ABSTRACT

LÓPEZ DE JUAN ABAD, BLANCA. Evaluation of Factors That Influence Dose Variability of Marek's Disease Vaccines (Under the direction of Dr. Isabel M. Gimeno).

Marek's disease (MD) vaccines are cell-associated and require special handling and care during administration. Vaccine dose is evaluated by plaque assay and is indicated as the number of plaque-forming units (PFU) per dose. The objectives of this study were to evaluate the dose variability within each vial of MD vaccines and to assess those factors (both from manufacturing and handling/administration of the vaccine) that could affect vaccine dose variability. Three experiments were conducted. Experiment 1 was to evaluate dose variability in thirty-six MD vaccine vials and the effect of manufacturing factors on dose variability. Vaccines were titrated 10 times. Dose variability was measured as the coefficient of variability (CV) calculated as standard deviation divided by average PFU and multiplied by 100. Our results show that all evaluated vaccines had levels of CV ranging from 10 to 34%. Variability exists regardless of manufacturer, vaccine serotype, and batch. Experiment 2 was conducted to evaluate the effect of infectivity rate (IR) on CV. IR was artificially reduced by adding non-infected chicken embryo fibroblast to the reconstituted vaccine before titration. Our results show that decreased IR results in higher CV. Experiment 3 was to evaluate the handling/administration factors (time and mixing during administration) on CV. Our results show that CV increases with time and that this effect is greater if vaccines are not mixed. Our study emphasizes the relevance of proper handling of MD vaccines and shows that dose variability can jeopardize the uniformity of vaccination in a flock and therefore the success of vaccination.

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Evaluation of Factors That Influence Dose Variability of Marek's Disease Vaccines

by
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DEDICATION

To my friends and family. Nothing would have been possible without your endless, unconditional support.

BIOGRAPHY

Blanca López de Juan Abad was born in Caracas, Venezuela where she lived during her childhood. After spending a year in Seoul, South Korea, she completed high school in the United States and continued her studies in biology at the Pennsylvania State University, where she attended for two years. She obtained a bachelor's degree in biology with a minor in chemistry from the University of North Carolina at Chapel Hill.

Blanca's interest in animals began from a young age and fueled her pursuit of science. During her studies, she has worked at small animal clinics as a veterinary assistant, as well as spending time working with other species. She later decided to continue her education and pursue a degree in Comparative Biomedical Sciences from North Carolina State University.

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CHAPTER 1

Bibliographical review

1. Marek's disease

1.1. Definition and relevance

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by an alpha-herpesvirus named Marek's disease virus (MDV) [1, 2]. It was first described by József Marek in 1907 [3] as a polyneuritis of old birds causing paralysis of legs and wings with little or no economic relevance. Since then, the disease has evolved and became a neoplastic disease of both young and old chickens causing high mortality, increased condemnations, and severe economic losses. Today, MD is characterized by the development of lymphomas in skin, nerves, and visceral organs [4]. However, MDV is able to induce a variety of other non-neoplastic syndromes affecting the brain (transient paralysis) [5], blood vessels (arteriosclerosis) [6, 7], eye (panophthalmitis) [8, 9], and immune system (immunosuppression) [10-13]. MD is one of the major concerns of the poultry industry as it could jeopardize intensive poultry production if not properly controlled, as it did in the early 1960's, when MD was a major cause of broiler condemnations and mortality of layers and breeders [14]. Since the late 1960's, MD has been successfully controlled by vaccination [15-17]. However, the efficacy of MD vaccines tends to decrease with time as MDV becomes more virulent [18, 19]. Since MDV was first isolated in 1968 [1], three pathotypes have been described: virulent (vMDV), very virulent (vvMDV), and very virulent plus (vv+MDV). Several factors might have contributed to such evolution. However, the fact that MD vaccines do not induce sterilizing immunity and only protect against the development of tumors is considered to be one of the driving factors [18]. Even when controlled, the cost of vaccination and of losses due to outbreaks (high mortality rate and condemnations associated with MD) is estimated to be 1-2 billion USD worldwide annually [20]. Most of the outbreaks in vaccinated flocks occur due to failures in the vaccination procedures

[21]. MD vaccines are cell-associated, requiring special considerations at the time of manufacturing, handling, and administration and errors in their administration are not uncommon.

1.2. Etiology – Cell associated nature and virus classification

MDV is an alpha-herpesvirus (*Gallid alphaherpesvirus 2*) that together with *Meleagrid herpesvirus 1* and *Gallid herpesvirus 3* form the genus *Mardivirus*, in the Alphaherpesvirinae subfamily of the *Herpesviridae* family. Historically, MDV has been classified into serotypes 1, 2 and 3. The successful sequencing of the serotypes shows that they are in fact three separate viruses that are closely related but have evolved independently. Thus, today they are classified into virus strains: *Meleagrid herpesvirus 1* (serotype 3 MDV) is the herpesvirus of turkeys (HVT), which is naturally nononcogenic and antigenically closely related to MDV [16]. *Gallid herpesvirus 3* (serotype 2 MDV) classifies the naturally nononcogenic virus isolated from chickens. Lastly, *Gallid herpesvirus 2* (serotype 1 MDV) represents all oncogenic MDV virus strains that have been attenuated by serial passage in cell culture and includes the mild (m), virulent (v), very virulent (vv), and very virulent plus (vv+) pathotypes. The virus is strictly cell-associated [22], a discovery that played a key role in isolating the etiological agent and the development of vaccines. Because the classification by serotypes is still the most widely used when referring to vaccines, we will use this classification in this thesis.

MDV can be seen in thin sections of infected cell cultures as hexagonal nucleocapsids 85-100 nm in diameter or as enveloped particles 150-160 nm in diameter. It can also be seen as enveloped particles in irregular 273-400 nm amorphous structures in feather follicle epithelium (FFE). The morphology of serotypes 2 and 3 resembles that of MDV 1 except in thin sections where HVT shows a unique crossed appearance [23]. The genomes of all *Herpesviridae* are

linear, double-stranded DNA molecules of 108-230 kbp [24]. The genomic structure of the 3 serotypes is typical for alphaherpesviruses with a unique-long (U_L) and a unique-short (U_S) sequence flanked by sets of inverted internal and terminal repeat sequences: the terminal repeat long (TR_L), internal repeat long (IR_L), internal repeat short (IR_S), and terminal repeat short (TR_S), respectively [24]. The genomes of representative strains of all serotypes have been sequenced, showing similarity in gene contents and linear arrangements. Differences between the 3 serotypes exist but there is conservation in the genome and shared homology at the DNA level. In addition, the ratio of guanine to cytosine (GC) was different among the serotypes where the ranges went from 43.9 to 47.6 to 53.6% in serotypes 1, 3 and 2 respectively [25, 26]. Differences within serotype 1 strains (Md5 (GenBank accession number AF243438), GA (AF147806, BAC clone of Md11 (AY510475) and of CVI-988 (DQ530348, 814 (JF742597), and LMS (JQ314003)) show similar gene structure. However, there are a few base pairs differences in length of the TR_L/IR_L regions between strains of varying virulence. In addition, there are various different single nucleotide polymorphisms (SNPs) between attenuated vs. non-attenuated strains [27, 28] that could be attributed to mutation by passage in cell culture [27, 28]. Genes responsible for the pathogenesis and immune responses are separated into those with homologues in alphaherpesviruses and those unique to serotype 1 MDV. Within genes with homologues in other alphaherpesviruses, there are two categories: a) Immediate early and early genes are expressed before the replication of the viral genome, they include intracellular proteins (ICP) 4, 0, 22, and 27 and are important for virus replication. B) Late genes are activated at viral DNA replication, they code virion structural proteins such as the glycoproteins (gB, gC, gD, gE, gH, gI, gK, gL, and gM), the nucleocapsid proteins, the envelope spike proteins and the tegument proteins which include VP16. The glycoproteins are important for cell infection, cell to

cell virus transfer and immune responses. gB is responsible for cell attachment and penetration [29] and cell to cell spread [30]. gC is synthesized by infected cells and is a major antigen for which an immune response is mounted. It is expressed on the cell surface and secreted from infected cells [31-34]. gD is not well understood but its expression is limited in FFE [35] and it is non-essential for horizontal transmission [36]. The remaining glycoproteins have not been well studied [36] but have been associated with pathogenesis, immune responses, and virus replication [37, 38] [39, 40]. Genes unique to MDV-1 are latency-associated transcripts (LATs), Marek's Disease Virus EcoQ (Meq), vIL-8, viral lipase (vLIP), pp38/pp24, the 1.8-kB gene family, telomerase RNA, and MDV-encoded microRNAs.

Marek's Disease Virus EcoQ (Meq), the oncogene of the virus, encodes for the 339 amino acid Meq protein that contains a basic leucine zipper (bZIP) domain at the N terminal and proline-rich repeat region at the C terminal resembling the *jun/fos* oncogene family and the *WT-1* tumor suppressor gene respectively [41]. The protein is expressed in lymphoid organs during lytic infection in peripheral blood mononuclear cells and during latent infection in CD4⁺ T cells [42]. It is also expressed in the nucleus of lymphoma and tumor cells [43-45] and during the S-phase [46]. The *Meq* gene is important for transformation, as is evident by its overexpression in rat cells resulting in morphological transformation, inhibition of apoptosis [43] and interactions with cell cycle regulator CDK2 [47]. The pathway for cell transformation is still poorly understood, but *Meq*'s essential role in individual cell level transformation is evident in studies where the *Meq* gene is deleted and oncogenicity disappears [48], making the virus an effective vaccine [49-51]. *Meq*-deleted viruses resulted in changes in gene expression [49] and reduced immunosuppressive effects [52]. In addition, the lack of interaction between *Meq* and the transcriptional co-repressor protein CtBP resulted in loss of oncogenicity of the virus [53].

vIL-8 is a virokinin located in the repeat region of the MDV genome. It is the viral homolog of interleukin-8 (IL-8) [54], is important in inflammation. vIL-8 targets mononuclear cells by chemotaxis. It is suggested to be important for the switch of infection from B to T lymphocytes because it attracts T cells to viral replication sites [55]. Deletion of the gene resulted in limited viral antigen expression, decreased level of lytic infection [56], but did not affect replication in the FFE [57]. Lack of vIL-8 also weakened activation of T cells, which led to less transformed cells. The result was decreased pathogenicity and decreased tumor incidence [57].

Viral Lipase (vLIP) is a secreted glycoprotein encoded by the *vLIP* gene. It shares homology to the lipase enzyme. It is likely involved in the recruitment of cells or in downregulating the activity of T and NK cells [58]. A mutant with the gene deleted lowered the incidence of MD and enhanced survival [58].

The pp38/pp24 complex is not well understood but is thought to play a role in apoptosis [59-61] and MDV latency and transformation [62]. *pp38* encodes for the 38 kD phosphoprotein which is expressed in lytically infected cells, lymphoblastoid cell lines and tumors. It is essential for cytolytic infection of B cells and maintenance of a transformed state [63, 64]. A mutant with the *pp38* gene deleted retained oncogenicity at low levels despite being impaired for *in vivo* replication in lymphoid organs, making *pp38* dispensable for tumor formation [63]. The *pp24* protein was detected in MDV infected CEF by immunoprecipitation but not by Western blot and thus was assumed to be associated to *pp38* [65]. Both *pp38* and *pp24* genes share the same promoter that transcribes *pp38*, *pp24* and the 1.8-kb mRNA. The combination of both genes is required for the activation of the promoter [66].

The 1.8 kb gene family, from which the transcripts are truncated in attenuated strains and not in pathogenic strains [67, 68]. The 1.8-kb mRNA contains two copies of the 132-bp repeat. Copies of the repeat increase as the virus becomes attenuated. The difference in the number copies produced by virulent vs. vaccine strains forms the basis of their differentiation by polymerase chain reaction (PCR) assays [69-71]. The 1.8-kb mRNA was not present in attenuated MDV, but when mutants lacking the gene family were inoculated into susceptible day-old chicks, lesions were formed, suggesting that the 132-bp repeat is not solely associated with attenuation [72].

Viral telomerase RNA (vTR) is encoded only by oncogenic serotypes, where there are two copies which are 88% similar to the chicken telomerase RNA (chTR). Telomerase protects against telomere shortening during mitosis and is elevated in many human cancers. It is associated with oncogenicity by its ability to induce lymphomas [73-75]. Mutants lacking vTR resulted in reduced tumor incidences and in smaller, less disseminated lymphomas [76, 77].

MicroRNAs are short RNAs that regulate gene expression post-transcription. In MDV-1, miRNAs are located in genomic repeat regions in two major clusters, one flanking the *Meq* gene and the other in the latency-associated transcript (LAT) [78]. They have been identified in viruses such as MDV, specifically 26 miRNAs in MDV-1, 36 miRNAs in MDV-2, and 28 miRNAs in HVT have been identified. Transcription of miRNAs depends on the viral strains as well as on the stage of infection [79, 80]. They have key roles in pathogenesis although their functions are not fully understood. Transcripts of some of them (*mdv1-miR-M4*, *mdv1-miR-M8*, and *mdv1-miR-M12*) were more abundant in tumors than in non-tumor sites of infected chickens [81]. *mdv1-miRNA-M4* is known to target *UL28* and *UL32*, viral genes involved in viral particle formation, as well as genes involved in lymphocyte proliferation and differentiation. It is the

most commonly found miRNA in tumors, accounting for 72% of all MDV microRNAs [82]. A mutant not expressing *mdv1-miR-M4* was unable to transform T cells [79]. *mdv1-miR-M7-5p* is expressed weakly expressed during virus reactivation [83], in lytically infected CEFs [84] and strongly in a lymphoblastoid cell line (MSB-1) [84], suggesting its role in latency.

In cell culture, viral penetration occurs 1-hour post attachment. Both *in vivo* and *in vitro*, subsequent spread of infection occurs by direct contact between uninfected and infected cells, the latter being mostly lymphocytes. Virus is transferred through intracellular bridges [85], but the interaction between the cells is not entirely understood. Virus replication rates vary among serotypes, virus strain and its passage level, cell type and temperature of incubation [86].

Three different interactions occur between the virus and host cell:

- a. During productive infection, viral DNA replicates, proteins are synthesized, and virus particles can be produced. This leads to intranuclear inclusion body formation and lysis of the cell and can cause necrobiotic lesions. This type of infection is called cytolytic [87] and can be of two types:
 - i. Fully productive infection occurs only in the feather follicle epithelium (FFE) where enveloped, infectious virions are formed [88].
 - ii. Productive-restrictive infection results in nonenveloped, noninfectious virions and occurs in the lymphoid organs and in cell culture. Most of the MDV genome is transcribed in infected fibroblasts [89-91].
- b. Latent infection is characterized by the presence of viral DNA and with minimum transcriptions of viral DNA and protein expression. MDV latency mostly is associated with CD4⁺ T cells, but both CD8⁺ T cells and B cells can also be latently infected [92-94]. *Mdv-miR-M7* is associated with latency [83, 84]. *vIL-8* impacts T lymphocytes and

therefore plays a role in latency [55]. *Meq* can also be expressed during latent infection in CD4+ T cells [42].

- c. The MDV genome can be reactivated from latently infected and tumor cells by inoculation of susceptible chickens, co-cultivation with permissive cells, and *in vitro* cultivation of latently infected lymphocytes. Transforming infection occurs only with infection by oncogenic serotype 1. The exact pathway to MDV oncogenesis is not clear, but the oncogene *meq* [43-45, 95-98], *vTR* [73-75], as well as *mdv1-miR-M4* [79, 81, 82], and *mdv1-miR-M8*, and *mdv1-miR-M1 2* [81] have been shown to be associated with oncogenicity. The pp38/pp24 complex is associated with maintenance of the transformed state [61-64]. *vIL-8* is related to tumor incidence [57].

1.3. Epidemiology

1.3.1. MDV-1

MDV serotype 1 is able to induce MD in its natural host, the chicken and also in quail, turkeys, and pheasants [99]. It is the only serotype able to induce tumors. It replicates in the epithelial cells of the keratinizing layer of the feather follicle epithelium (FFE) as enveloped, infectious viral particles [100, 101]. The reason for the FFE production of cell-free MDV is not well understood [102]. MDV spreads by horizontal transmission, the major source being shed the feathers and dander contaminated by such virus shed from the keratinized FFE cells [103, 104]. Infection is highly contagious through the respiratory tract via inhalation. Shedding begins 15 days post infection [105] and lasts for the duration of the host's life [106].

1.3.2. MDV-2

MDV serotype 2 infects its natural host, the chicken, but does not induce tumors. It may also infect turkeys when inoculated as embryos. It replicates in the FFE and spreads horizontally in the same way as MDV-1.

1.3.3. MDV-3

HVT, serotype 3, infects the natural host of turkeys as well as chickens and does not induce tumors in either one. It replicates in the FFE of both species but only efficiently spreads among turkeys.

Attenuated serotype 1, serotype 2, and serotype 3 are used as vaccines. Vaccination controls for disease manifestation (clinical signs) but does not prevent replication and shedding in infected chickens. Infectious MDV is able to survive in poultry house dust for at least 205 days at room temperature and longer at colder temperatures [107]. Despite being poorly understood, it is thought that dander wraps the cell-free virions and therefore protects them from degradation, explaining its persistence in the environment [102]. This resistance in the environment's dust makes it ubiquitous and able to infect all flocks very early in life. HVT is ubiquitous in turkey farms. Vertical transmission does not occur [108-110].

1.4. Pathogenesis

Pathogenesis of MDV is complex and varies depending on several factors involving both the host (genetic susceptibility, maternal antibody status, vaccination status, sex, and age at time of exposure) and the virus (virulence and dose). The most complex pathogenesis occurs in unvaccinated, susceptible chickens without maternal antibodies against any of the serotypes of MDV and challenge with a vv+MDV. The sequence of infectious events in those cases consists of 1) early cytolytic, 2) latent, 3) secondary cytolytic and 4) transformation and are as follows:

After infection via the respiratory tract by inhalation of virus, it is presumed that phagocytes engulf the virus and transport it to lymphoid organs (bursa of Fabricius, thymus and spleen) within 24-36 hours of infection [111, 112]. First, productive cytolytic infection is established in these organs 3-6 days post infection (dpi). The initial target are B lymphocytes [113] and this infection leads to necrosis and inflammation of the lymphoid organs resulting in lymphodegenerative syndromes and in early immunosuppression. It has been recently demonstrated that MDV is capable of destroying lymphocytes in both bursa of Fabricius and thymus by apoptosis [114]. The initial cytolytic infection of B lymphocytes and the resulting inflammatory response activates the resting T cells, making them susceptible to MDV infection. It is in activated T cells that a switch from cytolytic infection to latency occurs. During latency, MDV integrates into the genome of CD4⁺ T cells to escape detection. There is no virus replication, minimal or no antigen expression and the number of viral copies per cell is low. The onset of latency is quick and evidence of cytolytic infection is cleared by 7-8 dpi. Latent infection can still be detected in lymphoid organs, peripheral blood lymphocytes (PBL) and feather pulp [115, 116]. Latently infected PBLs will transport MDV to other tissues such as the eye, brain and peripheral nerves as early as 6 dpi [9, 117]. MDV can reactivate in these lymphocytes and mount an acute inflammatory response responsible for the development of panophthalmitis, neuritis, and transient paralysis respectively. Reactivation of the virus results in a second phase of productive cytolytic infection by 14 dpi affecting lymphoid and visceral organs with focal cell death and inflammation. This late cytolytic infection is characterized by high viral gene expression and replication which will severely dysregulate the immune responses and cause late immunosuppression [13, 118].

Latently infected lymphocytes also carry MDV to the FFE. Reactivation of MDV in the FFE results in the development of virions. The FFE is the only infected tissue where virus replication yields infectious cell-free virus [119].

Lastly, between 14 and 21 dpi, latently infected T cells, mostly CD4+, can undergo lymphoproliferative changes due to neoplastic transformation which results in lymphoma and tumor formation in the visceral organs. Here, the number of MDV copies increases and there is high expression of the oncogene *meq* but expression of most other viral antigen is low. Finally, later in the pathogenesis, MDV is able to produce changes in the blood vessels leading to arteriosclerosis [6, 7].

The presence of maternal antibodies and/or vaccination protects against the development of lymphodegenerative syndromes and early immunosuppression, panophthalmitis, transient paralysis, neuritis, and arteriosclerosis. They do not protect against replication of MDV in the FFE and transmission and late immunosuppression. When vaccination protocols are adequate for the intensity of the challenge, vaccination is very efficient at controlling the development of tumors as well.

1.5. Clinical and pathological manifestation

Clinical signs include lymphoid neoplasms, transient paralysis [5], lymphodegenerative syndromes [4], panophthalmitis [8, 9], arteriosclerosis [6, 7] and immunosuppression [10-12]. Each is described below:

- a. Lymphodegenerative syndromes occur during the early cytolytic infection in the lymphoid organs and in most cases is not accompanied by clinical signs. In very severe cases, when unvaccinated, susceptible chickens lacking maternal antibodies are challenged with vv+MDV, the result can be sudden death, but it is unclear whether the

cause of death is the lymphodegenerative syndrome or the concurrent transient paralysis that these birds normally develop. Active replication of MDV in the lymphoid organs at 5-6 dpi causes cytolysis of lymphocytes, hyperplasia of reticular cells, accumulation of macrophages and infiltration by granulocytes. The bursa of Fabricius, thymus and spleen become necrotic, enlarged or atrophic. This syndrome occurs only in unvaccinated chickens with no maternal antibodies.

- b. Transient paralysis is characterized by the sudden development of flaccid paralysis of the neck and limbs that can either completely resolve or kill the infected chicken within 24-48 hours. This syndrome is seen only in unvaccinated chickens without maternal antibodies at 8-12 dpi. It occurs when MDV reactivates in latently infected lymphocytes in the brain inducing vasculitis and vasogenic edema, which are responsible for the clinical signs of transient paralysis (TP) [5, 117, 120, 121]. It is common to observe atrophy of the bursa and thymus in the affected chickens.
- c. Panophthalmitis is normally accompanied by unilateral or bilateral blindness. MDV carried to the eye will cause lesions involving the iris, ciliary body and choroid layer induced by hypertrophy of endothelial cells, vasculitis and infiltration of lymphocytes, plasma cells, macrophages and heterophils. Inflammatory lesions in the eye can progress to complete destruction of the retina [9]. This will occur in nonvaccinated chickens without maternal antibodies.
- d. Other inflammatory lesions: Some inflammatory and degenerative lesions induced by MDV do not show clinical signs. Infiltration of the nerves (neuritis) can occur 7-8 dpi. Skin lesions such as dermatitis (perifollicular and follicular) and degeneration in the FFE can occur due to MDV.

- e. Immunosuppression: Although poorly understood, immunosuppression induced by MDV (MDV-IS) can be categorized into early, late unrelated to tumors, and late related to tumors. Early MDV-IS occurs because of MDV replication in lymphoid organs which leads to lymphoid organ atrophy and lymphocyte destruction. It only occurs in nonvaccinated chickens without maternal antibodies. Late MDV-IS unrelated to tumors occurs when humoral and cellular immune responses are dysregulated by MDV. It occurs in chickens with maternal antibodies even when properly vaccinated in the face of vv+MDV [13, 118]. It is particularly important since an improper response in the face of other diseases is detrimental to the health of the chicken. Lastly, late MDV-IS related to tumors occurs due to transformed lymphocytes T CD4+CD8- which cannot properly function. Vaccination programs that control the development of tumors will be able to control this type of immunosuppression as well.
- f. Neoplastic syndromes: the development of tumors is the most obvious manifestation of MD. Symptoms vary depending on the location of the tumors (nerves, viscera, skin). If nerves are affected, paralysis of limbs, neck and ataxia may occur. Lesions include nerve enlargement, discoloration, edema, or lack of striation. Tumors in the viscera can occur in any organ. Skin lesions are erythema, and tumors of the feather skin and non-feathered skin. MD tumors are composed by a heterogenous population of mononuclear cells including large lymphoblast, small lymphocytes, and macrophages [122]. Tumor cells have been found to be CD4+CD8-meq+CD30+ [122, 123].
- g. Arteriosclerosis is initiated by early lesions in the endothelial and muscular cells of the blood vessels caused by MDV [7]. However, arteriosclerotic lesions are not evident until later in the pathogenesis. Lesions are characterized by infiltrates of lymphocytes,

macrophages and cholesterol deposits. It only occurs in unvaccinated chickens with no maternal antibodies.

1.6. Control

1.6.1. Biosecurity

Management strategies surrounding biosecurity are an integral part of controlling MD. MDV is only transmitted horizontally by contaminated dust and dander. Protected within the dead cells of the epidermis, it can survive for long periods of time in the environment. Because vaccines take 5-7 days to confer protection, it is imperative to implement biosecurity measures that reduce contamination in the environment and delay challenge. Ideally, management of air using filters and positive pressure will minimize the number of virion particles by controlling the air that enters and leaves the facilities. Although these biosecurity measures have been successful in the case of SPF flocks, where MD has been eradicated, this practice is not economically feasible in commercial farms. Disinfection and cleaning of the housing facility, allowing a down time between flocks, and use single age housing can help minimize exposure to newly arriving chicks.

1.6.2. Genetics

Before the onset of vaccination, genetics was the most relevant method for MD control as it was recognized that certain genetic lines were resistant to MD while others were very susceptible [124]. Susceptible lines of chickens had a shorter latent period and greater incidence of visceral lesions than resistant lines [22]. Selection for chickens that are more resistant to MD is still a goal of breeder companies, but with the success of vaccination, focus on developing resistant genetic lines has fallen behind. The main strategies include mass selection (breeding from survivors) and family selection (using pedigreed offspring as breeders) [125]. Genetic

susceptibility to MD is separated by two groups of genes: those of the major histocompatibility complex (MHC) and non-MHC genes. MHC related genes control B-haplotype of the chickens, where having certain B-haplotypes (B21) granted resistance while having others (B19) made them susceptible. This difference in genetic resistance also plays a role in vaccination, as certain vaccines are more efficacious depending on serotype of vaccine and chicken susceptibility [126-128]. Non-MHC genes' role is seen in line 6 (resistant) and line 7 (susceptible) chickens which are both homozygous for MHC B² allele but differ in MD susceptibility [129]. Other areas of the genome are related to resistance to MD and evaluation of which loci are involved is underway [130-137].

1.6.3. Vaccines

The central strategy for control is vaccination, first introduced in the late 1960s [15-17]. Without the development of vaccines, the poultry industry would not stand as it does today, as losses would render it economically unviable. The success of MD vaccination can be appreciated by the decrease of losses from MD condemnations in the USA. Broiler condemnations due to skin leukosis, which almost exclusively refers to MD, were 1.5% in 1970 and reached its lowest of 0.00069% in 2016 (Figure 1) [86]. The extraordinary success of MD vaccines however has a dark side as they might contribute to the evolution of MDV towards more virulence. MD vaccines protect against tumor induction and mortality but do not stop virus replication and shedding. The lack of sterilizing immunity might have contributed to select those MDV isolates best fit to replicate in immunized birds and that can overcome vaccine immunity. The emergent more virulent MDVs are capable of overcome vaccine-induced immunity and require the use of novel more efficacious vaccines [18, 19].

2. MD vaccines

2.1. History

As the poultry industry started intensive methods of production in the late 50's, a new pathotype of MDV emerged (vMDV) and it was causing catastrophic losses that neither biosecurity nor genetics were able to control. The need for a protective vaccine prompted various governments to start research lines on Marek's disease at various institutions such as Houghton Poultry Research Station (now part of the Pirbright Institute) in the United Kingdom and the USDA Regional Poultry Research Laboratory (now the Avian Disease and Oncology Laboratory (ADOL)) in East Lansing, MI. Three vaccines were developed within the span of four years. The first vaccine was an attenuated serotype 1 virus named HPRS-16 [15]. HPRS-16att was used commercially in Europe and the United States in the early 70s until the introduction of HVT [16, 138]. HPRS-16 is still available in some markets but its use is limited since the 1970s. The naturally nononcogenic herpesvirus HVT was isolated in 1970 [4, 5] and the FC126 strain was licensed for use in the United States in 1971 (reviewed in [139]), overshadowing HPRS-16att and still being used today. The next vaccine came from an attenuated oncogenic serotype 1 virus: the strain CVI-988. It was introduced in 1971 [17, 140] at the Central Veterinary Institute (CVI) in the Netherlands. To date, it is the most protective vaccine against highly virulent MDV and is widely used. There are still a few countries where CVI-988 is not authorized (i.e. India) or its use is restricted (Costa Rica and Chile). In the USA, the use of CVI-988 was not authorized until 1994 [139].

The evolution of MDV towards more virulence decreased the efficacy of the existing vaccines. In the 1980's, the emergence of vvMDV strains such as RB-1B [141], Md5 and Md11 [4] was causing increased condemnations and a novel vaccination protocol combining vaccines

of two different serotype was taken. Such strategy is known as protective synergism [142] and occur when HVT is combined with vaccines of the serotype 2 that was first characterized in 1978 [143, 144]. SB-1 was the first strain of the serotype 2 to be characterized as nononcogenic and to show protection against challenge [143]. More serotype 2 strains were isolated [145, 146] but 301B/1[146] and SB-1 are the only ones licensed for use as vaccines in the United States.

When the vv+ pathotype emerged in the early 90s, 10 years after bivalent HVT+SB-1 had been in use, it forced USA to implement the use of CVI-988, as it was shown to offer superior protection to HVT alone or bivalent HVT+SB-1 against vv+ pathotypes [147]. CVI-988 had been used for almost 2 decades in Europe by the time its use was approved in the USA, where safety concerns delayed its use. CVI-988 had low levels of oncogenicity when first isolated and was attenuated by 35 passages in cell culture (p35). However, it was still able to spread horizontally [140] and there were concerns for the possibility of reversion to virulence or even safety for humans. Attempts to further attenuate it to meet the requirements for licensing in the USA resulted in two cloned variants at higher passages, clone C [148] and clone CIR6 [149]. They were licensed but were less protective than original CVI-988 [150] and did not protect against vvMDV [139]. Other serotype 1 strains have been attenuated in cell culture following the initial publication [17, 144, 151-156], but only CVI-988 gained popularity as a vaccine. In the USA, an attenuated serotype 1 virus came from the vv pathotype Md11 strain (the licensed Md11/75C/R2/23) provided high levels of protection [157] but was still less effective than CVI-988. Because of the superiority of CVI-988 as a vaccine, the use of the vaccine spread across Europe (license obtained in Germany in 1984 and in France in 1989), to the USA in 1994 [139], and to other regions of the world although still there are countries that either ban or restrict its use.

The interest to develop recombinant vaccines against MD started in the early 90's [158, 159] but it was not until the last decade that several recombinant vaccines became licensed and are now widely used worldwide. Most of the existing recombinant vaccines are vector vaccines using HVT [158, 160-170]. In addition to vector vaccines, a recombinant vaccine using a serotype 1 CVI-988 with insertion of the long terminal repeat (LTR) of the reticuloendotheliosis virus (REV) has been recently licensed to be used in the US [171, 172].

2.2. Classification (cell associated vs. lyophilized, serotypes, recombinant vs conventional)

As stated in section 1.3, the vaccine viruses can be classified into species, but have been historically classified into three serotypes and is the most widespread classification. MD vaccines can be also classified based on other criteria: cell associated vs. lyophilized, and conventional vs recombinant.

Serotype 3 is the herpesvirus of turkeys (HVT), which are naturally nononcogenic. First identified in turkey kidney cell cultures [173] or in cultures inoculated with turkey blood [16], the virus is antigenically closely related to MDV [16]. HVT is the only virus available from which an effective, cell-free vaccine can be manufactured. Infected cell cultures can be sonicated, and enough cell-free virus can be collected to produce a lyophilized product. The cell-associated form is still more protective and preferred, but the lyophilized vaccine may be beneficial in countries with difficulties to maintain the cold chain. It is also the only vaccine available for small or backyard flocks. HVT vaccine is the most economically viable vaccine to produce, and strain FC126 is widely used. HVT replicates in CEF and DEF [174]. In the last decade, HVT has been widely used as vector recombinant vaccines. Although serotype 3 strains replicates in the FFE, transmission of HVT among chickens is minimal, if any [175]. Shedding

of HVT among turkeys occurs readily and it was recently demonstrated that most commercial turkey flock are infected with HVT within the first week of life [176].

Serotype 2 are naturally nononcogenic viruses isolated from chickens. SB-1 was the first strain of this serotype be characterized as nononcogenic and to show protection against challenge [143]. Protection conferred by serotype 2 strains by themselves is low [143]. However, combination of serotype 2 and serotype 3 strains in bivalent vaccines results in a much higher protection than either serotype alone. This feature is known as protective synergism [142]. Serotype 2 vaccine viruses are combined with HVT and other viruses to produce bivalent or trivalent vaccines. The viruses grow well in CEF and exist only as cell-associated vaccines. Serotype 2 vaccines replicate in the FFE and transmit readily among chickens.

Serotype 1 vaccines are oncogenic viruses that have been attenuated by serial passage in cell culture. Attenuation means a loss of oncogenicity, measured by gross or microscopic tumor induction in chicks, without losing antigenicity. The number of passages in cell culture required to attenuate a strain depends on its original virulence. The more virulent the strain the higher the number of passages in cell culture needed for attenuation [177]. The serotype 1 vaccine viruses have been adapted to grow in CEF and are exclusively used as cell-associated vaccines. Studies have demonstrated that there are differences in the level of antigenicity of various CVI-988 depending on their origin [178-180]. Equally, differences have been shown on the ability to replicate in FFE and spread by contact [179].

Conventional MD vaccines are live vaccines made using either nononcogenic strains from serotypes 2 and 3 or attenuated from serotype 1. Besides conventional vaccines, the use of MD vaccine viruses as vectors for polyvalent vaccines is advantageous for multiple reasons. They simultaneously protect against MD and against the inserts that could be from one or

various viruses. They establish lifelong protection against MD and the inserts and reduce cost and time of labor by requiring a single, one-time vaccination. Genetically engineered recombinant vaccines using either serotype 1 or 3 as vectors are well established. Serotype 3 is the most widely used vector with inserts of infectious bursal disease (rHVT-IBDV) [160-163], Newcastle disease virus (rHVT-NDV) [158, 164-166] laryngotracheitis (rHVT-LT) [167, 168] avian influenza (rHVT-AI) [169, 170] or inserts of various infectious agents (i.e. Newcastle disease and infectious bursal disease). In addition to vector vaccines, a recombinant vaccine using a serotype 1 CVI-988 with insertion of the long terminal repeat (LTR) of the reticuloendotheliosis virus (REV) has been recently licensed to be used in the US [171, 172].

Although not licensed yet, there are many other recombinant vaccines using MDV that have shown various levels of protection. Recombinants of HVT expressing MDV-1 glycoproteins gA and gB as well as NDV HN and F proteins were efficacious in protecting against ND and MD [181]. Serotype 1 is also used as a vector, with inserts that express the NDV F protein [182, 183], IBDV VP2 protein [184], infectious bronchitis S1 subunit of the S protein [185] and highly pathogenic avian influenza H5N1 HA gene [186]. The use of bacterial artificial chromosome (BAC) clones has also been successful at creating vectors of serotype 3 [187, 188], serotype 2 [189] and serotype 1 [51, 190]. A serotype 2 recombinant using SB-1 strain expressing IBDV VP2 was able to protect against challenge with IBDV, with hope that inserts of other diseases could be expressed from the same loci [191]. A vaccine using serotype 1 vvMDV strain Md5 with the *meq* oncogene deleted protects against both tumors and late immunosuppression induced by vv+MDV [48, 118, 192, 193]. Furthermore, it has been used to develop vector vaccines by inserting the glycoprotein B of the ILTV [194]. The use of fowlpox as a vector with the insert of the MDV gB has been developed and is licensed in the USA [159,

195, 196]. Although protection of this vaccine in chickens bearing maternal antibodies against MDV is not good, it has synergism with HVT and it is possible to use in bivalent vaccines free of cells conferring higher protection than HVT alone [159, 196].

2.3. Vaccination strategies

2.3.1 *In ovo* vs SC

Since chickens get exposed to MDV very early in life, it is optimal that vaccines are administered as early as possible. Vaccines can be administered *in ovo* at 18 days of embryonation or immediately after hatch [197, 198]. Traditionally, MD vaccination was done at day of age and it is typically administered in the neck region subcutaneously or in the leg intramuscularly. This process was automated by devices to reduce cost, delivering vaccines to 2 to 3 thousand chicks per hour [150]. An advantage of this vaccination protocol is that vaccines may be administered solely to females after sexing in the case of egg-type chickens. However, one-day-old vaccination is time consuming and expensive as it requires labor, is prompt to errors, and there is the potential for horizontal pathogen transmission due to contamination of the needle, which is not changed or disinfected [199]. Today, *in ovo* vaccination has become the preferred method for broilers and broiler breeders in the USA and its use is expanding worldwide [200]. *In ovo* vaccination is done by insertion of a 1 inch needle along the length of the egg into the amniotic sac (18 ED) or directly into the embryo (19ED) [198]. It was shown to provide protection against challenge by Sharma and Burmester [198]. More importantly, chickens vaccinated *in ovo* showed greater resistance to challenge than chickens vaccinated post-hatch when challenge occurred within the first days [198, 201]. *In ovo* vaccination hastened the onset of protective immunity [202] and did not negatively impact hatchability [198], was compatible with vaccines all three serotypes [202] and was successful in the face of maternal antibodies

[203]. Furthermore, *in ovo* vaccination with HVT hastens immunocompetence in chickens embryos [204]

2.3.2. Revaccination

Revaccination of chickens at 1-3 weeks of age after initial vaccination became common practice despite lack of evidence for its advantages and standard protocols. The older literature failed to advocate for this practice, as no greater protection was conferred from two *vs* a single vaccination (reviewed in [150]), and vaccination after challenge was ineffective in laboratory trials [205]. The only case for this practice was to protect chicks that had been erroneously missed during the initial vaccination. Recently, however, revaccination was shown to increase protection against early challenge [206-208]. It resulted in two replication phases of vaccine viruses and a lower replication phase of the challenge virus [208]. It has also been shown that for revaccination to succeed, the second vaccine administered should inherently be more protective than the first (ex: HVT first, CVI-988 second) and both vaccines should be administered before challenge [207]. The recommended protocol is to administered both vaccines prior to MDV challenge, the first *in ovo* at 18ED and the second subcutaneously at hatch [206].

2.3.3. Adjuvants and immunomodulators

The use of immunomodulators, stabilizers of the immune system, alongside MD vaccines is a strategy used to potentially improve vaccine efficacy. Adjuvants, which enhance the vaccine's ability to induce a protective immune response, are available but not widely used.

Adjuvants used with MD vaccines include:

- a) Acemannan, a polysaccharide, is used to stimulate production of nitric oxide in macrophages [209] and to increase MD protection and has been shown to enhance

protection conferred by MD vaccines [210]. It has been licensed in the US since 1992 but is not currently in use.

- b) The use of cytokines IFN- γ , IL-2 and IL-18 as adjuvants, which possess strong antiviral and immunomodulatory properties, is common against various other pathogens (i.e. infectious bursal disease virus [211]). The deliverance of cytokines by injections is problematic due to their short half-life, thus development of other delivery methods (i.e. liposomes, recombinant viral vectors, plasmid DNA) is being explored. Still, its use is still experimental as the expression of cytokines in specific amounts and at specific time periods cannot be regulated by current methods. So far, IFN- γ was able to enhance protective efficacy of HVT against vvMDV by lowering tumor development occurrence, viral replication and MDV genome load when a recombinant chicken interferon gamma was expressed via a plasmid alongside the HVT vaccine [212]. Expression of IL-2 by an HVT/IL-2 recombinant was able to increase the neutralizing antibody response against HVT but did not increase protection against vMDV challenge nor did it promote growth of the chicken [213]. The use of chicken myelomonocytic growth factor (cMGF) was able to prolong chicken survival, reduce viremia and tumor incidence and improve protection by HVT when delivered by a fowlpox viral vector [214].
- c) Toll-like receptors (TLR) are innate receptors capable of identifying pathogen-associated molecular patterns (PAMPs) and of inducing immune responses, such as cytokine production. Ligands for TLRs have been used as vaccine adjuvants in other species and against various pathogens. The administration of a synthetic TLR3 ligand polyriboinosinic polyribocytidylic [poly(I:C)] in conjunction with an HVT vaccine

enhanced protection against incidence of tumors but did not inhibit viral shedding [215]. Using LPS and CpG oligodeoxynucleotides (ODN) as ligands for TLR4 and TLR21 respectively, administered prior to challenge, delayed disease onset and reduced MDV genome load [216].

- d) HVT, due to its protective synergism with SB-1 [142, 143], and with the recombinant fowlpox expressing glycoprotein B (rFPV/gB1) [196] as well as its ability to activate the innate immune system and hasten maturation, is in itself an adjuvant [204].

2.3.4. Pitfalls on MD vaccination

Due to the cell-associated nature of MD vaccines, special care is required when handling, storing and administering vaccines. Several factors can create suboptimal conditions for cell viability and therefore cause vaccination to fail. Any interruption of the cold chain will result in dead cells and therefore lowered protection. When receiving and storing vials, steps must be taken to ensure vials remain covered in liquid nitrogen so the vials remain frozen until the time of reconstitution. Monitoring of liquid nitrogen and proper record-keeping is necessary. Vials may also be stored in the inverted position to detect thawing. When it is time to reconstitute the vaccine, the vial must be thawed slowly, introducing into a lukewarm water bath (26-28 ° C) for short intervals (less than 60 seconds) and putting on ice once thawed. Before opening, outside surfaces are disinfected. Vaccine is transferred into sterile diluent using 18 g needles and mixed continuously to prevent cell flocculation and sedimentation. Over-dilution of vaccines to reduce cost of vaccination has been shown to reduce protection against MD [116] and adding antibiotics to the vaccine can negatively affect the vaccine titers and should be discouraged [217, 218]. Vaccine should be administered within the shortest amount possible, not exceeding 90 minutes and maintained under refrigeration and continuous mixing at all times. The largest possible

gauge needle will prevent disruption to cell membrane and improve viability. Vaccine should be handled following manufacturer recommendations to prevent confound variables.

2.3.5. Assays to titrate MD vaccines

MDV will grow in chickens, chicks, chick embryos and cell culture. With chickens, the virus is usually preserved in its frozen splenocytes. The chickens must be SPF and free of chicken infectious anemia virus (CIAV). The virus is most easily assayed in cell culture, once MDV is adapted to the type of cell, it will grow with differing responses depending on the combination of cell type and virus serotype. The optimum type of cell depends on the serotype. CKC and DEF are ideal for serotype 1 while CEF are ideal for serotypes 2 and 3. CEF can also be used to grow attenuated serotype 1 or serotype 1 adapted to grow in CEF.

Infection of cell cultures will result in a cytopathic effect (CPE) of characteristic plaques which can be used for the quantification of virus [1, 2, 219, 220]. The changes seen in the plaques include clusters of rounded degenerating cells, the area of cell death, multinucleate cells, and the presence of intranuclear type A inclusion bodies (cytoplasmic aggregates of stable substances, which represent viral multiplication in a cell). The presence and different combination of these changes, as well as the plaque size, vary with the serotype and the cell type on which the virus is seeded [1, 145, 220-224]. In most combinations, plaques are <1mm in diameter and are counted under magnification, normally at 10x [225].

Plaque assay is the *in vitro* quantitative assay for determining the vaccine titer [226]. It requires a monolayer of the appropriate cell type, which is obtained by seeding at least 1.0×10^6 secondary CEF or 4.0×10^6 secondary CEF per 60-mm cell culture dish [226]. Cells may be infected anywhere from 0 to 48 hours for secondary CEF and 24 to 72 hours for primary CKC [226]. Cell culture medium standard is Eagle's Basal Medium (BME), or McCoy/Leibovitz

modified glutamine and supplemented with 2 to 4% heat-inactivated calf serum [226]. The confluent monolayer is inoculated with ten-fold dilutions of cell-associated virus made by dilution of vaccine in appropriate diluent, following protocol of reconstitution (Figures 2 and 3). Once inoculated, cell culture dishes are gently swirled to ensure even distribution of virus onto the cell monolayer. The infected cells are then incubated in 4-5% CO₂ and a temperature ranging from 37 to 39° C [226]. Media is changed every 48 hours and CPE is analyzed daily (Figure 4). CPE is enumerated 3-5 dpi for HVT and up to 8 dpi for serotype 1 [226]. Growth of PFU depends on cell type and serotype, but daily evaluation of the cell cultures is needed to avoid development of secondary plaques that may confound the titration.

3. Vaccination failures and factors influencing MD outbreaks

Efficacy of MD vaccines is generally high reaching up to 97-99% [150]. However, sporadic outbreaks of MD in vaccinated flocks can cause severe economic losses and are of great concern to the poultry industry. There are different factors that can contribute to vaccine failures which can be divided into three different groups: those affecting the vaccine dose administered, normally associated with flaws in the storing/handling/administration of the vaccines [21]; those affecting replication of the vaccine in the chickens (interference of vaccination with maternal antibodies, interference with other vaccines); and those affecting the strength of the immune responses elicited against the vaccine virus (early challenge with MDV, other immunosuppressive diseases, and/or increased virulence of MDV)

3.1. Problems associated to poor management of the vaccine and/or vaccination procedures resulting in decreased dose

Improper storing, handling, and administration techniques of the vaccine ampoules can lead to insufficient vaccine titers to induce immunity. Reduced vaccine doses have been

demonstrated to have a negative effect on protection and the extent of such negative effect depends on the sex, genetic of the chickens, age at challenge, and virulence of challenge virus [21, 116, 227]. In a 2003 study of multiple ampoules in use at a Dutch hatchery, Landman demonstrated that the dose administered to chickens was below the mandatory titers in the European Union (10^3 PFU/chicken). Authors show that initial titers in the ampoules were, in all cases, above the required dose when titrated under optimal conditions and concluded that improper liquid nitrogen management was the problem [21]. Furthermore, a comparison of optimal vaccination procedures with actual practices performed showed common deficiencies such as: inadequate vaccine records, incorrect water bath temperature, removal of ampoules from liquid nitrogen, slow ampoule thawing time, failure to agitate the vaccine, failure to vaccinate within the recommended 1 hour and lack of consistency to vaccination of chicks [228].

Initial studies by Colwell *et al.* in 1975 [229] tested the effect of physical factors such as temperature, pH and osmolality of diluent on vaccine virus survival measured in PFU compared to its control at optimum conditions. Despite limitations such as procedural differences (thawing the vaccine under cold running water rather than with a room temperature water bath) and testing commercial diluents available at the time, the study gives insights into those factors affecting MD vaccines viability. The results showed that temperature plays a role on virus survival and that holding the vaccine diluent at 0 degrees Celsius was optimal regardless of initial diluent temperatures into which the vaccine was reconstituted [229]. The temperature of the diluent to which the vaccine was added also played a role, though some diluents performed best when vaccine was mixed at 25 degrees C and others when mixed at 0 degrees C. In a different study [230], it was found that reconstituting the vaccine in a refrigerated diluent (4 degrees C) rather than a room temperature diluent could lower the titers by 20%. Osmotic pressure in the diluent

was manipulated from 0 to 1000 mOsm/kg by addition of 0 to 3.18% sodium chloride respectively. There was a correlation between increased osmotic pressure and decreased virus survival [229]. Pressures above 475 mOsm/kg severely depressed virus survival, where the normal range of commercial diluents was 288 to 372 mOsm/kg [229]. All negative impacts of physical factors such as holding time, temperature and physical stress were worsened in the presence of a high osmotic pressure [229]. Additives which raised osmotic pressure had the most negative effect on virus survival [229]. Virus survival decreased with the physical stress of mechanical mixing. Survival was highest when mixed by manually inverting tubes, then decreased with stronger mechanical mixing by vortex for 1 second, and was lowest when passed through a syringe and 20 G needle [229]. Using an automated machine to vaccinate could affect cell integrity if pressure is too high, lowering titers [21].

Over diluting MD vaccines is a common practice within the broiler chicken industry to reduce cost and it negatively impacts the vaccine dose that chickens receive. Over-dilution of vaccines has been shown to reduce protection against MD as well as lowering relative body weights [116]. The extent of the negative effect of overdiluting vaccines depended on the vaccine used, the challenged virus, and the sex of the chickens, females being more susceptible than males [116]. Other practices, such as adding antibiotics to the reconstituted MD vaccines can affect negatively the dose and should be discouraged [217, 218].

Others actions such as leaving ampoules in the thawing bath for as long as 15 minutes can result in a 50% titer loss, and even leaving it for 5 min resulted in a 15% titer loss [230]. An incorrect water bath temperature (too hot at 40 degrees C or too cold at 17 degrees C) instead of the ideal room temperature (27 degrees C) could reduce titers by 25% [230]. Failure to rinse the ampoule with diluent when reconstituting could lower titers by 15% [230]. Removing an

ampoule from LN2 for as little as 2.5 min, and then replacing it to be reconstituted later could result in a 75% titer loss [230]. Prompt administration of the vaccine also affects vaccine titers, when the titers were measured at 30 min vs 2 hours, titers were 15% higher [230].

3.2. Factors influencing vaccine replication on the chickens

Replication of vaccine virus depends on the type of vaccine used (serotype and origin), route and age of vaccination, dose of vaccine administered and the combination of vaccines used [116, 178, 231]. Vaccine replication *in vivo* can be tested by obtaining samples of feather pulp at 1 week of age and testing using real time PCR [116].

3.3. Factors influencing the strength of the immune responses elicited by the vaccine

3.3.1. Early challenge. Exposure to MDV from infectious virus in dander and dust in the housing facility, either from previous flocks where litter is not properly sanitized or from multi-age farms where chickens are in close proximity [232], is an important cause of vaccine breaks. Infection with MDV occurs shortly after chicks arrive to their housing facilities [16] [233]. Onset of immunity by the vaccine virus takes at least 7 days [234] and therefore, immunity has not been established by the time of infection [235]. Intensive biosecurity measures to decrease levels of MDV are not prioritized economically, but the use of filtered air, positive pressure housing and disposal of biological material (manure, carcasses) are options for reducing MDV contamination in poultry farms [236]. Thus, it is beneficial to vaccinate embryos *in ovo*, as it provides better protection to early challenge with MDV at hatching [198, 201].

3.3.2. Highly virulent MDV challenge. As indicated in previous sections (1.6.3 and 2.1), the emergent more virulent MDVs cannot be successfully protected by HVT or

by HVT+SB-1 and requires vaccination with CVI-988 [18]. If the vaccine used is not efficient against the existing challenge MDV, it will result in a “vaccine failure”, even when vaccines have been administered correctly and they have replicated well in the chickens. There are some strategies that can be used to increase the efficacy of a vaccine such as *in ovo* vaccination, revaccination, and use of adjuvants as are described in section 2.3.

3.3.3. Other diseases inducing immunosuppression. Any factor negatively influencing cellular immune responses will reduce or prevent MD vaccine-induced protection. One extensively studied immunosuppressive agent that impairs MD vaccine-induced immunity is CIAV [237]. CIAV severely decreases the ability of the chickens to mount a cellular-immune response and coinfection with MDV results in increased incidence and severity of MD [238, 239] and vaccine failures [240-244]. Furthermore, CAV can stay latent in chickens and reactivate when the estrogen production increases, resulting in MD outbreaks at the onset of egg production [245]. Stress has also been shown to interfere with MD protection. Administration of corticosterone in chickens that were well protected against MD for months resulted in an outbreak of MD [246]. Other factors like mycotoxins could also play an important role as they severely decrease cell-induced immunity.

FIGURES

Figure 1. Condemnation rate due to skin leukosis between 1970 and 2016. There has been a gradual decrease in broiler condemnations due to skin leukosis, which almost exclusively refers to MD, from 1.5% in 1970 to its lowest of 0.00069% in 2016 (Figure 1) [86].

Three serial 10-fold dilutions made immediately after reconstitution

Figure 2. Reconstitution of MD vaccines for plaque assay titration. Vaccine vial is reconstituted according to manufacturer recommendation. Ten-fold dilutions of reconstituted cell-associated virus are made in 10 duplicates.

Dil 1: # plaques counted x 1 = PFU/mL
 Dil 0: # plaques counted x 10 = PFU/mL
 Dil -1: # plaques counted x 100 = PFU/mL
 Dil -2: # plaques counted x 1000 = PFU/mL

Figure 3. Dilutions of reconstituted MD vaccines in 10 duplicates. Once vaccine vial is reconstituted according to manufacturer recommendation, ten-fold dilutions are made in 10 duplicates in order to quantify CPE accordingly.

Figure 4. Timeline of titration by plaque-assay of MD vaccines. A confluent monolayer of 2.0×10^6 secondary CEFs is inoculated with respective duplicates of diluted reconstituted vaccine. Media is changed every 48 hours and CPE is analyzed daily with inverted microscope and enumerated before the onset of secondary PFUs.

CHAPTER 2

Evaluation of factors that influence dose variability of Marek's disease vaccines¹

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1. Abstract

Marek's disease (MD) vaccines are cell-associated and require special handling and care during administration. Vaccine dose is evaluated by plaque assay and is indicated as the number of plaque-forming units (PFU) per dose. The objectives of this study were to evaluate the dose variability within each vial of MD vaccines and to assess those factors (both from manufacturing and handling/administration of the vaccine) that could affect vaccine dose variability. Three experiments were conducted. Experiment 1 was to evaluate dose variability in thirty-six MD vaccine vials and the effect of manufacturing factors on dose variability. Vaccines were titrated 10 times. Dose variability was measured as the coefficient of variability (CV) calculated as standard deviation divided by average PFU and multiplied by 100. Our results show that all evaluated vaccines had levels of CV ranging from 10 to 34%. Variability exists regardless of manufacturer, vaccine serotype, and batch. Experiment 2 was conducted to evaluate the effect of infectivity rate (IR) on CV. IR was artificially reduced by adding non-infected chicken embryo fibroblast to the reconstituted vaccine before titration. Our results show that decreased IR results in higher CV. Experiment 3 was to evaluate the handling/administration factors (time and mixing during administration) on CV. Our results show that CV increases with time and that this effect is greater if vaccines are not mixed. Our study emphasizes the relevance of proper handling of MD vaccines and shows that dose variability can jeopardize the uniformity of vaccination in a flock and therefore the success of vaccination.

Keywords: Marek's disease, vaccines, titers, variability, plaque assay, dose

Abbreviations:

AI = avian influenza

CEF = chicken embryo fibroblasts

CKC = chicken kidney cells

CV = Coefficient of variability

DMSO = dimethylsulphoxide

EDTA = Ethylenediaminetetraacetic acid

HVT = herpesvirus of turkeys

IBDV = infectious bursal disease virus

IR = infectivity rate

LT = laryngotracheitis

LTR = long terminal repeat

MD = Marek's disease

MDV= Marek's disease virus

NDV = Newcastle disease virus

PFU = Plaque forming units

REV = reticuloendotheliosis

rHVT = recombinant HVT

SPAFAS = Specific Pathogen Free Avian Supplies

SPF= specific pathogen free

STDEV = standard deviation

USA = United States of America

2. Introduction

Marek's disease (MD), a highly contagious neoplastic disease in chickens is caused by Marek's disease virus (MDV). It is controlled by widespread vaccination of chickens using live attenuated cell-associated virus beginning in the late 1960's [15-17]. Even with proper control methods, MD has an annual economic impact of \$1-2 billion on the poultry industry worldwide [247].

The *Mardivirus* genus to which MDV belongs includes three species: *Gallid alpha-herpesvirus 2* or MDV, *Gallid alpha-herpesvirus 3*, and *Meleagrid alpha-herpesvirus 1* or herpesvirus of turkeys (HVT) [248]. Previously, the virus species were classified as serotypes 1, 2, and 3 respectively. This is the terminology that is most generally used in the poultry industry, thus will be used in this paper. Serotype 1 includes all pathogenic strains (very virulent plus, very virulent, virulent, and mildly virulent) [18, 19, 249]. Serotype 2 are naturally nononcogenic strains [143]. Lastly, serotype 3 are turkey strains which are naturally nononcogenic [16, 250].

Vaccines are made using any one of the three serotypes or a combination of them. The pathogenic serotype 1 strains are attenuated by passaging in chicken kidney cells (CKC) or chicken embryo fibroblasts (CEF) harvested from specific pathogen free (SPF) chickens or in duck embryo fibroblasts until pathogenicity is lost but immunogenicity remains. Serotype 2 strains are always used in bivalent vaccines with HVT as this combination of both serotypes results in protective synergism [144, 251]. Serotype 3 strains are used both as a monovalent vaccine and also in combination with either of the other serotypes as bivalent or all together as trivalent vaccines. Only serotype 3 strains may be lyophilized, but lyophilized vaccines are not as efficient as the cell-associated vaccines since they interact with maternal antibodies. Cell-associated vaccines are the gold-standard for controlling MD.

Vaccine protocols consist of administering reconstituted vaccine *in ovo* to embryos at 17-19 days of embryonation or to chickens subcutaneously at day of age. Vaccine-induced immunization takes 5-7 days and MDV challenge occurs often within the first days of life. Early challenge from leftover dander in poultry houses requires early vaccination and most commercial broiler chickens are vaccinated *in ovo*. A recent estimation (Villalobos, personal communication) indicates that 88% of the embryonated eggs for broilers in North America (USA and Canada) are vaccinated *in ovo*. A practice that is used in all regions of the world, yet the percentage varies (55% in Latin America, 11% in Europe, and 6% in Asia Pacific).

The vaccine is prepared by seeding the virus in CEFs, which are harvested after approximately 48 hours of incubation post-infection using EDTA and trypsin to detach the cells, resuspended in freezing media with dimethylsulphoxide (DMSO) and growth medium, and then gradually frozen in glass ampoules to be stored in liquid nitrogen [252]. Besides conventional vaccines, genetically engineered recombinant vaccines using either serotype 1 or 3 as vectors are available to protect against other avian diseases as well as MD. Serotype 3 is widely used as a vector with inserts of infectious bursal disease (rHVT-IBDV) [160-163], Newcastle disease virus (rHVT-NDV) [158, 164-166] laryngotracheitis (rHVT-LT) [167, 168], avian influenza (rHVT-AI) [169, 170] or inserts of multiple infectious agents (i.e. Newcastle disease and infectious bursal disease) (patent US20170306353A1). In addition to vector vaccines, a serotype 1 (CVI-988) with insertion of the long terminal repeat (LTR) of the reticuloendotheliosis virus (REV) has been recently licensed to be used in USA [171, 172].

Titration of the vaccines is done by plaque assay [226, 253]. The virus is incubated in cell culture until plaques are ready to be counted. Plaques are checked daily, but a general range of 3-5 days post inoculation for serotype 3, and 5-7 days post inoculation for serotypes 1 and 2 is

acceptable [252]. Each plaque counts as 1 regardless of size. Each dish of the titration is manually counted using an inverted microscope. The total number of plaque-forming units (PFU) is calculated and multiplied by the dilution factor and by the dose volume to obtain a total number of PFU/dose. The virus content of each batch is determined after filling and freezing the ampoules and the vaccine product is appropriately labeled for number of doses and dilution rate required per container [252]. The virus content assay and the titer used in the efficacy study will determine the recommended dose for each batch. In the USA, the minimum standard required dose is 1,000 PFU [254] but this does not guarantee protection when challenged with MDV of high virulence [116, 231]. The vaccine seed virus' protection potential varies and must be determined using protection tests (50% protective dose test and protection index test). The necessary dose required to immunize a flock will also depend on when challenge occurs and the dose of challenge virus [255-257], the challenge virus and its pathogenicity [116, 205], the genetics of the chickens [156], as well as its levels of maternal antibody [235, 258, 259]. When using recombinant MDVs, protective dose considerations include not only immunization against MDV but also against the insert.

Vaccine titers can be affected by a variety of factors and proper management and strict adherence of vaccine procedure protocols are critical for successful vaccination. Altering the reconstituted vaccine by the addition of antibiotics, other vaccines or over diluting to minimize cost is acknowledged in 37-50% of countries in a study involving 43 countries [260, 261]. In addition to these hurdles, the cell-associated nature of MDV poses a challenge at the time of administration. Reconstituted vaccines must be kept cold to preserve cell integrity and also agitated to maintain cell suspension homogeneity, as the cells will sediment and flocculate. Failures in the management and administration of MD vaccines will lead to reduce viable

vaccine virus and uneven dose uniformity, resulting in chickens who develop MD despite being vaccinated [21].

It was our hypothesis that due to the cell-associated nature of MD vaccines, there is an inherent variability of vaccine dose even within the same vial and that could negatively affect the vaccine dose that chickens receive. The objective of this study was to identify and evaluate the potential dose variability of cell-associated MD vaccines and the factors that influence such variability. In particular, it was our objective to evaluate factors during the manufacturing (infectivity rate, serotype, batch, and manufacturer) and during handling/administration (time and homogenization of the vaccine during reconstitution).

3. Materials and Methods

Chicken embryo fibroblast and vaccines. Primary chicken embryo fibroblasts (CEF) were obtained from Specific Pathogen Free Avian Supplies (SPAFAS) Inc. (Charles River Laboratory Inc., IL). Primary CEF were expanded in cell culture before they were frozen and stored in liquid nitrogen until they were used as secondary CEF. Vaccines used in this study are all commercially available. Specifically, strain FC126 for serotype 3 or herpesvirus of turkey (HVT) [16] and strain CVI-988 for serotype 1 [140] were used. All vaccines used were frozen and stored in liquid nitrogen, reconstituted by thawing in a lukewarm water bath (37°C) for a few seconds, putting on ice immediately until reconstitution, transferred with a 18-21 G needle to the diluent recommended by the manufacturer and diluted following manufacturer recommendations. Magnetic stirrers (Round, 2.54 x 0.95 cm) mixed the vaccine suspension continuously at an estimated speed of 1750-2000 rpm (Fisher Scientific magnetic stirrer, setting 7-8) .

Plaque assay and vaccine titration. In 35mm cell-culture dishes, 2×10^6 secondary CEFs were seeded with Leibovitz/McCoy Medium modified with glutamine media. Growth media was

supplemented with 4% calf serum for 24 hours until a confluent monolayer culture was formed. Then, a maintenance media supplemented with 2% calf serum was used and changed every other day for the rest of the study. Cells were incubated at 37.0 °C and 5.0% CO₂. Cell culture was inoculated with MD vaccine virus and titrated by plaque assay as reported [226, 253]. Briefly monolayer cultures of CEF were infected with one dose of vaccine (which was as manufacturer recommended), and with 3 serial 10-fold dilutions. Each vaccine was titrated 10 times independently. Growth of the virus was evaluated by assessing the cytopathic effect of MDV on CEF under inverted microscope. Titration was evaluated by counting the number of plaque-forming units (PFU) 3-6 days post infection as needed depending on growth of each virus. The maximum, minimum and average PFU was calculated by combining the 10 data points per vaccine, calculating the average (avg), standard deviation (STDEV) and range. Coefficient of variability (CV) is the ratio of standard deviation (STDEV) to the mean and reported in percentage ($STDEV / (\text{mean PFU per dose}) \times 100$). The lower the CV percentage, the less variability found in the vaccine.

Evaluation of infectivity rate. Infectivity rate (IR) was defined as the percentage of live cells in a vaccine that are infected with viable vaccine virus. Cells are obtained from the vaccine suspension at the time of reconstitution. Cell suspension is treated with Trypan Blue and loaded unto hemocytometer. Live cells are distinguished from dead cells by color, as dead cells will take up Trypan blue stain through a damaged membrane. IR was calculated as the average PFU per dose/ Number of alive cells at the time of titration.

Experimental design. Three experiments were conducted. Experiment 1 was conducted to assess dose variability within MD vaccines and to evaluate the effect of manufacturer, serotype, and batch. Titration of 36 vaccines of four manufacturers, 2 serotypes (1 and 3), and various

vaccines batches were evaluated. All vaccines were titrated 10 times in duplicates under optimal conditions (the vial was put on ice immediately after removing from the liquid nitrogen tank, it was thaw in a water bath at 37°C for 10-15 seconds and then kept in ice until reconstitution, diluted on the recommended diluent by the manufacturer using a 18-21g needle, rinsed the vial and the cup, mix the vaccine thoroughly with the diluent and titrated immediately) and CV was calculated. Batches refers to different serial numbers of the same vaccine serotype from the same manufacturer. These factors of vaccine manufacturing were compared for their effect on CV. Experiment 2 was conducted to evaluate the effect of IR on dose variability. IR was artificially decreased by adding non-infected CEF to the reconstituted vaccine. Vaccine was then titrated under optimal conditions and after adding non-infected CEF to the reconstituted vaccine in a 1:2 ratio and a 1:5 ratio before titrating. Number of live cells were calculated before titration in all cases. Experiment 3 was conducted to evaluate the effect of time and mixing of the vaccine on dose variability. Each vaccine was titrated under optimal conditions and 1-hour post reconstitution under two conditions: continuous mixing with magnetic stirrer and lack of mixing at room temperature (21°C).

Analysis of the data and statistical analysis. In order to analyze differences in CV among groups and the effect of different factors, our data were modified into frequencies. A subjective CV threshold of 20% was established to differentiate among low variability ($CV < 20\%$) and high variability ($CV \geq 20\%$). Frequencies of vaccines with low variability ($CV < 20\%$) vs the total number of vaccines titrated for each category were included in the analysis and presented in this paper. Frequencies were analyzed using Fisher's exact test (<http://vassarstats.net/index.html>). A binomial test (vassarstats.net/binomialX.html) was used to analyze the difference between CV frequencies in Experiments 2 and 3. The level of statistical significance considered was $p < 0.05$.

4. Results

Dose variability in cell-associated Marek's disease vaccine (Table 1). Vaccine dose variability was found in 36 vials of vaccines representing four different manufacturers, two serotypes (1 and 3), and multiple batches. CV varied between 10% and 34%. Twenty-two of the evaluated vaccines had CV equal or lower than 20% (low variability), while 14 of them had CV higher than 20% (high variability).

Titer ranges in cell-associated Marek's disease vaccines (Figure 1). The minimum, maximum and average PFU per dose for each of the 36 vials of vaccines representing different manufacturers, serotypes and batches is presented (Figure 1). When considering the maximum PFU per dose, all vaccines met minimum required dose for licensing in the United States (1,000 PFU/dose). When the average is considered, one sample (A-1-2) fell below the minimal PFU per dose. When considering the minimum reported PFU, three of the vaccines (A-1-2, C-3-4, B-3-1) did not meet the required titers and two vaccines (B-3-2, B-3-6) were at 1,000 PFU/dose.

Effect of vaccine manufacturer, vaccine serotype, and vaccine batch on dose variability (Table 2). The effect of several factors on the CV has been evaluated. For the statistical analysis CV data has been converted into frequencies. Results show the frequency of vaccines with low variability ($CV < 20\%$) vs the total number of vaccines titrated for each category. No differences in variability between manufacturers, serotypes, and batches were detected ($p < 0.05$)

Effect of infectivity rate on dose variability (Table 3). Addition of non-infected CEF to the reconstituted vaccine resulted in decreased IR. There was a trend to increase dose variability (measured as CV) as IR decreased. However, differences among vaccines were observed. For most vaccines (A-1-2, B-3-1, D-3-6, D-3-3, and D-3-5) adding CEF at 2X resulted in increased CV. However, for others (D-1-2 and D-3-4), CV was only increased after CEF were added at 5X.

For the statistical analysis CV data has been converted into frequencies. Results show the frequency of vaccines with low variability ($CV < 20\%$) vs the total number of vaccines titrated for each category. Variability significantly increased as the IR decreased ($p < 0.05$).

Factors of vaccine handling/administration on dose variability: time and mixing of the vaccine (Table 4). The effect of time and mixing of the vaccine is presented in Table 4. Dose variability measured as CV increased with time and this effect was greater when vaccines were not mixed. Differences among vaccines were found. While in vaccine C-3-1 CV increased from 11.6 to 20.2 and 49.5 at T1 mixing and T1-not mixing, respectively, no major effect was observed in vaccines C-3-2 and D-3-1. For the statistical analysis CV data has been converted into frequencies. Results show the frequency of vaccines with low variability ($CV < 20\%$) vs the total number of vaccines titrated for each category. Variability increased significantly with time. Lack of mixing resulted in higher variability although differences were not significant between T0-T1 mixing and T0-T1 not mixing.

5. Discussion

Results from this study confirm that dose variability due to the cell-associated nature of the vaccine makes it impossible to provide a uniform dose to all chickens in a flock, even in those vaccinated from the same vaccine vial. In our study, every vaccine vial was titrated 10 independent times and dose variability calculated as the coefficient of variability ($CV = \text{standard deviation} / \text{average PFU} \times 100$). Our results demonstrated that CV varied from 10% - 34% and occurred regardless of manufacturer, vaccine serotype, and vaccine batch. Infectivity rate, time after reconstitution and mixing during administration impacted variability.

The requirement for licensing MD vaccines in the U.S. is a virus titer per dose of least 1,000 PFU [254]. However, most biologic manufacturers exceed this requirement as a precaution against lack of protective immunity, partly to counteract the reduction in the dose that occurs for the normally handling of the vaccine but also because every vaccine requires different levels of PFU to achieve the maximum protection [178, 231]. Our results emphasize that MD vaccine doses should be given as a range of dose more than average PFUs, as dose variability will affect the dose of vaccines that chickens receive. In the current study we have not evaluated the effect of a CV on protection but the effect of CV on the vaccine dose. However, the effect of vaccine dose on protection has been well studied [116, 231]. While all the vaccines were above the required minimum PFU at maximum dose, 3 vaccines were below and 2 were at the minimum required level at the minimum dose. Our results were obtained under optimal conditions and as soon as the vaccine was reconstituted. Other factors decreasing titers will have even more of a detrimental effect.

Following the protocol for vaccine reconstitution properly is crucial to ensure vaccination efficacy [230, 262] as it ensures virus survival. Maintaining cell viability of the vaccines is the primary challenge with cell associated vaccines, as cells are sensitive and labile to temperature, osmotic pressure, osmolality, physical manipulation, and pH [229]. The addition of antibiotics can also reduce titers [218]. Furthermore, over-diluting the vaccines, a common practice in many countries to reduce cost, leads to reduced MD protection and has an overall negative effect [116]. As these factors can negatively impact vaccine dose, a vaccine with a high CV will be at further risk of lacking the required minimum protective dose when its titers are lowest.

A concept introduced in the present study is the vaccine's infectivity rate (IR = % of cells infected with viable vaccine virus). The fragility of infected cells, even under optimal conditions,

poses challenges in evaluating IR. Flow cytometry was initially performed in order to quantify live and total number of cells (IR ranged from 1% till 46% with an average of 14%), but due to the long incubation periods of the protocol, we could not ensure that the vaccine virus was not dying and skewing results (data not shown). Therefore, IR for each vaccine was measured as the average number of PFU/# live cells at the time of titration. There are multiple factors that could influence IR at the time of vaccine manufacturing such as vaccine virus, cell culture media, variability within the CEFs batches, and cell culture media and conditions. In our study IR had an effect on the CV, the lower the IR the higher the CV. Recently there has been a trend for vaccine companies to increase the number of doses per vial. Commercially, it is highly desirable that each vial contains 4,000 doses instead of 2,000 or 1,000 vaccine doses. While increasing the number of doses per vial will not have any negative effect in the CV of that vaccine batch if IR is high, it could be very detrimental if the IR is low as higher total of cells will be required to reach higher PFUs, overloading the suspension, promoting improper homogenization and increasing the CV.

Vaccine manufacturers recommend using the reconstituted vaccine within one hour. In this study we have demonstrated that both titers and CV are affected by time. In our study, CV increases when mixing of the vaccine was not performed, albeit no significant differences were detected between titrations after one hour of reconstitution mixing and not mixing. The lack of significance could be partly due to the low number of vaccines titrated and further studies including more vaccines are warranted. Death of infected cells with time, clumping, and sedimentation of cells might be responsible for our results. MD vaccines are unstable suspension of cells, they tend to sediment and flocculate, further worsening dose uniformity. Since infected cells form syncytia, they tend to coalesce more than noninfected cells and they are more

susceptible to sedimentation. Furthermore, infected cells are more susceptible to external factors and viability will be lower than noninfected cells. Maintaining cell viability during and after reconstitution and achieving proper homogenization of the cell suspension should be a priority in ensuring proper vaccination. *In ovo* vaccination greatly reduces the time to administer a bag of vaccine which will be beneficial for both titers and CV. However, to maintain the cells in suspension, a modification to the current diluent formula that prevents sedimentation while refrigerated is a potential solution. Alternatively, adding mechanisms that allow mechanical mixing of the suspension to the vaccination equipment will reduce the negative effect on CV.

This study demonstrates for the first time that there is an inherent, large variability of the vaccine dose within a vaccine vial in MD vaccines. Such variability can have detrimental effects on the immunization of the flock as some birds will receive suboptimal vaccine doses even when guidelines for vaccination have been strictly followed. Our results highlight the need to review current titration protocols and the way titers are understood. The average PFU/dose does not provide enough information and a titer range or a CV should be included. Based on our results, a vaccine titration should be done in several independent replicates to truly assess the titer range. In the current study we did 10 independent replicates but further studies are warranted to determine the minimum number of replicates necessary to assess the titer CV. Because reduced IR has a negative effect on CV, manufacturers should be discouraged from any practice that could reduce further IR, such as increasing dose numbers per vial of vaccine when initial IR is low. Our results also emphasize the need of optimizing handling and administration of the vaccine since time and/or improper homogenization of the vaccine increase CV. Further research on how to minimize dose variability in MD vaccines are warranted.

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6. Tables

Table 1. Dose variability in cell-associated Marek's disease vaccines.

Vaccine Code ¹	Vaccine ²	PFU av ³	CV ⁴
B-3-11	HVT	5120 (4400-5800)	10
B-3-10	HVT	2057 (1625-2375)	11
B-3-10	HVT	2713 (2350-3000)	11
D-3-5	rHVT	6410 (5100-7500)	11
A-1-2	CVI-988	860 (690-1000)	13
B-3-11	HVT	4960 (4300-5900)	13
B-3-11	HVT	4850 (3300-5600)	14
B-3-5	rHVT	1540 (1300-1900)	15
D-1-2	CVI-988	3000 (2300-3900)	15
D-3-6	rHVT	4765 (3350-5800)	15
B-3-10	HVT	2765 (2150-3500)	16
B-3-11	HVT	5100 (4100-6100)	17
C-3-3	rHVT	4010 (2900-5000)	17
D-3-3	rHVT	2370 (1900-3100)	17
A-1-3	CVI-988	5081(3567-6400)	18
B-3-11	HVT	4800 (3700-6300)	18
C-3-1	rHVT	3980 (3300-4550)	18
A-1-1	CVI-988	1605 (1150-2050)	19
B-3-1	rHVT	2395 (1600-3200)	19
B-3-1	rHVT	2426 (1750-2950)	19
C-3-2	rHVT	6595 (4200-9700)	19
D-3-7	rHVT	3070 (2000-3600)	20
D-3-1	rHVT	4115 (1700-5300)	21
A-3-1	HVT	2495 (1800-3700)	22
B-3-1	rHVT	2848 (1967-3967)	22
B-3-4	rHVT	2960 (2000-4100)	22
B-3-8	HVT	3340 (2300-4700)	22
B-3-9	HVT	2520 (1500-3400)	24

Table 1 (continued).

D-3-4	rHVT	2210 (1500-3100)	24
B-3-7	rHVT	2420 (1600-3800)	26
B-3-2	rHVT	1600 (1000-2300)	28
B-3-3	rHVT	2860 (1800-4100)	32
B-3-6	rHVT	2120 (1000-3000)	32
C-3-4	rHVT	1500 (800-2500)	32
B-3-1	rHVT	1647 (700-2700)	34
C-1-1	CVI-988	3685 (2000-7000)	34

¹ Vaccine code indicates the manufacturer (A-D), serotype (1 and 3), and the batch number

² Commercial CVI-988 and HVT (both wild type and recombinant, rHVT) vaccines were titrated.

³ Average plaque forming units (PFU) of titrations and the minimum and maximum PFU value in parenthesis. Each vial was titrated 10 times

⁴ Coefficient of variability of PFU per vial. CV was calculated as the standard deviation divided by average PFU multiplied by 100. For the purpose of this study, we will consider $CV \leq 20\%$ to be normal and $CV > 20\%$ to be high

Table 2. Effect of various factors on CV.

Factor ¹		CV ≤ 20% ²	CV interval ³	P value ⁴
Manufacturer	A	3/4	13-22	0.8
	B	11/20	10-34	
	C	3/5	17-34	
	D	5/7	11-24	
Serotype	1	4/5	13-34	0.6
	3	18/31	10-34	
Batch	A-1	3/3	13-19	0.7
	C-3	3/4	17-32	
	D-3	4/6	11-24	
	B-3-1	2/4	19-34	0.1
	B-3-10	3/3	11-16	
	B-3-11	5/5	10-18	

¹Several factors were evaluated for their effect on CV. Manufacturer includes companies A-D, serotypes were 1 and 3. Batches refers to different batches from the same vaccine. Various batches of four different vaccines (A-1, C-3, D-3, and B-3) were titrated. In addition several vials from the same batch were titrated for vaccine B-3.

²A CV ≤ 20% is considered acceptable for the purpose of this study. The frequencies are shown as the number of individual titrations that resulted in a CV of or below 20% out of the total number of titrations of vaccines performed. Comparisons within each factor were done by a Fisher's Exact Probability Test. Results indicate there were not any statistically significant (P<0.05) differences.

³The interval shows the minimum and maximum CV values for each titration of vaccine in %

⁴Fisher exact test was calculated to evaluate differences among frequencies. Comparisons were done for manufacturer, serotype, batches of vaccines in which only one vial per batch was titrated, and batches of vaccines in which multiple vials were titrated. Significance was considered at p<0.05.

Table 3. Effect of adding non infected chicken embryo fibroblast to the reconstituted vaccine on infectivity rate and dose variability.

Vaccine code ¹	Reconstituted vaccine ²		Adding 2X CEF ³		Adding 5X CEF ⁴	
	IR	CV	IR	CV	IR	CV
A-1-2	2.15	13	1.08	18	NA	NA
D-1-2	5.5	15	2.9	14	1.29	20
B-3-1	0.69	19	0.35	53	0.03	37
D-3-6	13.61	19	6.81	22	NA	NA
D-3-5	18.3	11	5.04	24	2.96	18
D-3-3	5.3	17	2.8	21	0.53	29
D-3-4	7.4	24	3.7	22	1.3	25
Statistical evaluation- Frequency of CV< 20% ^d						
Reconstituted vaccine		Adding 2X CEF		Adding 5X		
6/7 ^a		2/7 ^b		1/5 ^c		

¹ Vaccine code indicates the manufacturer (A-D), serotype (1 and 3), and the batch

² Vaccine was diluted following manufacturer's recommendations. Infectivity rate (IR) was calculated as the average of PFU per vial divided by the number of alive cells. Dose variability was calculated as coefficient of variability (CV = standard deviation divided by average PFU and multiply by 100)

³ Non infected chicken embryo fibroblast (CEF) were added to the reconstituted vaccine at 2X or 5X. NA = information not available.

⁴ Binomial test was calculated to evaluate differences among proportions. Different lowercase superscripts indicate that differences were statistically significant p<0.05

Table 4. Effect of mixing reconstituted vaccine and timing of administration on dose variability.

Vaccine code ¹	CV T0 ²	CV T0-T1 mixing ³	CV T0-T1 no mixing ⁴
A-1-1	17	22	30
B-2-1	19	34	35
C-3-1	11	20	50
C-3-2	17	15	17
D-3-1	21	19	21
A-1-2	17	32	26
A-1-3	17	27	35
Statistical evaluation- Frequency of CV < 20% ⁵			
	T0	T0-T1 mixing	T0-T1 no mixing
	6/7 ^a	3/7 ^b	1/7 ^b

¹ Vaccine code indicates the manufacturer (A-D), serotype (1 and 3), and the batch

² T0= titration was done immediately after reconstitution. Dose variability was calculated as coefficient of variability (CV = standard deviation divided by average PFU and multiply by 100)

³ T0-T1 = titration was immediately after reconstitution and one hour later and reconstituted vaccine was continuously mixed by magnetic stirring. Dose variability was calculated as coefficient of variability (CV = standard deviation divided by average PFU and multiply by 100)

⁴ T0-T1 no mixing = titration was immediately after reconstitution and one hour later and reconstituted vaccine was not mixed. Dose variability was calculated as coefficient of variability (CV = standard deviation divided by average PFU and multiply by 100)

⁵ Binomial test was calculated to evaluate differences among proportions. Different lowercase superscripts indicate that differences were statistically significant p<0.05

7. Figures

Figure 1. Maximum, average, and minimum PFU per dose of Marek's disease vaccines.

Thirty-six MD vaccines were titrated 10 independent times and for each vaccine the maximum

(A), average (B), and minimum (C) PFU per dose is presented. In the USA, the minimum required PFU per dose is 1,000. While the requirements are met when considering the maximum PFU/dose and for most vaccines when considering the average PFU/dose, five vaccines were below the required level at the minimum PFU/dose.

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