 2001; Burnside, *et al.*, 2006; Cebrian, *et al.*, 1982; Izumiya, *et al.*, 2001; Kishi, *et al.*, 1991; Tulman, *et al.*, 2000).

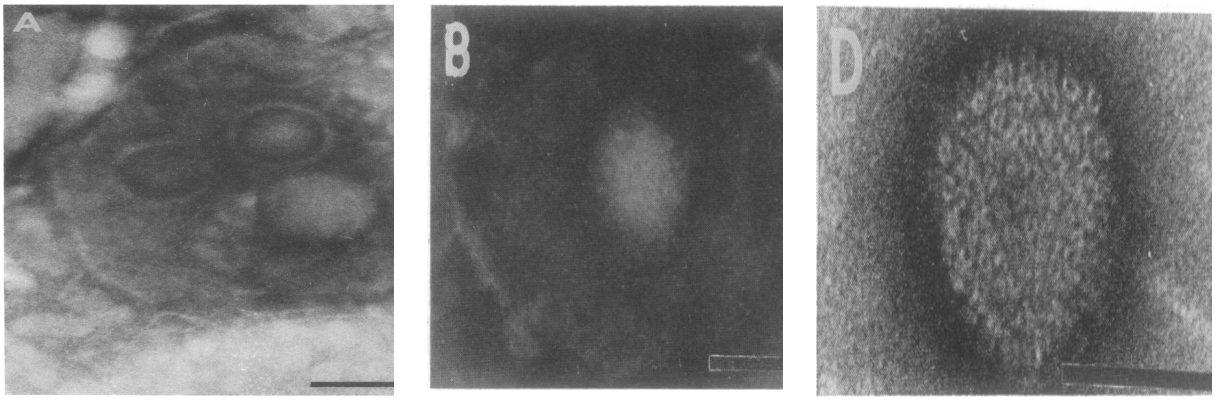

Figure 1.1 Partially purified Marek's disease stained with silicotungstic acid. A: three Marek's disease virus nucleocapsids in a single envelope; B: enveloped Marek's disease virus; D: naked Marek's disease virus nucleocapsid. The bar at the bottom right hand corner of each electron photomicrograph represents 100 nm.(Lee, *et al.*, 1969)

The replication of the herpes simplex virus is completed within the nucleus. In the nucleus the granular areas represent the foci in which the various components of the viral particle become spatially arranged so as to present the electron microscopic appearance of a central body and a

single outer membrane. The membranes are actually the process of formation at many sites in the aggregate of granules. Viral particles become enclosed by a second membrane before release from the nucleus, and they form at an intra-nuclear site removed from the primary template area where the internal body and initial membrane are believed to differentiate (Morgan, *et al.*, 1959).

Virus may enter into the cytoplasm upon disruption of the nucleus but this is not the sole mechanism of egress. Reduplication of the nuclear membranes probably enables the virus to gain release into the cytoplasm without disruption of the nucleus. New membranes are laid down behind the virus as it passes into the cytoplasm. Within the cytoplasm, the virus is generally lodged in walled vacuoles (Morgan, *et al.*, 1959).

1.6.3 Susceptibility to chemical and physical agents

MDV obtained from the skin of infected chickens was inactivated when treated for ten minutes at pH 3 or 11, or when stored for 2 weeks at 4 ˚C, 4 days at 25 ˚C, 18 hours at 37 ˚C, 30 minutes at 56 ˚C, or 10 minutes at 60 ˚C (Calnek and Adldinger, 1971). Dander, litter and feathers from infected chickens are infectious and presumably contain cell free virus from the feather follicle epithelium bound to cellular debris. The infectivity of such materials was retained for 4 - 8 months at room temperature possibly due to the viral particles being protected by the characteristic double envelope (Hlozanek, *et al.*, 1973; Witter, *et al.*, 1968). Dust stored at 37 °C remained infective for 90 days with no apparent decline in infectivity over this period (Blake, *et al.*, 2005). Several chemical disinfectant solutions (chlorine, quaternary ammonium compound, organic iodine, cresylic acid, synthetic phenol, and sodium hydroxide) were able to destroy infectivity of dried feathers within a 10-minute treatment period (Calnek and Hitchner, 1973). The most successful disinfecting agent is a combination of formaldehyde vapour and a preparation based on iodine bound to organic carriers (Hlozanek, *et al.*, 1977). In litter, the survival of virus may be affected adversely by increased humidity (Witter, *et al.*, 1968).

1.6.4 Virus-cell interactions

The virus-cell relationship of MDV is complex, and cell-free virus is recovered only from the feather follicle epithelium, while in all other tissues, the virus is generally cell-associated. Virus-cell interactions are mainly two types. The first one is productive infection where viral

replication and death of the cells occurs. Productive infection is subdivided into two types: fully productive and restrictively productive infection. The second main type of interaction is non-productive infection, which is further classified into two types: non-productive latent infection and non-productive neoplastic infection (Payne, 2004). These types of infection are summarised below.

1.6.4.1 Fully productive infection

In this kind of infection, virus replication is complete and enveloped virus produced and released resulting in death of the cells (Biggs, 2001). This kind of infection occurs in the feather follicle epithelium where large number of fully infectious viral particles are produced (Calnek*, et al.*, 1970a; Calnek*, et al.*, 1970b).

1.6.4.2 Restrictively productive infection (Abortive)

In these cases, fully infectious virus is not released from the cell. However, the viral genome expression may range from virus specific antigens to enveloped intracellular virions, which are rare. Cytolytic infection occurs later. Enveloped virions are generally only found in feather follicle epithelium of infected birds. However, they can be found occasionally in other cells and tissues of young antibody-free chicks (Frazier and Biggs, 1972).

1.6.4.3 Non-productive latent infection

This is a non-productive infection. Hybridization with viral DNA probes or methods that activate the viral genome only can detect the latent infection (Campbell and Woode, 1969). It is well established that all serotypes of MDV can persist in the bird for lifetime in latent form in lymphocytes. The latently infected lymphocytes contain less than five copies of viral DNA and it is not possible to detect viral antigens or virions in these cells (Calnek*, et al.*, 1981; Payne, 1985),

1.6.4.4 Non-productive neoplastic infection

In this phase, the viral genome persists in lymphoid cells resulting in immortalization of the cells with some limited antigenic expression. By the transcription and expression of a number of genes, neoplastic infection can be differentiated from latent infection (Sugaya*, et al.*, 1990). The target cell are T-cells, even though transformed cells lack the major T-cell antigen (Ross*, et al.*, 1977).

1.7 Epidemiology

1.7.1 Host

Chickens are the natural host for MD. All chickens including game fowl, jungle fowl and native breeds are susceptible to MDV infection and tumour development (Cho and Kenzy, 1975a; Grewal and Balwant, 1976; Kenzy*, et al.*, 1964); however turkeys, quail and pheasants are also susceptible. MD typical lesions like lymphomas and nerve lesions are rarely found in susceptible pheasants and related species such as the Black Francolin (Harriss, 1939; Jungherr, 1939; Pettit*, et al.*, 1976). In Japan and in far east region of Russia, MD has also been reported in white fronted geese (Murata*, et al.*, 2007).

1.7.2 Transmission

Horizontal transmission between chickens is the only significant means of MDV-1 and MDV-2 spread (Calnek and Hitchner, 1973). Transmission of HVT is horizontal in turkeys but very limited in chickens (Cho, 1976; Cho and Kenzy, 1975b). MDV is transmitted by direct or indirect contact, most effectively through the respiratory route (Biggs and Payne, 1967; Calnek and Hitchner, 1969). Epithelial cells in the keratinizing layer of the feather follicle are the main source of contamination as these cells reproduce fully infectious virus (Calnek*, et al.*, 1970). Feather and dander of MDV infected birds are infectious and dust of poultry housings remains infected for several months at 20-25 ˚C and for years at 4 ˚C (Calnek, 1986; Schat*, et al.*, 1982). Healthy birds commonly acquire infection through aerosols, or contaminated farm personnel. The daily infection rate has been estimated to be 8.4 % per day in unvaccinated broiler chickens and 0.8 % per day in vaccinated broiler chickens between 5 and 15 days. Darkling beetles (*Alphitobius diaperinus*) also inertly transmit the disease (Eidson*, et al.*, 1966). Vertical transmission of MDV does not occur (Calnek, 1986; Rispens*, et al.*, 1972a; Schat*, et al.*, 1982a). Transmission from dam to progeny due to external egg contamination also does not occur because at incubation temperatures and humidity virus does not survive until hatching (Calnek and Hitchner, 1973b). However, experimental transmission is accomplished by inoculation of day old, genetically susceptible chicks with blood, tumour suspension or cell free virus by virtually any parenteral route. The virus can be transmitted by intratracheal instillation or inhalation exposure using cell free virus preparations (Schat and Nair, 2008).

1.7.3 Incubation period

The incubation period is the time elapsed between exposure to a pathogenic organism and when symptoms and signs are first apparent. The incubation period of the disease differs with the virus strain and dose, route of infection, maternal antibody status, phenotype, age and sex of the chickens (Calnek and Witter, 1997). The incubation period is 3 to 4 weeks in broilers whereas in layer and breeders the disease occurs within 16 to 20 weeks of age (Purchase, 1985). However, MD outbreaks sometimes occur at 3 to 4 weeks in unvaccinated layer chickens though most of the serious cases commence following 8 to 9 weeks of age (Kreager, 1998).

Under experimental conditions, cytolytic infection can be found at 3 - 6 days post infection (dpi) and is followed by atrophy of thymus and bursa within 6 - 8 dpi (Calnek, 2001). Transient paralysis is usually found between 8 - 18 dpi (Witter*, et al.*, 1999), however in field cases this usually occurs at 6 - 12 weeks of age. Induction of tumours has been reported as early as 10 to 14 days after inoculation of virus infected cells in maternal antibody negative chicks (Witter*, et al.*, 1980).

Sire and dam effects have a significant impact on the susceptibility of the offspring to MD. Females are more susceptible to the disease than males (Biggs, 1968; Payne and Biggs, 1967; Purchase and Biggs, 1967). Earlier it had been reported that older adult chickens have strong age resistance to the induction of MD tumours but a recent report states that adult chickens raised under specific pathogen free conditions showed greater tumour occurrence when challenged with newly evolved strains (Witter, 1998a; Witter, 1998b). Maternal antibody delays appearance of the disease, transient paralysis can be reproduced constantly only in chickens lacking maternal antibodies (Kenzy*, et al.*, 1973). The early mortality syndrome is also reduced markedly in chickens with maternal antibodies (Witter*, et al.*, 1980).

1.7.4 Morbidity and Mortality

Before 1970, prior to use of vaccines the mortality rate in layers and breeders was 25 - 30 %, sometimes up to 50 %. The incidence of disease was up to 50 % in layers and 10 % in broilers. After the introduction of vaccine the mortality rate was reduced to 5 % in commercial layers and 0.1 % to 0.5 % in broilers (Purchase, 1985).

The incidence of MD is highly variable depending on many factors such as the virulence of the virus, natural and acquired immune status, sex and age of host, and dose and route of exposure in the field. Mortality is generally equal to morbidity while a small percentage of sick birds have been reported to recover from clinical signs (Biggs and Payne, 1967).

Milder strains of MD mainly cause peripheral nerve lesions, but the more virulent MDV pathotypes more often induce higher mortality and more visceral lymphomas and have a tendency to more often break through genetic host resistance or immunity induced by vaccination (Biggs*, et al.*, 1965; Witter*, et al.*, 1980).

Doses may influence disease frequency under natural conditions even though the MD response in genetically susceptible birds given virulent virus was found to be maximal even when a limiting dilution of virus was inoculated (Smith and Calnek, 1974).

Sire and dam effects have a significant impact on the susceptibility of the offspring to MD. Females often die earlier and experience higher losses than males (Biggs, 1968; Payne and Biggs, 1967; Purchase and Biggs, 1967).

The presence of maternally derived antibodies in young chicks can significantly protect them against early challenge with MDV (Baigent and Davison, 2004). *In ovo* or neonatal vaccination of chicks with HVT, MDV-2 / HVT bivalent vaccine or Rispens/CVI988 MDV-1 vaccine also protects birds against vMDV, vvMDV or vv+MDV respectively (Baigent and Davison, 2004).

Newly hatched chicks and older chickens are both susceptible to infection with MDV (Calnek, 2001), but cytolytic infection resolves more rapidly in older chickens (Buscaglia*, et al.*, 1988).

1.8 Pathogenesis

The pathogenesis of MD involves a complex life cycle and Calnek (Calnek, 2001) proposed four phases of MD pathogenesis: Early cytolytic phase, latent phase, late cytolytic and immunosuppressive phase and proliferative phase. The cytolytic form is characterized by the expression of viral antigens, formation of naked nuclear virions and cell-associated infectivity. This occurs in lymphoid tissues and, to a lesser extent, parenchymatous tissues and results in cell death by apoptosis. During latent and tumour stages the viral genome persists in lymphoid cells that express no viral antigen or show limited viral expression resulting in the

immortalization of the cells (Calnek, 1986). The mode of MDV replication changes according to a defined pattern depending on changes in virus-cell interactions at different stages of the disease and in different tissues. All chickens are susceptible to infection with MDV and shed virus, even resistant genotypes which do not develop clinical MD (Cole, 1968; Hansen*, et al.*, 1967). These are summarised in Figure 1.2 and described in more detail below.

1.8.1 Early cytolytic phase

The virus enters into the host via the respiratory tract and reaches the lymphoid organs by migrating macrophages within 24 - 36 hours of initial infection. Within 3 - 6 days, cytolytic infection is present in the B cells of spleen, thymus and bursa of Fabricius (Baigent and Davison, 1999; Barrow*, et al.*, 2003; Calnek, 2001; Schat and Nair, 2008).

This necrotizing infection causes an acute inflammatory response following an invasion of many cell types including macrophages, thymus-derived (T) - and B-lymphocytes, and various granulocytes (Payne and Roszkowski, 1973). With activation, T-lymphocytes become susceptible to infection themselves (Calnek, 1986). The activation of T cells during the early cytolytic phase is important for establishing infection in the target cells for latency and transformation (Schat, 1987).

The consequence of this phase is a transient atrophy of the lymphoid organs, particularly the thymus and bursa (Calnek, 2001). The atrophy may become permanent (transient or permanent immunosuppression) or the birds may recover within 8 - 14 dpi which depends on the virulence of the virus (Calnek*, et al.*, 1979). The vv strains (such as Md5) and vv+ strains (such as RK-1) generally cause more severe atrophy then less virulent strains (Calnek, 1998). The level of infection is generally similar in susceptible and genetically resistant chicken breeds during the early cytolytic period (Fabricant*, et al.*, 1977; Kaiser*, et al.*, 2003).

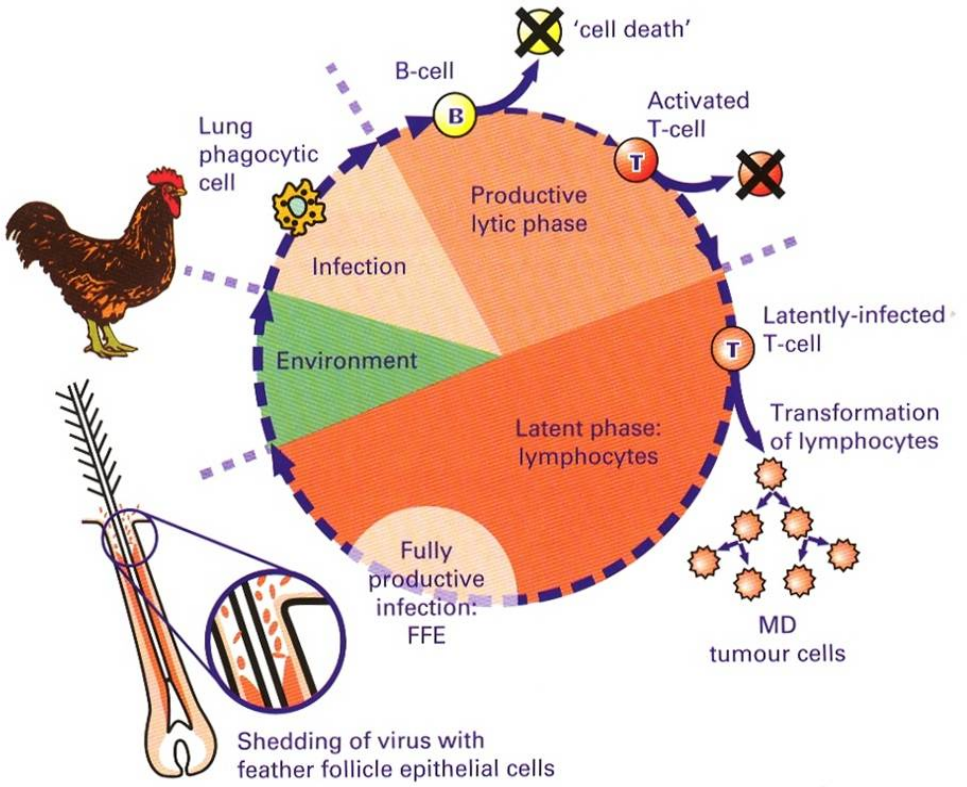

Figure 1.2 Stages in the cycle of MD pathogenesis. Birds acquire infection by inhalation of infectious virus shed from the feather follicle epithelium (FFE). Following early replication in the lungs, the virus replicates in the lymphoid organs. B-cells go through a lytic infection, resulting in the activation of T-cells, which are target cells of 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 (Baigent and Davison, 2004).

1.8.2 Latent phase

The infection becomes latent after 6 - 7 dpi probably due to the development of an adaptive immune response. The latency is deferred by the ablation of cell-mediated immunity (CMI) or infection with more virulent virus (Buscaglia*, et al.*, 1988; Yunis*, et al.*, 2004). Mainly latently infected cells are CD4+ T cells, though CD8+ T cells and B cells can also be involved (Calnek*, et al.*, 1984; Shek*, et al.*, 1983). Resistant birds and susceptible birds infected with vv and vv+ strains may exhibit permanent immunosuppression after the second or third week and the infection in genetically resistant birds may last for life (Schat and Nair, 2008; Witter*, et al.*, 1971).

1.8.3 Late cytolytic and immunosuppressive phase

The inflammatory changes in the lymphoid organs, a direct response to cytolytic infection of B-cells, resolve from 7 - 14 dpi (Baigent and Davison, 2004). However, in MD susceptible birds, a second wave of semi-productive infection and cytolysis from 14 - 21 dpi has been reported (Calnek, 1986). This coincides with permanent immunosuppression and occurs just before transformation. It is suggested that latently infected cells carry the virus to these lymphoid tissues, where it becomes reactivated due to a secondary wave of immunosuppression (Calnek, 1986). It is not clear what precipitates this phase. However, possible theories involve dysfunction of CTL or CD4+ T-cells due to viral mediated factors as they maintain latent infection (Calnek, 1986, 2001; Cardin*, et al.*, 1996).

1.8.4 Proliferative phase

This phase consists of tumour development and from 21 dpi death may occur because of lymphoma. There is a multifaceted tumour composition, consisting of a combination of neoplastic, inflammatory and immunologically devoted and non-devoted cells (Payne and Roszkowski, 1972). T and B cells are both present and the T cells are generally $CD4^+$ cells expressing TCRαβ1 or TCRαβ2 and MHC class II (Schat*, et al.*, 1991). The infection of transformed cells is non-productive *in vivo* and *in vitro*. The activation of T cells in response to the lytic infection of B cells is an important occurrence in the pathogenesis of MD by providing a profuse supply of cells that are the natural target cells for transformation (Calnek, 1986; Schat*, et al.*, 1982b). The probable factors required for transformation are susceptibility to infection, intrinsic or extrinsic control of virus replication (latency), cell division to incorporate virus genome and expression of viral oncogenes, activation of cellular oncogenes or oppression of the initiation of apoptosis (Schat and Nair, 2008).

1.9 Clinical signs

Clinical signs have changed over decades mainly because of the changing epidemiology of MD (Morrow and Fehler, 2004) and other diseases caused by avian leukosis virus (ALV) (such as lymphoid leukosis (LL) and myeloid leukosis) and reticuloendotheliosis virus (REV) which can induce tumour formation and may be difficult to distinguish from MD (Payne and Venugopal, 2000; Witter*, et al.*, 1970b). In 1970, guidelines for the pathologic diagnosis of MD and its differentiation from LL were described which were based on visible differences regarding distribution of tumours, and occurring cell type and conformation of the tumours

and in 1978, differential mortality and lesion responses to REV infection described in MD-resistant and susceptible chicken cell lines (Scofield*, et al.*, 1978; Siccardi and Burmester, 1970).

Clinical signs related to MD differ in accordance with the particular syndrome. Chickens with fowl paralysis or MD lymphoma syndromes may show signs, but only some are particular to MD (Biggs, 1968). MD clinical signs may occur from 4 weeks of age in chickens, however they are normally seen between 12 to 14 weeks of age and occasionally later (Nair*, et al.*, 1995). Two forms of MD exist, classical and acute. The classical form of the disease was described by Marek in 1907 which was a chronic, neuropathic condition (Marek, 1907b). On the other hand the acute form of the disease, which was more highly pathogenic, was first reported in 1950's (Benton and Cover, 1957).

In the classical form of the disease, neural signs occur over a few weeks or many months and mortality rate rarely exceeds 10 – 15 %. Incoordination or stilted gait may be observed first in MD affected chickens because locomotory disturbances can be detected easily. Paralysis and dilation of the crop and/or gasping also occur due to involvement of the vagus nerve (Nair*, et al.*, 1995). A specific characteristic syndrome of classical MD is a bird with one leg stretched forward and the other back which is the result of unilateral paresis or paralysis of the leg (Calnek and Witter, 1997). In the classical form of transient paralysis, at 8 - 12 days following exposure to the virus, affected chickens exhibit varying degrees of ataxia and flaccid paralysis of the neck or limbs from which they rapidly recover after 1 - 2 days, although after a few weeks the recovered chickens may be affected with MD lymphomas (Schat and Nair, 2008). Often, after recovery from classical transient paralysis, some birds develop torticollis 18 - 26 days after viral exposure which is termed as persistent neurologic syndrome (Gimeno*, et al.*, 1999). This can be induced by partially attenuated MDVs which no longer induce transient paralysis (Gimeno*, et al.*, 2001b).

In the acute form of MD, formation of lymphomas in visceral organs is the usual sign with an incidence rate of frequently 10 – 30 % which can increase up to 70 % in major outbreaks (Nair*, et al.*, 1995). Chickens with lymphomas may show few signs and become depressed and comatose before death. Other chickens may appear clinically normal but still have extensive neoplastic involvement after being euthanized. In cases with a prolonged course, weight loss, paleness, anorexia and diarrhoea can be observed. In the acute form of transient

paralysis death can occur within 24 - 72 hours following the onset of paralytic signs and is also associated with oedema of the brain (Witter, *et al.*, 1999).

An early paralysis/ mortality syndrome between 9 - 20 days post challenge is another specific syndrome of this form of disease where infected birds died over 2 - 3 days in experimental condition (Renz, *et al.*, 2012; Walkden-Brown, *et al.*, 2006). Birds with ocular involvement may show unilateral or bilateral blindness, grey discoloration of iris, irregular iris border and corneal opacity (Ficken, *et al.*, 1991; Witter, 1997). Early mortality syndrome results in very high mortality usually between 8 - 16 dpi in chickens infected with virulent strains and 10 - 14 dpi with vvMDV and vv+MDV where chickens suffered permanent immune suppression and severe atrophy of lymphoid organs was also recognised (Nair, *et al.*, 1995; Witter, 1997; Witter, *et al.*, 1980). Fabricant *et al*. (1978) reported that MDV has been found to be involved in atherosclerosis in chickens. However, in the almost 100 years since the initial description of MD, the clinical picture of the disease has changed - the chronic polyneuritis that was prevalent until 1925 was accompanied by visceral lymphoma from 1925 – 1950, and from 1950 onwards more aggressive and faster developing tumours were observed. During the past 25 years, MDV virulence has continued to increase and the clinical picture of the disease has changed again, so that along with the still prevalent visceral lymphoma and neurological symptoms, severe brain oedema and acute deaths, even in fully vaccinated animals, are observed (Osterrieder, *et al.*, 2006; Witter, 1998b). Recently Wajid *et al.* (2013) described clinical signs related to MD in Australia where they found that clinical signs typically commenced with depression or unwillingness to move followed by leg dragging/knuckling over, drooping of wings, mild opisthotonos and then paralysis. Paralysis during the later episodes typically involved chickens having one leg stretched forward and the other backward, together with flaccid paralysis or paresis of necks and wings.

REV can induce lymphoid tumours 15 - 20 weeks of age in turkey and infrequent nerve lesions that can be confused with MDV (Payne and Venugopal, 2000). Peripheral nerve lesions are not a feature of LL but they are found frequently in MD and REV (Beyer, *et al.*, 1980; Witter, *et al.*, 1970b). Death due to LL rarely occurs before 14 wk of age and is more frequent around the time of sexual maturity (Siccardi and Burmester, 1970). Bursal lesions due to MD are different from those due to LL. With MD, there is atrophy or diffuse gross enlargement whereas LL shows nodular tumours (Zelnik, 2004).

1.10 Pathology

1.10.1 Gross lesions

Enlargement of one or more peripheral nerves is the main finding of the classical form of MD. The most commonly affected nerves are the brachial and sciatic plexus and nerve trunks, celiac plexus, abdominal vagus and intercostal nerves, which enlarge to two to three times their normal size. The affected nerves lose their normal cross-striations and glistening white appearance and have a greyish or yellowish oedematous appearance (Goodchild, 1969; Payne and Biggs, 1967; Witter and Schat, 2003).

Lymphoma formation is the characteristic finding of the acute form of MD. The location of lymphoma is dependent on the virulence of the strain, age and genetics of chickens, eg. tumour enlargement in the spleen and liver are common in older birds (Goodchild, 1969). Lymphomas can occur in various organs including the liver, spleen, heart, gonads, lung, bursa, muscle, iris and peripheral nerves, proventriculus, serosa, kidneys, thymus, intestines, skin, pancreas and adrenals. Lymphomas present as diffuse enlargements or nodular growths of different size and with grey or white discolouration (Biggs, 2001; Calnek, 2001; Payne, 1985; Renz*, et al.*, 2012; Walkden-Brown*, et al.*, 2013b). Figure 1.3 shows gross MD lesions.

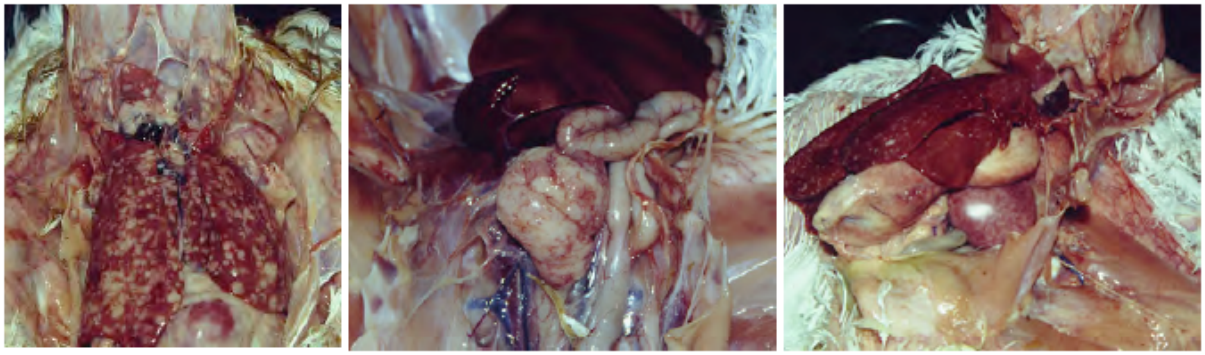

Figure 1.3 Typical gross Marek's Disease Lymphomas of the liver (left), ovary (middle) and liver and spleen (right). (Source – T. Islam)

The highest incidence of gross MD lymphomas also is often found in the gonads, particularly in the ovary. In testes, it is very hard to detect lymphomas grossly because the background colour of the testes is identical to that of lymphoma tissue. Moreover, bilateral enlargement of the testes due to lymphoma and lymphocyte infiltration is unlikely to score as MD except when it is extreme. Under experimental conditions, severe thymic and bursal atrophy are the

most consistent findings on *post mortem* examination, sometimes no trace of thymus can be found. The following Table 1.3 compares the incidence of MD lymphomas in different organs in maternal antibody negative SPF layer chickens (Renz*, et al.*, 2012) and maternal antibody positive broiler chickens, challenged with v and vvMDV (Walkden-Brown*, et al.*, 2013b).

Table 1.3 Overall distribution of MD lymphomas in different organs with the incidence rate

Organ	Incidence of lesions (%) in Ab-positive broilers (Walkden-Brown*, et al.*, 2013)	Incidence of lesions (%) in Ab-negative SPF layers (Renz*, et al.*, 2012)
Ovary	68.6	92.0
Liver	55.9	37.2
Spleen	37.3	11.7
Heart	17.8	5.1
Lung	13.6	1.5
Thymus	11.9	5.8
Kidney	5.9	19.7
Muscle	3.4	4.4
Eye	2.5	0.7
Bursa	1.7	2.9
Skin	1.7	1.5

1.10.2 Microscopic Lesions

Microscopic changes, such as the proliferation of lymphoid cells, have been observed in the peripheral nerves, central nervous system (CNS) and visceral organs (Payne, 1972). Acute neurological lesions were reported in chickens infected with vv+ MDV strains resulting in early mortality within 2 weeks of infection (Swayne*, et al.*, 1988, 1989). Three main types of

lesions were identified in peripheral nerves of chickens affected by both classical and acute forms of the disease:

- Proliferative (Type A) lesions are frequently observed in chickens that die early from MD. They consist of masses of different sized lymphocytes, reticular cells and lymphoblasts with lymphoid tumour development (Payne, 1972).
- Inflammatory (Type B) lesions are observed in chickens in which MD has a more chronic course. They are characterised by infiltration of small lymphocytes, plasma cells and small numbers of macrophages (Payne, 1972).
- Chronic minor lesions (Type C) are small and comprise lightly scattered lymphocytes and plasma cells. Chronic minor lesions are seen in bird with no clinical signs of MD (Payne, 1972).

In the brain the initial lesions include vascular elements; endotheliosis occurs at 6 dpi and is followed by a moderate to severe infiltration of lymphocytes and macrophages around blood vessels and scattered all over with neurophil (Gimeno*, et al.*, 2001a).

Lymphomatous lesions in the visceral organs are more consistently abundant than those in the nerves. The types of lymphoproliferative lesions present in the nerves are similar to those in the visceral organs. The cellular composition of the tumours is the same in most organs though the gross lesions may differ (Payne, 1972). Lesions in the skin are localized around infected feather follicles and in the dermis, compact aggregates of proliferating cells, few plasma cells and histiocytes may be seen (Helmboldt*, et al.*, 1963; Payne and Biggs, 1967). Mononuclear infiltration of the iris is the most regular change in the eye though infiltrates may also be found in the eye muscles (Jungherr and Hughes, 1965).

1.11 Diagnosis

For a number of reasons the diagnosis of clinical disease is complicated in practice. Firstly, for MD there are actually no pathognomic gross lesions. Gross lesions of MD can be similar to those of other neoplasms and distinct conditions characterised by tumours in visceral organs or grossly enlarged nerves. MDV, ALV and REV are common in commercial poultry resulting in concurrent infection (Davidson and Borenstein, 1999), causing difficulties in diagnosis, which depends on virological techniques to differentiate between the diseases.

MD diagnosis in the field is mainly based on clinical signs and examination of gross and microscopic lesions in tissues. MDV infections, which do not induce obvious pathological changes, can be confirmed by laboratory based virological techniques.

1.11.1 Clinical Diagnosis

Diagnosis of clinical MD on the basis of clinical signs and gross pathology is complex. Although enlarged peripheral nerves and visceral lymphomas are common in MD they don't always occur. Therefore, additional factors, such as age and lesion distribution, should be considered in the *post-mortem* diagnosis of MD. Chickens might be diagnosed provisionally as having MD if at least one or more of the following situations are found (Schat and Nair, 2008):

- Leukotic enlargement of peripheral nerves.
- Lymphoid tumours in various tissues (liver, heart, gonad, skin, muscle and proventriculus) in birds under 16 weeks of age.
- Visceral lymphoid tumours in birds 16 weeks or older that lack tumorous enlargement of the bursa of Fabricius.
- Iris discoloration and pupil irregularity.

1.11.2 Laboratory diagnosis

Laboratory diagnosis of MD is, therefore, essentially based on pathological confirmation of the disease together with the positive identification of MDV. The principal methods to identify the presence of infection are isolation of the virus, demonstration of viral DNA or antigens in tissues, and detection of antibodies (Schat and Nair, 2008).

1.11.2.1 Histopathology

Affected tissues, fixed in formalin or fresh frozen, are used to prepare paraffin and cryostat sections, respectively. Impression smears of tumors also may be used. Essential diagnostic features may be seen in routine histologic sections stained with hematoxylin and eosin or touch preparations stained with methyl green pyrolin or Shorr's stain (Siccardi and Burmester, 1970). Cellular and viral antigens can be demonstrated in frozen, or in some cases, paraffin sections by immunohistochemistry. A mixed population of small to large lymphocytes, lymphoblasts, plasma calls, and macrophages are typically found in MD tumors and nerve lesions (Payne and Biggs, 1967).

However, the characteristics of the lesions may not be similar in all cases and may not be distinctive enough for a definitive diagnosis. Single or dual infections of chickens with avian leukosis virus (ALV) and / or MDV also have important consequences for the correct differential diagnosis of MD or lymphoid leukosis (LL) due to the similar pathology induced by these viruses (Biggs and Payne, 1976). In addition, the special skills and experience required for histopathological evaluation might not be available in many laboratories.

1.11.2.2 Isolation and identification of virus

Virus can be isolated from a range of infected tissues (blood lymphocytes, heparinized whole blood, splenocytes, or tumour cells) after 1 - 2 dpi or 5 days after contact exposure and throughout the life of the birds (Adldinger and Calnek, 1973; Phillips and Biggs, 1972; Witter, 1984). For primary isolation of MDV, chicken kidney cells (CKC) or duck embryo fibroblast (DEF) cells are most favourable cell cultures and have been preferred over chicken embryo fibroblasts (CEF) cells (Sharma, 1998). The viral replication rate is not influenced by the genetic susceptibility of the specific pathogen free (SPF) chickens used for the preparation of CKC (Spencer, 1969). On the other hand, chicken embryo kidney cells (CEKC) and CEF have often resulted in very low virus isolation rates (De Laney*, et al.*, 1995; De Laney*, et al.*, 1998; Lee*, et al.*, 1999). Schat (2005) used splenocytes infected with either of the two strains of MDV (vv RB-1B and vv+ RK-1) to compare the efficacy of CKC, CEF and CEKC for virus isolation and concluded that CKC are superior to CEF and CEKC (Schat, 2005).

1.11.2.3 Assays for detection of MDV

There are several assays available which can be divided in two groups- serological tests (A) and molecular tests (B).

A. Serological tests (based on antigen protein recognition)

There are following serological tests available

- **Agar gel precipitation (AGP) assay:** This is a simple test where serum reacts with MDV antigen (Chubb and Churchill, 1968). The presence of either MDV antigen or MDV specific serum can be identified by this test. The antigen suspension, or even tips obtained straight from small feathers, are placed in wells prepared in an agar layer containing salt and buffer. Serum is placed in adjacent wells and the samples are left to diffuse and react for 24 - 72 hours in a humidified atmosphere. A positive reaction can be identified by the lines of

precipitate in the agar between the wells. In the precipitate, the MDV antigen A predominates; this was soon identified as a homologue of herpes virus glycoprotein C (gC) (Binns and Ross, 1989). The virulent and vaccine strains of MDV-1 can be differentiated by AGP (Zanella*, et al.*, 2000; Zelnik, 2004) but the sensitivity is poor compared with that of ELISA and DNA hybridization (Davidson*, et al.*, 1986).

- **Direct or Indirect immunofluorescent assay:** This test is also used for identifying MDV specific antibodies or antigens in samples (Purchase and Burgoyne, 1970). The use of monoclonal antibodies has greatly expanded the utility of immunofluorescent assay for detailed analysis of MDV and HVT antigens and detection and differentiation of MD viruses (Dorange*, et al.*, 2000; Ikuta*, et al.*, 1982; Lee*, et al.*, 1983).

- **Enzyme-Linked Immunosorbent Assay (ELISA):** This is a more sensitive serological test for identifying the MDV-specific antibodies (Cheng*, et al.*, 1984). There are two related reason that ELISA has not found widespread diagnostic use. First, MD vaccines contain suspensions from the live infected CEF on which they have been cultured, and this can induce antibodies against antigen expressed by CEF. Therefore this often results in high background readings in ELISA, especially if the coating MDV antigens used in the ELISA have been prepared using CEF (Zelnik, 2004). Secondly, MDV is an immunosuppressive virus that depletes the antibody producing B cells and causes a reduction in titres of MDV specific antibodies (Zelnik, 2004). Zelnik *et al*. (2004) found that ELISA results are more reliable when a cell culture system other than CEF (e.g. CKC) is used for the production of the MDV coating antigen (Zelnik*, et al.*, 2004).

B. Molecular tests (based on DNA sequence)

- **Polymerase Chain Reaction (PCR) Assays:** The polymerase chain reaction (PCR) is a scientific procedure in molecular biology to duplicate a single target sequence of DNA repeatedly producing thousands to millions of copies of a specific DNA sequence. Developed in 1983 by Kary Mullis (Mullis*, et al.*, 1986), PCR is now a universal and frequently crucial procedure used in medical and biological research labs for a variety of applications (Saiki*, et al.*, 1985; Saiki*, et al.*, 1988). These include DNA cloning for sequencing, functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints; and the detection and diagnosis of infectious diseases.

The method depends on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers containing sequences corresponding to the target region and a DNA polymerase are key components to facilitate selective and repeated amplification. As PCR progresses, the generated DNA itself utilized as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

In most PCR, a heat-stable DNA polymerase is used. Usually this is *Taq* polymerase, initially isolated from the bacterium *Thermus aquaticus*. This DNA polymerase enzymatically builds a new DNA strand from individual nucleotides by using single-stranded DNA as a template. Specific primers that initiate and terminate the DNA synthesis are included in the reaction mix. Thermal cycling is used to initiate repeated amplification of the target sequence.

A typical PCR solution comprises several components and reagents (Sambrook and Russel, 2001). These components are:

- A DNA template: The DNA to be copied, usually extracted and purified from blood or other tissue.
- Primers: Single stranded oligonucleotides that match exactly the beginning and end of the DNA target sequence. These are generated synthetically and provide the specificity of the PCR reaction.
- A DNA polymerase (e.g. *Taq* polymerase): To synthesise the DNA.
- dNTPs (Deoxyribonucleotide triphosphates): The building blocks from which the *Taq* polymerase can synthesise new DNA. These are added in excess amount.
- A buffer solution: Creates an optimal environment for the reaction to occur in.
- Divalent cations such as magnesium or manganese ions, which work as a co-factor for correct function of DNA polymerase. Generally Mg^{2+} is used, but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, since high Mn^{2+} concentration increases the error rate during DNA synthesis (Pavlov*, et al.*, 2004)
- Potassium ions providing a monovalent cation. They affect the denaturing and annealing temperatures of the DNA and enzyme activity. Increase in salt concentrations slows down denaturation of long DNA products so short products will amplify preferentially.

In general, PCR consists of a series of 20 - 40 repeated temperature changes, called cycles, with each cycle commonly consisting of 3 discrete temperature steps, (Figure 1.4). The cycling is frequently preceded by a single temperature step that is called "hold" at a high temperature (> 90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers (Rychlik*, et al.*, 1990). The steps of each cycle are briefly described below-

- Initialization step: This step consists of heating the reaction to a temperature of 94 – 96 °C that is held for 1 – 9 minutes, which is only necessary for DNA polymerases, which need heat activation by hot-start PCR (Sharkey*, et al.*, 1994).
- Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94 – 98 °C for 20 – 30 seconds. From this single-stranded DNA molecules are achieved through "melting" of the DNA template by disrupting the hydrogen bonds between complementary bases. This separates the two complementary strands of double stranded DNA into two single strands.
- Annealing step: The reaction temperature is lowered to 50 – 65 °C for 20 – 40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3 - 5 degrees Celsius below the Tm of the primers used. This step allows the DNA to cool and re-anneal. Since there are many more molecules of primer than there are of DNA, it is more likely that stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.
- Extension/elongation step: The temperature at this step depends on the DNA polymerase used; *Taq* polymerase has its optimum activity temperature at 75 – 80 °C (Chien*, et al.*, 1976; Lawyer*, et al.*, 1993), and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that correspond to the template in 5' to 3' direction, compressing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the growing DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to

be amplified. Under optimum conditions, at each extension step, the amount of DNA target is doubled, leading to exponential amplification of the specific DNA fragment.

- Final elongation: This single step is occasionally performed at a temperature of 70–74 °C for 5 – 15 minutes following the last PCR cycle to make sure that any remaining single-stranded DNA is completely extended.
- Final hold: This step at 4 – 15 °C for an indefinite time may be engaged for temporary storage of the reaction.

To check whether the PCR generated the anticipated DNA fragment, agarose gel electrophoresis is employed for size separation of the PCR products. The size of PCR products is determined by comparison with a DNA ladder, which contains DNA fragments of known size, run on the gel alongside the PCR products. Results are based on size discrimination, which may not be very precise. Marginal variations in reaction components, thermal cycling conditions, and mispriming events during the early stages of PCR may greatly affect the yield of the amplified product (Wu*, et al.*, 1991). Due to poor resolution, gels may not be able to resolve these variabilities in yield. End point detection is very time consuming and labor intensive. Moreover, in a typical standard end point PCR experiment, all responses saturate at a similar level so the end point measurement is not directly related to the initial amounts of target molecules present in the samples. Thus the test is not fully quantitative and is really only useful to differentiate a positive from a negative sample. Figure 1.5 showing the plateau phase is where traditional PCR takes its measurement, also known as end-point detection.

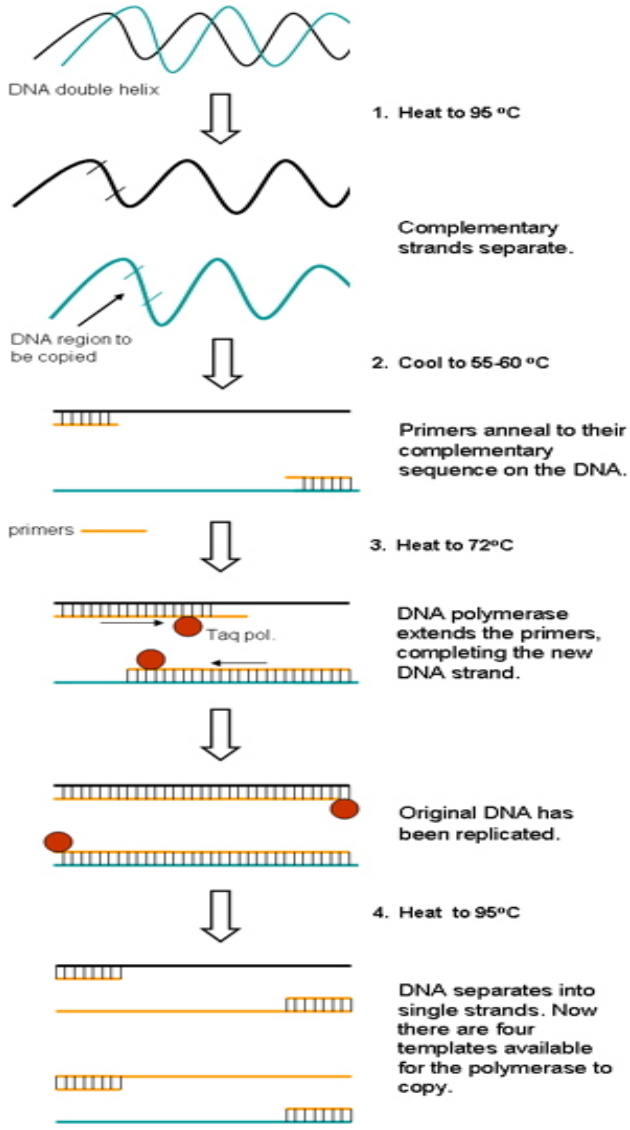

Figure 1.4 Schematic drawing of the PCR cycle. (1) Denaturing at 94 – 96 °C. (2) Annealing at ~60 °C (3) Elongation at 72 °C. Four cycles are shown here.

- **Real time PCR:** Real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (qPCR), is a laboratory procedure based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. qPCR enables both detection and quantification for one or more specific sequences in a DNA sample. The quantity can be either an absolute number of copies or a relative amount. The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in real time. Real time PCR requires a fluorescent reporter that binds to the product formed and reports its presence by fluorescence (Kubista*, et al.*, 2006). A number of probes and dyes are available. Higuchi *et al.* (1992), used the

common nucleic acid stain ethidium bromide, which becomes fluorescent upon intercalating into DNA. Classical intercalators, however, interfere with the polymerase reaction, and asymmetric cyanine dyes such as SYBR Green I and High Resolution Melting dyes (HRM dyes) such as EvaGreen, LCGreen®, SYTO® 9 and BEBO have become more popular (Bengtsson*, et al.*, 2003; Zipper*, et al.*, 2004). The commonly used probes are TaqMan® probes, LNA® Double-Dye probes, Molecular Beacon probes, Hybridization probes, MGB Eclipse® probes (Vandesompele).

The reporter generates the fluorescence signal, which reflects the amount of product formed. Fluorescent reporter probes detect only the DNA containing the probe sequence; therefore, use of the reporter probe significantly increases specificity, and enables quantification even in the presence of non-specific DNA amplification. The method relies on a DNA-based probe with a fluorescent reporter at one end and a quencher of fluorescence at the opposite end of the probe. The close proximity of the reporter to the quencher prevents detection of its fluorescence; breakdown of the probe by the 5' to 3' exonuclease activity of the *Taq* polymerase breaks the reporter-quencher proximity and thus allows unquenched emission of fluorescence, which can be detected after excitation with a laser. An increase in the product targeted by the reporter probe at each PCR cycle therefore causes a proportional increase in fluorescence due to the breakdown of the probe and release of the reporter (Lee, 1993; Livak*, et al.*, 1995).

The amplification curve or primary growth curve usually shows the increase of fluorescence level on the Y axis, compared with the run cycle number on the X axis. Each amplification curve consists of at least three distinct phases: 1) an initial lag phase, 2) an exponential phase, and 3) a plateau phase (Wilhelm and Pingoud, 2003). During the initial cycles, the signal is weak and cannot be differentiated from the background (Figure 1.5). As the amount of product accumulates, a signal develops that initially increases exponentially. Thereafter the signal levels off and saturates. The signal saturation is due to the reaction running out of some critical component. This can be the primers, the reporter, or the dNTPs (Kubista*, et al.*, 2001). In addition, the number of polymerase molecules may be limiting, in which case the exponential amplification converts to linear amplification.

In real-time PCR the response curves are differentiated during the exponential phase of the reaction when conditions are not limiting. Under these conditions the amount of product

obtained at the critical threshold number of cycles is directly related to the initial amount of target sequence in the template. The test is thus quantitative for the initial amount. Real-Time PCR focuses on the exponential phase because it provides the most precise and accurate data for quantitation. Within the exponential phase, the real-time PCR instrument calculates two values. The Threshold line is the level of detection at which a reaction reaches a fluorescent intensity above background. The PCR cycle at which the sample reaches this level is called the Cycle Threshold, Ct (Wilhelm*, et al.*, 2001). The Ct value is used in downstream quantitation or presence/absence detection. By comparing the Ct values of samples of unknown concentration with a series of standards, the amount of template DNA in an unknown reaction can be accurately determined. Figure 1.5 shows the exponential growth phase, where qPCR takes its measurement.

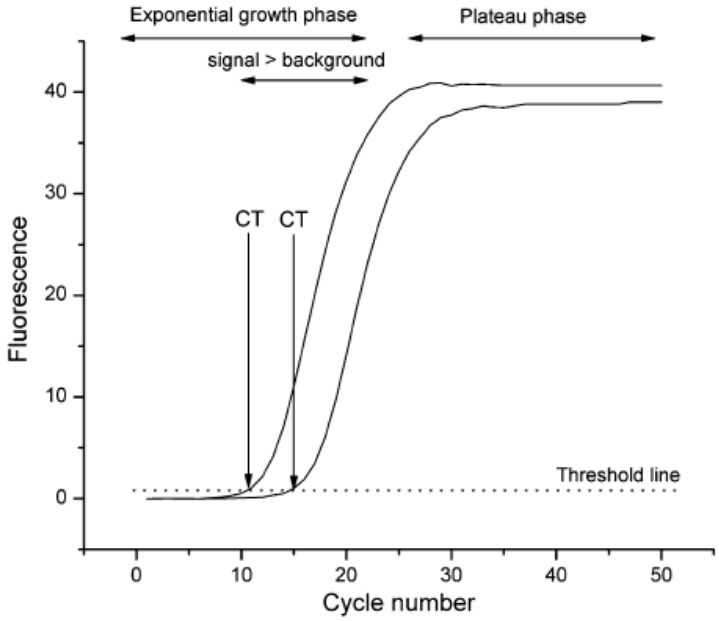

Figure 1.5 Real-time PCR response curves. A threshold level is set sufficiently above background and the number of cycles required to reach threshold, Ct, are registered (Kubista*, et al.*, 2006).

The efficiency of a PCR assay can be estimated from a standard curve based on serial dilution of a standard, which can be a purified PCR product or a purified plasmid that contains the target sequence (Figure 1.6) (Rutledge and Cote, 2003). The Ct values of the diluted standards are read out, and plotted versus the logarithm of the sample concentrations, number of template copies (N0) or dilution factor. The data are fitted to the equation:

Ct = slope log (N0) + Ct (1)

The PCR efficiency (E) is calculated from the slope as:

E= 10(-1/slope) – 1

If the slope of the standard curve is -3.32 then the PCR is 100 % efficient.

With 100 % efficiency, a 2x dilution gives a ΔCt of 1 between each dilution (each cycle the amount of amplification is doubled). With 100 % efficiency, a 10x dilution gives a ΔCt of 3.2 values between each dilution (every 3.2 cycles the amount of amplification is 10 fold higher). The efficiency of the PCR should be 90 - 100%. Therefore, a perfect assay would have a slope between – 3.6 and – 3.1 (Dorak, 2006).

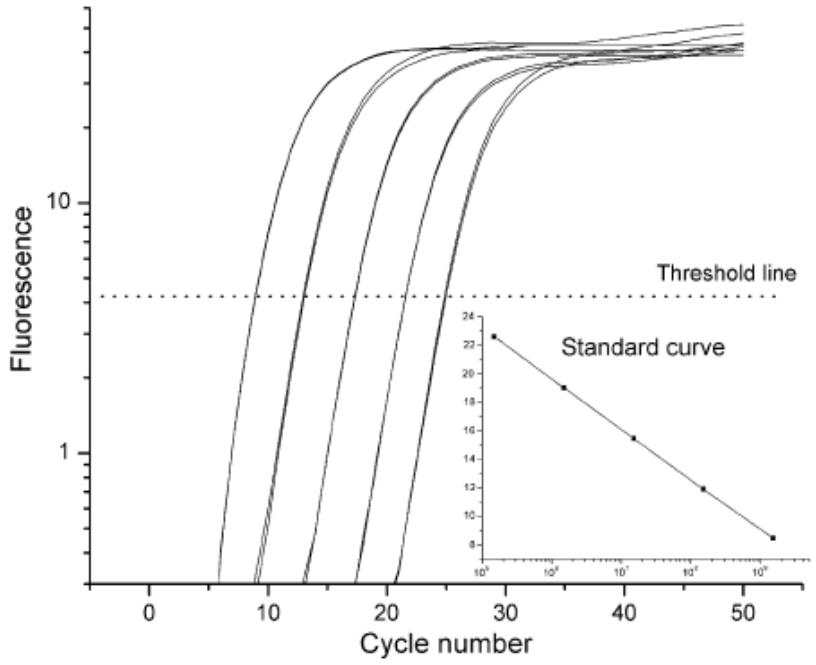

Figure 1.6 Real-time PCR standard curve inset in an amplification plot of the same standards. Real-time PCR amplification curves are shown on a logarithmic scale for five standard samples in a 10-fold serial dilution series. The crossing points with threshold line are the C_t values. In the inset the C_t values are plotted against the logarithm of the initial number of template copies in the standard samples (Kubista, *et al.*, 2006)

Real-time PCR in MDV research: Quite a lot of modern fluorescent detection strategies are now available to perform sensitive and accurate DNA/RNA analyses. Many scientific papers and reviews have been published on human and animal viruses certifying the real-time

technology, and most strategies for the detection of viral nucleic acids are based on the use of the TaqMan® system (Niesters, 2001).

Complete DNA sequences of all three serotypes of MDV genomes are available (Afonso*, et al.*, 2001; Izumiya*, et al.*, 2001; Kingham*, et al.*, 2001; Tulman*, et al.*, 2000), which allows the use of PCR-based methods of specific detection of MDV. In recent times qPCR assays have been developed to detect MDV load in various infected tissues and to investigate the replication kinetics of MDV and related vaccine strains in feathers, dust and lymphoid tissues, correlation between protection and vaccine load, the association between replication rates of viral genomes and virulence and the impact of specific gene deletion on replication (Baigent*, et al.*, 2005a; Baigent*, et al.*, 2006a; Baigent*, et al.*, 2006b; Islam*, et al.*, 2006a; Islam*, et al.*, 2006b; Yunis*, et al.*, 2004). Recently Walkden-Brown *et al*. (2013a) showed that detection and quantitation of MDV-1, HVT, and MDV-2 in poultry house dust using qPCR is robust, sensitive, reproducible, and meaningful, both biologically and commercially.

The assays as described by Baigent *et al*. (2005a) and Islam *et al*. (2006a) use TaqMan® probes for generating the fluorescent signal whereas Abdul-Careem *et al.* (2006) use the SYBR® Green chemistry. With these assays, the reliable detection and quantitation of all three serotypes in a single sample is possible. More recently, Renz *et al.* (2013) reported two molecular qPCR assays that reliably detect, quantify and differentiate Australian pathogenic strains of MDV-1 from the non-pathogenic vaccinal Rispens/CVI988 serotype 1 MDV. Nowadays, real-time PCR has become a most useful molecular technique for the consistent detection, differentiation and quantitation of MDVs in a wide range of samples. It has thus become an essential tool for the diagnosis and monitoring of MD in experimental and field situations.

1.12 Prevention & Control

1.12.1 Vaccination

Vaccination is the preferred control procedure worldwide for MD in breeder and layer chickens. The first practical vaccine for MD was developed by Churchill *et al.* (1969a). This vaccine was based on the oncogenic HPRS-16 strain of serotype 1 MDV that had been attenuated by serial passages using chicken kidney cell culture (Churchill*, et al.*, 1969a). This vaccine was replaced by a new vaccine, naturally avirulent HVT vaccine (FC126 strain),

which showed to be highly effective in preventing MDV (Burmester, *et al.*, 1972; Purchase and Okazaki, 1971a). An attenuated vaccine strain, which is an MDV-1 isolate of low pathogenecity, designated CVI988 but also called the Rispens strain, was developed and shown to be protective in both laboratory and field trials (Rispens, *et al.*, 1972a; Rispens, *et al.*, 1972b). In earlier studies, the protection level against MD was similar with HVT and Rispens/CVI988 (Maas, *et al.*, 1982; Vielitz and Landgraf, 1971; von Bülow, *et al.*, 1976) but in more recent studies the Rispens/CVI988 vaccine proved to provide better protection against highly virulent challenge strains of MDV (Buscaglia, *et al.*, 2004; Witter, *et al.*, 1995b).

All the vaccines protect against mortality, clinical signs and gross MD lesions, but none of them provides complete protection against infection, replication and shedding of the challenge virus. The HVT, bivalent HVT + MDV-2 and CVI988 vaccines are the most widely used MD vaccines in present time. In moderately contaminated areas broilers are not vaccinated because the incubation period of MD is longer than the lifespan of broilers. On the other hand, broilers are vaccinated usually with HVT or with the bivalent HVT + MDV-2, vaccine in heavily infected areas where MDV strains of high virulence occur such as in the USA. Most breeders and layers are vaccinated with the Rispens/CVI988 vaccine strain either alone or in combination with other vaccine serotypes, usually HVT (Bublot and Sharma, 2004). In problem areas where the monovalent vaccines are give poor protection, the use of bivalent or polyvalent vaccine is recommended for the successful prevention of disease due to virulent MDV (Ben-Nathan and Lustig, 1990). Bivalent vaccines based on combinations of HVT and either SB-1 (Schat and Calnek, 1978) or 301B/1 (Witter, 1987) strains of serotype 2 MDV. A polyvalent vaccine composed of Md11/75C, HVT and SB-1 viruses protected chickens better against a multi isolate challenge with five highly virulent MD challenge viruses (Witter, 1982). All vaccine types are protective but to varying degrees. HVT, mainly strain FC126 (Witter, *et al.*, 1970c) continues to be extensively used because it is effective and economical to produce and combines well with other products. Even though both cell-free and cell-associated forms of HVT are available, cell-associated form is widely used because it is more effective than cell-free virus in the presence of maternal antibodies (Witter and Burmester, 1979). Rispens/CVI988 is an attenuated serotype 1 vaccine which was first isolated by Rispens in 1972 (Rispens, *et al.*, 1972a). Because of some safety problems with low-level oncogenicity in highly MD-susceptible chicken lines as reported by von Bulow (1977a), the vaccine was launched much later in 1994 in the USA. Present preparations of this vaccine are

considered to be the 'gold standard' as it offers superior protection even against the strains which are classified as very virulent plus (Davison and Nair, 2005; Witter, 2002). Recently a novel MDV serotype 1 vaccine was introduced in Australia using strain BH16. This vaccine has comparable efficacy to the CVI988 vaccine (Karpathy*, et al.*, 2003) but was only tested against a comparatively mild MDV strain (MPF57).

The vaccine is administered by either the subcutaneous route or intramuscularly in day old chicks. This vaccine is administered using a semi-automated device developed to expedite the process, so that one operator can deliver vaccine to between 2000 - 3000 chicks per hour (Witter, 2001). However, most major commercial broiler hatcheries use the *in ovo* vaccination, where live vaccine viruses are administered to embryonated egges before hatching usually at around embryonation day 18 (Bublot and Sharma, 2004). The concept of *in ovo* vaccination was first examined by inoculating HVT vaccine into SPF chickens (Sharma and Burmester, 1982). Consequent studies discovered that MDV-1 and MDV-2 vaccines, and vaccines against a number of other pathogens, could be administered *in ovo* (Karaca*, et al.*, 1998; Reddy*, et al.*, 1996; Sharma, 1986). Recently it has shown that a single injection of a multivalent *in ovo* vaccine (MIV) containing five live vaccine viruses (MDV-1, MDV-2, HVT, IBDV, FPV) was effective in immunizing chickens against multiple pathogens (Sharma*, et al.*, 2002).

1.12.1.1 Responses to vaccination

Serotype 1 MDV vaccinal virus has been found in the blood lymphocytes at 4 days post vaccination (dpv) with peak levels at around 14 dpv (Baigent*, et al.*, 2005b). Serotype 2 virus was recovered at 5 - 6 dpv with peak levels at around 6 - 21 dpv (Calnek*, et al.*, 1979; Witter, 1984). In contrast HVT was first detected at 2 dpv (Prasad and Spradbrow, 1980; Witter, 1984) with peak levels achieved at 37 dpv (Islam*, et al.*, 2006b).

In laboratory and field experiments, there is no evidence of clinical disease or depression of body weight due to MD vaccines. However, the CVI988 strain proved to be pathogenic for genetically susceptible Rhode Island Red Chickens if vaccinated at a high dose equivalent to 10 times the field dose of vaccine. The virus caused symptoms of classical MD in up to 28.5 % of the inoculated chickens with gross lesions limited to the peripheral nerves (von Bülow, 1977).

Serotype 1, 2 or 3 vaccine strains do not interfere with cellular or humoral immune responses to other antigens even if inoculated into embryos (Sharma, *et al.*, 1984; Witter, 1984); however, a recent study showed that HVT has immunosuppressive effects that reduce T and B lymphocytes (Islam, *et al.*, 2002). Antibodies are induced in the birds within 1 - 2 weeks ensuing vaccination and remain at reasonably stable titres throughout the lifespan of the chickens (Melchior, 1973; Witter, 1982). However, active antibody titres following vaccination are lower in chickens with maternal antibodies than in chickens without maternal antibodies (Spencer and Gavora, 1980).

1.12.1.2 Mechanism of protection

Protection against pathogenic MDV challenge is fully efficient by 1 - 2 weeks after vaccination (Eidson and D.P.Anderson, 1971). The genetic background of chickens and the type of vaccine also affect the immunity following vaccination (Landgraf and Vielitz, 1978; Vielitz and Landgraf, 1987). Vaccinated chickens were adequately protected against challenge with pathogenic MDV although vaccine virus might no longer be easily isolated from them and the protective efficacy is 85 to 95 % in both laboratory and commercial practice (Witter and Offenbecker, 1978). Synergism has been detected among all three MDV vaccines. Mixtures of two vaccine viruses provided better protection than single one and in some cases addition of a third virus was able to improve the efficacy of the combination of two vaccine viruses. However, the best synergistic effect has been noted between serotype 2 and 3 vaccines and has since been widely used successfully in the field (Calnek, 1983; Calnek, *et al.*, 1983; Cheng, *et al.*, 1984; Schat, *et al.*, 1982b; Witter, 1984).

All live MD vaccines induce anti-viral humoral and cell-mediated immunity. Specific antibody reduces the level of MDV infection by reducing spread from cell to cell but cannot induce sterile immunity. Thus vaccinated chickens can still become infected and both vaccine and pathogenic MDV can co-exist in the same host (Hiramoto, *et al.*, 1996; Purchase, *et al.*, 1971b). Cell mediated immune responses are more significant in the protective effects of the MD vaccines than the humoral immune response. It has been shown that preventing function of the bursa (the principal source of B lymphocytes which drive the humoral immune response) by irradiation or by performing bursectomy that there was no effect on the level of protection provided to chickens vaccinated with attenuated MDV (Else, 1974; Sharma and Witter, 1975).

Neonatal vaccination with Rispens/CVI988 decreased the titre of MDV reisolated from $CD4^+$T cells, which are the main target cells for latent infection in lymphoid cells (Hiramoto, *et al.*, 1996). Vaccination with Rispens/CVI988 can also induce specific cytotoxic activity of $CD8^+$T cells against several MDV antigens (Omar, 1997; Omar and Schat, 1996; Omar, *et al.*, 1998). However, CD4+ and CD8+T cell responses induced by Rispens/CVI988 are not essential for anti-tumour immune mechanisms (Morimura, *et al.*, 1998).

However, the exact mechanisms of the anti-tumour effects of MD vaccines are unknown but likely protection mechanisms are summarised in Figure 1.7.

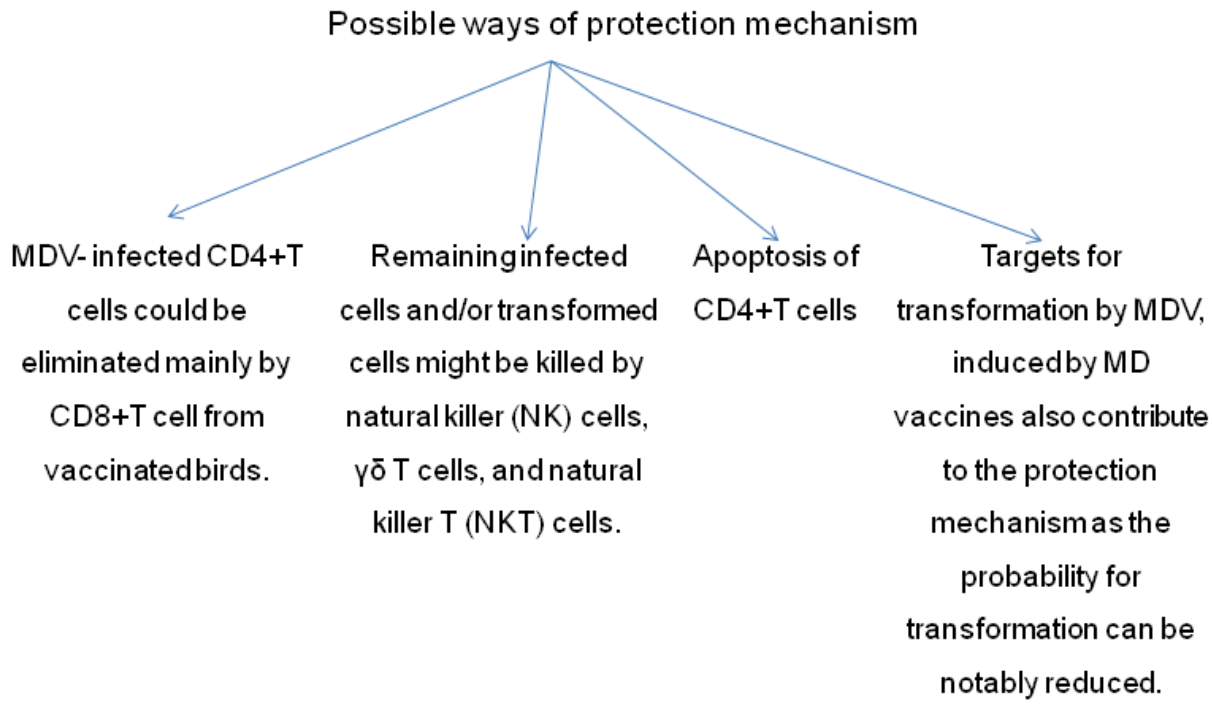

Figure 1.7 Possible mechanisms of vaccinal protection against MD (Morimura, *et al.*, 1998)

1.12.1.3 Factors affecting vaccine efficacy

- **Vaccine Dose:** Vaccine doses may vary depending on the vaccine type and the virulence level of MDV against which protection is sought. The typical dose of MD vaccine varies between 2,000 - 6,000 plaque forming units (pfu) per chicken (Schat and Nair, 2008) but commercial vaccines often exceed 10,000 pfu per dose as their potency varies (Payne, 1985). Eidson *et al.* (1978b) showed that although higher doses (over 4,000 pfu) accelerate the initiation of viraemia and reduce the time necessary for the development of protective

immunity, they do not provide any improvement in protection (Eidson, *et al.*, 1978a; Yoshida, *et al.*, 1973). Islam *et al.* (2007a) showed that HVT vaccine doses ranging from 125 - 64,000 pfu induced protection over a range from 66 to 100 % when chickens were challenged 5 days after vaccination. Some data are available showing that repetitive vaccination results in earlier or higher titre antibody response but this is possibly caused by an increased vaccine dose which is known to produce similar effects (Benda and Hlozanek, 1975; Eidson, *et al.*, 1978b; Melchior, *et al.*, 1973). Revaccination at 7 - 12 days continues to be practiced in Europe and is sporadically used in the United States, but the laboratory studies does not validate the efficacy of this procedure (Schat and Nair, 2008).

- **Maternal antibodies:** The presence of maternal antibody, the antibody that is transferred from hens to the chicks via the egg can interfere with the development of immunity following vaccination with live virus. Maternal antibodies decrease the efficacy of cell associated vaccines but do not abolish the protective effect (Calnek and Smith, 1972). Adverse effects of homologous maternal antibodies were alike for serotype 2 and 3 vaccines, but were significantly higher for an attenuated very virulent serotype 1 vaccine (Witter, 1984). However, cell-free serotype 3 vaccine is more susceptible to neutralization by maternal antibodies than cell-associated vaccine (Sharma and Graham, 1982b; Witter and Burmester, 1979). Moreover, heterologous antibody is less inhibitory than homologous antibody. For that reason, breeder chickens are typically vaccinated with a vaccine of different serotype to that of their progeny, most likely in industry broiler breeders are vaccinated with Serotype 1 vaccines while progeny are vaccinated with HVT or serotype 2 partly to prevent maternal antibody (mab) inhibition of vaccine (Calnek and Smith, 1972; Chubb and Churchill, 1969; Sharma and Graham, 1982).
- **Vaccination to challenge interval:** Islam *et al.* (2007a) showed that though vaccine dose has significant effects on the level of protection against challenge with MDV, the interval between vaccination and challenge is a more important factor of protection . If the interval is short between vaccination and exposure to the virulent virus then the protection level is likely to be low (Islam, *et al.*, 2007a; Okazaki, *et al.*, 1970). Early exposure is certainly one of the most important causes of excessive MD in vaccinated flocks because it takes at least 7 days to establish solid immunity after vaccination, and field exposure usually occurs very soon after placement of chickens (Basarab and Hall, 1976; Witter, *et al.*, 1970a). Vaccination is ineffective at the time of challenge or following challenge and for that reason vaccines are

administered at hatching in order to get better protection against early exposure (Ianconescu, *et al.*, 1971). If the challenge takes place at the same time of vaccination or at 2 or 3 dpv afterward the protection level found very poor. When challenge occurs with virulent serotype 1 virus at 5 - 8 dpv, then better protection is achieved from vaccines of all three serotypes (Okazaki, *et al.*, 1971; Sharma and Burmester, 1982a; Witter, *et al.*, 1984).

- **Stress:** Stress from various other infectious diseases and other causes may possibly influence the efficacy of vaccination. Sharma (1984) showed that IBDV interfered with the induction of immunity against MD using HVT vaccine. Von Bulow (1977b) showed that contamination of MD vaccines with REV interfered with induction of antibodies to serotype 3 MD vaccine. Concurrent vaccination of chickens with serotype 3 and attenuated reovirus vaccines results in poor protection against natural MDV infection in the field compared with vaccination with serotype 3 alone (Rosenberger, 1983). In addition, physiological and environmental stresses such as handling may decrease immunity after 20 weeks of age (Landgraf, *et al.*, 1981).
- **Sex:** The sex of chicken is also an important determinant of vaccine efficacy. It has been shown that although unvaccinated females are more susceptible to MD than males, females appeared to respond to vaccination quicker than males (Spencer and Gavora, 1980).
- **Route of administration:** Route of vaccination also appears to influence the maintenance of vaccinal immunity. Studies investigating MD vaccine administration via the oral, intranasal, and intraocular routes showed that these routes were unsuccessful (Purchase, 1971; Rispens, *et al.*, 1972a). The most effective route is subcutaneous, although intramuscular and intra-abdominal administrations of vaccines are also used (Purchase, 1971; Rispens, *et al.*, 1972a). Recently, the *in ovo* route involving the vaccination of embryonated eggs at days 17 to 19, has become the dominant method of vaccination in the broiler industry (Avakian, *et al.*, 2000; Ricks, *et al.*, 1999).

1.12.2 Genetic resistance

Hutt and Cole (1947) showed that selection for genetic resistance represents a sensible approach for the control of Marek's disease. Since then, poultry breeders have integrated genetic resistance to MD in their selection programs (Hutt and Cole, 1953). Resistance against MD infection is not associated with unwanted production traits (Biggs, *et al.*, 1968), and Ameli *et al.* (1992) showed that genetic resistance is correlated with higher egg production and egg weight .

In case of selection, family selection or progeny testing may be more appropriate than mass selection for commercial breeders to avoid high loss of genetic material on initial challenge exposure (Bacon, *et al.*, 2001). By mass selection susceptibility is reduced from 76 % to 8 % in 6 generations (Maas, *et al.*, 1981), however by family selection Cole (1968) developed two lines N and P from Cornell Randombred control stock with susceptibilities of 96 % and 4 % after 4 generations.

There are two groups of genes, which influence MD resistance: 1) MHC (major histocompatibility complex) genes and 2) Non-MHC genes. The MHC is a region of chicken microchromosome 16 containing three closely linked regions known as B-F (encoding class I MHC proteins), B-L (encoding class II MHC proteins) and B-G (encoding class IV MHC proteins).

Selection based on blood typing depends on the close relationship between MD resistance and certain alleles, particularly B^{21} of the B-F region of the MHC (Briles, *et al.*, 1983; Briles, *et al.*, 1977). This kind of selection procedure makes the production of resistant stocks in populations containing a specific allele for resistance simpler (Gomez, *et al.*, 1991). However, the value of selection for MHC associated markers may differ significantly between commercial lines and crosses (Blankert, *et al.*, 1990; Hartmann, *et al.*, 1992).

The inbred line 6 and line 7 chickens are both homozygous at the B locus for the B2 allele but differ significantly in MD susceptibility proving that non-MHC genes or other MHC genes may also be involved in resistance (Crittenden, *et al.*, 1972). The MHC effects were considered less significant than non-MHC effects in studies on several commercial lines (Groot and Albers, 1992).

It is reported that genetically resistant chickens are protected by vaccination to a greater extent than more susceptible strains (Spencer, *et al.*, 1974). The chicken lines of different B-haplotypes do not respond to vaccination in the same way that they respond to natural challenge. Although the chickens of the congenic (animals which are genetically constructed to differ only at one particular locus) lines B15/B21 or B21/B21 were resistant to the JM strain of MDV-1 without prior MD vaccination, after vaccination with HVT the B5 and B12 homozygotes showed greater resistance to MD challenge, with the B15 and B21 showing intermediate levels of resistance (Bacon and Witter, 1992; Bacon and Witter, 1993).

Genetic resistance was the first method used to control the MD (Cole, 1968). With present situation of progressively wider MD vaccination possibly driving MDV to evolve to higher levels of virulence, there is renewed potential for using genetic selection for MD resistance.

1.12.3 Management procedures

Following strict biosecurity practices is a major tool in the control of MD. Chickens are most susceptible to MDV infection during early stages of their life, so it is most important to eliminate and/or at least reduce the risk of MDV exposure on a farm by averting the entrance of MDV into poultry housings and preventing the contamination of the environment (Gimeno, 2004). In order to best prevent MD and co-infection diseases a farm should follow the following general biosecurity procedures (DuPont, 2013).

- Breeder farms should be sited at least five miles from any commercial farms.
- Maintain 'all-in all-out' systems, single age buildings etc.
- Use protective, farm only, clothing to prevent pathogen spread.
- Staff movements should be as limited as possible.
- Use regularly refilled foot dips, charged with a suitable disinfectant (e.g. Hyperox® or Virkon® S).
- Effective waste disposal and removal of used litter from the site is essential.
- Avoid the potential spread of infection from diseased carcasses by on-site incineration.
- Cleaning and disinfection should include houses, equipment and surroundings.

1.13 Status of MD and its control in Australia

As in other parts of the world, MD is endemic in much of Australia and historically had required vaccination in layers and breeders, but not broiler chickens. The status of MD and its control have been well summarised by Jackson (Jackson, 2000a, 2000b). First vaccine introduced in Australia to control MD successfully was HVT vaccine in 1971. Due to maternal antibody interference problem with cell free HVT vaccine and contamination of HVT with REV, resulted introduction of the serotype 2 MD vaccine (Maravac) in 1978. Between 1992 and 1997, MD caused massive outbreaks in both the layer and broiler industries as conventional Australian vaccines and vaccination programs failed to control MD in the imported genotypes. Mortalities in the range of 20 – 40 % of birds were common in layers and breeder flocks (Cumming*, et al.*, 1998; Groves, 1995). The problem in layers and broiler

breeders was brought under control by the importation of seed for the Rispens/CVI988 vaccine (attenuated MDV-1) in 1997 and MD remains well controlled by this vaccine at present. Broilers had not traditionally been vaccinated against MD in Australia, but during 1992 - 97 clinical MD was appearing in birds from 35 days onwards, associated with reduced flock productivity, typically around 8 points (0.08) in feed conversion ratio (FCR), and increased intercurrent disease. These problems were generally responsive to vaccination with HVT and in 1996 two Embrex® machines were imported for in-ovo vaccination of broiler eggs, with several more imported subsequently. All major Australian companies use the technology. This, coupled with the production from 1997 onwards of high titre cell-associated HVT vaccine, helped to bring the immediate problem in broilers under control, although at considerable cost. Since 1996 imported strains of HVT (FC126, and NBSL S.AR) are now widely used as vaccines for broiler chickens in cell-associated form administered *in ovo*. Layer birds and broiler breeders are all vaccinated exclusively with the introduced CVI988/ Rispens, either alone, or in conjunction with HVT as a bivalent vaccine. The local BH16 attenuated serotype 1 vaccine is not used commercially despite being at least as protective as CVI988/Rispens (Karpathy*, et al.*, 2002, 2003). However, the HVT vaccine had broken down many years earlier in the USA and other countries, and may be destined to do so in Australia. Very virulent MDV strains, against which HVT confers only partial protection first identified in Australia in 1985 (McKimm-Breschkin*, et al.*, 1990) and isolated from subsequent outbreaks of MD in vaccinated birds (De Laney*, et al.*, 1995; Zerbes*, et al.*, 1994). In consequent formal challenge experiments using titrated doses of a local isolate of MDV (MPF57), failure of HVT to provide complete protection was repeatedly demonstrated (Islam*, et al.*, 2007a; Islam*, et al.*, 2006; Islam*, et al.*, 2002). These findings are consistent with evolution of Australian MD viruses towards greater pathogenicity in the face of HVT vaccination. This process is likely to have accelerated since the introduction of wider HVT vaccination of broilers in the late 1990s. Therefore, in order to avoid future outbreaks of MD in Australia and to minimize the evolution of MDV towards greater virulence in the face of 'imperfect' vaccines (Atkins*, et al.*, 2012; Gandon*, et al.*, 2001), effective monitoring systems for MD are needed which enable early detection of MDV infection and will predict the behaviour of the disease in chicken populations as vaccine efficacy declines. However, tactical vaccination based on monitoring of MDV-1 rather than routine vaccination may reduce selection pressure for increased virulence in MDV-1 (Walkden-Brown*, et al.*, 2013a).

1.14 History and details of the Rispens/CVI988 MD vaccine

1.14.1 History of the Rispens/CVI988 vaccine

Rispens/CVI988 was first described by Rispens *et al.* (1972a) as an attenuated vaccine strain of MDV-1 which proved protective in both laboratory and field trials. Initially these authors isolated a Marek's disease virus of low pathogenicity from a flock of susceptible chickens that did not show severe clinical symptoms of MD.

In laboratory trials, Rispens *et al*. (1972a) initially used the 26th passage of CVI988 strain in DEF but this induced microscopic lesions of MD in chickens so the authors used it at a passage level of 35 in DEF which avoided this and also successfully produced A antigen in tissue culture supernatants. Eventually, CVI988 was shown to be effective and safe in both laboratory and field trials where there was no adverse effect on body weight and egg production, yet it generated good immunization with very low early mortality (Rispens*, et al.*, 1972a; Rispens*, et al.*, 1972b). Although the first serial passages of CVI988 were performed using DEF, the virus strain was later adapted to CEF (Maas*, et al.*, 1982) and today these strains are widely used in MDV vaccination programs. In 1973, Rispens/CVI988 applied to have the vaccine licensed in The Netherlands and afterward applied for licences in other European and Asian countries. Later, von Bulow (1977a) showed that CVI988 was mildly pathogenic for genetically susceptible Rhode Island Red chickens if injected at 10 times the recommended dose. Finally, in the early 1990s the Rispens/CVI988 vaccine was first licensed in USA after it was evident that this vaccine offered the best protection against vv MDV (Witter, 1992; Witter, 1998b). In 1996, Witter (Witter, 1996) described the most recent pathotype of MDV, i.e. vv+ MDV, against which the Rispens/CVI988 vaccine also offers protection (Baigent*, et al.*, 2006a; Witter*, et al.*, 1995). De Boer *et al.* (1986; 1981) described that under experimental conditions the protective efficacy of the CVI988 clone C was greater than that of the commercial Rispens/CVI988 vaccine based on PD_{50} assays.

1.14.2 Earlier transmission studies with the Rispens/CVI988 vaccine

In 1972, Rispens *et al.* (1972a) showed that CVI988 virus spreads directly from vaccinated flocks to unvaccinated contact chickens. They placed 35 unvaccinated chickens with 190 vaccinated chickens in isolated conditions to monitor the contact transmission of the vaccinal

virus. They kept the birds for 2 years. Antibodies were found in all vaccinated (from 3rd week) and contact birds (from 5th week). Antibody levels were high throughout the observation period and they did not differ significantly from those found after a natural infection. Since strain CVI988 virus spread readily to unvaccinated contacts, an experiment was performed to determine whether repeated contact passage would result in reversion to virulence. Rispens *et al.* (1972a) vaccinated 25 antibody free MD susceptible chickens with 3,000 pfu of the 35th DEF passage of the Rispens/CVI988 vaccine by intramuscular administration. After 3 weeks, 25 unvaccinated chickens were placed in contact with the vaccinated chickens. After another 3 weeks, the 25 vaccinated chickens were separated from the first contact group and another 25 unvaccinated chickens were placed in contact with the first contact group. This procedure was repeated 5 times during a period of 22 weeks. Contact infection was monitored by periodic virological and serological assays and specific *post-mortem* examination was performed at the termination of the experiment. The results confirmed that the vaccine virus spread readily from one contact group to another without any MD lesions (Rispens, *et al.*, 1972a).

To confirm the spreading capacity of CVI988, Rispens *et al.* (1972a) performed an additional experiment. In this experiment an equal number of vaccinated (5 birds) and unvaccinated (5 birds) antibody free birds were placed in same room with strict isolation. Five birds were injected with 3000 pfu of Rispens/CVI988 virus in the muscle at one day of age. The virus could be reisolated from buffy coat cells from 2 out of 5 contact chickens at 4 weeks. During week 5 week, virus was reisolated from buffy coat (1/5), feather tips (3/5). Serology results were positive for all 5 in-contact birds at 5 and 6 weeks after placement with vaccinated chickens (Rispens, *et al.*, 1972a). Subsequent publications report that both a plaque purified clone of CVI988 (988C) at a passage level 65 and CVI988 at passage 42 showed very limited transmission between birds (Witter, *et al.*, 1987; Witter, *et al.*, 1995)

1.14.3 Molecular characteristics of Rispens/CVI988 vaccine

Comparative analysis of the genomic sequence of the three MDV serotypes revealed that only the serotype 1 genome encodes a number of unique genes such as Marek's EcoRI-Q (*meq), pp38, vIL8,* and *telomerase* (Chen, *et al.*, 1992; Cortes and Cardona, 2004; Cui, *et al.*, 2004a; Cui, *et al.*, 1991; Jones, *et al.*, 1992; Parcells, *et al.*, 2001). *Pp38* was first recognized as a compound phosphorylated protein by monoclonal antibody (Mab) H19 or M21 (Ikuta, *et al.*,

1985; Lee, *et al.*, 1983) and pp38 of CVI988 varies from all other MDV-1 viruses in H19-epitope because the Mab H19 responded with all serotype 1 viruses except Rispens/CVI988 (Cui, *et al.*, 2004b). Pp38 of all MDV-1 isolates share the terminal 65 amino acids (aa) and the pp38 of Rispens/CVI988 is different from all other pathoypes by one amino acid i.e. all pathogenic serotype have the amino acid glutamine which is changed to arginine in Rispens/CVI988 because this virus differs from MDV-1 pp38 by a single nucleotide Guanine (G) instead of Adenine (A) (Cui, *et al.*, 1999; Lee, *et al.*, 2005).

As there is only limited information available on genetic variability within a given serotype of MDV, PCR differentiation between MDV isolates of the same serotype is difficult. Visible electrophoretic changes between virulent MDV isolates and their attenuated counterparts were evident in the *Bam* HI-H fragment of the MDV genome, as an expansion of this region (Fukuchi, *et al.*, 1985; Maotani, *et al.*, 1986). The expansion is due to multiplication of 132 base pair (bp) repeats that are present in virulent strains as only two or three copies, while in highly passaged viruses their number is variable and can reach more than ten (Ross, *et al.*, 1993). This feature has facilitated the development of a simple PCR test to discriminate between virulent and passaged viruses (Becker, *et al.*, 1993; Silva, 1992). PCR analysis of the expansion of the 132 bp repeats through multiple *in vitro* passages appears to be a consistent method for recognizing Rispens/CVI988 strain. However, expansion of the 132 bp repeats is simply a marker of attenuation and not the cause (Silva, *et al.*, 2004). A further Rispens/CVI988-specific insertion is characterized by a single 116 bp repeat upstream of the MDV ICP4 ORF and can be easily detected by PCR (Majerciak, *et al.*, 2001). Variability in the MDV specific *meq* gene has also been reported, however, early reports suggested that this polymorphism cannot be used to distinguish vaccine and virulent viruses (Chang, *et al.*, 2002a).

Based on the results of gene deletion studies (Lupiani, *et al.*, 2004) and knowledge of its transforming properties (Liu, *et al.*, 1998), the *meq* gene has been shown to be the main oncogene for MDV-1. The *meq* gene is 1020 base pairs (bp) long and Meq, its gene product, is a 339 aa protein (Jones, *et al.*, 1992). The Meq protein has two major domains: the N-terminal basic leucine zipper (bZIP) domain and the C-terminal proline-rich domain (Jones, *et al.*, 1992; Qian, *et al.*, 1996). In the case of Meq, the C-terminal domain includes a proline rich region that is a potent transcriptional transactivator (Chang, *et al.*, 2002a; Qian, *et al.*, 1995).

The attenuated vaccinal isolate CVI988 has a 177 - 180 bp insertion in the *meq* gene that makes it different from oncogenic MDV-1 and may be responsible for the non-oncogenicity of CVI988 (Chang*, et al.*, 2002b; Lee*, et al.*, 2000; Spatz and Silva, 2007). Renz (2008) also reported *meq* sequence polymorphism between Australian isolates of MDV-1 (MPF57, 04CRE, 02LAR, FT158, Woodlands1) and CVI988. The overall length of the *meq* gene for all five Australian isolates was 1197 bp whereas for CVI988 it was 1200 bp. Thus all five Australian isolates had an insertion of 177 bp behind position 575 whereas CVI988 has an insertion of 180 bp. The Australian insertions were homologous with the CVI988 insertion apart from deletion of the first 3 bases and a single base variation at position 646 in the Australian isolates, aligned to position 649 of CVI988. Whereas the reference isolate CVI988 showed a 'C' at this particular position, all Australian MDV-1 isolates showed a 'G'.

The five Australian isolates encode 398 aa while CVI988 encodes 399 aa. The insertion of 59 aa which is present in all the Australian isolates, is similar to that of CVI988 following the extra proline insertion separately from a single amino acid variation at position 216.

1.15 Conclusions

In 1907, more than 100 years ago Jozsef Marek first described the form of polyneuritis in four cockerels. It was an additional 60 years before the causative agent of MD was identified and once identified, it was a very short time until the first live attenuated vaccine was developed. Since then, despite the widespread use of such vaccines MD has persisted as a disease of concern for the poultry industry. Within 10 years of the introduction of vaccines, outbreaks of MD were reported and hypervirulent pathotypes of MDV were isolated from vaccinated poultry and it is widely accepted that vaccination has contributed to the evolution of virulence in MDV (Atkins*, et al.*, 2012). The failure of vaccinal protection in turn led to use of different vaccines and combinations, for instance Rispens/CVI988 and bivalent and trivalent vaccines, some of which went on to fail also as virulence increased. In some areas only the Rispens/CVI988 vaccine, either alone or in combination with another serotype, is now an effective measure. However, CVI988, probably the most efficacious MD vaccine so far, is derived from a serotype 1 MDV that is weakly oncogenic in susceptible genotypes of chickens. This has led to the important question: what we will do if hypervirulent MDV pathotypes evolve that can break through the protection provided by CVI988.

To properly understand how vaccination may contribute to the evolution of virulence of MDV an understanding of the kinetics of virus replication, shedding and transmission in hosts co-infected with pathogenic and vaccinal MDV is required (Atkins, *et al.*, 2011; Atkins, *et al.*, 2012). To date such interaction is well understood for HVT and MDV-2 vaccines (Islam, *et al.*, 2008; Islam, *et al.*, 2007b; Islam, *et al.*, 2006b; Walkden-Brown, *et al.*, 2013b). With the recent development of quantitative molecular tests that differentiate the Rispens/CVI988 vaccine from wild type MDV in Australia (Renz, *et al.*, 2013) it is now possible to investigate the interaction between pathogenic MDV and the most widely used serotype 1 vaccine in the world, Rispens/CVI988.

In this thesis I will firstly examine the kinetics, shedding profile and transmissibility of current commercial strains of the Rispens/CVI988 vaccine, and subsequently investigate the effects of co-infection with this vaccine and a vvMDV isolate in the same host when the two viruses are administered at various intervals relative to each other.