FEATHER TIP MONITORING

OF MAREK'S DISEASE VIRUS

IN EXPERIMENTAL AND COMMERCIAL SETTINGS

by

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fulfillment of the requirements for the degree of Master of Science in Animal Science

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ABSTRACT

Marek's disease (MD) is a lymphoproliferative disorder in chickens caused by an alphaherpesvirus, Marek's disease virus (MDV). MD has been controlled successfully for a half-century by vaccination with various non-oncogenic related viruses, but outbreaks still occur.

Field monitoring of MDV vaccine and challenge strains has potential utility for both commercial producers and researchers. Feather tip sampling offers the possibility of measuring virus loads in field settings in a non-invasive manner. To assess the potential of feather tip monitoring, end-point and qPCR were used to evaluate the presence of vaccine and challenge viruses under both experimental and commercial settings. Using a controlled experimental environment, levels of vaccine and challenge virus were determined in chickens that were vaccinated with herpesvirus of turkeys (HVT; monovalent), HVT + SB-1 (bivalent), or HVT + SB-1 + CVI988 (trivalent). Half of the vaccinated chickens were challenged with the T. King very virulent plus (vv+) strain of MDV, and the other half were left unchallenged. In general, differences in virus loads were observed early in life; i.e., during the first three weeks, but were not evident later when all viruses were present in feather tips and infections became more complex. Also, in general, qPCR confirmed end-point PCR results in almost every instance, indicating that end-point PCR is adequate for feather tip monitoring.

In non-challenged chickens, HVT detection was highest in HVT vaccinates, lower in bivalently-vaccinated chickens, and lowest in trivalently-

vaccinated chickens. This pattern was expected and likely reflects competition among infecting viruses early after exposure. However, in T. King-challenged chickens, infections were more complicated. First, vaccination decreased the amounts of T. King detected in feather tips during the first three weeks of life compared to chickens that were not vaccinated. Therefore, vaccination with virtually any regimen diminished early infection with the T. King vv+ virus. This could be due to multiple factors including viral interference, stimulation of innate immune responses, or induction of acquired immune responses. Second, in T. King-challenged chickens, detection of vaccine viruses was decreased compared to unchallenged chickens. Thus, the presence of mixed infections complicated the kinetics of early viral infections regardless of whether vaccine or challenge viruses were being measured. While we found that HVT levels were lower in challenged chickens, we did not observe differences in the level of HVT among the various vaccine groups as was observed in non-challenged chickens. That we did not detect differences in HVT accumulation in feather tips among vaccine groups could be due to the low number of individuals sampled or to the timing of sampling related to kinetics of viral passage through chickens.

With regard to commercial chickens, we monitored thirty commercial flocks from week 2 through week 7 of life with regard to vaccine and field challenge viruses in feather tips. In these commercial flocks, all MDV serotypes were detected. Only serotype 2 and 3 vaccines were used for vaccinating these flocks, and therefore, serotype 1 viruses (MDV1) detected resulted from infection with naturally occurring MDV1 field strains. The pattern of PCR products generated when detecting MDV1 indicated that the naturally occurring field challenge strains were varied and mixed and

differed from standard laboratory MDV1 stocks. One noteworthy finding of this study was that during the third week of life, MDV1 detection decreased markedly in all 30 flocks, while detection of vaccine strains did not. This may be due to natural killer cell responses, which would be expected at this time, and may provide some insight into one mechanism of vaccine-induced protection. Also, all maternal antibodies decrease during this time, and infections with various other viruses occur that might have an impact on MDV detection. Finally, latency of MDV1 might also take place during this time. Overall, correlating performance data with detection of viruses in feather tips and with disease outcome proved difficult. As expected, leukosis condemnations were higher in older flocks, but the presence of MDV1 or vaccine strains in feather tips during grow-out did not foretell eventual leukosis condemnations or flock performance. While feather tip monitoring may not be a good predictor of performance, it should be useful for confirming that commercial flocks have been vaccinated.

Chapter 1

LITERATURE REVIEW

Marek's disease (MD) is a lymphoproliferative disease affecting poultry. MD is caused by Marek's disease virus (MDV), an α-herpesvirus that is ubiquitous to poultry, spreads horizontally, and can cause tumor formation in visceral tissues (70). MD was first described in 1907 by a veterinarian named József Marek, after whom the disease was named (42). The first cases in the US were reported in 1914 (70). During the late twentieth century, there were increases in the severity of MD, but in general the disease has been controlled successfully by vaccines.

1.1 Serotypes

MDV is classified into three serotypes (8). Serotype one includes all of the oncogenic strains and their attenuated forms and is further divided into mild (m), virulent (v), very virulent (vv), and very virulent plus (vv+) pathotypes (65). Serotype two includes all non-oncogenic strains (18), and serotype three consists of herpesvirus of turkeys (HVT) (69).

1.2 Genome Organization

MDV and HVT are α-herpesviruses and have similar genome structures to herpes simplex virus (HSV). As shown in Figure 1.1, the genome consists of a unique long and a unique short region, which are both flanked by inverted and terminal repeat regions (16, 23). The genome of MDV1 is 180 kb long, which is longer than that of

herpes simplex virus (HSV) (152 kb) and about the same size as Epstein-Barr virus (EBV) (170 kb) (38, 62). The genome of MDV2 is 165 kb and the genome of HVT is 160 kb long (2, 32), both of which are smaller than the MDV1 genome. Most of the differences in genome structure among herpesviruses lie in the repeat regions of these genomes. The RL of MDV1 is 30% longer, and the RS of MDV1 is twice as long as the corresponding regions of the HSV. Differences, such as insertions and deletions, among MDV serotypes are mostly found in and adjacent to RL and RS regions (2).

Figure 1.1 **Genome organization of Marek's disease herpesvirus. Unique long (U_L) and short (U_S) regions are flanked by inverted and terminal repeat long and short regions (IR_L, IR_S, TR_L, and TR_S).**

1.3 The ICP4 gene

The MDV ICP4 gene was used for genome quantitation by PCR in this study. The ICP4 gene is encoded in the inverted repeat flanking the unique short region (3). The coding sequence, which is 4,245 nucleotides, is highly conserved among various strains (3, 61). There are many transcriptional regulatory sites upstream and downstream of the ICP4 translational start site (3). A long open reading frame is positioned 5' to and in frame with the conventional ICP4 coding sequence, and therefore, it is possible that ICP4 products longer than the conventional one exist although these have not been definitely detected using Western blotting and immunoprecipitation (3). Also, the ICP4 promoter region in MDV1 CVI988 strain has insertions and duplications compared to ICP4 promoters of other MDV1 strains (34).

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The function of ICP4 in MDV is still not fully understood. Transfection of the ICP4 gene into lymphoblastoid cells increased the expression of pp38 and pp24 genes, suggesting that the protein is a transactivator (48). Antisense oligodeoxynucleotides, designed to bind to translation initiation regions of ICP4, inhibited MSB1 lymphoblastoid cell proliferation and soft agar colony formation (71), further supporting the role of ICP4 in transactivation. In HSV, ICP4 functions in activation and repression of transcription, and it is assumed that ICP4 has similar functions in MDV (21).

1.4 Phases of Infection

MDV infection is divided into four phases. These phases are not fixed, and the onset and duration of each phase depends on several factors such as the age of the chicken and the pathotype of the strain. The first phase is cytolytic infection and results in degenerative changes in chickens. This phase is 3-6 days post-infection (PI) and causes atrophy of the spleen, bursa, and thymus (10). The primary target cells are B cells. Chickens may recover in 8-14 days PI, but depending on the nature of the MDV strain, early mortality can take place during this time as well as the appearance of leg lesions and paralysis. During the second phase of infection, the virus becomes latent. Latency takes place 6-7 days PI, and during this time, immune responses develop (10). The third phase of infection is secondary cytolytic infection, which occurs following reactivation from latency. During this phase, permanent immunosuppression develops. Lastly, lymphoid cells can become transformed. Tumors can first be detected 2-4 weeks PI (10). Highly virulent strains do not necessarily follow all four phases, and chickens can die during the first cytolytic infection. In the feather follicle epithelium (FFE), cytolytic infection always takes

place even during latency (12). FFE is the only location where complete virus replication occurs resulting in shed of fully infectious particles (11, 43). It is believed that MDV is transferred to FFE by infected lymphocytes at about seven days PI (19).

1.5 Increase in Virulence Over Time

The virulence of the serotype one strains has increased over the years. Field strains isolated between 1987 and 1995 have been characterized using a pathotyping procedure developed at the East Lansing, MI Avian Disease and Oncology Laboratory (65, 68). The procedure compares the virulence of each field isolate to known strains (MD5 and JM) with regard to ability to break through vaccination with HVT or HVT + SB-1 (68). This comparison allows for the field strains to be ranked according to relative virulence. For field strains that were isolated between 1987 and 1995, there was a trend in the change of virulence (65). During the time period 1987-1989, there were 20 virulent and 80 very virulent MDV strains isolated, during 1990-1992 there were 7 virulent, 71 very virulent and 21 very virulent plus strains isolated, and during 1993-1995 there were 8 virulent, 58 very virulent and 33 very virulent plus strains isolated (65). Therefore, within approximately one decade, the number of virulent strains isolated from the field declined, while the number of very virulent strains increased. Very virulent plus strains were not detected prior to 1987, but 33 were isolated by 1995.

1.6 History of Vaccination

HVT has been used as a vaccine in commercial settings since the 1970s. HVT is still very widely used as an MD vaccine because it is effective, inexpensive to make, and combines well with other vaccine viruses. HVT is a ubiquitous,

nonpathogenic virus of turkeys (69). It is classified as a third serotype of MDV. HVT is nonpathogenic in chickens, but it does replicate, albeit less efficiently (15). The viremia of HVT in chickens is associated with its ability to induce protection against virulent strains of MDV. Infection with HVT is persistent and thus the immunity is long-lasting (15).

As MDV increased in virulence over the years, serotype two vaccines began to be used, usually in association with HVT (55, 66, 67). SB-1 is a serotype 2 MDV strain that was isolated and characterized in 1978 (56). The strain is used in combination with serotype 1 or 3 during vaccination, since the combined strains induce much higher protection than monovalent vaccines (57, 67). This effect was termed protective synergism (67) and was most evident between serotypes 2 and 3.

Attenuated forms of serotype one strains have been used in Europe since the 1970s and in the United States since the early 1990s when the virulence of MDV1 increased to very virulent plus (50, 51, 64). CVI988, an attenuated MDV1, was isolated in the Netherlands in 1972 from healthy chickens, which suggested that chickens harbored MDV of low pathogenicity (51). Initially, this strain caused minor microscopic lesions, and it was then passaged in cell culture to further decrease its virulence. This virus provided excellent protection against virulent strains of MDV, even shortly after the vaccination (51).

MDV vaccination does not prevent infection and latency, but it does prevent tumor formation and causes a sharp decrease in viral replication during the first cytolytic phase of infection (13, 57). Vaccination also results in antibody production (27, 45). There are 35 virus-specific proteins and more than half of them are glycosylated and can act as antigens (63). Maternal antibodies decrease the

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severity of MD in infected chickens (9, 20, 47), but also can interfere with vaccination (14, 37, 59).

1.7 Measuring MDV by PCR

Recently, research has addressed the relationship between the amount of challenge and vaccine virus present in organs and feather tips and the outcome of the infection (4-6, 25, 28, 29). Initially, quantitative PCR (qPCR) methods were developed for the detection of each MDV serotype in spleen, peripheral blood lymphocytes (PBL), and feather tips (6, 29, 49). qPCR is a very rapid, accurate, and sensitive way of measuring the amount of MDV, and is used in diagnostics of other pathogens. The qPCR results on detection of all three serotypes of MDV were compared to end-point PCR results, and it was found that the majority of test results were consistent, the exception being a few that were negative by end-point PCR but positive by q-PCR (29).

In order to improve the understanding of pathogenesis, spread, vaccination and diagnosis of MDV, absolute quantitation assays were developed that measured the number of MDV genomes per number of chicken cells (4, 6, 28, 30, 49). Serotype specific genes such as *meq* for MDV1, DNA polymerase for MDV2, and SORF1 for HVT, were used to measure the MDV genomes, and collagen or ovotransferrin were used to measure the number of chicken cells. It has been reported that the amount of MDV in feather tips was very high, and that it was predictive of the amount of MDV in lymphoid tissues 10 days post infection (6).

Feather tip collection is also very easy and non-invasive and since the viral load in feather tips is high and representative of the remainder of the chicken (6),

feather tip monitoring became a tool that is favorably used to detect and diagnose MDV in chickens.

There have been several attempts to relate the amount of MDV and/or HVT to the disease outcome, but to date, these studies have been inconclusive (5, 24, 31). The difficulty of distinguishing among serotype one pathogenic and vaccine strains still complicates this work (5). All of the reported studies were done on chickens that were experimentally vaccinated at day one of age, and then challenged at different times. This differs from field situations, in which chickens are typically vaccinated during embryogenesis, and exposed to challenge when placed in grow-out houses. If vaccine and challenge virus monitoring is to be used to predict disease outcome, it must be relevant for field situations.

Chapter 2

MDV AND HVT IN FEATHER TIPS OF EXPERIMENTAL CHICKENS

2.1 Introduction

Marek's disease condemnations can be very costly to commercial growers and to processors. Currently, there is no early, easy, and accurate way of testing chickens for MDV infection other than looking for disease signs such as "red leg" and paralysis, which is a visible reddening of the shanks that sometimes accompanies MD. However, many chickens do not exhibit "red leg" but still develop tumors in visceral tissues. An early and simple way of testing chickens for MDV infection is needed, and such a test could also be useful for determining if chickens have been adequately vaccinated against MD. Feather tips may be useful for monitoring infection of chickens with vaccine and challenge viruses. We hypothesized that during infection, feather tip sampling could be used to monitor the presence of vaccine and challenge viruses and that information learned from monitoring feather tips could be used to predict the disease outcome. For these studies, we attempted to mimic field conditions.

2.2 Methods

2.2.1 Experimental Design

An MDV shedder trial was designed (Figure2.1) and executed as follows. Embryonated broiler eggs were received from a commercial source and hatched at the University of Delaware. In order to prepare chickens that would be a source of challenge virus in experimental houses, 25 chicks were inoculated intraabdominaly at

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hatch with ~5×10^5 peripheral blood lymphocytes obtained from T. King-infected chickens. The "shedder chicks" were wing-banded and placed in a clean colony house, and after two weeks, were examined for "red leg". Another group of embryonated broiler eggs was obtained from the same commercial hatchery and incubated at the University of Delaware. These eggs were vaccinated by hand at 18 days of incubation with a full dose of HVT (Merial Select, JZ350), HVT+SB1 (Merial Select, JV321), or HVT+SB1+Rispens (Intervet, 02766007). Eggs were sprayed with 70% ethanol and a hole was punctured at top of each egg by a 20 gauge needle. Vaccine was injected by 20 gauge needle syringe by inserting the needle until an embryo was hit, or about 1 inch deep. At hatch, twenty chicks from each vaccine group were wing-banded. Ten chicks from each vaccine group were placed in a house with shedders, and the other ten from each group were placed in clean colony house with no shedders. Also, ten non-vaccinated chicks were placed in each colony house as challenge controls. During the trial, chickens were checked twice daily.
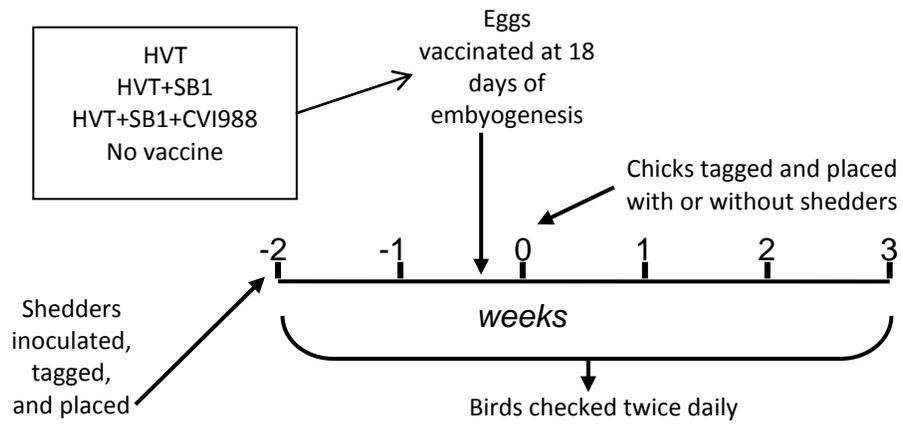
Figure 2.1   **MDV shedder trial design showing the time of vaccination and the placement times of MDV1 shedder chickens and the experimental chickens.**

2.2.2   Sample Collection

Feather tip samples from the axial feather tract were collected daily from day 2 to 18 from every chicken. For approximately the first week, the axial tract contained mostly down, and after that time period, true feathers began growing in and were collected. Each day, 5-8 feathers were plucked from each chicken, and approximately 1 cm of each feather tip was cut (Figure 2.2) and collected into a microfuge tube. The rest of the feather was discarded, and scissors were cleaned with 70% ethanol before next sample was collected. All samples were collected inside the colony houses and stored at -80°C. Samples from non-challenged chickens were collected before those from challenged chickens to prevent contamination of the clean colony house.

Figure 2.2   Picture of a typical axial feather. The red line signifies approximate location of cut and size (1 cm) of feather tip collected.

2.2.3   Sample Processing

DNA isolation from the feather tip samples was done using proteinase K digestion followed by phenol-chloroform extraction. Proteinase K buffer is 50 mM EDTA, 5% SDS, 100 mM Tris-HCL pH 8.0, and 400 ng/mL proteinase K. 500 uL of the pK buffer was added to each microfuge tube containing feather tips. The tubes were vortexed and centrifuged to insure that all feather tips were in solution and not adhered to the side of the microfuge tube. The tubes were incubated at 55°C overnight. The next day, buffer from the tubes was transferred to new pre-labeled tubes, and 500 uL of phenol:chloroform:isoamyl alcohol (25:24:1) pH 6.7 was added. The mixture was gently vortexed and centrifuged at 18,000 rcf for 5 minutes. The top aqueous layer was transferred to a new tube, and an equal amount of chloroform was added. Again, the mixture was gently vortexed and centrifuged at 18,000 rcf for 5 minutes. The top aqueous layer was transferred to a new tube, and two volumes of cold 95% ethanol were added. Tubes were inverted to mix the solutions, and DNA was pelleted by centrifuging at 18,000 rcf for 15 minutes. The DNA pellets were washed with cold 70% ethanol, resuspended in water, and stored at -80°C for further analysis.

2.2.4   End-Point PCR

ICP4-promoter (ICP4-p) primers (Table 2.1) were used to identify MDV1 and distinguish between T. King and CVI988 in feather tips. The primers, designed by Dr. Mark Parcells (34), amplify a 750-bp product from T. King and three products from CVI988 (634 bp, 750 bp and 800 bp) (Figure 2.3). Three bands can be visualized when the two viruses are at equivalence.
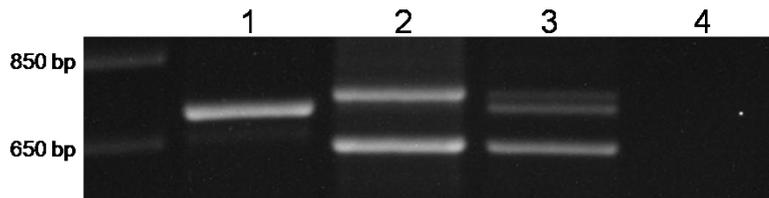
Figure 2.3   PCR amplification of T. King and CVI988 using ICP4-p primers. Primers were designed to amplify a portion of the ICP4 promoter that differs in size between virulent serotype 1 MDV strains and CVI988 vaccine. A band of ~750 bp is amplified from feather tip DNA of chickens infected with T. King (lane 1), while three bands (634 bp, 750 bp, and 800 bp) are amplified from CVI988 vaccinated chickens (lane 2). All three bands can be visualized in samples where CVI988 and T. King are at equivalence (lane 3). There is no amplification in negative control (lane 4). The DNA for the negative control came from feather follicles from uninfected chickens.

The PCR was done using the GeneAmp PCR System 9700 cycler with an annealing period of 1 minute at 65°C and an elongation period of 1.5 minutes at 72°C. All samples were analyzed on a 1% agarose gel, and pictures were taken using the AlphaInnotech AlphaImager.

HVT was identified in feather tips of chickens using end-point PCR and HVT ICP4 primers (Table 2.4), which amplify a 453 bp product from the HVT ICP4 gene (Figure 2.4). The GeneAmp PCR System 9700 was used to amplify HVT with an annealing time of 1 minute at 65°C and an elongation time of 1.5 minutes. All samples were analyzed on a 1% agarose gel, and the pictures were taken using the AlphaInnotech AlphaImager.
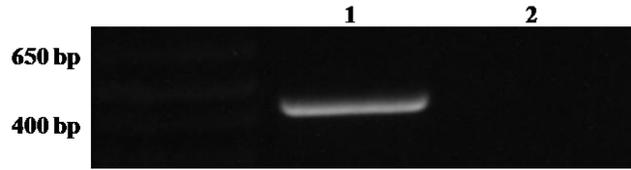
Figure 2.4 **PCR amplification of HVT using HVT-ICP4 primers. Primers amplify a 453 bp product from the ICP4 gene. PCR product from HVT-infected CEF DNA is shown in lane 1, and from uninfected CEF DNA is shown in lane 2.**

2.2.5 Quantitative PCR

HVT070 is a gene located in the U_L region of HVT (accession number: NC 002641) that does not have homologues in MDV (2). Since there is just one copy of this gene per HVT genome, and since this gene is unique to HVT, it was chosen to quantitate HVT by qPCR. A region of 896 bp (nucleotides 116501-117397) was amplified by end-point PCR using the HVT-70 forward and reverse primers (Table 2.1). This amplicon was inserted into a TOPO TA cloning vector by topoisomerase, and the resulting products were grown in *E. coli*, according to the TOPO TA Cloning Kit manual instructions (Invitrogen). Plasmids were purified using Qiagen Midi Prep kit (Qiagen) according to the manufacturer's instructions, and sequenced for confirmation. qPCR primers were then designed to amplify a 63-bp region contained in the original 896-bp sequence (Table 2.1).

Chicken ovotransferrin (accession number: Y 00407) was used to measure the number of chicken genomes in each sample (33). A 1-kb region (nucleotides 4001-5001) was amplified by end-point PCR using forward and reverse primers, and inserted in the TA Cloning vector by topoisomerase (Invitrogen) according to the manufacturer's instructions. The resulting plasmid was propagated in *E. coli* and purified by a Qiagen Midi Prep kit (Qiagen) according to the manufacturer's instructions. The plasmid was sequenced to confirm that it contained the desired sequences. qPCR primers used to measure ovotransferrin were previously described and amplify a 71-bp region (Table 2.1) (4).

An AB 7500 Fast Real Time System was used for amplification and detection of HVT and ovotransferrin using qPCR under standard mode with an annealing temperature of 60^oC. Each reaction contained Maxima SYBR Green/ROX

qPCR Master Mix (Fermentas Life Sciences), forward and reverse primers (0.4 μM), DNA template (100 ng), and nuclease-free water.

A dilution series was done on the HVT-70 plasmid ranging from 2×10^7 to 20 copies, and a standard curve was generated based on the Ct value for each dilution reaction. This standard curve was then used to determine the amount of HVT in each experimental sample based on the Ct value of that sample. A similar dilution series was done with the ovotransferrin plasmid, and a standard curve was generated. This standard curve was then used to determine the number of chicken genomes in each sample by correlating the Ct value of the sample to the standard curve.

Each sample was run in triplicate. All results with a Ct value ≥ 35 were considered negative. The number of HVT genomes was determined by the HVT-70 standard curve. This number was then divided by the number of chicken genomes in that sample, as determined using the ovotransferrin standard curve, and multiplied by 10,000 to represent the number of HVT genomes per 10,000 chicken cells.

Table 2.1   Primers used for PCR assays.

Target Name	Type of PCR	Primer Name	Primer Sequence	Amplicon Size (bp)
MDV1	End-point	ICP4-p-F	GTGCATATCTGCAAGTAAACAAAACT	varies by strain
Kaplan and Schat, 2000		ICP4-p-R	GTTCTCATATCCCCTGAACCGTACTC	
SB-1	End-point	SB-1-F	GCGATGGAACAGAGGTTCCT	746
		SB-1-R	CGACGTGGTGCTTGATGA	
HVT (ICP4)	End-point	ICP4-F	GGATTGAAGGTAAACGGGGT	453
		ICP4-R	GTGACGCAGCTTCTATAACG	
Chicken Ovotransferrin	End-point	OVO-F	GAACACTTCTCTGCCGTTAT	1000
Jeltsch et al., 1992		OVO-R	GAATCAGCCAATAGCACTCC	
HVT (HVT070)	End-point	HVT-70-F	GGAGAGTGATTGCAGGAATC	896
		HVT-70-R	ACGTAGTTGGTCTGATGGCG	
Chicken Ovotransferrin	Quantitative	qOVO-F	CACTGCCACTGGGCTCTGT	71
Baigent et al., 2005		qOVO-R	GCAATGGCAATAAACCTCCAA	
HVT	Quantitative	qHVT-70-F	AGCAATACGACGACGGACAGT	63
		qHVT-70-R	GGTCGCGATAGCAATTTTGG	

2.3   Results

2.3.1   MDV1 in Feather Tips

In feather tips of chickens co-housed with shedders, T. King could be detected in samples as early as day 2 of age, with a significant increase noted on day 7. Detection on day 2 of age is considered early for MDV. The true feathers do not grow in during the first week; therefore, we collected the down. The down could have contained a great deal of material from the dust inside the colony house, resulting in amplification of virus deposited from shedders. Therefore, we cannot be certain when T. King first appeared in feather tips in this experiment. To eliminate this problem, chicks could be washed prior to each sample collection.

CVI988 was first detected on day 10 in chickens not exposed to T. King and on day 12 in chickens exposed to T. King (Figure 2.5 and 2.6, respectively). By day 16, CVI988 could be detected in all chickens that were vaccinated with CVI988 and continued to be detected through day 18, the time at which the last samples were collected.
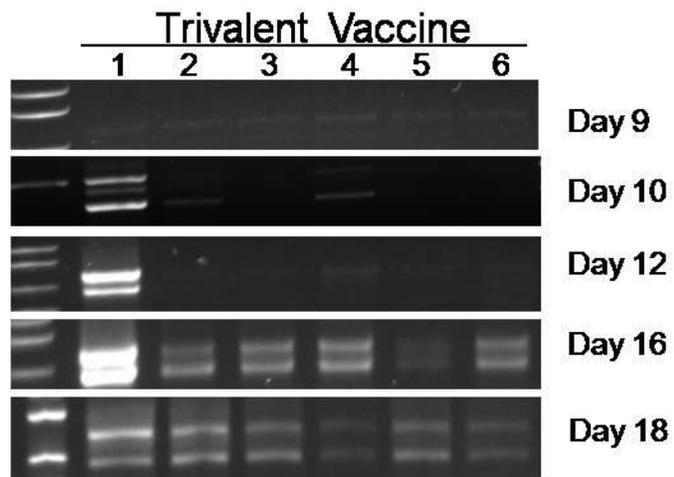
Figure 2.5 **MDV1 amplification using ICP4-p primers on six trivalently vaccinated chickens not exposed to MDV1 challenge. CVI988 is first detected on day 10 in lane 1. By day 16, CVI988 is detected in every chicken sampled.**
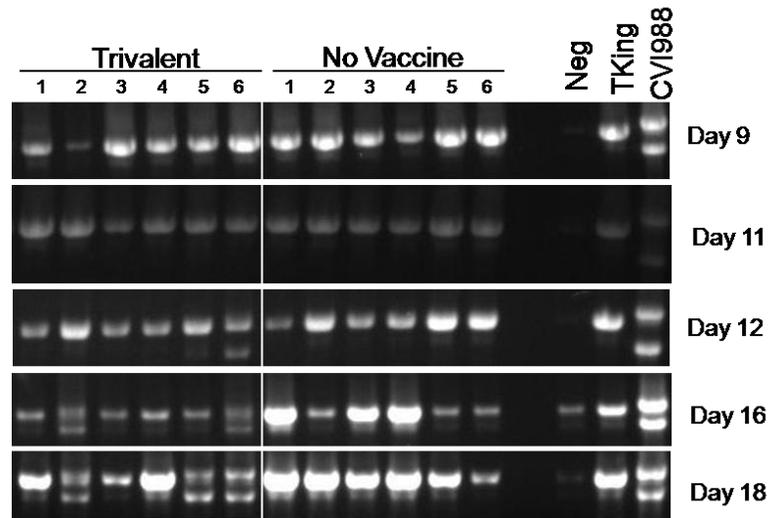
Figure 2.6 **MDV1 amplification with ICP4-p primers on six trivalently and unvaccinated chickens that were exposed to T. King shedders. DNA from feather tips of a chicken that was not vaccinated or exposed to MDV was used as a negative control. T. King and CVI988 controls came from vaccine DNA. CVI988 is first detected on day 12 in chickens exposed to T. King challenge.**

We observed that during the first 18 days, more T. King appeared to be detected in feather tips of chickens that were not vaccinated (Figure 2.7). Generally, more T. King virus was detected in the unvaccinated chickens than the chickens vaccinated with any of three vaccines, except in a few cases, where the amount of T. King in feather tips of vaccinated chickens seemed to reflect that in unvaccinated chickens (Figure 2.7). This confirmed that vaccination does not prevent infection, replication, or shed of virus. However, it does appear to reduce the amount of virus in the feather tips and accordingly the amounts available for transmission to other chickens. There seemed to be no difference in the amount of T. King detected among different vaccine groups, but this may be hard to interpret from end-point PCR results (Figure 2.7). In addition, within the same individual chicken, the amount of MDV in feather tips is inconsistent from day to day (Figure 2.7). There could be a difference among feathers that were collected on those days. Finally, with regard to CVI988 detection, all three bands could only be visualized in samples where CVI988 and T. King were at equivalence, and if one virus was even slightly more abundant, it would appear to be amplified much more efficiently than the other virus.
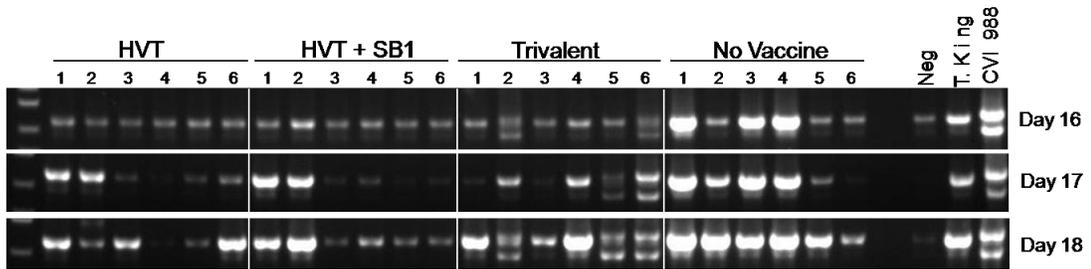
Figure 2.7   **MDV1 amplification using ICP4-p primers on feather tip samples from chickens vaccinated with HVT, HVT+SB1, or trivalent, and unvaccinated chickens. All chickens were exposed to T. King shedders.   Six individual chickens were examined from each vaccine group over the time course. DNA from feather tips of a chicken that was not vaccinated or exposed to MDV was used as a negative control. T. King and CVI988 controls came from vaccine DNA.**

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2.3.2   HVT in Feather Tips

HVT was first seen in feather tips of chickens at about two weeks of age. At that time, the amount of HVT in chickens exposed to shedders appeared less than in chickens not exposed to shedders. By day 18, the effect of T. King on the amount of HVT in feathers was not so obvious (Figure 2.8). This suggests that early post infection, interaction with a challenge virus complicates HVT infections.

a) T. King Challenged Chickens

b) Non-challenged Chickens
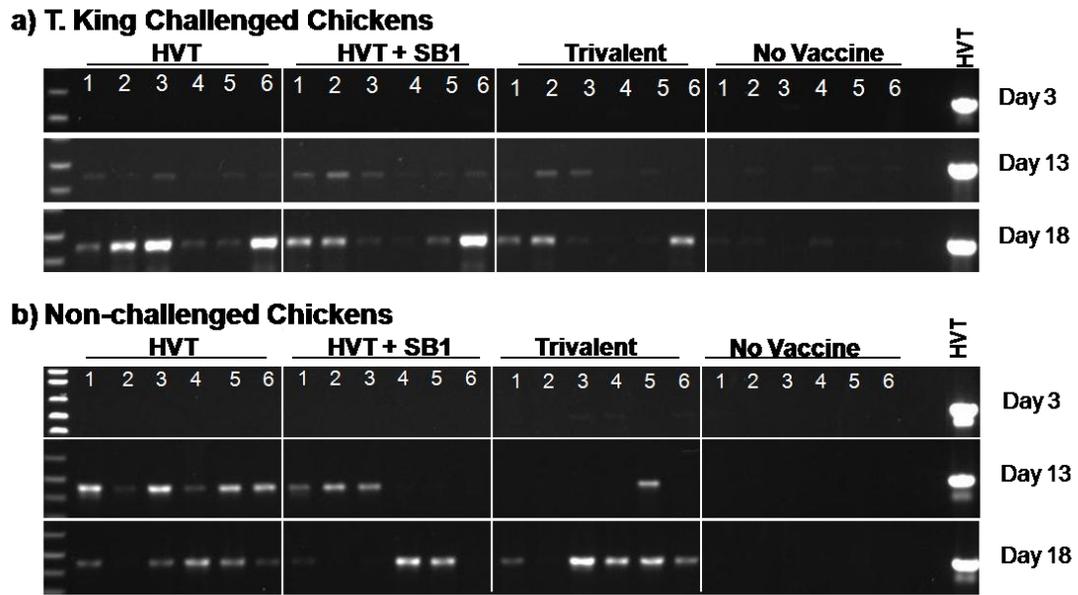
Figure 2.8   HVT amplification using HVT-ICP4 primers on feather tip samples
             from chickens vaccinated with HVT, HVT-SB1, trivalent vaccine
             and unvaccinated chickens. Six individual chickens from each
             group were used throughout the experiment. HVT control came
             from HVT vaccine DNA.

The amount of HVT in feather tips differed among different vaccine groups in non-challenged chickens (Figure 2.8). More HVT was detected in HVT-vaccinated chickens than in bivalently vaccinated chickens, and the least amount was seen in trivalently vaccinated chickens early after vaccination. This suggests that co-infection of a chicken is complex. This pattern was not seen in T. King challenged chickens, suggesting that challenge further complicates the kinetics of HVT infections during this time period.

In order to more accurately measure the amount of HVT in feather tips and to more definitively show the effect of MDV on HVT abundance, qPCR was done. The qPCR results were closely correlated with the end-point PCR results for the majority of chickens examined (Figure 2.9). This finding supports the idea that end-point PCR reactions are semi-quantitative. qPCR results supported the previous observation that T. King challenge decreased the amount of HVT found in feather tips on day 13 post challenge. qPCR results also confirmed that in chickens not challenged with T. King challenge, the amount of HVT detected was greater in HVT-vaccinated chickens than in bivalently-vaccinated chickens, and the least was detected in trivalently-vaccinated chickens.

T. King Challenge – HVT Vaccinated

	Bird 1	Bird 2	Bird 3	Bird 4	Bird 5	Bird 6
Genomes	102	22	160	8	18	55

No Challenge – HVT Vaccinated

	Bird 1	Bird 2	Bird 3	Bird 4	Bird 5	Bird 6
Genomes	115,574	13	40,070	104	26,374	9,436

T. King Challenge - HVT + SB-1 Vaccinated

	Bird 1	Bird 2	Bird 3	Bird 4	Bird 5	Bird 6
Genomes	105	332	23	26	17	107

No Challenge - HVT + SB-1 Vaccinated

	Bird 1	Bird 2	Bird 3	Bird 4	Bird 5	Bird 6
Genomes	9,100	18,984	21,907	95	50	39

T. King Challenge – Trivalently Vaccinated

	Bird 1	Bird 2	Bird 3	Bird 4	Bird 5	Bird 6
Genomes	29	410	30	3	10	17

No Challenge – Trivalently Vaccinated

	Bird 1	Bird 2	Bird 3	Bird 4	Bird 5	Bird 6
Genomes	178	132	18	53	149,005	29

Figure 2.9   **HVT amounts in feather tips of 13 day old chickens. Three different vaccinate groups were analyzed (HVT, HVT+SB-1, and triv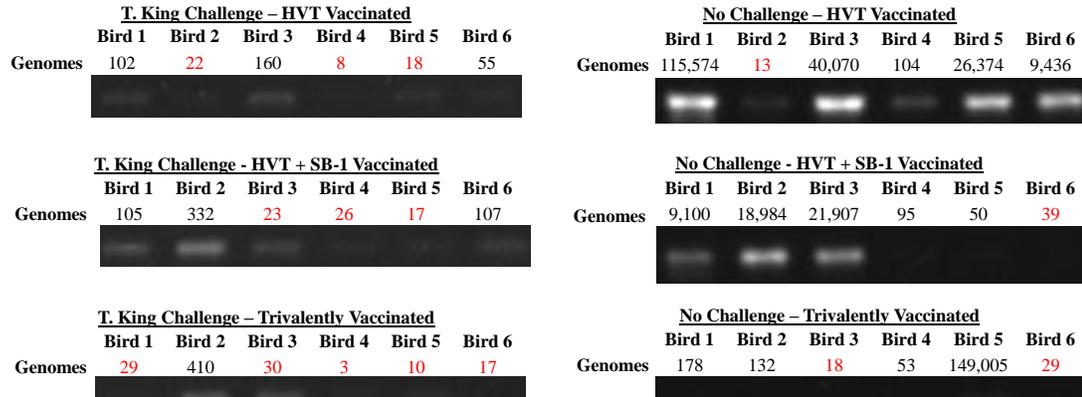alent) as was one unvaccinated group. One set of chickens was exposed to T. King shedder challenge, while the other set was not. HVT amounts are expressed as HVT genomes per 10,000 chicken cells. The red numbers represent amounts that were below Ct threshold (Ct $\geq$ 35).**

2.4 Discussion

We quantified MDV and HVT in the feather tips of young chickens that were vaccinated at 18 days of embryogenesis, and challenged by contact at one day of age by being co-housed with MDV1-infected chickens. Previous work on detection of MDV and HVT in feather tips was done mostly with older chickens that were vaccinated at hatch and challenged at different times after vaccination (4-6, 24, 28-31). The route of challenge is typically intraabdominal, which eliminates/alters the natural sequence of the infection. There was no correlation between the virus load in feather tips and the disease outcome reported by these studies. Our experimental design was similar to commercial settings and natural exposure, and accordingly we reasoned that it might provide a different perspective since their immune systems of young chickens are still developing during the early stages of life.

2.4.1 MDV1 Detection in Feather Tips of Experimental Chickens

MDV1 detection was analyzed in experimentally vaccinated and challenged chickens by end-point PCR. The amounts of T. King in feather tips of individual chickens seemed to vary from day to day. This is probably due to the differences in how many feathers were infected, how much virus was present at the beginning of the infection and how rapidly the virus replicated in those feathers.

The initial appearance of T. King, the MDV1 challenge virus used in this experiment, in the feather tips could not be determined in this study. T. King was detected in feather tip samples from 2-day-old chicks. It is unlikely for the virus to travel to the feather follicle and replicate to detectable amounts by two days post infection. It was reported by Schat that after intratracheal inoculation, 24-36 hours are

required for the virus to reach lymphoid organs (70), and challenge virus is expected to reach feather tips even later. A plausible explanation for our results is that large amount of dust that had accumulated in the chicken houses was lodged in the down of the 2-day-old chicks. Thus, our early PCRs probably detected MDV1 deposited from shedders. By day 7, the intensity of the bands generated by end-point PCR increased, and it is likely that approximately 7 days post placement is the time at which T. King was first detected in the experimental chickens. This is also the time when real feathers emerge, which greatly facilitates feather tip sampling. Thus we believe that T. King was first detected in feather tips on day 7 in this experiment.

Based on the intensities of the bands generated by end-point PCR, the amount of T. King was lower in chickens that were vaccinated with any of the three vaccines compared to unvaccinated chickens. Even though it is known that vaccination does not protect against MDV infection, it does lower the amount of MDV detected in the feather tips at this early time post-infection. It is possible that T. King competed with vaccine strains for lymphocytes, and thus its accumulation appeared less in vaccinates compared to unvaccinated chickens. Competition among viruses has been described before for other viruses (44, 72), and for MDV by John Dunn (22). Mechanisms and details of competition among vaccine and challenge viruses are unclear and apt to be complicated. In addition, immune responses triggered by the vaccine strains are likely to have affected replication and establishment of T. King in vaccinated chickens. Since this experiment was terminated when the chickens were 18 days old, it is not known if the levels of T. King in feather tips of vaccinated chickens would eventually be similar to the levels observed in unvaccinated chickens. Indeed, one study reported that at 56 days post-challenge, MDV1 levels in feather tips were

not significantly different between HVT vaccinated and unvaccinated chickens (28). However, in another study, MDV1 detection in PBL was reported to be lower in HVT-vaccinated chickens after day 28 compared to the unvaccinated chickens (31). Finally, both HVT and bivalent vaccination initially reduced MDV1 amount in dander, but after 35 days post challenge, no significant difference in the amount of MDV1 in dander between vaccinated and unvaccinated chickens was observed (30). Therefore, MDV1 seems to reach a plateau after a certain time during infection, and we expect that if our experiment had been carried out for a longer period of time, we would have seen less difference between the amounts of MDV1 in feather tips from vaccinated versus unvaccinated chickens at later time points.

CVI988 vaccine virus can also be detected by the MDV1 end-point PCR assay that we used. In this system, multiple bands are amplified from the vaccine virus, compared to one band that is amplified from T. King. This allows the distinguishing between CVI988 and T. King. Using end-point PCR, which is semi-quantitative, however, observing all bands in co-infected chickens is difficult unless the viruses are present in roughly equal amounts. Since one virus is usually in greater abundance than the other, in practice, usually only the band/s of the virus in higher abundance is/are detected.

CVI988 first appeared in feather tips of experimental chickens on day 10 in the absence of challenged shedders, and on day 12 in chickens exposed to T. King shedders. It appeared that T. King delayed the appearance of CVI988 in feather tips, an observation that can be explained by the competition between the two viruses (44, 72). It is possible that T. King and CVI988 were competing for lymphocytes or for some limiting factors in these herpesvirus infections. T. King is known to induce

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severe lymphocyte depletion particularly in young chickens. Interference with the establishment of CVI988 infection would result in a longer period of time for the virus to reach the minimal threshold necessary for the detection.

In order to accurately evaluate these observations seen by end-point PCR, a qPCR method should be developed. qPCR on MDV1 has been described recently, but the reported primers do not distinguish between CVI988 and challenge strains. Obtaining qPCR primers that distinguish among serotype 1 strains is a challenge because sequence differences that could be used to design strain-specific primers are not obvious.

2.4.2 Detection of HVT in Feather Tips of Experimental Chickens

HVT was first detected in feather tips of vaccinated chickens during the second week of life by end-point PCR. This has been reported by others in dander and PBL as well (28, 30). On day 13, more HVT was detected in feather tips of non-challenged chickens compared to T. King-challenged chickens, suggesting that T. King inhibits establishment of HVT infections in feather tips. As mentioned previously for CVI988, this could be due to competition for lymphocytes between HVT and T. King and to lymphocyte depletion by the T. King challenge virus. Interestingly, by day 18 post infection the amount of HVT appeared similar in both groups of chickens. It is possible that by day 18 some type of immune response is triggered that reduces the effect of T. King on HVT, allowing HVT to replicate to normal levels. Others have reported that in chickens vaccinated at hatch and challenged by one of three Australian MDV1 isolates (MPF57, 02LAR, or FT158) at day 5, the amount of HVT detected in feather tips fourteen days after challenge was higher when chickens were co-infected with MDV1 challenge virus (30). Different

experimental designs, sampling times, and virus strains used offer explanations for differences between our results and those of Islam et al. (30).

The relative abundance of HVT in feather tips was affected by the presence of other vaccine strains, SB-1 and CVI988, an observation that was apparent when feather tips of non-challenged chickens were examined. The amount of HVT is lower in HVT + SB-1-vaccinated chickens and even lower in trivalently vaccinated chickens compared to HVT only-vaccinates. This suggests that interactions occur in vivo among the MDV vaccine strains. As discussed previously, competition for lymphocytes or for factors that limit infections could be at play, and as the number of strains in co-infections increased, the abundance of each individual virus decreased. It would be interesting to compare the amount of SB-1 virus in chickens that were vaccinated bivalently to the amount in chickens that were vaccinated trivalently, and one would predict that amount of SB-1 would decrease with the addition of CVI988 vaccine strain. The effect of SB-1 and CVI988 on the HVT amounts is not observed in the T. King-challenged chickens. T. King is a very virulent plus MDV1 strain that has profound effects on lymphocytes, including causing lymphocyte depletion and lymphoid tissue atrophy. The effect of T. King infection in vaccinated chickens complicates the establishment of various infections in ways that are not clear.

Differences in HVT detection in feather tips were first noticed using the end-point PCR. To determine if valid comparisons of these differences could be made, a quantitative PCR assay was developed. We found that the qPCR results were very similar to the end-point PCR results. This suggests that the end-point PCR that we used was semi-quantitative, as long as it was performed with care and precision.

Since the development of qPCR assays can be difficult and expensive, once validated, an end-point PCR provides a reasonable alternative for large scale screening.

Chapter 3

MDV AND HVT IN FEATHER TIPS OF COMMERCIAL CHICKENS

3.1   Introduction

Developing an understanding of the baseline of detection of MDV challenge virus(es) and vaccines in commercial broiler chickens under production conditions is of great interest and importance to producers and researchers. This information should reveal differences between experimental and commercial settings, and it may uncover information with regard to the sorts of challenges present in commercial settings and the effectiveness of currently used vaccines. The baseline might also provide a way to predict MD early in life before investments in production inputs have been made. To develop and evaluate the baseline level of vaccine and challenge viruses in commercial broiler flocks, we collaborated with Mountaire Farms, CEVA BIOMUNE, and AviServe LLC.

3.2   Methods

3.2.1   Experimental Design

Thirty-commercial flocks were sampled over one grow-out period, which is the time from hatch to processing (Table 3.1). Actual grow-out periods varied among the commercial flocks sampled depending on the time of chick placement and on the time of processing, with some flocks being processed as younger chickens and some as older ones.

Feather tips were collected from six chickens from each flock weekly, and at the time of feather tips sample collection, each chicken was necropsied, examined

for lesions, and sampled for other purposes by our collaborators. In this trial, primary flight feathers were collected as a source of feather tips. Baigent et al. and Calnek et al. have shown that there was no significant difference in the load of MDV among different feather tracts (4, 11). The sample collection and necropsy took place at the University of Delaware Lasher Lab at the Elbert N. and Ann V. Carvel Research and Education Center in Georgetown, Delaware, and the samples were processed at the Delaware Biotechnology Institute in Newark, Delaware.

Table 3.1   **List of the 30 commercial farms, age of chickens at each sampling time, and the MD vaccination program used by that farm.**

	Age at Time of Sample Collection (Days)			Vaccination Program
Flock 1	16	23	38	0.33X HVT + SB-1
Flock 2	15	22	37	0.33X HVT + SB-1
Flock 3	12	19	48	0.33X HVT + SB-1
Flock 4	16	23	38	0.33X HVT + SB-1
Flock 5	15	22	37	0.33X HVT + SB-1
Flock 6	16	23	38	0.33X HVT + SB-1
Flock 7	16	23	45	0.33X HVT + SB-1
Flock 8	16	23	38	0.33X HVT + SB-1
Flock 9	13	20	34	0.33X HVT + SB-1
Flock 10	13	20	46	0.33X HVT + SB-1
Flock 11	15	22	44	0.33X HVT + SB-1
Flock 12	12	19	34	0.33X HVT + SB-1
Flock 13	15	22	45	0.33X HVT + SB-1
Flock 14	15	22	38	0.33X HVT + SB-1
Flock 15	12	19	34	0.33X HVT + SB-1
Flock 16	12	19	48	0.33X HVT + SB-1
Flock 17	16	23	45	0.33X HVT + SB-1
Flock 18	16	23	45	0.5X Innovax ILT + 0.33X SB-1
Flock 19	15	22	44	0.5X Innovax ILT + 0.33X SB-1
Flock 20	12	19	48	0.5X Innovax ILT + 0.33X SB-1
Flock 21	13	20	47	0.5X Innovax ILT + 0.33X SB-1
Flock 22	12	19	48	0.5X Innovax ILT + 0.33X SB-1
Flock 23	13	20	47	0.5X Innovax ILT + 0.33X SB-1
Flock 24	15	22	44	0.5X Innovax ILT + 0.33X SB-1
Flock 25	15	22	44	0.5X Innovax ILT + 0.33X SB-1
Flock 26	16	23	45	0.5X Innovax ILT + 0.33X SB-1
Flock 27	15	22	45	0.5X Innovax ILT + 0.33X SB-1
Flock 28	16	23	45	0.5X Innovax ILT + 0.33X SB-1
Flock 29	13	20	47	0.5X Innovax ILT + 0.33X SB-1
Flock 30	12	19	48	0.5X Innovax ILT + 0.33X SB-1

3.2.2 Sample Processing and Analysis

DNA was isolated from each feather tip sample using proteinase K digestion and phenol:chloroform extraction and subjected to end-point PCR analysis, as described in the methods section of Chapter 2 with the exception that the SB-1 reactions were done at annealing temperature of 60°C. HVT end-point PCR was done using the HVT-ICP4 primers, SB-1 was done using SB-1 primers, and MDV1 was done using ICP4-p primers described earlier (Table 2.1).

3.2.3 Performance Data Analysis

Performance data was obtained from the producer and organized in an Excel spreadsheet. For each flock, the European Production Efficiency Factor (EPEF) was calculated using the formula EPEF = (livability% X weight kg) / (age X adjusted feed conversion). EPEF is not commonly used by producers in America, but is readily used in Europe. The use of EPEF in this study was done because it incorporates several important aspects of production into one statistic. High EPEF numbers are desired. EPEF, however, can be deceiving because the increase in age of chicken is linear while the increase in weight is exponential as the chicken gets older. In different Excel sheets, data was ordered by age or by EPEF to identify any patterns or correlations with leukosis condemnation.

3.3 Results

HVT was detected in the feather tips of chickens from all chicken flocks throughout the entire grow-out period, but it was not detected in every chicken (Figure 3.1). This finding is consistent with our previous work. The number of chickens that were positive for HVT in feather tips did vary a little over the grow-out period and appeared to decline by the seventh week of production. However, in general,

approximately 40% of chicken samples were positive for HVT in the feather tips

between 2 and 7 weeks of age.

Figure 3.1 **HVT virus detection by end-point PCR in feather tips of commercially raised chickens. The ages of chickens sampled are at the x-axis, and the percentage of chickens where HVT was detected is at the y-axis. N is the number of farms represented by each time point. HVT is detected in feather tips of some chickens, but not all, during the entire grow-out period.**

SB-1 detection was analyzed in the same feather tip samples described for HVT detection (Figure 3.2). In these samples, the frequency of SB-1 seemed to increase from week two through three, peaked during the third week of life, and then slightly decreased during week four. During this time, the detection of SB-1 was more frequent then the detection of HVT. During weeks 5-7, SB-1 is detected in nearly every chicken.
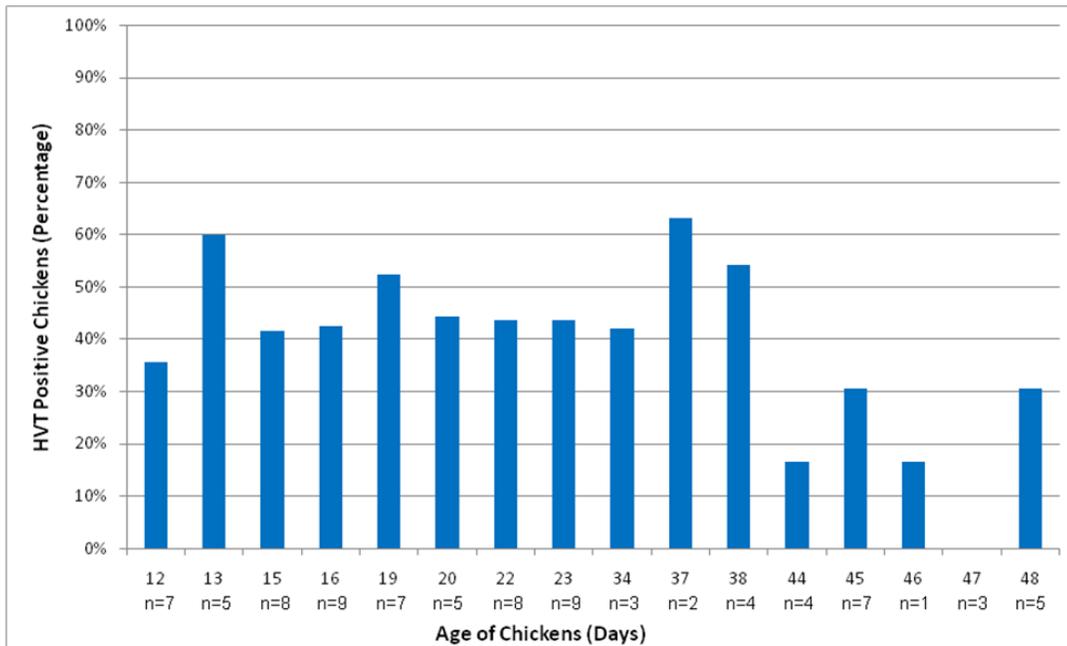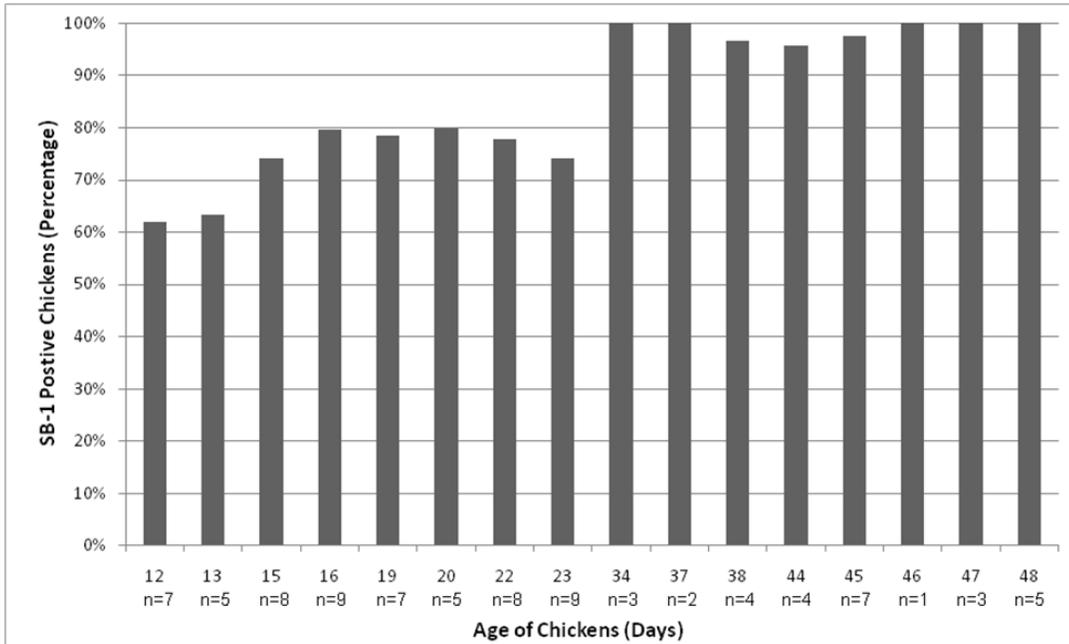
Figure 3.2   **SB-1 virus detection by end-point PCR in feather tips of commercially raised chickens. The ages of chickens sampled are at the x-axis, and the percentage of chickens where SB-1 was detected is at the y-axis. N is the number of farms represented by each time point. SB-1 is detected in feather tips of some chickens, but not all, during the whole grow-out period.**

MDV1 was also detected in these same samples by end-point PCR and the results are shown in Figure 3.3. The chickens in this experiment are from commercial chicken flocks, therefore, none were experimentally challenged with MDV1. Since none of these flocks were vaccinated with CVI988, serotype 1 viruses detected in these commercial chickens are derived from the field, which indicates that MDV1 strains are present in the grow-out houses during normal production conditions. Variations in the sizes of end-point PCR products visualized on agarose gels were particularly noteworthy among flocks and even within a flock.   To determine if this variation was unique to currently obtained field samples, end-point PCR amplification for various stock MDV1 strains obtained from Dr. Mark Parcells was done (Figure 3.3). The trend observed was that multiple bands appeared to be amplified among less virulent strains; whereas, only a single band was amplified from highly virulent strains. Also, when multiple bands were present, one band consistently appeared more intense than the others. There was no similarity between the band patterns observed from commercial chicken samples and those from the stock MDV1 strains.
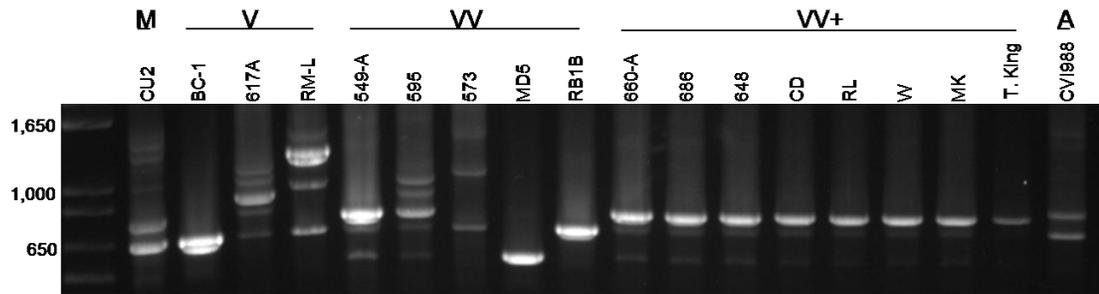
Figure 3.3   Amplification of various MDV1 stock strains by end-point PCR. Multiple bands are observed in less virulent strains, while only a single band is observed in the more virulent strains.

The detection of MDV1 in commercial chickens over time appeared to parallel a typical pattern of MDV infection. MDV1 was detected at 12 days of age, but then, at about three weeks of age, MDV1 could not be detected in feather tip samples obtained from these flocks. By the end of week 5, the MDV1 was again apparent, with levels that appeared to approach those originally observed.
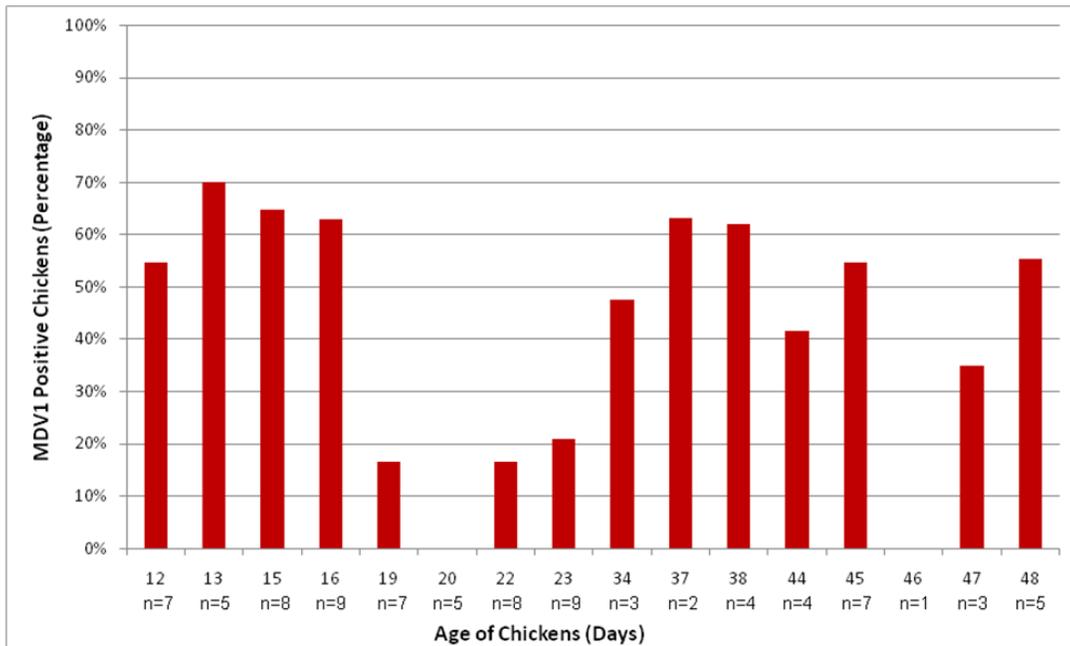
Figure 3.4   MDV1 detection by end-point PCR in feather tips of commercially raised chickens. The ages of chickens sampled are at the x-axis, and the percentage of chickens where MDV1 was detected is at the y-axis. N is the number of farms represented by each time point. MDV1 is detected in feather tips of some chickens, but not all. The detection dropped during third week of life, but came back and leveled off until the end of grow-out period.

Performance data was generated by the processing company, and organized in Excel spreadsheets. We calculated EPEF for every flock and incorporated these data into the datasets. In order to find correlations among the performance data; detection of MDV1, MDV2, and HVT; and leukosis condemnations, the data was sorted by either age at slaughter or by EPEF, as is shown in Table 3.2 and Table 3.3, respectively.

Based on sorting the data by age of chickens at slaughter, the flocks with leukosis condemnations are grouped within the oldest category. As would be expected, the trend is that older chickens have a higher chance of exhibiting signs of leukosis compared to younger chickens. Based on sorting the data by EPEF, flocks with leukosis condemnations (older chickens) also had a relatively high EPEF.

Correlation between the frequency and relative abundance of HVT, SB-1, and MDV1 in feather tips of these flocks to leukosis condemnations was also attempted. However, no pattern could be observed with regard to the frequency of detection of MDV1 viruses since MDV1 detection was quite uniform among flocks. Also, there were no apparent correlation between the relative amount of any MDV detected and leukosis condemnations. For example, Flock 21, a flock with leukosis condemnations, has a relatively low abundance of SB-1 at 2 weeks, but so does Flock 23, a flock showing high overall performance. On the other hand, Flock 25 and Flock 26, two flocks with leukosis condemnations, showed relatively high abundance of SB-1 at 2 weeks. Likewise, correlations between the relative abundance of HVT in feather tip samples, EPEF, and eventual leukosis condemnations were not evident.

Table 3.2   Performance data for the 30 commercial flocks that is ordered by the age of the chickens on the day of slaughter. The red lines separate small, medium and large sized chickens. Highlighted numbers are of flocks with leukosis condemnation.

Grower Name	Age	Program	Rank	Liv %	Avg Wt	Wt kg	Adj FC	EPEF	Lek %
Flock 6	41.0	S	11	0.97	4.59	2.08	1.800	272.82	0.00%
Flock 15	41.0	S	15	0.97	4.82	2.19	1.793	288.58	0.00%
Flock 14	42.0	S	16	0.97	4.86	2.20	1.829	278.09	0.00%
Flock 9	42.0	S	21	0.97	4.74	2.15	1.861	266.88	0.00%
Flock 1	42.0	S	19	0.96	4.67	2.12	1.858	261.26	0.00%
Flock 4	42.0	S	7	0.97	5.15	2.34	1.765	306.12	0.00%
Flock 13	42.4	S	3	0.96	5.15	2.33	1.756	301.03	0.00%
Flock 5	43.0	S	20	0.91	4.73	2.14	1.869	242.93	0.00%
Flock 2	43.0	S	14	0.95	5.25	2.38	1.813	290.40	0.00%
Flock 8	43.0	S	10	0.97	5.00	2.27	1.775	287.61	0.00%
Flock 12	44.0	S	2	0.97	5.39	2.45	1.749	308.77	0.00%
Flock 7	51.0	L	3	0.94	6.78	3.07	1.907	298.31	0.00%
Flock 11	51.8	L	1	0.93	7.04	3.20	1.925	298.86	0.00%
Flock 10	52.0	L	4	0.97	6.89	3.13	1.941	300.85	0.00%
Flock 17	52.1	L	6	0.95	7.04	3.19	1.938	300.80	0.00%
Flock 16	53.0	L	9	0.96	6.86	3.11	1.962	287.32	0.00%
Flock 3	53.3	L	11	0.94	6.67	3.02	2.016	263.75	0.00%
Flock 18	59.0	L	2	0.95	8.44	3.83	1.886	327.29	0.00%
Flock 25	59.9	L	1	0.96	8.66	3.93	1.867	338.54	0.02%
Flock 23	60.0	L	8	0.94	8.60	3.90	1.914	318.28	0.00%
Flock 30	60.0	L	13	0.96	8.43	3.83	1.957	311.72	0.32%
Flock 20	60.8	L	16	0.94	8.55	3.88	1.927	310.83	0.00%
Flock 24	61.0	L	3	0.97	8.86	4.02	1.886	338.80	0.00%
Flock 21	61.0	L	5	0.93	8.88	4.03	1.926	319.06	0.05%
Flock 29	61.0	L	11	0.96	8.64	3.92	1.943	318.22	0.00%
Flock 22	61.8	L	15	0.93	8.93	4.05	1.956	312.59	0.00%
Flock 28	62.0	L	2	0.97	9.01	4.09	1.878	340.24	0.00%
Flock 26	62.0	L	6	0.96	9.14	4.15	1.898	339.59	0.64%
Flock 19	62.0	L	17	0.95	8.76	3.98	1.976	307.21	0.00%
Flock 27	62.0	L	19	0.91	8.82	4.00	1.993	295.48	0.00%

Table 3.3   Performance data for the 30 commercial flocks that is ordered by the EPEF value. Highlighted numbers are of flocks with leukosis condemnation.

Grower Name	Age	Program	Rank	Liv %	Avg Wt	Wt kg	Adj FC	EPEF	Lek %
Flock 5	43.0	S	20	0.91	4.73	2.14	1.869	242.93	0.00%
Flock 1	42.0	S	19	0.96	4.67	2.12	1.858	261.26	0.00%
Flock 3	53.3	L	11	0.94	6.67	3.02	2.016	263.75	0.00%
Flock 9	42.0	S	21	0.97	4.74	2.15	1.861	266.88	0.00%
Flock 6	41.0	S	11	0.97	4.59	2.08	1.800	272.82	0.00%
Flock 14	42.0	S	16	0.97	4.86	2.20	1.829	278.09	0.00%
Flock 16	53.0	L	9	0.96	6.86	3.11	1.962	287.32	0.00%
Flock 8	43.0	S	10	0.97	5.00	2.27	1.775	287.61	0.00%
Flock 15	41.0	S	15	0.97	4.82	2.19	1.793	288.58	0.00%
Flock 2	43.0	S	14	0.95	5.25	2.38	1.813	290.40	0.00%
Flock 27	62.0	L	19	0.91	8.82	4.00	1.993	295.48	0.00%
Flock 7	51.0	L	3	0.94	6.78	3.07	1.907	298.31	0.00%
Flock 11	51.8	L	1	0.93	7.04	3.20	1.925	298.86	0.00%
Flock 17	52.1	L	6	0.95	7.04	3.19	1.938	300.80	0.00%
Flock 10	52.0	L	4	0.97	6.89	3.13	1.941	300.85	0.00%
Flock 13	42.4	S	3	0.96	5.15	2.33	1.756	301.03	0.00%
Flock 4	42.0	S	7	0.97	5.15	2.34	1.765	306.12	0.00%
Flock 19	62.0	L	17	0.95	8.76	3.98	1.976	307.21	0.00%
Flock 12	44.0	S	2	0.97	5.39	2.45	1.749	308.77	0.00%
Flock 20	60.8	L	16	0.94	8.55	3.88	1.927	310.83	0.00%
Flock 30	60.0	L	13	0.96	8.43	3.83	1.957	311.72	0.32%
Flock 22	61.8	L	15	0.93	8.93	4.05	1.956	312.59	0.00%
Flock 29	61.0	L	11	0.96	8.64	3.92	1.943	318.22	0.00%
Flock 23	60.0	L	8	0.94	8.60	3.90	1.914	318.28	0.00%
Flock 21	61.0	L	5	0.93	8.88	4.03	1.926	319.06	0.05%
Flock 18	59.0	L	2	0.95	8.44	3.83	1.886	327.29	0.00%
Flock 25	59.9	L	1	0.96	8.66	3.93	1.867	338.54	0.02%
Flock 24	61.0	L	3	0.97	8.86	4.02	1.886	338.80	0.00%
Flock 26	62.0	L	6	0.96	9.14	4.15	1.898	339.59	0.64%
Flock 28	62.0	L	2	0.97	9.01	4.09	1.878	340.24	0.00%

3.4   Discussion

Previous MDV and HVT analysis of feather tips of experimentally infected chickens has failed to correlate virus loads in feather tips with disease outcome, however, these studies were not done on chickens raised under commercial settings. In our study, MDV1, SB-1, and HVT were monitored in the feather tips of chickens from 30 commercial flocks samples over an entire grow-out period. Results were compared to performance data that was generated by the processing company to determine if correlations among MDV detection, flock performance, and/or leukosis condemnations could be made.

3.4.1   Detection of HVT in Feather Tips

In commercial flocks, HVT was detected in feather tip samples of chickens from all farms examined throughout the grow-out period, but it was not detected in every chicken. This is not surprising considering that HVT does not replicate in chicken feather follicle epithelium as well as MDV does (15, 17). Detection of HVT does confirm that the chickens were vaccinated. We observed an interesting variation in detection of HVT over the course of grow-out period, with a decline during week 7 of life. The significance of this observation is not clear, and it might be related to the lower number of flocks examined during week 7 compared to previous weeks. The differences in the number of flocks analyzed during later time points are due to the differences in time of slaughter of each flock. Others have reported that in PBL, HVT quantities remained consistent throughout a 40-day experiment (28).

3.4.2 Detection of SB-1 in Feather Tips

The SB-1 vaccine strain was also detected in feather tips from all flocks throughout the grow-out period. For the first four weeks of life, SB-1 was not detected in every chicken, and the highest average levels seemed to be during week three. From week five through the end of the experiment, SB-1 was detected in 100% of chickens samples, except for a slight decline between weeks six and seven. This percentage of positive chickens was much higher than that of HVT or MDV1, and reflects the reported presence of SB-1 in chicken dander (30). Detection of SB-1 in flocks confirms that chickens were vaccinated.

3.4.3 Detection of MDV1 in Feather Tips

MDV1 was detected in feather tip DNA from commercial chickens, indicating that MDV1 viruses are present in commercial houses. However, the observed patterns of amplified products did not match the pattern of CVI988, suggesting that these MDV1 viruses are field strains. Also, the gel patterns of MDV1 viruses detected were different among different chicken flocks and even within a single flock, suggesting that several MDV1 strains were present in typical commercial chicken houses. And, multiple bands could be detected in samples from some chickens, suggesting that they were infected with multiple viruses. Upon analysis of various MDV1 stock strains obtained from Dr. Mark Parcells, it was apparent that multiple bands are typically amplified from relatively mild MDV1 strains, whereas single bands were amplified in samples from all very virulent plus strains. Also, in the strains characterized by multiple bands, one usually predominated. Thus, many of the field chickens and the stock mild isolated appeared to be mixed infections. Single bands were amplified for the MD5 and BC1 strains, and while bands of the same sizes

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are also amplified from several other strains, they were not the predominant bands in those samples.

The observation that different patterns are amplified among and within flocks could mean that commercial chickens are infected by milder strains of MDV1 and/or they are co-infected by multiple strains. It was difficult to match PCR patterns from commercial chickens to the patterns from the various MDV1 stock strains; thus, the MDV1 field strains detected in commercial chickens may be different from the laboratory stocks that we currently have. The diversity of field strains is very high.

A striking observation was made regarding MDV1 detection in feather tip samples from commercial flocks obtained during week 3. More specifically, on days 19, 22, and 23, the detection of MDV1 was very low compared to the other days sampled, with no MDV1 detected on day 20 by end-point PCR. The number of flocks sampled on day 19 was 7, on day 20 was 5, on day 22 was 8, and on day 23 was 9. By day 34, detection increased back to original levels.

A drop in the mean amount of MDV1 in PBL samples on day 21 post challenge has been observed by others, but differences between samples taken on days 14, 21, and 28 was not statistically significant (28). Judging by the standard error of the means on day 21, there appears to be a large variation in MDV1 abundance in PBL among the three sampled chickens. It would be interesting to do this analysis using a larger sample size.

Similar phenomenon has been observed for several strains of avian reoviruses, where virus titers dropped below detection during week three (52).

Both HVT and SB-1 seem to have a peak in detection around this three-week time, suggesting that they might have an effect on disappearance of MDV1 from

feather tips. HVT and SB-1 both increase the activity of natural killer (NK) cells in chickens (26, 54, 58), with this activity being highest during weeks 2-4, with a peak at week 3. Also, both viruses together had a synergistic effect on NK cell activity (26). Interestingly, the increase in NK cell activity was not observed for MDV1. Activated NK cells could target and induce apoptosis of infected and tumor cells. Also, host immune responses to MDV in feather tips have been reported (1). Therefore, it is plausible that stimulation of NK cell activity by HVT and SB-1 results in a transient disappearance of MDV1 from feather tips at approximately three weeks of age.

We speculate that NK cells attach MDV1-infected cells, an activity that may be partly responsible for the shift of the virus into latency. If this was to happen, majority of infected cells would be killed and replication of new virus would be inhibited. Once the activity of NK cells decreases after week three or four, MDV1 could reactivate, infect new cells, and be localized in feather tips again. NK cells can be activated in several ways and their targeting of MDV-infected cells and tumor cells in chicken has to vary between MDV1 and the vaccine strains since HVT and SB-1 detection remained the same during week 3. It is tempting to propose that differences in detection of HVT, SB-1, and MDV1 during week 3 are related to NK cell activity and reflect a key way that HVT and SB-1 vaccines protect chickens from MD.

Some supporting evidence for these ideas comes from studies on the JMV-1 cell line (36), which is a lymphoblastoid cell lines established from JMV (MDV1)-induced tumor. Cell-free supernatant from JMV-1 cells protects chickens from JMV (MDV) challenge, coccidiosis, and prevents REV tumor formation (35, 39). *In vitro*, it was shown that incubation of spleen cells with the JMV-1 supernatant increased NK cell activity (36). It is possible that NK activity induced by the JMV-1 supernatant

was responsible for protection of chickens from JMV-induced MD, REV tumorigenesis, and coccidiosis.

Another explanation for the decrease in detection of MDV1 in feather tips during week three is the decrease in maternal antibodies. Maternal antibodies to several avian viruses including infection bursal disease virus (IBDV) and chicken anemia virus (CAV) decrease by week 3 to the level at which infections by these viruses can take place (7, 40, 41, 53, 60). We know that the chickens studied here were, indeed, infected with IBDV (data not shown) (52). IBDV and CAV cause thymic atrophy (70), which decreases the abundance of lymphocytes that are available for the continuous infection by MDV1. The reason why MDV2 and MDV3 were not affected by this is not clear.

A controlled chicken trial could be done to study the effect of other viruses on MDV1 amounts in feather tips. In this trial, commercial eggs would be obtained and vaccinated with HVT + SB-1 just like in the commercial experiment. The chicks would be exposed to both MDV1 and IBDV challenge post-hatch. Another group of eggs would be vaccinated the same way, but chicks would be exposed to MDV1 only. MDV1 detection would be monitored in the feather tips over time to see if a similar drop in MDV1 detection takes place during week 3 in chicks exposed to IBDV challenge compared to the ones that were not.

A final explanation for why the detection of MDV1 decreased during week 3 is that MDV1 went latent at that time in the commercial chickens studied here. It was previously reported that latency takes place prior to the third week post-challenge in 3-week-old chickens that were exposed to MDV1 by inoculation (70). This experimental design was quite different compared to our study. In younger

chickens that were challenged by exposure to MDV1 shed, latency seems to take place around week 3, at which time the amounts of Meq and Meq splice-variants are very high and indicative of latency (46). Both MDV2 and MDV3 are thought to undergo latency, but it seems to be very short. This rapid latent stage might explain why the detection of MDV1 decreases at week 3, while the detection of MDV2 and MDV3 does not.

3.4.5 Performance Data Analysis

The performance data on the 30 flocks was analyzed and compared to the detection of HVT, SB-1 and MDV1 in feather tips to determine if feather tip monitoring could predict the flock performance. This proved to be difficult. While we did observe that leukosis condemnations occurred more frequently in older chickens, this is expected since older chickens have a longer time to develop tumors. One of the difficulties in assessing correlations between feather tip monitoring and flock performance was that out of the 30 commercial flocks studies, only four had leukosis condemnations. Among these four flocks, there was no consistency in production efficiency criteria such as livability %, average weight, and feed conversion. In addition, there was no consistency in relative abundances of HVT, SB-1, or MDV1 among the farms that had leukosis condemnation and those that did not. The sample collection took place during fall season, and Marek's disease is low during this time. As an alternative, samples could be collected during the times of high Marek's disease such as spring season, which would ensure that more than four flocks would have leukosis condemnation.

Thus we are left with following conclusion. We can tell that viruses are present in the feather tips of commercial chickens, determine how much virus is

present, and assess how these virus loads change over time; however, we cannot predict flock performance by feather tip monitoring.

We are left wondering about the plethora of MDV1 strains present in feather tips of vaccinated, commercial chickens in the absence of apparent MD. More work needs to be done to characterize these viruses. Finally, we are intrigued by the apparent synchrony of MDV1 field infections in vaccinated chickens, particularly with regard to the circumstances that occur at approximately week three of field infection and result in a striking but transient drop in the accumulation of MDV1 in feather tips.

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