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MAREK'S DISEASE VIRUS SUPERINFECTION:
THE EFFECT OF VIRAL DOMINANCE AND
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MAREK'S DISEASE VIRUS SUPERINFECTION: THE EFFECT OF VIRAL DOMINANCE AND EXPOSURE INTERVALS

By

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ABSTRACT

MAREK'S DISEASE VIRUS DUAL INFECTION: THE EFFECT OF VIRAL DOMINANCE AND EXPOSURE INTERVALS

By

John Richard Dunn

Marek's disease vaccination protects chickens against the development of disease, but does not prevent infection or transmission of infectious virus. Previous studies have demonstrated the presence of multiple strains of Marek's disease virus (MDV) simultaneously circulating within poultry flocks, leading to the assumption that individual birds are repeatedly exposed to a variety of virus strains in their lifetime. Initial experiments were designed to evaluate whether chickens could be infected simultaneously with two fully virulent serotype 1 MDV strains, using two similar (rMd5 and rMd5//38CVI) and two different (JM/102W and rMd5//38CVI) virus pairs. Bursa, feather follicle epithelium (FFE), spleen, and tumor samples were collected at multiple time points to determine the frequency and distribution of each virus present following simultaneous infection using pyrosequencing, immunohistochemistry and quantitative PCR.

Following simultaneous dual challenge, mixtures of both virus strains were present in bursa, FFE and tumors samples in 35 of 84 birds (42%). Dominance, as defined by a majority frequency in samples regardless of inoculation order, was consistently demonstrated for the more virulent rMd5//38CVI strain when challenged with the less virulent JM/102W strain. Dominance was not observed between the two similar strains. Both viruses were readily detected in individual bursa and feather

follicles from 16 of 23 birds (70%), indicating that the presence of one virus did not exclude the second virus. Results from tumors demonstrated differences between the predominance of each strain within multiple tumors from individual birds, suggesting but not proving a possible polyclonal origin. These effects may be relevant under field conditions as no significant differences in dominance were detected in vaccinated.

Exposure to multiple MDV strains is presumably more likely to occur over time in natural conditions, so an additional set of experiments were designed to evaluate the effect of time between challenges on the effect of superinfection using the same virus pairs and similar sampling protocol. The effect of time interval was strong as superinfection was observed in 82 of 149 (55%) FFE samples following short interval challenge (24 hours) compared to only 6 of 121 (5%) samples following long interval challenge (13 days). Significant dominance was detected again using JM/102W and rMd5//38CVI following a short interval challenge, but detection was unapparent following the longer challenge interval. Tumor samples generally consisted of a single predominant virus, but both viruses were detected again within a small portion of tumors. No significant differences were detected between virus frequencies in vaccinated versus unvaccinated chickens.

Viral dominance and temporal relationships may be important factors that influence the outcome of coinfection under field conditions, including the potential outcome of emergence or evolution of more virulent strains. Understanding what conditions exclude superinfection may help slow the evolution and establishment of new strains within poultry flocks.

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KEY TO ABBREVIATIONS

ADOL	Avian Disease and Oncology Laboratory
dpi	Days post infection
DNA	Deoxyribonucleic acid
FFE	Feather follicle epithelium
HVT	Turkey herpesvirus (vaccine)
IHC	Immunohistochemistry
J	JM/102W
M1	rMd5
M2	rMd5//38CVI
Mab	Maternal antibody
MD	Marek's disease
MDV	Marek's disease virus
pp38	MDV phosphoprotein pp38
qPCR	Quantitative polymerase chain reaction
S1	Serotype 1 MDV
S2	Serotype 2 MDV
S3	Serotype 3 MDV
SNP	Single nucleotide polymorphism
VCN	Virus copy number
vMDV	Virulent MDV
vvMDV	Very virulent MDV

Chapter 1

LITERATURE REVIEW

Relevance

Superinfection is defined as an additional infection occurring during the course of an existing infection. Superinfection with two virulent Marek's disease virus (MDV) strains is likely an important step in the pathway towards establishment of evolved strains within a population. Demonstrating whether superinfection occurs and the conditions that promote infection and transmission of a second MDV strain would provide knowledge to help slow evolution of the virus within poultry flocks.

Marek's disease background

Marek's disease (MD) is a lymphoproliferative disease in chickens caused by an alphaherpesvirus, MDV. The disease is typically characterized by enlarged nerves and visceral tumors composed of transformed T cells. Marek's disease (MD) remains an important problem in poultry throughout many parts of the world, due to condemnation losses and the cost of vaccination. As recently as 2002, an outbreak of Marek's disease in Indonesia was reported to have killed 2.8 million chickens from two major chicken suppliers with an estimated loss of $21M for the breeders (90). MDV is classified into three serotypes: serotype 1 (S1) includes pathogenic strains and their attenuated variants, serotype 2 (S2) includes all apathogenic chicken strains, and serotype 3 (S3) includes turkey herpesvirus (HVT), which is also apathogenic in chickens (12).

Serotypes 2 and 3 have been used as vaccines, as well as several attenuated strains of S1. Although vaccination has successfully been used to prevent disease, the

vaccines do not prevent against infection or transmission of the virus. Witter et al. reported that in birds vaccinated with HVT at hatch challenged with JM/102W at day 15, MDV was not isolated from vaccinated and challenged birds until 14 days after challenge, compared to 3 days in non-vaccinated challenged birds (104). Calnek et al. similarly reported on the effects of vaccination with HVT (500 pfu) at hatch followed by JM-10 virus (500 pfu) infection 17 days later (21). According to the authors, splenomegaly associated with JM-10 infection was prevented, viremia levels were markedly reduced, and expression of virus internal antigens in lymphoid organs was completely prevented. Thus, if vaccinated birds are less susceptible to virus replication, can one still demonstrate S1 superinfection in such populations?

Marek's disease pathology

Factors that influence pathogenesis of MD include age at exposure, maternal antibody, genetic constitution, age resistance, strain of virus, sex, and preceding or concurrent infections with immunosuppressive agents. Superinfection may be an additional factor that influences the pathogenesis of MD. Infection with MDV can be divided into four phases: 1) Early cytolytic infection, 2) Latent infection, 3) Late cytolytic infection, and 4) Transformation. The stages of infection with serotype 1 MDV have been described in multiple reviews (7, 66, 70, 71).

Early cytolytic infection occurs in the primary lymphoid organs, consisting of the bursa of Fabricius (bursa), thymus and spleen. The cytolytic infection appears at approximately 3 days post infection, peaking at 5-7 days and resolving by about 2 weeks (72). Schat et al. reported that embyronal bursectomy eliminated early cytolytic

infection, although not latent infection (83). Shek et al. supported this finding by demonstrating that B cells were the principal targets of MDV during early cytolytic infection (85). Early cytolytic infection is subdued or absent in birds with maternal antibody (15, 72) or prior vaccination (17, 82, 87).

At approximately 7-8 days post infection, early cytolytic infection switches to latent infection, defined as the presence of viral DNA in the absence of viral transcripts and proteins. Viremia refers to the latently infected peripheral blood lymphocytes. The viral genome persists with no viral or tumor antigen expression, and there is no virus production except following reactivation. Interestingly, the predominant lymphocyte for latent infection are activated T cells, with only a few latently infected B cells (22). It is unclear what causes the switch from cytolytic infection to latency, but reactivation of the viral genome can occur by both *in vitro* cultivation and by inoculation of susceptible chickens.

A second cytolytic phase of infection has been reported from 14-21 dpi, affecting the thymus, bursa, kidney, adrenal gland, proventriculus, and some epithelial tissues including FFE (16). Calnek and Hitchner first reported the predilection of MDV to the FFE, describing fluorescent staining of antigen varying from a few stained cells in the superficial layers to heavy staining several cell layers deep surrounding the feather shaft (20). Replication of MDV in the FFE is fully productive and creates large numbers of enveloped, fully infectious virions (18). As early as 2 weeks after inoculation or exposure, MDV is spread in the feather dust to other chickens as the keratinized layer of infected epithelial cells is sloughed and shed (17). Shedding appears to peak at approximately 3-5 weeks and continues indefinitely (94, 106).

Infected feather dust can remain infectious in a poultry house for at least several months at 20-25°C and for years at 4°C (17, 58). The fully productive replication of MDV in FFE has made feather tips a useful source for virus detection (18, 35, 36, 74).

Within feathers, lymphoid lesions have been described that developed in the feather pulp. Fujimoto et al. described two types of lesions: R-type (non-tumorous response) and T-type (tumorous proliferation) (39). Ten years later, Moriguchi et al. further classified the lesions into three types: R_1-type – variable infiltration by small lymphocytes; R_2-type – edema and infiltration by plasma cells and small lymphocytes; and T-type – proliferation usually by medium lymphocytes or blast cells (64). Cho et al. have confirmed similar types of lesions using feather pulp cytology, and concluded feather pulp was an effective diagnostic and prognostic tool for MD incidence (29). The relationship between virus load in feather pulp and pathogenesis has not been demonstrated, but Baigent et al. recently reported quantitation of MDV genomes in feather tips with copy numbers considerably greater than in the spleen and peripheral blood lymphocytes (3).

Lymphoid proliferation can begin as early as 1 week post infection and can produce grossly visible tumors from as early as 2 weeks post infection (73). Tumors typically consist of pleomorphic lymphoid cells, ranging from small lymphocytes to large blast cells. Common sites affected include spleen, heart, gonads, liver, kidneys, proventriculus, intestine, lung, thymus, bursa, skeletal muscles, skin, and peripheral nerves. The skin, therefore, is associated with both dermal lymphocytic infiltration and FFE late cytolytic infection. Ross et al. reported that although nearly all T cells in MD lymphomas contain the MDV genome, most cells appear latent because only a small

number of cells produce transcripts (80). The authors reported that among others, Meq transcripts were abundant in purified lymphoma cell populations, but pp38 transcripts were not. Using a knockout recombinant virus, Lupiani et al. demonstrated that Meq is involved in lymphocyte transformation, but not lytic replication in chickens (62). Gimeno et al. showed that MD tumors have much higher amounts (~100-fold) of MDV DNA compared to latently infected tissues (42).

There is current debate on the clonality of MD tumors. Delecluse et al. performed *in situ* hybridization to examine MDV genome structure and its relationship to host DNA in MD lymphomas (37). The authors reported that tumors were monoclonal based on the identical number of hybridization signals of interphase nuclei and the identical pattern of chromosomal integration in metaphase spreads. In contrast, Burgess and Davison have suggested a polyclonal tumor origin based on their analysis of T cell receptor (TCR) variable β chain gene family expression (13). A recent abstract presented by Cheng et al., also suggested that MD tumors were not necessarily clonal based on TCR mRNA spectratyping (24). In an experiment evaluating expansion of a 132-bp repeat region, Silva et al. demonstrated differences in virus populations in two tumors from one bird (86). Pathology also favors polyclonal tumors, as there are lymphoid proliferations in many tissues at a very early time without evidence for a single focus and subsequent metastasis.

Evolution of Marek's disease

A major concern facing the poultry industry is the evolutionary trend of MDV towards greater virulence (95). Two waves of evolution have been described in a recent

review (65). The first wave of virus evolution from mild (mMDV) to virulent (vMDV) strains was during the 1950's and was likely due to the transformation of the industry to highly intensive poultry practices. The continuous availability of large populations of susceptible birds is certainly a major contributor. Subsequent evolution has been attributed to the introduction of successive generations of MD vaccines (92). Because MD vaccines fail to produce a sterilizing immunity, viruses are able to replicate and shed within vaccinated hosts.

Evolution of strains within a flock could begin from an internal or external source. A more virulent mutated virus may develop spontaneously within a resident bird, or a more virulent virus could arrive from an outside source, such as introduction of new birds or from a neighboring flock via fomites or contaminated personnel. By either source, this new virus needs the proper fitness traits to become established and for transmission to other birds. Once multiple strains become established in a flock, birds may become repeatedly exposed to superinfection.

What properties are necessary for one virus strain to have a competitive advantage over another strain? Virulence has historically been defined by oncogenicity. The pathotyping classification has been used to designate differences in virulence of field viruses based on frequency of MD lesions induced in vaccinated and unvaccinated birds. Techniques for pathotyping have evolved and were recently reviewed by Witter et al. (98). Pathotyping became an important classification of viruses not long after increased virulent forms of MDV were initially reported (5). Early classifications by Biggs et al. differentiated strains as classical or acute, with the term acute based on higher morbidity and mortality, shorter time until onset, and a higher incidence of

visceral tumors (11). Biggs et al. later included additional criteria for this designation based on histological lesion scores (9). Witter first designated the virulence of MDV strains based on responses in vaccinated chickens, using a cut-off value of HVT protection (77%) (93). Several years later he included prototype isolates as a comparison against field isolates to better standardize the pathotype designations from other laboratories (97). Specifically, vvMDV (very virulent) isolates were those with MD lesions in HVT-vaccinated, susceptible birds at a rate greater than a prototype vMDV(virulent) isolate, such as JM or GA. Other groups began using these techniques, but the procedures were variable with different prototype viruses and chicken lines used for making the designation of vvMDV (14, 50, 51, 89). Witter recognized an increased virulence of MDV strains in the early 1990's in bivalent-vaccinated chickens, leading to four current pathotype designations: m (mild), v (virulent), vv (very virulent), and vv+ (very virulent plus) (95). It still remains difficult to distinguish between similar strains or to be certain that two strains do not differ.

Perhaps virulence is not defined by oncogenicity, but by the increased potential for replication and/or transmission. Citing the work of Gandon et al., Nair has suggested that since MDV vaccine does not provide sterile immunity, a partially effective vaccine may be leading to virus evolution with increased transmission potential and adaptation to vaccinated hosts (40, 65). The topic of replication potential and its relationship to virulence has been the topic of several recent papers. Yunis et al. infected resistant and susceptible chickens with v and vv+ MDV strains and measured copies of DNA or RNA transcripts during 1-10 days post infection (dpi) using qPCR and qRT-PCR (109). Viral DNA increased with initiation of viral transcription for both

virus strains and chicken strains. The virulent virus became latent at 6 dpi, but the vv+ virus never went into latency in both chicken lines during the 10 days. The prolonged virus replication and presence of viral transcripts for the vv+ virus throughout 10 days could cause more severe damage and atrophy of lymphoid organs.

Using quantitative RT-PCR and PCR to measure lytic viral transcription and DNA replication, Jarosinski et al. showed significantly higher levels of viral replication for the vv+ strain RK-1 in two chicken lines compared with the v strain JM-16 (57). Calnek et al. demonstrated a prolonged phase of cytolytic infection at 7-8 dpi for three vv+ isolates (RK-1, 584A, 648A) compared with two v isolates (JM16, GA5) (19). Gimeno et al. compared higher and lower protective vaccine pairs originating from the same MDV strain and found better *in vivo* replication for the higher protective strains (45).

Past studies have correlated viremia to tumor development (106). Recent work using quantitative PCR assays have confirmed the associations. Gimeno et al. evaluated the load of MDV in peripheral blood at 3 weeks with subsequent development of MD tumors and found chickens that developed tumors had significantly higher MDV load in peripheral blood compared to chickens that did not develop tumors (41). The authors also noted that HVT virus load in peripheral blood at 3 weeks had no correlation with subsequent tumor development. In a similar study using quantitative PCR, Islam et al. reported the mean MDV copy number in peripheral blood was greater in chickens subsequently exhibiting gross MD lesions than in chickens with no lesions at all four time points of 14, 21, 28, and 35 days post challenge (55). The bottom line may be that evolution is driven by the ability of replication, which is related to oncogenicity.

Marek's disease transmission

Transmission of MDV occurs through the production of fully infectious virions in the feather follicle epithelium (FFE) that are spread in the feather dust as early as 2 weeks after exposure and then inhaled by other birds (18). Carrozza et al. demonstrated the virus in the debris of dead stratified epithelial cells and moulted feathers with infected cells attached (23). The authors proposed that highly infectious but labile cell-free virus particles were associated with skin debris, whereas keratin-wrapped particles were less infectious but more stable in the environment. Shedding appears to peak at approximately 3-5 weeks and continues indefinitely at quantities as high as 10^9 virus copies per chicken per day, irrespective of vaccination status (53, 94, 106). Infected feather dust can remain infectious in a poultry house for at least several months at 20-25°C and for years at 4°C (17, 58). The continuous transmission, long survivability of the virus, and large population of birds favor a significant viral load within a poultry house.

Evidence of multiple strains within a flock

There is evidence that multiple strains of MDV can circulate simultaneously within the same flock. Biggs et al. reported on the epidemiology of MD in six flocks from a broiler female grandparent line (10). Virus isolates were propagated from five flocks, and then 25 isolates were examined and classified for their pathogenicity as either apathogenic, classical or acute. Interestingly, four of the five flocks had strains

present from more than one level of pathogenicity. Two flocks, including a single house from one flock, had strains present from all three levels of pathogenicity.

In a follow-up study the next year, Biggs et al. recorded mortality from a flock of 8000 female broiler breeders ages 8-22 weeks to determine the effect from supply flock, rearing house/pen, and production house/pen (8). The study was based on two supply flocks, placed in pens at three rearing houses, and then at 8-9 weeks each pen was divided and placed in one of two production houses. The authors reported that only the rearing house pen of origin significantly affected the incidence of MD. Further, the authors concluded the levels of mortality were likely determined by the events occurring at the time of primary infection and the variable incidence of MD was due to a complex interrelationship between strains of virus of variable pathogenicity. This mixture resulted in natural vaccination by apathogenic strains prior to exposure to infection with pathogenic strains in some groups and not in others.

Jackson et al. followed up on the interrelationship between strains of variable pathogenicity (56). The authors confirmed the presence of multiple pathotypes circulating within the same flock and that the environment during the first 8-9 weeks of life was of greatest importance in subsequent mortality from MD. MD mortality was closely associated with the sequence of infection and frequency of isolation of viruses of differing virulence. For example, three pens that experienced high mortality had early isolates that were of the acute type, whereas two different pens with low incidence of MD had apathogenic strains that predominated throughout early and late isolations.

Witter et al. evaluated the virulence of multiple isolates (2-3) from two separate flocks and found very similar pathotypes (95). These isolates were all from tumor-

bearing birds which may have created a bias towards isolation of more virulent strains. This also suggests, however, that a single virus strain may have the ability to become dominant. The studies described above provide evidence for multiple strains of MDV circulating with a flock, but don't address the possibility of multiple strains within the same bird.

Evidence of superinfection following S2 or S3 infection

Superinfection has been demonstrated following initial infection with MD vaccine strains. Witter et al. showed that S1 virus was isolated when MDV was challenged after initial infection with S3 turkey herpesvirus (HVT) (104). Okazaki et al. evaluated the temporal relationship between HVT vaccination and MDV challenge (69). The authors concluded that HVT was fully protective when administered at least one week before challenge, and the vaccine gave partial protection when administered simultaneously with challenge at one day of age (25% MD vs 90% MD in unvaccinated challenged group).

Calnek et al. infected birds with S2 SB-1 and S3 HVT-4, followed by JM-10, to study the effect of vaccination on early pathogenesis (21). Viremia was present, although reduced following subsequent challenge.

Cho et al. reported evidence of coinfection with acute (Id-1) and mild (HN) MDV strains following various intervals between challenge, although HN was later classified as a nonpathogenic S2 strain (28, 81, 93) Cho and Kenzy reared several chicken lines in pens with contaminated litter from two groups of birds that had been challenged with either Id-1 or HN MDV strains (27). Blood was collected weekly from

1-6 weeks post exposure and strains were identified by viremia based on plaque morphology. The acute strain alone was first to be identified in all chicken lines at 1 or 2 weeks post exposure. By three weeks, dual infection was identified in several birds which generally persisted through 6 weeks. In a few cases the mild strain was singly identified beginning at 3 weeks. The authors concluded that infection with one strain, whether acute or mild, did not exclude subsequent infection by the other strain.

In another study, Cho challenged birds by subcutaneous injection singly and dually with Id-1 and HN strains of MDV to compare viremia responses between resistant and susceptible lines of chickens (26). Based on plaque morphology, Cho identified both strains in susceptible and resistant birds. In susceptible birds, isolates with both strains were present at 1 week continuing through termination at 8 weeks, with gradual reduction from 6 mixed isolates down to 1 mixed isolate at 8 weeks. In the resistant line of birds, the mixed isolates were present at 1 week but only lasted through 4 weeks, after which only the mild strain was isolated at 6 and 8 weeks. Interestingly, in the resistant line, the viremia persisted in all birds singly challenged with the mild strain, but challenge with the acute strain resulted in a decrease in viremic birds after 2 weeks.

Cho then studied the relationship of MDV simultaneous challenge in blood and feather follicles using the same strains described above, Id-1 and HN (25). Based on plaque morphology, dual viremia was detected in all of the susceptible chickens at 2, 4, and 6 weeks post challenge, but in less than half of the resistant chickens. Not all feather tip extracts had detectable virus, but consistent with his prior experiment,

feathers with dual virus maturation had more pathogenic than apathogenic MDV in susceptible chickens and more apathogenic MDV in resistant chickens.

Superinfection following attenuated S1 infection

In addition to S1 superinfection following initial infection with S2 or S3 strains, superinfection has also been demonstrated following initial infection with attenuated S1 strains. Churchill et al. illustrated that S1 viruses can cause infection by infecting birds with live attenuated S1 virus (HPRS-16/att) followed by more virulent strains (30). Rispens soon after demonstrated infection after initial challenge with the avirulent CVI988 strain (79). Witter and Kreager also recently demonstrated infection and MD lesions after initial infection with a series of fully attenuated S1 strains, with increased MD prevalence proportionate to the virulence of subsequent challenge strains (100).

Superinfection with two fully virulent MD strains

Laboratory-based evidence that superinfection can occur with two fully virulent S1 MDV strains is limited. Ianconescu et al. attempted to evaluate the effect of early low virulent natural exposure on subsequent challenge with virulent JM MDV (48). Nonvaccinated farm-reared chickens were removed from the farm and challenged with JM at 5, 7 or 12 weeks of age. Uninoculated groups developed MD tumors and antibodies after isolation, confirming natural exposure on the farm. At 12 weeks, farm-reared chickens inoculated with JM had no difference in tumor incidence compared to unchallenged birds. Thus, there was no clear evidence that superinfection occurred or had an effect.

Witter and Gimeno studied the affect of MDV superinfection in adult birds, specifically asking the question if chickens exposed to MDV early in life were susceptible to a highly virulent strain as an adult (99). Virus exposed chickens were vaccinated with HVT at hatch plus challenged with JM/102W at 5 weeks. When virus exposed chickens were challenged with a highly virulent strain as adults at 18 weeks, they generally failed to develop transient paralysis or tumors. Not only were the virus exposed groups refractory to lesion indication and transient paralysis, but the viremia responses and transmission to contacts appeared minimal at best, indicating that these birds had a much reduced susceptibility to infection. The authors concluded that late outbreaks in the field are not likely due to superinfection, but are more likely triggered by unknown environmental factors. Thus, with a long time interval between exposures, both studies failed to detect any significant superinfection.

Reports from the field have suggested that superinfection of virulent MDV strains is possible, based on apparent adult-to-adult transmission of vv+ MDV with very late outbreaks occurring, even after molt (59). It would seem that superinfection must occur for the establishment of new strains within flocks.

Superinfection in other herpesviruses

Superinfection has been studied using other herpesvirus models. Richter et al. recently evaluated feline herpesvirus type 1 (FeHV-1) superinfection in cats latently infected with wild-type FeHV-1 (77). Nine SPF cats were inoculated with wild-type FeHV-1 and superinfected 8 months later with a recombinant FeHV-1 virus with green fluorescent protein substituted for glycoprotein G (rFeHV-1ΔgG/GFP). The

recombinant virus produced a temporary local lymphadenopathy, but otherwise produced no clinical or ocular signs of infection. Cyclophosphamide-dexamethasone treatment was administered 16 months after superinfection to stimulate reactivation, which was successful for the underlying wtFeHV-1 infection but no reactivation of the superinfecting recombinant virus was detected. The study unfortunately lacked a control group of cats infected only with rFeHV-1ΔgG/GFP to confirm both strains were fully pathogenic.

A study of patients in Malawi infected with human herpesvirus-8 (HHV-8), also known as Kaposi sarcoma-associated herpesvirus, analyzed sequence variation at 3 loci of the virus genome (6). The authors determined there was significant intraperson/intersample and intrasample sequence polymorphisms in 60% of patients with amplifiable HHV-8 DNA, implying HHV-8 superinfection.

A review of multiple infection of Epstein-Barr virus (EBV), a γ-herpesvirus, in healthy humans cited 9 references documenting multiple infections ranging from 2-100% prevalence among tested individuals (91). In the current study, the authors developed a genotyping technique based sequence variation in the EBV LMP-1 gene and found 2 of 9 subjects harboring multiple EBV infections. One subject typed for two different genotypes among simultaneously collected saliva and blood specimens at two time points.

Rickabaugh et al. constructed a recombinant murine gammaherpesvirus-68 (MHV-68) that overexpresses a viral replication and transcription activator (78). Along with a reduction in disease, the recombinant MHV-68 had faster replication kinetics *in vitro* and *in vivo*, and when simultaneously challenged to mice with wild-type MHV-68

was dominant and protected from secondary infection. This reference suggests that inherent dominance, or an inherent fitness trait, of one virus over another may be important in the outcome of infections with more than one virus strain.

A study evaluating *in vitro* recombination potential in cells superinfected with two bovine herpesvirus-1 (BoHV-1) recombinants demonstrated a relatively high level of superinfection and recombination when viruses were administered within two hours, but very little superinfection or recombination after two hours (63). The authors suggested there was an establishment of a barrier which reduced or prevented superinfection within the same cell, thus preventing the generation of recombinant viruses. This is an important fact that may influence the rate of virus evolution depending on infection interval *in vivo*. In addition to potential effect of inherent dominance, temporal effects may also be an important factor governing the outcome of superinfection.

Prior obstacles

Prior obstacles have made the study of virulent S1 coinfection difficult using DNA and antigen as methods for critical analysis, particularly because of the difficulty distinguishing between S1 viruses. Previous studies have relied on plaque morphology or changes in total MD lesions to make assumptions about coinfection. The recent development of mutant strains with slight differences in the MDV phosphoprotein pp38 gene has provided a convenient method for distinguishing between two similar (and two different) fully virulent strains. The function of the pp38 gene, located in the IR_L and U_L region, is not completely understood, but several studies have contributed to our

current knowledge. Xie et al. concluded that pp38, in addition to ICP4 and meq were involved in maintenance of MDV-transformed MSB1 lymphoblastoid cells (107). Cui and Qin described immunodepressive effects of pp38 (32). Several experiments have indicated that pp38 may be an important part of MDV reactivation. Infection of QT35 cells, a cell line latently infected with serotype 1 MDV, with HVT activates pp38 expression (108). The gene is also expressed in productively infected cells including the FFE (33, 49, 67, 68, 76). Gimeno et al. used a mutant virus lacking pp38 (rMd5Δpp38) to demonstrate that pp38 was necessary to establish cytolytic infection in B cells, but not FFE (43). The authors found that pp38 was also necessary to produce an adequate level of latently infected T cells and to maintain the transformed status *in vivo* by preventing apoptosis.

The recombinant rMd5 strain was generated by Reddy et al. using overlapping cosmid clones produced from wild type Md5, a vvMDV strain (75, 93, 103). The recombinant virus rMd5//38CVI was subsequently produced by Lee et al. using the rMd5 cosmid clones, substituting the pp38 gene from CVI988/Rispens (60). Thus, these two viruses share the same rMd5 backbone and are only different in the pp38 gene which differs by two single nucleotide polymorphisms (SNPs). Differentiation between these two viruses is possible by detecting the SNPs coupled with the availability of monoclonal antibodies that detect the difference. The two strains were also useful based on their similar pathogenicity, as reported by Gimeno et al., in which there were no differences in the frequency of lymphoproliferative lesions between the viruses at either 6 or 15 wpi (43). Nearly all other S1 MDV strains share the same pp38 sequence as rMd5 and therefore could all be differentiated from rMd5//38CVI. In this study,

JM/102W was chosen to pair with rMd5//38CVI as the second virus pair because of differing pathogenicity. The parental strain, JM, was isolated by Sevoian in 1962 (84), and later cloned by 3X endpoint passage in DEF and designated as JM/102W (88). JM/102W was pathotyped as vMDV by Witter and is a principle prototype strain frequently used in MD experiments (93, 95).

Monoclonal antibodies (Mab) were the other critical technology used to detect and differentiate the viruses, in addition to sequence alone. According to early published pp38 sequence data, all serotype 1 MDV strains have identical bases in the pp38 gene except bases #320 and #326 (31, 38). CVI988, however, is the only serotype 1 MDV strain that is not reactive with pp38-specific Mab H19 (61, 105). Using DNA sequencing from the pp38 gene of additional strains, Cui et al. determined the molecular basis for the difference in the antigenic specificity of H19 and T65 antibodies for the pp38 gene (34). Mab H19 was specific to base pair #320, and was only positive when adenine (A) was present. All serotype 1 MDV strains except CVI988 were positive for H19 and had A at bp #320. Mab T65 was specific to base pair #326, and was only positive when guanine (G) was present. T65 was positive for CVI988 which had G present at base pair #326. Most serotype 1 MDV strains were positive for H19 only, having A at both base pairs #320 and #326, however, a few field strains have A at base pair #320 and G at base pair #326, such as GA22, and thus are positive for both H19 and T65. Pyrosequencing was designed to detect the differences at base pair #320, thus measuring the frequency of A (JM/102W or rMd5) vs. G (rMd5//38CVI) in DNA samples.

Goals of current study

Superinfection of multiple virulent MDV strains seems necessary to allow the establishment of evolved MDV strains within a flock. Understanding what events are necessary for superinfection to occur will increase our understanding of the conditions necessary to allow viruses to interact within a host and evolve to greater virulence or prevalence in a population.

This current study had several primary goals. 1) To demonstrate whether two virulent S1 MDV strains could successfully co-infect and replicate within individual birds. This goal was evaluated in the chapter 2 pilot study and validated in chapter 3. 2) To determine whether one virus could dominate another in terms of replication, transmission, and tumor formation. Understanding the potential phenomenon of dominance under the conditions of simultaneous infection is essential to evaluate data from non-simultaneous superinfection studies. 3) To demonstrate whether superinfection was still possible after delaying the time between challenges to 24 hours and 13 days. Detection of dominance was evaluated with respect to increasing time interval. 4) To compare effects in maternal antibody positive birds vaccinated with HVT to determine the relevance under field conditions.

Hypotheses

Based on these goals, the following hypotheses were made:

- Following simultaneous challenge with two fully virulent S1 strains, both strains will replicate, transmit to other birds and lead to tumor formation.
- Following simultaneous challenge, both strains will have equal chance of transmission and tumor formation.

- Following short and long interval challenge, the first virus will be present in greater frequency in bursa, feather follicles, and tumors relative to the second inoculated virus.
- Vaccination will mute the effects of both viruses leading to equal mixtures in bursa, feather follicles and tumors following short and long interval challenges.

Chapter 2

PILOT STUDIES (I-III)

Pilot I. Methods to qualitatively and quantitatively distinguish selected serotype 1 MDV in dual-infected chickens

Abstract

Marek's disease virus (MDV) can spread between vaccinated birds within a flock, leading to potential superinfection by the same or multiple strains of MDV. This study was designed to evaluate methods to distinguish two nearly identical recombinant strains of serotype 1 MDV (rMd5 and rMd5//38CVI) in the same tissue sample, with focus on the sensitivity and specificity of each assay. Methods used to differentiate the viruses included immunohistochemistry (IHC) using monoclonal antibodies specific for each strain, as well as pyrosequencing. 112 chickens were infected with one or both viruses. IHC and pyrosequencing were 100% specific for samples from single-infected birds. Using IHC, both virus strains were able to be detected in birds dually infected, however, the relative intensity of staining for each virus was variable between birds. Pyrosequencing was also able to detect both viruses in dually infected birds and results correlated well with IHC. IHC and pyrosequencing were successful for detecting both viruses in dually infected birds and will be used in further studies to understand the effects of serotype 1 MDV superinfection.

Introduction

Marek's disease (MD) is a lymphoproliferative disease of poultry caused by MD virus (MDV), an alphaherpesvirus. The pathogenesis of infection with a single virulent

strain of MDV has been extensively studied; however, the pathogenesis of infection with multiple virulent MDV strains has not been studied in depth. Infection with an initial serotype 1 MDV followed by a second infection with a serotype 1 MDV (superinfection) is assumed to occur in commercial poultry raising conditions. Chickens are presumably being repeatedly exposed with MDV from their environment. It is unclear what effect each virus has on the other during coinfection. Studying the relationship between dual infection with multiple viruses *in vivo* would further increase our understanding of MDV pathogenesis. Current technologies such as immunohistochemistry (IHC) and pyrosequencing would be key elements in characterizing the effects of one virus on the other.

This project incorporated two different virus pairs to study the effect of two similar viruses on each other. The first virus pair included rMd5 and a recombinant Md5 virus containing pp38 from MDV Rispens strain (rMd5//38CVI) developed by Lee (60). Her study found that there were no significant differences between the two strains with *in vivo* replication, antibody response, or tumor inductions. Specific monoclonal antibodies exist for detection of each strain with IHC.

The goal of this pilot study was to evaluate specific methods to distinguish the selected viruses using the tests described in the following section. If successful, these methods would be used for an extensive set of studies examining the effect of superinfection at multiple time intervals, and comparing the effect of vaccination.

Materials and Methods

Chickens

This experiment used $15I_5x7_1$ white leghorn chickens, a F1 hybrid cross of MD susceptible $15I_5$ males and 7_1 females (2). This particular cross has been a common choice of birds for Marek's disease experiments because both parental lines are >99% inbred and are highly susceptible to MD, while the F1 birds have strong hybrid vigor. Chicks were maternal antibody negative, as birds were hatched from an in-house SPF breeder flock that had not been exposed to MDV vaccination. Chicks were challenged at day of age before placement in Horsfall-Bauer negative pressure isolators.

Viruses

Two recombinant MD viruses were used for this experiment: rMd5 and rMd5//38CVI. The origins of the two recombinant viruses come from the wild-type Md5 virus, isolated by Witter in 1977 (103). Md5 is a vvMDV, and is considered a principle prototype strain used commonly for MD experiments, including as a control in the ADOL pathotyping assay (93). Reddy et al. used Md5 to construct a library of overlapping cosmid clones which generated a recombinant Md5 (rMd5) when transfected into MDV-susceptible cells (75). The authors concluded that i*n vitro* growth properties were the same between Md5 and rMd5, and development of visceral tumors were similar between groups.

The recombinant virus rMd5//38CVI was produced by Lee et al. using the rMd5 cosmid clones to substitute the pp38 gene from CVI988/Rispens in order to determine whether the pp38 gene from CVI988/Rispens was related to the protective properties of the vaccine (60). The authors, however, found the biological properties of rMd5//38CVI were similar to the wild-type rMd5 virus with regards to *in vivo*

replication, antibody response and tumor induction. Gimeno et al. reported no differences in the frequency of lymphoproliferative lesions between rMd5 and rMd5//38CVI at either 6 or 15 wpi (43). Based on these two studies, the virus pair rMd5 and rMd5//38CVI were chosen for the experiments in the following chapters to simulate re-infection with an identical virus on the assumption that there was no significant differences in the pathology of the two viruses.

Immunohistochemistry testing

Immunohistochemistry (IHC) is the process of localizing specific proteins in a tissue section by using antibodies specific to antigens in the tissue. There are several different methods of visualizing the antigen-antibody interaction, most commonly by conjugating the antibody or a secondary antibody to an enzyme such as peroxidase, or by tagging with a fluorophore such as fluorescein. Both methods were used in these experiments and will be described below.

Peroxidase staining of thymus, bursa, spleen, and FFE tissue sections was performed using the Vectastain ABC kit.[1] The kit was designed on the principles of the high affinity of the avidin glycoprotein for the biotin vitamin (46, 47). The kit includes biotinylated affinity-purified horse anti-mouse IgG (H + L) antibody, blocking serum (normal horse serum), avidin DH, and biotinylated horseradish peroxidase H. Colorizing was achieved using the Vectastain DAB substrate kit for peroxidase.[2] This kit includes 3, 3'- diaminobenzidine (DAB), buffer stock solution, and hydrogen peroxide. Frozen samples were dried under vacuum and fixed in acetone before

[1] Vector Laboratories, Inc., Burlingame, CA

[2] Vector Laboratories, Inc., Burlingame, CA

staining. Slides were later rehydrated and blocking serum was added to the fixed tissue from the serum of the same species the secondary biotinylated antibody was made from (horse) to reduce background or unspecific staining. The primary antibody was then added, which was H19 (IgG monoclonal antibody specific for all S1 MDV pp38 genes except CVI988 Rispens) or T65 (IgG monoclonal antibody specific for CVI988 Rispens pp38 gene). After washing, the biotinylated secondary antibody was added, incubated, and washed. Next, the avidin and biotinylated horseradish peroxidase were added which form high affinity complexes between each other and the biotin conjugated to the secondary antibody. After incubating and rinsing, the DAB solution was added including the buffer and hydrogen peroxide which reacts with the horseradish peroxidase and produces a dark brown color.

Monoclonal antibodies: H19, T65

The MDV phosphoprotein pp38 gene, located in the IR_L and U_L region, was utilized for virus differentiation in the following chapters of experiments. The function of the pp38 gene is not completely understood, but several studies have contributed to our current knowledge. Xie et al. concluded that pp38, in addition to ICP4 and meq were involved in maintenance of MDV-transformed MSB1 lymphoblastoid cells (107). Cui and Qin described immunodepressive effects of pp38 (32). Several experiments have indicated that pp38 may be an important part of MDV reactivation. Infection of QT35 cells, a cell line latently infected with serotype 1 MDV, with HVT activates pp38 expression (108). The gene is also expressed in productively infected cells including the FFE (33, 49, 67, 68, 76). Gimeno et al. used a mutant virus lacking pp38 (rMd5Δpp38)

to demonstrate that pp38 was necessary to establish cytolytic infection in B cells, but not FFE (43). The authors found that pp38 was also necessary to produce an adequate level of latently infected T cells and to maintain the transformed status *in vivo* by preventing apoptosis.

According to early published pp38 sequence data, all serotype 1 MDV strains have identical bases in the pp38 gene except bases #320 and #326 (31, 38). CVI988, however, is the only serotype 1 MDV strain that is not reactive with pp38-specific Mab H19 (61, 105). Using DNA sequencing from the pp38 gene of additional strains, Cui et al. determined the molecular basis for the difference in the antigenic specificity of H19 and T65 antibodies for the pp38 gene (34). Mab H19 was specific to base pair #320, and was only positive when adenine (A) was present. All serotype 1 MDV strains except CVI988 were positive for H19 and had A at bp #320. Mab T65 was specific to base pair #326, and was only positive when guanine (G) was present. T65 was positive for CVI988 which had G present at base pair #326. Most serotype 1 MDV strains were positive for H19 only, having A at both base pairs #320 and #326, however, a few field strains have A at base pair #320 and G at base pair #326, such as GA22, and thus are positive for both H19 and T65.

The experiments described in the following chapters utilized this difference in the pp38 gene for detection of individual viruses from the virus pairs described above. JM/102W and rMd5 have A at both base pair #320 and #326 and thus were positive for H19 only. rMd5//38CVI has G at both base pairs #320 and #326 and thus was positive for T65 only. Pyrosequencing was designed to detect the differences at base pair #320,

thus measuring the frequency of A (JM/102W or rMd5) vs. G (rMd5//38CVI) in DNA samples.

Pyrosequencing assay

Pyrosequencing is a DNA sequencing technique that relies on the following steps to detect pyrophosphate released from nucleotide incorporation: 1) DNA fragments containing the sequence of interest are amplified using standard PCR. One of the primers used for amplification is biotin-labeled for immobilization to sepharose beads. The sequencing primer is designed within five bases from the single nucleotide polymorphism (SNP) of interest **(Figure 2-1)**. 2) The amplified PCR product is mixed with a sequencing primer and incubated with DNA polymerase, ATP sulfurylase, luciferase, apyrase, adenosine 5' phophosulfate (APS) and luciferin. 3) Deoxyribonucleotide triphosphates (dNTPs) are added one at a time and if the dNTP is complementary to the template strand, DNA polymerase catalyzes the incorporation which releases pyrophosphate (PPi) in an amount equimolar to the amount of incorporated dNTP. 4) The PPi in combination with APS is converted to ATP by ATP sulfurylase. The ATP activates luciferase which mediates the conversion of luciferin to oxyluciferin which generates visible light proportional to the amount of ATP. The light is detected by a charge coupled device chip which produces a peak on the pyrogram proportional to the number of dNTPs incorporated. Apyrase degrades unincorporated nucleotides and ATP before the next dNTP is added.

Pyrosequencing was performed on a PSQ 96MA system.[3] Three primers specific for the pp38 gene were used for each reaction, consisting of the forward, biotinylated reverse, and sequencing primers **(Figure 2-1)**. The sequencing primer was designed using SNP Primer Design from Pyrosequencing AB version 1.0.1.[4] Samples results are reported as failed when no sequence is detected. The samples may either not have adequate virus present (e.g. negative control samples) or there may have been either insufficient or excessive total DNA present.

Pyrosequencing validation

Pyrosequencing was validated for sensitivity and accuracy using plasmid constructs. The validations included 1) testing the accuracy of known mixtures of plasmids, 2) determining the lowest level of virus copy number for detection on gel electrophoresis 3) determining the accuracy of DNA amplified by nested PCR from known mixtures of low quantity, and 4) comparing the accuracy of pure plasmid mixtures compared to plasmid mixtures diluted in chicken DNA.

To test the accuracy of known mixtures of plasmids, two plasmids were generated containing the pp38 genes of rMd5 and rMd5//38CVI using the A6 cosmid from rMd5 and DNA isolated from rMd5//38CVI, respectively. Cloning was performed using the TOPO Cloning Reaction Kit and Transforming One Shot Competent E. coli, according to manufacturers instructions.[5] PCR and gel electrophoresis were performed from three bacterial colonies of each clone to confirm positive reaction, using MdCV-F

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[3] Qiagen, Hilden, Germany

[4] Applied Biosystems Inc., Foster City, CA

[5] Invitrogen Corporation, Carlsbad, CA

and MdCV-R2 primers **(Figure 2-2)**. Plasmid DNA was purified with the QIAprep MiniPrep using the manufacturer's protocol for a microcentrifuge column tube.[6] DNA was then sequenced on ABI 3100 Genetic Analyzer to confirm correct SNPs.[7] Two plasmids were selected containing the pp38 genes from rMd5 and rMd5//38CVI, respectively. The first two sets of independent reconstructions (trial 1 & 2) were performed using only the plasmids, each diluted to 10ng/ul **(Table 2-1)**. From each dilution, 2ul was used in a 50ul PCR reaction volume, and 40ul was used for pyrosequencing. Pyrosequencing was run in duplicate for both reconstruction mixtures and indicated that in some cases greater than 10% of the lower frequency virus was needed for detection. rMd5 was over-represented in both mixtures **(Figure 2-3)**.

To determine the lowest level of detection for passing results, a similar reconstruction mixture was created using a different set of plasmids provided by Robert Silva which included prMd5 pp38-1 and L233-6 pp38 Rispens, specific for pp38 genes of rMd5 and rMd5//38CVI, respectively. Stocks were serially diluted in chicken DNA (isolated from DF-1 cells; 5ng/ul) from $1x10^6$ copies/ul to 1 copy/ul for both plasmids, to mimic the conditions of the experiment when DNA samples will contain both virus and chicken DNA. Pyrosequencing will fail without the quantity of PCR product necessary to produce a band on gel electrophoresis. A gel was run following PCR and the lightest band present for both plasmids indicated 1000 copies/ul necessary for band detection **(Figure 2-4)**. Pyrosequencing analysis confirmed passing results for 1000 copies/ul and failure to detect sequence at higher dilutions (data not shown).

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[6] Qiagen, Hilden, Germany

[7] Applied Biosystems Inc., Foster City, CA

The same set of mixtures was used to determine the accuracy of DNA amplified by nested PCR from known mixtures of low quantity. The next two lower dilutions (100 copies/ul & 10 copies/ul) were mixed together in known frequencies similar to Table 2-1. A nested PCR was run for the mixture using pp38-R and MdCV-R outside primers and MdCV-F and MdCV-BiotinR inside primers. The same mixtures were also run on a single PCR using the inside primers, and as expected all samples failed pyrosequencing since bands were not detected on the gel. However, double amplification using the nested PCR assays led to passing results. Results were less accurate then after a single PCR assay, but both viruses were still able to be detected in all samples **(Figure 2-5)**.

Finally, an additional reconstruction was made using plasmid DNA and tested by pyrosequencing following nested PCR both with and without background chicken DNA (5ng/ul). The plasmids were mixed together in the same mixtures as described above, and 10ul of each was used by itself in the nested PCR or mixed with 10ul of chicken DNA. The same PCR primers were used as described above for the nested PCR. The presence of chicken DNA did not alter the results, although results again were less accurate after nested PCR than following single PCR **(Figure 2-6)**.

Experimental design

Twenty chickens were singly-infected with rMd5 or rMd5//38CVI, or dually-infected with both viruses at hatch **(Table 2-2)**. Tissue samples (spleen, bursa, thymus) were collected from 5 birds (3 control birds) at both 4 and 6 dpi for IHC and DNA isolation **(Table 2-3)**. Feather follicle epithelium samples were similarly collected from

5 birds (3 control birds) at 21 days post infection for IHC only. Serial sections of thyms, bursa, spleen, and FFE were cut and stained with H19 (specific for rMd5) and T65 (specific for rMd5//38CVI) monoclonal antibodies. IHC results were scored subjectively as negative, or 1-4+ based on the amount of staining within follicles and total number of follicles. Gross necropsy and histopathology were performed on the remaining 5 birds (3 control birds) at 8 weeks post infection to compare lesions between groups.

Results

The pathogenicity of single and dually-infected chickens was compared between the three groups at termination **(Table 2-4)**. Every bird from each of the three groups was diagnosed with Marek's disease based on lymphoid atrophy with nerve enlargement and visceral tumors. Heart and spleen lymphomas were prevalent, and all infected birds had one or both of this type of tumor. All three groups plus the control group were terminated early at 41 dpi due to the death of all five birds from the rMd5//38CVI group by 41 days. The rMd5 group had 2 deaths by 41 days, and the dual infected group had one death.

Immunohistochemistry staining of sections from 4 dpi lacked sufficient quality to interpret the results, but did not appear to have any staining. Staining at 6 and 21 dpi was specific, as predicted, for birds singly infected **(Figures 2-7, 2-8; Tables 2-5, 2-6)**. Both virus strains were able to be detected in birds dually infected, however, the relative intensity of staining for each virus was variable between birds **(Figures 2-9, 2-10)**. There were birds within each group that did not stain for either antibody and was likely

due to inoculation failure or slow virus replication. N/A indicates that no sample was available for that particular bird.

Pyrosequencing results were similarly 100% specific in singly-infected birds. All birds that had no staining by IHC subsequently failed with pyrosequencing, since no virus sequence was detected. In dual infected birds, pyrosequencing results correlated with IHC results, except for one bird that had weak IHC staining and failed by pyrosequencing. Both viruses were present by pyrosequencing from the bird that had dual IHC staining.

Summary and Conclusion

Pyrosequencing validation of sensitivity demonstrated the detection of virus at quantities as low as 1000 copies/ul. The next two higher log dilutions, however, were also detected by using nested PCR before pyrosequencing. Results from plasmid mixtures illustrated a linear relationship of pyrosequencing results with slight over-representation of rMd5. Results from chapters 2 and 3 categorize the pyrosequencing data according to which virus was predominant, thus minimizing the effect of over-representation. Since not all reconstruction replicates successfully identified mixtures with frequencies of 10% suggests results indicating the presence of only one virus may not reveal small proportions of the second virus.

Immunohistochemistry and pyrosequencing were both 100% specific for rMd5 and rMd5//38CVI based on results from singly infected birds. Both strains were detected in dually infected birds, although relative intensity was variable between birds. Interestingly, both virus strains were detected by IHC in dually infected birds in only

one of four positive staining birds at both 6 and 21 dpi. Results from one bird at 6 dpi (M6392) were positive for IHC and not by pyrosequencing, which indicated that IHC staining may be more sensitive than pyrosequencing. The methods that were developed and tested in this pilot experiment were considered sufficient for differentiating between rMd5 and rMd5//38CVI and were used for all subsequent experiments.

Pilot II. Pathotyping of rMd5 and rMd5//38CVI

Abstract

Pathotyping was performed to confirm that both Md5 recombinant viruses used in the following studies were of similar virulence. Two trials of two replicates each were performed because the prototype v and vv MDV strains had uncharacteristic results in trial 1. The prototype control strains were adequately protected against in trial 2, and results showed statistically similar virulence between rMd5 and rMd5//38CVI, although both were significantly less protected using bivalent vaccination compared to prototype vv strain Md5. Pathotype designations were not made due to prototype virus inconsistencies between replicates and trials. Results of virulence rank were nearly identical between rMd5, rMd5//38CVI and historical results of Md5. Despite inconsistencies, rMd5 had the highest percentage of MD lesions from all MDV strains in both replicates from trials 1 and 2.

Introduction

The proposed model to study the effects of superinfection and simultaneous dual infection with the "same" virus will use rMd5 and rMd5//38CVI. It is therefore important to confirm that these two viruses are as similar in pathogenicity as possible. Two previously reported studies found that there were no significant differences between the two strains with *in vivo* replication, antibody response, or tumor inductions (43, 60).

Pathotyping is one additional method for evaluating the similarity between two virus strains. The philosophy and a description of pathotyping methods was recently reviewed by Witter et al. (98). Pathotyping became an important classification of viruses not long after increased virulent forms of MDV were initially reported (5). The classification has been used to designate differences in virulence based on frequency of disease induced in vaccinated and unvaccinated birds. Early classifications by Biggs et al. differentiated strains as classical or acute, with the term acute based on higher morbidity and mortality, shorter time until onset, and a higher incidence of visceral tumors (11). Biggs et al. later included additional criteria for this designation based on histological lesion scores (9). Witter first designated the virulence of MDV strains based on responses in vaccinated chickens, using a cut-off value of HVT protection (77%) (93). Several years later he included prototype isolates as a comparison against field isolates to better standardize the pathotype designations from other laboratories (97). Specifically, vvMDV (very virulent) isolates were those with MD lesions in HVT-vaccinated, susceptible birds at a rate greater than a prototype vMDV(virulent) isolate, such as JM or GA. Other groups began using these techniques, but the procedures were variable with different prototype and chicken lines used for making the designation of vvMDV (14, 50, 51, 89). Witter recognized an increased virulence of MDV strains in the early 1990's in bivalent-vaccinated chickens, leading to four current pathotype designations: m (mild), v (virulent), vv (very virulent), and vv+ (very virulent plus) (95).

rMd5//38CVI and rMd5 are assumed to be the same pathotype as Md5 (**vv**) based on the previous studies, but neither have been officially pathotyped. The purpose

of this subphase was to pathotype rMd5 and rMd5//38CVI in order to give further evidence to the assumption that rMd5 and rMd5//38CVI are pathogenically similar.

Materials and Methods

Chickens

This experiment used maternal antibody positive $15I_5x7_1$ white leghorn chickens, a F1 hybrid cross of MD susceptible $15I_5$ males and 7_1 females (2). Birds were hatched from an in-house SPF breeder flock, separated into treatment groups and placed in Horsfall-Bauer negative pressure isolators.

Viruses

The viruses pathotyped included rMd5 and rMd5//38CVI, which were described in pilot 1. Prototype control viruses used were JM/102W and Md5 which were also described in pilot 1. Briefly, the two recombinant Md5 viruses being tested share the same rMd5 backbone and are only different in the pp38 gene. The viruses were previously shown to have no differences in the frequency of lymphoproliferative lesions at either 6 or 15 wpi (43).

Experimental design

The ADOL pathotyping assay was used for this study. Two prototype MDV strains, (JM/102W and Md5) and the unknown strains (rMd5 and rMd5//38CVI) were inoculated in three lots (17 birds each) of $15I_5x7_1$ antibody positive chickens:

unvaccinated, HVT-vaccinated, and bivalent-vaccinated (HVT + SB-1) **(Table 2-7)**. Vaccination (2000 pfu) was administered at hatch and challenge (500 pfu) at 5 days post vaccination. Termination and necropsy were performed at 56 days post challenge and a diagnosis of MD was made based on gross lesions consisting of nerve enlargement and/or visceral tumors. Suspicious lesions were confirmed by histopathology. One isolator (6 birds) of unvaccinated/unchallenged control birds was included in each replication. A second full replication was performed following the first challenge. Two complete trials were completed (four total replicates).

Chi-square analysis was used to compare the similarity of responses between replicates as well as to measure the significance of response in vaccinated birds compared to the prototype control viruses.[8] Virulence rank was calculated for each virus, defined as the mean of the % protection by HVT and bivalent vaccines subtracted from 100.

Results

Trial 1

The percentage of MD positive birds was consistent between all replicates except for two lots, where higher mortality was seen in the second replicate **(Table 2-8)**. The percentage of MD positive birds challenged with the prototype control viruses varied somewhat from previously reported studies under the same conditions. Due to variability in prototype virus results, viruses were not assigned a pathotype designation and a second trial was performed.

[8] SAS V9.1, Cary, NC

Trial 2

The percentage of MD positive birds was consistent between all replicates **(Table 2-8)**. Bivalent protection against Md5 was unusually high, so rMd5 and rMd5//38CVI were both significantly less protected by bivalent vaccination **(Table 2-9)**. When compared with each other, there was no significant difference in protection between rMd5 and rMd5//38CVI in each group of vaccinated birds. Virulence rank was calculated using the summary of results from trials 1 and 2. Results from rMd5 (57.5) and rMd5//38CVI (58.5) were well in line with historical results reported from Md5 (58) by Witter (95). At the same time, these results also confirm the significant difference in virulence between JM/102W and rMd5//38CVI, an additional virus pair used in chapters 3 and 4.

Summary and Conclusion

Trial 2 suggested there was no significant difference in pathogenicity between rMd5 and rMd5//38CVI in vaccinated birds, while also confirming significant difference in virulence between the virus pair JM/102W and rMd5//38CVI used in chapters 3 and 4. Although statistically similar to each other, rMd5 and rMd5//38CVI both were significantly more virulent than Md5 in trial 2, suggesting the recombinant strains might not be pathogenically equivalent to the parent strain. Both viruses were nearly identical to historical results from Md5 based on virulence rank. It is not known what caused the uncharacteristic reduced protection of the prototype virus strains in trial 1, but further trials should be conducted to confirm results from trial 2 before officially

designating pathotypes for rMd5 and rMd5//38CVI. Despite the inconsistencies, rMd5 had the highest percentage of MD lesions from all MDV strains in both replicates from trials 1 and 2, even though there were no statistically significant differences in trial 2.

Pilot III. A comparison between MDV production in feather pulp versus feather follicle epithelium (FFE)

Abstract

Although Marek's disease viral load in chickens can be assessed by quantifying the viral DNA in homogenized feather tips, this value may not correlate to the viral load being shed. This study was designed to determine what portion of the feather tip tissue is most reflective of virus load in the shed dander by comparing feather follicle epithelium (FFE) cells, adhered to the exterior of the shaft, to interior cells within the feather pulp. Using multiplex quantitative PCR, the FFE cells from infected birds had significantly higher viral copy number (VCN) per host cell than from feather pulp, and were more comparable to the VCN measured from feather dander. These results suggest that DNA isolated from FFE is more reflective of actual shed virus than VCN measured from the feather pulp.

Introduction

Several recent studies have been performed by other groups analyzing the MDV load in feather tips (1, 3, 4, 35, 36, 52, 54). Despite the success quantifying DNA in these studies, there has been no evidence that the quantity of DNA in the feather pulp is related to the quantity of virus that is shed via the FFE.

Islam et al. have reported on MDV DNA extracted from isolator dust (54). Dust samples were collected between days 9 and 58 after placement and DNA was extracted from 5mg of dust and quantified as viral copy number per mg of dust. MDV was first detected in dust at 7 dpi and quantity peaked around 35-40 dpi. The quantity of virus

shed in dust, however, has not been directly correlated with the quantity of virus within the feather follicle epithelium.

The objectives from chapters 3 and 4 were to characterize the difference in pattern and distribution of specific virus antigen for single infection vs. superinfection in FFE, and to compare the quantity and frequency of shed viruses based on DNA isolated from FFE. This experiment was designed to help understand the relationship of DNA quantity isolated from feather pulp and FFE, and whether DNA isolated from FFE correlates to the DNA isolated from dust. The results were used to determine which samples would be collected in the subsequent set of experiments to accurately represent virus being shed to other birds.

Materials and Methods

Chickens

This experiment used maternal antibody negative $15I_5x7_1$ white leghorn chickens, a F1 hybrid cross of MD susceptible $15I_5$ males and 7_1 females (2). Maternal antibody positive chickens were used for one group that was challenged with a vv+ MDV strain to protect the birds from early mortality. Birds were hatched from an in-house SPF breeder flock, separated into treatment groups, challenged and placed in Horsfall-Bauer negative pressure isolators.

Viruses

Four viruses representing three pathotypes (v, vv, vv+) were compared in this experiment, including a partially attenuated vv+ strain. The lower virulent strains

JM/102W (v) and rMd5 (vv) have already been described (see chapter 1). The two higher virulent strains used in this study were derived from 648A, a field strain isolated in 1995 that has been designated as a prototype vv+ strain (95). A highly virulent low passage working stock (p8) was used, as well as a partially attenuated strain at passage 50 (96). Gimeno et al. reported that 648A p50 had 100% persistent neurological disease but slightly reduced tumors and absence of transient paralysis, compared to lower passage strains (44). Three contact birds exposed to chickens challenged with 648A p50 had no viremia, suggesting a lack of transmission from the partially attenuated strain.

Quantitative PCR assay

The standard polymerase chain reaction (PCR) has been adapted to allow quantitative measurements of amplification following each cycle in a technique known as quantitative PCR (qPCR), or real-time PCR. The standard PCR is a molecular technique that amplifies a selected portion of DNA exponentially with each repeated cycle that includes denaturation, annealing, and elongation steps. The denaturation step (~95°C) melts the double-stranded DNA strand into single-stranded DNA. The annealing step cools down the DNA (~65°C) which allows the primers to bind to the single-stranded DNA. The DNA is then heated (~72°C) to activate Taq polymerase, a DNA polymerase which synthesizes a new DNA strand complementary to the single-stranded DNA template. A final elongation step is generally programmed which elongates any remaining single-stranded DNA. Thirty PCR cycles are commonly used,

followed by agar gel electrophoresis to confirm that the correct sized PCR fragment has been amplified.

qPCR follows the same principles as standard PCR except the target amplified DNA fragment is quantified following every cycle. This quantification can be absolute or relative by normalizing to DNA input or another gene, such as a host cellular gene. Detection of the DNA fragment for quantification is enabled by the use of an oligonucleotide probe that fluoresces when bound to the complimentary DNA. Experiments described in the following chapters used a TaqMan® probe (fluorogenic 5′ nuclease chemistry) which has a reporter fluorescent dye on the 5' end and a quencher dye on the 3' end. The close proximity of the quencher dye reduces fluorescence from the reporter dye when intact. During the annealing step, the probe binds downstream from one of the primer sites and is cleaved during elongation when the upstream primer is extended. Cleavage of the quencher dye from the reporter dye increases the fluorescence from the reporter dye, and removes the probe for primer extension to continue to the end of the template. The advantages of using TaqMan chemistry is that specific hybridization is required between the probe and DNA strand to allow fluorescence which reduces background signal, and probes can be labeled with different reporter dyes to allow more than one type of quantification in the same reaction.

The experiments described in this and the following chapters used a multiplex reaction performed on an ABI 7500 Real-Time PCR System[9]. One probe was specific to the MDV pp38 gene and the other specific to an endogenous reference, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Both probes used TAMRA™

[9] Applied Biosystems Inc., Foster City, CA

(tetramethylrhodamine) quencher dyes. The probe specific to MDV used a green emitter (500-549nm) reporter dye, 6FAM™ (6-carboxyfluorescein, excitation 492, emission 515nm) whereas the probe specific to GAPDH used a yellow emitter (550-584nm) reporter dye, VIC™ (excitation 538, emission 554nm). The following conditions were used: 1) 50°C – 2 minutes; 2) 95°C – 10 minutes; 3) 95°C – 15 seconds; 4) 60°C – 1 minute with steps 3-4 repeated 40 times. The primers and probes used for the pp38 and GAPDH sequences are listed below.

pp38 primers and probe:

MdCv-F: 5'-GTGATGGGAAGGCGATAGAA-3'

MdCv-TMR: 5'-TCGTCAAGATGTTCATTCCCTG-3'

MC-TMP: 5'FAM-TACCGCCTGAGCCCCGGAGG-3'TAMRA

GAPDH primers and probe:

GDH-TM2.5: 5'-ACAGAGGTGCTGCCCAGAA-3'

GDH-TM2.3: 5'-ACTTTCCCCACAGCCTTAGCA-3'

GDH-TMP2: 5'VIC-TCATCCCAGCGTCCACT-3'TAMRA

To isolate DNA from the FFE and feather pulp, the following procedure was used. Approximately 5-6 feathers were plucked from the feather tract and placed intact in a microcentrifuge tube (Tube #1) containing 300uL of cell lysis buffer. Once all samples were collected, the microcentrifuge tubes were shaken at a moderate speed for approximately 2 hours on an orbital shaker at room temperature to allow lysis of FFE cells adhered to the outside of the feather shaft. After shaking, feathers were removed from the lysis buffer and the feather shaft was cut into small pieces to expose the feather

pulp and placed in a second microcentrifuge container (Tube #2) containing 300uL of cell lysis buffer. Both microcentrifuge tubes were incubated overnight. Tube #1 was used to represent DNA isolated from FFE cells and Tube #2 represented DNA isolated from the feather pulp. Additional DNA purification was done using the PureGene DNA Purification Kit, according to manufacturer instructions.[10] Results were reported as viral copy number per host cell (VCN/host cell) by dividing quantity results from virus gene pp38 by quantity results from host gene GAPDH, factoring for two copies of each host gene per cell.

DNA was isolated from feather dust using a commercially available handheld shop vacuum. Individual collections were made using a standard commercially available paper coffee filter placed over the end of the collection hose. Birds were vacuumed together as groups (dust pooled) or individually for isolators 6-17. The negative pressure from the vaccum created a small cup in the middle of the coffee filter containing the feather dust. This cup containing the feather dust was inverted into the top of a 50mL centrifuge tube and the lid closed. The outer portions of the coffee filter outside of the lid were torn off. At the end of the collections, the tubes were centrifuged at approximately 500rpm to collect the dust at the bottom of the tubes. After spinning, the paper filter was removed and 300uL of cell lysis buffer was added to the tubes. Tubes were mixed and the contents were transferred to a microcentrifuge tube where overnight incubation and DNA purification proceeded in the same way as the FFE and feather pulp samples. Results were also reported as VCN/host cell, assuming that a significant number of intact keratinized epithelial cells were present in samples.

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[10] Gentra Systems, Inc., Minneapolis, MN

Experimental design

Birds were inoculated with one of four different challenge viruses (or control) and sampled at 11, 21, and 31 days post-challenge **(Table 2-10)**. Birds with the same challenge virus were housed in isolators with 10 birds each (lots 1-5) and in addition 10 birds were infected with rMd5 and housed individually in their own isolator unit (lots 6-17). For individually housed birds, an extra bird control bird was kept in each isolator for socialization until the first sampling was initiated. Birds were housed individually to prevent contamination of dander samples with infected dander from other birds. Sampling consisted of pulling feathers from the cervical and subhumeral feather tracts and collecting dust at each time point, as well as skin sections saved for immunohistochemistry. Dust samples were pooled from birds grouped in the same isolator, whereas feather samples were kept separate from individual birds. Multiplex quantitative PCR was performed as described above.

A paired t-test was calculated to determine the statistical significance in the difference of the VCN/host cells between each group (FFE vs feather pulp). The relationship between FFE, feather pulp and dust in individual birds was measured using Pearson correlation, along with a t-test to determine the difference in viral quantity between averages for FFE, feather pulp and dust.

Results

Based on the average virus copy number (VCN) per host cell for all samples separated by sampling time, the FFE cells had a significantly higher virus load

compared to cells isolated from the feather pulp **(Figure 2-11)**. These differences were statistically significant based on a paired t-test for all viruses tested at all three time points **(Table 2-11)**. Results from day 31 post-challenge were inconsistent with the first two samplings with respect to VCN from FFE and feather pulp cells **(Figure 2-11)**. The protocol for processing of the feathers was deviated for the day 31 samples, and feathers remained in cell lysis buffer overnight, instead of 2 hours, before removing and cutting the shaft to isolate DNA from feather pulp. Results suggest that this prolonged interval may have caused disintegration of the feather shaft and dilution of FFE DNA with feather pulp DNA. An attempt to validate the day 31 samples was made using laser microdissection to isolate and quantitate DNA from individual cells from the FFE and feather pulp (methods not described), but results were inconsistent **(Figure 2-12)**.

Dust samples were analyzed in the same way as feather tissue samples. In general, VCN/host cell was low at 11 days post-challenge, peaking by 21 days and decreasing again by 31 days based on averages from the isolators with multiple birds infected with the same virus. Birds infected with the vv+ MDV strains, however, increased linearly from 11 to 31 days without peaking at 21 days post-challenge **(Figure 2-13)**. The VCN/host cell was considerably higher for the FFE and dust, compared to the feather pulp **(Figure 2-14)**.

Analysis from birds infected with the same virus but housed individually showed variable results at each of the three samplings, but a similar average trend in dust samples as birds housed together **(Figure 2-15)**. Results from birds housed alone also showed a much higher quantity of VCN/host cell in the FFE and dust compared to feather pulp, although all three were significantly different at 21 days post challenge

(FFE*Dust p=0.0037; FFE*Pulp p<0.0001; Dust*Pulp p=0.0060) **(Figure 2-16)**. Interestingly, VCN/host cell was higher in the FFE cells in the individual birds than from dust samples which was the opposite relationship seen in pooled samples from groups of birds housed together **(Figure 2-14)**.

A Pearson correlation analysis in individually-housed birds indicated a significant positive correlation between FFE and dust (0.39; p=0.0387), including all three time points. A partial correlation with the effect of sampling day removed had even a high correlation between FFE and dust (0.64, p=0.0007). A significant correlation was not observed with feather pulp, likely due to low values. If the day 31 samples are removed, the Pearson correlation was quite strong among all three sample types (FFE*Dust 0.72, p=0.0005; FFE*Pulp 0.54, p=0.0262; Dust*Pulp 0.72; p=0.0018).

Summary and Conclusion

Based on the current data, the VCN/host cell appears to be quite high from DNA isolated from FFE and dust, compared to the lower quantity present from cells within the feather pulp. These results suggest that based on techniques used in this study, FFE VCN is more reflective of actual shed virus than VCN measured from the feather pulp, and may be the preferable method for measuring true quantity of shed virus from individual chickens rather than by using homogenized feather tips. As a result of these findings, sampling from subsequent experiments was done as described in this experiment, isolating DNA from cells adhered to the outside of the feather shaft, to

reflect the frequency and quantity of shed virus from individual birds following dual infection.

Table 2-1: Reconstruction mixtures used for pyrosequencing validation

Plasmid 1 (rMd5 pp38)		Plasmid 2 (rMd5//38CVI pp38)	
%	Volume (ul)	%	Volume (ul)
0	0	100	10
10	1	90	9
20	2	80	8
30	3	70	7
40	4	60	6
50	5	50	5
60	6	40	4
70	7	30	3
80	8	20	2
90	9	10	1
100	10	0	0

Table 2-2: Challenge outline

Lot	# chickens	Challenge strain	Dose
Lot 1	12	Uninfected controls	500 pfu/bird
Lot 2	20	rMd5 only	500 pfu/bird
Lot 3	20	rMd5//38CVI only	500 pfu/bird
Lot 4	20	rMd5 + rMd5//38CVI	500 pfu/virus/bird

Table 2-3: Sample outline

Sampling Date	Tissue	Challenge	# Birds
4 days	Thymus, Bursa, Spleen	Control	3
		rMd5	5
		rMd5//38CVI	5
		Dual	5
6 days	Thymus, Bursa, Spleen	Control	3
		rMd5	5
		rMd5//38CVI	5
		Dual	5
21 days	FFE	Control	3
		rMd5	5
		rMd5//38CVI	5
		Dual	5
56 days	Gross necropsy	Control	3
		rMd5	5
		rMd5//38CVI	5
		Dual	5

Table 2-4: Gross lesions at termination

Bird#	Challenge	K/D[1]	DPC	TA[2]	BA	Vag	Bra	Sci	Tumors	Diag
M6794	Control	K	41							X
M6795	Control	K	41							X
M6796	Control	K	41							X
M6779	rMd5	K	41	4+	4+	2+			Ht	MD
M6780	rMd5	K	41	2+	2+	3+	2+	2+	Ht	MD
M6781	rMd5	D	32	4+	4+	2+	2+	2+	Spl	MD
M6782	rMd5	D	37	4+	4+	1+			Spl	MD
M6784	rMd5//38CVI	D	41	4+	4+	2+			Ht, Spl	MD
M6785	rMd5//38CVI	D	32	4+	4+			2+	Spl	MD
M6786	rMd5//38CVI	D	35	4+	4+		2+	2+	Ht	MD
M6787	rMd5//38CVI	D	33	4+	4+			1+	Ht, Spl	MD
M6788	rMd5//38CVI	D	36	4+	4+		2+	2+	Spl	MD
M6769	Dual infection	K	41	4+	4+			1+	Ht, Spl	MD
M6770	Dual infection	D	21	4+	3+		2+	3+	Spl	MD
M6771	Dual infection	K	41	4+	3+			2+	Ht, Spl	MD
M6772	Dual infection	K	41	4+	4+		2+	2+	Ht, Spl	MD
M6773	Dual infection	K	41	4+	4+	2+	1+	1+	Ht, Spl	MD

[1] Abbreviations: Killed or Died (K/D), Days post challenge of death (DPC), Thymus atrophy (TA), Bursa atrophy (BA), Vagus nerve enlargement (Vag), Brachial nerve enlargement (Bra), Sciatic nerve enlargement (Sci), Diagnosis (Diag), Nonspecific mortality (X), Marek's disease (MD), Heart (Ht), Spleen (Spl)

[2] When present, thymus and bursa atrophy and nerve enlargement were classified on a scale from 1+ (mild) to 4+ (severe)

Table 2-5: IHC and pyrosequencing results from samples collected 6 dpi.

Bird#	Challenge	Immunohistochemistry[1]						Pyrosequencing		
		H19			T65			% rMd5 : % rMd5//38CVI		
		Thy[2]	Bur	Spl	Thy	Bur	Spl	Thy	Bur	Spl
M6738	Control	-	-	-	-	-	-	F	F	F
M6739	Control	-	-	-	-	-	-	F	F	F
M6740	Control	-	-	-	-	-	-	F	F	F
M6723	rMd5	1+	-	1+	-	-	-	100:0	100:0	100:0
M6724	rMd5	2+	2+	2+	-	-	-	100:0	100:0	100:0
M6726	rMd5	+	+	2+	-	-	-	100:0	100:0	100:0
M6727	rMd5	2+	2+	2+	-	-	-	100:0	100:0	100:0
M6783	rMd5	-	-	-	-	-		F	F	F
M6728	rMd5//38CVI	-	-	-	2+	2+	2+	0:100	0:100	0:100
M6729	rMd5//38CVI	-	-	-	2+	2+	2+	0:100	0:100	0:100
M6730	rMd5//38CVI	-	-	-	2+	2+	2+	0:100	0:100	0:100
M6731	rMd5//38CVI	-	-	-	-	-	-	F	F	F
M6732	rMd5//38CVI	-	-	-	3+	2+	2+	0:100	0:100	0:100
M6391	Dual infection	2+	2+	2+	-	-	-	100:0	100:0	100:0
M6392	Dual infection	-	-	-	1+	-	1+	F	F	F
M6393	Dual infection	2+	2+	2+	-	-	-	100:0	100:0	100:0
M6394	Dual infection	-	-	-	-	-	-	F	F	F
M6395	Dual infection	2+	2+	2+	1+	1+	1+	95:5	99:1	99:1

[1] Lesions are expressed on a scale from 1+ (scattered staining) to 4+ (extensive staining)

[2] Abbreviations: F = failed sample (no sequence), Thy = Thymus, Bur = Bursa, Spl = Spleen

Table 2-6: Immunohistochemistry results from FFE samples collected 21 dpi.

Bird#	Challenge	FFE[1]	
		H19	T65
M6766	Control	-	-
M6767	Control	-	-
M6768	Control	-	-
M6751	rMd5	2+	-
M6752	rMd5	2+	-
M6753	rMd5	2+	-
M6754	rMd5	2+	-
M6755	rMd5	2+	-
M6756	rMd5//38CVI	-	2+
M6757	rMd5//38CVI	N/A	N/A
M6758	rMd5//38CVI	-	1+
M6759	rMd5//38CVI	-	3+
M6760	rMd5//38CVI	-	2+
M6741	Dual infection	-	-
M6742	Dual infection	1+	-
M6743	Dual infection	3+	-
M6744	Dual infection	-	2+
M6745	Dual infection	2+	1+

[1] Lesions are expressed on a scale from 1+ (scattered staining) to 4+ (extensive staining)

[2] Abbreviations: FFE = Feather follicle epithelium, N/A = Not available

Table 2-7: Challenge outline for pathotyping assay. Chickens were unvaccinated or vaccinated with 2000 pfu of either HVT or Bivalent vaccine (HVT + SB-1) at hatch. Chickens were challenged with 500 pfu of virus at 5 days post hatch. Two replicates were performed.

Lot	# chickens	Vaccination	Challenge strain
1	6	None	Uninfected controls
2	17	None	JM/102W
3	17	HVT	JM/102W
4	17	HVT + SB-1	JM/102W
5	17	None	Md5
6	17	HVT	Md5
7	17	HVT + SB-1	Md5
8	17	None	rMd5
9	17	HVT	rMd5
10	17	HVT + SB-1	rMd5
11	17	None	rMd5//38CVI
12	17	HVT	rMd5//38CVI
13	17	HVT + SB-1	rMd5//38CVI

Table 2-8: % Marek's disease (MD) in replicates 1 and 2 (R1 & R2) from trials 1 and 2.

		% MD in chickens vaccinated with					
		None		HVT		HVT + SB-1	
Trial	Isolate	R1	R2	R1	R2	R1	R2
1	JM/102W	82	88	41	53	33	35
	Md5	100	100	82	82	24	59*
	rMd5	100	100	71	53	29	65*
	rMd5//38CVI	100	100	94	94	13	24
2	JM/102W	100	100	18	0	12	6
	Md5	100	100	59	76	18	18
	rMd5	100	100	76	47	53	65
	rMd5//38CVI	100	100	65	82	53	41

*Percent MD significantly different by chi-square tests from that of replicate trial R1

Table 2-9: % Protection from trials 1 & 2 plus combined results

Trial	Isolate	% Protection in chickens vaccinated with[1]		Virulence Rank[2]
		HVT	HVT + SB-1	
1	JM/102W	53 ^{A}	66	
	Md5	18 ^{B}	59 ^{A}	
	rMd5	38 ^{AB}	53 ^{A}	
	rMd5//38CVI	6 ^{B}	81 ^{B}	
2	JM/102W	91 ^{A}	91	
	Md5	32 ^{B}	82 ^{A}	
	rMd5	38 ^{B}	41 ^{B}	
	rMd5//38CVI	26 ^{B}	53 ^{B}	
All	JM/102W	72 ^{A}	79	24.5
	Md5	25 ^{B}	71 ^{A}	52.0
	rMd5	38 ^{B}	47 ^{B}	57.5
	rMd5//38CVI	16 ^{C}	67 ^{A}	58.5

[1] Significant differences of percentages within vaccination groups are indicated by different superscript letters

[2] Virulence rank is defined as the mean of the % protection by HVT and bivalent vaccines subtracted from 100.

Table 2-10: Challenge outline

Lot	# chickens	Chicken strain	Challenge strain	Challenge	Sampling
1	10	$15I_5x7_1$ MAb-	JM/102W	500 pfu/bird	11, 21, 31 dpi
2	10	$15I_5x7_1$ MAb-	rMd5	500 pfu/bird	11, 21, 31 dpi
3	10	$15I_5x7_1$ MAb+	648A (p8)	500 pfu/bird	11, 21, 31 dpi
4	10	$15I_5x7_1$ MAb-	648A (p50)	500 pfu/bird	11, 21, 31 dpi
5	5	$15I_5x7_1$ MAb-	Control		11, 21, 31 dpi
6	1	$15I_5x7_1$ MAb-	rMd5	500 pfu/bird	11, 21, 31 dpi
7	1	$15I_5x7_1$ MAb-	rMd5	500 pfu/bird	11, 21, 31 dpi
8	1	$15I_5x7_1$ MAb-	rMd5	500 pfu/bird	11, 21, 31 dpi
9	1	$15I_5x7_1$ MAb-	rMd5	500 pfu/bird	11, 21, 31 dpi
10	1	$15I_5x7_1$ MAb-	rMd5	500 pfu/bird	11, 21, 31 dpi
11	1	$15I_5x7_1$MAb-	rMd5	500 pfu/bird	11, 21, 31 dpi
12	1	$15I_5x7_1$ MAb-	rMd5	500 pfu/bird	11, 21, 31 dpi
13	1	$15I_5x7_1$ MAb-	rMd5	500 pfu/bird	11, 21, 31 dpi
14	1	$15I_5x7_1$ MAb-	rMd5	500 pfu/bird	11, 21, 31 dpi
15	1	$15I_5x7_1$ MAb-	rMd5	500 pfu/bird	11, 21, 31 dpi
16	1	$15I_5x7_1$ MAb-	Control		11, 21, 31 dpi
17	1	$15I_5x7_1$ MAb-	Control		11, 21, 31 dpi

Table 2-11: Results of paired t-test statistics for the mean difference of FFE minus feather pulp VCN/host cells by virus and by days post challenge

Virus	Days post challenge	# Paired samples	Mean difference (FFE – Pulp)*	P-value**
All viruses	11	22	984	<0.0001
All viruses	21	38	1,212	<0.0001
All viruses	31	35	153	0.0041
JM/102W	All samples	25	1,276	<0.0001
rMd5	All samples	25	812	<0.0001
648A p8	All samples	22	659	0.0010
648A p50	All samples	23	277	0.0171

*VCN host cell

**Using paired t-test

Figure 2-1: Sequence of pp38 gene indicating pyrosequencing primer binding sites

ATGGAATTCGAAGCAGAACACGAAGGGCTGACGGCGTCTTGGGTCG
CCCCCGCTCCCCAGGGTGGAAAAGGGGCGGAGGGCCGCGCAGGGGTCGCC
GACGAGGCAGGGCATGGGAAAACAGAAGCGGAATGCGCCGAGGACGGCGA
GAAATGCGGGGACGCCGAGATGAGCGCTTTGGATCGGGTCCAGAGGGACC
GGTGGAGATTCAGT[TCTCCGCCCCCTCACTCT][1]GGAGTCACGGGGAAGGGG
GCTATTCCAATAAAGG[GTGATGGGAAGGCGATAGAA][2]TGCCAGGAGCTAAC
CGGAGAG[GGAGAGTGGCTGTCA][3]C[A/G][4]GTGGG[A/G][5]GGAGCTACCGCCTGA
GCCCCGGAGGTCAGGGAATGAACATCTTGACGAAAGTCGGTATGCGAAACA
AACCGAAAGGGGTAGCTCTACGGGGAAAGAAGAGG[GAGATGGTATGAAGC
AGATG][6]GGGGAGCTTGCCCAGCAGTGC[GAAGGAGGAACATATGCGGA][7]CTT
GCTTGTCGAAGCAGAGCAAGCTGTTGTACATTCCGTTCGCGCATTAATGCTG
GCCGAAAGACAAAACCCAAATATATTGGGGGAGCATTTGAATAAAAAACG
GGTTCTTGTACAACGACCCCGTACTATTCTATCCGTGGAGTCAGAGAATGCA
ACAATGCGTTCTTATATGCTGGTTACATTGATCTGTTCTGCAAAATCATTATT
ACTAGGATCGTGCATGTCATTTTTCGCTGGTATGTTAGTCGGTAGAACGGCA
GACGTAAAAACACCATTATGGGATACTGTATGTTTGTTAATGGCTTTCTGTG
CAGGCATTGTCGTTGGGGGAGTGGATTCTGGGGAGGTGGAATCTGGAGAAA
CAAAATCTGAATCAAAT

Standard pyrosequencing reaction:

[2]Forward primer (MdCv-F): 5'-GTGATGGGAAGGCGATAGAA-3'

[3]Sequencing primer (MdCv-PyroSeq): 5'-GGAGAGTGGCTGTCA-3'

[4] SNP used in pyrosequencing assay (bp #320)

[5]Additional SNP in pp38 gene (bp #326)

[6]Reverse primer (MdCv-BiotinR): 5'-CATCTGCTTCATACCATCTC-3'

Nested PCR pyrosequencing reaction:

Additional outside primers

[1]Forward primer (pp38-R): 5'-TCTCCGCCCCCTCACTCT-3'

[7]Reverse primer (MdCV-R): 5'-TCCGCATATGTTCCTCCTTC–3'

Figure 2-2: Gel analysis of pp38 gene cloned into plasmid. Lane 1 = molecular-weight marker of 100-bp ladder. Lanes 2-4 = rMd5 pp38 plasmid. Lanes 5-7 = rMd5//38CVI pp38 plasmid

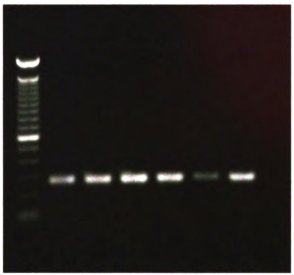


Figure 2-3: Averaged pyrosequencing results from two sets of independent plasmid mixtures (trial 1 & 2) each run in duplicate. Standard deviations between the four sets of results are indicated.

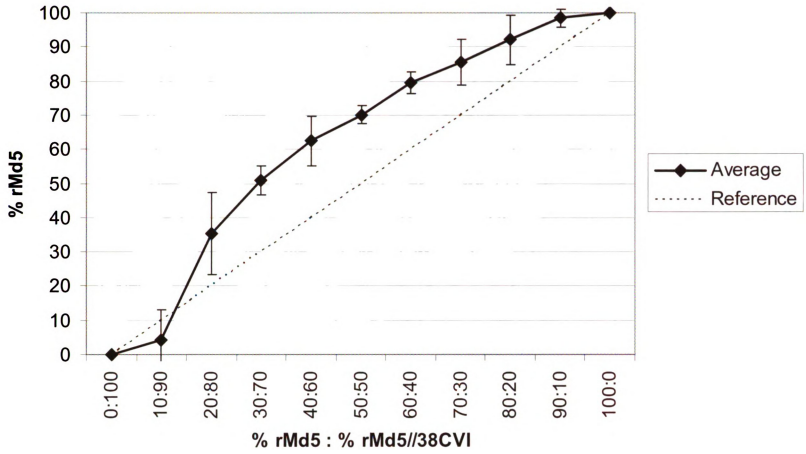
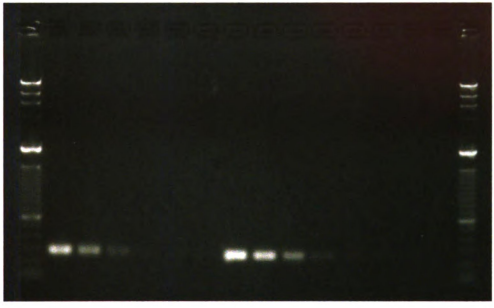


Figure 2-4: Gel analysis of pp38 gene plasmid serial dilutions. Lanes 1 & 16 = molecular-weight marker of 100-bp ladder. Lanes 2-7 = serial log dilutions of rMd5 pp38 plasmid in chicken DNA (100,000 copies/ul in lane 2 down to 1 copy/ul in lane 7). Lanes 8-15 = serial log dilutions of rMd5//38CVI pp38 plasmid in chicken DNA (same dilutions as lanes 2-7).



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 2-5: Pyrosequencing results following nested PCR of low plasmid quantities

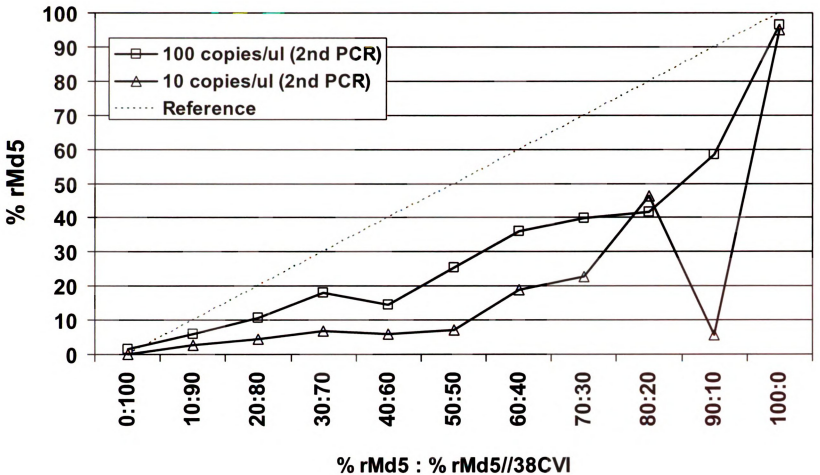


Figure 2-6: Pyrosequencing results following nested PCR comparing the effect of using samples diluted in chicken DNA

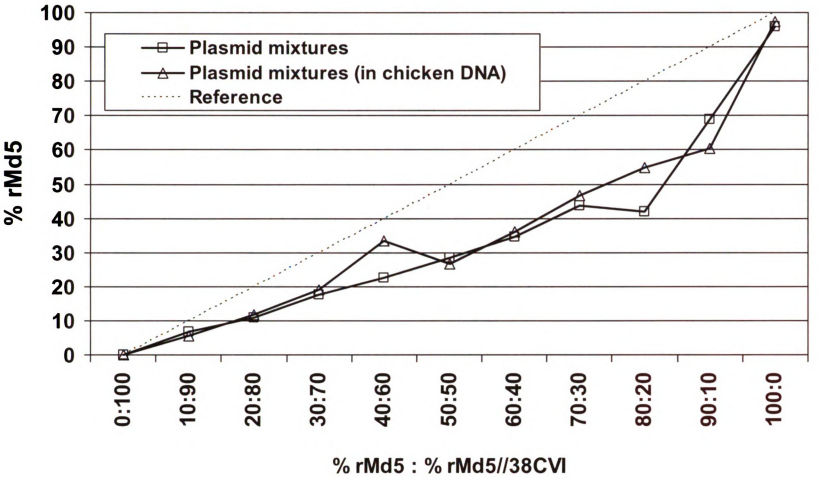


Figure 2-7: Serial bursa samples stained with monoclonal antibodies H19 and T65 at 6 dpi from a bird singly infected with rMd5 (on top) and rMd5//38CVI (on bottom)

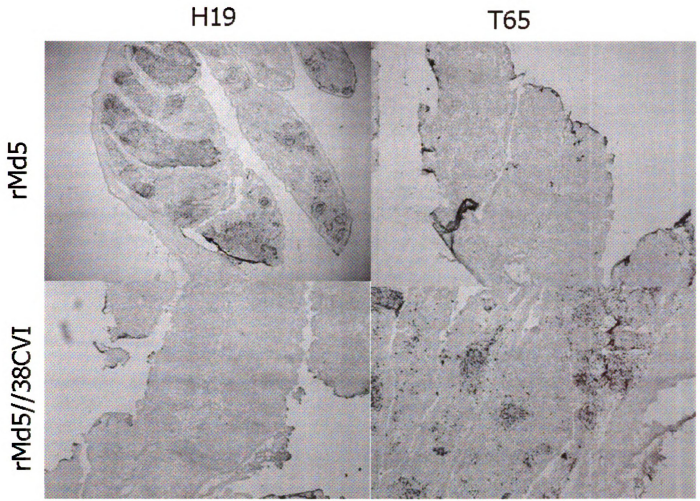


Figure 2-8: Serial FFE samples stained with monoclonal antibodies H19 and T65 at 21 dpi from a bird singly infected with rMd5 (on top) and rMd5//38CVI (on bottom)

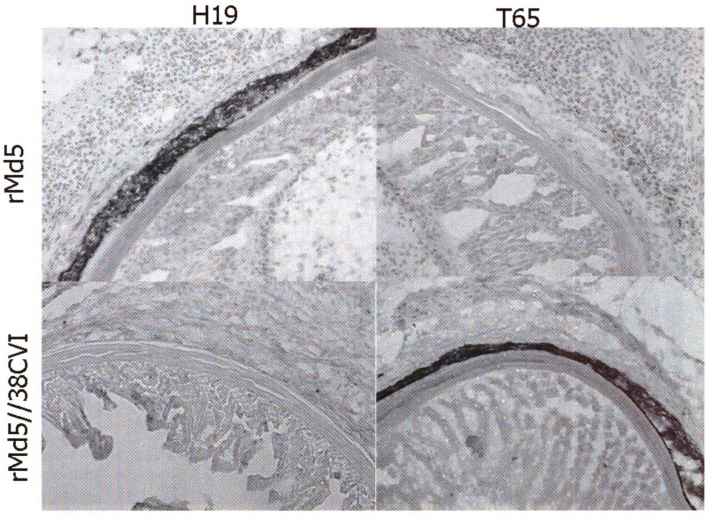


Figure 2-9: Serial bursa samples stained with monoclonal antibodies H19 and T65 at 6 dpi from a bird dually infected with rMd5 and rMd5//38CVI

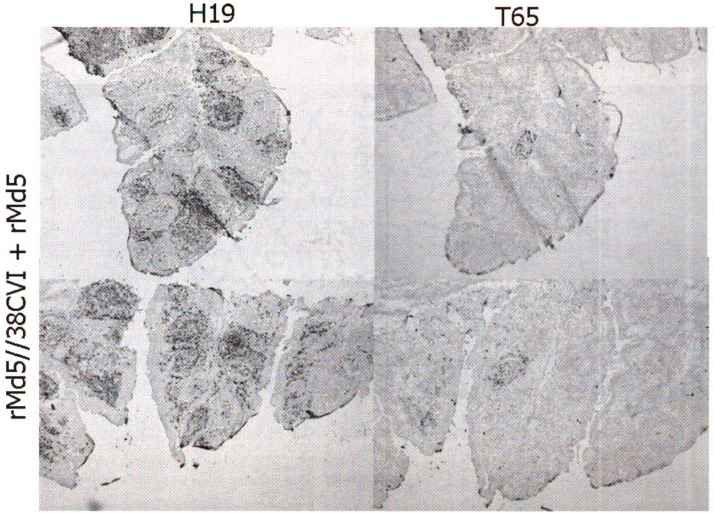


Figure 2-10: Serial FFE samples stained with monoclonal antibodies H19 and T65 at 21 dpi from a bird dually infected with rMd5 and rMd5//38CVI

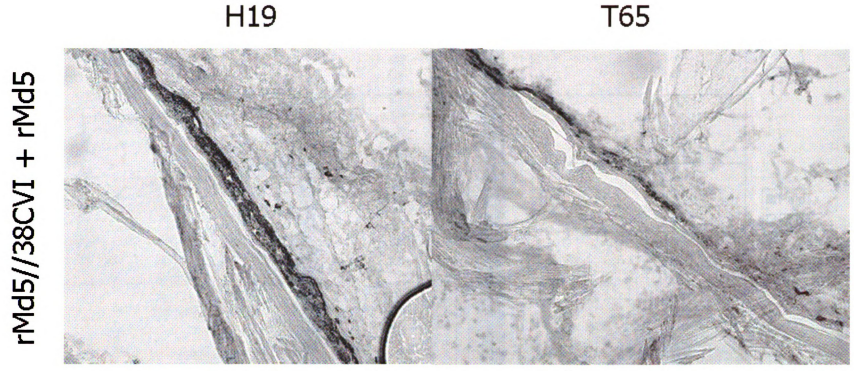


Figure 2-11: Average copy number for all viruses combined comparing feather pulp and FFE by sampling time

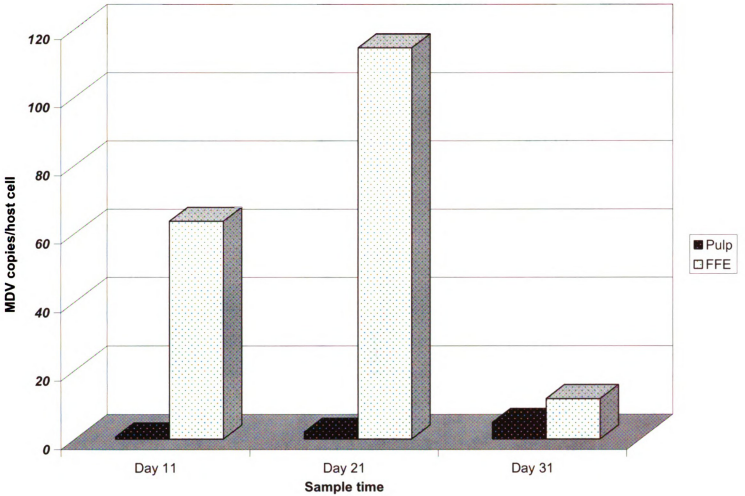


Figure 2-12: Laser microdissection results from sections of frozen feather follicles collected at 31 days post challenge

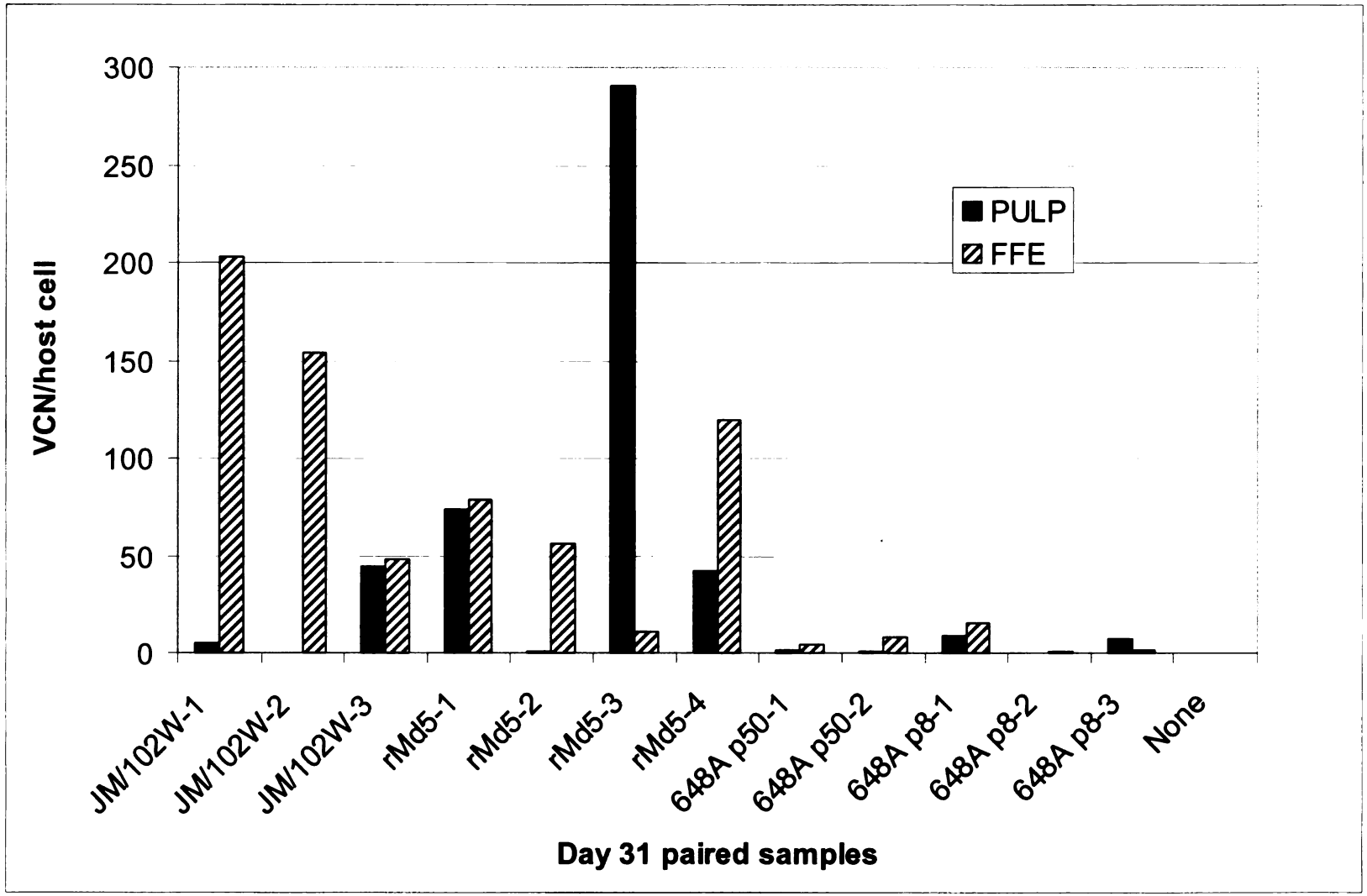


Figure 2-13: Virus copy number/cell in DNA isolated from pooled dust samples based on virus and sampling time (10 birds/group)

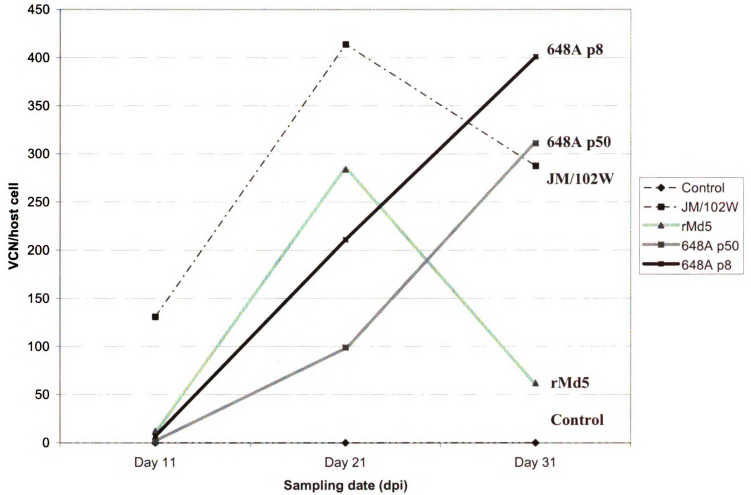


Figure 2-14: Viral copy number group comparisons for each virus based on sampling time. FFE and pulp copy numbers are averages based on individual samples from each group. Dust quantities are based on one pooled sample from all birds at each time point.

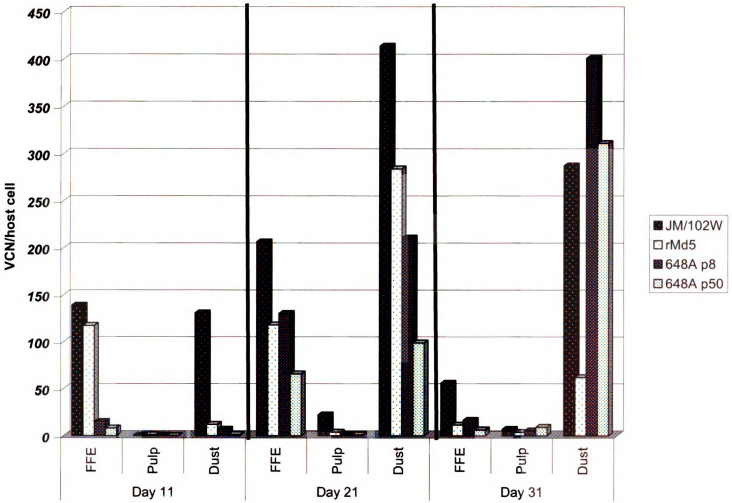


Figure 2-15: Virus copy number over time in individually-housed chickens, averaged from 2 control birds and 7 rMd5-challenged birds. Error bars indicate one standard deviation.

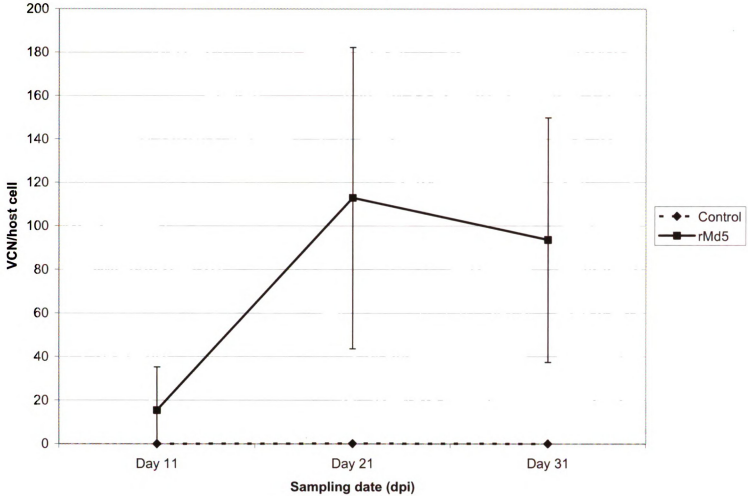
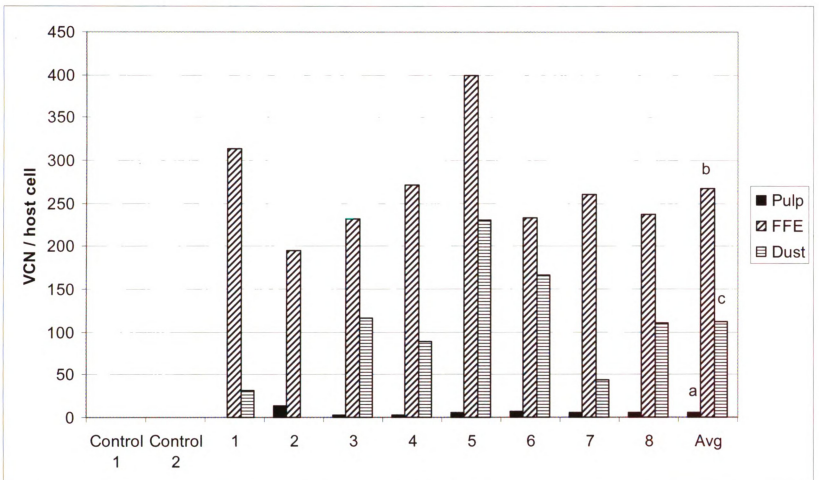


Figure 2-16: Virus copy number comparison for individually housed chickens at 21 days, challenged with rMd5. Significant results based on the average are indicated by different lowercase letter.



Chapter 3

DEMONSTRATION OF VIRAL DOMINANCE FOLLOWING SIMULTANEOUS CHALLENGE OF TWO FULLY VIRULENT MAREK'S DISEASE VIRUS STRAINS

Abstract

Marek's disease vaccination protects chickens against the development of disease, but does not prevent infection or transmission of infectious virus. Previous studies have demonstrated the presence of multiple strains of Marek's disease virus simultaneously circulating within poultry flocks, leading to the assumption that individual birds are repeatedly exposed to a variety of virus strains in their lifetime. A series of four experiments were designed to test whether coinfection was possible with two fully virulent S1 MDV strains, using two similar (rMd5 and rMd5//38CVI) and two different (JM/102W and rMd5//38CVI) virus pairs. Bursa of Fabricius, feather follicle epithelium, spleen, and tumor samples were collected at multiple time points to determine the frequency and distribution of each virus present following simultaneous infection using pyrosequencing, immunohistochemistry and quantitative PCR. Virus dominance was significant between the second virus pair, but was not consistent between the two similar strains. Both viruses were readily detected in individual bursa and feather follicles in 16 of 23 birds (70%), indicating that the presence of one virus did not exclude the second virus. Results from tumors demonstrated differences between the predominance of each strain within multiple tumors from individual birds, suggesting a possible polyclonal origin. These effects may be relevant under field conditions as no significant differences were detected between vaccinated and unvaccinated chickens. Viral dominance may be an important factor that influences the

outcome of coinfection under field conditions, including the potential outcome of emergence or evolution of more virulent strains.

Introduction

Marek's disease (MD) is a lymphoproliferative disease in chickens caused by an alphaherpesvirus, Marek's disease virus (MDV). The disease is typically characterized by enlarged nerves and visceral tumors composed of transformed T cells. MDV is classified into three serotypes: serotype 1 (S1) includes pathogenic strains and their attenuated variants, serotype 2 (S2) includes all apathogenic chicken strains, and serotype 3 (S3) includes turkey herpesvirus (HVT), which is also apathogenic in chickens (12). S2 and S3 have been used as vaccines, as well as several attenuated strains of S1. Although vaccination has successfully been used to prevent disease, the vaccines do not prevent against infection or transmission of the virus. Thus, as early as 2 weeks after inoculation or exposure, MDV is spread in the feather dust to other chickens as the keratinized layer of infected epithelial cells is sloughed and shed (17). Shedding appears to peak at approximately 3-5 weeks and continues indefinitely (94, 106). Infected feather dust can remain infectious in a poultry house for at least several months at 20-25°C and for years at 4°C (17, 58).

As a result of continual shedding and virus stability in the poultry house environment, it is assumed that most poultry houses are rich in infectious virus. Chickens raised in a commercial setting are likely exposed to MDV repeatedly and continuously over their lifetime. It is unknown whether chickens are susceptible to more than one virulent strain (coinfection), either simultaneously or later in time

(superinfection). Superinfection may be in the form of re-infection with the same strain or with a novel strain. Although superinfection may be more likely, if the virus load is high enough, newly placed chicks may be exposed to multiple strains virtually simultaneously.

Evidence exists for the simultaneous presence of more than one strain of MDV in a chicken flock. Biggs et al. reported on the epidemiology of MD in six flocks from a broiler female grandparent line (10). Virus isolates were propagated from five flocks, and then 25 isolates were examined and classified for their pathogenicity as either apathogenic, classical or acute. Four of the five flocks had strains present from more than one level of pathogenicity. Two flocks, including a single house from one flock, had strains present from all three levels of pathogenicity. A study by Jackson et al. confirmed the presence of multiple pathotypes circulating within the same flock, and concluded that the environment during the first 8-9 weeks of life was of greatest importance in subsequent mortality from MD (56). Witter et al. evaluated the virulence of multiple isolates (2-3) from two separate flocks and found very similar pathotypes (95). These isolates were all from tumor-bearing birds which may have created a bias towards isolation of more virulent strains. This also suggests, however, that a single virus strain may have the ability to become dominant.

Vaccine studies have shown that coinfection can occur in chickens with virulent and avirulent MDV strains. Witter et al. showed that S1 virus was isolated when MDV was challenged after initial infection with S3 HVT (104). Okazaki et al. concluded that HVT was fully protective when administered at least one week before challenge, and the vaccine gave partial protection when administered simultaneously with challenge at

one day of age (25% MD vs 90% MD in unvaccinated challenged group). (69). Calnek et al. infected birds with S2 SB-1 and S3 HVT-4, followed by JM-10, to study the effect of vaccination on early pathogenesis (21). Viremia was present, although reduced following subsequent challenge. Cho et al. reported evidence of coinfection with acute (Id-1) and mild (HN) MDV strains following simultaneous challenge, although HN was later classified as a nonpathogenic S2 strain (28, 81, 93) After contact exposure to both strains, Cho and Kenzy concluded that infection with one strain, whether acute or mild, did not exclude subsequent infection by the other strain (27). After simultaneous challenge by injection, Cho et al. reported isolates from both strains were present in susceptible birds at 1 week continuing through termination at 8 weeks, with gradual reduction from 6 mixed isolates down to 1 mixed isolate at 8 weeks (26). Cho also reported the presence of both viruses in feather tip extracts following simultaneous challenge (25). Coinfection with attenuated S1 strains and virulent S1 strains has also been documented. Churchill et al. infected chickens with live attenuated S1 virus (HPRS-16/att) and demonstrated that subsequent challenge with more virulent S1 strains can cause infection (30).

Given the evidence of multiple strains of MDV within a flock, the question remains whether simultaneous exposure with fully virulent strains will result in simultaneous infection. Possible outcomes of simultaneous infection include independent growth of each strain, total exclusion of a lesser strain, or dominance without complete exclusion. If the phenomenon of dominance exists between different strains, this may lead to decreased replication and/or transmission of the subordinate strain and may affect the contribution of each virus in tumors. The potential selection

of dominant strains within individual chickens may be an important mechanism in the evolution of virus strains within a flock. Simultaneous infection of multiple strains and subsequent dominance of one strain over the other may lead to transmission of only the dominant virus to neighboring chicken flocks or future flocks exposed to contaminated litter.

Prior obstacles have made the study of virulent S1 coinfection difficult using DNA and antigen as methods for critical analysis, particularly because of the difficulty distinguishing between S1 viruses. Previous studies have relied on plaque morphology or changes in total MD lesions to make assumptions about coinfection. The recent development of mutant strains with slight differences in the pp38 gene has provided a convenient method for distinguishing between two similar fully virulent strains (rMd5 and rMd5//38CVI). The recombinant rMd5 was generated by Reddy et al. using overlapping cosmid clones produced from wild type Md5 (75). The recombinant virus rMd5//38CVI was produced by Lee et al. using the rMd5 cosmid clones, substituting the pp38 gene from CVI988/Rispens (60). Thus, these two viruses share the same rMd5 backbone and are only different in the pp38 gene which differs by two single nucleotide polymorphisms (SNPs). Differentiation between these two viruses is possible by detecting the SNPs coupled with the availability of monoclonal antibodies that detect the difference. The viruses were also useful based on their similar pathogenicity, as reported by Gimeno et al., in which there were no differences in the frequency of lymphoproliferative lesions between the viruses at either 6 or 15 wpi (43).

This study had two hypotheses. First, following simultaneous challenge with two fully virulent S1 strains, both strains will replicate, transmit to other birds and lead

to tumor formation. Second, following simultaneous challenge, both strains will have equal chance of transmission and tumor formation. Understanding the potential phenomenon of dominance under the conditions of simultaneous infection is essential to evaluate data from non-simultaneous superinfection studies. All experiments were carried out using two virus pairs, the first being a pair with similar pathogenicity (rMd5 and rMd5//38CVI, described above) and the second being a pair of different pathogenicity (JM/102W and rMd5//38CVI). Samples were collected and analyzed from bursa during early cytolytic infection, feather follicle epithelium during late cytolytic infection, and spleen and tumors following transformation. Maternal antibody positive, vaccinated chickens were used in the last set of experiments to test any observed effects in conditions relevant to the normal poultry environment.

Materials and Methods

Chickens

White leghorn $15I_5x7_1$ chickens, a F1 hybrid cross of MD susceptible $15I_5$ males and 7_1 females, were used in these experiments (2). Maternal antibody negative (Ab-) chickens were used in experiments 1 and 2 to prevent any potential masking of significant effects. Ab- chickens were reared from an SPF breeding flock with no MD vaccinations or exposure. The flock was negative for MDV antibodies by routine surveillance tests. Maternal antibody positive (Ab+) chickens were used in experiments 3 and 4 to reduce mortality and simulate field conditions. Ab+ chickens were reared from breeder hens vaccinated at hatch with 2000 plaque forming units (pfu) of HVT and at 25 weeks with 2000 pfu of SB1 and Md11/75C viruses for exposure to all three

serotypes. Both flocks were also negative for exogenous avian leukosis virus and reticuloendotheliosis virus from routine surveillance testing. All birds were maintained in negative pressure Horsfall-Bauer isolators. Experiments were approved by the ADOL Animal Care and Use Committee.

Viruses

Two virus pairs were used for simultaneous dual infection: 1) JM/102W and rMd5//38CVI; 2) rMd5 and rMd5//38CVI. The recombinant rMd5 was generated by Reddy et al. using overlapping cosmid clones produced from wild type Md5, a vvMDV strain (75, 93, 103). The recombinant virus rMd5//38CVI was produced by Lee et al. using the rMd5 cosmid clones to substitute the pp38 gene from CVI988/Rispens in order to determine whether the pp38 gene from CVI988/Rispens was related to the protective properties of the vaccine (60). Thus, these two viruses share the same rMd5 backbone and are only different in the pp38 gene. The two pp38 genes differ by two single nucleotide polymorphisms (SNPs) which was the basis for differentiation by IHC and pyrosequencing. The viruses were also chosen based on their similar pathogenicity, as reported by Gimeno et al., in which there were no differences in the frequency of lymphoproliferative lesions between the viruses at either 6 or 15 wpi (43).

The third virus used was JM/102W. The parental strain, JM, was isolated by Sevoian in 1962 (84), and later cloned by 3X endpoint passage in DEF and designated as JM/102W (88). JM/102W was pathotyped as vMDV by Witter and is a principle prototype strain frequently used in MD experiments (93, 95). JM/102W was chosen to

pair with rMd5//38CVI to study dual infection of two MDV strains of differing pathotype.

Experiment 4 compared superinfection results between vaccinated and unvaccinated chickens. For this experiment, chicks were vaccinated at hatch with 2000 pfu of S3 HVT (strain FC126/2) vaccine (101, 102).

Monoclonal antibodies

The MDV pp38 gene, located in the IR_L and U_L region, was utilized for virus differentiation by immunohistochemistry (IHC) and pyrosequencing. All S1 MDV strains reportedly have identical bases in the pp38 gene except bases #320 and #326 (31, 38). Cui et al. determined that monoclonal antibody (Mab) H19 was specific to base pair #320, and was only positive when adenine (A) was present, whereas Mab T65 was specific to base pair #326, and was only positive when guanine (G) was present (34). JM/102W and rMd5 used in this study have A at both base pair #320 and #326 and thus were positive for H19 only. rMd5//38CVI has G at both base pairs #320 and #326 and was positive for T65 only.

Immunohistochemistry testing

Serial sections of frozen bursa and feathered skin were cut at -25°C and dried overnight under vacuum at room temperature. Slides were then fixed in acetone for 45 minutes, air dried, and stored at room temperature. Peroxidase staining of bursa and FFE tissue sections was performed using the Vectastain ABC kit, as described by the

manufacturer.[11] Slides were loaded onto a DakoCytomation Autostainer, where they were rinsed with PBS and then incubated for 20 minutes with 150 uL of normal blocking sera (horse) from the same species the secondary biotynlyated antibody was made from.[12] Slides were rinsed once with PBS and serial sections of each tissue were incubated for 30 minutes with either 150 uL of H19 (1:3200) or T65 (1:2000) monoclonal antibody. Slides were rinsed three times with PBS and then incubated for 30 minutes with 150 uL of the biotinylated secondary antibody. Slides were again rinsed three times with PBS followed by a 30 minute incubation with 150 uL of the avidin and biotinylated horseradish peroxidase (ABC complex). Slides were then rinsed three times with PBS and then stained for 7 minutes with 150 uL of the DAB solution including the buffer and hydrogen peroxide which reacts with the horseradish peroxidase and produces a dark brown color. Two rinses followed and then slides were counterstained with hematoxylin and coverslipped.

Virus Isolation

Viral plaque assays were performed using spleen cell suspensions plated on duck embryo fibroblasts (DEF). Whole spleens were collected at termination (day 56) and placed in 2 mL of Leibovitz McCoy (LM) cell media. Samples were kept on ice and processed immediately following the termination. Spleen samples were rinsed in PBS, placed inside folded gauze pads, and mashed using a syringe handle. Approximately 5 mL of LM media was rinsed through the cheese cloth to collect cells. 100 uL of the cell suspension was added to 1 mL freezing media (55% - 4% LM media,

[11] Vector Laboratories, Inc., Burlingame, CA

[12] Dako, Glostrup, Denmark

25% - calf serum, 20% - Dimethyl sulfoxide [DMSO]) and the samples were stored at -80°C until later plated on DEF.

Cell suspensions were thawed 2-6 weeks later, counted and plated on 2-4 secondary DEF cultures. After 7 days, cultures were fixed with an alcohol-acetone mixture (6 parts acetone and 4 parts 95% alcohol) and stored at -20°C until they were stained and read. Fixed cell culture dishes were rehydrated and incubated for 30 minutes with the same primary antibodies used for immunohistochemistry (H19 or T65), half of the plates with one antibody and half with the other. Plates were washed with three rinses of distilled water followed by three rinses with PBS and then incubated for 30 minutes with a fluorescein-conjugated goat anti-mouse IgG (H + L) secondary antibody[13], purified by gel filtration. Plates were washed again with three rinses of distilled water and PBS and then plaques were counted using a fluorescent microscope.

Pyrosequencing assay

Pyrosequencing was performed with bursa, FFE, spleen and tumor samples on a PSQ 96MA system, according to the manufacturer's protocol.[14] Briefly, biotinylated PCR product specific to the MDV pp38 gene was generated using a standard PCR with a biotinylated reverse primer (forward primer 5'-GTG ATG GGA AGG CGA TAG AA-3', reverse primer 5' Biotin-CAT CTG CTT CAT ACC ATC TC-3'). The PCR product was then captured on streptavidin-coated beads by shaking binding buffer (10 mM Tris-HCl, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20), streptavidin sepharose beads, and PCR product at 1400 rpm for 10 minutes. Vacuum probes were used to collect the

[13] MP Biomedicals, LLC, Solon, OH

[14] Qiagen, Hilden, Germany

beads for transfer to 70% ethanol, denaturation solution (0.2M NaOH), and wash buffer (10mM Tris-Acetat, pH 7.6) to denature and remove the non-biotinylated DNA strand. The beads were then transferred to a new 96-well plate containing annealing buffer (20 mM Tris-Acetat, 2 mM $MgAc_2$) and the sequencing primer (5'-GGA GAG TGG CTG TCA-3'), designed using SNP Primer Design from Pyrosequencing AB version 1.0.1.[15] Nucleotides were added one at a time and incorporation was measured and quantified by light generated by successful elongation. The resulting sequence reported the frequency of each nucleotide that was incorporated at the location of the SNP. Pyrosequencing results were analyzed from all samples with passing results, meaning from samples in which sequence was detected. Samples that failed either did not have adequate virus present (e.g. negative control samples) or there was either insufficient or excessive total DNA present.

Quantitative PCR assay

Quantitative PCR (qPCR) using a multiplex reaction was performed on FFE samples using an ABI 7500 Real-Time PCR System[16]. One TaqMan® probe (fluorogenic 5′ nuclease chemistry) was specific to the MDV pp38 gene and the other specific to an endogenous reference, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Both probes used TAMRA™ (tetramethylrhodamine) quencher dyes. The probe specific to MDV used a green emitter (500-549nm) reporter dye, 6FAM™ (6-carboxyfluorescein, excitation 492, emission 515nm) whereas the probe specific to GAPDH used a yellow emitter (550-584nm) reporter dye, VIC™ (excitation 538,

[15] Applied Biosystems Inc., Foster City, CA

[16] Applied Biosystems Inc., Foster City, CA

emission 554nm). The following conditions were used: 1) 50°C – 2 minutes; 2) 95°C – 10 minutes; 3) 95°C – 15 seconds; 4) 60°C – 1 minute with steps 3-4 repeated 40 times. Each well contained primers and FAM-TAM probe for the MDV pp38 gene (forward primer 5'-GTG ATG GGA AGG CGA TAG AA-3', reverse primer 5'-TCG TCA AGA TGT TCA TTC CCT G-3', TaqMan probe 5'FAM-TACCGCCTGAGCCCCGGAGG-3'TAMRA) and primers and VIC-TAM probe for the cellular gene GAPDH (forward primer 5'-ACA GAG GTG CTG CCC AGA A-3', reverse primer 5'-ACT TTC CCC ACA GCC TTA GCA-3', TaqMan probe 5' VIC-TCA TCC CAG CGT CCA CT-3' TAMRA). A standard curve with 10-fold serial dilutions of plasmids containing MDV pp38 or GAPDH was used to calculate absolute copy numbers of each gene per sample. For the calculation of averages, outliers were removed if the value was greater than 1.5 times the interquartile range (the difference between the 25^{th} and 75^{th} percentile). No more than two values were removed per group. Results were expressed as the viral copy number per host cell.

Experimental design

Four experiments were designed to characterize coinfection in simultaneously challenged chickens **(Table 3-1)**. All four experiments utilized JM/102W, rMd5 and rMd5//38CVI virus strains challenged alone or in combination. Data from each virus pair was analyzed separately as parts A & B of each experiment. Single-challenged control birds were used to confirm the specificity of each assay and as a comparison for quantitative differences compared to dual-infection. All virus and vaccine inoculations

were administered intra-abdominally (IA), with each dose divided into two sites. Specifics of each experiment are detailed below.

Experiment 1 – Twenty $15I_5x7_1$ Ab- chickens were singly-challenged with each virus or dually-challenged with both viruses at hatch, except one group of uninfected chickens used as controls. Bursa samples were collected from 6 birds that were removed and euthanized from each lot at 4 and 6 days post infection (dpi). The remaining 8 birds from each lot were euthanized at 21 dpi and feathers and skin sections were collected from the subhumeral feather tract for DNA and IHC testing, respectively.

Experiments 2 & 3 – These two experiments were identical except experiment 3 used Ab+ chickens to reduce early mortality. Groups of seventeen chickens were singly-challenged with each virus or dually-challenged with both viruses at hatch to increase the probability of acquiring visceral tumors. Surviving chickens were euthanized at 56 dpi and samples were collected. Tumors were collected for pyrosequencing and qPCR testing, and spleens were collected for virus isolation. In experiment 2, only visceral tumors were collected for analysis. In experiment 3, all chickens with visceral tumors were sampled plus enlarged nerves were randomly collected from additional chickens without visceral tumors in order to collect samples from a minimum of eight birds per lot.

Experiment 4 – This experiment consisted of two lots of seventeen $15I_5x7_1$ Ab+ chickens dually-challenged with virus pair 1, and another two lots dually-challenged with virus pair 2. Vaccinated lots received IA inoculation of 2000pfu of HVT at hatch. Virus challenge was administered at 6 days for both vaccinated and unvaccinated groups. Feathers were collected from each chicken at three time points: 13, 26, and 63 dpi. Tumors were also collected at the time of the last feather collection. All feathers and tumor samples were analyzed from tumor-bearing chickens. In addition, feather samples were analyzed from remaining non tumor-bearing chickens chosen in ranking order of wing band numbers to have a minimum of eight birds per lot.

Data analysis

IHC results were analyzed by comparing individual bursa and feather follicles from serial sections stained with H19 or T65 monoclonal antibodies. The percent of follicles staining for each virus or both were contrasted. Pyrosequencing results compared the frequency of each virus present within all bursa, FFE or tumor DNA samples that had passing results (sequence was detected). Differences in frequency (dominance) between viruses in each experiment were analyzed by a paired t-test. Virus isolation results were analyzed using the Wilcoxon rank-sum test due to low numbers of samples in most groups. The analysis compared plaque counts for each virus in dual-challenged chickens with single-challenged chickens challenged with the same virus. Differences in virus copy numbers from qPCR results were compared by ANOVA for significant differences between viruses based on challenge group and sampling day. Significant results were followed by Duncan post hoc analysis to

determine which groups were different. P-values less than 0.05 were considered significant. Data were analyzed with the statistical program SAS 9.1 (SAS Institute Inc., Cary, NC).

Results

Bursa and FFE

Experiment 1 was designed to compare the differences between virus frequency and distribution for both virus pairs during early and late cytolytic infection in the bursa and FFE, respectively. Data was not included from day 4 bursa samples due to a lack of staining in all but one IHC sample and lack of virus sequence detection in all but 4 samples by pyrosequencing. In experiment 1A, virus mixtures were detected in 2 of 5 bursa samples by pyrosequencing, and 2 of 3 samples with staining by IHC **(Table 3-2)**. Seven of eight FFE samples appeared as mixtures by at least one assay, although pyrosequencing did not detect mixtures from two of these birds (M6890 & M6882). A majority of individual feather follicles were found to stain for both viruses following dual challenge and viruses were frequently present within the same region of the follicle **(Figure 3-1)**. The frequency of rMd5//38CVI was significantly greater than JM/102W in bursa and FFE samples from dual-challenged chickens ($p=0.0100$).

In experiment 1B, virus sequence was detected from 4 bursa samples from chickens dual-challenged with rMd5 and rMd5//38CVI **(Table 3-3)**. Both viruses were detected from 3 of the 4 by both pyrosequencing and IHC. In most cases, only one virus was detected within individual bursa follicles, although both viruses were detected in a small number of follicles **(Figure 3-2)**. Both viruses were also detected from 5 of 7

FFE samples by both pyrosequencing and IHC. The frequency of rMd5 was significantly greater than rMd5//38CVI in bursa and FFE samples (p=0.0006).

The individual contributions of each virus to qPCR results from each sample were calculated based on pyrosequencing frequencies. Combined qPCR results from experiments 1A & 1B showed that in the bursa, dual-challenge did not change the average copy number/host cell of each virus compared to single challenged birds based on ANOVA (p=0.0968) **(Figure 3-3)**. In contrast, qPCR results from FFE samples illustrated a significant decrease in average copy number/host cell for both viruses in experiment 1A, but only one virus in experiment 1B (p=0.0009) **(Figure 3-4)**.

Tumors

The purpose of experiments 2 and 3 were to look for evidence of virus dominance in tumors. Experiments 2 and 3 were identical except Ab+ birds were used in the latter experiment to increase survival until sampling. Virus sequence was detected from 14 tumors from 8 birds dually-challenged with JM/102W and rMd5//38CVI in experiments 2 and 3 **(Table 3-4)**. Of these 14 tumor samples, 10 samples were positive for both viruses. Seven tumors from 5 birds favored JM/102W and 7 tumors from 5 birds favored rMd5//38CVI. Two of four birds with multiple tumors had virus frequencies that were markedly different between tumors and in fact were predominant for different viruses. Virus plaque counts were unusually low from spleen cell suspensions, but of 6 samples that stained positive, two stained for only JM/102W and four stained only for rMd5//38CVI. There were no significant

differences between plaque counts for each virus in single versus dual-challenged chickens (p=0.3152).

In chickens dually-challenged with rMd5 and rMd5//38CVI in experiments 2 and 3, virus was detected in 17 tumor samples from 11 chickens **(Table 3-5)**. Both viruses were detected from 3 samples from 2 birds and rMd5 was favored in all three. In the remaining 14 tumors, rMd5 was detected exclusively. More than one tumor was tested for 5 birds and unlike with samples from virus pair 1, all frequencies were similar within individual birds. Virus plaques stained only for rMd5 from spleen cells isolated from 6 of 7 chickens. The remaining chicken had plaques present from both viruses, although twice as many were positive for rMd5. There were no significant differences between plaque counts for each virus in single versus dual-challenged chickens (p=0.2413).

Effect of vaccination

Experiment 4 was designed to confirm potential virus dominance in vaccinated birds. After simultaneous challenge with JM/102W, the frequency of rMd5//38CVI was significantly higher compared to JM/102W in the FFE and was nearly exclusively present in both unvaccinated and HVT-vaccinated chickens (p<0.0001) **(Table 3-6)**. There was no significant difference in virus frequencies between vaccinated and unvaccinated chickens in FFE samples (p=0.3466). rMd5//38CVI was dominant in all 14 tumors, including 4 tumors in which both viruses were present. Interestingly, four chickens had mixed infections in heart tumors, in contrast to the exclusive

rMd5//38CVI detected from FFE, and frequencies differed between multiple tumors from the same bird.

Both viruses were detected in DNA isolated from FFE in 6 of 9 unvaccinated chickens and 3 of 9 vaccinated chickens dually-challenged with rMd5 and rMd5//38CVI **(Table 3-7)**. Mixtures frequencies were quite consistent between the three time points in individual birds. In contrast to experiments 1-3, however, rMd5//38CVI was dominant compared to rMd5 as it was present in higher frequency for all birds within mixtures and those with only one virus present ($p<0.0001$). Similar to virus pair 1, there was no significant difference in virus frequencies between vaccinated and unvaccinated chickens in FFE samples ($p=0.1602$). Three of eight tumors had substantial mixtures of both viruses detected and similar virus frequencies were detected between all tumors and FFE samples in respective birds.

Quantitative PCR results from both virus pairs were highly variable, but except for three day 26 samples of rMd5//38CVI, there were no significant differences between virus quantity in vaccinated versus unvaccinated chickens **(Figure 3-5)**.

Discussion

Data from experiments 1-4 provided evidence that two virulent S1 MDV strains could be present simultaneously in chickens. Mixtures of both strains were relatively common in bursa and feather follicles in birds challenged with either virus pair. Interestingly, virus mixtures were also present within at least 21 of 53 individual tumors. The phenomenon of viral dominance was also demonstrated. For the first time, certain virulent S1 virus strains were shown to have a competitive advantage over

others **(Table 3-8)**. In birds simultaneously challenged with JM/102W and rMd5//38CVI, all bursa samples and 6 of 8 FFE samples from experiment 1A favored rMd5//38CVI whether samples were composed of one or both virus strains. The same was true in experiment 4A in which rMd5//38CVI was nearly exclusively dominant in FFE samples from both vaccinated and unvaccinated chickens, although it is unclear why the predominance was more one-sided compared to experiment 1. JM/102W used in experiments 1 and 4 came from different lots, and perhaps minor variations in titer estimates could have significant effects in this type of challenge model. The dominance of rMd5//38CVI was statistically significant in both experiments.

Dominance may be more difficult to detect using two strains of similar virulence. The majority of bursa and FFE samples from chickens simultaneously challenged with rMd5 and rMd5//38CVI in experiment 1 consisted of virus mixtures, with 6 of 7 favoring rMd5. The remaining non-mixtures also favored rMd5. In experiment 4, both viruses were again detected from most FFE samples (6 of 9 birds), although in this experiment rMd5//38CVI was present in higher proportions in both vaccinated and unvaccinated chickens. Although one virus tended to have a slight edge of the other within an experiment, the predominant virus was not consistent between experiments. If both viruses had similar inherent dominance, this may account for the fact that mixtures of both viruses in bursa and FFE samples were more prevalent with the similar viruses (63% of birds) compared to the more different viruses (28% of birds) in all experiments.

The presence of one virus strain within bursa and feather follicles apparently provided no exclusion from infection with the second virus. IHC confirmed the

presence of both viruses within individual follicles. In feather follicles, a very high percentage of follicles stained for both viruses and many times the positive cells occupied the same proximity **(Figure 3-1)**. In most cases, dominance of a particular virus within a chicken as evident by pyrosequencing results was consistent with proportions of each strain within individual follicles (data not shown).

Viral dominance was less evident in tumor samples, although tumors originate from rare individual transformation events and may be less appropriate for measuring dominance. It was interesting that with the rMd5 and rMd5//38CVI virus pair, the frequency of viruses in tumors was very consistent between multiple tumors in individual birds, and was also very consistent compared to FFE samples from the same bird. On the other hand, the JM/102W and rMd5//38CVI virus pair led to some differences in the virus frequency between tumors in the same bird, suggesting that these tumors may have a polyclonal origin. There is current debate on the clonality of MD tumors. Some have reported that tumors were monoclonal based on the identical number of hybridization signals of interphase nuclei and the identical pattern of chromosomal integration in metaphase spreads (37). In contrast, other reports have suggested a polyclonal tumor origin based on their analysis of T cell receptor (TCR) variable β chain gene family expression and spectratyping (13, 24). Another study also found differences in 132-bp repeat expansion between two tumors from one bird, suggesting two separate transforming events (86). It is difficult to conclude why the two virus pairs in the current study had different results in tumors, but in the case of the rMd5 and rMd5//38CVI virus pair, the results would suggest that tumors may be the result of multiple transforming events, possibly from transformed cells containing more

than one virus. The presence of virus of both viruses within a tumor sample, however, does not necessarily identify the virus responsible for transformation. These experiments may be a useful model for future studies of clonality of tumors.

Vaccination seemingly had no effect on the detection of dominance between virus strains for either pair. Since FC126 (HVT) protects better against JM/102W than Md5, it seemed likely that vaccination would benefit rMd5//38CVI (95). This was difficult to assess, however, since rMd5//38CVI was already significantly dominant to JM/102W in the absence of HVT. The two similar viruses were likely protected to the same degree by HVT, preventing any advantage of one virus over the other.

The topic of replication potential and its relationship to virulence has been the topic of several recent papers. Following challenge with v and vv+ MDV strains in resistant and susceptible birds, Yunis et al. found that the virulent virus became latent at 6 dpi, but the vv+ virus never went into latency in both chicken lines during 10 days. (109). The prolonged virus replication and presence of viral transcripts for the vv+ virus throughout 10 days could cause more severe damage and atrophy of lymphoid organs.

Jarosinski et al. showed significantly higher levels of viral replication for the vv+ strain RK-1 in two chicken lines compared with the v strain JM-16 (57). And Calnek et al. demonstrated a prolonged phase of cytolytic infection at 7-8 dpi for three vv+ isolates (RK-1, 584A, 648A) compared with two v isolates (JM16, GA5) (19).

If virulence is partially defined by replication potential, this would give a clear advantage to whichever virus can replicate faster during coinfection. Presumably when administered simultaneously, both viruses have equal chances for replication within

bursa follicles. The collection of FFE samples from the same birds at three time points showed the relative stability of virus frequencies over time, indicating that any competition that occurred early on was finished and established by 13 days. The proportions of each virus that were present at day 13 in the FFE changed very little during the next two samplings, regardless of which virus was more predominant at the first samplings. The two similar viruses in this study did not have a significant advantage over each other, yet coinfection with the two differing viruses was consistently dominated by the virus of higher virulence. Whether an advantage is the result of preventing coinfection in the same cells is unclear and was not answered by this study. The possible correlation between dominance and replication ability requires additional confirmation.

Viral dominance may be an important factor that influences the outcome of MDV coinfection under field conditions. This effect may be especially important in the selection and propagation of more virulent strains within a poultry flock. Additional studies are necessary to elucidate the effect of dominance in strains of similar virulence and to evaluate the effect in conditions of non-simultaneous challenge (superinfection). Although a weak vaccination was intentionally used, the results suggest that the effect of dominance may be relevant under field conditions.

Table 3-1: Challenge outline

Exp	Lot	# chickens	Maternal antibody[1]	Vaccine	Challenge strain	Challenge age	Dose	Sampling age
1	1	20	Negative		Uninfected controls			4, 6, 21 days[2]
	2	20	Negative		JM/102W only	1 day	500 pfu/bird	4, 6, 21 days
	3	20	Negative		rMd5 only	1 day	500 pfu/bird	4, 6, 21 days
	4	20	Negative		rMd5//38CVI only	1 day	500 pfu/bird	4, 6, 21 days
	5	20	Negative		JM/102W + rMd5//38CVI	1 day	500 pfu/virus/bird	4, 6, 21 days
	6	20	Negative		rMd5 + rMd5//38CVI	1 day	500 pfu/virus/bird	4, 6, 21 days
2	1	10	Negative		Uninfected controls			56 days
	2	17	Negative		JM/102W only	1 day	500 pfu/bird	56 days
	3	17	Negative		rMd5 only	1 day	500 pfu/bird	56 days
	4	17	Negative		rMd5//38CVI only	1 day	500 pfu/bird	56 days
	5	17	Negative		JM/102W + rMd5//38CVI	1 day	500 pfu/virus/bird	56 days
	6	17	Negative		rMd5 + rMd5//38CVI	1 day	500 pfu/virus/bird	56 days
3	1	10	Positive		Uninfected controls			56 days
	2	17	Positive		JM/102W only	1 day	500 pfu/bird	56 days
	3	17	Positive		rMd5 only	1 day	500 pfu/bird	56 days
	4	17	Positive		rMd5//38CVI only	1 day	500 pfu/bird	56 days
	5	17	Positive		JM/102W + rMd5//38CVI	1 day	500 pfu/virus/bird	56 days
	6	17	Positive		rMd5 + rMd5//38CVI	1 day	500 pfu/virus/bird	56 days
4	1	10	Positive		Uninfected controls			13, 26, 63 days[3]
	2	17	Positive		JM/102W + rMd5//38CVI	6 days	500 pfu/virus/bird	13, 26, 63 days
	3	17	Positive	HVT[4]	JM/102W + rMd5//38CVI	6 days	500 pfu/virus/bird	13, 26, 63 days
	4	17	Positive		rMd5 + rMd5//38CVI	6 days	500 pfu/virus/bird	13, 26, 63 days
	5	17	Positive	HVT	rMd5 + rMd5//38CVI	6 days	500 pfu/virus/bird	13, 26, 63 days

[1] All chickens were strain $15I_5x7_1$

[2] Bursa was sampled from 6 birds on 4 and 6 dpi, and the remaining 8 birds were removed and FFE sampled at 21 days.

[3] All birds were sampled at each time point

[4] HVT vaccine was administered at hatch with the dose 2000 pfu

Table 3-2: Experiment 1A. Pyrosequencing and IHC results from bursa (6 dpi) and FFE (21 dpi) samples in chickens single or dual-challenged with JM/102W (J) and/or rMd5//38CVI (M2). Pyrosequencing results show the ratio of each virus present in DNA isolated from the tissue. IHC results are the percentage of positive-staining follicles for one or both viruses. Samples that failed pyrosequencing are indicated by an F.

Challenge strains	Sampling day	Bird #	Tissue	Pyrosequencing (Frequency of each virus)	IHC[1] (% follicles staining for each virus)		
				%J:%M2	J only	Mixed	M2 only
Controls (averaged per group)							
None	6	Avg (6 birds)	Bursa	F	0%	0%	0%
J	6	Avg (3 birds)	Bursa	97:3	8%	0%	0%
M2	6	Avg (4 birds)	Bursa	0:100	0%	0%	12%
Dual-challenged (individual birds)							
J, M2	6	M6898	Bursa	11:89[2]	0%	1%	2%
J, M2	6	M6886	Bursa	6:94	1%	2%	16%
J, M2	6	M6899	Bursa	0:100	0%	0%	30%
J, M2	6	M6895	Bursa	0:100	0%	0%	0%
J, M2	6	M6896	Bursa	F	0%	0%	0%
J, M2	6	M6884	Bursa	0:100	0%	0%	0%
Controls (averaged per group)							
None	21	Avg (8 birds)	FFE	F	0%	0%	0%
J	21	Avg (8 birds)	FFE	98:2	98%	0%	0%
M2	21	Avg (8 birds)	FFE	0:100	0%	0%	98%
Dual-challenged (individual birds)							
J, M2	21	M6890	FFE	0:100	0%	29%	71%
J, M2	21	M6882	FFE	0:100	0%	23%	77%
J, M2	21	M6893	FFE	99:1	86%	0%	0%
J, M2	21	M6881	FFE	17:83	0%	92%	8%
J, M2	21	M6888	FFE	45:55	0%	100%	0%
J, M2	21	M6883	FFE	70:30	0%	100%	0%
J, M2	21	M6900	FFE	43:57	0%	0%	0%
J, M2	21	M6892	FFE	7:93	0%	80%	20%

[1] Number of bursa and feather follicles analyzed per sample averaged 286 (range 138-433) and 9 (range 2-18), respectively.

[2] Frequency of rMd5//38CVI was significantly greater than JM/102W in bursa and FFE samples from dual-challenged birds by paired t-test ($p=0.0100$).

Table 3-3: Experiment 1B. Pyrosequencing and IHC results from bursa (6 dpi) and FFE (21 dpi) samples in chickens single or dual-challenged with rMd5 (M1) and/or rMd5//38CVI (M2). Pyrosequencing results show the ratio of each virus present in DNA isolated from the tissue. IHC results are the percentage of positive-staining follicles for one or both viruses. Samples that failed pyrosequencing are indicated by an F.

Challenge strains	Sampling day	Bird #	Tissue	Pyrosequencing (Frequency of each virus)	IHC[1] (% follicles staining for each virus)		
				%M1:%M2	M1 only	Mixed	M2 only
Controls (averaged per group)							
None	6	Avg (6 birds)	Bursa	F	0%	0%	0%
M1	6	Avg (6 birds)	Bursa	97:3	25%	0%	0%
M2	6	Avg (4 birds)	Bursa	0:100	0%	0%	12%
Dual-challenged (individual birds)							
M1, M2	6	M6911	Bursa	83:17[2]	11%	1%	0%
M1, M2	6	M6909	Bursa	F	0%	0%	0%
M1, M2	6	M6912	Bursa	F	0%	0%	0%
M1, M2	6	M6901	Bursa	76:24	7%	1%	1%
M1, M2	6	M6913	Bursa	97:3	40%	0%	0%
M1, M2	6	M6905	Bursa	21:79	0%	3%	3%
Controls (averaged per group)							
None	21	Avg (8 birds)	FFE	F	0%	0%	0%
M1	21	Avg (6 birds)	FFE	97:3	67%	0%	0%
M2	21	Avg (8 birds)	FFE	0:100	0%	0%	98%
Dual-challenged (individual birds)							
M1, M2	21	M6908	FFE	91:9	8%	92%	0%
M1, M2	21	M6902	FFE	84:16	0%	100%	0%
M1, M2	21	M6903	FFE	98:2	No sample		
M1, M2	21	M6907	FFE	88:12	25%	75%	0%
M1, M2	21	M6920	FFE	82:18	15%	85%	0%
M1, M2	21	M6915	FFE	88:12	0%	100%	0%
M1, M2	21	M6910	FFE	98:2	100%	0%	0%

[1] Number of bursa and feather follicles analyzed per sample averaged 286 (range 138-433) and 9 (range 2-18), respectively.

[2] Frequency of rMd5 was significantly greater than rMd5//38CVI in bursa and FFE samples from dual-challenged birds by paired t-test ($p=0.0006$)

Table 3-4: Experiments 2A & 3A. Virus isolation and pyrosequencing results from single and dual-challenged chickens with JM/102W (J) and/or rMd5//38CVI (M2). Tumors were used for pyrosequencing, except negative controls which were spleen samples. Pyrosequencing results show the ratio of each virus present in DNA isolated from the tissue. Virus isolation results are the average virus plaque count/10^6 cells for each virus. Samples that failed pyrosequencing are indicated by an F.

Exp	Challenge strains	Sampling day	Bird#	Virus isolation Avg Plaque Count/10^6 cells[1]		Pyrosequencing Frequency of each virus in tumors %J:%M2					
				J	M2	Ner[2]	Hrt	Spl	Gon	Liv	Kid
Controls (averaged per group)[3]											
2	None	56						F (3)			
	J	56					96:4 (4)		99:1 (3)		100:0 (1)
	M2	56					0:100 (5)				
3	None	56		0 (10)	0 (10)			F (8)			
	J	56		6 (1)	0 (1)	100:0 (3)		100:0 (1)	100:0 (4)		
	M2	56		0 (7)	8 (7)	0:100 (5)				0:100 (1)	0:100 (1)
Dual-challenged (individual birds)											
2	J, M2	56	M6984				0:100				
	J, M2	56	M6988				89:11				
3	J, M2	56	Q1825			F			69:31		68:32
	J, M2	56	Q1828	0	2	F					
	J, M2	56	Q1829			77:23			F		
	J, M2	56	Q1830	9	0				0:100		
	J, M2	56	Q1832	0	18	3:97	13:87		9:91		
	J, M2	56	Q1834	0	0	66:34	23:77		100:0		
	J, M2	56	Q1835	0	10						
	J, M2	56	Q1837	2	0						
	J, M2	56	Q1838	0	25	70:30			0:100		

[1] Spleens were used for virus isolation. Each sample consisted of 1-2 plates/monoclonal antibody/bird.

[2] Tumor abbreviations: Enlarged nerve (Ner), Heart (Hrt), Spleen (Spl), Gonad (Gon), Liver (Liv), Kidney (Kid)

[3] The numbers of control group virus isolation or tumor samples of each type are indicated within parentheses

Table 3-5: Experiments 2B & 3B. Virus isolation and pyrosequencing results from single and dual-challenged chickens with rMd5 (M1) and/or rMd5//38CVI (M2). Tumors were used for pyrosequencing, except negative controls which were spleen samples. Pyrosequencing results show the ratio of each virus present in DNA isolated from the tissue. Virus isolation results are the average virus plaque count/10^6 cells for each virus. Samples that failed pyrosequencing are indicated by an F.

Exp	Challenge strains	Sampling day	Bird#	Virus isolation Avg Plaque Count/10^6 cells[1]		Pyrosequencing Frequency of each virus %M1:%M2					
				M1	M2	Ner[2]	Hrt	Spl	Gon	Liv	Kid
Controls (averaged per group)[3]											
2	None	56						F (3)			
	M1	56					99:1 (1)				
	M2	56					0:100 (5)				
3	None	56		0 (10)	0 (10)			F (8)			
	M1	56		9 (9)	1 (9)	99:1 (5)	100:0 (1)		100:0 (1)		
	M2	56		0 (7)	8 (7)	0:100 (5)				0:100 (1)	0:100 (1)
Dual-challenged (individual birds)											
2	M1, M2	56	13090				100:0				
	M1, M2	56	13093				100:0				
	M1, M2	56	13099				69:31				
	M1, M2	56	13100				97:3				
3	M1, M2	56	Q1842	4	0	100:0		98:2			
	M1, M2	56	Q1844	7	0	100:0	100:0				
	M1, M2	56	Q1847			F					
	M1, M2	56	Q1848	40	0	99:1	100:0		100:0		
	M1, M2	56	Q1852	12	0	100:0			F		
	M1, M2	56	Q1853	32	0	100:0			100:0		
	M1, M2	56	Q1855	3	0	73:27	72:28				
	M1, M2	56	Q1857	32	13	100:0					

[1] Spleens were used for virus isolation. Each sample consisted of 1-2 plates/monoclonal antibody/bird.

[2] Tumor abbreviations: Enlarged nerve (Ner), Heart (Hrt), Spleen (Spl), Gonad (Gon), Liver (Liv), Kidney (Kid)

[3] The numbers of control group virus isolation or tumor samples of each type are indicated within parentheses

Table 3-6: Experiment 4A. Pyrosequencing results from multiple FFE and/or tumor samples in vaccinated and unvaccinated chickens challenged simultaneously with JM/102W (J) and rMd5//38CVI (M2). Pyrosequencing results show the ratio of each virus present in DNA isolated from the tissue. Samples that failed pyrosequencing are indicated by an F.

			Pyrosequencing (Frequency of each virus) %J:%M2							
			FFE			**Tumors**[2]				
Challenge strains	**Vaccination**[1]	**Bird#**	**Day 13**	**Day 26**	**Day 63**	**Hrt**	**Spl**	**Gon**	**Liv**	**Kid**
J, M2		R5364	0:100[3]	0:100	0:100					
J, M2		R5365	0:100	0:100	0:100	15:85	0:100			
J, M2		R5366	0:100	0:100	0:100					
J, M2		R5367	0:100	0:100	0:100					
J, M2		R5369	0:100	0:100	0:100	31:69	0:100		0:100	0:100
J, M2		R5370	5:95	0:100	10:90					
J, M2		R5371	0:100	0:100	0:100	14:86				
J, M2		R5372	0:100	6:94	0:100					
J, M2		R5377	0:100	0:100	0:100	0:100				
J, M2	HVT	R5383	0:100	0:100	F			0:100		
J, M2	HVT	R5384	F	0:100	0:100					
J, M2	HVT	R5385	0:100	0:100	0:100	0:100			0:100	0:100
J, M2	HVT	R5386	0:100	0:100	0:100	4:96				
J, M2	HVT	R5387	0:100	0:100	0:100					
J, M2	HVT	R5388	0:100	0:100	0:100					
J, M2	HVT	R5389	0:100	0:100	0:100			F		
J, M2	HVT	R5390	0:100	0:100	0:100					
J, M2	HVT	R5391	0:100	0:100	0:100					
J, M2	HVT	R5396	0:100	0:100	10:90			27:73		

[1] HVT was administered at hatch (2000 pfu) followed by 500 pfu of each challenge strain at 6 days of age

[2] Tumor abbreviations: Heart (Hrt), Spleen (Spl), Gonad (Gon), Liver (Liv), Kidney (Kid)

[3] Frequency of rMd5//38CVI was significantly greater than JM/102W in bursa and FFE samples from dual-challenged birds by paired t-test ($p<0.0001$). No significant difference between vaccinated and unvaccinated results by t-test ($p=0.3466$)

Table 3-7: Experiment 4B. Pyrosequencing results from multiple FFE and/or tumor samples in vaccinated and unvaccinated chickens challenged simultaneously with rMd5 (M1) and rMd5//38CVI (M2). Pyrosequencing results show the ratio of each virus present in DNA isolated from the tissue. Samples that failed pyrosequencing are indicated by an F.

			Pyrosequencing (Frequency of each virus) %M1:%M2							
			FFE			**Tumors**[2]				
Challenge strains	**Vaccination**[1]	**Bird#**	**Day 13**	**Day 26**	**Day 63**	**Hrt**	**Spl**	**Gon**	**Liv**	**Kid**
M1, M2		R5174	16:84[3]	17:83	17:83	0:100				
M1, M2		R5175	19:81	12:88	6:94					
M1, M2		R5176	24:76	21:79	22:78					
M1, M2		R5177	35:65	20:80	18:82					
M1, M2		R5178	0:100	0:100	0:100			0:100		3:97
M1, M2		R5182	F	0:100	0:100	0:100				
M1, M2		R5184	15:85	15:85	33:67	25:75				
M1, M2		R5187	0:100	0:100	7:93	13:87				
M1, M2		R5189	23:77	27:73	F	54:46				
M1, M2	HVT	R5191	0:100	0:100	0:100					
M1, M2	HVT	R5193	21:79	4:96	17:83					
M1, M2	HVT	R5194	F	0:100	0:100					
M1, M2	HVT	R5195	0:100	0:100	0:100					
M1, M2	HVT	R5196	F	0:100	0:100	3:97				
M1, M2	HVT	R5197	8:92	9:91	71:29					
M1, M2	HVT	R5198	0:100	0:100	0:100					
M1, M2	HVT	R5201	21:79	33:67	37:63					
M1, M2	HVT	R5204	0:100	0:100	10:90					

[1] HVT was administered at hatch (2000 pfu) followed by 500 pfu of each challenge strain at 6 days of age

[2] Tumor abbreviations: Heart (Hrt), Spleen (Spl), Gonad (Gon), Liver (Liv), Kidney (Kid)

[3] Frequency of M2 was significantly greater than M1 in FFE samples from dual-challenged birds by paired t-test ($p<0.0001$). No significant difference between vaccinated and unvaccinated results by t-test ($p=0.1602$).

Table 3-8: Pyrosequencing summary – viral predominance

Exp	Tissue	JM/102W	rMd5//38CVI	p-value
1	Bursa & FFE	23%	77%	0.0100
2/3	Tumors	42%	58%	0.4398
4	FFE	1%	99%	<0.0001
4	Tumors	6%	94%	<0.0001
Exp	**Tissue**	**rMd5**	**rMd5//38CVI**	**p-value**
1	Bursa & FFE	82%	18%	0.0100
2/3	Tumors	95%	5%	0.4398
4	FFE	20%	80%	<0.0001
4	Tumors	12%	88%	<0.0001

Figure 3-1: Experiment 1. Immunohistochemistry staining of serial FFE sections 21 dpi from a chicken simultaneously challenged with JM/102W and rMd5//38CVI. The sections on the left and right were stained with H19 and T65 monoclonal antibodies, respectively.

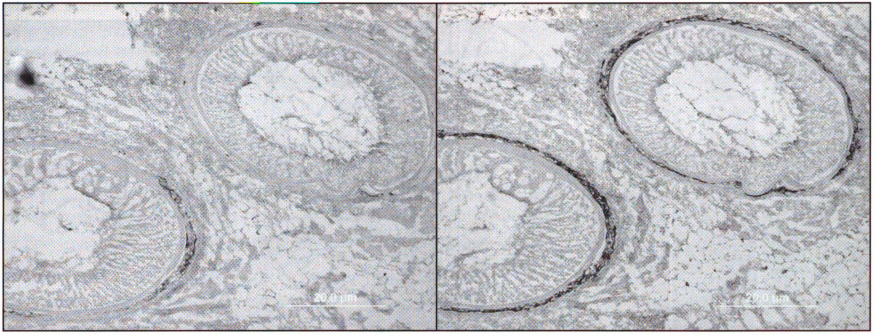


Figure 3-2: Experiment 1. Immunohistochemistry staining of serial bursa sections 6 dpi from a chicken simultaneously challenged with rMd5 and rMd5//38CVI. The sections on the left and right were stained with H19 and T65 monoclonal antibodies, respectively. Black circles surround follicles that stained for rMd5 virus only, white circles stained for rMd5//38CVI virus only, and white/black circles stained for both viruses.

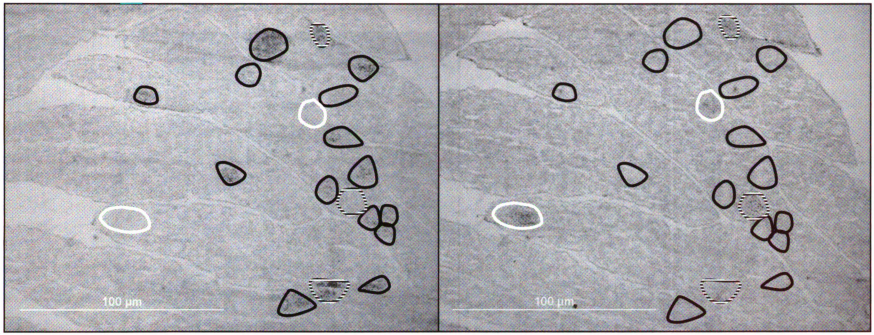


Figure 3-3: Experiment 1. Average quantitative PCR results from DNA isolated from bursa sections sampled at 6 dpi from birds singly or dually infected with JM/102W (J), rMd5 (M1), or rMd5//38CVI (M2). Error bars indicate one standard deviation. Two of 20 samples were removed as outliers as described in the methods on page 88. No significant differences between groups were observed by ANOVA (p=0.0968)

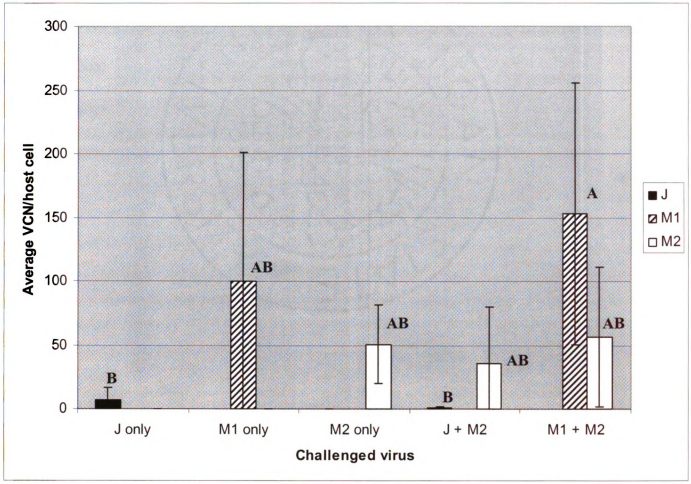


Figure 3-4: Experiment 1. Average quantitative PCR results from DNA isolated from FFE sections sampled at 21 dpi from birds singly or dually infected with JM/102W (J), rMd5 (M1), or rMd5//38CV1 (M2). Error bars indicate one standard deviation. Three of 29 samples were removed as outliers as described in the methods on page 88. Groups with different letters indicate significant differences by ANOVA (p=0.0009).

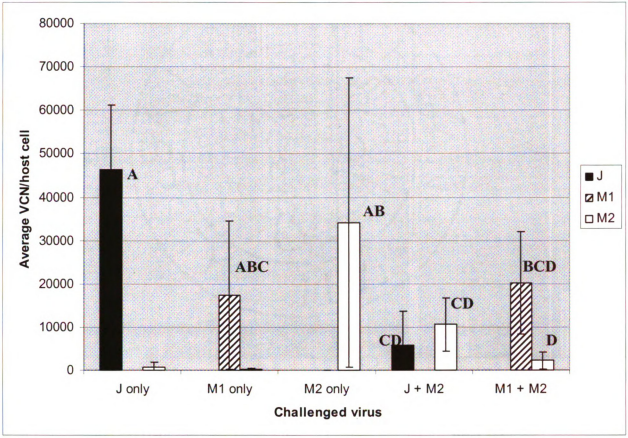
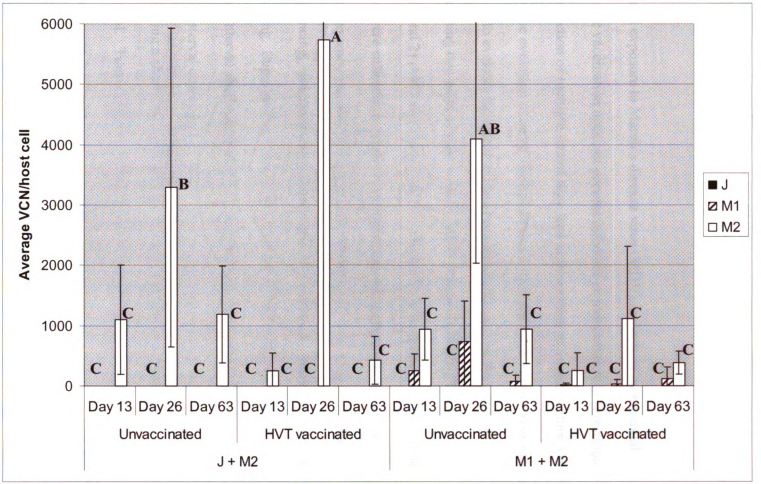


Figure 3-5: Experiment 4. Average quantitative PCR results from DNA isolated from FFE sections sampled at 13, 26, and 63 dpi from birds vaccinated or unvaccinated with HVT and dually infected with either JM/102W (J) and rMd5//38CVI (M2), or rMd5 (M1) and rMd5//38CVI (M2). Error bars indicate one standard deviation. 13 of 103 samples were removed as outliers as described in the methods on page 88. Groups with different letters indicate significant differences by ANOVA ($p<0.0001$).



Chapter 4

THE EFFECT OF THE TIME INTERVAL BETWEEN EXPOSURES ON THE SUSCEPTIBILITY OF CHICKENS TO SUPERINFECTION WITH MAREK'S DISEASE VIRUS STRAINS

Abstract

Repeated exposure to Marek's disease virus (MDV) is likely in commercial chickens because vaccination does not prevent infection or transmission of infectious virus. Superinfection of multiple strains may lead to interaction of viruses within a host and encourage the evolution of more virulent strains within the flock. Four experiments were performed to evaluate the effect of time between challenges on the effect of superinfection using two pairs of fully virulent MDV strains: 1) JM/102W and rMd5//38CVI , and 2) rMd5 and rMd5//38CVI. Feather follicle epithelium, spleen, and tumor samples were collected at single or multiple time points from the same bird to determine the frequency and distribution of each virus present following superinfection using pyrosequencing, immunohistochemistry, and virus isolation. The effect of time interval was strong. Superinfection was observed in 82 of 149 (55%) FFE samples following short interval challenge (24 hours) compared to only 6 of 121 (5%) samples following long interval challenge (13 days). Dominance was detected between the first virus pair following a short interval challenge, but was unapparent following the longer challenge interval. Tumor samples generally consisted of a single predominant virus, but both viruses were detected within a small portion of tumors. Vaccination with HVT had no significant effect on the virus frequency for either virus pair or challenge time interval, suggesting these results may be significant in field conditions. In cases where birds are exposed within shorter periods of time or where the initial exposure is weak or

delayed, mixed infections are likely to occur regardless of vaccination status or inoculation order. These studies demonstrate that mixed infections under some conditions can lead to shedding of the second virus a prerequisite for the establishment of the second virus strain in the population.

Introduction

Marek's disease virus (MDV) is an alphaherpesvirus that causes lymphoproliferative lesions in chickens. One of the greatest concerns facing the poultry industry is the evolutionary trend of MDV towards greater virulence (95). The evolution of Marek's disease (MD) was recently reviewed (65). The first wave of virus evolution from mild (mMDV) to virulent (vMDV) strains was during the 1950's and was likely due to the transformation of the industry to highly intensive poultry practices. Subsequent evolution has been attributed to the introduction of successive generations of MD vaccines (92). The more recent evolution is likely due to the fact that MD vaccines fail to produce a sterilizing immunity, allowing viruses to replicate and shed within vaccinated hosts. The early and later evolution waves, however, both rely on the continuous availability of large populations of susceptible birds.

Transmission of MDV occurs through the production of fully infectious virions in the feather follicle epithelium (FFE) that are spread in the feather dust as early as 2 weeks after inoculation or exposure (18). Shedding appears to peak at approximately 3-5 weeks and continues indefinitely at quantities as high as 109 virus copies per chicken per day, irrespective of vaccination status (53, 94, 106). Infected feather dust

can remain infectious in a poultry house for at least several months at 20-25°C and for years at 4°C (17, 58). The continuous transmission, long survivability of the virus, and large population of birds favor a significant viral load within a poultry house.

Previous studies have confirmed that multiple strains of MDV can circulate simultaneously within the same flock. One study propagated 25 isolates from five poultry flocks and found that four of the five flocks had strains present from more than one level of pathogenicity (apathogenic, classical, or acute) (10). Two flocks, including a single house from one flock, had strains present from all three levels of pathogenicity. Another study examined isolates from a flock at different time points and found that Marek's disease (MD) mortality was closely associated with the sequence of infection and frequency of isolation of viruses of differing virulence (56). For example, three pens that experienced high mortality had early isolates that were of the acute type, whereas two different pens with low incidence of MD had apathogenic strains that predominated throughout early and late isolations.

The studies described above provide evidence for multiple strains of MDV circulating with a flock, but don't address the possibility of multiple strains within the same bird. Superinfection is defined as an additional infection occurring during the course of an existing infection. In this study, the term is meant to describe infection with one MDV strain followed by a later infection with a different MDV strain. Establishment of a second strain not only implies infection, but requires the necessary fitness traits for that strain to compete with the first virus. After the strain infects one cell, it must be able to replicate and spread to other cells. Conditions that may facilitate superinfection include internal sources of new mutant strains from within a bird or

external sources of new strains brought in from other flocks. Superinfection with two virulent Marek's disease virus (MDV) strains is likely an important step in the pathway towards establishment of evolved strains within a population. Chapter 3 demonstrated that coinfection was possible when viruses were administered simultaneously and dominance was observed between viruses of different pathotypes. In field conditions, a time interval between exposures is more likely than simultaneous exposure.

Laboratory-based evidence that superinfection can occur with two fully virulent S1 MDV strains is limited. Ianconescu et al. attempted to evaluate the effect of early natural exposure on subsequent challenge with virulent JM MDV (48). Nonvaccinated farm-reared chickens were removed from the farm and challenged with JM at 5, 7 or 12 weeks of age. Uninoculated groups developed MD tumors and antibodies after isolation, confirming natural exposure on the farm. At 12 weeks, farm-reared chickens inoculated with JM had no difference in tumor incidence compared to unchallenged birds. Thus, there was no clear evidence that superinfection occurred or had an effect. A more recent study evaluated the effect of superinfection in adult birds using known virus challenge for early and late exposure (99). Virus exposed chickens were vaccinated with HVT at hatch plus challenged with JM/102W at 5 weeks. When virus exposed chickens were challenged with a highly virulent strain as adults at 18 weeks, they generally failed to develop transient paralysis or tumors. Not only were the virus exposed groups refractory to lesion indication and transient paralysis, but the viremia responses and transmission to contacts appeared minimal at best, indicating that these birds had a much reduced susceptibility to infection. The authors concluded that late outbreaks in the field are not likely due to superinfection, but are more likely triggered

by unknown environmental factors. Thus, with a long time interval between exposures, both studies failed to detect any significant superinfection. Reports from the field have suggested that superinfection of virulent MDV strains is possible, based on apparent adult-to-adult transmission of vv+ MDV with very late outbreaks occurring, even after molt (59). Since most birds in a commercial flock are presumably infected with MDV early after placement, it would seem that superinfection must occur for any new strain to gain access and become established within a bird and flock.

Superinfection has been studied using other herpesvirus models. A recent study evaluated feline herpesvirus type 1 (FeHV-1) superinfection in cats latently infected with wild-type FeHV-1 (77). Nine SPF cats were inoculated with wild-type FeHV-1 and superinfected 8 months later with a recombinant FeHV-1 virus with green fluorescent protein substituted for glycoprotein G (rFeHV-1ΔgG/GFP). The recombinant virus produced a temporary local lymphadenopathy, but otherwise produced no clinical or ocular signs of infection. Cyclophosphamide-dexamethasone treatment was administered 16 months after superinfection to stimulate reactivation, which was successful for the underlying wtFeHV-1 infection but no reactivation of the superinfecting recombinant virus was detected. The study unfortunately lacked a control group of cats infected only with rFeHV-1ΔgG/GFP to confirm both strains were fully pathogenic.

This study had two hypotheses. First, following short (24 hours) and long (13 days) interval challenge, the first virus will be present in greater frequency in bursa, feather follicles, and tumors relative to the second inoculated virus. Second, vaccination will mute the effects of both viruses leading to equal mixtures in bursa,

feather follicles and tumors following short and long interval challenges. The short interval was potentially more reflective of rapid exposure to more than one strain in field conditions, whereas the long interval was considered sufficient time to illustrate the effect of delayed challenge. The experiments in this chapter also were designed to determine whether the virus dominance observed in chapter 3 was still evident when one virus was given a head start. The final experiment in this chapter included maternal antibody positive birds vaccinated with HVT to determine the relevance of these events to field conditions.

Superinfection of multiple virulent MDV strains seems necessary to allow the establishment of evolved MDV strains within a flock. Understanding what events are necessary for superinfection to occur will increase our understanding of the conditions necessary to allow viruses to interact within a host and evolve to greater virulence or prevalence in a population.

Materials and Methods

Chickens

The experiments used $15I_5x7_1$ white leghorn chickens, a F1 hybrid cross of MD susceptible $15I_5$ males and 7_1 females (2). Maternal antibody negative (Ab-) chickens were used in experiments 1 and 2, which were reared from an SPF breeding flock housed in isolators that have received no MD vaccinations or exposure. The flock was negative for MDV antibodies by routine surveillance tests. Maternal antibody positive (Ab+) chickens were used in experiments 3 and 4, reared from breeder hens vaccinated at hatch with 2000 plaque forming units (pfu) of HVT and at 25 weeks with 2000 pfu of

SB1 and Md11/75C viruses for exposure to all three serotypes. Both flocks were also negative for exogenous avian leukosis virus and reticuloendotheliosis virus from routine surveillance testing. All birds were maintained in negative pressure Horsfall-Bauer isolators. Experiments were approved by the ADOL Animal Care and Use Committee.

Viruses

Three viruses were used to create two virus pairs: 1) JM/102W and rMd5//38CVI; 2) rMd5 and rMd5//38CVI. JM/102W is a clone originating from the parental strain JM, and has been pathotyped as a vMDV (84, 93, 95). The two recombinant viruses originate from the wild type Md5 strain, a vvMDV strain (93, 103). rMd5 was produced from the wild type strain using overlapping cosmid clones (75). JM/102W and rMd5 were both paired against rMd5//38CVI, produced using the rMd5 cosmid clones with substitution of the pp38 gene from CVI988/Rispens (60). The ability to differentiate the pp38 gene of CVI988/Rispens from other S1 MDV strains, allowed differentiation of these two virus pairs within a mixture. The first virus pair was chosen based on differences in pathotype. The second virus pair was chosen based on their similar pathogenicity, as reported by Gimeno et al., in which there were no differences in the frequency of lymphoproliferative lesions between the viruses at either 6 or 15 wpi (43).

Experiment 4 compared superinfection results between vaccinated and unvaccinated chickens. For this experiment, chicks were vaccinated at hatch with 2000 pfu of S3 HVT (strain FC126/2) vaccine (101, 102).

Monoclonal antibodies

The MDV pp38 gene, located in the IR_L and U_L region, was utilized for virus differentiation by immunohistochemistry (IHC) and pyrosequencing. According to early published pp38 sequence data, all serotype 1 MDV strains have identical bases in the pp38 gene except bases #320 and #326 (31, 38). Cui et al. determined that monoclonal antibody (Mab) H19 was specific to base pair #320, and was only positive when adenine (A) was present, whereas Mab T65 was specific to base pair #326, and was only positive when guanine (G) was present (34). JM/102W and rMd5 used in this study have A at both base pair #320 and #326 and thus were positive for H19 only. rMd5//38CVI has G at both base pairs #320 and #326 and thus was positive for T65 only.

Immunohistochemistry testing

Immunohistochemistry (IHC) testing was performed using previously described methods (see Chapter 3). Briefly, feathered skin sections were cut at -25°C, dried overnight, and fixed in acetone for 45 minutes before storage. Peroxidase staining for pp38 antigen was performed using the Vectastain ABC kit, as described by the manufacturer.[17] A DakoCytomation Autostainer was used for staining, where slides were rinsed (all rinses were done with PBS) and then incubated for 20 minutes with 150 uL of normal blocking sera (horse).[18] Slides were rinsed once and then incubated for 30 minutes with either 150 uL of H19 (1:3200) or T65 (1:2000) monoclonal antibody.

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[17] Vector Laboratories, Inc., Burlingame, CA

[18] Dako, Glostrup, Denmark

Slides were rinsed three times and then incubated for 30 minutes with 150 uL of the biotinylated secondary antibody. Slides were rinsed three times followed by a 30 minute incubation with 150 uL of the avidin and biotinylated horseradish peroxidase (ABC complex). Slides were then rinsed three times and stained for 7 minutes with 150 uL of the DAB solution including the buffer and hydrogen peroxide which reacts with the horseradish peroxidase and produces a dark brown color. Two rinses followed and then slides were counterstained with hematoxylin and coverslipped.

Virus isolation

Viral plaque assays were performed using spleen cell suspensions plated on duck embryo fibroblasts (DEF), as previously described (See Chapter 3). Briefly, whole spleens were collected at termination and mashed using a syringe handle to produce a single cell suspension. Approximately 100 uL of the cell suspension, in Leibovitz McCoy (LM) media, was added to 1 mL freezing media (55% - 4% LM media, 25% - calf serum, 20% - Dimethyl sulfoxide [DMSO]) and the samples were stored at -80°C until later plated on DEF.

Cell suspensions were thawed 2-6 weeks later, counted and plated on 2-4 secondary DEF cultures. After 7 days, cultures were fixed with an alcohol-acetone mixture (6 parts acetone and 4 parts 95% alcohol) and stored at -20°C until they were stained and read. Fixed cell culture dishes were rehydrated and incubated for 30 minutes with either H19 or T65 monoclonal antibodies. Plates were washed with three rinses of distilled water followed by three rinses with PBS and then incubated for 30 minutes with a fluorescein-conjugated goat anti-mouse IgG (H + L) secondary

antibody[19], purified by gel filtration. Plates were washed again and then plaques were counted using a fluorescent microscope.

Pyrosequencing assay

FFE and tumor samples were analyzed by pyrosequencing to determine the percentage of each nucleotide present in an A/G SNP at base pair #320 of the MDV pp38 gene. This SNP was used to distinguish rMd5//38CVI (dGTP) from JM/102W and rMd5 MDV strains (dATP). DNA samples were purified using the Gentra Puregene Genomic DNA Purification Kit.[20] The assay was performed using a PSQ 96MA system, according to the manufacturer's protocol, as described previously (See Chapter 3).[21] Briefly, biotinylated PCR product specific to the MDV pp38 gene was generated using a standard PCR with a biotinylated reverse primer (forward primer 5'-GTG ATG GGA AGG CGA TAG AA-3', reverse primer 5' Biotin-CAT CTG CTT CAT ACC ATC TC-3'). In experiments 3 and 4, DNA degradation led to low yield of PCR product and failing pyrosequencing results. A nested PCR was performed on these samples first using an additional set of outside primers (forward primer 5'-TCT CCG CCC CCT CAC TCT-3', reverse primer 5'-TCC GCA TAT GTT CCT CCT TC–3'). The PCR product was then captured on streptavidin-coated beads by shaking binding buffer, streptavidin sepharose beads, and PCR product at 1400 rpm for 10 minutes. Beads were transferred to 70% ethanol, denaturation solution, and wash buffer to denature and remove the non-biotinylated DNA strand. The beads were then

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[19] MP Biomedicals, LLC, Solon, OH

[20] Qiagen, Hilden, Germany

[21] Qiagen, Hilden, Germany

transferred to a new 96-well plate containing annealing buffer and the sequencing primer (5'-GGA GAG TGG CTG TCA-3'), designed using SNP Primer Design from Pyrosequencing AB version 1.0.1.[22] Nucleotides were added one at a time and incorporation was measured and quantified by light generated by successful elongation. The relative percentage of each nucleotide incorporated at the location of the SNP was reported and interpreted as the frequency of each virus within the DNA sample. Pyrosequencing results were analyzed from all samples with passing results, meaning from samples in which sequence was detected. Samples that failed either did not have adequate virus present (e.g. negative control samples) or there was either insufficient or excessive total DNA present.

Quantitative PCR assay

Quantitative PCR (qPCR) using a multiplex reaction was performed on an ABI 7500 Real-Time PCR System, as described previously (see Chapter 3)[23]. Briefly, each well contained the purified DNA sample, primers and FAM-TAM probe specific for the MDV pp38 gene (forward primer 5'-GTG ATG GGA AGG CGA TAG AA-3', reverse primer 5'-TCG TCA AGA TGT TCA TTC CCT G-3', TaqMan probe 5'FAM-TACCGCCTGAGCCCCGGAGG-3'TAMRA) and primers and VIC-TAM probe specific for the cellular gene GAPDH (forward primer 5'-ACA GAG GTG CTG CCC AGA A-3', reverse primer 5'-ACT TTC CCC ACA GCC TTA GCA-3', TaqMan probe 5' VIC-TCA TCC CAG CGT CCA CT-3' TAMRA). The following conditions were used: 1) 50°C – 2 minutes; 2) 95°C – 10 minutes; 3) 95°C – 15 seconds; 4) 60°C – 1

[22] Applied Biosystems Inc., Foster City, CA

[23] Applied Biosystems Inc., Foster City, CA

minute with steps 3-4 repeated 40 times. A standard curve with 10-fold serial dilutions of plasmids containing MDV pp38 or GAPDH was used to calculate absolute copy numbers of each gene per sample. For the calculation of averages, outliers were removed if the value was greater than 1.5 times the interquartile range (the difference between the 25^{th} and 75^{th} percentile). On average, one value was removed per group (range 0-3). Results were expressed as the viral copy number per host cell.

Experimental design

A total of four experiments were designed to analyze superinfection following short and long time intervals. Each experiment consisted of two parts (A/B); Part A used virus pair 1 (JM/102W and rMd5//38CVI) and part B used virus pair 2 (rMd5 and rMd5//38CVI).

Experiments 1 & 2

Experiment 1 examined superinfection in FFE using Ab- chickens. Experiment 1A consisted of 19 groups of 10 chickens, examining the frequency of JM/102W and rMd5//38CVI in feathered skin sections **(Table 4-1)**. Viruses were administered alone or together following short (1 day) or long (13 day) intervals in forward and reverse order. Samples were collected 28 days post infection of the second virus challenge. Skin sections were frozen for IHC and DNA isolated from the outside of the feather shafts for pyrosequencing. Contact chickens were also included as an additional method to determine which viruses were being shed in mixed infections. Three "donor" birds were transferred from each of the four lots of dually infected chickens and

transferred to the contact "recipient" isolator of newly hatched chickens. The donor birds remained in the contact isolators for 1 week and were then removed for sampling along with the rest of the main group of birds. Thus, donor birds were transferred at approximately 21 days after the second challenge during the time of peak shedding and were removed at 28 days post infection of the second challenge. Contact recipient birds were sampled at 28 days post exposure. Experiment 1B was identical to 1A, except using virus pair 2 (rMd5 and rMd5//38CVI).

Experiment 2 examined superinfection in tumors using Ab- chickens. Experiment 2 was the same basic design as experiment 1, except this experiment was designed to determine the frequency of each virus in tumors and latently infected lymphocytes **(Table 4-1)**. Samples consisted of DNA isolated from tumors and enlarged nerves for pyrosequencing and spleen suspensions for measuring viremia. Samples were collected 56 days post inoculation of the second virus challenge. Seventeen birds were used in challenged groups instead of 10 birds because it was expected that at least half of the infected chickens would not produce visceral tumors. Tumors and enlarged nerves were collected from all surviving birds at the time of termination with visceral tumors. Multiple tumors were collected when present. If less than 10 birds per group had visceral tumors, nerves were sampled from birds with nerve enlargement randomly in order of low to highest wing band number until at least 10 birds were sampled from each group. Some groups did not have enough surviving birds to meet this requirement. Spleen was sampled from uninfected control chickens. Contact chickens were hatched and exposed in the identical procedure as experiment 1, except donor birds were returned to their original isolator following one week of

housing with recipient birds since sampling of the main group of birds was not until 4 weeks later. Contact recipient birds were sampled at approximately 56 days post exposure. Experiment 2A was terminated early at 5.5 weeks (short and long intervals) due to higher than expected mortality in groups challenged with rMd5//38CVI. Experiment 2B was also terminated early due to high mortality with the short interval and long interval groups terminated at 4.5 and 5.5 weeks, respectively.

Experiments 3 & 4

Experiment 3 examined superinfection over three time points in Ab+ chickens, comparing multiple FFE and tumor samples collected from individual birds **(Table 4-2)**. Multiple sampling from the same bird allowed changes to be analyzed over time in virus frequency within individual birds. Viruses were again administered alone or together following short (1 day) or long (13 day) intervals in forward and reverse order. Feathers were sampled at three time points (day 14, 27, and 70) and tumors were collected on day 70 at termination. DNA isolated from the outside of feather shafts was analyzed by pyrosequencing and qPCR, and tumors were analyzed by pyrosequencing. The first sampling on day 14 was used to confirm the presence of the first inoculated virus before inoculation of the second virus. Seventeen birds were used in singly infected control groups, but dually infected chicken groups were increased to 34 to further increase the number of visceral tumors, since five of eight dual-challenged groups from experiment 2 had 3 tumors or less. Multiple tumors were sampled when present and sampling only consisted of visceral tumors. Contact birds were used again to provide further evidence of the frequency of virus shedding in mixed infections, but

the method of exposure was altered. Instead of physically transferring three donor birds, the air outtake tubing from the isolator of inoculated donor birds was re-routed to the air intake from the isolator of the uninfected recipient chicks at 21 days post infection of the second virus in donor birds. Contacts were only exposed to isolators of birds challenged with the long interval in this experiment, and were only sampled for feathers and tumors at one time point (day 75). Contact chicks were three weeks old at initial exposure, compared to 1 day old in experiments 1 and 2, due to availability of chickens.

Experiment 4 used vaccinated Ab+ chickens to determine the relevance of effects in conditions more closely similar to field conditions. Experiment 4 had similar inoculation and sampling schedule as experiment 3, except due to vaccination at hatch, all virus inoculation and sampling dates were delayed 5 days compared to experiment 3. Since experiment 4 was used to compare results from vaccinated and unvaccinated dual-challenged chickens, there were no single-challenged control groups.

Data analysis

The pyrosequencing procedure resulted occasionally in some background noise that was reflected in virus frequencies from single-challenged control birds that were less than 100%. Therefore, results from single-challenged control birds were used as standards for interpretation of results from dual-challenged birds. To determine if one virus was exclusively predominant in a sample from dual-challenged birds, cut-off values were assigned by age-matched single-challenged control birds based on the lowest frequency value at each sampling day for each experiment. For example, on day

14 of experiment 3A, the frequency of dATP from chickens single-challenged with JM/102W ranged from 95-100% and samples single-challenged with rMd5//38CVI were all 100%. Samples from dual-challenged chickens were then considered predominant for JM/102W or rMd5//38CVI if the frequency of dATP was >95% or dGTP was 100%, respectively. Samples not predominant for either virus were considered mixtures.

Chi-square analyses were used to analyze 1) the association between virus challenge interval and predominance of the first inoculated virus in FFE samples in experiments 1-4; 2) The association between vaccination status and virus predominance in FFE samples in experiment 4; and 3) the association between virus predominance in multiple tumors from individual birds in experiments 3 & 4. The Fisher's exact test was used when expected values were less than 5 in any group. Significant results were based on p-value <0.05.

Virus isolation results from experiment 2 were analyzed using the Wilcoxon rank-sum test due to low numbers of samples in dual-challenged chickens. P-values were reported based on two-tailed tests comparing plaque counts for each virus in dual-challenged chickens with single-challenged chickens challenged with the same virus on the same day. One-tailed tests were used to analyze the reduction of plaque numbers for the second virus inoculated following long interval challenge.

Results

Feather follicle epithelium

Data from uninfected and single-challenged control birds indicated that both IHC and pyrosequencing assays were highly specific in FFE samples from all experiments. In experiment 1, the presence of both virus strains was common for both virus pairs following short interval challenge **(Table 4-3)**. In experiment 1A, mixtures were common in individual follicles following challenge with JM/102W and rMd5//38CVI, with relatively equal distribution of each strain within mixed samples **(Figure 4-1)**. In experiment 1B, rMd5 was more prevalent than rMd5//38CVI, within single and also in the few dual-staining follicles. Pyrosequencing results from the same birds were generally very similar to that of IHC except in experiment 1B with rMd5//38CVI challenged first, in which most samples that were positive for rMd5//38CVI only by IHC were detected as mixtures by pyrosequencing **(Figure 4-2)**. Short interval contact birds had virus frequencies very similar to the frequencies seen in the donor bird groups for both IHC and pyrosequencing. The contact group from donors inoculated with rMd5//38CVI first in experiment 1B had a higher frequency of the first virus (rMd5//38CVI) compared to the inoculated group. Results from qPCR analysis were extremely variable within short and long interval challenge groups (data not shown). There was no clear pattern or significance between virus quantities in single-challenged compared to dual-challenged chickens.

Long interval challenge in experiment 1 led to markedly different results compared to the short interval **(Table 4-3)**. Based on IHC, all follicles stained only for the first virus in both experiment 1A and 1B, regardless of which virus strain was administered first. Pyrosequencing results were similar although the second virus was detected in mixture with the first virus from one bird in two groups. IHC results from

contact recipient birds similarly were positive for the first virus only, although virus staining was very low in 3 of 4 groups. Virus sequence was undetected by pyrosequencing from all three groups with low staining.

Experiment 3 introduced samplings at multiple times to (1) investigate the influence of time on the interaction between viruses in FFE and (2) confirm the results of experiment 1. Overall, 102 of 106 samples (96%) had the same virus predominance at all three sampling time points **(Table 4-4)**. Following short interval challenge, the rMd5//38CVI strain was present as the majority strain when administered with JM/102W, as JM/102W was only present in FFE from two birds. Mixtures were also rare following short interval challenge with rMd5 and rMd5//38CVI strains, but both strains had relative equal chance of exclusive majority within FFE of individual birds.

Following long interval challenge, the first virus administered was the exclusive majority for both virus pairs in all but one sample **(Table 4-4)**. In contact recipient birds to long interval challenge, however, the second virus was much more prevalent. Contact birds were exposed to donor birds that both passed and failed initial FFE testing used to confirm the presence of the first virus challenged. In donor birds that failed the initial test, the second virus was present in 42 of 95 birds (44%) **(Table 4-5)**.

Quantitative PCR results from experiment 3 were again highly variable, but of particular interest was the low replication of JM/102W strain in all single and dual-challenged birds (data not shown).

Tumors

Experiment 2 was similar in design to experiment 1 but focused on the virological analysis of tumors. Data from uninfected and single-challenged control birds in experiment 2 indicated that both pyrosequencing and virus isolation assays were highly specific **(Tables 4-6 & 4-7)**. Due to high mortality, the number of samples per group was quite low in many cases. Following short interval challenge, 8 of 10 tumors were positive for both viruses among both virus pairs **(Tables 4-6 & 4-7)**. Tumors from short interval contact recipient birds consisted of mixtures as was seen in short interval donor birds, but there were more tumors that were predominant for a single virus. Virus isolation results were similar to pyrosequencing results for both virus pairs in dual-challenged and contact recipient birds with nearly identical proportions of samples positive for each virus as in pyrosequencing. Due to low sample numbers in dual-challenged chickens, average plaque counts for each virus were compared between single and dual-challenged chickens using the Wilcoxon rank-sum test. In experiment 2B, there were no significant differences between the plaque counts from viruses challenged on day 1 or day 2 in single versus dual-challenged chickens.

Following the long interval challenge, the first virus was exclusively predominant in 16 of 16 (100%) tumors in experiment 2A by pyrosequencing, whereas experiment 2B included 3 of 8 (37%) tumors that were positive for only the second challenged virus. Whereas long interval contact birds were similar to donor birds in experiment 2B with the second virus present in 10 of 24 tumors (42%), the presence of the second virus in 3 of 22 tumors in experiment 2A was unexpected. Similar as with FFE results, contact birds were exposed to donor birds that failed initial testing in which the second virus was prevalent **(Table 4-5)**. In virus isolation, all but one sample in

experiment 2A were positive for the first virus only and all contact recipient birds were positive for this first virus only. In experiment 2A, there were no significant differences in plaque counts for viruses administered on day 1 in single vs. dual challenged birds, however there was a significant reduction in plaque count for viruses administered on day 14 in dual-challenged birds compared to single control birds (p-values 0.0333 & 0.0476).

Multiple tumors were compared from individual birds either dual-challenged or contact exposed from experiment 2 **(Table 4-8)**. Only 1 of 24 birds with multiple tumors had differences in virus frequency >10% between tumors.

Experiment 3 was designed as a replicate of experiment 2, except using only pyrosequencing **(Table 4-9)**. A major difference in experiment 3 was the use of nested PCR prior to pyrosequencing due to DNA degradation. The result was a reduction in specificity as high levels of background noise were detected in 13 of 84 tumors (15%). Following short interval challenge rMd5//38CVI was exclusively predominant in 19 of 21 tumors when challenged with JM/102W, whereas both virus were present in tumors following challenge with rMd5 and rMd5//38CVI with mild favor towards the latter strain. Tumors were not present from the long interval groups in experiment 3B, but only the first inoculated virus was present following long interval challenge in experiment 3A. Unlike results from donor birds, tumors from long interval contact birds in experiment 3A tended to favor rMd5//38CVI as opposed to the first virus inoculated.

Effects of vaccination

Experiment 4 compared the effects of vaccination on results observed from the first three experiments. In the FFE, there were no significant differences between vaccinated and unvaccinated chickens for either virus pairs or challenge intervals **(Table 4-10)**. Results were consistent with experiment 3, demonstrating a preference for rMd5//38CVI in short interval challenge with JM/102W and a loss of this effect following long interval challenge.

As expected, fewer tumors were obtained from the vaccinated chickens than unvaccinated chickens, yet after short interval challenge the presence of the second challenged virus was relatively consistent within each challenge group **(Table 4-11)**. rMd5//38CVI was present in greater frequency in virus pair 1, but dominance was not detected with virus pair 2. Following long interval challenge, 3 of 23 tumors were detected as mixtures, but all others were exclusively composed of the first virus challenged. Quantitative PCR results from experiment 4 were highly variable, and similar to experiment 3 was a noticeable low replication of JM/102W strain compared to rMd5 and rMd5//38CVI strains. There were no significant differences in virus quantity between vaccinated and unvaccinated birds **(Figure 4-3)**.

Unvaccinated birds in experiment 4 also served as a replicate for earlier experiments, except challenge was administered at day 6 instead of day 1. Short interval FFE results were most similar to experiment 3 results, demonstrating a strong dominance of rMd5//38CVI over JM/102W. When JM/102W was administered first, rMd5//38CVI was present in 10 of 10 samples with JM/102W only present in 5 of 10. When rMd5//38CVI was challenged first, 10 of 10 were positive for rMd5//38CVI and zero for JM/102W. Neither virus was favored with the rMd5 and rMd5//38CVI virus

pair. Similar to both earlier experiments, nearly every sample was exclusively predominant for the first challenged virus following long interval challenge for both virus pairs (41 of 43 samples). Tumor samples were nearly the same as FFE samples, as was the case in earlier experiments.

Summary Data

A chi-square analysis was performed to test the independence between time interval of challenge and the exclusive predominance of the first virus in the FFE by pyrosequencing. Results were stratified by virus pair and combined from all four experiments comparing the number of samples with the second inoculated virus present based on inoculation order **(Table 4-12)**. This table focused on the second virus as its presence was the main determinant for judging the effect of time interval and the detection of dominance. In the FFE, the presence of the second virus was significantly different following challenge with JM/102W and rMd5//38CVI for both short and long interval challenge. This difference was based on order of inoculation as the second virus was only present in significant quantity when rMd5//38CVI was the second virus. No significance was seen following challenge with rMd5 and rMd5//38CVI. The presence of the second virus in FFE samples was strongly dependent on the challenge interval for both virus pairs, regardless of the order of inoculation ($p<0.0001$). Results were nearly identical in tumors, except significant differences for the JM/102W and rMd5//38CVI virus pairs were only seen following short interval challenge.

Some variation was detected between experiments in this study. Most noticeable of these variations was the difference in the degree of dominance for

rMd5//38CVI following short interval challenge between experiment 1 and subsequent experiments. In general, rMd5//38CVI was generally present in greater frequency in later experiments within both virus pairs.

Discussion

Chapter 3 demonstrated that dominance should be considered as a factor that influences the outcome of superinfection. Virulence, as measured by pathotype, was a significant factor that may have defined dominance. The JM/102W strain, a vMDV strain, was subordinate when challenged simultaneously with the rMd5//38CVI strain, a recombinant of a vvMDV strain. When viruses of similar virulence were challenged simultaneously (rMd5 and rMd5//38CVI), there was no clear strain that was consistently dominant. The experiments in the current chapter were designed to 1) evaluate the effect of time with the outcome of superinfection and 2) evaluate the strength of dominance when the same two virus pairs were challenged following different time intervals.

With the short interval challenge (24 hours), dominance measured in the FFE was detected when JM/102W was paired with rMd5//38CVI **(Table 4-12)**. The dominance of rMd5//38CVI over JM/102W was most evident in experiments 3 and 4, although it was also weakly evident in experiment 2 when analyzed with results from contact recipient birds. Similar to chapter 3 results, dominance was weak and inconsistent when birds were challenged with rMd5 and rMd5//38CVI.

The effect of time was strong compared to dominance in FFE. Any dominance detected following the short interval was inapparent following long interval challenge

(13 days) with both virus pairs. FFE samples were nearly all exclusively predominant for the first challenged virus, regardless of virus pair or which virus was challenged first. Chi-square analysis confirmed a strong association between challenge interval and predominance of the first virus challenged ($p<0.0001$).

The second virus had opportunities for infection, even following a long interval between challenges. In experiments 1, 3 and 4, there were a total of five birds in which the second virus was present in the FFE by pyrosequencing after the long interval. Of greater importance, however, was the presence of the second challenged virus in cases when birds failed early infection with the first virus challenged on day 1. Experiments 3 & 4 focused on the probability of superinfection following confirmation of infection with the first challenged virus, but analysis of samples that failed this confirmation revealed an abundance of the second challenged virus in FFE samples **(Table 4-11)**. These birds were most likely the source of the second challenged virus that was present in many of the long interval contact birds in experiment 3. The reason for the lack of response to the first challenged virus in some birds is unknown. Viruses were challenged with two separate injections to minimize administration failures, yet somehow some birds appeared to have the effective dose diminished. Thus, conditions that favor the second challenged virus in the field include short interval between exposures and in chickens with low dose or delayed exposure to the first virus. Birds infected in the field are likely to be exposed to different levels of virus. If the initial exposure is weak enough, superinfection and establishment of the second virus may be more likely.

Results from contact birds showed a close relationship between virus frequencies present in the FFE of donor birds and frequencies present in the FFE of the contact recipient birds. This indicated that the FFE pyrosequencing results from donor birds were reflective of the frequency of viruses being shed and exposed to contact chickens. Viruses shed in the highest frequency from donor birds usually had the advantage in contact birds. Data from additional passages through contact birds would be very useful for tracking the competition in future generations to see if mixtures remain or if one virus becomes mostly exclusive.

Tumors were collected and analyzed in experiments 2, 3 and 4. Results of virus frequencies were similar to results seen in FFE with the presence of both viruses following short interval challenges and the predominance of the first virus with long interval challenges. Three tumors were predominant for the second challenged virus following long interval challenge in experiment 2B, but the infection status of these birds at the time of the second challenge was not tested. Similarly, the second challenged virus was present in contact birds from experiments 2 and 3 exposed to long interval challenged donor birds, but again, the initial infection status of the donor birds was unknown. In experiments 3 and 4, the necessity of using a nested PCR for tumor samples unexpectedly reduced the specificity of the assay in single-challenged control chickens. Overall, the data following dual-challenge was consistent with results from experiment 2, but mixtures were interpreted with caution. Results were included in the overall summary, but not for the comparison of multiple tumors within individual birds. Early studies suggested that MDV tumors were of a monoclonal origin (37), but more recent studies have suggested a polyclonal tumor origin (13, 24, 86). The presence of

lymphoid proliferations in many tissues early in infection would favor polyclonal tumors as well as the presence of both viruses within a single tumor. And although most chickens with multiple tumors in experiment 2 were predominant for the same virus in each tumor (24 of 25), there was one case of tumors of different predominance within individual birds. However, the presence of more than one virus in a tumor does not necessarily provide enough evidence to imply both viruses were causative agents for the tumor. Rather, if a single cell containing both viruses became transformed, it would be difficult to know which strain was involved in the transformation. The existence of a virus-specific transformation marker would be necessary to answer this question.

It was interesting to note that vaccination had no apparent effect on virus frequencies in short or long interval dual infections. HVT was expected to mute the effects of the first challenged virus significantly enough to reduce differences detected between the first and second virus. Yet dominance was still detected between JM/102W and rMd5//38CVI following short interval challenge, and the first virus remained significantly predominant following long interval challenge. The results suggest that these effects may both be relevant under field conditions although this has only been demonstrated with the use of weak vaccine.

This study provides evidence that MDV superinfection can occur following short periods of time between exposures, but the probability is drastically reduced following a longer interval of two weeks between challenges. In cases where birds are exposed within shorter periods of time or where the initial exposure is weak or delayed, mixed infections are likely to occur regardless of vaccination status or inoculation order. These studies demonstrate that mixed infections under some conditions can lead

to shedding of the second virus a prerequisite for the establishment of the second virus strain in the population.

Superinfection is likely an important step in the establishment of evolved strains within a population. The sequence of events may begin by the generation of a mutant strain within an infected chicken or from an outside source introduced into a poultry flock. By either origin, evolution won't occur unless conditions exist for replication and transmission of the second virus strain. This work provides examples of conditions that would promote establishment of the second virus. Future work should include additional virus pairs of similar and different virulence to confirm the phenomenon of dominance. In addition, gaining a better of understanding of how delayed challenge excludes the second virus and determining a more precise time interval for the exclusion would provide important knowledge that may be used to help slow virus evolution in the field. The lack of effect by vaccination should be tested with stronger vaccines to confirm relevance under field conditions.

Table 4-1: Outline of treatment groups for experiments 1 and 2. Differences in chicken numbers and sampling dates for experiment 2 are indicated in parentheses.

Lot	# chickens[1]	Maternal antibody[2]	Challenge strain[3]	Challenge Age (days)	Dose	Sampling age (days)[4]
1	10	Negative	Uninfected controls			30 (58)
2	10 (17)	Negative	JM/102W only	1	500 pfu/bird	30 (58)
3	10 (17)	Negative	rMd5//38CVI only	1	500 pfu/bird	30 (58)
4	10 (17)	Negative	JM/102W only	2	500 pfu/bird	30 (58)
5	10 (17)	Negative	rMd5//38CVI only	2	500 pfu/bird	30 (58)
6	10 (17)	Negative	JM/102W, rMd5//38CVI	1, 2	500 pfu/virus/bird	30 (58)
7	10 (17)	Negative	rMd5//38CVI, JM/102W	1, 2	500 pfu/virus/bird	30 (58)
8	10	Negative	Uninfected controls			42 (70)
9	10 (17)	Negative	JM/102W only	1	500 pfu/bird	42 (70)
10	10 (17)	Negative	rMd5//38CVI only	1	500 pfu/bird	42 (70)
11	10 (17)	Negative	JM/102W only	14	500 pfu/bird	42 (70)
12	10 (17)	Negative	rMd5//38CVI only	14	500 pfu/bird	42 (70)
13	10 (17)	Negative	JM/102W, rMd5//38CVI	1, 14	500 pfu/virus/bird	42 (70)
14	10 (17)	Negative	rMd5//38CVI, JM/102W	1, 14	500 pfu/virus/bird	42 (70)
15	10	Negative	Uninfected controls			30 (58)
16	10 (17)	Negative	Lot 6 contacts	1	Contact	30 (58)
17	10 (17)	Negative	Lot 7 contacts	1	Contact	30 (58)
18	10 (17)	Negative	Lot 13 contacts	1	Contact	30 (58)
19	10 (17)	Negative	Lot 14 contacts	1	Contact	30 (58)

[1] Differences in chicken numbers for experiment 2 are indicated in parentheses.

[2] All chickens were strain $15I_5x7_1$

[3] Experiments 1 and 2 each had two parts using different virus pairs. Experiments 1A & 2A used JM/102W and rMd5//38CVI, shown in this outline. Experiments 1B & 2B used rMd5 and rMd5//38CVI which was identical except rMd5 was used in place of JM/102W.

[4] Sampling age for experiment 2 are indicated in parentheses.

Table 4-2: Outline of treatment groups for experiments 3 and 4.

Exp	Lot	# chickens	Maternal antibody[1]	Vaccine[2]	Challenge strain[3]	Chal. age (days)	Sampling age (days)
3	1	10	Positive		Uninfected controls		14, 27, 70
	2	17	Positive		JM/102W only	1	14, 27, 70
	3	17	Positive		rMd5//38CVI only	1	14, 27, 70
	4	17	Positive		JM/102W only	2	14, 27, 70
	5	17	Positive		rMd5//38CVI only	2	14, 27, 70
	6	17	Positive		JM/102W only	14	14, 27, 70
	7	17	Positive		rMd5//38CVI only	14	14, 27, 70
	8	34	Positive		JM/102W, rMd5//38CVI	1, 2	14, 27, 70
	9	34	Positive		rMd5//38CVI, JM/102W	1, 2	14, 27, 70
	10	34	Positive		JM/102W, rMd5//38CVI	1, 14	14, 27, 70
	11	34	Positive		rMd5//38CVI, JM/102W	1, 14	14, 27, 70
	12	17	Positive		Lot 10 contacts	22	75
	13	17	Positive		Lot 11 contacts	22	75
4	1	10	Positive		Uninfected controls		19, 32, 75
	2	10	Positive	HVT	Uninfected controls		19, 32, 75
	3	17	Positive		JM/102W, rMd5//38CVI	6, 7	19, 32, 75
	4	17	Positive	HVT	JM/102W, rMd5//38CVI	6, 7	19, 32, 75
	5	17	Positive		JM/102W, rMd5//38CVI	6, 19	19, 32, 75
	6	17	Positive	HVT	JM/102W, rMd5//38CVI	6, 19	19, 32, 75
	7	17	Positive		rMd5//38CVI, JM/102W	6, 7	19, 32, 75
	8	17	Positive	HVT	rMd5//38CVI, JM/102W	6, 7	19, 32, 75
	9	17	Positive		rMd5//38CVI, JM/102W	6, 19	19, 32, 75
	10	17	Positive	HVT	rMd5//38CVI, JM/102W	6, 19	19, 32, 75

[1] All chickens were strain $15I_5x7_1$

[2] Vaccines in experiment 4 were administered on day 1 of age at 2000 pfu/bird, followed by first inoculation 5 days later.

[3] Experiments 3 and 4 each had two parts using different virus pairs. Experiments 3A & 4A used JM/102W and rMd5//38CVI, shown in this outline. Experiments 3B & 4B used rMd5 and rMd5//38CVI which was identical except rMd5 was used in place of JM/102W. Dose was 500 pfu/virus/bird.

Table 4-3: Experiment 1 – FFE. IHC and pyrosequencing results of FFE samples from single-challenge, dual-challenged, and contact chickens exposed to JM/102W (J) and/or rMd5//38CVI (M2) [Exp 1A] or rMd5 (M1) and/or M2 [Exp 1B]. Results are shown from birds with passing results only (except groups in which all samples failed). Samples with failed virus sequence detection are indicated by an F.

Footnotes:

[1] Based on pyrosequencing results from experiment 1A single-challenged control birds, samples were considered predominant for J if frequency was >90% for J and predominant for M2 if frequency was 100% for M2. Samples were otherwise considered mixtures.

[2] Based on pyrosequencing results from experiment 1B single-challenged control birds, samples were considered predominant for M1 if frequency was >90% for M1 and predominant for M2 if frequency was 100% for M2. Samples were otherwise considered mixtures.

Table 4-3 continued:

	Lot	Challenge strains	Inoculation day(s)	Sampling day	IHC (% follicles staining for each virus, averaged by group)				Pyrosequencing (% birds predominant for each virus)			
					n	1st only	1st & 2nd	2nd only	n	1st only	1st & 2nd	2nd only
EXPERIMENT 1A	**Controls**											
	1	None		30	10	0	0	0	9	F	F	F
	2	J	1	30	9	100	0	0	8	100	0	0
	3	J	2	30	4	100	0	0	5	100	0	0
	4	M2	1	30	6	83	0	0	7	100	0	0
	5	M2	2	30	4	100	0	0	5	100	0	0
	6	None		42	10	0	0	0	10	F	F	F
	7	J	1	42	10	99	0	0	6	83	17	0
	8	J	14	42	10	96	0	0	10	100	0	0
	9	M2	1	42	8	100	0	0	8	100	0	0
	10	M2	14	42	6	100	0	0	5	100	0	0
	Dual-challenged[1]											
	11	J, M2	1, 2	30	9	12	55	33	9	11	33	56
	12	M2, J	1, 2	30	7	14	29	57	7	14	29	57
	13	J, M2	1, 14	42	8	95	0	0	9	89	11	0
	14	M2, J	1, 14	42	4	100	0	0	6	100	0	0
	Contact exposed to lot											
	11			29	7	44	37	29	9	22	56	22
	12			29	10	8	41	51	10	20	30	50
	13			29	10	100	0	0	10	100	0	0
	14			29	9	22	0	0	7	F	F	F
EXPERIMENT 1B	**Controls**											
	1	None		30	6	0	0	0	10	F	F	F
	2	M1	1	30	10	100	0	0	9	100	0	0
	3	M1	2	30	4	100	0	0	4	100	0	0
	4	M2	1	30	7	100	0	0	7	100	0	0
	5	M2	2	30	3	100	0	0	3	100	0	0
	6	None		42	10	0	0	0	10	F	F	F
	7	M1	1	42	7	99	0	0	5	100	0	0
	8	M1	14	42	9	99	0	0	9	100	0	0
	9	M2	1	42	4	92	0	0	4	100	0	0
	10	M2	14	42	10	85	0	0	9	100	0	0
	Dual-challenged[2]											
	11	M1, M2	1, 2	30	5	77	8	0	4	75	0	25
	12	M2, M1	1, 2	30	7	0	0	100	7	0	86	14
	13	M1, M2	1, 14	42	5	100	0	0	7	100	0	0
	14	M2, M1	1, 14	42	5	95	0	0	7	86	14	0
	Contact exposed to lot											
	11			29	5	96	4	0	4	100	0	0
	12			29	8	63	9	28	8	63	12	25
	13			29	10	10	0	0	4	F	F	F
	14			29	10	20	0	0	4	F	F	F

Table 4-4: Experiment 3 - FFE. Number of birds with FFE samples positive by pyrosequencing based on order of inoculation for each virus in single, dual-challenged or contact chickens exposed to JM/102W (J) and/or rMd5//38CVI (M2) [Exp 3A] or rMd5 (M1) and/or M2 [Exp 3B]. Results are shown from birds with passing results (except groups in which all samples failed). Samples with failed virus sequence detection are indicated by an F.

	Lot	Challenge strains	Inoculation day(s)	Sampling day	# birds predominant for each virus on day 70[1]				# samples that changed predominance between three sampling dates
					n	1st only	1st & 2nd	2nd only	
EXPERIMENT 3A	**Controls**								
	1	None		70	3	F	F	F	
	2	J	1	70	6	6	0	0	0
	3	J	2	70	6	6	0	0	0
	4	J	14	70	5	5	0	0	0
	5	M2	1	70	6	6	0	0	0
	6	M2	2	70	7	7	0	0	0
	7	M2	14	70	3	3	0	0	0
	Dual-challenged[2]								
	8	J, M2	1, 2	70	16	0	0	16	0
	9	M2, J	1, 2	70	8	6	2	0	2
	10	J, M2	1, 14	70	5	4	1	0	1
	11	M2, J	1, 14	70	12	12	0	0	0
	Contact exposed to lot								
	10	J, M2	1, 14	70	9	0	1	8	0
	11	M2, J	1, 14	70	9	9	0	0	0
EXPERIMENT 3B	**Controls**								
	1	None			3	F	F	F	
	2	M1	1	70	7	7	0	0	0
	3	M1	2	70	7	7	0	0	0
	4	M1	14	70	11	11	0	0	0
	5	M2	1	70	6	6	0	0	0
	6	M2	2	70	8	8	0	0	0
	7	M2	14	70	9	9	0	0	0
	Dual-challenged[3]								
	8	M1, M2	1, 2	70	10	2	1	7	1
	9	M2, M1	1, 2	70	8	4	0	4	0
	10	M1, M2	1, 14	70	12	12	0	0	0
	11	M2, M1	1, 14	70	0				
	Contact exposed lot								
	10	M1, M2	1, 14	70	9	7	1	1	0
	11	M2, M1	1, 14	70	8	8	0	0	0

[1] Long interval analysis only includes results from FFE samples that passed at all three time points

[2] Based on results from single-challenged control birds, FFE samples were considered predominant for J if frequency was >95% on day 14, >90% on day 27 or >87% on day 70 for J. FFE results were considered predominant for M2 if frequency was 100% on day 14, >97% on day 27, or 100% on day 70 for M2. Samples were otherwise considered mixtures.

[3] Based on results from single-challenged control birds, FFE samples were considered predominant for M1 if frequency was 100% on day 14, >97% on day 27 or >86% on day 70 for M1. FFE results were considered predominant for M2 if frequency was 100% on day 14, day 27, or day 70 for M2. Samples were otherwise considered mixtures.

Table 4-5: Experiments 3 & 4 – Failed initial results. Number of birds positive by pyrosequencing for each virus inoculated in long-interval dual-challenged chickens from those that had failed results from the first sampling taken immediately before inoculation of the second virus.

Exp	Challenge strains[2]	Vaccine days	Inoculation day(s)	Sampling day	FFE[1]					Tumors			
					Pyrosequencing (# birds predominant for each virus on day 70)				# samples that changed predominance between second and third samplings	Pyrosequencing (# tumors predominant for each virus on day 70)			
					n	1st only	1st & 2nd	2nd only		n	1st only	1st & 2nd	2nd only
3A	J, M2		1, 14	70	21	3	5	13	3	6	2	0	4
	M2, J		1, 14	70	15	14	1	0	2	4	2	2	0
3B	M1, M2		1, 14	70	8	7	0	1	0	0			
4A	J, M2		6, 19	75	7	1	3	3	3	2	1	0	1
	J, M2	HVT	6, 19	75	13	3	3	7	4	1	1	0	0
	M2, J	HVT	6, 19	75	10	10	0	0	0	0			
4B	M1, M2		6, 19	75	3	0	0	3	0	2	0	0	2
	M1, M2	HVT	6, 19	75	7	4	1	2	1	1	0	0	1
	M2, M1		6, 19	75	1	1	0	0	0	0			
	M2, M1	HVT	6, 19	75	10	10	0	0	0	0			

[1] Analysis only includes results from FFE samples that failed at the first FFE sampling.

[2] Challenge strains used were JM/102W (J), rMd5 (M1) or rMd5//38CVI (M2)

Table 4-6: Experiment 2A – Tumors. Pyrosequencing (tumor) and virus isolation (spleen) results from single and dual-challenged chickens challenged with JM/102W (J) and/or rMd5//38CVI (M2) and contact recipient chickens.

Lot	Challenge strains	Inoculation day(s)	Sampling day[3]	Pyrosequencing[1] (# tumors predominant for each virus)				Virus isolation[2] (# of samples positive for each virus)				Avg Plaque Count/10^6 cells for each virus	
				n	1st only	1st & 2nd	2nd only	n	1st only	1st & 2nd	2nd only	1st	2nd
Controls													
1	None		41	10	F	F	F	10	0	0	0	0	0
2	J	1	41	7	7	0	0	10	9	0	0	15	0
3	J	2	41	9	9	0	0	10	9	0	0	25	0
4	J	14	41	6	6	0	0	9	6	0	0	10	0
5	M2	1	41	4	4	0	0	10	6	0	0	12	0
6	M2	2	41	0				2	1	0	0	5	0
7	M2	14	41	4	4	0	0	10	7	0	0	4	0
Dual-challenged													
8	J, M2	1, 2	41	5	0	4	1	3	0	0	0	0	0
9	M2, J	1, 2	41	2	0	2	0	1	0	0	0	0	0
10	J, M2	1, 14	41	13	13	0	0	9	6	0	1	9	7
11	M2, J	1, 14	41	3	3	0	0	3	2	0	0	28	0
Contact exposed to lot													
8			42	8	2	3	3	6	1	2	2	27	9
9			42	10	7	2	1	9	6	1	1	14	13
10			42	9	6	2	1	7	7	0	0	11	0
11			42	13	13	0	0	8	6	0	0	9	0

[1] Results are shown from birds with passing results (except failed [F] uninfected control spleen samples). Based on results from single-challenged control birds, samples were considered predominant for J if frequency was >98% for J and predominant for M2 if frequency was 100% for M2. Samples were otherwise considered mixtures.

[2] Each sample consisted of 1-2 plates/monoclonal antibody/bird.

[3] Experiment was terminated early due to higher than expected mortality among several groups.

Table 4-7: Experiment 2B – Tumors. Pyrosequencing (tumor) and virus isolation (spleen) results from single and dual-challenged chickens challenged with rMd5 (M1) and/or rMd5//38CVI (M2) and contact recipient chickens.

				Pyrosequencing[1] (# tumors predominant for each virus)				**Virus isolation**[2] (# of samples positive for each virus)				Avg Plaque Count/10^6 cells for each virus	
Lot	Challenge strains	Inoculation day(s)	Sampling day[3]	n	1st only	1st & 2nd	2nd only	n	1st only	1st & 2nd	2nd only	1st	2nd
Controls													
1	None		32	6	F	F	F	5	0	0	0	0	0
2	M1	1	32	9	9	0	0	9	9	0	0	197	0
3	M1	2	32	8	8	0	0	9	9	0	0	432	0
4	M2	1	32	6	6	0	0	7	7	0	0	211	0
5	M2	2	32	4	4	0	0	8	7	0	0	265	0
6	None		39	5	F	F	F	NS[4]					
7	M1	1	39	4	4	0	0	NS					
8	M1	14	39	1	1	0	0	NS					
9	M2	1	39	4	4	0	0	NS					
10	M2	14	39	2	2	0	0	NS					
Dual-challenged													
11	M1, M2	1, 2	32	1	0	1	0	1	0	1	0	250	657
12	M2, M1	1, 2	32	2	0	1	1	3	0	3	0	144	171
13	M1, M2	1, 14	39	3	2	0	1	NS					
14	M2, M1	1, 14	39	5	3	0	2	NS					
Contact exposed to lot													
11			41	4	4	0	0	8	3	0	1	10	11
12			41	6	2	0	4	6	0	0	0	0	0
13			42	11	5	0	6	7	3	0	3	23	21
14			42	13	9	2	2	9	4	0	0	44	0

[1] Results are shown from birds with passing results (except failed [F] uninfected control spleen samples). Based on results from single-challenged control birds, samples were considered predominant for M1 if frequency was >95% for M1 and predominant for M2 if frequency was 100% for M2. Samples were otherwise considered mixtures.

[2] Each sample consisted of 1-2 plates/monoclonal antibody/bird.

[3] Experiment was terminated early due to higher than expected mortality among several groups.

[4] No spleen samples (NS) were available from the sampling on day 39.

Table 4-8: Experiment 2 – Comparison of multiple tumors. Pyrosequencing results of birds with multiple tumors in experiment 2. Birds were dual-challenged with either JM/102W (J) and rMd5//38CVI (M2) or rMd5 (M1) and M2. Results from contact birds are indicated. Pyrosequencing results show the ratio of each virus present in DNA isolated from the tumors.

					Pyrosequencing Frequency of each virus in tumors %1st virus:%2nd virus		
Exp	Challenge strains	Challenge	Sampling day	Bird#	Nerve	Heart	Gonad
	J, M2	1,2	58	Q2024	38:62	68:32	47:53
Contact to	J, M2	1,2	58	Q2102	0:100	0:100	
Contact to	J, M2	1,2	58	Q2108	91:9	92:8	80:20
	M2, J	1,2	58	Q2054	48:52	57:43	
Contact to	M2, J	1,2	58	Q2116	0:100	0:100	
Contact to	M2, J	1,2	58	Q2117	0:100		0:100
Contact to	M1, M2	1,2	58	Q1695	0:100	0:100	
Contact to	M1, M2	1,2	58	Q1700	0:100	0:100	
Contact to	M2, M1	1,2	58	Q1707	0:100		0:100
Contact to	M2, M1	1,2	58	Q1717	100:0		100:0
	J, M2	1,14	70	Q2035	100:0	100:0	
	J, M2	1,14	70	Q2037	100:0		100:0
	J, M2	1,14	70	Q2045	100:0	100:0	100:0
	J, M2	1,14	70	Q2046	100:0		96:4
Contact to	J, M2	1,14	70	Q2136	83:17	75:25	
Contact to	J, M2	1,14	70	Q2137	100:0	99:1	
Contact to	M2, J	1,14	70	Q2153	0:100	0:100	
Contact to	M2, J	1,14	70	Q2157	0:100	0:100	
Contact to	M2, J	1,14	70	Q2161	0:100	0:100	
Contact to	M2, J	1,14	70	Q2162	0:100	0:100	
Contact to	M1, M2	1,14	70	Q1735	100:0	100:0	
Contact to	M1, M2	1,14	70	Q1738	99:1	100:0	
Contact to	M2, M1	1,14	70	Q1748	99:1	100:0	
Contact to	M2, M1	1,14	70	Q1749	0:100	0:100	

[1] Spleens were used for virus isolation. Each sample consisted of 1-2 plates/monoclonal antibody/bird.

[2] Tumor abbreviations: Enlarged nerve (Ner), Heart (Hrt), Spleen (Spl), Gonad (Gon), Liver (Liv), Kidney (Kid)

[3] The numbers of control group virus isolation or tumor samples of each type are indicated within parentheses

Table 4-9: Experiment 3 - Tumors. Number of birds with tumor samples positive by pyrosequencing based on order of inoculation for each virus in single, dual-challenged or contact chickens exposed to JM/102W (J) and/or rMd5//38CVI (M2) [Exp 3A] or rMd5 (M1) and/or M2 [Exp 3B]. Results are shown from birds with passing results (except groups in which all samples failed). Samples with failed virus sequence detection are indicated by an F. A nested PCR was used as described on page 120.

	Lot	Challenge strains	Inoculation day(s)	Sampling day	n	# tumors predominant for each virus on day 70[1]: 1st only	1st & 2nd	2nd only
EXPERIMENT 3A	**Controls**							
	1	None		70	2	F	F	F
	2	J	1	70	2	1	1	0
	3	J	2	70	3	1	2	0
	4	J	14	70	2	0	1	1
	5	M2	1	70	1	1	0	0
	6	M2	2	70	7	7	0	0
	7	M2	14	70	6	6	0	0
	Dual-challenged[2]							
	8	J, M2	1, 2	70	9	0	0	9
	9	M2, J	1, 2	70	12	10	0	2
	10	J, M2	1, 14	70	2	2	0	0
	11	M2, J	1, 14	70	5	5	0	0
	Contact exposed lot							
	10	J, M2	1, 14	70	5	0	2	3
	11	M2, J	1, 14	70	8	7	1	0
EXPERIMENT 3B	**Controls**							
	1	None			3	F	F	F
	2	M1	1	70	14	13	1	0
	3	M1	2	70	7	5	1	1
	4	M1	14	70	16	14	2	0
	5	M2	1	70	6	5	1	0
	6	M2	2	70	9	8	1	0
	7	M2	14	70	11	10	1	0
	Dual-challenged[3]							
	8	M1, M2	1, 2	70	19	4	2	13
	9	M2, M1	1, 2	70	13	8	1	4
	10	M1, M2	1, 14	70	0			
	11	M2, M1	1, 14	70	0			
	Contact exposed lot							
	10	M1, M2	1, 14	70	0			
	11	M2, M1	1, 14	70	0			

[1] Long interval analysis only includes results from tumors with passing FFE results at all three time points.

[2] Tumor samples were considered predominant for single viruses if frequency of J was >98% or M2 was 100%. Tumors were otherwise considered mixtures.

[3] Tumor samples were considered predominant for single viruses if frequency of M1 was >87% or M2 was >96%. Tumors were otherwise considered mixtures.

Table 4-10: Experiment 4 - FFE. Number of birds positive by pyrosequencing for each virus inoculated in vaccinated and unvaccinated dual-challenged chickens by order of inoculation for each virus. FFE samples are sorted by those which the same virus was predominant at each sampling and those that changed over time. Tumor results are sorted based on similar or different results in tumors from the same bird.

Lot	Challenge strains[2]	Vaccine days	Inoculation day(s)	Sampling day	Pyrosequencing[1] (# birds predominant for each virus on day 75) n	1st only	1st & 2nd	2nd only	χ^2 p-value	# samples that changed predominance between three sampling dates
1	J, M2		6, 7	75	10	0	5	5	0.2841	2
2	J, M2	HVT	6, 7	75	11	0	3	8		2
3	M2, J		6, 7	75	10	10	0	0	1.000	1
4	M2, J	HVT	6, 7	75	11	11	0	0		
5	M1, M2		6, 7	75	9	7	0	2	0.5413	1
6	M1, M2	HVT	6, 7	75	10	6	1	3		2
7	M2, M1		6, 7	75	10	7	1	2	0.2013	
8	M2, M1	HVT	6, 7	75	9	9	0	0		
9	J, M2		6, 19	75	6	5	1	0	0.0877	1
10	J, M2	HVT	6, 19	75	1	0	1	0		1
11	M2, J		6, 19	75	13	13	0	0	1.000	
12	M2, J	HVT	6, 19	75	5	5	0	0		
13	M1, M2		6, 19	75	12	11	0	1	0.4022	1
14	M1, M2	HVT	6, 19	75	8	8	0	0		
15	M2, M1		6, 19	75	12	12	0	0	1.000	
16	M2, M1	HVT	6, 19	75	6	6	0	0		

[1] Long interval analysis only includes results from FFE samples that passed at all three time points

[2] Challenge strains used were JM/102W (J), rMd5 (M1) or rMd5//38CVI (M2)

Table 4-11: Experiment 4 - Tumors. Number of birds with tumor samples positive by pyrosequencing based on order of inoculation for each virus in single, dual-challenged or contact chickens exposed to JM/102W (J) and/or rMd5//38CVI (M2) [Exp 3A] or rMd5 (M1) and/or M2 [Exp 3B]. Results are shown from birds with passing results (except groups in which all samples failed). Samples with failed virus sequence detection are indicated by an F. A nested PCR was used as described on page 120.

Lot	Challenge strains[2]	Vaccine days	Inoculation day(s)	Sampling day	# tumors predominant for each virus on day 75[1]			
					n	1st only	1st & 2nd	2nd only
1	J, M2		6, 7	75	5	1	0	4
2	J, M2	HVT	6, 7	75	6	0	1	5
3	M2, J		6, 7	75	2	2	0	0
4	M2, J	HVT	6, 7	75	3	2	1	0
5	M1, M2		6, 7	75	9	3	1	5
6	M1, M2	HVT	6, 7	75	3	1	1	1
7	M2, M1		6, 7	75	4	1	0	3
8	M2, M1	HVT	6, 7	75	3	3	0	0
9	J, M2		6, 19	75	3	3	0	0
10	J, M2	HVT	6, 19	75	0			
11	M2, J		6, 19	75	4	2	2	0
12	M2, J	HVT	6, 19	75	0			
13	M1, M2		6, 19	75	7	7	0	0
14	M1, M2	HVT	6, 19	75	4	3	1	0
15	M2, M1		6, 19	75	3	3	0	0
16	M2, M1	HVT	6, 19	75	2	2	0	0

[1] Long interval analysis only includes results from tumors with passing FFE results at all three time points.

[2] For virus pair 1, tumor samples were considered predominant for single viruses if frequency of J was >98% or M2 was 100%. For virus pair 2, tumor samples were considered predominant for single viruses if frequency of M1 was >87% or M2 was >96%. Tumors were otherwise considered mixtures.

Table 4-12: Summary table. Combined pyrosequencing data displaying the number of samples where the second-challenged virus was present in FFE or tumor samples.

Virus challenge[1]	Exp 1	Exp 3	Exp 4	Exp 4 HVT	Totals (%)[2]	χ^2 p-value
Number of samples with second inoculated virus present / Total samples						
FFE Short						
J, M2	8/9	16/16	10/10	11/11	45/46 (98%)*	<0.0001
M2, J	6/7	2/8	0/10	0/11	8/36 (22%)*	
M1, M2	1/4	8/10	2/9	4/10	15/33 (45%)	0.7238
M2, M1	7/7	4/8	3/10	0/9	14/34 (41%)	
				Group total:	**82/149 (55%)**[A]	
FFE Long						
J, M2	1/9	1/5	1/6	1/1	4/21 (19%)*	0.0152
M2, J	0/6	0/12	0/13	0/5	0/36 (0%)*	
M1, M2	0/7	0/12	1/12	0/8	1/39 (3%)	1.0000
M2, M1	1/7	-	0/12	0/6	1/25 (4%)	
				Group total:	**6/121 (5%)**[B]	**<0.0001**

Virus challenge[1]	Exp 2	Exp 3	Exp 4	Exp 4 HVT	Totals (%)	χ^2 p-value
Tumors Short						
J, M2	5/5	9/9	4/5	6/6	24/25 (96%)*	<0.0001
M2, J	2/2	2/12	0/2	1/3	5/19 (26%)*	
M1, M2	1/1	15/19	6/9	2/3	24/32 (75%)	0.2422
M2, M1	2/2	5/8	3/4	0/3	10/17 (59%)	
				Group total:	**63/93 (68%)**[A]	
Tumors Long						
J, M2	0/13	0/2	0/3	-	0/18 (0%)	0.1517
M2, J	0/3	0/5	2/4	-	2/12 (17%)	
M1, M2	1/3	-	0/7	1/4	2/14 (14%)	1.0000
M2, M1	2/5	-	0/3	0/2	2/10 (20%)	
				Group total:	**6/54 (11%)**[B]	**<0.0001**

[1] Viruses challenge are listed in order of inoculation with the following viruses: JM/102W (J), rMd5 (M1), or rMd5//38CVI (M2)

[2] Significant differences by chi-square analysis between pairs of results are indicated by (*). Significant differences between group totals based on sample interval are indicated by different superscript letters.

Figure 4-1: Experiment 1 - Distribution of short interval virus frequencies within exclusively predominant and mixed samples analyzed by IHC

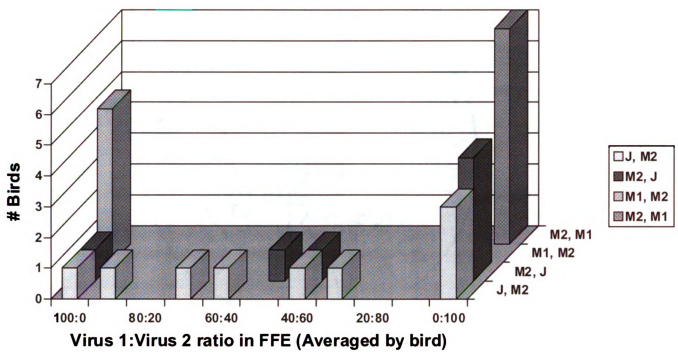


Figure 4-2: Experiment 1 - Distribution of short interval virus frequencies within exclusively predominant and mixed samples analyzed by pyrosequencing

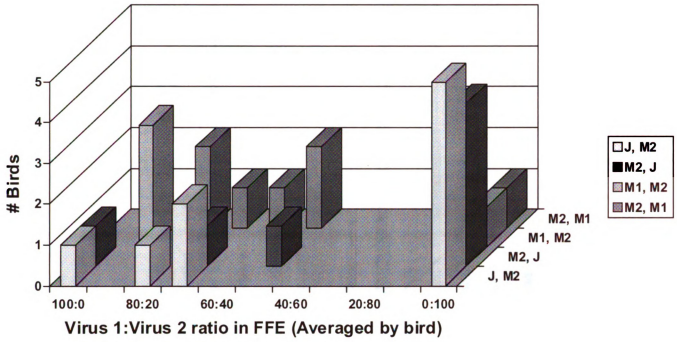
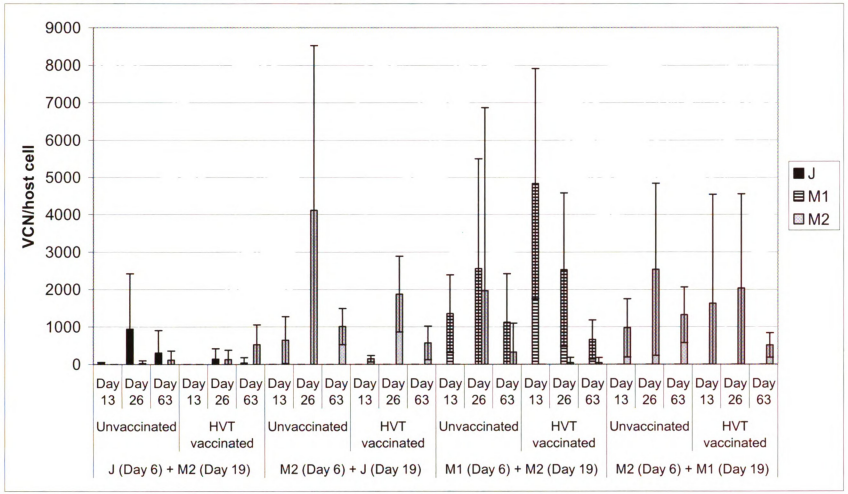


Figure 4-3: Experiment 4 – Results of qPCR from FFE in vaccinated or unvaccinated chickens dually-challenged with JM/102W (J) and rMd5//38CVI (M2), or rMd5 (M1) and rMd5//38CVI (M2). 28 of 270 samples were removed as outliers as described in the methods on page 122. Bars represent one standard deviation.



Chapter 5

SUMMARY AND FUTURE STUDIES

The first chapter provided an introduction to Marek's disease with a review focused on the likelihood of multiple exposures to MDV during the lifetime of a chicken. Evidence was presented demonstrating multiple virus strains simultaneously present within a flock. The presence of multiple strains in a flock increases the probability of multiple exposures to chickens and the possibility of infection with more than one strain (superinfection). Despite anecdotal field evidence, there is little laboratory evidence, demonstrating that two fully virulent S1 MDV strains can both replicate and transmit within individual birds. Recent technology using recombinant viruses gives us the opportunity to now identify individual virulent strains within mixed populations. Superinfection is likely an important mechanism in the establishment of evolved strains within a population; therefore, understanding the conditions that promote infection with a second virus may be important for knowing how to slow the evolution of MDV in the field.

The second chapter included three pilot studies evaluating the methods and conditions used in the following chapters. Pilot 1 validated the sensitivity and specificity of pyrosequencing and IHC, confirming the use of these techniques for accurately distinguishing between virus pairs. Superinfection studies were based on two virus pairs – one with similar virulence and the other of different virulence. Pilot 2 evaluated the pathogenicity of strains selected for the two virus pairs by pathotyping, confirming the similarity between rMd5 and rMd5//38CVI as well as the dissimilarity between JM/102W and rMd5//38CVI. Pilot 3 validated the sampling methods used in

later chapters, isolating DNA from cells adhered to the outside of the feather shaft, to reflect the frequency and quantity of shed virus from individual birds following dual infection.

The third chapter demonstrated the phenomenon of viral dominance, as detected in simultaneously dual-challenged chickens. For the first time, certain virulent S1 virus strains were shown to have a competitive advantage over others. There was no consistent dominance detected between the two similar strains, but the higher virulent rMd5//38CVI was significantly dominant over the less virulent JM/102W strain. For both virus pairs, the presence of one virus strain within bursa and feather follicles apparently provided no exclusion from infection with the second virus. Interestingly, virus mixtures were present in a large portion of tumors. Simultaneous challenge with JM/102W and rMd5//38CVI strains led to some differences in the virus frequency between tumors in the same bird, suggesting that these tumors may have a polyclonal origin. Results from HVT-vaccinated birds suggest that the effect of dominance may be relevant under field conditions.

The fourth chapter evaluated the effect of dominance following a short (24 hours) and long (13 days) challenge interval. Dominance was again detected for rMd5//38CVI when challenged with JM/102W following the short interval, regardless of which strain was inoculated first. Any detection of dominance was lost, however, following the long challenge interval as most FFE and tumor samples were exclusively predominant for the first inoculated virus. Tumor samples generally consisted of a single predominant virus, but both viruses were detected within a small portion of tumors. Vaccination with HVT had no effect on the virus frequency for either virus pair

or challenge time interval, suggesting these results may be significant in field conditions.

Several significant findings were demonstrated in these experiments.

1) This study established that superinfection with two virulent S1 MDV strains is possible and indeed prevalent following simultaneous or short interval dual-challenge. Both viruses can be simultaneously present within bursa, FFE and tumor tissues, and frequently found within the same individual bursa and feather follicles. Many times both strains were present within the same overlapping regions of each follicle.
2) The phenomenon of virus dominance was demonstrated in at least one virus pair, establishing a potential mechanism for the establishment and transmission of evolved strains within individual birds.
3) The effect of delayed challenge was strong and masked the effect of dominance observed in shorter intervals. The presence of the second virus in certain conditions indicates superinfection may be especially significant in instances when time between exposures is short and viruses are of similar virulence. Understanding what events are responsible for the eventual exclusion of superinfection may help to slow the progress of evolution within the field.
4) Many tumor samples were composed of both virus strains and in a few instances multiple tumors within individual birds were composed of differing strain frequencies. These results are consistent with but do not prove a polyclonal origin of tumors.

5) Although a weak vaccine was used, vaccination had no effect on virus dominance or the effect of challenge interval on virus frequencies. This suggests that the effects in these studies may be relevant to field conditions.

The experiments described in this study have prompted several additional questions that deserve follow-up studies.

1) The confirmation of viral dominance using more virus pairs of differing and similar virulence. The evidence of dominance between rMd5//38CVI and JM/102W was compelling, but only represents one virus pair. An evaluation of additional virus pairs would help confirm which characteristics contribute most to dominance. Replication appears to be closely related to virulence and should be evaluated with reference to dominance.
2) Narrowing down the time interval necessary to exclude the second challenged virus. We observed a significant effect between the presence of the second virus after short interval (24 hours) versus long interval (13 days) challenge. An experiment designed to evaluate the effect with challenge intervals between 2-12 days would help to contribute to determining the cause of exclusion.
3) Serial passages through contact recipient birds. Results from contact recipient birds were correlated with the donor recipient birds, but tended to include fewer mixtures. In field conditions, establishment of new strains may take place over several generations as less frequent strains grow towards greater frequency. Evaluating potential changes over multiple generations may provide further knowledge on mechanisms for viral evolution.

4) Increased or varied dosages. Results from chapters 3 and 4 demonstrated differences between the extent of dominance for rMd5//38CVI when challenged with JM/102W. Experiments were performed over a period of several years and the later experiments used different virus lots compared to earlier experiments. The increased apparent dominance of rMd5//38CVI may have been related to differences in effective dosages between the first and second virus. New experiments with higher total dosages and differing dosages for each strain would help elucidate the effect of virus fitness compared to starting dosage.
5) Superinfection within individual cells. Superinfection was demonstrated within individual tissues and both strains were observed in overlapping regions within bursa and feather follicles. The methods used in the study do not provide information on whether virus was present within the same cells. Flow cytometry would be a suitable method to demonstrate multiple strains within individual cells if a suitable marker was available for differentiation. Unfortunately, MDV pp38 is poorly expressed in tumor cells, so this could not be evaluated in the current study.
6) Recombination of MDV strains. The evolution of MDV strains may be caused by the establishment of dominant strains following mutation or introduction of new strains into a poultry flock. Recombination between multiple strains may be another source of virus evolution within birds. There is little or no evidence of recombination within the MD system.
7) Determining the clonality of MD tumors. The experiments designed in this study may be an appropriate model for studying clonality of MD tumors. Simultaneous

dual challenge with both virus pairs produced mixed tumors that could be analyzed with additional assays, such as TCR spectratyping.

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