The Avian Herpesvirus mdv1-miR-M4 microRNA is a miR-155 Analog and Increases Susceptibility of Young Chicks to *Salmonella* Enteriditis

by

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ABSTRACT

Gene regulation at the post-transcriptional level can be controlled by small segments of RNA called microRNAs (miRNAs). Previous studies have shown that Marek's disease virus (MDV) has a variety of different miRNAs in its genome. A particularly important miRNA found in the virulent serotype 1 of MDV (MDV1) is MDV1-miR-M4. This miRNA has been shown to be necessary for the cancer caused by MDV and the level of its expression correlates with virulence of different MDV1 strains. Marek's disease can be prevented by a vaccine based on a similar virus called herpesvirus of turkeys (HVT). Recombinant versions of HVT containing MDV1-miR-M4 proved to elevate viral loads in inoculated chickens compared to the parent HVT virus. This suggested that MDV1-miR-M4 may cause a reduction in immune function and enable more facile MDV infection to occur. We chose to investigate this hypothesis by studying *Salmonella* infections in chicks vaccinated with a HVT as well as with two different recombinant HVT vaccines: one based on MDV1-miR-M4 (rHVT-M4) and the second containing all of miRNAs proximal to MDV1-miR-M4 (rHVT-meqmiRs). Half of each group was treated with a commercial *Salmonella* vaccine and challenged with *Salmonella* Enteriditis (SE) after 1 week. Spleens harvested from the chickens were assayed for the presence of SE after 7 days. The chickens which were vaccinated with either rHVT-M4 or rHVT-meqmiRs proved to be more susceptible to the SE challenge than those vaccinated with the HVT parent. These studies suggest that one function of MDV1-miR-M4 is to cause the immunosuppression linked to MDV1 strains thereby contributing to MDV1 virulence.

Chapter 1

INTRODUCTION

1.1 Introduction to Marek's Disease

Poultry plays an important role in world economies and is especially important to the state of Delaware. Far more chickens than humans live on the Delmarva Peninsula. One of the biggest threats to the global poultry industry is Marek's disease (MD). In its classical form, MD interferes with nerve function. Uneven gait, lameness, and torticollis (twisted neck) are all visual indicators of the neuropathology of MD. Once the carcasses of the birds are inspected, tumors may be found in many tissues, including lungs, liver, heart, spleen, and kidney. MD is the most common clinical neoplastic condition of any organism (Payne, 1985). This disease is caused by Marek's disease virus (MDV), which is a herpesvirus. MDV and related viruses are grouped into three serotypes. Serotype 1 includes all virulent types of virus and their attenuated derivatives. Serotype 2 includes all naturally occurring non-oncogenic strains that infect chickens. Serotype 3 is a related poultry herpesvirus that is found naturally in turkeys (Hirai, 2001). MDV was the first virus known to induce cancer, and this breakthrough back in the 1960's was a landmark discovery in our understanding of oncology. This extremely infectious virus is found all over the world and is ubiquitous on poultry farms. In a now outdated analysis, MD has been estimated to cause up to $1-2 billion of annual losses to the poultry industry (Morrow, 2004). If an extremely virulent strain that cannot be controlled by the current vaccines emerges, the economic costs of MD could be catastrophic to the industry. Combined

with the fact that there is no cure for MD, the massive potential economic loss explains why control or prevention of the disease is imperative.

1.1.1 Pathogenesis

MDV is an alphaherpesvirus. Other examples of alphaherpesviruses are HSV-1 and HSV-2, which cause herpesvirus infections in humans; Varicella zoster virus, which causes chicken-pox and shingles; and Epstein-Barr virus, which causes mononucleosis. Once an alphaherpesvirus enters a host cell, its linear double-stranded DNA is replicated in the nucleus, and the viral genes are transcribed (Hirai, 2001). MDV is an atypical alphaherpesvirus in that it is lymphotrophic. It establishes latency in lymphocytes quickly. During this time, only genes in the latency activated region are expressed, and the DNA is circular.

MD is an extremely contagious disease that causes a great number of symptoms. The disease is usually spread among chickens by inhalation of feather dander present in the environment (Hirai, 2001). The virus initially targets B cells and activated T cells located in the spleen, thymus, feather follicles, and other tissues. Infection of the feather follicle cells is responsible for spread of the disease once the feathers and feather dander are shed into the environment. Eradication of the virus is practically impossible because it is stable in dust and feathers, which may remain after routine disinfection of poultry farms. In fact, dried feathers stored at 4º C contained infectious virus even after 10 years (Payne, 1985).

Within a week after inhalation of the infectious MDV, the next stage of the infection process begins when the virus enters a latency phase within activated T cells. The length of the latency period is variable and in some cases, infection never actually results in tumor formation. More typically, however, a cytolytic phase begins, which

causes abnormal development of T cells resulting in the manifestation of lymphomas starting at about three weeks post infection. Regardless of whether they develop symptoms, infected chickens will continue to deposit feather dander containing MDV into their environment (Hirai, 2001). Thus, entire flocks must be vaccinated since asymptomatic birds can be carriers and spread infections throughout the population.

1.1.2 Marek's Disease Vaccines

In 1970, the first MD vaccine was introduced and was made from herpesvirus of turkeys (HVT), which is antigenically related to MDV (Hirai, 2001). HVT does not cause tumors in chickens and is not associated with neuropathy. The lack of pathogenesis of HVT and its similarity to MDV led to its use as a vaccine in much the same way that a human smallpox vaccine was first prepared from the less virulent cow pox. Wide-scale introduction of HVT vaccines into the poultry industry resulted in a 20-fold decrease in mortality caused by MD (Payne, 1985). Nonetheless, outbreaks still occurred, and so in 1983, a bivalent vaccine was introduced. This bivalent vaccine was comprised of HVT and SB-1, a serotype 2 strain of MDV. The respite provided by the bivalent MD vaccine continued until the 1990s when a highly virulent strain of MD emerged (Hirai, 2001). This isolate was known as vv+MDV and was not controlled completely by the bivalent MD vaccine. The use of the serotype 1 vaccine CVI988 has helped with controlling vv+ MDV challenges (Hirai, 2001).

The need to address continuing evolution of MDV toward increasing virulence prompted the development of recombinant DNA (rDNA) vaccines. This line of research has led to mixed results. Gene deletions have been explored to see which genes affect immunity and which target virulence. Some labs including our own have looked at live virus vectors based on HVT (Hirai, 2001). To date, none of the newly

developed recombinant MD vaccines have outperformed the best commercially available vaccines in a variety of challenge trials.

1.2 MicroRNAs

MicroRNAs (miRNAs) were first found in *Caenorhabditis elegans* in 1993 by Victor Ambros' lab. They are approximately 21-24 nucleotide RNAs that are processed from precursor transcripts containing hairpin loops. Transcription of miRNAs by RNA polymerase produces a long primary microRNA (pri-miRNA), which is capped and polyadenylated. Processing of a pri-miRNA by the ribonuclease-like enzyme, Drosha, produces a 60-70 nucleotide RNA hairpin (pre-miRNA). Pre-miRNAs can be transported to the cytoplasm where Dicer, a second ribonuclease-like enzyme, processes them into double-stranded RNA. One strand of this miRNA provides the template for the RNA-induced silencing complex (RISC) to recognize complementary mRNA sequences. The second strand, known as the passenger strand, is usually degraded. The RISC complex lowers levels of the target protein by a number of different mechanisms depending upon the degree of complementarity of the miRNA and the mRNA. Argonaute, an endonuclease component of the RISC complex, can cleave the mRNA, or the mRNA may become less stable, or translation may be repressed. Among these mechanisms, the most common is believed to be destabilization of mRNA.

Since their discovery, much work has been done to assess miRNA functions. MiRNAs are now believed to function in organ and immune system development as well as in the promotion or suppression of cancer. One important miRNA is miR-155, which is involved in the differentiation of both B- and T- lymphocytes (Rodriguez, 2007). Viral versions of this miRNA function in the pathogenesis of MD, a

herpesvirus-induced T-cell lymphoma of chickens, and in Kaposi's sarcoma (caused by Kaposi's sarcoma herpesvirus [KSHV]), an opportunistic cancer that is particularly important in humans with Human Immunodeficiency Virus (HIV) infections (Osterreider, 2006). In fact, miRNAs from both MDV and KSHV share a seed sequence with miR-155. Seed sequences are approximately 6-7 nucleotides in length and can help identify functional miRNA homologues in different species (Appasani, 2008). A recent search of Mirbase revealed that more than 43 other miRNAs have been identified in MDVs (Erin Bernberg, personal communication). The MDV analog of miR-155 is called mdv1-miR-M4 and has been found to be necessary for tumor formation (Zhao, 2008). In solid tumors formed by a highly pathogenic strain of the virus T. King, MDV1-miR-M4 is very highly expressed, suggesting that MDV1-miR-M4 plays a causative role in tumor formation or growth (Morgan, 2008). Indeed, very recent research has confirmed the importance of mdv1-miR-M4 in the oncogenicity of MDV through analysis of a series of deletion mutants (Zhao et.al, 2011). Deletion of the miRNA cluster containing mdv1-miR-M4 resulted in a virus that did not induce tumors. Deleting only mdv1-miR-M4 or mutating a single nucleotide within its sequence also resulted in derivatives that were not oncogenic. Thus, mdv1-miR-M4 has been shown to play a key role in MD.

In MDV, the gene *meq* is closely associated with a cluster of miRNAs. The Meq protein has structural similarities to the Jun/Fos family of transcriptional activators (Anobile, 2006). Jun/Fos family members have been implicated in the progression of cancer. MDV1-miR-M4 is one of seven miRNAs that are located just upstream of *meq*. Another cluster of miRNAs is located near the latency associated

region (LAT), which is comprised of genes primarily expressed during MDV latency. This cluster consists of four miRNAs (Burnside, 2006) as shown in Figure 1.

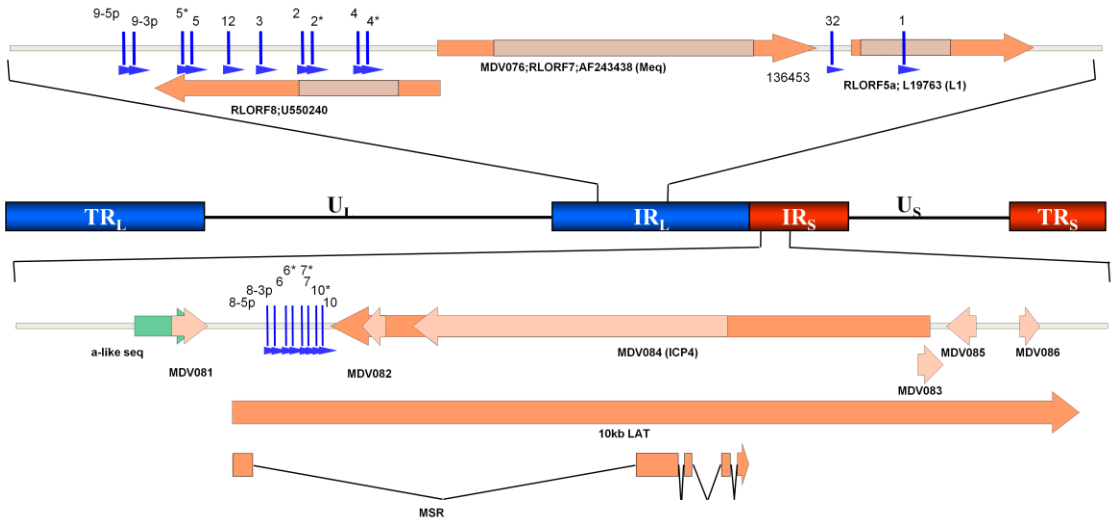

Figure 1: MicroRNA map of Marek's Disease Virus

Why do viruses encode miRNAs? Sullivan groups virus-encoded miRNAs into two classes (Kincaid and Sullivan, 2012). Some are similar in structure to host miRNAs and some are specific to the viruses. The ones that resemble those found within uninfected cells can manipulate cellular regulation and processes to support the needs of the virus regardless of the expense to the host. An important function of these miRNAs is to help the virus evade immune responses of the host. Another important function of these miRNAs is to keep the infected cell from undergoing apoptosis. Cell death will also spell the death of the virus so prolonging host survival is important to maximize production of virus. Formation of tumors is unlikely to be

something that the virus is programmed to cause, but rather one side effect of miRNA-induced inhibition of cellular apoptosis (Kincaid and Sullivan, 2012).

1.2.1 MiRNAs in the Field Of Marek's Disease Virus Vaccines

Work is ongoing in the field of MD to investigate the use of miRNAs to improve vaccination efficacy. Insertion of the mdv1-miR-M4 miRNA into the herpesvirus of turkeys (HVT) genome generated a recombinant HVT-M4 construct (Morgan, 2008). Preliminary results indicate treatment of chicks with HVT-M4 results in higher loads in spleens of re-isolatable virus post inoculation compared to the HVT parent virus. Expression of MDV1-miR-M4 may increase lymphocyte differentiation resulting in more lymphocytes to support HVT propagation, a scenario that could augment immune responsiveness to MDV (Morgan, 2008). Improved versions of vaccines are important to the future of poultry husbandry as more virulent and pathogenic versions of MDV are emerging and may limit the effectiveness of current vaccines.

Moving forward, research is focused on determining functions of the miRNAs in MD. In addition, miRNAs have been discovered in related avian viruses that infect turkeys (HVT) and ducks (duck enteritis virus or DEV) (Yao et. al., 2012). Candidate targets of these miRNAs can be predicted using target prediction software, but experimental validation has not been reported. However, as more information on the targets of MD miRNA analogs becomes available, it will be easier to identify which genes are most important for each of the various MDV miRNAs.

MiR-155 probably plays a key role in the response to *Salmonella* infections in rodents since it is required for normal immune function in mice. Transgenic mice lacking miR-155 fail to respond normally to *Salmonella* infections as well as to

Citrobacter infections (Rodriguez, 2007). Interestingly, when transgenic mice are engineered to overexpress miR-155 in B cells, high grade lymphomas result (Clare, 2013).

1.3 The Importance of *Salmonella* Infections in the Poultry Industry

Salmonella species are members of the gram negative rod enterobacteriaceae group. These bacteria can flourish within a wide range of temperatures from 35ºC to 42ºC, meaning that they can infect a wide range of organisms including poultry and humans. Thus, the implications of *Salmonella* infections in poultry cross the boundary between animal science and human health. Salmonella is not only a problem for farmers, but also for the consumers and producers of the processed poultry. Salmonellosis has been a public health problem since World War II. It causes approximately 76 million illnesses, 325,000 hospitalizations and 5000 deaths in the US alone each year. Estimating the actual costs associated with *Salmonella* infections is difficult because of the need to include effects such as lost work days, hospitalization, and medication, but a figure of nearly $800 million per year has been proposed by the USDA-FSIS (Russell, 2012). Poultry associated cases make up 10 to 20% of that figure.

1.3.1 Types of Poultry Salmonellosis

Several *Salmonella* induced diseases are important in poultry. The most common symptom of *Salmonella* infections in poultry is diarrhea (Avian Disease Manual, 2013). Some particularly important pathologies are pulloram disease, fowl typhoid, Arizonosis, and parathyphoid infection. Each of these conditions can weaken chickens and cause stunting in addition to death.

Pulloram disease, caused by *Salmonella pullorum* is transmitted through eggs, and especially affects young chicks. Although there are few clinical signs in adults, the young may appear weak with diarrhea, respiratory signs occur, and mortality can result. After extensive losses in a flock, the clearest signs of pulloram disease are stunted survivors. Losses occur quickly so post-mortem diagnosis is common although positive agglutination tests using antisera are good indicators. The most effective form of diagnosis is by isolation and identification of the *S. pulloram* bacteria. Prevention is the key to controlling pulloram disease. Losses can be prevented by rigorous cleaning and disinfection of poultry houses between flocks. Hatcheries that are compliant with the National Poultry Improvement Plan can help ensure that chicks are *Salmonella* free (Avian Disease Manual, 2013).

Another contributor to poultry deaths due to *Salmonella* is fowl typhoid. Fowl typhoid has many similarities to pullorum disease, but the causal bacterium is *Salmonella gallinarum*. The two types of *Salmonella* will cross-agglutinate. Fowl typhoid can be transmitted through the eggshell, and it has a higher incidence among adults. Mortality is significant, but other clinical signs include stunting and diarrhea. Lesions occur with a concomitant enlargement of spleen and kidneys. There is a paling of the bird, and enteritis occurs in the anterior of the small intestine. Diagnosis can be made through isolation of the bacteria from tissue samples, and control is usually through prevention and disinfection (Avian Disease Manual, 2013).

Arizonosis is an egg-transmitted disease, and outbreaks occur mainly in turkeys. The causative agent is *Samonella enterica* ser. Arizona. Embryos may be infected by carrier birds with ovarian infections. Adult birds may also be shedders, so the disease spreads laterally as well. Excessive morbidity occurs in affected flocks,

and diarrhea, ataxia, trembling, and torticollis occur as well. Poor growth is one of the most common clinical signs of Arizonosis along with blindness and central nervous system problems. Adults show fewer symptoms than young poultry. Lesions cause a yellowed, enlarged liver and turbidity in the eyes. To ensure a proper diagnosis, Arizonosis must be identified through procurement and culture of the causative agent. Control is best done by eradicating infected breeder flocks and using antibiotics. Hygienic practices in the hatchery and in grow-out operations are key to prevention of Arizonosis (Avian Disease Manual, 2013).

Paratyphoid infection can occur in a variety of birds, reptiles, and mammals including humans. The causative *Salmonella parathyphi* bacteria are intermittently shed in feces, and so these organisms can spread to anything that comes in contact with infected fecal matter. In birds, clinical signs of infection include diarrhea, shivering, and somnolence along with high morbidity and mortality. The etiological agent can be isolated from a variety of organs and used for diagnosis. Control includes minimizing exposure through cleaning, keeping eggs sanitary, not adding new birds to a brood, eliminating potential carriers, and inoculating day-old chicks with antibiotics. Prevention is paramount since paratyphoid infections are difficult to treat, and the infections are not easily eliminated (Avian Disease Manual, 2013).

1.3.2 Control of *Salmonella* in Poultry by Antibiotics

Salmonella can be controlled by the use of antibiotics, but use of antibiotics in the poultry industry has become controversial. In the U. S., in the past, standard practice has been to treat commercially produced chickens with antibiotics to kill bacteria (Bell and Kyriakides, 2002). As is true with many rapidly reproducing species, *Salmonella* have developed the ability to evade commonly used antibiotics.

Environmental activists and the popular press have argued that the overuse of antibiotics in the poultry industry may increase antibiotic resistance among strains of *Salmonella*, a situation that could negatively affect human health. Resistance can be overcome by the development of new antibiotics to a limited extent, but multiple resistant serovars have emerged, and alternative control methods would be welcomed by the industry and by the environmental movement. Many in the poultry industry are working to raise chickens in antibiotic-free conditions.

1.3.3 *Salmonella* Vaccines

The use of vaccines against *Salmonella* infection was first proposed as early as 1971 in mice, and, in that case, a live attenuated version of one strain of *Salmonella* provided protection against multiple serovars. Studies on newly hatched chicks provided similar results (Knivett and Stevens, 1971). Since that time, a great deal of research has been devoted to discovering commercial vaccines that can provide protection against *Salmonella* infections. In general, live *Salmonella* vaccines are more effective than inactivated vaccines (Russell, 2012). For example, the commercial vaccine, SALMUNE (Ceva Biomune Animal Health), contains a live *Salmonella typhimurium* bacteria which has been rendered permanently attenuated by chemical mutation (http://www.ceva.us/us/Products/Poultry, 2013). Despite the variety of approaches to vaccine preparation that have been explored in the industry, more effective vaccination strategies would be beneficial.

Eradication of *Salmonella* in the poultry industry is difficult because *Salmonella* is so pervasive in poultry production including vertical transmissions, transmissions through feed, through semen, from rodents infesting living quarters, and in processing. Both chemical and physical means are used to destroy the bacteria and

sanitize hatcheries. Dangerous biocides such as hydrogen peroxide, ozone and chlorine gas often need to be employed to ensure disinfection. Failure to control the bacteria in the flock can create conditions that lead to subsequent human infection with *Salmonella*. One of the prime places for spreading of *Salmonella* is through the scalding process in which contaminated fecal matter can be spread. Other processing steps such as defeathering and evisceration also allow *Salmonella* spread. Despite research aimed at eliminating *Salmonella* contamination in each of the steps by a plethora of methods, *Salmonella* reach the food supply more often than the industry and consumers would like (Russell, 2012).

Chapter 2

MATERIALS AND METHODS

2.1 Salmonella Strains

The *Salmonella* strains used in the study were *Salmonella* Enteritidis (SE). The nalidixic acid resistant SE strain, PT3, was originally isolated by John Rosenberger and was obtained from Rolf Joerger. This strain was used to challenge the chicks at six days of age. A nalidixic acid sensitive SE strain was obtained from Rolf Joerger as a control for plating. SALMUNE vaccine was obtained from the commercial manufacturer CEVA Bioimmune (Lenexa, KS).

2.2 Recombinant Vaccine Preparation

The constructs of rHVT-M4 and rHVT-meqmirs, which were used in the challenge experiments, were prepared by Amy Anderson (Morgan, 2013). The coding sequences for mdv1-miR-M4 and mdv1-meqmirs regions were amplified from MDV1 strain RBIB using PCR. The resulting amplicons were inserted into a specialized transfer vector called pVEC48 provided by Intervet International (Boxmeer, NL). pVEC48 contains a non-essential region US10 which can then be used to insert the mdv1-miR-M4 and mdv1-meqmirs into the HVT genome. *NotI* sites in the pVEC48 allow selective removal of non-essential vector sequences prior to transfection with HVT, and this creates a scenario wherein only recombinant HVT can replicate. The homologous HVT region from the transfer vector was then combined with HVT-Sce DNA in a double reciprocal recombination event to insert the mdv1 miRNA into

HVT. Individual plaques resulting from the calcium phosphate transfection were purified and amplified to produce large quantities of the recombinant HVT's. Northern blots probed with a ^{32}P labeled antisense mdv1-miR-M4 DNA demonstrated incorporation of the miRNAs into the recombinant HVT.

2.3 Chickens

Fertile specific pathogen-free eggs were obtained from Sunrise Farms and incubated in the Charles C. Allen Jr. Biotechnology Laboratory. Initial *in vivo* studies were done in Mountaire-Andersen glove-port isolators using BSL-3 containment conditions.

2.4 Media

Tryptic soy broth (TSB) was obtained from Difco and was produced from enzymatic digestion of casein and soybean meal. TSB provided support for the growth of bacteria. Available nitrogen sources such as amino acids and peptides provide nutrition for growth. The broth contained sodium chloride to help maintain osmotic equilibrium as well. Difco Xylose Lysine Deoxycholate (XLD) agar is a selective growth medium that can be used for the isolation of *Salmonella* species. The appearance of the XLD agar is red in color because the pH is 7.4 and a colored indicator is present in the medium. *Salmonella* colonies appear as red colonies with central black dots on this medium. XLD media contains a variety of additives which can be used to differentiate different bacterial strains. The red color for *Salmonella* derives from the fact that these bacteria can ferment the xylose in the agar which lowers the pH of the medium and makes the indicator phenol red change color to yellow. The lysine is then metabolized once the xylose is depleted which raises the

pH and reestablishes a red color. *Salmonella* metabolize thiosulfate in the medium to hydrogen sulfide, which produces the characteristic black dots in the colonies. Difco Buffered Peptone water was used to digest the cecas and spleen samples from the chickens. This diluent is buffered at high pH and contains peptone, which provides carbon, nitrogen, minerals, and vitamins, all of which are necessary for bacterial growth. Osmotic balance is maintained by the addition of sodium chloride. The peptone water sample is added to the TSB for enrichment of the samples prior to plating on XLD.

To make XLD plates both selective and differentiating, naladixic acid (NA) was used. NA is a quinolone antibacterial agent that is effective against gram-negative bacteria. NA acts by inhibiting DNA gyrase, which is part of the DNA replication machinery for the cell. Many strains of *Salmonella* are resistant to NA, as is our challenge strain for the study. Many vaccines for *Salmonella* are sensitive to NA and will not grow on media containing it. SALMUNE did not grow on XLD plates containing 50 ug of naladixic acid/ml over the first 24 hours. These plates were prepared by making a stock aqueous solution of naladixic acid at a concentration of 25 mg/ml and adding this to the media prior to plating. Small colonies from the SALMUNE begin to form after this 24-hour period. For this reason, all results in this study contain data produced within the first 24 hours after plating, and control strains were always plated.

2.5 Safety Precautions

Salmonella was handled only in special hoods, which were thoroughly decontaminated between uses using ultraviolet light and alcohol solutions. All samples were taped closed prior to transportation so that in the event of an accident

none of the material would escape containment. Incubators used were marked as containing *Salmonella* and researchers protected themselves from direct contact with any samples by means of proper personal protective equipment.

2.6 *In vivo* Protocols

Fertile eggs were placed in an incubator for 18 days before *in ovo* vaccination, and 25% were inoculated with rHVT-M4, 25% with rHVT-allmeqmiRs, 25% with HVT parent, and 25% with saline. The eggs remained in the incubator until hatching at which point half of each group was given PBS (saline solution) and half was given the CEVA SALMUNE vaccine, which was administered orally at the suggested dose. The birds were placed in isolators at the Allen Lab and fed with antibiotic-free feed in a temperature-controlled environment. Approximately 20-40 birds were placed in each isolator and sorted by group so that no cross-contamination could occur. They were then challenged at day 7 with 1.7×10^9 cfu/chick of *Salmonella* Enteritidis for the August trial and 6.9×10^8 cfu/chick for the October trial. To obtain the challenge solutions, a loopful of a frozen culture of a NA resistant strain of the bacteria was placed in 10 ml of TSB containing 50 ug/ml of nalidixic acid and left to shake overnight at 200 RPM. This solution was diluted and plated on TSA plus 50 ug/ml of nalidixic acid as well as XLD plus 50 ug/ml of nalidixic acid. Plates were incubated for 24 hours at 37º C and counted to determine the concentration of bacteria in the overnight cultures. The challenges were administered orally to the birds at the doses indicated above. The chickens were placed back into the isolators where they remained for one week. At the termination of the experiment, spleens were harvested and placed in stomacher bags filled with 4 ml of 1% Difco peptone water. Spleens were pulverized so that the tissue was homogenous. The liquid was drawn from the

opposite side of the stomacher bag with a pipette and placed in 15 cc tubes, which were kept on ice.

Spleen samples were enriched with 5 ml of TSB and grown overnight in a shaking incubator at 37º C operating at 250 RPM. A 50 ml sample was removed and plated on XLD containing 50 ug/ml of NA. NA-resistant *Salmonella* strain, NA-sensitive strain, and the SALMUNE vaccine were also enriched and incubated overnight then were concurrently plated on the same medium to serve as controls. Each of these platings was done in duplicate. The samples were scored after 24 hours of incubation. An individual chicken was scored as positive if at least one black colony denoting *Salmonella* was found on one plate. Typically multiple colonies were present. If one plate of a duplicate plating was negative, it was left in the incubator to see if it would eventually become positive. Controls of NA-resistant and NA-sensitive strains were always plated alongside experimental samples to ensure that the plates were prepared and handled properly.

Chapter 3

RESULTS

3.1 Optimizing the SE Plate Assay

XLD plates containing 50 ug/ml NA were appropriate for discriminating the NA-resistant challenge strain (PT3) from an NA-sensitive strain (Fig. 2 A and B). We found, however, that the density of bacteria on the plate affected the appearance of the colonies. When a NA-resistant strain was plated at very high density, the bacteria formed a lawn. Initially, the lawn appeared to have black colonies, but over time as the medium became depleted, the lawn appeared to be a cloudy yellow with a black outline (Fig. 2C). We found that it was important to score plates after 24 hours of incubation. More prolonged incubation times allowed the SE-sensitive strain to form microcolonies.

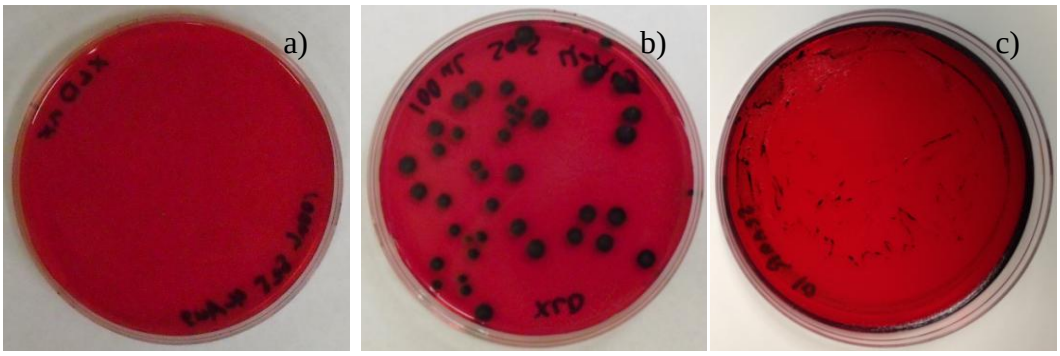

Figure 2: XLD plates containing naladixic acid plated with a) a naladixic acid sensitive strain of SE, b) a naladixic acid resistant strain of SE and c) a higher concentration of naladixic acid resistant SE.

3.2 Optimizing the *in vivo* Assay

A pilot trial was done to compare cecum and spleen as sites for *in vivo* sampling. We also compared times of sample collection from inoculated chickens as well as several challenge doses. All unchallenged chickens were negative for SE as expected. We found that among the SE-challenged chickens, all cecal samples were positive regardless of whether the samples were obtained at 7 or 14 days post challenge and regardless of the challenge dose over the range of 10^6 cfu/chick to 10^8 cfu/chick (Figure 3). Thus, use of cecal samples did not allow for the differentiation of challenge doses, which was the basis for this experiment.

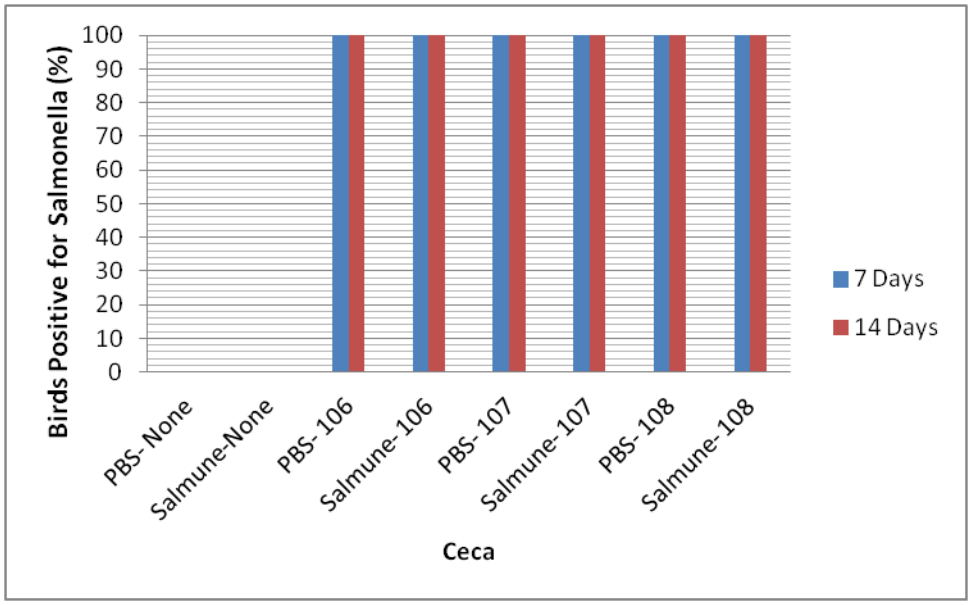

Figure 3: Percentage of birds positive for *Salmonella* in SE challenges with mock and SALMUNE using cecal extract as the substrate for plating.

Results for the spleen samples were more promising (Figure 4). As expected, spleen samples were negative for chickens that were not challenged with SE. For the SE-challenged chickens, the results depended on whether the birds were previously mock-vaccinated or vaccinated with SALMUNE. For the mock-vaccinated chickens, SE was more readily recovered if the samples were taken at 7 days post-challenge compared to 14 days post-challenge. Approximately 65% of the birds were positive among those receiving a dose of 10^6 cfu/chick. At the higher doses of 10^7 and 10^8 cfu/chick, all of the chickens were positive for SE at the 7-day sampling time. For the SALMUNE-vaccinated chickens, considerably less SE was recovered, which indicated that SALMUNE was an effective vaccine against early exposure to SE. In fact, at the 7-day sampling, none of the SALMUNE vaccinates challenged with 10^6 or 10^7 cfu/chick yielded SE in spleen samples using our assay. At the 10^8 cfu/chick challenge dose, approximately 40% of the birds had SE in their spleens at the 7-day time point. At the 14-day time point, more birds were positive for SE in their spleens with roughly 20% of the chickens receiving the 10^6 cfu/chick dose being positive and 40% of the chickens receiving the higher doses being positive. Among the SALMUNE vaccinates, we never recovered SE in more than 40% of the chickens.

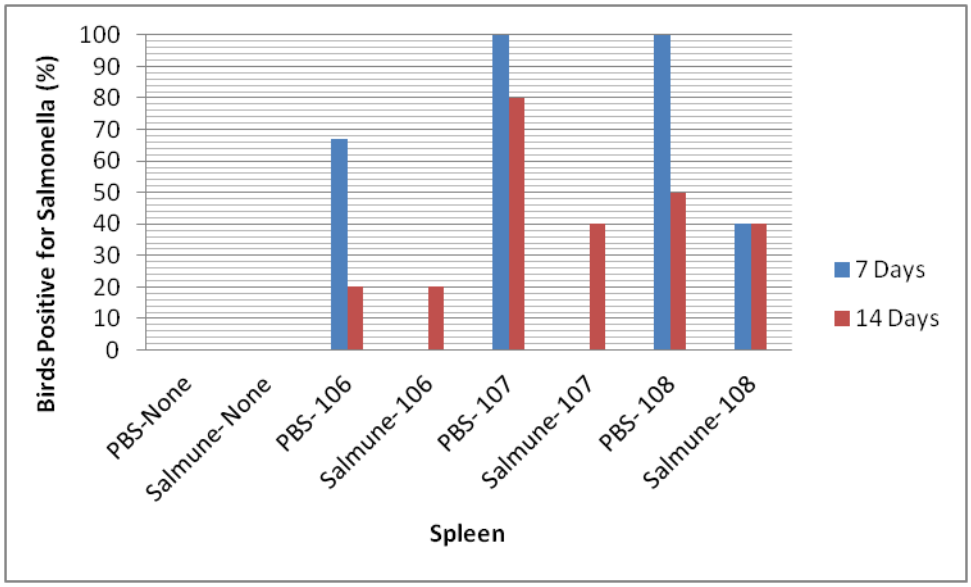

Figure 4: : Percentage of birds positive for *Salmonella* in SE challenges with mock and SALMUNE using spleen extract as the substrate for plating.

From this pilot experiment, we concluded that we would use a high dose of SE, harvest *in vivo* samples from spleens, and sample at 7 days post-challenge. In subsequent experiments, the addition of mdv1-miR-M4 may affect susceptibility to SE challenge, and therefore, it was very important to determine *in vivo* conditions that would allow us to see a range of SE susceptibilities.

3.3 Effect of MDV1 meq microRNAs on SE Susceptibility

For this experiment, embryos were vaccinated at 18 days of embryogenesis with either HVT parent, recombinant HVT containing mdv1-miR-M4 (rHVT-M4), or recombinant HVT containing all of meq microRNAs (rHVT-all). A control group of chickens was not vaccinated *in ovo*. At hatch, half of each *in ovo* group was vaccinated with SALMUNE and half was given PBS only. Five days later, chickens

were challenged with NA-resistant SE, and spleen samples were taken 7 days later. This experiment was done twice, with one difference between the experiments being the dose of SE that was used for challenge. Results from these experiments are presented in Figures 5 and 6 and in Table 1.

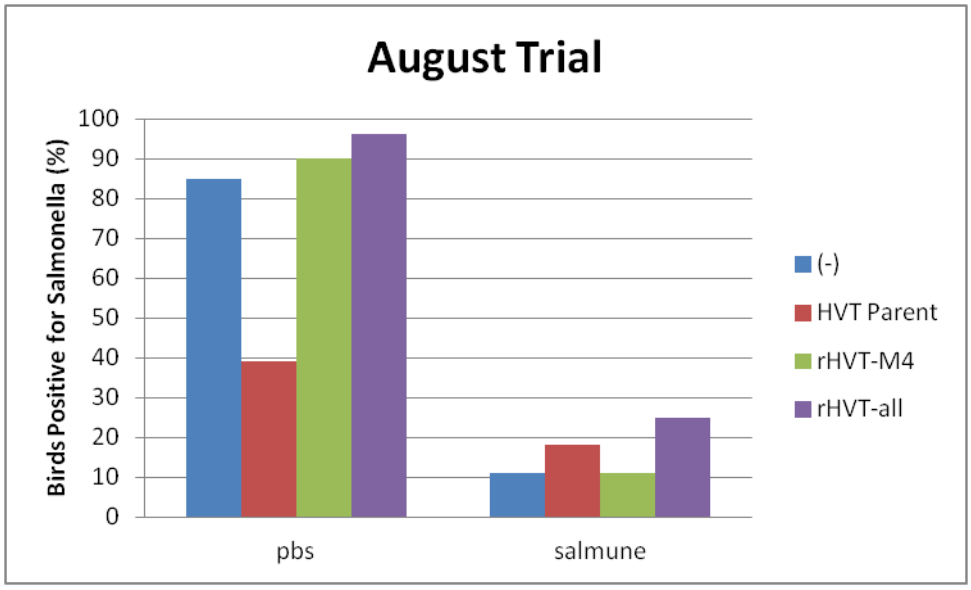

Figure 5: Percentage of birds positive for *Salmonella* in the August SE challenge trial with HVT and rHVTs using a dose 1.7 x 10^9 cfu/chick.

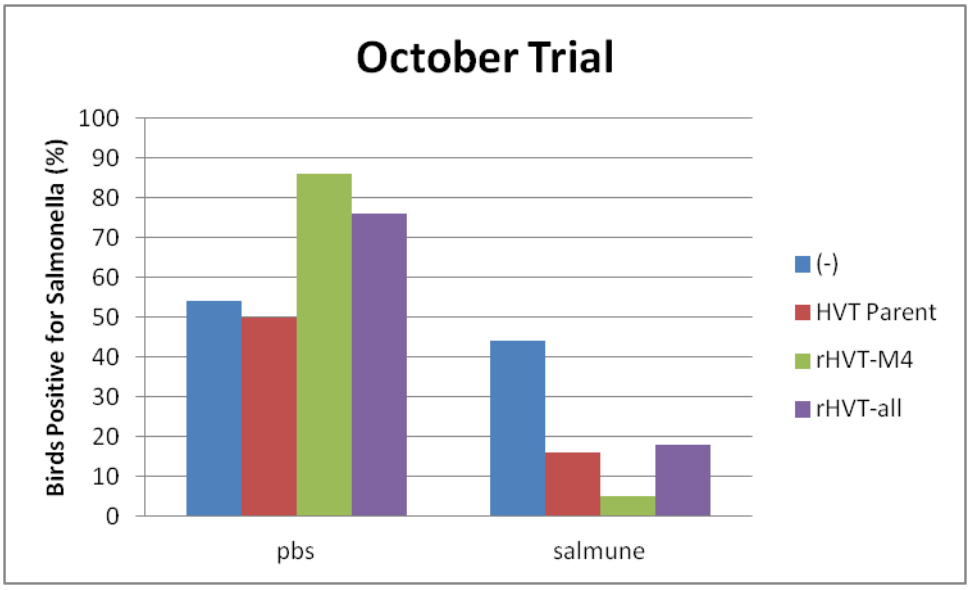

Figure 6: Percentage of birds positive for *Salmonella* in the October SE challenge trial with HVT and rHVTs using a dose with 6.9 x 10^8 cfu/chick assayed 1 week later.

Table 1: Tabular representation of *Salmonella* positive individuals from the August and October SE challenge trials with HVT and rHVTs.

	# birds positive for SE per total birds (%)			
	August		October	
Trt.	PBS	SALMUNE	PBS	SALMUNE
(-)	17/20 (85)	2/18 (11)	21/39 (54)	17/39 (44)
HVT parent	9/23 (39)	4/22 (18)	20/40 (50)	6/38 (16)
rHVT M4	17/19 (90)	2/18 (11)	36/42 (86)	2/38 (5)
rHVT all	21/22 (96)	5/20 (25)	29/38 (76)	7/38 (18)
	1.7 x 10^9 cfu/chick		6.9 x 10^8 cfu/chick	

In the higher challenge instance (August trial; challenge dose 1.7 x 10^9 cfu/chick), we found that chicks not receiving the SALMUNE vaccine were very susceptible to SE challenge in our trial. HVT alone given as an *in ovo* vaccine

provided some protection against the SE challenge at hatch. This has been observed by other investigators for a variety of challenge scenarios (Mark Parcells, personal communication; John K. Rosenberger, personal communication) and probably reflects the stimulation of innate immunity in general by HVT *in ovo* vaccination. In the August trial, about 85% of the unvaccinated chickens were susceptible to SE; whereas, about 40% of the HVT vaccinates yielded SE post-challenge. We did not observe this effect so strongly in the October trial. It was unexpected that only about 50% of the birds in the control group receiving no *in ovo* vaccination or SALMUNE vaccine were positive for SE post-challenge, and based on other experiments that we have done (including the pilot experiments shown in Fig 3), we expected to isolate SE from 80-100% of those chickens. We did use a somewhat lower dose in the October trial (6.9 x 10^8 cfu/chick versus 1.7 x 10^9 cfu/chick) and, in addition, the assay for this group was done nearly one week after the other assays because of closure of the laboratory due to Hurricane Sandy. It is possible that the extra time that these samples were stored prior to assay resulted in a lower percentage of SE recovery.

Chickens that were *in ovo* vaccinated with recombinant HVTs containing either mdv1-miR-M4 or the entire cluster of meq microRNAs were more susceptible to SE challenge than those vaccinated with the HVT parent. This increased susceptibility was also observed in the October trial. This is a very interesting result and has led us to hypothesize that mdv1-miR-M4 may be a player in immune suppression that characterizes MDV1 strains (see discussion). For SALMUNE-vaccinated chickens, we did not see significant differences among groups *in ovo* vaccinated with either the HVT parent or either of the two recombinant vaccines.

Chapter 4

DISCUSSION

MiRNAs can serve a number of functions such as regulate the post-transcriptional expression of genes. We examined the function of a subset of mdv1 miRNAs.

The miRNAs that flank the mdv1 *meq* gene include mdv1-miR-M4, which is an analog of miR-155. The special role of miRNA-155 in the immune response has been studied in mice against several different types of bacteria (Rodriguez, 2007). The decreased immune response shown by transgenic mice that lack miRNA-155 compared with normal mice towards *Salmonella* challenges is compelling evidence for this hypothesis. This argues for a role for miRNA-155 in immune protection of normal mice from bacterial infection. To clarify the role that miRNA-M4 plays in immune response, we examined the phenotype of the rHVT-M4 vaccine in chickens challenged with *Salmonella*. We recognized that a number of possible outcomes were possible since immune pathways are highly complex. On one hand, the recombinant vaccines could stimulate the immune system and provide enhanced protection against *Salmonella* infection in the chicks. On the other hand, mdv1-miR-M4 may interfere with immune responses and be a means by which serotype 1 MDV evades the immune response and causes immunosuppression.

Our experiments provided evidence that the commercial SALMUNE vaccine works because few SALMUNE-vaccinated birds were positive even with the very severe challenge dose that we used. SALMUNE administered along with HVT

provided better protection against SE than HVT alone, but worse than SALMUNE only. A possible reason for the high level of positive birds is the fact that HVT vaccination itself is rough on the immune system. With repeated vaccinations of young chickens in a short period of time, the individual may become somewhat compromised. In fact, the accompanying materials for SALMUNE actually indicate that the birds should not be exposed to stressful conditions during and after vaccination (CEVA Biomune product literature, 2012).

A significant finding is that HVT only is more effective than the recombinant HVT-M4 or HVT-all vaccines. Addition of miRNAs to the HVT vaccine decreased generalized immune protection as measured by the SE challenge model. This finding suggests that mdv1-miR-M4 may be interfering with immune responsiveness. Thus, mdv1-miR-M4 may function in immunosuppression as has long been observed for mdv1. This finding is consistent with other aspects of serotype 1 MDV strains such as the requirement of MDV1-miR-M4 for oncogenicity, its seed sequence similarity with the immune system regulating miR-155, and the fact that MDV1-miR-M4 correlates with virulence among MDV1 strains. Additionally, chickens inoculated with rHVT-M4 exhibit increased virus loads in peripheral blood compared to chickens inoculated with parent HVT. Further investigations into the merits of vaccines with miRNA constructs should be conducted. In a future study, we will compare miRNA-155 inserts directly with the miRNA-M4. We will also see if the miRNA-M4 is important through use of the allmeq-mirs without the miRNA-M4. This will help highlight the possible interplay between miRNAs and whether the response is weighted by the miRNA-M4.

In conclusion, we have shown that expression of mdv1-miR-M4 in the context of an HVT vaccine increases susceptibility of young chicks to SE. We believe mdv1-miR-M4 plays a major role in immunosuppression induced by virulent mdv1 strains. Our attempts to improve the *Salmonella* immunity by using HVT and modified HVT vaccines proved to only decrease the protection of the chickens from infection. Of significance is the finding that adding HVT reduces infection much more than using the modified rHVT-M4 or rHVT-all vaccines. This raises interesting questions about the interplay between the various miRNAs in infected cells. The results suggest that other modified HVT vaccine constructs with and without the miRNA-M4 region should be prepared and tested for their effectiveness in the SE challenge experiments. Such experiments may shed light on the interrelationships of the various miRNAs present in MDV. A direct comparison of the effect of miRNA-M4 and miRNA-155 may also be possible by incorporating miRNA-155 into the constructs. Such a study may also be worthy as a topic of future research.

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