



# Marek's Disease Virus



## ABOUT THE AUTHOR



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Dr. Isabel Gimeno received her Veterinary Degree (1995), MS (1996), and PhD (1999) from Universidad Complutense de Madrid, Spain. She is a Diplomate of the American College of Poultry Veterinarians since 2002. She conducted postdoctoral research in Centro de Investigacion en Sanidad Animal (CISA), Spain and in the USDA-ARS Avian Disease and Oncology Laboratory (ADOL), East Lansing, MI, USA. She is currently an associate professor in the College of Veterinary Medicine, North Carolina State University. Since 1994, her main area of research is Marek's disease focusing on pathogenesis, diagnosis, and control. She has authored or co-authored 45 peer reviewed publications, 5 book chapters, over 60 other publications, and has given more than 40 invited presentations in various countries. She has been supporting the poultry industry with Marek's disease diagnosis and control strategies throughout her professional career.

## SUMMARY

- Marek's disease (MD) continues to be a problem for the poultry industry worldwide due to continuous evolution of the field virus strains and emergence of increasingly pathogenic strains.
- Newly emergent Marek's disease viruses (MDVs) are capable of overcoming vaccine immunity and can be very immunosuppressive.
- To control development of MD tumors, it is necessary to delay MDV challenge in the farms by using proper biosecurity practices and optimizing vaccination protocols and techniques.
- Because of the cell-associated nature of the vaccines, management of vaccination is complex and requires special training and continuous monitoring. Monitoring can be done by auditing vaccine storage and vaccine preparation procedures periodically at the hatchery, by titrating MD vaccines in specialized laboratories, and by evaluating vaccine replication in the feather pulp at one week of age.
- If MD occurs, diagnosis should be done in a multi-step process taking into consideration epidemiological, clinical, and pathological data. Diagnosis often cannot be reached at the farm and various laboratory techniques could be used to confirm it. Histopathology, real time PCR, and immunohistochemistry are the most useful techniques.
- In addition to confirmation of MD diagnosis, it is important to evaluate the cause. Auditing the vaccination process, assessing field challenges, measuring protection, and determining pathotype are all possible diagnostic tools.
- The immunosuppressive abilities of the vv+MDV (MDV-IS) are of great concern because they can jeopardize cell-mediated immune responses against other diseases. MDV-IS is difficult to diagnose since it can occur in the absence of lymphoid organ atrophy and/or tumors. It is also difficult to control since vaccine protocols highly effective against MDV-induced tumors do not always protect against late-MDV-IS. Controlling MDV-IS will be one of the major challenges for the poultry industry in the future. Critical points about MD are summarized in **Table 9**.
- ***After a full investigation of any Marek's outbreak, selecting the most protective vaccine or vaccine combination is of utmost importance.***

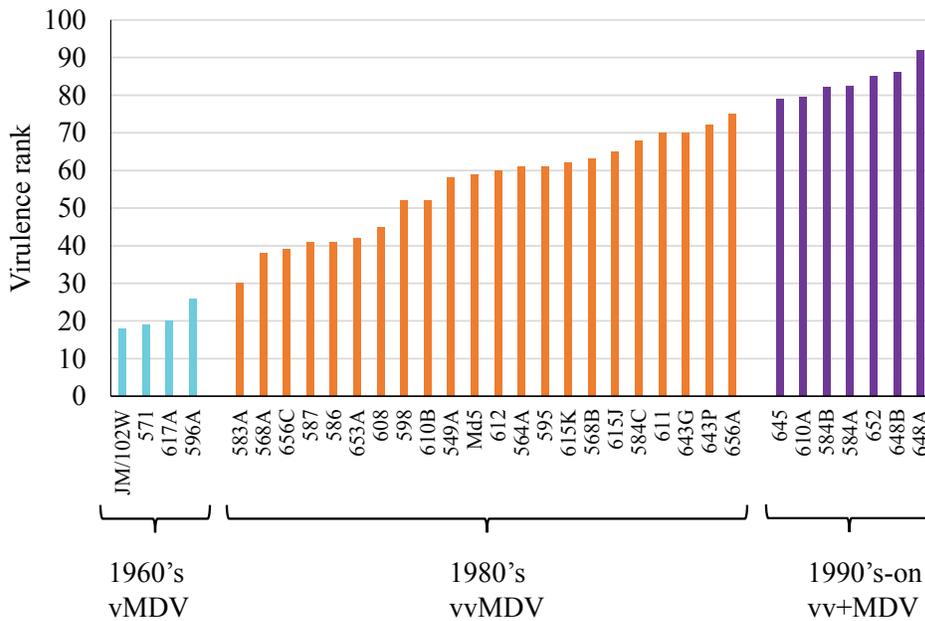
# INTRODUCTION

Marek's disease (MD) is a lymphoproliferative disease of chickens induced by a herpesvirus, Marek's disease virus (MDV). MD is a major threat for the poultry industry because of the economic consequences in the absence of proper methods of control. MD has been successfully controlled by vaccination since 1968 (11, 36). However, MDV has evolved towards more virulence and the new emergent viruses not only are capable of breaking vaccine immunity but also they are very immunosuppressive. In this technical bulletin, we will review those aspects of the disease that are relevant for the diagnosis and control of MD in broiler breeders.

MD has evolved since it was first described by Josef Marek in 1907 (27). Initially MD was described as a polyneuritis characterized by inflammation in peripheral nerves that affected old birds and did not produce high mortality. In the 1960's, as the poultry industry grew and became more intensive, MD became a worldwide problem. Instead of inflammatory in nature it was characterized by the development of tumors (lymphomas) not only in the peripheral nerves but also in the viscera and skin. Furthermore, it affected younger birds and in some cases caused very high mortality. The poultry industry, as we know it today, could have not developed without proper control of MD. In the USA, the first vaccine that was introduced in the market was the herpesvirus of turkeys (HVT) in 1970 (36). HVT was a success and kept MD under control for more than a decade. However, in the 1980's outbreaks of MD in HVT vaccinated chickens started to occur and a new vaccine (HVT+SB-1) was introduced (7, 33, 37). This new vaccine was based on the protective synergism that occur when HVT is administered with serotype 2 MDV SB-1 strain. In the 1990's, outbreaks of MD in HVT+SB-1 vaccinated chickens occurred and strain CVI988 (Rispens) was introduced (30, 38). In other regions of the world (e.g. Europe), Rispens was introduced as early as 1972 and its use has continued (where it is permitted) either alone or in combinations with HVT or HVT+SB-1. Today, MD is characterized by the development of lymphoma in nerves, skin, and viscera that can occur even in vaccinated chickens. In addition to tumors, infection with MDV induces a variety of non-neoplastic syndromes (neurological, ocular, vascular and the most important of all immunosuppression).

One of the major factors for the evolution of MD is that the etiological causative agent, MDV, has evolved towards more virulence. Witter (39) evaluated the virulence of 35 MDV strains isolated between 1960 and 1997. A pathotyping assay based on the ability of MDV isolates to break vaccine immunity was developed and viruses were ranked from 0 to 100, 100 being the highest possible virulence. **Figure 1a** shows the result of that study and demonstrates how isolates from 1960's were virulent Marek's disease virus (vMDV), the isolates from 1980's were of increased virulence or very virulent Marek's disease virus (vvMDV), and the isolates from 1990's were of the highest virulence or very virulent plus Marek's disease virus (vv+MDV).

**Figure 1a:** Evolution of MDV.

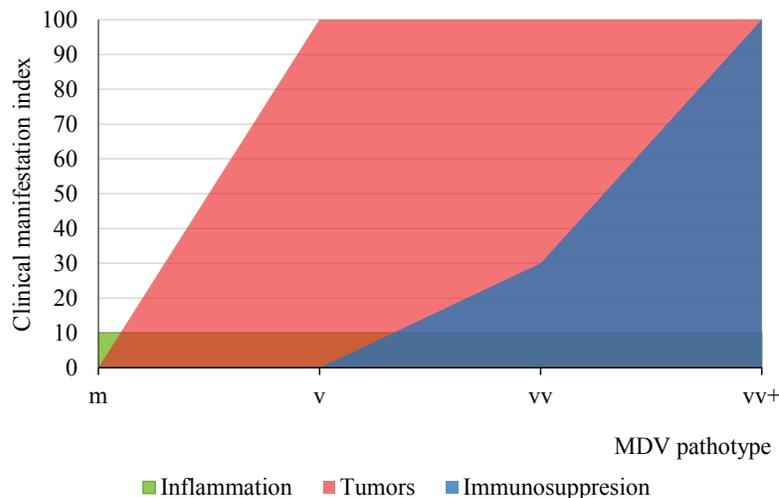


v=virulent, vv=very virulent, vv+=very virulent plus

From: R.L. Witter. Increased virulence of Marek's disease virus field isolates. *Avian Diseases*, 41:149-63, 1997.

Besides breaking vaccine immunity, MDV has acquired other features. Viruses occurring before 1960's were of moderate virulence Marek's disease virus (mMDV). Those viruses were unable to induce tumors and only capable of inducing moderate inflammation. As mMDV progresses into vMDV, viruses acquire the ability to induce tumors. The latest feature acquired by the viruses is the ability to cause immunosuppression which started with the vvMDV but has become greater in the vv+MDV (**Figure 1b**).

**Figure 1b:** Consequences of MDV evolution for MD.



m=moderately virulent, v=virulent, vv=very virulent, vv+=very virulent plus

MDV-induced immunosuppression (MDV-IS) is very complex, difficult to diagnose in the field (it can occur in the absence of lymphoid organ atrophy or tumors), and difficult to control. Vaccine protocols that protect efficiently against MDV-induced tumors do not always protect against late-MDV-IS associated with dysregulation of the immune responses. MDV-induced immunosuppression can affect negatively productive parameters (18, 24), efficacy of vaccination programs against other diseases (5, 6, 14, 24, 26, 28, 29, 31), mortality, and condemnations unrelated to tumors.

MDV is a unique virus. **Table 1** summarizes the aspects of the etiology, epidemiology, and pathogenesis of MDV that have important implications in the diagnosis and control of MD.

**Table 1.** Key points on MDV infection to understand diagnosis and control.

MDV bio-characteristic		Implications in DDx	Implications in control
Etiology	MDV is cell- associated		Management of vaccines is complex
	MDV has an oncogene (meq)	It can induce tumors in chickens as young as 3 weeks of age  Detection of meq in tumors by immunohistochemistry is a diagnostic criteria	Very efficient recombinant vaccines can be produced by deletion of meq in serotype 1 MDV strains (experimental phase)
Epidemiology	MDV is ubiquitous	Even healthy chickens are infected with oncogenic MDVs. Detection of oncogenic MDV alone does not have any diagnostic value	Delay challenge by reducing MDV load in the farm is critical
	MDV transmission is horizontal through infected dander and occurs regardless of vaccination and diseases status		Vaccination does not protect against infection or transmission
Pathogenesis	MDV establishes latency in lymphocytes	Latently infected cells have very few copies of the virus	
	MDV induces lymphomas in chickens	Latently infected cells have high number of copies of virus. Real time PCR can be used for diagnosis  Lymphomas are CD4+CD8-meq+ and can be diagnosed by immunohistochemistry	Proper vaccination protects against tumors
	MDV dysregulates the immune responses in absence of tumors and lymphoid organ atrophy	Immunosuppression due to MDV is very difficult to diagnose	Immunosuppression of MDV is not well controlled by current methods of vaccination
	MDV induces tumors in nerves and viscera	Tumors in nerves is a valid criteria for diagnosis	

**MDV is a cell-associated herpesvirus.** This feature helps MDV to avoid the immune system and complicates greatly the management of MD vaccines that are cell-associated as well. MDV has acquired a very efficient system for transmitting from chicken to chicken. It replicates actively in the feather follicle epithelium and **transmits into the environment and to other chickens through infected dander.** Dead skin cells protect the virus from the environment and that allows MDV to persist in the farm for a long time. Furthermore, once the chicken is infected with MDV it will be infected for life and will continuously release viruses into the environment by contaminated dander and dust which spreads through the air. It is important to remember that **vaccination against MD protects against the development of tumors but not against the infection or transmission of MDV.** Under commercial conditions, most if not all chickens get exposed to MDV early in life by inhaling air, dust and infected dander containing the virus. This is very important for diagnosis and control. The fact that field strains of MDV are found in a chicken does not have any diagnostic value since most chickens will be infected even if they never develop MD. Furthermore, it is critical for its control to reduce MDV load in the farm by proper cleaning and disinfection to delay the age or time of infection with MDV as much as possible. Also, mixed age and multi-age rearing farms should be discouraged as the challenge levels will usually be very high on these premises.

**MDV establishes latency in lymphocytes.** When birds are properly protected against MD, exposure with field strains of MDV result in latent infection in the birds' lymphocytes without causing lesions. However, if birds are not immunized correctly against MD, latently infected lymphocytes can become neoplastic and tumors will develop. One of the major challenges in the diagnosis of MD is to differentiate chickens that have tumors due to MDV from those that have tumors induced by retroviruses but they are also latently infected with MDV. In other words, we need to differentiate between tumors induced by MDV and tissues latently infected with MDV.

**MDV is able to dysregulate the immune responses of the chickens.** This can lead to severe immunosuppression that can jeopardize the immunity of the chicken against other diseases and negatively affect livability and productive parameters.

## DIAGNOSIS

Differential diagnosis of tumor diseases in poultry differs greatly from the diagnosis of other diseases. The most relevant challenges in the diagnosis are included in **Table 2** and detailed information has been recently reviewed (42). ***The diagnosis of tumor diseases should be done in a multi-step approach taking into consideration epidemiological information (age), clinical signs, and gross lesions. In some cases, this information would be enough to make a proper diagnosis at the farm. However, most times diagnosis has to be confirmed at the laboratory.***

**Table 2.** Challenges in the differential diagnosis of tumor diseases.

MDV infection and development of tumors (MD) are not synonymous. Most chickens are infected with oncogenic MDV but never develop MD.
Different viruses can induce tumors that grossly look very similar (Reticuloendotheliosis Virus [REV], Avian Leukosis Virus [AVL], MDV).
There are spontaneous tumors that grossly and microscopically are identical to tumors induced by viruses.
There are several non-neoplastic diseases that can be confused with tumors (i.e. peripheral neuropathy, Hepatitis E).
MDV infection can result in non-neoplastic syndromes such as immunosuppression that is very difficult to detect under field condition.

The most useful techniques to confirm a diagnosis of MD are histopathology, real time PCR, and immunohistochemistry. Histopathology is very helpful in identifying the type of tumor cells (lymphoma versus other tumor types) and distributions of lesions. However, in many cases histopathology cannot confirm the diagnosis and other techniques (real time PCR and immunohistochemistry) are necessary for a definitive diagnosis. A summary of the criteria that should be used in the diagnosis of tumor diseases is presented in **Table 3**.

**Table 3.** Differential diagnosis of Marek's disease with other tumor and non-tumor diseases of poultry.

Age (weeks)	Gross lesions	Differential Diagnosis	Test to confirm diagnosis	
			Aid in the diagnosis	Confirm diagnosis
<14	Enlarged nerves	Marek's disease Peripheral neuropathy	Histopathology	Real time PCR
	Tumors in viscera + Enlarged nerves	Marek's disease	Not necessary in most cases	Not necessary in most cases
	Tumors in viscera	Marek's disease Acutely transformed retrovirus	Histopathology Immunohistopathology	Real time PCR
>14	Enlarged nerves	Marek's disease Peripheral neuropathy Reticuloendotheliosis (neuritis)	Histopathology	Real time PCR
	Tumors in viscera + Enlarged nerves	Marek's disease REV-induced tumors	Histopathology Immunohistochemistry	Real time PCR
	Tumors in viscera	Marek's disease ALV-induced tumors REV-induced tumors Spontaneous tumors	Histopathology Immunohistochemistry	Real time PCR
	Tumors in the Bursa of Fabricius	Marek's disease ALV-induced tumors REV-induced tumors	Histopathology Immunohistochemistry	Real time PCR

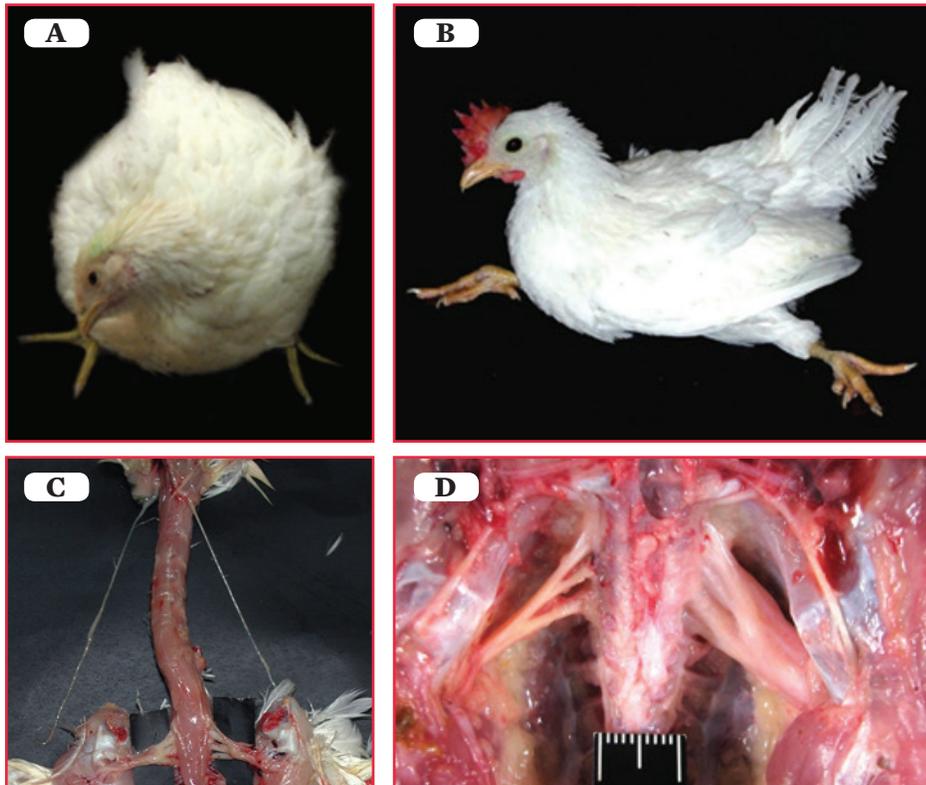
**Diagnosis at the farm**

**Age.** MDV can induce tumors in chickens as young as 3 weeks but retroviruses (ALV and REV) takes longer to induce tumors (normally not before 14 weeks of age and sometimes much later).

**Clinical signs.** MDV can induce neurological clinical signs such as paralysis, torticollis, and ataxia (**Figure 2a & b**) that do not happen in chickens infected with retroviruses (ALV and REV).

**Gross lesions.** Peripheral nerve enlargement (**Figure 2 c & d**) is one of the most characteristic lesions of MD. If it is accompanied by tumors in viscera, diagnosis of MD can usually be done at the farm. However, if peripheral nerves are enlarged in absence of tumors, MD needs to be differentiated from peripheral neuropathy (PN) (1) that has been described only in egg-type chickens.

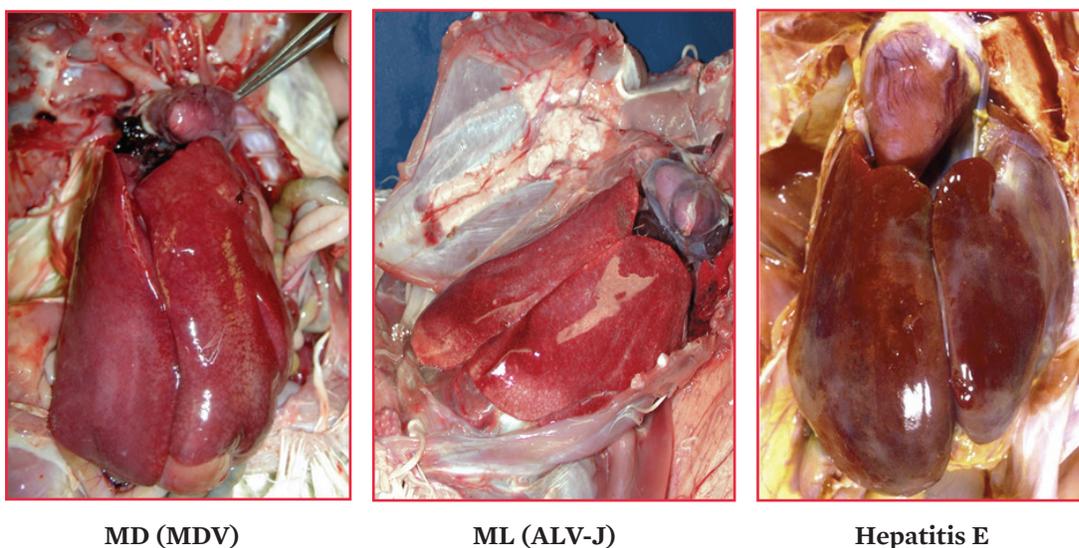
**Figure 2.** Neurological clinical signs (a and b) and peripheral nerve enlargement (c and d) induced by MDV.



Figures 2a and 2b from I.M. Gimeno and A.R. Pandiri, *Virus-induced immunosuppression: Marek's disease virus infection and associated syndromes*. In *Immunosuppressive Diseases of Poultry*, ed I.M.Gimeno, Servet, Zaragoza, Spain.

Tumors in viscera are common to all virus-induced tumors in chickens and do not help much in the differential diagnosis (**Figure 3**). In meat-type chickens, it is not uncommon for MDV to induce only visceral tumors with no visual/gross enlargement of nerves, in these cases diagnosis should be confirmed by real time PCR or immunohistochemistry. Furthermore, non-neoplastic lesions such as those induced by Hepatitis E virus/Big Liver and Spleen Disease (BLS) in liver and spleen could be confused with tumors by gross inspection.

**Figure 3.** Tumors in viscera (DDx with other tumor and non-tumor diseases).



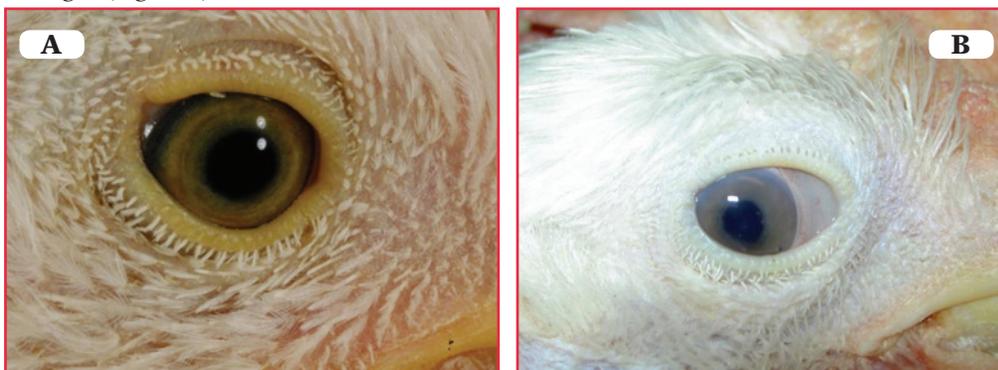
Skin lesions are very characteristic of MD and help greatly in the diagnosis (**Figure 4**). The most characteristic lesions in the skin are nodular tumors in the feathered skin (**Figure 4a**). However, tumors in shanks, comb, and wattles can also occur (**Figure 4b**). A unique lesion named “Alabama Red Leg” can occur in the shank of meat type chickens (**Figure 4c**).

**Figure 4.** Skin lesions caused by MDV.



MDV is able to induce a panophthalmitis, a condition that can affect all structures in the eye (**Figure 5**). The best described is pale iris (grey eye - *right*) and irregular pupil. However, MDV can also induce cataracts, opacity of the cornea, destruction of the retina, and pectinitis. Eye lesions are not common but could aid in the diagnosis if present.

**Figure 5.** Eye lesions induced by MDV. A normal eye can be seen on the left (Figure a) and a MDV affected eye on the right (Figure b).



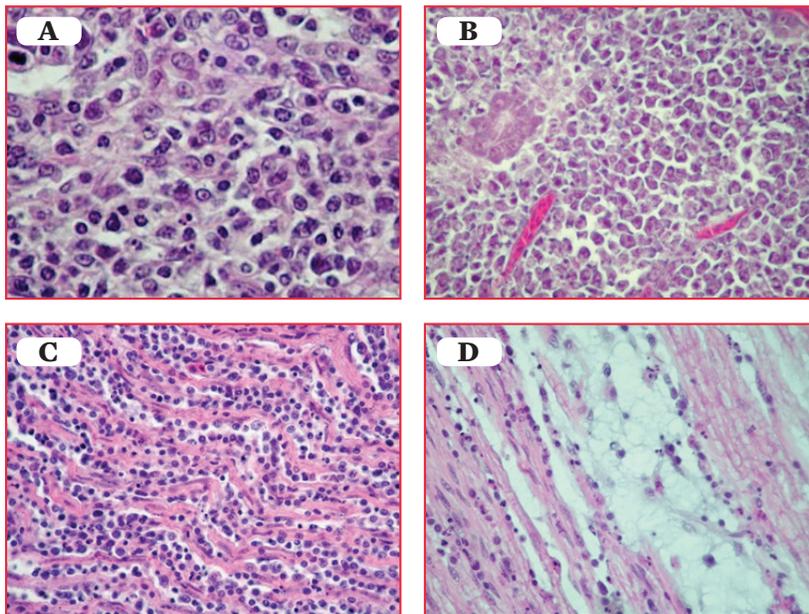
*These images were obtained from Gimeno and Pandiri (2013). Marek's disease. In Immunosuppressive diseases of poultry. Ed. I.M. Gimeno. Editorial Servet, Zaragoza, Spain pp123-152*

Tumors in the bursa of Fabricius can occur in MDV although they are rare. Whenever tumors in the bursa occur, histopathology samples should be collected as well as samples for virological assays. Histopathology will help to differentiate MD from retrovirus-induced tumors. Virological assays will help to confirm if the lesion is due to an exogenous retrovirus or if it is a spontaneous tumor.

## Diagnosis at the Laboratory

**Histopathology.** Samples should be collected in 10% buffered formalin with a volume sample: formalin of 1:10. Histopathology can confirm that the lesion is indeed a lymphoma and sometimes can differentiate between lymphoma induced by MDV or by retroviruses (ALV and REV). The main criteria to confirm a MD tumor by histopathology are the locations of lesions (tumors in peripheral nerves are pathognomonic) and the heterogeneous population of cells in the tumors (**Figure 6**). The major difficulty in the diagnosis of MD tumors is that **MDV can induce inflammatory lesions in healthy chickens and sometimes they can be confused with tumors**. This is particularly true in the case of the nerves since MDV is able to induce two types of lesions: type A (neoplastic and confirmatory of MD) and type B (inflammatory and cannot be used to diagnose MD) (**Figure 6**). In the latter case confirmation of the diagnosis by other techniques is necessary.

**Figure 6 .** Histopathology for MD diagnosis (A: Typical MD tumors composed by a heterogenous population of cells; B: Tumor induced by ALV characterized by a homogenous cell population; C: Type A lesion (tumors) induced by MDV; D: Type B lesion (edema and plasma cell infiltration) in a nerve induced by MDV).



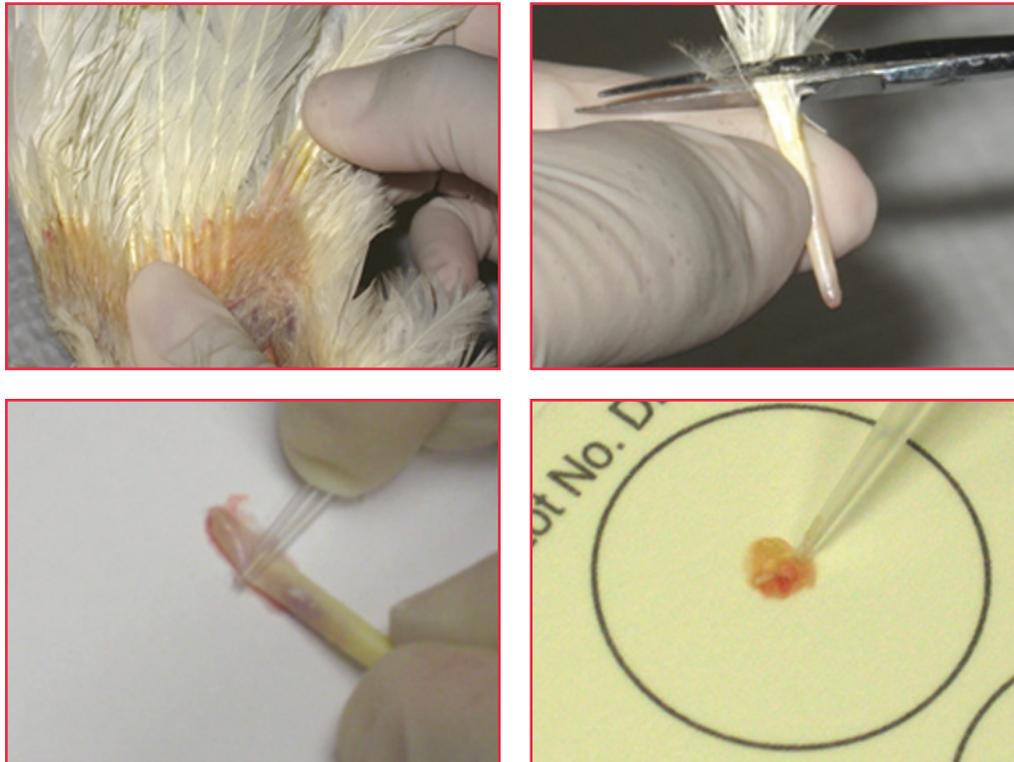
Histopathology of tumors in bursa of Fabricius is very useful to differentiate between MD and tumors of other etiologies. While MDV induced interfollicular tumors, retroviruses and spontaneous tumors are intrafollicular. **Since it is not possible to differentiate between bursal lesions induced by exogenous retroviruses (ALV and REV) and spontaneous tumors, in case of intrafollicular tumors further virological confirmation is needed.**

Diagnosis of poultry tumor diseases by histopathology only is complex and not always possible. It is very important that a complete set of samples (eye, bursa, peripheral nerves, skin, visceral organs, etc.) are submitted to diagnostic laboratories with experience in poultry tumor diseases.

**Immunohistochemistry (IHC).** The phenotype of tumor cells can be evaluated by immunohistochemistry and can aid in the diagnosis. MD tumors are positive for T cell markers (CD3). In addition, they are CD4+CD8- and meq+. By contrast tumors induced by retroviruses and spontaneous tumors are positive for B cell markers (IgM) and they are negative for T cell markers or the MDV oncogene meq. Most cell markers do not work well in samples fixed in paraffin and require samples that have been frozen in liquid nitrogen.

**Real time PCR. Quantification of the MDV genome in the tumor is a valid diagnostic criterion for MD. Tumors induced by MDV have large numbers of MDV DNA copies. By contrast, tumors induced by retroviruses or spontaneous tumors that have latent infection with MDV will have very low load of MDV DNA. Diagnosis of MD by real time PCR can be done using tumors, nerves, feather pulp, and blood (13). Samples can be maintained frozen at -70C or they can be collected in FTA® cards. Details on how to collect samples in FTA® cards from feather pulp are illustrated in Figure 7 (images obtained from <http://www.aaap.info/frequently-asked-questions-on-viral-tumor-diseases>). In particular, blood and feather pulp samples can be used for an early diagnosis of MD as early as 3 weeks of age before chickens develop any clinical signs or lesions (13, 16).**

**Figure 7.** Collection of samples for IHC and real time PCR.



**Virus isolation/Identification of retroviruses. Isolation of MDV does not have any diagnostic value since healthy chickens are normally infected with field strains of oncogenic viruses.** However, it is a necessary step if pathotyping assays need to be performed. Isolation of MDV can be done from peripheral blood white cells (buffy coats), splenocytes, and tumors. Cells need to be alive to ensure viability of MDV. Spleen and tumor samples need to be processed immediately after the euthanasia of the chickens. Cells suspensions of spleen and tumors can be frozen in liquid nitrogen using dimethyl sulfoxide (DMSO). Frozen cell suspensions can be shipped to a diagnostic laboratory in dry ice. Blood samples can be shipped to the diagnostic lab refrigerated overnight. Alternatively, buffy coats can be isolated, frozen in DMSO and shipped frozen in dry ice.

Isolation and characterization of retrovirus is necessary in most cases to confirm the presence of an exogenous ALV or infectious REV. ALV and REV may be isolated from fresh tissues, plasma or serum. Samples can be stored at -70°C (-94°F). Isolation of the virus will confirm infection but demonstrating their role on the development of tumors requires further testing. Since most of the commercial poultry lines are free of exogenous retroviruses, detection of exogenous retroviruses in such flocks is a cause for alarm.

**Nucleic-acid based techniques to detect retroviruses.** The best way to discriminate between lymphomas induced by exogenous ALV, by REV, or spontaneous lymphomas is to demonstrate clonal insertion and c-myc alterations using molecular techniques. However, so far the most common molecular diagnosis tool for retroviral detection is the standard qualitative PCR assays on DNA samples to demonstrate the proviral DNA of REV and the several different ALV subgroups (1, 16, 17). Tumor samples can be maintained frozen at -70C or they can be collected in FTA® cards.

**Serology.** Several serologic tests may be used to detect serum antibodies against MDV or retroviruses. Serological testing for MDV specific antibodies in commercial flocks is of limited value since all flocks have been vaccinated at hatch or exposed to pathogenic MDV. Serological testing has value to help in the diagnosis of retroviruses, especially in commercial flocks that are known to be free of exogenous retroviruses.

## CONTROL

Biosecurity, genetics, and vaccination are the three major points of control for MD and it is imperative to optimize them for a proper control of the disease. ***The most important aspect of biosecurity is to delay exposure and infection with field MDV as much as possible. Vaccines take 5-7 days to induce proper protection and it is very important that chickens do not get exposed and infected before they are fully protected. Adequate biosecurity measures such as down period between flocks, cleaning and disinfection, restriction of visits, showers, one single age flock, avoiding contact with older farms or dust carried by the wind from neighboring farms, etc. should be implemented to delay an early infection.***

**Genetics** was the first method developed for the control of MD. Since 1962 (12), it is known that selection for certain MHC-haplotypes results in birds more resistance to suffer MD. Since then, MD resistance has been included as a trait for selection by the genetic companies. Furthermore, numerous studies have been done to identify other areas of the genome involved in MD resistance and knowledge in this area is expanding fast (9, 10).

***Vaccination has been the cornerstone in the protection against MD since 1968. Due to the cell-associated nature of MD vaccines, management of the vaccine and vaccination process is a complex and delicate task. In addition, to develop a vaccination program there are several aspects that need to be considered: vaccine type, vaccine dose, age/route of vaccination, and single vaccination vs. re-vaccination.***

**Vaccine types.** Classification of MD vaccines is summarized in **Table 4**.

**Table 4.** Classification of MD vaccines.

Criteria	Types	Description
Serotypes	1	Serotype 1 MDV strains that have been attenuated by serial passages in cell culture (conventional) or by genetic modification (recombinant). Strain CVI988 or Rispens is the most widely serotype 1 MD vaccine used. Recombinant serotype 1 MDV vaccines are still under experimental phase.
	2	Serotype 2 MDV strains are naturally non-oncogenic strains of virus isolated in chickens (i.e. SB-1 and 301B) that are used in combination with serotype 3 vaccine in bivalent vaccines or with serotypes 1 and 3 vaccines in trivalent vaccines. They do not protect well against MDV if used by themselves and they are never used alone.
	3	Serotype 3 MDV strains are naturally non-oncogenic strains of virus isolated in turkeys (normally referred to as Herpesvirus of Turkey or HVT). There are two types of HVT in the market conventional (not genetically altered) and recombinant (rHVT) carrying various genes of other viruses (infectious bursal disease virus [IBDV], Newcastle disease [ND], avian influenza [AI], infectious laryngotracheitis virus [ILTV]).
Cell-associated nature	Cell-associated (frozen in liquid nitrogen)	Most commonly used vaccines of all three serotypes both conventional and recombinant.
	Lyophilized (freeze-dried)	Only available for conventional HVT. It confers less protection than cell-associated vaccines but it does not require storing in liquid nitrogen (LN2).
Method of attenuation	Conventional	Vaccines that have been attenuated by serial passages in cell culture or they are naturally non-oncogenic. No modification in the genome has been done.
	Recombinants	Vaccines that have been genetically modified. Currently there are several recombinant vaccines that use HVT as vector (i.e. rHVT-ILT, rHVT-IBDV, rHVT-ND, rHVT-AI).

Most MD vaccines are cell-associated. Only conventional HVT can be found lyophilized (freeze-dried) free of cells. Lyophilized HVT vaccine is commonly used in small flocks of chickens or in those countries where the cold chain is not secure (and liquid nitrogen is not available). However, protection conferred by lyophilized HVT is lower than that conferred by conventional cell-associated (vaccine frozen in liquid nitrogen) HVT vaccines. The cell-associated nature of MD vaccines makes them difficult to manage. Resuspension and constant mixing of the reconstituted vaccine is critical to ensure that infected cells are distributed uniformly. It is critical to perform vaccine management and preparation procedures following the manufacturer's recommendations, and for these procedures to be audited on a regular basis.

Cell-associated vaccine can be classified based on various criteria. According to serotype, MD vaccines are classified as serotype 1 (i.e. CVI988 or Rispens), serotype 2 (i.e. SB-1, 301B), and serotype 3 (HVT) vaccines. ***Serotype 1 MD vaccines are the most protective vaccines against early challenge with MDV.*** The use of serotype 1 MD vaccines, however, is still not allowed in some countries and in those cases the next protective vaccine protocol is the combination of serotypes 2 and 3 vaccines (i.e. HVT+SB-1). Serotype 3 MD vaccines or HVT is the most widely used vaccine for broiler chickens but it has to be used in combination with other serotypes to provide enough protection in broiler breeders. Although no protective synergism has been found experimentally between serotype 1 MD vaccines and other serotypes, strain CVI988 is frequently combined with HVT or with HVT+SB-1. In some broiler growing areas that have high challenges with MDV, combining serotype 3 MD vaccine with either serotype 2 or Rispens is needed to maximize protection against MD.

Vaccines can also be classified as conventional or recombinants. Conventional vaccines are those that have not been genetically modified and include viruses that are not oncogenic (serotypes 2 and 3) or poorly oncogenic viruses that have been attenuated by serial passages in cell culture (serotype 1). There are several recombinant MD vaccines licensed that use HVT as a vector. They are normally referred to as recombinant HVT or rHVT or vectored vaccines. Currently in the market there are rHVT including inserts of Newcastle disease virus (rHVT-ND), infectious laryngotracheitis virus (rHVT-LT), infectious bursal disease virus (rHVT-IBD), and avian influenza virus (rHVT-AI). rHVTs have the advantage of targeting two different antigens (MDV and the insert of another virus) in one single injection and they can be administered in ovo. **However, it is critical to remember that each rHVT is different and they differ from the original HVT as well.** Again, it is very important to follow the manufacturer's recommendations to get the best protection possible and avoid potential failures as a result of interference problems between conventional and recombinant vaccine products.

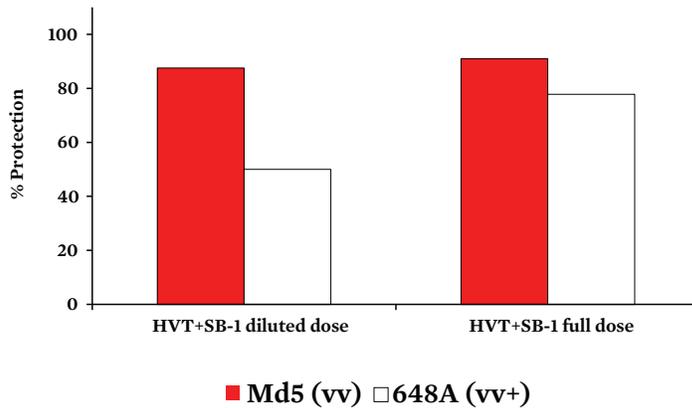
It is imperative to be cautious in combining MD vaccines. Joint administration of serotypes 2 and 3 lead to a beneficial protective synergism that has been well studied. In addition serotype 1 vaccines can be administered with vaccines of serotypes 2 and 3 without a negative effect. **However, rHVTs should never be mixed among themselves or with a conventional HVT** because only one of them will grow (likely the conventional HVT) and protection against the exogenous insert of rHVTs that do not grow will not occur. Likewise, if you are using a HVT in ovo, you cannot then use a rHVT post-hatch or interference will occur. **MD vaccines should not be mixed with vaccines against other diseases or some additives (antibiotics, vitamins, supplements, etc.) unless specifically advised by the manufacturers of the vaccines.**

Finally, it is important to remember that each vaccine **is unique, even if they have the same name.** The number of plaque forming units (PFUs) is determined to evaluate the concentration of virus in a vaccine, and the amount of PFU's necessary to achieve the maximum protection for a given vaccine depends on each vaccine. For this reason, **it is critical to follow manufacturer's recommendations.** Differences among vaccine CVI988 (Rispen) from various manufacturers have been described (21, 40). Likewise, differences among conventional HVTs from various origins, and between conventional HVTs and recombinant HVTs also exist (23). Furthermore each rHVT is unique even if they use the same HVT as a vector with different inserts or if they have different vectors with similar inserts.

### **Handling of Vaccines**

Vaccine dose (**Figure 8**). Cell-associated MD vaccines are labile and difficult to handle. Many factors can affect cell viability and vaccine titers. To minimize this problem, vaccine manufacturers tend to include titers of vaccines much higher than 1500 PFU, which is the minimal dose necessary for licensing MD vaccines in the USA (34). If vaccination is done properly and following manufacturer's recommendations, the dose of vaccine that birds received should be appropriate. However, it is very common that errors in the management and administration of the vaccine results in administration of titers well below the protective level for that particular vaccine. This is particularly true in the broiler industry where diluting vaccines to reduce cost is not an uncommon procedure.

**Figure 8.** Effect of vaccine dose on MD vaccines protection (Red bars indicate Md5 which is very virulent MDV (vvMDV); White bars indicate 648A which is very virulent plus MDV (vv+MDV). Gimeno, et al. 2011 Avian Dis. 55:263-272.

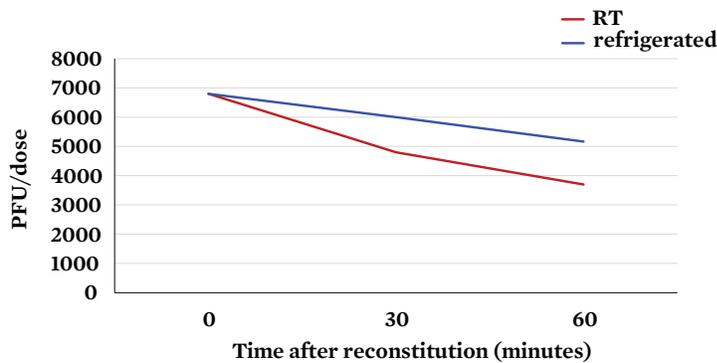


The negative effects of receiving suboptimal doses depends on several factors; the pathogenicity of the challenge or field MDV, the sex of the animals, and the particular vaccine used being the most relevant (18). The severity of the negative effect is most when challenge occurs early, with vv+MDV, and in female chickens (in both commercial layers and broiler breeders, females have been shown to be more susceptible to MDV) (18). In addition, the negative effects of administering low doses depend also on the vaccine used. While some vaccines are able to protect well against early challenge with vv+MDV at low dose, others require higher doses to be able to protect (35). **Administration of suboptimal doses should be always discouraged because even in the absence of tumors, productive parameters can be affected** significantly as a result of partial incomplete protection and/or MDV's induced immunosuppression (18).

**Factors that Negatively Impact Vaccine Dose**

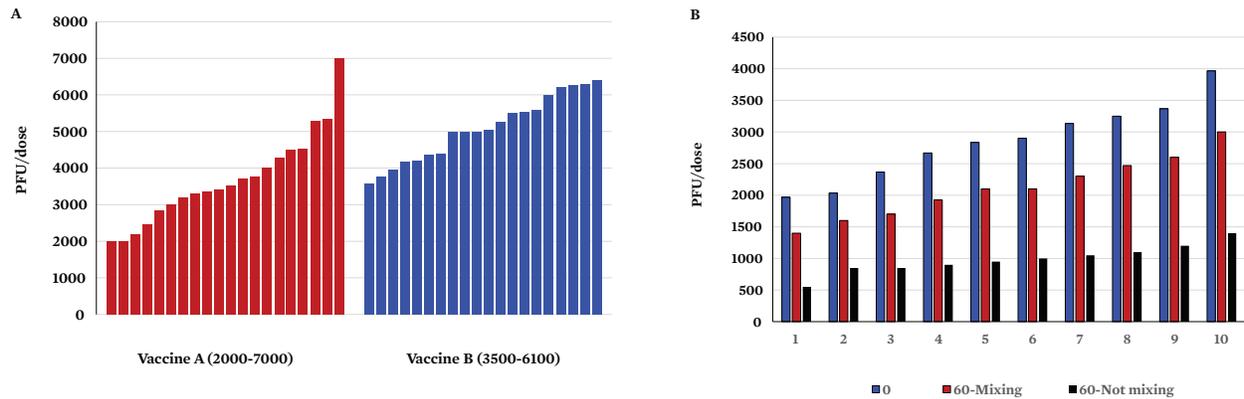
There are several factors that negatively affect vaccine dose. The most relevant are time (Figure 9), mixing of the vaccine (infected cells should be uniformly distributed in the diluent) (Figure 10), addition of antibiotics (Figure 11), and the dilution of the vaccine to reduce cost.

**Figure 9.** Effect of time on vaccine dose (RT = Room temperature; Room temperature: reduced to 55% within 1h; Refrigeration: reduced to 76% within 1h).

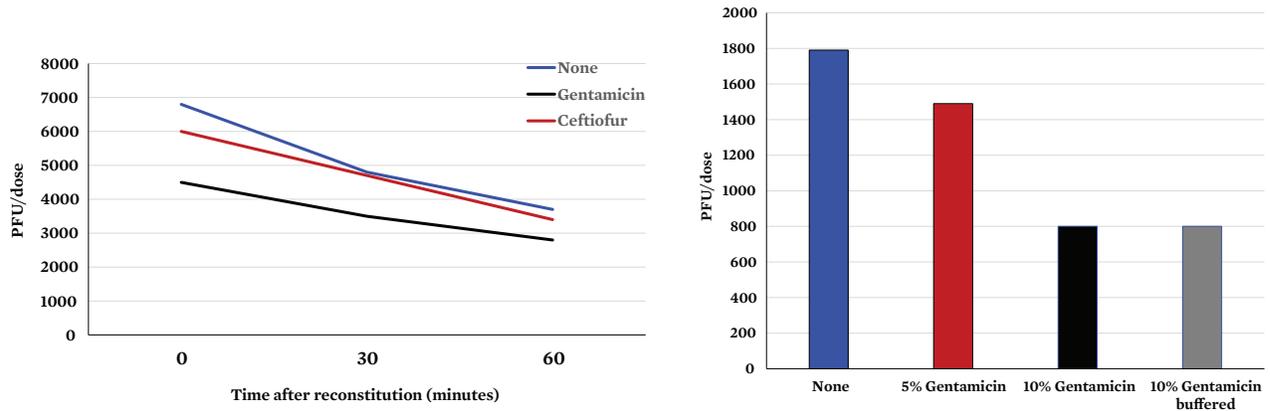


Room temperature: reduced to 55% within 1h  
 Refrigeration: reduced to 76% within 1h

**Figure 10.** Effect of mixing MD vaccine during vaccination on vaccine titers (A: Vaccine A indicated in red bars shows that PFU per dose varied from 2000-7000 while Vaccine B indicated in blue bars shows that PFU per dose varied from 3500-6100; B: Vaccine was titrated ten times (blue bars), held at room temperature for 1 hour without mixing (black bars) and held at room temperature for 1 hour with mixing (red bars)).



**Figure 11.** Effect of antibiotics on vaccine titers.



**Age/Route of vaccination.** In ovo vaccination (injection at 18 days of embryonation during transfer) has become a widespread practice in the last decades. In the USA, all broiler chickens and most broiler breeders are vaccinated against MD in ovo. In ovo vaccination against MD confers better protection against early challenge with MDV and it has a positive effect on the development of the chicken embryo immune system (22). ***In ovo administration of any of the currently available MD vaccines provides better protection than subcutaneous administration at 1 day of age*** (17, 19). Furthermore, it has been recently demonstrated that in ovo administration of HVT accelerates maturation of the chicken embryo immune system resulting in chicks capable of responding better not only to an early challenge with MDV, but also to non-related antigens.

**Revaccination.** Administration of a second vaccine of MD can improve protection against early challenge with very pathogenic field strains (vv+ MDV) and in certain circumstances such as in areas with heavy concentrations of neighboring poultry farms, multiple age farms, farms reusing litter, and breeder chicks traveling long distances. Both vaccines should be administered before challenge occurs. It is recommended that first vaccine is administered in ovo and the second vaccine at day of age. Key points in re-vaccination are summarized in **Table 5**.

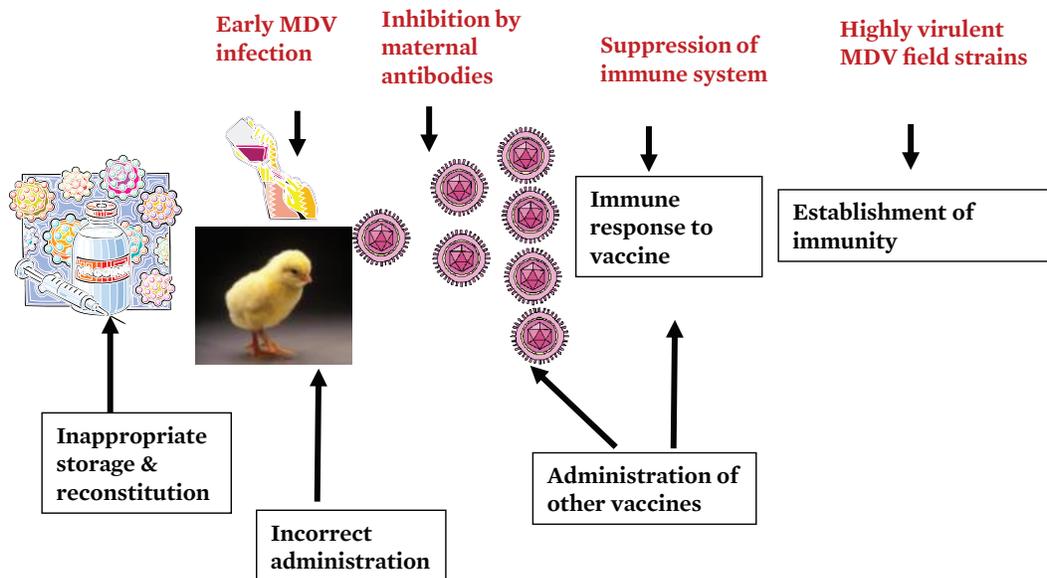
**Table 5.** Basic concepts of double vaccination (Gimeno et al., 2012 Avian Dis. 56:295-305, Gimeno et al., 2012 Avian Pathology, 41:59-68).

Better effect if second vaccine is more protective than the first vaccine
Best protocol: first vaccine in ovo and second vaccine at day of age
Rationale: administration of HVT in ovo hasten maturation of immune system
Vaccination after field exposure has occurred does not have any value

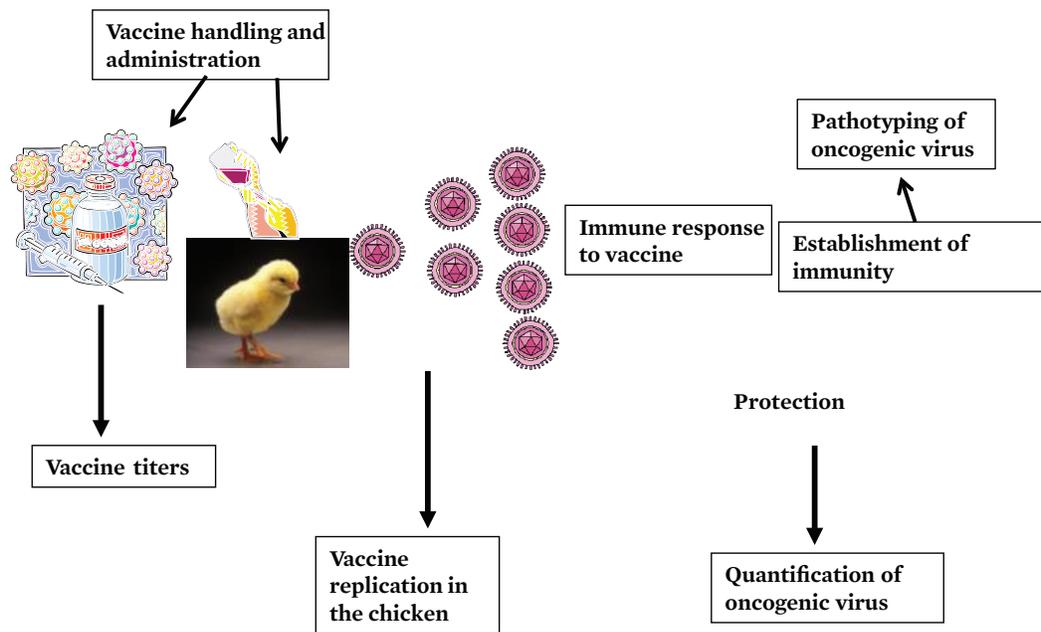
## DISSECTION OF AN OUTBREAK

Once an outbreak of MD has been confirmed, it is important to evaluate all critical steps that might have led to failures in immunization. **Figure 12** shows the critical points in immunization failures and **Figure 13** the checkpoints that can be done.

**Figure 12.** Critical points in immunization failure.



**Figure 13.** Areas to evaluate during immunization.



## MONITORING VACCINATION

**Auditing vaccination:** MD vaccines are cell associated and require special care for storage, reconstitution, and administration. MD vaccines are stored in liquid nitrogen at  $-196^{\circ}\text{C}$  ( $-384.8^{\circ}\text{F}$ ). Thawing of the vaccine needs to be done in lukewarm water ( $27^{\circ}\text{C}$ ;  $80^{\circ}\text{F}$ ) and should take about 30-60 seconds (continuously agitate the vial/vials to aide in thawing). It should then be immediately reconstituted (within 30-60 seconds) in the proper MDV diluent supplied by the manufacturer. ***MD vaccines are unstable suspensions of cells and improper mixing results in poor uniformity of the dose. Furthermore, periodic shaking of the vaccine is necessary to avoid cell sedimentation.*** Reconstituted vaccine should be maintained under refrigeration and be used within a short time (30-60 minutes). Addition of antibiotics, other additives, or other vaccines to MD vaccines diluents could severely affect vaccine titers. Contamination of the vaccine with bacteria should be avoided by maintaining sterile mixing techniques and hygiene in the hatchery's vaccine preparation room. Correct needle size should be used when mixing the vaccine into the diluent. Needles of too small gauge (smaller than 18 gauge) can damage cell-associated cells and viability of the vaccine virus.

When an outbreak of MD occurs, companies should conduct a vaccine audit to make sure that MD vaccines were handled properly and none of the steps mentioned above were compromised. The points that need to be checked are summarized in **Figure 14** and **Table 6**.

**Figure 14.** Vaccine auditing points in the hatchery.

**Storage**



Correct storage of cell associated MD vaccine in liquid nitrogen is essential to the viability of the vaccine.



Vaccine vials should be stored and delivered inverted in liquid nitrogen. The vaccine vial on the left is normal with the vaccine frozen at the base. The vial on the right shows vaccine in the cap due to thawing and re-freezing. This vaccine will be damaged and should not be used.

**Thawing  
(water bath)**



Centigrade



Fahrenheit

**Reconstitution**



Using sterile technique to mix the vaccine.



Vaccine bag made up and ready to be used. The vaccine bag also contains dye and the time of mixing readily apparent.

**Administration  
(monitoring chicks)**



Checking chicks (no vaccine administered as indicated by the absence of dye color).



Checking chicks (vaccinated chick as indicated by dye present under the skin).

**Table 6.** Critical checkpoints for auditing MD vaccination at the hatchery.

Step	Checkpoints
Receiving and storing of vaccine	Vaccine covered with LN2 (check regularly and keep records)
	Batch number of vaccines recorded
	Vials stored inverted in the LN2 tank to detect possible thawing
Thawing of vaccine	Water bath should be clean (careful with disinfectants)
	Place vial in ice immediately after removing from LN2 tank
	Thaw vaccine in lukewarm water for few seconds and maintain it in ice
	Dry the vial to avoid contamination of the vaccine when open
Reconstitution	Use sterile recommended vaccine diluent (pH marker)
	Use sterile gloves to reconstitute and manage the vaccine
	Use needles 18 gauge or wider to remove the vaccine from the ampoule
	Rinse the ampoule with diluent to ensure transferring all the vaccine to the diluent
	Mix well the vaccine in the diluent
	Record the time when the vaccine has been reconstituted
	Do not add anything else to the diluent unless it is known not to damage the vaccine.
	If using colorant to monitor vaccination in 1 day-old chickens, make sure colorant is not contaminated with bacteria
	Keep sterile conditions to avoid contaminating the reconstituted vaccine with bacteria
	Do not over dilute the vaccine. Use dose recommended by the manufacturer
Administration	Maintained the reconstituted vaccine under refrigeration
	Mix the vaccine often (at least every 10-15 minutes). Cells tend to precipitate and need to be resuspended
	Ensure vaccine guns or in ovo vaccination equipment is sterile but without disinfectant residues
	Use 20 gauge or wider needles to vaccinate and ensure that needles are not clogged. Ensure vaccine flow.
	If using colorant in the vaccine to vaccinate 1-day-old chickens ensure that the vaccine is properly administered
	Do not use the reconstituted vaccine for longer than 30-60 minutes
	Check cell viability at the beginning and at the end of the administration of one bag of reconstituted vaccine
General	Provide training to people involved in managing MD vaccines
	Ensure proper training on maintaining sterile techniques
	Monitor vaccine contaminations in vaccine samples, vaccine guns, water bath, etc

**Titration of vaccines (plaque assay).** MD vaccines can be titrated by plaque assay (32). It is important that titration is done not only directly from the vial but also from the reconstituted vaccine. Unfortunately, the latter is difficult since this technique requires cell culture facilities and very few, if any, hatcheries will have them. Important facts on vaccine titrations are summarized in **Table 7**. Counting live cells can be an alternative for assessing the management of vaccines at the hatchery. Although this technique does not provide information on how much vaccine virus is present in the vaccine, it can at least provide indirect evidence of poor management if the number of dead cells is very high or if it increases rapidly after reconstitution.

**Table 7.** Important facts on vaccine titrations.

It gives the number of PFU per administered dose
It should be done from the resuspended vaccine
Vaccines are cell suspensions and there is variability of doses within a vial (range of doses)
Vaccine titration should be done in replicates (10-20 replicates)
Results can vary from laboratory to laboratory depending on cell culture protocols
It should be done in laboratories with experience in Marek's disease cell culture

**Vaccine replication** can be assessed by evaluating vaccine viral DNA in feather pulp or in spleen. Important facts about monitoring vaccination by real time PCR are summarized in **Table 8**.

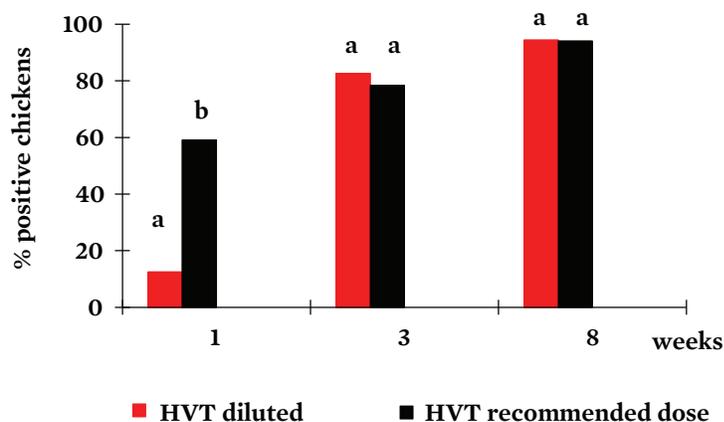
**Table 8.** Important facts about monitoring vaccination by real time PCR.

The technique can differentiate between serotypes and also between CVI988 and oncogenic MDVs
Samples should be collected at 1 week of age
The best samples to collect are feather pulp and spleen (blood samples give false negatives)
The percentage of positive chickens depends on the vaccine used (serotype and origin of the vaccine), route/age at vaccination, vaccine dose administered, combinations of vaccines used
It provides information on how vaccine was administered
It does not provide information on how well immunized the flock is
It should be done in laboratories with experience. This is particularly relevant when monitoring CVI988 in the field as the technique to differentiate it from oncogenic viruses requires very stringent conditions

The use of blood is not recommended as many false negatives have been reported (13). Results obtained from spleen and feather pulp samples are very compatible with the feather pulp having the logistical advantage of being able to be collected from a live chicken (2, 13). It is critical that samples from individual chickens are collected. Samples can be stored frozen at -70°C (-94°F) or they can be collected in FTA® cards and maintained at room temperature.

**The best time to monitor vaccination is 1 week of age** (18, 20). At this time, it is possible to identify differences between chickens receiving a full dose of vaccine versus those receiving a sub-optimal (18, 20). By 3 weeks of age, vaccine virus can be found in most chickens regardless of the administered vaccine dose (3, 18, 20) (**Figure 15**).

**Figure 15.** Monitoring vaccine replication by real time PCR (Modified from Gimeno et al. Avian Dis. 2011, 55:263-72). Figure shows the percentage of chickens with detectable levels of HVT and HVT DNA load in feather pulp at 1, 3, and 8 weeks of age following vaccination.



The percentage of chickens in which vaccine can be detected at 1 week of age depends on the age at vaccination, vaccine dose, and vaccine strain used. It is important to consider all those factors in the interpretation of the results.

Primers specific for each serotype have been reported (25) and are commonly used to differentiate between vaccines of serotypes 2 and 3 and viruses of serotype 1. When serotype 1 CVI988 vaccine strain is used, it is necessary to use primers that are specific for CVI988 and do not amplify other serotype 1 MDVs (4, 20). This technique requires very stringent conditions and special care in conducting the technique and interpreting results is necessary.

#### ***Monitoring early infection with oncogenic MDV***

After administration of MD vaccines, it takes about 5-7 days to achieve the maximum level of protection. Infection with oncogenic MDVs in the farms often occurs earlier than 5-7 days and this might jeopardize the vaccine efficacy. ***It is possible to assess if early infection has occurred in a flock by evaluating MDV DNA load in the feather pulp, spleens, and blood of chickens at 1 week of age*** (16, 18, 20). It is critical that samples from individual chickens are collected. Samples can be stored frozen at -70°C or they can be collected in FTA® cards and maintained at room temperature.

#### ***Monitoring protection/early diagnosis***

It has been recently demonstrated that ***evaluating MDV DNA load in feather pulp or in blood samples as early as 3 weeks of age can be used to predict protection in a flock*** (16, 18, 20). If blood samples are collected, it is important that the anticoagulant used is EDTA. It is critical that samples from different birds do not get mixed. Samples can be stored frozen at -70°C or they can be collected in FTA® cards and maintained at room temperature.

In flocks that are properly protected against MD, most chickens have low MDV DNA load, compatible with levels of latency. However, in flocks that are not protected adequately many chickens have high viral DNA load at levels comparable to MDV-induced tumors.

#### ***Pathotyping MDV***

Although several attempts have been made to find molecular markers for virulence, at the moment the only way to pathotype MDV isolates is by biological assays. The gold standard assay is based on measuring the ability of MDVs to break vaccine immunity conferred by various MD vaccines:

- virulent MDVs (vMDVs) are protected by HVT
- very virulent MDVs (vvMDVs) are protected by HVT+SB-1, and
- very virulent plus MDVs (vv+MDVs) are protected by Rispens (CVI988) (39, 41).

This assay is done in susceptible SPF (specific pathogen free) chickens with maternal antibodies and require the use of prototype MDV strains for each pathotype (JM, Md5, and 648A for v, vv, and vv+, respectively) (39, 41). The assay is time consuming and require infrastructure that are available in only a few laboratories. However, when cases of increased virulence are suspected it is recommended to investigate if the vaccine protocol used can indeed protect against those particular isolates. If pathotyping is necessary, samples could be submitted to OIE (World Organization for Animal Health) reference laboratories for MD.

Alternatives to the gold standard pathotyping assay, such as lymphoid organ atrophy (8), neuropathotyping (15), and viral DNA load (43) have been described. All the alternative assays can easily differentiate between v and vv+ but they fail to separate vv from vv+MDVs (41). Further studies to simplify MDV pathotyping and make it more readily available to other laboratories are warranted.

**Table 9.** Key points to remember about MD.

<b>MD is a complex disease that evolve with time</b>
<b>The two most relevant consequences of MDV infection are tumors and immunosuppression</b>
<b>MDV transmit through infected feathers and skin dander and persist in the farm. Under commercial conditions every chicken is exposed to MDV and infected for life</b>
<b>Delaying challenge by proper biosecurity measures is critical in the control of the disease</b>
<b>Proper vaccination program can control the development of MDV-induced tumors</b>
<b>The main reasons for breaks in vaccinated birds are mistakes in handling MD vaccine and early challenge with MDV in the farm</b>
<b>People handling MD vaccines need to be properly trained and periodic auditing of the procedures should be done</b>
<b>Vaccination should be done carefully and following manufacturer's recommendations</b>
<b>MD diagnosis can be challenging. There are several laboratory techniques available to confirm a diagnosis of MD</b>
<b>If an outbreak of MD is confirmed, an investigation of the outbreak should be done to identify the causes and implement proper measures in the future</b>
<b>Investigation of an outbreak involves auditing at the hatchery, monitoring replication of the vaccine in birds, early diagnosis, and pathotyping.</b>
<b>As of today, we don't have proper methods for detection or control of immunosuppression induced by MDV</b>

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# GLOSSARY OF TERMS

<b>AI</b>	avian influenza
<b>ALV</b>	avian leukosis virus
<b>ALV-J</b>	avian leukosis virus strain J often referred to as J-virus
<b>Ataxia</b>	the loss of full control of bodily movements
<b>Atrophy</b>	the wasting away of body tissues or organs
<b>Attenuation</b>	to make a bacteria or virus less virulent
<b>Bursa of Fabricius</b>	specialized organ found in birds
<b>CD4</b>	cluster of differentiation 4 is a glycoprotein found on the surface of immune cells
<b>CD8</b>	cluster of differentiation 8 is a trans-membrane glycoprotein that serves as a co-receptor for T cell receptor
<b>DDx</b>	differential diagnosis
<b>Diluent</b>	a substance used to dilute something
<b>DMSO</b>	dimethyl sulfoxide, a solvent
<b>Dysfunction</b>	abnormality or impairment in the function of a specified bodily organ or system
<b>Dysregulation</b>	abnormality or impairment in the regulation of a metabolic, physiological, or psychological process
<b>Edema</b>	swelling caused by excess fluid trapped in the tissues
<b>Epidemiology</b>	deals with the incidence, distribution, and possible control of diseases and other factors relating to health
<b>Etiological</b>	the cause, set of causes, or manner of causation of a disease
<b>Exogenous</b>	developing from an outside organism
<b>Haplotype</b>	a group of genes within an organism that was inherited together from a single parent
<b>Heterogeneous</b>	diverse in character or content
<b>Histopathology</b>	the study of the changes in tissues caused by disease
<b>Homogeneous</b>	of the same kind
<b>IBDV</b>	infectious bursal disease virus
<b>IgM</b>	immunoglobulin M, an antibody that is produced by B cells
<b>ILTV</b>	infectious laryngotracheitis virus
<b>Immunohistochemistry</b>	the process of selectively imaging antigens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues
<b>Immunosuppression</b>	the partial or complete suppression of the immune response
<b>In ovo vaccination</b>	vaccination of the chick inside the egg
<b>Latency</b>	the state of existing but not yet being developed
<b>LN2</b>	liquid nitrogen

<b>Lymphocyte</b>	a white blood cell with a single round nucleus, occurring in the lymphatic system
<b>Lymphoid organ</b>	an organ where lymphocytes are concentrated
<b>Lymphoma</b>	tumor(s)
<b>Lymphoproliferative</b>	lymphocytes are produced in excessive quantities
<b>Lyophilized</b>	freeze-dried
<b>MD</b>	Marek's Disease
<b>MDV</b>	Marek's Disease Virus
<b>MDV-IS</b>	Marek's Disease Virus-induced immunosuppression
<b>Meq</b>	an oncogene in virulent strains of MDV that is detected by some diagnostic assays
<b>mMDV</b>	mildly virulent Marek's Disease Virus
<b>ND</b>	Newcastle disease
<b>Neuritis</b>	inflammation of a peripheral nerve or nerves, usually causing pain and loss of function
<b>Non-neoplastic</b>	not growing abnormally
<b>OIE</b>	World Organization for Animal Health
<b>Oncogenic</b>	tumor causing
<b>Panophthalmitis</b>	inflammation of all structures of the eye
<b>Pathogenesis</b>	the manner of development of a disease
<b>Pathognomonic</b>	specifically characteristic or indicative of a particular disease or condition
<b>Peripheral neuropathy</b>	is damage to or disease affecting nerves, which may impair sensation, movement, gland or organ function, or other aspects of health, depending on the type of nerve affected
<b>PFUs</b>	plaque forming units
<b>Polyneuritis</b>	any disorder that affects the peripheral nerves collectively
<b>Precipitate</b>	to cause a substance to be deposited in solid form from a solution
<b>Real-time PCR</b>	real-time polymerase chain reaction (qPCR) is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR)
<b>Recombinant vaccines</b>	a vaccine produced through recombinant DNA technology
<b>Resuspension</b>	a renewed suspension of insoluble particles after they have been precipitated. suspension
<b>Retrovirus</b>	a single-stranded positive-sense RNA virus with a DNA intermediate and, as an obligate parasite, targets a host cell
<b>REV</b>	reticuloendotheliosis virus
<b>Serology</b>	disease diagnosis via blood serum

<b>Serotype</b>	a strain of microorganism that can be identified through blood serum
<b>SPF</b>	specific pathogen free
<b>Splenocytes</b>	consist of a variety of cell populations such as T and B lymphocytes, dendritic cells and macrophages, which have different immune functions
<b>Synergism</b>	cooperation of two or more agents
<b>Titration</b>	a process of figuring out how much of a substance is in a substance with a known volume
<b>Torticollis</b>	a condition in which the head becomes persistently turned to one side, often associated with painful muscle spasms
<b>Ubiquitous</b>	found everywhere
<b>Virological assay</b>	a test used to diagnose a virus
<b>vMDV</b>	virulent Marek's Disease Virus
<b>vvMDV</b>	very virulent Marek's Disease Virus
<b>vv+MDV</b>	very virulent plus Marek's Disease Virus





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