REVIEW OF LITERATURE
II. REVIEW OF LITERATURE

2.1 History and Etiology

Marek's disease (MD) was first described by Jozsef Marek in 1907 (Marek, 2007) as fowl paralysis (polyneuritis) and was later shown to be associated with lymphoproliferation in various organs. The clinical disease has similarities to avian leukosis and it was about 40 years before, two conditions were differentiated and the causative agent, the Marek's disease virus (MDV), was identified (Pastoret, 2004). In the 1960's, this disease was named in honour of Jozsef Marek and his contributions to the field (Biggs, 2001). MD drew much attention in the beginning, during the 1950's when the world poultry industry rapidly expanded and intensification of poultry production took place in the 1960’s and it was identified as one of the major threats to the industry causing significant economic losses (Pastoret, 2004). With subsequent intense research, the causative agent of the disease was identified as a herpesvirus (Churchill and Biggs, 1967) and a great deal of knowledge on the virus was obtained. Subsequently, vaccines were developed against MD and successfully used, leading to a dramatic worldwide reduction in economic losses caused by this disease (Witter, 2001). Control of MD by vaccination is considered as a milestone in veterinary medicine and MD vaccine is the first commercial vaccine against a virus (Davison, 2003). MD has also been suggested as a model to study mechanisms of carcinogenesis and cancer regression, as well as a model to explore the influence of host genetics on susceptibility or resistance to infectious diseases (Pastoret, 2004).
Marek’s disease virus is an alpha-herpesvirus, genetically similar to herpes simplex and varicella zoster viruses. However, some of its biological properties resemble those of gamma-herpesviruses, such as Epstein-Barr and Kaposi sarcoma (Baigent and Davison, 2004). MDV, like other herpesviruses, consists of a linear double-stranded DNA genome with at least 103 distinguishable genes (Osterrieder and Vautherot, 2004). Genomes of MDV as well as the other two members of the genus Mardi virus represent a class “E” genomic organization similar to that of herpes simplex virus (HSV) type 1, the prototype member of the subfamily Alphaherpesvirinae (Osterrieder and Vautherot, 2004). In MDV genome, there are two unique short and long regions (US and UL) each of which is flanked by the terminal and internal long repeats (Osterrieder et al., 2006). Non-enveloped virions of MDV have a diameter of about 100 nm (Nazerian and Burmester, 1968) while the diameter of enveloped particles may range from 273 to 400 nm (Calnek et al., 1970).

Marek’s disease virus was originally classified in the subfamily of Gammaherpesvirinae based on its ability to induce lymphoproliferation, but it was re-classified and included in the subfamily of Alphaherpesvirinae based on sequence homology (Davison et al., 2002). Within this subfamily, MDV is classified under the genus Mardi virus. There are three members included within this genus; Gallid herpesvirus 2 (GaHV-2), Gallid herpesvirus 3 (GaHV-3) and Meleagrid herpesvirus 1 (MeHV-1) (Faquet et al., 2004). In the old classification, these species were known as the MDV type 1 or serotype-1, MDV type 2 or Serotype-2 and turkey herpes virus (HVT) or Serotype-3, respectively. The genome of GaHV-2 is the largest (174,076 bp) followed by GaHV-3 (164,270 bp) and MeHV-1 (160,673 bp) (Kingham et al., 2001). Gallid
herpesvirus 2, the causative agent is cell-associated in all the organs except in the feather follicle where enveloped infectious virions egress from the body (Purchase et al., 1976). Since MDV and HVT are the most commonly used names for GaHV-2 and MeHV-1 respectively, the old nomenclature i.e., MDV and HVT will be used throughout this thesis in order to avoid confusion.

Within the MDV species, there are various strains classified into different groups, referred to as pathotypes, based on the particular strain's ability to overcome immunity induced by different types of MD vaccines. Accordingly, there are several pathotypes of MDV; namely virulent (vMDV), very virulent (vvMDV), and very virulent plus (vv+MDV) (Witter, 1997). While almost all commercially produced chickens in the world are vaccinated against MD, no vaccine induces immunity which prevents shedding of infectious virus. The continuous pool of the vaccine virus has allowed the virus to evolve into strains with potentially higher virulence in order to overcome the selection pressure exerted by vaccination and other approaches such as genetic selection for enhanced resistance to MD. As a result, there have been periodic MD outbreaks in vaccinated flocks necessitating the introduction of more efficacious vaccines (Witter, 1997).

2.2 Incidence

The presence of MD and/or MDV has been reported from different states in India, like Orissa (Pradhan and Nayak, 1973), Punjab (Grewal and Singh, 1976), Assam (Baruah and Kwatra, 1979), Uttar Pradesh (Goel et al., 1980), Andhra Pradesh (Reddy et al., 1980 and Haribabu, 1986) Arunachal Pradesh, Tripura (Verma et al., 1989) and Tamilnadu, Karnataka and Maharashtra (Raja et al., 2009).
2.3 Types of vaccines

Vaccines against MD were first introduced in 1970s, have brought enormous benefits to poultry production and without them the modern poultry industry would not have survived the attack of MD. Vaccines have been prepared from virus of serotypes 1, 2 and 3. The HVT (Serotype-3), bivalent HVT + MDV-2 (i.e., Serotype-3+Serotype-2) and CVI988 (Serotype-1) vaccines are the most widely used MD vaccines today around the world (Bublot and Sharma, 2004). Serotype-1 vaccines have not been licensed for commercial production and use in India.

2.3.1 Serotype-1 MDV vaccines

Churchill et al. (1969) described a vaccine prepared from an attenuated virulent serotype-1 MDV (HPRS-16). The virus was attenuated by serial passages using chicken kidney cell cultures. Reversion to virulence was not noticed.

Biggs et al. (1970) attenuated HPRS-16 strain by 40 serial passages in chick kidney to be used as vaccine. They undertook four trials involving more than 19,000 birds and compared the results with those of controls. They confirmed that vaccinated groups had low incidence of MD.

Rispens et al. (1972) inoculated MDV-1 isolate of low pathogenicity into day-old MD-susceptible chicks which protected them from mortality and showed no gross pathological lesions. They developed an attenuated vaccine strain, designated CVI988 also called the Rispens strain which was shown to be protective in both laboratory and field trials.
Jackson *et al.* (1974) studied in detail the efficiency of attenuated HPRS-16 strain and reported that the body weight of vaccinated chicken at eight weeks of age were 5.6 per cent higher than that of the unvaccinated chicken. They also observed that vaccinated birds produced more eggs.

Bullow (1977) vaccinated the chicks with attenuated HPRS-16 strain and reported protection against virulent JMV strain challenge. He concluded that attenuated MDV-1 strains had produced mild pathogenecity in chickens of higher genetic susceptibility.

Witter (1982) described an experimental vaccine prepared from an attenuated very virulent strain designated MDll/75C and Witter and Lee (1984) noticed that this vaccine was highly neutralized *in vivo* and was relatively ineffective in chickens with either homologous or heterologous maternal antibodies.

Karpathy *et al.* (2002) studied the safety and efficacy of MD vaccine developed from an Australian isolate of very virulent MDV. They took one of the two cloned passages of the attenuated strain BH-16 for vaccine development and carried out a comparative protection trial and immunosuppressive potential assay on the BH-16 vaccine and on CVI988 Rispens vaccine using commercial and SPF chicken. They showed that these vaccines induced comparable levels of protection when used as monovalent or multivalent vaccines and that protection achieved with the monovalent vaccine was lower. They observed no gross tumour formation and no histological evidence of damage to either the bursa of Fabricius or the thymus. They concluded that BH -16 vaccine was safe and at least it was as protective as the Rispens vaccine against three virulent MD challenge viruses.
2.3.2 Serotype-2 MDV vaccines

Serotype-2 MDV is naturally occurring infectious virus, but is nonpathogenic or only weakly pathogenic and non-oncogenic to chickens.

Jackson et al. (1977) used non pathogenic MDV-19 strain as a vaccine and they found that the protection conferred by MDV-19 in the presence or absence of HVT maternal antibody was comparable to that conferred by NSW 1/70 strain in the absence of maternal antibody.

Schat and Calneck (1978) reported optimal protection of SB-1 strain against virulent JMV (MDV-1) strain. Whereas, Witter (1985) reported that SB-1 strain was efficacious against challenge with most virulent MD viral strains and spreads readily by contact.

2.3.3 Serotype-3 HVT vaccines

Kawamura et al. (1969) first isolated turkey herpes virus (HVT) from tissue cultures of normal turkeys. Later, Witter et al. (1970) isolated the FC 126 strain of HVT from a commercial turkey flock and characterized the virus as non-pathogenic and antigenically related to MDV.

Okazaki et al. (1970) described the efficacy of the FC 126 strain as a vaccine. They reported that HVT vaccine administered parenterally with a low dose of 600 PFU protected the chicks against virulent JM strain of MDV-1. Further studies by Okazaki et al. (1971) showed that vaccination with one or more weeks before challenge with MD virus gave no protection and simultaneous vaccination and challenge gave partial
protection. They suggested that the birds should be vaccinated as early as possible preferably on the day they hatch.

Purchase et al. (1971) conducted 17 field trials with HVT-FC126 strain as a vaccine against MD. The virus offered more than fifty per cent protection against MD in all the trials. The incidence of MD in unvaccinated birds was greater than two per cent. The vaccine virus persisted in the protected birds but did not spread significantly to unvaccinated birds. They noticed that 2,000 PFU or 10,000 PFU dose per chick was equally effective in offering protection but results with 500 PFU/chick were inconclusive. Subsequently, Purchase et al. (1972) recorded that the average mortality was reduced from 19.2 to 2.0 per cent and the degree of protection averaged to 80.8 per cent.

Churchill and Carrington (1973) provided evidence that chicks with no maternal antibody to MDV or HVT, when vaccinated with graded doses of HVT, developed both viraemia and antibody response. Chicks with maternal antibody to MDV responded in a similar manner to chicks with no maternal antibody. However, chicks with maternal antibody to HVT, when vaccinated with HVT, demonstrated a prompt viraemia but showed a delay of one to two weeks in the antibody response when compared with antibody free chicks.

Kilgore and Brokken (1973) protected day old chicks with a lyophilized turkey herpes virus in doses ranging from 10 to 10,000 PFU. The vaccines effectively reduced the incidence of MD and a satisfactory protection was provided by doses of 211 PFU and above.
Zanella et al. (1973) also conducted experimental and field trials using the freeze-dried FC-126 HVT vaccine. According to them, one to two PFU induced viraemia and antibody response and a dose of 20 to 100 PFU was protective in 90 per cent of chicks.

Eidson et al. (1974) conducted vaccination trials against MD using cell-free HVT vaccine. Three strains of chicks with maternal antibodies to HVT and MDV were vaccinated on day one with various doses of cell-free HVT. Chicks withstood challenge exposure regardless of the mode of exposure. Protection was recorded even when the vaccine dose was as small as 250 PFU/chick.

Eidson et al. (1975) noticed that the cell-free HVT was as efficacious as cell-associated HVT vaccine with regard to protection against MD lesions and mortality. However, the maternal antibodies interfered with cell-free HVT vaccine and they reported that the influence of maternal antibodies could be easily overcome by doses of 300-1000 PFU or more of HVT vaccine.

Witter et al. (1976) showed that chickens vaccinated at hatching with high doses of HVT developed viremia that peaked in titer around 12th day and gradually declined. They also found that HVT infection induced mild microscopic lympho-proliferative lesions in the gonads and nerves. These lesions were more prominent around the 12th day and then regressed. HVT viremia and lesions were both dose-dependent and were less in chickens with maternal antibodies against Marek's Disease Virus (MDV). Sequential studies on chickens vaccinated with HVT and challenged with MDV showed that chickens were protected against the earliest detectable MD viremia and lymphoproliferative lesions attributed to MD. They concluded that HVT protected
against productive MDV infection in the thymus and against cell-associated viremia that were evidence for an anti-viral immune response.

Cho (1977) studied HVT induced viraemia at three weeks intervals through 21 weeks of age in individual chickens of experimental (WSU-VS) and commercial strains (C-WL) of White Leghorns vaccinated with graded doses of HVT. They noticed that the viraemia titres were significantly lower in the C-WL than in the WSU-VS birds despite the same dose of vaccination. Both the strains of chicken exhibited significantly lower viraemia titres when vaccinated with 240 PFU than with 1,480 or 6,600 PFU. This indicated possible genetic differences between the two strains of chickens in susceptibility.

Donahoe and Kleven (1977) observed that HVT prevented tumour formation by MSB-1, a MD tumour cell line, whereas, 21.4 per cent of unvaccinated controls had tumours.

Eidson et al. (1978) noticed that the maternal antibodies to HVT, inhibited induction of viraemia but also made the antibody responses less uniform and delayed. The combined effect of HVT maternal antibody on viraemia and antibody development plus early exposure to MDV resulted in tumour development in chicken given a low dose of the HVT vaccine.

Landgraf and Vielitz (1978) tested HVT-FC 126 (MDV-3), CVI988 (MDV-1) vaccine and experimental JMV vaccine in laboratory and field trials for protection against MD. They carried out the test with 780 SPF chicks and 3200 commercial white Leghorn
chicks with maternally derived antibodies to HVT and MDV-1. Both vaccines showed increased protection with an increased interval between vaccination and challenge. The significant protection (> 80%) resulted with both vaccine viruses after the 8th dpv. JMV vaccine (embryo adapted strain JMV-A-164) provided a 40 to 60 per cent protection against MD depending on the time of challenge but it completely protected birds against the highly pathogenic JMV tumour cell inoculum. Hyper immunisation of adult birds with JMV vaccine did not reduce the mortality in their progeny from MD.

Cauchy et al. (1979) vaccinated six different groups of one day old chicks with the HVT vaccine at doses of 0, 4, 16, 64, 256 and 1024 PFU and challenged the birds on the ninth day of age with an acute strain of MDV. They observed the gross and microscopic lesions in nerve and gonads that were combined for quantitative expression of MDHV pathogenicity. A slight response was obtained in birds vaccinated with high doses of HVT (256 and 1024 PFU) and the oncogenic product of MDHV strain was fully expressed in unvaccinated chicks. A moderate response to challenge with MDHV was obtained after vaccination with graded doses of HVT vaccine.

Fuzikava et al. (1979) carried out the field trials in 20,424 birds in 10 farms to evaluate the efficacy of lyophilized vaccine prepared from HVT in comparison with a cell associated HVT vaccine. They found no difference in protection rate between the two vaccines. They inoculated two groups of chickens with or without maternal antibody with lyophilized HVT vaccine and examined for the appearance and persistence of antibody by both AGPT and immuno-fluorescence antibody technique (IFA). They noticed that there was no significant difference between the two groups in the rate of chicken positive
for precipitating antibody after vaccination, but IFA indicated that antibody production was delayed to a great extent in the group with a maternal antibody titre of 320 and higher even if the antibodies are derived from MDV infection and this delay had disappeared by the seventh week after vaccination.

King et al. (1981) noticed that homologous maternal antibodies delayed the development of vaccine virus viraemia and inhibited vaccinal immunity as judged by protection against challenge with the virulent JM-10 strain of MD virus at seven dpi.

Choi et al. (1983) compared the efficacy of two avirulent MD viruses, MDV-19 and CVI988 and one HVT in chicks derived from the parents vaccinated with HVT and naturally exposed to field MDV. They inoculated a graded dose of two strains of avirulent MDV and one strain of HVT into groups of one day old layer birds derived from parents vaccinated with HVT. They noticed that the median protective dose was lowest with CVI988 strain of MDV (104 PFU) than the MDV-19 (736 PFU), being highest with HVT (6187 PFU).

Thornton (1985) noticed that HVT vaccine should not contain less than 1,000 PFU per dose, throughout its shelf life or more if this has been shown to be necessary in protection trials.

Afzal et al. (2001) vaccinated day-old broilers with Rispens strain vaccine, HVT or combination of HVT and Rispens and observed for weight gain, mortality and humoral and CMI responses. They noticed that mortality rate did not differ between any of the vaccinated groups and unvaccinated controls. They also found that the CMI response of
different groups were also non-significant as measured by reaction to photo-
haemagglutinin.

Islam *et al.* (2007) found that the HVT vaccine dose had a significant impact on PI, but vaccination to challenge interval appeared to have greater impact on the protective efficacy of vaccination. A fourfold increase in challenge virus dose from 50 PFU to 200 PFU increased the mortality rate and incidence of MD.

### 2.3.4 Bivalent and Polyvalent vaccines

Witter (1982) studied protection by attenuated polyvalent vaccines against highly virulent strains of MDV. They attenuated Md11 strain of MD virus by 75 cell culture passages and designated it as Md11/75 and it provided good protection against challenge with Md5 and most other highly virulent MD viruses tested, but was less efficacious against JM/102W strain, a prototype MD virus that was protected against well by HVT and SB-1 vaccines and the efficacy was lower in chicks with HVT antibody. They also showed that a polyvalent vaccine composed of Md11/75C, HVT and SB-1 viruses protected chickens better against a battery of five highly virulent MD challenge viruses. The data suggested that vaccinal immunity may have been partially viral strain specific.

Calnek *et al.* (1983) administered a bivalent MDV consisting of HVT and SB-1 to one group of chicks and the other group with HVT only and showed that the bivalent vaccine uniformly protected the chickens better than HVT alone between 12 and 16 -20 weeks of age when serious MD losses occurred. They observed that total mortality in bivalent vaccine ranged from 0.39-1.26 per cent whereas that in HVT vaccinated group not exposed to SB-1 varied from 1.92-7.44 per cent. They observed that SB-1, when
given in conjunction with HVT, exerted a profound effect on MD mortality on each of the farms. They concluded that MD losses were reduced approximately 3-13 folds in groups vaccinated with HVT + SB-1 compared with groups receiving only HVT.

Witter *et al.* (1984) studied the efficacy of trivalent (Md 11/5 C+ SB-1+HVT), bivalent (SB-1+HVT) and HVT alone as vaccines against MD and compared the performance in commercial broiler flocks in four trials involving 11 farms. They found that chicken receiving polyvalent vaccine had lower leucosis (MD) condemnation rate than chickens vaccinated with HVT alone and also stated that bivalent vaccines containing either 1:500 or 200 PFU of SB-1 virus were equally effective. They concluded that HVT needed to be supplemented with small amounts of SB-1 to obtain the benefits of protective synergism.

Witter *et al.* (1985) conducted four vaccination trials with layer and broiler breeder flocks with trivalent (6,000 PFU of HVT + 1,000 PFU of SB-1 + 1,000 PFU of Md 11 / 75 C) and bivalent (6,500 PFU HVT + 1500 PFU SB-1) vaccines and compared the results with those vaccinated with 8000 PFU of HVT alone. They concluded that polyvalent vaccines were more effective than HVT.

Pruthi *et al.* (1989) studied the efficacy of the bivalent vaccine prepared by combining inactivated MDV and HVT. They compared the efficacy of the bivalent vaccine with HVT and inactivated MDV separately in white leghorn chicks vaccinated on day one then challenged at 21 days with fowl blood infected with virulent MDV. They observed that the bivalent vaccine appreciably delayed mortality resulting from MD and elicited the highest protective efficacy as judged on the basis of MD specific mortality
and percentage occurrence of lesions. They concluded that the protective efficacy of bivalent vaccine was higher than either HVT or inactivated MD vaccine.

Friedman et al. (1992) vaccinated one-day-old chicks with HVT, bivalent HVT+SB-1 and Rispens vaccines and evaluated the immune response and resistance to MD. They found temporary depletion of B-lymphocyte activity of varying intensity and temporary decreased resistance to pathogenic *E. coli* infection mainly in HVT+SB-1 and Rispens vaccinated chicks.

Young (1993) studied the efficacy of Australian commercial Type 2 and Type 3 (HVT) vaccines either alone or in combination and found that the efficacy of these vaccines was considerably diminished by the presence of maternal antibody. He observed that vaccine registration trials were usually carried out in SPF birds, where protective indices of 80 per cent were common. Whereas, in birds with maternal antibody the same vaccines provided inadequate protection against challenge with virulent Type 1 virus, giving protective indices of less than 60 per cent. He also observed that no protective benefit could be demonstrated for the Type 2 vaccine used alone.

Young (1996) studied the efficacy of currently available local and imported vaccines, alone and in combination, against challenge by virulent MDV-1 in the presence of serotype-1 and Serotype-3 maternal antibodies. He observed that both HVT and C/R6 provided good protection in SPF birds, but that protective indices were considerably reduced in commercial layer birds with maternal antibody. He opined that the best vaccine combinations were HVT + C/R6 or HVT + Maravac (an Australian Type 2 vaccine).
Witter (1998b) made an attempt to develop an improved vaccine against the Marek's disease through the selection of optimum viral strains. Seven attenuated serotype-1 strains and 22 avirulent serotype-2 strains, alone and in combination with the FC126 strain of serotype-3 were screened for protective efficacy against challenge with virulent and very virulent MD viral strains. He evaluated these three viruses alone and in various combinations and compared with commercially available vaccines, including FC126, bivalent (FC126 + SB-1), and CV1988/C. He concluded that two of these viruses 301 B/1 (Serotype-2) and Md11nS/R2 (Serotype-1) were exceptionally protective compared to the prototype vaccine strains, four monovalent and polyvalent vaccines based on the performance of the new vaccines. He found a better protection than bivalent vaccine and noted a protective synergism commonly between Serotype-2 and Serotype-3 but only sporadically between serotype-1 and serotype-2 or between serotype-1 and serotype-3.

Buscaglia et al. (2004) evaluated the protective efficacy of various vaccine viruses in combination against challenge with a vv MDV called NULP-1. They found that HVT didn't protect satisfactorily against any of the virulent strains, but CV1988/Rispens vaccine alone or in combination with Serotype-2 and/or Serotype-3 vaccine strains enhanced protection significantly against NULP-1. They concluded that Serotype-2 plus Serotype-3 provided significant protection when challenged with the virulent strain.

Chang et al. (2010) reported that the genetic line significantly influenced MD incidence and days of survival post-MDV infection after vaccination of chickens (P <
0.01). The protective indices against MD varied greatly among the lines with a range of zero up to 84 per cent. They also reported that non-MHC host genetic variation significantly affects MD vaccine efficacy in chickens.

2.4 Meq gene

Jones et al. (1992) reported a MDV gene encoding a protein with homology to the leucine-zipper class of nuclear oncogenes closely related to the fos/jun family of oncogenes which contained a proline-rich domain characteristic of transcription factors in regulating viral latency or oncogenesis. This gene designated Meq, maps to the long repeat of MDV and is one of the few genes that are highly expressed in MDV-induced T-cell tumors.

Liu et al. (1998) suggested that Meq antagonizes apoptosis through regulation of its downstream target genes involved in apoptotic and/or anti-apoptotic pathways.

Lupiani (2004) generated an rMd5Delta Meq virus by deleting both the copies of Meq gene from the genome of a very virulent strain of MDV. They indicated that Meq is dispensable for in vitro virus replication essential for latency. They showed that the rMd5DeltaMeq virus was fully attenuated in chickens because none of the infected chickens developed MD associated lymphomas, suggesting that Meq is involved in lymphocyte transformation.

Li et al. (2011) suggested that the Meq gene played an important role not only in tumor formation but also in inducing immunosuppressive effects in MDV-infected chickens.
2.5 Effect of thawing temperature and duration of thawing on vaccine titre

Halvorson and Mitchell (1979) reported that very subtle deviations from the explicit directions accompanying Marek's vaccine can cause dramatic losses of vaccine potency which resulted in 14 to 97 per cent loss of vaccine titer.

Morrow and Fehler (2004) noted that cell associated MD vaccines must be kept at -196°C during the transport and until used. Thawing the ampoules at temperatures of more than 28°C or prolonged incubation before dilution, could cause damage to (or destruction of) the vaccine virus.

Geerligs and Hoogendam (2007) investigated the effect of the thawing procedure on the live virus titer of the CVI988 vaccine. They showed that directly after thawing in the water baths at 27°C and 37°C, with or without agitation, the live virus titers for CVI988 were all in the same range. After incubation of the thawed virus at both temperatures for 15 min, the live virus titers were still in the same range. A decrease in live virus titers were seen after incubation for four hr. Live virus titration of the vaccine in diluent in different temperatures revealed that the highest titers were found with diluent at a temperature of 30°C to 37°C and the lowest titers in diluent at a temperature of 5°C.

2.5.1 Thawing temperatures of different commercial Marek’s Disease vaccines

Thawing temperatures advised by the manufactures of cell associated vaccines in major poultry producing countries is listed follow.
<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Vaccine trade name*</th>
<th>Company</th>
<th>Contents</th>
<th>Thawing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Poulvac Marek CVI</td>
<td>Fort Dodge Animal Health USA</td>
<td>CVI988 live virus</td>
<td>27°C to 37°C</td>
</tr>
<tr>
<td>2</td>
<td>Cell Associated Vaccine HVT &amp; SB-1</td>
<td>Ventri Biologicals India</td>
<td>FC126+SB-1</td>
<td>35-37°C</td>
</tr>
<tr>
<td>3</td>
<td>Cell associated SB-1</td>
<td>Ventri Biologicals India</td>
<td>SB-1</td>
<td>32°C to 38°C</td>
</tr>
<tr>
<td>4</td>
<td>Marek's disease vaccine</td>
<td>Merial select USA</td>
<td>CVI988 STRAIN + FC-126 STRAIN</td>
<td>24°C to 30°C</td>
</tr>
<tr>
<td>5</td>
<td>Marek’s disease vaccine</td>
<td>Merial select USA</td>
<td>Rispens CVI988 Strain</td>
<td>15-25°C</td>
</tr>
<tr>
<td>6</td>
<td>Marek’s disease vaccine</td>
<td>Merial Canada Inc</td>
<td>SB-1</td>
<td>20° to 30°C</td>
</tr>
</tbody>
</table>

* Note. All these are cell associated vaccines and they are stored at -196°C

2. 6 Detection of Marek’s disease virus by PCR

Bumstead et al. (1997) described quantification of MDV in chicken lymphocytes using PCR with fluorescence detection. The assay determined the numbers of viral genomes present in samples by PCR amplification of a portion of the viral genome for a restricted number of cycles. Fluorescent-tagged primers were used for the PCR amplification, which allows quantification of the fluorescent product.

Handberg et al. (2001) developed a method to detect MDV serotype-1 and serotype-3 by specific PCR from various tissue samples of chicken experimentally
inoculated with MDV serotype-1 or serotype-3. The serotype-1 strains, CVI988 and RB-1B could be detected in feather follicle epithelium up to 56 and 84 days post-inoculation respectively, while the MDV-3 serotype was detected until 42 dpi. MDV-1 was detected in buffy-coat, spleen, liver, skin, feather tips and ovaries. The detection of MDV in feather tips appeared to be as sensitive as co cultivation of buffy coat.

Islam et al. (2004) developed quantitative real-time PCR (qPCR) assays for the three serotypes of MDV. They employed chicken α2 (VI) collagen gene as internal control for the quantitation of MDV. To reduce the cost and time, assays for MDV-1 and the internal control were combined into a duplex assay. They found that MDV qPCR assay was 10 to 100 fold more sensitive than standard PCR.

Baigent et al. (2005a) optimised and validated, real-time PCR to enable quantitation of MDV genomes as copy number per million host cells. The duplex PCR measured the virus Meq gene and host ovo-transferrin gene in a single reaction, enabling correction for differences in amount of sample DNA added.

Baigent et al. (2005b) detected and measured CVI988 load in live chickens from 10 days onwards. Virus load in feather tips was predictive of virus load in lymphoid tissues where immune responses would occur. They found feather tips to be a better choice than blood in terms of CVI988 genome load and ease of sampling.

Abdul-Careem et al. (2006) detected and quantified MDV load in feather tips targeting Meq gene of MDV by SYBR Green real-time PCR. They found that real time PCR is 2.5–10 times more sensitive than the conventional PCR technique. They detected
the MDV genome in feather tips of infected birds by 7 dpi and noted that the number of MDV copies peaked by day 14pi decreased by gradually by 28 dpi.

Islam et al. (2006) found that vaccination with HVT vaccine provided protection against clinical MD but did not preclude infection with wild-type MDV. They observed that the quantity of MDV detected in circulating lymphocytes during the early period after infection was a useful predictor of subsequent clinical MD later in the life. They quantified MDV and HVT copy numbers in PBL using real-time PCR between days 5 and 35 pc and related this to subsequent development of gross MD lesions. They recorded mortality and scored all dead and euthanized chicken for gross MD lesions. They found that there was an increase in HVT load in PBL between days 7 and 37 post-vaccination, with a marked increase between days 7 and 16 and again between days 30 and 37. They observed a steady increase in MDV load to 35 dpc. They concluded that the mean MDV copy number (log 10) was greater in chickens subsequently exhibiting gross MD lesions (5.05±0.21).

Kuhnlein et al. (2006) reported the presence of MD virus in feather tips, feather pulp at 12 dpe. They noted that frequency reached a maximum at 27 dpe and then decreased and at 80 dpe, when the experiment was terminated, no viral DNA was detected in the feather pulp of the surviving chickens (82%). They found that quantification of the viral genome titers in feather tip extracts at 27 and 38 dpe revealed a positive correlation with the presence of MD lesions, but only in the declining phase (38 dpe) and not at the peak (27dpe) of the viral titer.
Baigent et al. (2007) measured the response of chickens to CVI988 vaccination by quantifying CVI988 genomes in the feather tips using a real-time PCR assay. They demonstrated that the CVI988 level in the feather tips is significantly influenced by the dose of CVI988 administered and correlates with protection against virulent MDV in an experimental challenge model. They reported that q-PCR assay was clearly a valid test to demonstrate the effect of the vaccine dose and the time of challenge on vaccine efficacy.

Islam and Walkden-brown (2007) detected MDV-1 and HVT viruses in dander filtered from isolator exhaust air from day seven and MDV-2 from day 12 after infection and thereafter until the end of the experiment at 61 days of age of the chickens. They found that there was no difference in shedding rate among the three MDV-1 isolates. They also noted that the daily shedding of MDV-1 increased sharply between days seven and twenty eight and stabilized thereafter at about $10^9$ virus copies per chicken per day, irrespective of vaccination status. Challenge with three different MDV-1 isolates markedly increased shedding of the vaccinal viruses HVT and MDV-2 in dander by 38 and 75 folds, respectively.

Islam et al. (2008) investigated the effects of the vaccination-to-challenge interval on vaccinal protection against MD, and the kinetics of MDV and HVT load in the spleen and feather dander using real-time quantitative PCR. They found that vaccinal protection increased from approximately 40 to 80 per cent with increasing vaccination to challenge interval between days two and seven but not thereafter. They detected MDV in both the spleen and dander on seven days pc and increased rapidly to approximately 21 dpc, after which levels plateaued, rose or fell gradually depending on treatment. They also reported
that HVT was also shed in significant amounts, one to two logs lower than for MDV; with a clear peak around 14 to 21 dpv.

Gimeno et al. (2008) challenged the HVT vaccinated and HVT+SB-1 vaccinated birds with virulent MDV. They sampled each chicken three times during the length of the experiment (3, 5 and 15 week post challenge [wpc]), and evaluated gross lesions in chickens that died and at the termination of the experiment. They also quantified MDV DNA and HVT DNA by real-time PCR. Chickens that developed MD by the end of the experiment had higher load of challenge MDV DNA than those that did not develop MD. They did not find any differences in the load of HVT DNA between protected and non protected groups at any time point of the study indicating load of challenge MDV DNA but not load of HVT DNA in blood can be used as criterion for early diagnosis of MD.

2.7 Assay of turkey herpes virus in vitro methods

Purchase (1971) found that the avian cultures were susceptible to low passaged MD virus (LMD), high passaged MD virus (HMD) and HVT but cultures varied in susceptibility and cytopathic responses. He opined that the CEF cells were less susceptible to LMD than DEF, were equally susceptible to HMD and were much more susceptible to HVT. The changes in CEF cells consisted mainly of small, spherical, highly refractile cells which tended to become multilayered, whereas, some detached from the monolayer and that HVT produced thin syncytia with many vacuoles and some rounded or polygonal cells.

Calneck et al. (1972) first described in vitro methods for assay of turkey herpes virus. They found that chicken embryo fibroblasts, chicken embryo kidney cells and
chicken kidney cells were the suitable cell types for assay of cultures. They observed that inoculation of cells while in suspension before plating was inferior to inoculation of a monolayer. Culture inoculated at 48 hr or more after plating were less sensitive than those inoculated after 24 hr of incubation. Cultures were incubated at 38 to 39°C. The inoculum was 0.05 ml per 60mm diameter Petri dish culture with an adsorption time of 30 min for virus. They plaques were enumerated on 5th dpi.

Ferris et al. (1974) inoculated 0.1 ml virus dilution onto a 24 hr old CEF monolayer and gave 90 min adsorption time with agitation of plate at 30 min interval. They fed the culture with the same medium as used for the initiation of the culture except that the concentration of bovine serum was two per cent. They changed the medium 24 hrs post-inoculation to one containing 0.5 per cent serum and compared the different parameters. They found the vaccine was more stable when reconstituted in SPGA compared with a balanced salt solution and ambient temperature of 24 - 28°C than 37°C. They opined that 24 hr CEF culture was more suitable than 48 hr old culture for titration of standard virus preparation. They observed no significant difference of plaque counts from day 4 till day 8.

Kaleta (1974) noticed that lower cellular densities drastically reduced the number of plaques, whereas, the use of higher cellular densities resulted in a slower decline of the plaque number. They also found that the type of the cell culture medium and the type of the buffer used did not seem to be very important factors for quantitative assays of HVT. Their data indicated that the plaque should be counted on the 5th dpi. They used two plates per dilution for all titration.
Lodetti et al. (1974) noticed that the highest titre of the virus was obtained by the use of stabilizer, sucrose phosphate glutamine albumin buffer and sucrose phosphate glutamine with 15 per cent calf serum.

Withell (1974) used the focus forming technique for the assay of HVT vaccines against MD. He diluted the virus in PBS containing 7.46 per cent sucrose and 0.06 per cent sodium glutamate. He opined that the formation of typical plaques under a liquid overlay was depended greatly on the extent to which culture had been moved during a medium change or during an interim examination where plaque counts were increased. He also stated that the single movement increased the observed titers by about 2.5 folds and daily movement by over 9-fold. He concluded that the foci of infection became visible 2-3 days PI and the maximum size and numbers were achieved after 5-7 dpi.

Landman and Verschuren (2003) analyzed 31 outbreaks of MD in vaccinated flocks. They determined the PFU of MD vaccine per chicken dose through *in vitro* assays on vaccine ampoules (2 to 5 per hatchery) and samples of reconstituted vaccine (approximately 22 per hatchery). They found that all 40 reconstituted vaccine samples showed PFU doses < 10³ per chicken dose. They concluded that the outbreaks of MD can happen due to insufficient content of vaccine virus (CVI988) in the MD cell associated vaccine.

### 2.8 Flow Cytometric analysis of CD4 and CD8 cell counts

Morimura et al. (1995) examined the flow cytometric profiles of CD4 and CD8 cells in MD affected birds. The expression of CD8 was down-regulated after infection with MDV and there was no difference in the expression levels of CD4 molecules
between normal and infected chickens. Northern blot analysis indicated that the down-regulation of CD8 occurred at the transcriptional level. These results suggest that both apoptosis of CD4 T cells and down regulation of CD8 molecules could contribute to the immuno-suppression caused by MDV.

Okada et al. (1997) found that double negative T-cells (CD3+ CD4- CD8-) and CD3- CD4- CD8- cells are transformed morphologically in addition to CD3+ CD4+ CD8- T-cells by MDV infection.

Baigent et al. (1998) used flow cytometric and immunocytochemical techniques to quantify, identify and locate MDV infected lymphocytes in lymphoid organs of infected two closely related lines of chicken, one susceptible to MD (line 72) and another resistant (line 61), by expression of the virus antigen pp38. They found that the mean number of pp38M lymphocytes in spleen, thymus and bursa was greater in line 72 than in line 61.

Gisela et al. (1998) determined the proportions of T-cells expressing CD4, CD8, and T Cell Receptor (TCR)s in the thymus and spleen by flow Cytometry. They observed that in the thymus, percentages of CD4+CD8- cells increased, CD4-CD8+ cells remained unchanged and CD4+CD8+ cells decreased between two and seven weeks of age. Age-associated shifts in TCR usage were observed in the single-positive, but not in the double-positive, T-cell populations of both thymus and spleen.

Morimura et al. (1998) determined the roles of T cell subsets in the protection mechanism in chickens vaccinated with an attenuated MDV (CVI988) vaccine. The
vaccinated chicken were depleted of either CD4⁺ or CD8⁺ T cells by neonatal thymectomy and further by injecting monoclonal antibodies against chicken CD4⁺ or CD8⁺ molecules and then challenged with an oncogenic MDV. These birds were effectively protected from MDV-induced tumors. However, virus titers in CD4⁺ T cells, which are the main target cells for MDV-latent infection and subsequent transformation, were much higher in CD8⁺ deficient vaccinated chickens than in untreated vaccinated chickens at the early stage of the latent phase. They suggested that CD8⁺ T cell responses induced by the MD vaccine are essential for anti-virus but not anti-tumor effects.

Schat and Xing (2000) found that Interferon-γ and nitric oxide were important for the control of virus replication during the lytic phase of MDV infection and were also important to prevent reactivation of MDV replication in latently infected and transformed cells. They reported that Cytotoxic T cells (CTLs) were the most important of the specific immune responses in MDV which could be detected as early as six to seven dpi.

Dennis et al. (2006) measured the CD4 and CD8 T cells and B lymphocytes in blood using flow cytometry from MD resistant and susceptible birds. MD resistant birds had a higher percentage of CD8 cells but not CD4 cells than the chickens of the susceptible lines. In contrast, susceptible lines had a greater percentage of B cells.

Changxin et al. (2009) showed that in the chickens receiving a single CVI988 vaccination, the CD3 subpopulation started to increase at seven dpv, reached a peak at 14 dpv and then declined to a level of unimmunized chicken. Compared with the results obtained with a single vaccination, the revaccination significantly extended the period of expansion of the CD3 subpopulation to two weeks. They found that the dynamics of the
patterns of the CD4 and the CD8 subpopulations were similar to those for the CD3 subpopulation in the groups receiving either a single vaccination or revaccination. Revaccination moderately increased the levels of CD3 and CD8 T-cell expansion in PBLs but significantly increased the level of CD4 T-cell expansion.

Kano et al. (2009) reported that both CD8α high TCR1+ and CD8α high TCR2+ cells could be induced by the CVI988 vaccination. They also suggested that CD8αhigh TCR1+ cells might be primed by the vaccination and specifically induced by the challenge with virulent strain of MDV during the latent phase of MD infection making CD8αhigh TCR1+ cell population one of the key factors involved in the protective mechanism induced by a vaccine strain, CVI988.

Parvizi et al. (2009) analyzed T cells from the spleens of MDV infected as well as age-matched controls by flow cytometry at 4, 10 and 21 dpi. There was an increase in the number of CD4+ T cells, as well as a significant increase in the expression of the viral meq gene in CD4+ T cells, which coincided with the presence of tumors in various organs of infected birds.

Li et al. (2011) noted that the percentage of CD8 T cells was drastically reduced on day 21 and 28 pi, following infection of chickens with GX-101,a virulent MDV-1. However of CD4 T cells was increased on days 14, 21 and 28 pi. The ratio of CD4 T cells to CD8 T cells in MDV-1 infected chicken was significantly higher than the control group on days 21 and 28 pi.
2.9 Safety test for MDV vaccines

Code of Federal Regulations (2007) states that, at least 25 one-day-old, SPF chickens shall be injected, by the s/c route with the equivalent of 10 chicken doses of vaccine virus (vaccine concentrated 10X). The chickens shall be observed each day for 21 days. Chickens dying during the period shall be examined; cause of death determined and the results are to be recorded.

(i) If at least 20 chickens do not survive the observation period, the test is inconclusive.

(ii) If lesions of any disease or cause of death are directly attributable to the vaccine, the vaccine is unsatisfactory.

(iii) If less than 20 chicks survive the observation period and there are no deaths or lesions attributable to the vaccine, the test may be repeated one time, provided that, if the test is not repeated, the serial shall be declared unsatisfactory.

Asean Standards for Animal Vaccines (2006) states that, at least 20 susceptible, one day-old-chickens, each inoculated with 10 doses of vaccine by the recommended route. At either 50 days or 120 days post-inoculation, all chickens are killed and examined histo-pathologically or grossly for lesions of MD. No lesions of MD must be detected in any of the chickens.

European Pharmacopoeia (2005) stipulates using not fewer than 10 chickens from SPF flock and not older than the youngest age recommended for vaccination. Administer by a recommended route and method to each chicken 10 doses of the vaccine. Observe the chickens at least daily for 21 days. The test is not valid if more than 20 per cent of the
chickens show abnormal clinical signs or die from causes not attributable to the vaccine. The vaccine complies with the test if no chicken shows notable clinical signs of disease or dies from causes attributable to the vaccine.

OIE terrestrial manual (2010) states that, ten doses of vaccine or a quantity of diluent equivalent to two doses of vaccine should be inoculated into separate groups of ten, one day-old SPF chickens. No adverse reactions should occur during a 21-day observation period.

2.10 Potency test for MDV vaccines

European Pharmacopoeia (2005) stipulates that, the quantity of the vaccine virus administered to each chicken is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine. Use for the test not fewer than 60 chickens of the same origin and from an SPF flock. Vaccinate by a recommended route not fewer than 30 chickens. Maintain not fewer than 30 chickens as controls. Challenge each chicken after nine days by a suitable route with a sufficient quantity of virulent MDV. Observe the chickens at least daily for 70 days after challenge. Record the deaths and the number of surviving chickens that show clinical signs of disease. At the end of the observation period kill all the surviving chickens and carry out examination for macroscopic lesions of MD. The test is not valid if;

— During the observation period after challenge fewer than 70 per cent of the control chickens die or show severe clinical signs or macroscopic lesions of MD,
— And/or during the period between the vaccination and challenge more than 10 per cent of the control or vaccinated chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

OIE Terrestrial manual (2010) insists vaccination of MD susceptible chickens at one day of age and challenge with sufficient virulent MDV eight days later to cause at least a 70 per cent incidence of MD in unvaccinated chickens. Two types of tests are used. In the protection index test, a single field dose (1,000 PFU) of vaccine is given and the incidence of MD in vaccinated birds is compared with that in unvaccinated birds. Protective indices should be greater than 80, i.e., vaccinated birds should show at least 80 per cent reduction in the incidence of gross MD, compared with unvaccinated controls.

2.11 Virus in Feather tips

Calnek and Hitchner (1973) described the feather follicle epithelium (FFE) as the only anatomical site in the bird where productive replication of MDV occurs and enveloped virions were found.

Davidson et al. (1986) reported that both MDV antigens and DNA were detected in the feather tips of MDV injected birds commencing on day 11 pi and the in-contact infected birds became positive two weeks later.

Levy et al. (1991) assessed the presence of HVT DNA in the feather tips of chickens vaccinated with HVT by dot blot hybridization with a probe specific for HVT and lacking homology to MDV DNA. Only small amounts of HVT DNA were detected in the feather tips of chickens that were vaccinated or left in contact with HVT vaccinated
chickens and when chickens were challenged with virulent MDV, HVT DNA was detected in the feather tips of vaccinated chickens and the largest amount was detected 35 days after vaccination. They identified MDV DNA in the feather tips of MDV challenged chickens from 25 to 45 days after challenge proving immunization of chickens with HVT did not prevent the replication of MDV in the feather tips but only diminished it.

Handberg et al. (2001) developed a method to detect MDV-1 and MDV-3 by specific PCR. They applied the method to various tissue samples from chicken experimentally inoculated with serotype-1 or serotype-3 MDV. They observed that serotype-1 strains of CVI988 and RB-1B could be detected in feather follicle epithelium up to 56 and 84 dpi respectively, while the MDV-3 serotype was detected until 42 days pi. In addition, they applied PCR to the samples collected from four commercial table egg layer flocks of young stock or pullets vaccinated with either MDV-1 or MDV-3 (HVT) vaccine, which had various clinical signs of MD and could detect MDV-1 in buffy-coat, spleen, liver, skin, feather tips and ovaries. They concluded that the detection of MDV in feather tips by PCR appeared to be as sensitive as co-cultivation of buffy coat cells in primary CEF.

Davidson and Borenshtein (2002) aimed at examining the efficacy of amplifying MDV and / or ALV-J feather tip DNA as compared to DNA purified from liver and spleen. They concluded that the PCR for MDV and ALV-5 using DNA from feather tips was more effective for diagnosis of naturally infected commercial chicken than using the liver and spleen.
Abdul-Careem (2008) characterized the cellular and cytokine responses as indicators of CMI response in FFE and associated feather pulp following immunization with either CVI988/Rispens or HVT against MD and found that that replication of these vaccine viruses started at 7 dpi, peaked by 21 dpi and subsequently, showed a declining trend. This pattern of viral replication, which led to the viral genome accumulation in feather tips, was associated with infiltration of T cell subsets particularly CD8 T cells into the feather pulp area and the expression of cytokine genes such as interferon-γ.

2.12 Histopathology in Marek's Disease

Jakowski et al. (1969) noted the early changes in bursa Fabricius from MD by inoculating SPF X Line seven hybrid birds by using four isolates of MD agents as whole blood from the infected donors that was the source of inoculum. They inoculated PBS dilutions of blood at 12th dpi and observed that bursae were dramatically smaller in all chicks receiving undiluted blood and comparatively small in those receiving dilutions of blood. They observed that histological changes in bursa revealed extreme follicular degeneration, reticular cell proliferation in the interfollicular spaces and invagination of surface epithelium that were typical for MD infection.

Jakowski et al. (1970) showed that the hematopoietic destructive lesions, characterized by necrosis and loss of architecture in the bursa of Fabricius and Thymus, were observed consistently in SPF X L7-F2 cross chickens inoculated at 1 day with a vvMDV. They found lesions in early necrosis of medullary portion of follicles and increased reticulum proliferation in bursa of Fabricius and focal area of medullary necrosis in thymus. They concluded that the absence of maternal protection to the MD
agent and overwhelming infection produces hematopoietic destruction in lymphoid, myeloid, and erythroid tissues.

Sharma et al. (1980) studied the effect of neonatal thymectomy on the pathogenesis of HVT in chickens. They surgically thymectomized and $\gamma$-irradiated the newly hatched chicks of the two genetic strains and then exposed them to HVT. They stated that the immunosuppressed chicken had more circulating HVT and had a slightly higher frequency of microscopic lesions in peripheral nerves than in the intact control chickens, although the intensity of lympho proliferation remained very mild and no gross lesions or clinical disease was noticed during the five week observation period. They concluded that the HVT generally caused no clinical disease in intact or immune depressed chickens.

Rana et al. (1984) found that HVT was safe for use in day old chicks. They observed that a dose containing 1,000 PFU of DEF propagated HVT FC 126 strain prevented development of symptoms, gross and microscopic lesions and mortality due to MD in 82 per cent of vaccinated chicks following challenge with a virulent strain of virus. They also found microscopic changes characterized by a mild paucity of lymphocytes and mild to moderate degree of retrogressive changes in the cortex and medulla that were confined to Bursa of Fabricius and only in a few cases, nerve showed mild degenerative changes.

Kobayashi et al. (1986) studied MD in Japanese Quails vaccinated with HVT. They observed that 43 of 220 quails exhibited microscopic lymphomatous lesions characterized by diffuse infiltration and proliferation of lymphoid cells of various sizes
found in lesions of duodenum, small intestine, spleen, liver, proventriculus and kidney that were similar to those of chickens with MD. They also found that there was a positive correlation between the incidence of lymphomatous lesions and MDV-specific antigen in feather tips and negative correlation between incidence of lymphomatous lesions and antibody against HVT in serum.

Pruthi et al. (1989) studied the pathology of MD in chickens vaccinated separately with three different vaccines, viz., HVT, inactivated MDV and a bivalent vaccine. They observed that MD-specific mortality following challenge was significantly lower (2.0 %) in bivalent vaccinated birds than in those vaccinated with either HVT (5.9 %) or IMDV (13.7 %) alone. The occurrence, extent and severity of gross lymphomas and lymphoproliferative lesions in visceral organs was lower in the bivalent vaccinated birds than in other groups and also that atrophy and degenerative changes were seen in unvaccinated and challenged birds but not in challenged and bivalent-vaccinated birds. They observed that mild degenerative lesions and slight RE cell hyperplasia occurred in HVT and Inactivated MDV inoculated and challenged birds. They concluded that low intensity and frequency of gross and histological lesions in bivalent-vaccinated birds following challenge was highly suggestive of better vaccine-induced resistance.

Calnek and Witter (1991) described the classical MD clinical signs, such as asymmetric progressive paralysis, drooping of limb, torticollis, vagal involvement leading to tilted growth with characteristic athlete posture. They also found that varying degrees of ataxia, blindness and grayish opacity of eye. They concluded that neoplastic
signs like, weight loss, paleness, anorexia was followed by diarrhoea in chronic cases by death of bird due to starvation and dehydration.

Gimeno *et al.* (1999) recorded neurological signs induced by MDV and identified for the first time persistent neurologic disease and late paralysis. They evaluated the clinical signs and histopathologic alterations of brain in two MDV strains infected birds and found that neurological response was greatly influenced by viral pathotypes. They concluded that central nervous system was an important target organ for MDV resulting in several distinct clinical manifestations and suggested that neurological responses in antibody free chickens might be a useful criterion for virus pathotyping.

Kamaldeep *et al.* (2007) studied the occurrence of MD in ten vaccinated poultry flocks of Haryana (India) from different geographical locations. They found that samples collected from all flocks showing MD outbreak had gross and histopathological lesions suggestive of MD. They observed the proliferation and infiltration of lymphoblasts and lymphocytes in the sections of liver, spleen, kidney, sciatic nerve and ovary of the affected birds.

Grzegorz *et al.* (2010) studied the occurrence of ICP4, pp38, Meq and LAT genes of MDV and its rate of replication in internal organs of infected chickens and found that the highest dynamics of MDV replication was noticed in the lymphoid organs, the bursa of Fabricius and thymus which indicated stringent association of MDV with lymphoid cells and tissues.