CHARACTERIZATION OF IMMUNE RESPONSES TO AVIAN REOVIRUS VARIANTS AND EVALUATION OF AN ATTENUATION METHOD

by

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(Under the Direction of Holly Sellers)

ABSTRACT

Avian reoviruses are causative agents of tenosynovitis and viral arthritis. Historically, vaccination with live attenuated and inactivated vaccines provided protection against vertical transmission in breeders and by providing maternal antibodies to progeny. Since 2011, the incidence of tenosynovitis has increased dramatically and commercial vaccines no longer provide adequate protection against the variant viruses emerging from clinical cases. The reovirus outer-capsid attachment protein, σC, contains neutralizing epitopes and is the target for molecular characterization by genotyping. There are currently at least seven genotypes recognized and each genotype contains multiple subgroups. The commercially available vaccines make up a sub-group within genotype one. Reovirus field isolates are now commonly included in custom made inactivated vaccines, but the duration of immunity provided by the inactivated custom vaccines in the absence of an antigenically homologous live prime is unknown. We carried out three studies to evaluate antibody responses and an attenuation method to address the inadequacy of commercial vaccines to provide protection against variants. In the first investigation, the antibody response was evaluated following vaccination with a combination of commercial live attenuated/inactivated

and/or a custom-made Genotype 5 vaccine. In the absence of a heterologous or homologous live prime, the antibody responses were significantly reduced compared either group that received the commercial live vaccine. In the second investigation, a variant pathogenic field isolate was attenuated by cold passage in a cell line and evaluated for pathogenicity. The cold-adapted virus was less pathogenic in day-old broiler chicks compared to the parent virus isolate. Results from this study provide evidence that cold-adaptation of avian reovirus variants in a cell line is suitable for a more rapid method of attenuation. In the third investigation, linear B-cell epitope mapping was performed on a commercial vaccine and two variants in order to identify the molecular basis behind the antigenic differences of commercial live vaccines and variants. Fewer B-cell epitopes within σC were observed in birds that received only live or inactivated vaccinations. For both of the variant viruses, a combination of live and inactivated vaccinations resulted in the most complex B-cell response to σC. Identification of the antibody binding sites on σC advances the understanding of how variant reoviruses escape vaccine-induced protection, and this information will enhance diagnostic evaluations, vaccine design, and vaccine selection.

INDEX WORDS: Viral arthritis, tenosynovitis, avian reovirus, variant reovirus, vaccines, epitope mapping, attenuation, cold adaptation, neutralizing antibodies

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The commercial poultry industry has developed from backyard flocks and small family farms into an integrated and highly efficient industry supplying eggs and meat to a growing population. In addition to diseases, economic losses result from poor management, inadequate housing, nutritional deficiencies, stock genetic traits, natural disasters, consumer preferences for farming practices, and international trade. Diseases of poultry spread from infected flocks, human traffic between farms, insects, and wild birds. The control of classic and emerging infectious diseases in poultry has many components. Biosecurity is a management practice focused on preventing disease spread between dense livestock populations, but other methods of disease control are necessary to reduce economic losses resulting from poultry diseases. Effective disease interventions must be mass applied with ease to flocks while not offsetting the cost/benefit ratio. The control of diseases in poultry improves product quality, animal welfare, sustainability of farming practices, and profit. Veterinary medicine has evolved alongside the industry to address continuously emerging challenges to poultry health by improving management practices, genetics, nutrition, and disease interventions. Lameness in broiler chickens results in economic losses due to mortality, culling, and plant condemnations. *Mycoplasma synovia, Staphylococcus spp.,* avian *Orthoreoviruses*, other bacterial and fungal agents can cause infections associated with lameness in commercial poultry. Reovirus infections are also associated with a variety of other clinical conditions including hepatitis, myocarditis, osteoporosis, decreased hatchability, poor flock

uniformity, and increased mortality (1-4). Historically, reovirus-induced lameness has been effectively controlled by vaccination. Despite the continued use of vaccines against reovirus, there has been an increased prevalence of disease in recent years. This review focuses on avian reovirus-induced tenosynovitis/viral arthritis disease and control in commercial poultry.

LITERATURE REVIEW

Clinical disease

In addition to tenosynovitis/viral arthritis, reoviruses in chickens are associated with malabsorption syndrome (MAS) and runting/stunting syndrome (RSS) (4-6). MAS clinical signs are femoral head fractures, osteoporosis, lack of flock uniformity, diarrhea, and undigested feed in droppings. In recent years, MAS has been better characterized by the presence of combinations of microbes in affected chickens, including hemolytic *E. coli,* enterovirus, adenovirus, parvovirus, reovirus, and bacteriophages (7, 8). Enteric ARV strains have been associated with central nervous system (CNS) symptoms including torticollis and tremor (9, 10). Avian reoviruses are causative agents of tenosynovitis/viral arthritis in commercial poultry, affecting both chickens and turkeys (11-13). Clinical signs and lesions in affected birds include lameness, swollen hocks and tendons, ruptured tendons, and poor uniformity. Reovirus-induced viral arthritis/tenosynovitis causes arthritis in the hock joints and tendonitis of the gastrocnemius, digital flexor, and/or metatarsal extensor tendons (14).

ARVs that cause tenosynovitis/VA vary in pathogenicity from subclinical infection to lameness and with or without mortality (15). Birds infected at an earlier age are more susceptible to developing clinical disease (16, 17). During replication, the virus is localized in the tendons and synovial membrane of the hock joint, but may also be present in respiratory, gastrointestinal, and reproductive tissues (18, 19). In response to infection, mononuclear cells infiltrate the synovial

membranes and fluid in hock joints, resulting in cartilage erosions and arthritis (2, 14). Tendon sheaths thicken due to fibrosis and in severe cases may rupture. After the immune system has eliminated the virus, chronic inflammation leads to tenosynovitis/viral arthritis clinical signs (14, 19). Myocarditis and lymphoid cell hyperplasia in the lungs, spleen, liver, cecal tonsils, and brain can be observed in chickens following infection with arthrotropic reoviruses (14, 20). Myocarditis in turkey poults has also been linked to ARV infection (21).

Etiology

The prefix "Reo" was proposed by Sabin in 1959 during investigation of related viral agents linked to, but not directly associated with, respiratory and enteric diseases (22). Avian reovirus is a member of the *Reoviridae* family. ARV is classified within the subfamily *Spinareovirinae* and belongs to the genus *Orthoreovirus*. Avian rotavirus is also a member of the *Reoviridae* family, belonging to the subfamily *Sedoreovirinae*, and associated with enteric disease in chickens and turkeys (23, 24). ARV and mammalian reoviruses (MRV) have a similar genomic organization, acid stability, resistance to lipid solvents and trypsin, and capsid morphologies, but they differ in their limitations of host range, replication characteristics, and antigenicity (22, 25, 26). Unlike ARVs, MRVs possess hemagglutination activity, are pathogenic in newborn mice, and most are not fusogenic and thus do not produce syncytia in cell culture (27).

ARV was first identified as the causative agent of viral arthritis in 1967 based on studies of pock lesions formed on the chorioallantoic membrane (CAM) inoculated chicken embryos that revealed the particle size, structure, nucleic acid type, and replication characteristics (11). ARV was isolated in 1954 by Fahey and Crawley during a study of respiratory disease (28). The agent caused synovitis in chickens and was differentiated from synovitis-causing *Mycoplasma synoviae* due to its resistance to tetracycline (29, 30). In 1969, Kerr and Olson described the viral arthritis

pathology characterized by lesions in the synovial membrane of the hock joint and tendon sheaths (14). van der Heide demonstrated that the virus responsible for tenosynovitis was also connected with the gastrointestinal and respiratory disease, including hydropericardium, myocarditis, and hepatitis (31). In 1975, van der Heide isolated a viral arthritis agent from Connecticut, identified as S1133 based on its accession number. The reovirus isolate was vertically transmitted to broiler progeny from infected broiler-breeders, and determined to be serologically related to isolates 2937, UMI 1-203, and Reo-25 by agar gel precipitation (AGP) (32). Van der Heide characterized the isolate as a reovirus based on electron microscopy morphology, determination of nucleic acid type, resistance to solvents, low pH, and stability following incubation at 56°C. Researchers in Italy, Japan, the Netherlands, Brazil, and Australia also conducted similar investigations in the late 1960's and early to mid-1970's to characterize viral arthritis/tenosynovitis agents affecting flocks in their countries (33-36). The identification of multiple serotypes in different countries and increased frequency of reports of tenosynovitis in the 1970's supported the need to control avian reovirus infections (37, 38). Commercial vaccines were developed and historically provided good protection (39). In recent years, the emergence of avian reoviruses, antigenically and genetically distinct from commercially available vaccines, has resulted in increased incidence of tenosynovitis cases in commercial poultry (40).

Biology of avian reovirus

Reoviruses are small, non-enveloped, icosahedral, double-capsid RNA viruses that infect a wide variety of hosts. ARV particles are typically 70nm or less in diameter and share similarities with MRV (26, 41-43). ARV resists inactivation from incubation for 60 minutes at 56°C, treatment with chloroform, exposure to pH 3.0 for 30 minutes, and culture with the DNA inhibitor IUDR (32).

Viral Genome

The ARV genome consists of 10 segments of dsRNA. SDS-PAGE migration patterns of genome segments can vary among ARV strains (44). The genome consists of three large segments (L1, L2, L3), three medium segments (M1, M2, M3), and four small segments (S1, S2, S3, S4) (25). The ARV genome codes for ten structural proteins (λA, λB, λC, μA, μB, μBC, μBN, σA, σB, and σC) and four non-structural proteins (μNS, σNS, p17, and p10) (26, 45, 46). Nucleic acid hybridization analysis of different S1133 attenuation passage mutants correlates with changes in the S1, M2, and M3 genome segments with decreased pathogenicity, and changes in the S2 and S3 with cold adaptation (47). ARV genome segments are monocistronic, with the exception of the S1 gene, which is functionally tricistronic, having three out of phase open reading frames that partially overlap and code for the structural protein σC, and the non-structural proteins p10 and p17 (45).

Replication

A proposed model for the replication of ARV has been described (48). σC binding and receptor-mediated endocytosis are followed by virus uncoating and escape from the endosome (49). Replication takes place in the cytoplasm. Virus cores transcribe the segmented genome into viral mRNAs in the capsid for translation by ribosomes into structural and non-structural proteins. The viral mRNAs serve as templates for minus strands of RNA that incorporate into progeny cores formed by newly synthesized viral proteins. Outer core proteins associate with assembled inner cores, completing morphogenesis. The recruitment of viral proteins and assembly of particles takes place in globular cytoplasmic vacuoles know as virus factories (50, 51). Prior to lysis, cell-to-cell fusion occurs, forming large, multi-nucleated cells called syncytia (33).

Viral Proteins

λA, λB, μA, and σA are proteins associated with the inner core of virus, while μB, μBC, μBN, σB, and σC are associated with the outer core (26, 52). λA is coded for by the L1 gene and forms the inner core that surrounds the viral genome segments. λB is coded for by the L2 gene and is the RNA-dependent RNA polymerase component of a transcriptase complex anchored to λA (53). λC is coded for by the L3 gene and spans from the inner core to the outer capsid (52). λC pentamers have a turret morphology extending from the core's five-fold axis and shares a similar function with the mammalian reovirus λ3 protein (41, 54). λC has autoguanylation and methylase activity that caps the 5' ends of viral mRNAs after transcription (52, 55).

μA is coded for by the M1 gene, part of the inner core transcriptase complex, and is believed to be an N-6 adenine-specific DNA methylase (56). The M2 gene segment codes for μB, a protein that is cleaved by an unidentified protease into the amino-terminal peptide μBN and carboxy-terminal protein μBC (52). μB, μBN, and μBC are structural outer-capsid proteins. μBC is involved with reovirus uncoating in the lysosome and release of core particles into the cytoplasm (57). M3 codes for the non-structural protein μNS and is a viral factory recruitment factor for the σNS and λA proteins (58, 59). Phylogenetic analysis of the M1, M2, and M3 genes suggests genome segment reassortment among co-circulating avian reoviruses (56). Reassortment can result in altered fitness, potentially affecting host range, pathogenicity, and virulence (60). While some regions of ARV M genes are conserved in MRV, there are dissimilarities in the size of these genes and the proteins they encode (61).

The S1 gene is tricistronic and codes for protein p10 from open reading frame one, p17 from open reading frame two, and σC from open reading frame three (45). Translation of the S1 gene is regulated by leaky scanning and atypical ribosome shunting mechanisms (62, 63). σC is a

minor outer capsid protein that exists as a homotrimer, binds to the host cell, and stimulates the production of neutralizing antibodies (26, 52, 64). Residues 151-326 form the C-terminal globular head of σC and the receptor-binding domain that has a similar topology to MRV σ1 (65, 66). The host cell receptor for ARV σC is unknown. Junctional adhesion molecule A (JAM-A) is the cellular receptor of the similar MRV minor outer capsid attachment protein σ1 (67). Genome reassortment among co-circulating ARV strains and evolution through the accumulation of point-mutations in the S1 gene variable regions confer enhanced pathogenicity and escape from vaccine-induced antibody neutralization (68-72).

ARV modulates host cell plasma membrane permeability responsible for cell-to-cell fusion during late replication via the p10 viroporin (73). p10 is a fusion-associated small transmembrane (FAST) protein that regulates syncytia formation and apoptosis (74). Localization of p10 in the host cell plasma membrane forms multimers in cholesterol-dependent membrane microdomains, resulting in fusion platforms (75). Degradation of p10 by ubiquitination and interaction with cellular lysosome-associated membrane protein 1 (LAMP-1) inhibits apoptosis of infected cells (74).

Conservation of cellular resources needed for viral replication and the prevention of apoptosis are associated with p17, a shuttling protein found in the cytoplasm and nucleus of infected cells (76). p17 modulates the cell cycle through interactions with cycle regulatory proteins (77). p17 activates cellular tumor suppressor protein p53, a transcription inducer that regulates the expression of proteins related to cell cycle arrest and apoptosis. (78). Cyclin-dependent kinase (CDK) inhibitor p21 is also activated by p17 and regulates products of p53 induction related to cycle arrest. Because p53 mutations are frequent in human cancers, investigation of reovirus p17 cell cycle modulatory mechanisms benefits the development and understanding of cancer

treatments (79). Additionally, p17 activates cellular autophagy and autolysosome formation that supports improved virus replication (80, 81).

σA is coded for by the S2 gene and is a sequence-independent dsRNA binding protein (82). σA blocks the dsRNA-dependent protein kinase PKR enzyme pathway from being activated and is linked to reovirus resistance to the cellular anti-viral interferon response induced by a caspase-independent mechanism (83-85).

σB is a major outer capsid protein coded for by the S3 gene segment and associates with μB and μBC in the cytosol prior to forming a complex that later coats virus cores (86). σB has also been suggested to function as an inflammatory gene expression modulator, but further investigation is needed to confirm these findings (87). σB is immunogenic, but does not elicit significant levels of neutralizing antibodies (88). A linear B-cell epitope for σB was previously identified, but has not been shown to be associated protection in regard to vaccination (89). The S4 gene segment codes for the non-structural protein σNS (90). σNS is localized in viral factories in association with μNS and binds ssRNA (58, 91). σNS mediates interactions between pre-genomic viral RNAs as segment assortment and packaging into viral cores takes place (92)

Immune response

Humoral immunity provides protection from clinical disease (93). σC elicits type-specific virus neutralizing-antibodies following live virus infection or vaccination (94). Inactivated vaccines with an adjuvant induce σC group-specific antibodies that can react with heterologous ARVs. Type-specific immunity protects against development of clinical signs to a greater extent than group-specific immunity (95). Broiler chicks are more susceptible to infection resulting in clinical disease, highlighting the importance of breeder vaccination (16). The transfer of maternal antibodies from immunized broiler-breeders protects broiler chicks from clinical disease when

challenged orally at one day of age (96). The half-life of reovirus vaccine maternal antibodies in broiler chicks is approximately five days post-hatch (97). Cellular immunity induced by live vaccination at an early age can protect broilers, but this method of vaccination is not common practice in the United States (98).

Recovery from infection with ARV relies on cell-mediated and humoral responses (93). A study conducted by Pertile *et al.* profiled lymphocyte populations in perivascular and superficial regions of the tarsal joint synovium in chickens experimentally infected with avian reovirus (99). Low counts of CD8+ T-cells observed between two and six days post infection (acute phase) are followed by increased counts of CD4+ and CD8+ T-cells at eight to fourteen days post infection (sub-acute phase). During the sub-acute phase, IgM+ B-cells and plasma cells surround T-cell aggregates in the perivascular region of the tarsal joint and CD8+ T-cells are found on articular cartilage surfaces. During the chronic phase, there are increasing amounts of T-cells and plasma cells in the tarsal joint perivascular and superficial regions, with lymphoid aggregates containing CD4+ T-cells in the perivascular region. Infiltrates of lymphocytes and macrophages form nodules and synovial membranes develop villous processes during the progression of disease (14, 100).

ARV has an immunosuppressive potential characterized by bursal atrophy, splenomegaly, depressed humoral response to infection with other viruses, and inhibition of viral oncogenesis due to Marek's disease herpesvirus infection (101-105). Additionally, vaccination against ARV can alter the immune response following Marek's disease vaccination and reduce protection. (106). Reovirus and rotavirus evade antiviral interferon responses by localizing or degrading interferon regulatory transcription factors, preventing downstream innate responses from destroying infected cells (107, 108).

Epidemiology

The association of ARV with infectious tenosynovitis began in the mid- 1950's when Fahey and Crawley isolated and characterized an agent from birds with respiratory disease (28). In the early 1960's, 8.2% of broiler condemnations in Maine were attributed to synovitis that did not correlate with *Mycoplasma synoviae* serology (109). Between the late 1960's and mid-1970's similar reports of viral arthritis/tenosynovitis in Italy, the Netherlands, Brazil, and Australia were made (31, 33-35, 110). Widespread sero-prevalence was observed in Italy and Canada (111, 112). Wood et al. suggested in a 1980 study that there were at least 11 serotypes based on isolates obtained from the U.S., Germany, Britain, and Japan (38).

Avian reoviruses are ubiquitous in poultry and can be isolated from healthy flocks (15). The majority of ARV research has been focused on disease in meat-type chickens. ARV is also associated with disease in commercial layer chickens and turkeys (113-115). Reoviruses isolated from turkeys have the potential to cause disease in chickens (12, 116). Additionally, avian reoviruses can be isolated from a variety of wild birds (117, 118).

ARV is transmitted horizontally and vertically. Horizontal transmission occurs through the fecal-to-oral route (119). Contact with infected birds, post-hatch exposure, or contact with contaminates in the poultry house can facilitate transmission (14, 120). Vertical transmission from infected broiler-breeders to progeny occurs when infected hens shed virus in the oviduct during egg production (1, 32).

Research in the 1970's provided serologic and pathologic characterization of the virus and disease (2). Maternal antibodies transferred to progeny chicks provided protection against oral challenge with the S1133 isolate in one-day-old birds (96). Focus on the development of a commercial reovirus vaccine lead to the development of inactivated and live-attenuated vaccines

for use in broiler-breeder vaccination programs (96, 121). Commercial avian reovirus vaccines became available in 1978 and provided good protection against disease in the U.S. for many years prior to the emergence of variant, antigenically unrelated viruses (37, 122). Despite the use of commercially available vaccines, there has been an increasing incidence of viral arthritis/tenosynovitis in chickens and turkeys in many countries (40, 123-126) from 2010 to present.

Diagnosis

ARV is a primary rule-out for flocks exhibiting lameness, reluctance to move and swollen hocks/tendons. Diagnostic evaluation is important for confirmation of the causative agent (127). Sampling of legs, hearts, intestines, and livers from suspect flocks should include birds that display clinical signs and birds that appear normal. Virus isolation from synovial fluid of the hock joint, tendon, liver, heart, and intestine can be carried out in chicken embryo liver cells (CELiC), chicken hepatocellular carcinoma cells (LMH), chicken embryo kidney cells (CEK), African green monkey kidney cells (VERO), and specific pathogen free (SPF) embryos (128-131). CELiC or LMH cells are preferred over other cell types (125, 132). Cytopathic effect (CPE) characterized by syncytia in cell culture and pock-like lesions, mortality, and hemorrhage in SPF embryos inoculated via the chorioallantoic membrane (CAM) are diagnostic features of ARV (133). Several serial passages may be necessary in these systems to achieve virus titers high enough for down-stream diagnostic evaluations (125). Following laboratory isolation of ARV, it may be beneficial to study the pathogenicity of isolates to confirm their role in the clinical disease observed in the originating flock (70). Pathogenicity studies are performed by footpad, oral, and subcutaneous inoculations with ARV field isolates (134).

Reverse transcriptase polymerase chain reaction (RT-PCR) is routinely utilized to amplify the region of the S1 gene encoding for Sigma C. Genetic characterization of reovirus isolates is performed by sequencing the RT-PCR product and phylogenetic analysis (69, 71). Phylogenetic comparison of isolates and grouping into genotype clusters is useful for the comparison of isolates to each other and vaccine strains (135). Six genotypes have been reported based on neighbor-joining phylogenetic analysis (125). A seventh genotype was recently identified from clinical case submissions at the University of Georgia Poultry Diagnostic and Research Center (PDRC) (personal communication). Because anti-σC antibodies neutralize ARV, the sequence of an isolate is useful for evaluating vaccination programs (122, 123). The timing of specimen collection from affected flocks is crucial because ARV may not be detectable in the tissues of lame birds if the infection has cleared.

ARV enzyme-linked immunosorbent assay (ELISA) is a commonly used test to measure reovirus antibodies. ELISA titers in breeder flocks can indicate response to vaccination or infection (136). Commercially available ELISA kits contain plates coated with whole virus and do not differentiate serotype-specific antibodies (137). Two-way cross virus neutralizations (VN) evaluate the serological relatedness of ARV strains (15, 38, 113, 133). Serum from vaccinated flocks can be evaluated using reference viruses and new isolates can be used to generate ARV-immune serum for serologic evaluation (138). The agar-gel-precipitin (AGP) and fluorescent antibody (FA) tests detect group-specific anti-ARV antibodies, but do not provide information about type-specific neutralizing antibodies (19, 96, 101, 139, 140).

Prevention and control

Reovirus vaccination of broiler-breeders provides maternal antibodies to progeny chicks and can confer protection against early challenge in the field (16, 96). In 1976, the 73rd CAM

passage of S1133 was used to vaccinate broiler-breeders at a company where there was a high prevalence of tenosynovitis in broilers, and exhibited promising protective effects (37, 141). Further attenuation of the S1133 strain resulted in the development of a non-pathogenic vaccine safe for young broiler-breeders (106). Attenuation was carried out by passaging the S1133 isolate 235 times in SPF embryos, followed by 65 passages in chicken embryo fibroblast (CEF) cells at 32C, and an additional 35 passages in CEF cells at 37C. Commercially available S1133 vaccines vary in attenuation and suitable application age (Table 1). A naturally non-pathogenic ARV strain derivative, designated 2177, is also used as an early-age vaccine (15). Commercially available inactivated vaccines are derived from the above strains and the 1733, 2408, SS412, and MSB isolates (15). Routinely, broiler-breeders are given numerous live vaccinations early in life, followed by multiple inactivated oil-emulsion vaccinations prior to the onset of egg production (126). Vaccination is most effective when the challenge virus and vaccine are antigenically homologous (39). Repeated injections of inactivated vaccine can reduce the specificity of neutralizing antibodies and allow for limited protection against heterologous strains (142). Commercially available live attenuated reovirus vaccines are limited and contain reovirus strains S1133 or 2177. Inactivated reovirus vaccines contain one of several reoviruses and may also include Infectious Bursal Disease Virus, Newcastle Disease Virus, and Infectious Bronchitis Virus antigens (Table 1.1).

Vaccination programs vary between companies, and in recent years, have commonly included autogenous inactivated vaccines in an attempt to protect against challenge with antigenic variants (20, 123, 143). σC characterization aids in the selection and consideration of ARV isolates for inclusion in autogenous vaccines (125). Vaccination programs combining antigenically heterologous strains may not elicit an adequate secondary immune response due to dissimilarities

in the neutralizing epitopes of σC recognized (122). Failure to achieve a strong secondary response results in the lack of long-lived B-cell responses required for long term transfer of protective maternal antibodies to progeny broilers. Regulations for the use of limited-license autogenous inactivated vaccines in the U.S. are outlined in the Code of Federal Regulations, Title 9 Section 113.113. The efficacy of live reovirus vaccines is tested by foot pad inoculation with a challenge virus three to four weeks post vaccination and observation for clinical signs (144). Challenge studies in broilers are also utilized to demonstrate vaccine efficacy (96, 122).

An experimental inactivated vaccine composed of representatives from four different genotypes of ARV protected against challenge with pathogenic viruses from the genotypes included in the vaccine (145). The likelihood of common epitopes in this vaccine may contribute to a robust immune response. Antigenic predictions and characterization of the conserved and variable σC epitopes among a variety of isolates and vaccine strains enhances the understanding of the relationship between genotype and serotype, providing the framework for enhanced characterization of clinical isolates and consideration of the peptides needed for inclusion in vaccines (123). A σC fragment consisting of amino acids 122-326 from the S1133 virus is highly immunogenic for the production of neutralizing antibodies and priming for a secondary vaccination, and comparable to vaccination with whole virus (146). The full length recombinant σC is weakly immunogenic compared to the 122-326 fragment and may be due to differences in protein folding, trimerization, or the presence of immunosuppressive elements (146, 147). In 2010, Goldenberg *et al.* reported the successful use of antigenic prediction tools, bioinformatics, and structural analysis methods that lead to the identification and laboratory validation of the S1133 GT1 vaccine outer capsid σC epitope ^{254}THIPSDL^{260} (148). In another study published in 2013, Yin *et al.* used a pepscan-mAb epitope mapping approach and identified the S1133 inner

capsid σC linear B-cell epitope ^{45}ELLHRSISDI^{54} (149). The identification of relevant σC B-cell epitopes responsible for a protective immune response refines important diagnostic targets for characterization of variant field isolates and development of effective vaccines (89, 149).

Alternative methods for the production and delivery of expressed σC show potential for reducing costs of vaccination associated with manufacturing and application. The expression of σC in yeast can protect against challenge when used as an oral vaccine (150). σC has also been expressed in transgenic plants, but vaccine efficacy has not been evaluated in this system (151). Modern techniques for the design and evaluation of vaccines, combined with better understanding of the epidemiology of ARV, will lead to more rapid, cost effective, and customizable ARV vaccines.

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TABLE

Table 1.1. Live and inactivated avian reovirus vaccines sold in the United States.

Company	Live/Inactivated Virus	Vaccine	Minimum Age of Administration	Adjuvant	Route of Administration	Strains
CEVA	Inactivated	CMV[A]		Water in oil emulsion	IM[B]	Field Isolate(s)
CEVA	Inactivated	Reomune 3	18-week-old chickens	Water in oil emulsion	SC[C]	Reo: S1133, Standard 2408, SS412
CEVA	Inactivated	Maximune 6	18-week-old chickens	Water in oil emulsion	SC	IBDV[F]: Standard, Delaware variant A, Delaware variant E Reo: S1133, Standard 2408, SS412
CEVA	Inactivated	Maximune 8	18-week-old chickens	Water in oil emulsion	SC	IBDV: Standard, Delaware variant A, Delaware variant E NDV[G]: B1 LaSota IBV[H]: Mass Holland 52, Mass 41 Reo: S1133, Standard 2408, SS412
Merck	Live	2177 Tenosynovitis Vaccine	Day of age or older		SC	Reo: 2177, natural, non-pathogenic strain
Merck	Live	Enterovax	Day of age by spray or 7-day-old by drinking water		SP[D]/DW[E]	Modified live S1133
Merck	Live	Teno-Vaxin	10 to 17 weeks of age		DW	Modified live S1133
Merck	Inactivated	Bursamate-Plus	18-week-old chickens	Water in oil emulsion	IM	IBDV: Standard, Delaware variant E, GLS, and AL-2 variants Reo: 1733 and 2408
Merck	Inactivated	Breedervac-Reo-Plus	3-week-old chickens	Water in oil emulsion	IM or SC	IBDV: Standard, Delaware variant A, Delaware variant E, and GLS variants Reo: 1733 and 2408
Merck	Inactivated	Breedervac-IV-Plus	3-week-old chickens	Water in oil emulsion	IM or SC	IBDV: Standard, Delaware variant A, Delaware variant E, and GLS variants NDV: Newcastle disease virus IBV: Massachusetts Reo: 1733 and 2408
Boehringer Ingelheim	Live	Tenosynovitis (frozen)	Day of age or older		SC	Reo: Modified live S1133
Boehringer Ingelheim	Live	Reoguard L (freeze-dried)	2-week-old chickens		DW	Reo: Modified live S1133
Boehringer Ingelheim	Inactivated	Bursa Guard Reo	12-week-old chickens		SC or IM	IBDV: Standard and Delaware variant E Reo: S1133, 2408, and MSB
Boehringer Ingelheim	Inactivated	Bursa Guard N-B-R	12-week-old chickens		IM	IBDV: Standard and Delaware variant E NDV: LaSota IBV: Mass and Ark Reo: S1133, 2408, and MSB

[A]Limited license, custom made vaccine
[B]Intramuscular injection
[C]Subcutaneous injection
[D]Spray vaccination
[E]Drinking water vaccination
[F]Infectious Bursal Disease Virus
[G]Newcastle Disease Virus
[H]Infectious Bronchitis Virus

CHAPTER 2

EVALUATION OF IMMUNITY IN SPF BIRDS VACCINATED WITH REOVIRUS S1133 AND GENOTYPE 5 AUTOGENOUS REOVIRUS VACCINES[A]

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SUMMARY

In recent years, there has been an increase in the prevalence of variant reoviruses isolated from clinical cases of viral arthritis/tenosynovitis in broilers from reovirus-vaccinated broiler breeder chickens. Many poultry companies elect to utilize variant reovirus field isolates in inactivated custom-made vaccines (CMV). This investigation was carried out to evaluate immunity in SPF birds vaccinated with commercial live and inactivated S1133 and a CMV containing a genotype 5 (GT5) reovirus field isolate. As expected, homologous live and inactivated vaccines elicit a higher antibody response compared to heterologous combinations as measured by ELISA and virus neutralizations. In addition, birds vaccinated with S1133 live followed by the GT5 CMV demonstrated a higher level of GT5-specific neutralizing antibody response compared to birds receiving only the inactivated S1133 and GT5 vaccines. Neutralizing antibodies were detected 12 weeks following the final vaccination, but were declining by the end of the study.

Key Words: Avian reovirus, tenosynovitis, viral arthritis, autogenous vaccine, serology.

Abbreviations: Avian reovirus (ARV), Double-stranded RNA (dsRNA), and genotype (GT).

INTRODUCTION

Avian reoviruses (ARV) can be isolated from the tendons of clinically affected chickens. ARVs belong to the family *Reoviridae,* genus *Orthoreovirus*. ARV is a non-enveloped virus composed of three large, three medium, and four small segments of double-stranded RNA (dsRNA) (1). The S1 genome segment is tricistronic with three partially overlapping reading frames (2). The minor outer-capsid protein σC is responsible for host cell attachment and elicits neutralizing antibody responses (3). The third open reading frame of the S1 segment is the most variable segment and codes for the reovirus outer capsid protein σC, making it a target for diagnostic evaluations (3, 4). Molecular characterization based on RT-PCR of the σC coding region of S1 can be used to

differentiate reoviruses into genotypes (5, 6). Phylogenetic analysis of σC currently classifies ARVs into seven different genotypes, with commercial vaccines only representing a sub-group within genotype 1 (7, 8).

Reovirus ELISA is commonly used to monitor vaccination and challenge antibody responses, but does not differentiate antigenic variants from vaccine strains (9, 10). Two-way cross virus neutralization tests can be used to characterize antigenic variants by serotype, but this method is resource-intensive due to the need to generate reference hyper-immune serum following the isolation of a variant (11, 12). Virus neutralizations are also used to evaluate type-specific neutralizing antibody responses in settings where vaccination programs include custom made vaccines (CMVs).

ARVs are horizontally and vertically transmitted (13, 14). Younger birds are more susceptible to developing clinical disease, and protection by vaccination relies on the passive transfer of neutralizing maternal antibodies from broiler-breeders to progeny broilers (15, 16). ARVs are associated with malabsorption syndrome and runting/stunting syndrome in chickens and have been identified as the causative agent of viral arthritis/tenosynovitis in commercial poultry causing economic losses in both chickens and turkeys (13, 14, 17). Clinical signs of ARV include poor flock uniformity, ruptured tendons, hydropericardium, myocarditis, and increased mortality (18). ARV can cause arthritis in the hock joints and tendonitis of the gastrocnemius, digital flexor, and/or metatarsal extensor tendons (19). ARVs are ubiquitous in commercial poultry and can be isolated from the gastrointestinal tract of healthy birds, with pathogenicity ranging from subclinical infection to lameness and mortality (12).

In recent years, the incidence of reovirus-induced tenosynovitis has increased dramatically, and commercial vaccines do not provide adequate protection against emerging antigenic variants

isolated from clinical cases of disease (5, 6, 20-23). Long-lasting humoral immunity in breeder flocks is required for the transfer of maternal antibodies to progeny (24, 25). Historically, vaccination against ARV has been effective, and utilized a combination of live and inactivated commercial vaccines. This method is commonly referred to as prime and boost vaccination, where birds are first vaccinated with a live-attenuated vaccine and then later given one or more inactivated vaccines prior to the onset of lay, resulting in increased B-cell responses. Many broiler companies utilize limited-license, inactivated CMVs produced with reoviruses isolated from flocks on their premises. Regulations for the use of limited-license CMVs in the U.S. are outlined in 9 CFR § 113.113. The duration of immunity following vaccination with antigenically dissimilar live and inactivated vaccines is not well understood. In this study, the reovirus antibody response was monitored in SPF chickens following vaccination with a combination of commercial S1133 live attenuated/inactivated and a bivalent genotype 5 (GT5) CMV.

MATERIALS AND METHODS

Chickens. White leghorn chickens were hatched from specific-pathogen-free embryos (Charles River Laboratories, North Franklin, CT) and housed in separate isolation rooms on pine shaving floors. 15 day-of-hatch chicks were placed in each of the three isolation rooms. The air supply to the bird rooms was HEPA filtered. Water and feed were provided *ad libitum* for the duration of the experiment. The animals were maintained and reared following good animal husbandry practices. All animal protocols were approved by the University of Georgia Institutional Animal Care and Use Committee (IACUC AUP A2013-09-022-Y1-A0).

Vaccines. The following commercial vaccines were used in this study: VA Chick Vac (Fort Dodge/Zoetis Parsippany-Troy Hills, NJ), an S1133 modified live vaccine, administered subcutaneously at full dose and an inactivated classic 4-way vaccine containing avian reovirus,

infectious bursal disease virus, Newcastle disease virus, and infectious bronchitis virus (AviPro® 442 ND-IB2-BTO2-Reo, Lohmann Animal Health) administered intramuscularly at full dose. A bivalent genotype 5 CMV was used to vaccinate birds intramuscularly in the breast at the full label dosage of 0.5ml.

Experimental design. A total of 45 birds were equally divided among three experimental groups to evaluate the classic and variant GT5 reovirus antibody responses, as well as the duration of those responses: 1) An inactivated 4-way (IBD, NDV, IBV, Reo S1133) plus autogenous GT5 at 12 weeks-of-age then GT5 at 18 weeks-of-age; 2) S1133 modified live vaccine at day-of-hatch, then repeated at 3 and 6 weeks-of-age, followed by an inactivated 4-way and GT5 at 12 weeks-of-age, followed by an additional GT5 vaccine at 18 weeks and 3) S1133 modified live vaccine at day-of-hatch, again at 3 and 6 weeks-of-age, followed by an inactivated 4-way plus GT5 at 16 weeks-of-age (Table 2.1).

Reovirus ELISA. Birds were bled at 3, 6, 10, and 12 weeks-of age, and then every two weeks until the 30 weeks-of-age endpoint of the study. Individual serum samples were evaluated by reovirus ELISA (IDEXX Laboratories, Inc., Westbrook, ME USA) and S1133 and GT5 virus neutralization (VN) assays *in vitro*. Reo ELISA and VN graphs were generated with GraphPad Prism (version 8.2.0 for Windows, GraphPad Software, La Jolla California USA).

Virus neutralization assays. VNs were performed by the beta-method previously described, with a few modifications (11). S1133 positive control serum was purchased from Charles River SPAFAS. Reovirus 94826 GT5 positive control serum was generated in SPF chickens using plaque purified, live virus administered by eye drop followed by inactivated virus given intramuscularly. Serum was heat-inactivated at 56°C for 45 minutes and 50µl of each individual serum sample was serially diluted in 50µl of 100 $TCID_{50}$/0.1ml chicken embryo liver cells (CELiC)

of the S1133 virus or the 94826 GT5 reference virus strain. Briefly, 2-fold serial dilutions of serum were made in 100 $TCID_{50}$/0.1ml CELiC of the S1133 virus or the 94826 Genotype 5 reference virus strain in 96-well plates and allowed to incubate at 37°C for 1 hour. Titrations of virus stocks and back-titrations of viruses diluted for VNs were performed in CELiC and fifty-percent endpoints were calculated by the Reed-Muench method (26). The VN titer was recorded as the reciprocal of the last dilution where cytopathic effect (CPE) was observed after 5 days of incubation. VN geometric mean titers (GMTs) were calculated for each treatment group at each time-point. The positive threshold for neutralizing antibodies was 5 Log_2 GMT.

RESULTS

Reovirus ELISA. At 18 week-of-age, four weeks after receiving the first inactivated commercial and custom made vaccines, birds in treatment group 1 were positive by reovirus ELISA, identified by GMTs > 400, and remained positive through 30 weeks-of-age (Fig. 2.1). The peak reovirus antibody response measured by ELISA for treatment group 1 was a GMT of 1060 at 22 weeks-of-age. Birds in treatment group 2 were positive by ELISA beginning at 16 weeks-of-age, and remained positive through 30 weeks-of-age. The peak reovirus antibody response measured by ELISA for treatment group 2 was a GMT of 7488 at 18 weeks-of-age. Birds in treatment group 3 were positive beginning at 18 weeks-of-age, and remained positive through 30 weeks-of-age, with a peak GMT of 3782 at 20 weeks-of-age.

Virus neutralizations. S1133 virus-neutralizing antibodies were first detected at 16 weeks-of-age in treatment group 1, having a 3 Log_2 GMT at this point in time (Fig. 2.2). S1133 virus-neutralizing antibodies remained detectable through 30 weeks-of-age in treatment group 1, but were only above the positive threshold of 5 Log_2 GMT at 24 weeks-of-age, having a titer of 5.3 Log_2 GMT at this time point. In treatment group 2, S1133 virus-neutralizing antibodies were first

detected at 3 weeks-of-age, having a titer of 1.4 Log_2 GMT at this time point. Treatment group 2 S1133 titers rose above 5 Log_2 GMT at 16 weeks-of-age, having a titer of 8.5 Log_2 GMT at this time point, and remained above 5 Log_2 GMT through 30 weeks-of-age. Treatment group 3 birds S1133 virus-neutralizing antibodies rose above 5 Log_2 GMT at 18 weeks-of-age, with a titer of 8.3 Log_2 GMT at this point in time, and remained above 5 Log_2 GMT through 30 weeks-of-age.

Treatment group 1 had GT5 virus-neutralizing antibodies titers of 5 Log_2 GMT or greater at 20, 22, 24, 26, and 28 weeks-of-age (Fig. 2.3). GT5 virus-neutralizing antibodies peaked at 22 weeks-of-age in treatment group 1, having a titer of 6.7 Log_2 GMT, but were below 5 Log_2 GMT at 30 weeks-of-age. Birds were positive for GT5 virus-neutralizing antibodies at 16 weeks-of-age and remained above 5 Log_2 GMT through 30 weeks-of-age. The treatment group 2 peak GT5 virus-neutralizing antibody response was at 22 weeks-of-age, with an 8.6 Log_2 GMT at this time-point. Treatment group 3 had GT5 virus-neutralizing antibodies above 5 Log_2 GMT at 18 weeks-of-age and remained positive through 30 weeks-of-age. The treatment group 3 GT5 virus-neutralizing antibody response peaked with an 8.3 Log_2 GMT at 24 weeks-of-age.

DISCUSSION

Historically, the protection of broilers against disease caused by ARVs has been reliant on the immunization of breeders using a combination commercial live and inactivated vaccines (15). ARV maternal antibodies titers in day-old broiler chicks are approximately one-third of the antibody titer in parent breeders, and have a half-life of 5 days, which is sufficient to provide protection during the time chicks are most susceptible to ARV (24, 25). In recent years, CMVs have been used to protect birds from challenge with variant reoviruses (22). These CMVs are typically applied after breeders have been vaccinated with a series of commercially available, antigenically dissimilar, classic live vaccines. The neutralizing antibody responses against

variants, following heterologous combinations of classic live and custom-made inactivated ARV vaccines, are not well understood. In this study, the duration of immunity for classic and variant reoviruses was evaluated following vaccination with only inactivated classic and custom vaccines, or a combination of live and inactivated vaccines in SPF layers.

Previous work demonstrated the onset of protective immunity against homologous challenge in broilers 3 weeks after vaccination of 45-week-old breeders (27). While this study did not evaluate protection by challenge of progeny, increases in virus neutralizing titers for S1133 and GT5 neutralizing antibodies were observed 2 to 4 weeks following inactivated commercial/GT5 CMV vaccinations prior to the onset of lay. In the present study, birds that received the live attenuated vaccine and inactivated commercial/GT5 CMV at 12 and 18 weeks had S1133 and GT5 neutralizing antibody titers that peaked two weeks before the birds that also received the live vaccine, but received only one inactivated vaccination at 16 weeks. This finding suggests that earlier protection against challenge or vertical transmission may be conferred by vaccinating broiler-breeders earlier in life, but may also result in an immune response that wanes earlier over time.

The neutralizing antibody response against S1133 was greater than the GT5 neutralizing antibody response, highlighting the benefit of using a homologous combination of live and inactivated vaccines over a heterologous combination. The neutralizing antibody response against GT5 was higher in birds that received live and inactivated vaccines compared to birds that received only inactivated vaccines. Consistent with the above finding, ELISA antibody titers following inactivated commercial/GT5 CMV vaccinations were higher in birds that were administered live vaccinations, compared to birds that did not receive any live vaccinations. To provide better

neutralizing antibody responses to variant strains represented in custom made inactivated vaccines, the development of antigenically relevant live attenuated vaccines may be beneficial.

For birds in treatment groups two and three, which received live and inactivated commercial/GT5 CMV vaccinations, the total antibody response started to decline in both groups at 26 weeks, while neutralizing antibody responses started to decline at 28 weeks of age. In this study, there was little difference in neutralizing antibody levels between birds that received 1 or 2 inactivated vaccines following the application of a series of live attenuated vaccination. While the timing and number of CMV vaccinations did not extend the presumptive protective level of GT5 neutralizing antibodies beyond 30 weeks, the data collected in this trial suggests there is some benefit to using heterologous vaccine combinations; however, homologous vaccination results in higher antibody titers. Commercial vaccination programs often utilize less-attenuated S1133 vaccine strains administered several times through the drinking water to pullets to elicit a more protective antibody response compared to that achieved by the use of highly attenuated S1133 vaccines (27-29).

Vaccination programs vary widely among companies. The duration of neutralizing antibody titers in the field needs to be further evaluated to better understand reovirus immunity. To evaluate protection in broilers against variant ARV viruses, the progeny of vaccinated broiler-breeders need to be challenged with the pathogenic variant(s) from which the CMV was derived. An extended field progeny challenge study could achieve this, and may better reflect the duration of immunity and efficacy provided by autogenous vaccine use throughout the period of lay.

In this study, the use of a commercial live vaccine resulted in the development of neutralizing antibodies even when a heterologous inactivated vaccination followed. Even though the duration of immunity was less than what was achieved by a homologous live and inactivated

vaccination, compared to birds that did not receive the live vaccine, the levels of GT5 neutralizing antibodies were higher in birds that were given live attenuated commercial vaccinations prior to immunization with inactivated commercial/GT5 CMV products. The development of a novel generation of commercial live-attenuated ARV vaccines based on contemporary strains will improve the duration of immunity, resulting in a reduction in vertical transmission, and an increase in the repertoire of maternal antibodies transferred to progeny that will reduce clinical disease in the field.

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TABLE

Table 2.1. Treatment groups and schedule of vaccinations.

Group[A]	Age at time of vaccination and vaccine(s) administered					
	Day-of-hatch	Week 3	Week 6	Week 12	Week 16	Week 18
1	None	None	None	CI[B] + CMV	None	CMV[C]
2	S1133 Live[A]	S1133 Live	S1133 Live	CI + CMV	None	CMV
3	S1133 Live	S1133 Live	S1133 Live	None	CI + CMV	None

[A]S1133 live-attenuated Va Chick Vac vaccine (Fort Dodge/Zoetis Parsippany-Troy Hills, NJ) administered as a full dose, subcutaneously in the back of the neck (S1133 live).

[B]Inactivated classic 4-way vaccine containing avian reovirus, infectious bursal disease virus, Newcastle disease virus, and infectious bronchitis virus (AviPro® 442 ND-IB2-BTO2-Reo, Lohmann Animal Health) administered intramuscularly in the breast at full dose (CI).

[C]Inactivated custom made vaccine (CMV) containing genotype 5, administered intramuscularly in the breast at full dose.

FIGURES

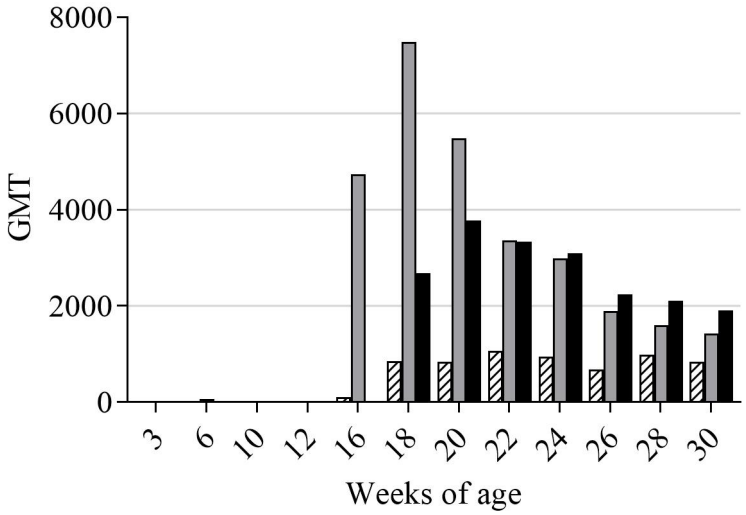

Fig. 2.1. Reovirus antibody responses measured by reovirus ELISA. Treatment group one received inactivated commercial and CMV vaccinations at 12 weeks-of-age, followed by an additional CMV vaccination at 18 weeks-of-age, and GMTs (geometric mean titers) are represented by striped bars. Treatment group two received S1133 live vaccinations at day-of-age, 3 weeks, and six weeks, followed by inactivated commercial and CMV vaccinations at 12 weeks-of-age, followed by an additional CMV vaccination at 18 weeks-of-age, and GMTs are represented by grey bars. Treatment group three received S1133 live vaccinations at day-of-age, 3 weeks, and six weeks, followed by inactivated commercial and CMV vaccinations at 16 weeks, and GMTs are represented by black bars. Reo ELISA GMTs at or above 400 are considered positive.

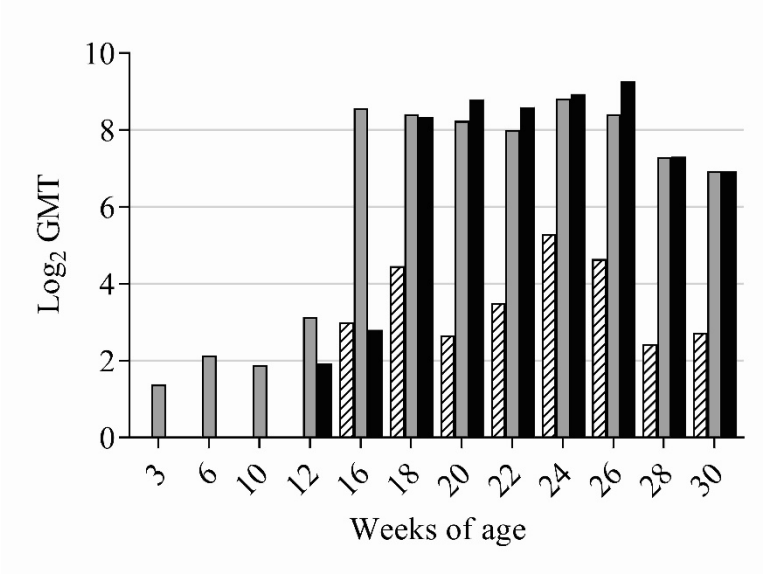

Fig. 2.2. Virus neutralization titers for reovirus S1133 represented as Log_2 GMT. Treatment group one received inactivated commercial and CMV vaccinations at 12 weeks-of-age, followed by an additional CMV vaccination at 18 weeks-of-age, and GMTs represented by striped bars. Treatment group two received S1133 live vaccinations at day-of-age, 3 weeks, and six weeks, followed by inactivated commercial and CMV vaccinations at 12 weeks-of-age, followed by an additional CMV vaccination at 18 weeks-of-age, and GMTs represented by grey bars. Treatment group three received S1133 live vaccinations at day-of-age, 3 weeks, and six weeks, followed by inactivated commercial and CMV vaccinations at 16 weeks, and GMTs represented by black bars. S1133 Log_2 GMTs at or above 5 Log_2 are considered positive.

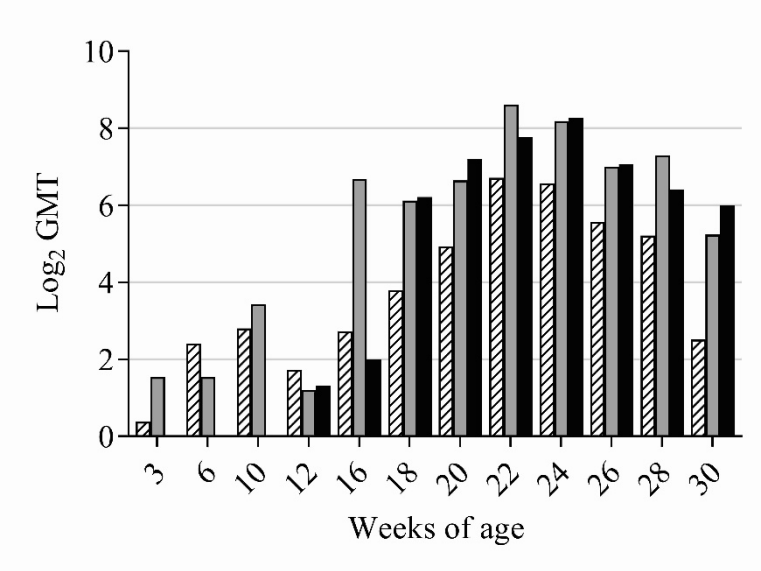

Fig. 2.3. Virus neutralization titers for reovirus genotype 5 represented as Log_2 GMT. Treatment group one received inactivated commercial and CMV vaccinations at 12 weeks-of-age, followed by an additional CMV vaccination at 18 weeks-of-age, and GMTs represented by striped bars. Treatment group two received S1133 live vaccinations at day-of-age, 3 weeks, and six weeks, followed by inactivated commercial and CMV vaccinations at 12 weeks-of-age, followed by an additional CMV vaccination at 18 weeks-of-age, and GMTs represented by grey bars. Treatment group three received S1133 live vaccinations at day-of-age, 3 weeks, and six weeks, followed by inactivated commercial and CMV vaccinations at 16 weeks, and GMTs represented by black bars. GT5 VN GMTs at or above 5 Log_2 are considered positive.

CHAPTER 3

COLD ADAPTATION OF AN AVIAN REOVIRUS GENOTYPE ONE VARIANT AND *IN VIVO* PATHOGENICITY STUDY IN BROILERS[A]

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SUMMARY

Avian reoviruses are causative agents of tenosynovitis and viral arthritis in chickens and turkeys. Control against disease caused by avian reoviruses in the U.S. is focused on the vaccination of broiler-breeder chicken flocks to provide maternal antibodies to progeny chicks that can confer protection against early challenge in the field. In recent years, variant reoviruses have been isolated from vaccinated flocks with clinical cases of disease, indicating the need for new commercially available vaccines. Historically, the commercial S1133 modified-live vaccines were attenuated by passaging the S1133 isolate 235 times in SPF embryos, followed by 65 passages in chicken embryo fibroblast (CEF) cells at 32°C, and an additional 35 passages in CEF cells at 37°C. Continuous passage of the virus in embryos and cell culture for attenuation was effective, but required years of passaging to produce safe, highly attenuated vaccine strains. In the current study, a variant avian reovirus isolate was cold-passaged to evaluate the utility for a more rapid attenuation of variant reovirus isolates. The variant reovirus isolate was serially passed 42 times in the LMH cell line at 32°C. The replication dynamics of the cold passaged virus were evaluated *in vitro* and enhanced replication at 32°C was observed compared to the original isolate. The cold-adapted virus was less pathogenic in day-old broiler chicks compared to the parent virus isolate. Results from this study provide evidence that cold-adaptation of avian reovirus variants in a cell line is suitable for a more rapid method of attenuation.

Key words: avian reovirus, attenuation, tenosynovitis, variant

Abbreviations: ARV = avian reovirus; CEF = chicken embryo fibroblast cells; CFR = code of federal regulations; CMV = custom made vaccine; dsRNA = double-stranded RNA LMH = chicken hepatocellular carcinoma cells; RT-PCR = reverse transcriptase polymerase chain reaction; SPF = specific pathogen free

INTRODUCTION

Avian reoviruses (ARVs) are ubiquitous in poultry and can be isolated from the gut in healthy chickens. Reovirus infections are also associated with a variety of other clinical conditions including hepatitis, myocarditis, osteoporosis, decreased hatchability, and increased mortality. However, ARVs are also causative agents of tenosynovitis/viral arthritis in commercial poultry, affecting both chickens and turkeys (1-3). Reovirus-induced viral arthritis/tenosynovitis causes arthritis in the hock joints and tendonitis of the gastrocnemius, digital flexor, and/or metatarsal extensor tendons (4). Clinical signs and lesions in affected broilers include swollen tendons, bruised hocks as a result of ruptured tendons, lameness, and poor flock uniformity. Economic losses from clinical disease are due to mortality, culling, and processing plant condemnations.

ARVs belong to the genus *Orthoreovirus*. The ARV is a non-enveloped virus and its genome consists of 10 segments of dsRNA coding for 10 structural and 4 non-structural proteins (5, 6). The S1 gene segment is tricistronic and the third open reading frame codes for the viral outer-capsid attachment protein, σC (7). σC binds to the host cell and stimulates the production of neutralizing antibodies (8, 9).

Reassortment of viral segments as well as point mutations in the variable regions of the S1 gene contribute to the evolution of reoviruses and emergence of variant viruses (10). Genetic characterization of ARV isolates is performed by amplification of the σC encoding region of the S1 gene by RT-PCR followed by sequencing and analysis (11). Currently, 7 genotypes have been identified, with the commercially available vaccines grouped together within a subgroup of Genotype 1 (12).

Historically, tenosynovitis and viral arthritis have been controlled by reovirus vaccination using a combination of modified-live and inactivated vaccines. Broiler-breeders routinely receive

several modified-live vaccines followed by one or several inactivated oil-emulsion vaccines prior to the onset of egg production (13). The use of reovirus vaccines in breeder flocks is intended to provide protection against disease in the breeders, eliminate vertical transmission, and provide protective maternal antibodies to progeny broilers (14, 15). Currently in the U.S., commercial reovirus live and inactivated vaccines belong to the same serotype (and genotype) and are antigenically different from field viruses isolated from clinical cases of tenosynovitis in recent years. In an effort to provide protection against emerging pathogenic variant ARVs, many poultry companies in the U.S. have elected to use inactivated custom-made vaccines (CMVs). Regulations for the use of limited-license CMVs in the U.S. are outlined in the 9 Code of Federal Regulations (CFR) § 113.113.

Protection is optimized when antibody responses are specific to field challenge viruses and the duration of immunity is sufficient to protect against disease in breeders and progeny before breeder flocks start egg production. A more robust and longer lived antibody response is achieved when live vaccinations are followed by homologous inactivated vaccinations (16). The development of new commercial live-attenuated vaccines is postulated to improve the antibody response to inactivated CMVs (17). The attenuated S1133 classic modified-live vaccines were derived from 235 serial passages in specific pathogen free (SPF) chicken embryos, followed by 65 passages in chicken embryo fibroblast (CEF) cells at 32°C, and an additional 35 CEF passages at 37°C (18). Passaging at reduced temperatures produced a highly-attenuated temperature sensitive mutant that replicated well at reduced temperatures, but had limited replication at avian host temperatures (19). This method was utilized to produce numerous S1133 vaccine seed stocks ranging from high to moderate levels of attenuation and immunogenicity.

Due to a need for new, antigenically relevant vaccines, cold adaptation of a variant reovirus isolate was evaluated in an avian cell line.

MATERIALS AND METHODS

Viruses. A variant reovirus, identified as CK/GA/117816 Tendon/2017, was isolated from the tendons of a flock of commercial broilers with ruptured tendons and increased condemnations at the processing plant. The genotyping performed on the isolate revealed a σC amino acid sequence that was 79.4% similar to the S1133 vaccine and belonged to a distinct subgroup within Genotype 1. Two-way cross neutralization tests revealed that the 117816 variant and the classic S1133 vaccine are serologically unrelated (20).

Cold adaptation and replication characteristics. The 117816 isolate was expanded in chicken embryo liver cells (CELiC) and identified as Li3. Li3 was passaged 42 times in a chicken hepatocellular carcinoma cell line (LMH) (ATCC® CRL-2117™) incubated at 32°C with 5% CO_2 and is designated as P42. For each passage, culture plates were observed for cytopathic effect, frozen and thawed three times, and the lysate was clarified by centrifugation for 10 minutes at 1500 RPM at 4°C. Media was removed from preformed monolayers of LMH cells in 35mm culture plates, then plates were inoculated with 0.2ml of clarified supernatant and incubated at 32°C for 1 hour. Following the 1-hour incubation, 2ml of Waymouth's medium (Gibco® MB752/1) was added to the plates. Each passage was incubated for 3 to 4 days before harvesting. LMH cells were maintained in Waymouth's medium with 10% fetal bovine serum at 37°C as previously described (21).

Replication characteristics of Li3 and P42 were evaluated by serial passage and titrations in LMH cells at 32°C, 37°C, 39°C, and 41°C. LMH cells were cultured in 35mm plates, and confluent monolayers were inoculated with an MOI of 0.01 of each virus, incubated for 1 hour at

each temperature then, maintenance media was added, and the plates were returned to either 32°C, 37°C, 39°C, or 41°C for 72 hours. Plates were frozen and thawed three times and the lysate was clarified by centrifugation for 10 minutes at 1500 RPM at 4°C. Clarified supernatant was then titrated by performing 10-fold serial dilutions in 96-well plates and transferred to LMH cell monolayers. Following incubation for 96 hours, the dilution endpoints of cytopathic effect in the plates were marked, and $TCID_{50}$ titers were calculated by the Reed and Muench method (22). The P42 virus was passed three times at 39°C to evaluate reversion to phenotype.

RT-PCR, sequencing and analysis. A molecular analysis of Li3 and P42 was performed to evaluate if the identity of σC was altered during passaging. RNA was extracted from clarified cell culture lysates using a RNeasy® kit (Qiagen®, Hilden, Germany). Using methods previously described, the σC encoding region of the S1 gene was amplified by RT-PCR using the S1 gene P1 and P4 primers (23). RT-PCR products were purified using a QIAquick® gel extraction kit (Qiagen®, Hilden, Germany). Purified DNA was submitted to Eurofins® Genomics (Louisville, KY) for Sanger sequencing. The Lasergene® software package (version 14 for Windows, DNASTAR®, Inc, Madison, WI) was used for contig assembly to generate consensus sequences and perform ClustalW multiple alignments of nucleotide and protein sequences.

Evaluation of pathogenicity in chickens. An *in vivo* study was performed in day-of-hatch broiler chicks to evaluate the pathogenicity of the Li3 and P42 viruses. Commercial broiler chickens were sourced from an in-house research flock lacking antibodies to reovirus and reared in pens on fresh pine shavings in isolation houses. Day-of-hatch chicks were bled for reovirus serology and reovirus ELISA (IDEXX®, Westbrook, ME) results confirmed the absence of reovirus antibodies. At placement, chicks were neck tagged with unique identification numbers. Water and feed were provided *ad libitum* for the duration of the experiment. The birds were maintained and reared

following good animal husbandry practices. The animal experiment was approved by the University of Georgia Institutional Animal Care and Use Committee (IACUC AUP A2016 11-011-R1).

Experimental design. Ninety healthy day-of-hatch chicks were randomly divided into 6 treatment groups of 15 birds each. Body weights were recorded and chicks were inoculated by footpad or oral routes with either P42, Li3 (positive control group), or PBS (negative control group) (Table 3.1). Groups of birds inoculated in the footpads were injected with either 10^4TCID_{50}/0.1ml virus (50ul per footpad) or phosphate buffered saline (PBS). Groups of birds inoculated via the oral route were administered 100µl of 10^4TCID_{50}/0.1ml virus or PBS.

Birds were monitored daily for clinical signs and tendon and footpad swelling. Measurements of body weights, digital flexor tendons, and footpads were taken every 7 days from days 7 through 28. Tendons and hearts were harvested on day 28 for histopathology. Tendons were scored microscopically based on a previously described method where the tendon score is the sum of the synovium, subsynovium, and additional features (1). Briefly, synovium scores where 0 = normal single squamous epithelium, 1 = single layer of hypertrophied synoviocytes, 2 = 2 to 4 layers of hyperplastic synoviocytes, and 3 = more than 4 layers of hyperplastic lymphocytes and subsynovium scores where 0 = less than 10 lymphocytes, 1 = 10 to 50 lymphocytes, 2 = 50 to 100 lymphocytes, and 3 = too numerous to count. Subsynovial lymphoid follicles, fibroplasia, and dilated blood vessels were additional features that were each given a score of 1 if present.

Statistical analysis. Analysis of the data and generation of figures was performed using GraphPad Prism® software (version 8.2.0 for Windows, GraphPad Software, La Jolla California USA). Multiple one-way ANOVAs were used to evaluate body weight suppression, tendon swelling, and

footpad swelling differences between treatment groups. The Mann-Whitney test was used to evaluate significance of median histologic tendon score differences between treatment groups. The level of significance was $P<0.05$.

RESULTS

Replication characteristics. The *in vitro* replication characteristics of the Li3 virus were altered following 42 passages in LMH cells at 32°C (Fig. 3.1). The cold passaged P42 virus replicated to the highest titer ($10^{4.3}$TCID$_{50}$/1ml LMH) when propagated at 32°. The titer of the P42 virus was reduced between 1.5 to 2 $\log_{10}$ at 37°C ($10^{2.7}$TCID$_{50}$/1ml LMH) and 39°C (10^{2}TCID$_{50}$/1ml LMH). Following three passages in LMH cells at 39°C, the cold passaged virus failed to produce a cytopathic effect, indicating temperature-sensitivity.

RT-PCR, sequencing and analysis. The 117816 σC nucleotide sequence identity after 42 passages at 32°C in LMH cells was identical to Li3.

Pathogenicity study. Clinical signs were not observed in the negative control PBS oral and footpad inoculated groups during the course of the study. At day-of-age, mean body weights were not significantly different between any treatment groups (Fig 3.2A). Day 7 body weights were significantly suppressed in the Li3 footpad inoculated group compared to all other groups, with a 19.4% mean reduction in body weight in the Li3 footpad inoculated group compared to the PBS footpad inoculated group (Fig. 3.2B). Mean body weights on days 14, 21, and 28 were significantly lower in the Li3 oral inoculated and Li3 footpad inoculated groups compared to all other groups (Fig. 3.2 C, D, E). Significant body weight suppression was observed in the Li3 oral inoculated group compared to the PBS oral and footpad inoculated groups on days 14, 21, and 28, and there was a respective 4.1%, 4.8%, and 5.9% reduction in mean body weight.

In the Li3 footpad inoculated group there was significant body weight suppression compared to the PBS oral and footpad inoculated groups on days 7, 14, 21, and 28, with a respective 19%, 17.7%, 16.1%, and 10.8% reduction in mean body weights.

Significant digital flexor tendon and footpad swelling was observed in birds challenged by footpad with Li3 at days 7, 14, 21, and 28, compared to the PBS footpad inoculated group (Fig. 3.3 and Fig. 3.4). Birds orally challenged with Li3 had significant digital flexor tendon swelling at day 28, with a 7% increase in swelling compared to PBS orally challenged birds (Fig. 3.3D). No significant tendon swelling was observed in any of the P42 inoculated birds compared to the PBS negative controls at any time point during this study. There was significant footpad swelling in the orally inoculated Li3 group on day 21 compared to the orally inoculated PBS group (Fig. 3.4C). No significant footpad swelling was observed in the P42 inoculated birds compared to the PBS negative control birds at any time point during this study, irrespective of route of challenge.

On day 28 in the P42 oral and footpad groups, swollen hocks were observed in several birds (Table 3.2). Additional and more severe clinical signs were observed in both of the Li3 groups. Additional clinical signs in the Li3 oral group include visible tendon swelling (1/17 birds) and hydropericardium (1/17 birds). The Li3 footpad group also had visible tendon swelling (1/16 birds). Inflamed hocks were present in the P42 oral (4/17 birds) and footpad (1/16 birds) groups, but were more severe in the Li3 oral (3/17 birds) and footpad (6/16 birds) groups.

Microscopic heart lesions characteristic of mild epicarditis and mild myocarditis were observed in the P42 oral (3/10 birds) and footpad (3/10 birds) treatment groups, but were observed in more birds in the Li3 oral (8/10 birds) and footpad (10/10 birds) groups, which had chronic lymphocytic epicarditis, and chronic lymphocytic myocarditis (Table 3.3). There were no lesions observed in hearts from the PBS oral and footpad groups. The P42 oral and footpad groups both

had mild tenosynovitis, but only the P42 footpad group had significantly higher microscopic tendon scores compared to the negative controls. The Li3 oral and footpad groups had severe and chronic lymphocytic tenosynovitis and significantly higher microscopic tendon scores than all other groups (Fig. 3.5 and Fig. 3.6). The Li3 footpad group had significantly higher tendon scores than the PBS treatment groups, but not significantly different from the Li3 oral inoculated group.

DISCUSSION

Compared to Li3, the P42 cold passaged derivative exhibited reduced *in vitro* replication at 37°C and 39°C, suggesting decreased replication fitness at avian host temperatures. This observation is supported by a previous study comparing replication characteristics of the pathogenic S1133 virus and the attenuated S1133 P100 vaccine strain that was passaged at 32°C during the course of attenuation. (24). The lack of genetic changes in the σC coding region of the S1 genome segment following cold passaging suggest stability of the P42 virus following cold passaging, providing support that this method may be suitable for attenuation.

In the present study, microscopic evidence of reovirus infection was identified in tendons from birds inoculated with the P42 virus, but was significantly reduced compared to birds inoculated with the Li3 virus. Past studies with S1133 observed a similar effect, wherein the highly attenuated S1133 P100 vaccine strain produced microscopic lesions in younger birds when administered subcutaneously or by footpad injection, but the pathogenicity was significantly reduced compared to the pathogenic S1133 parent virus (25, 26). Furthermore, birds orally inoculated with P42 exhibited fewer microscopic tendon lesions compared to birds that were orally inoculated with Li3 or footpad inoculated with P42. This finding suggests P42 may be more suitable for younger birds when administered via drinking water and warrants further investigation.

Pathogenicity was evaluated by inoculating birds by the oral and footpad routes as both routes of inoculation have been used in previous challenge studies. The footpad route of inoculation is the method outlined for efficacy testing in the Title 9 CFR § 113.332 (27, 28). Day-old chicks were used for initial evaluation of pathogenicity of Li3 and P42, but evaluation of the pathogenicity of these viruses in older birds would provide information about the potential utility of P42 as a vaccine in older birds. The P42 virus has not been evaluated in older birds or tested for safety, immunogenicity, or efficacy, so these are the next logical steps in evaluating P42 (29). An *in vivo* back-passage study to evaluate reversion to virulence would also aid in further evaluating the phenotypic stability and safety of P42. The historical shortcoming of temperature-adapted and traditional live-attenuated vaccines is the time it takes to reach attenuation by passaging in laboratory systems, a process that can take years (30). Attenuation by cold passaging in a cell line aims to shorten the turnaround time between isolation of a variant and the production of a live-attenuated vaccine to protect against antigenically related and clinically relevant ARVs.

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TABLES

Table 3.1. Treatment groups.

Treatment group number	Inoculum	Challenge route
1	Li3[A]	Oral
2	Li3	Footpad
3	P42[B]	Oral
4	P42	Footpad
5	PBS	Oral
6	PBS	Footpad

[A]Original 117816 isolate (Li3).

[B]Cold-passaged 117816 virus (P42).

Table 3.2. Day 28 Clinical signs.

	Frequency of clinical signs at day 28				
Treatment Group	Inflamed Hocks	Bilateral Hemorrhaging	Visible Tendon Swelling	Hydro-pericardium	Abdominal Bruising
PBS Oral	0/17	0/17	0/17	0/17	0/17
PBS Footpad	0/16	0/16	0/16	0/16	0/16
P42 Oral	4/17	0/17	0/17	0/17	0/17
P42 Footpad	1/16	2/16	0/16	0/16	0/16
Li3 Oral	3/17	2/17	1/17	1/17	0/17
Li3 Footpad	6/16	2/16	1/16	0/16	1/16

Table 3.3. Pathogenicity study microscopic heart lesions.

Treatment group number	Inoculum/challenge route	Number of hearts with lesions[A]
1	Li3 oral	8/10
2	Li3 footpad	10/10
3	P42 oral	3/10
4	P42 footpad	3/10
5	PBS 0ral	0/10
6	PBS footpad	0/10

[A]Ten hearts from each treatment group were collected at necropsy on day 28 for histopathology.

FIGURES

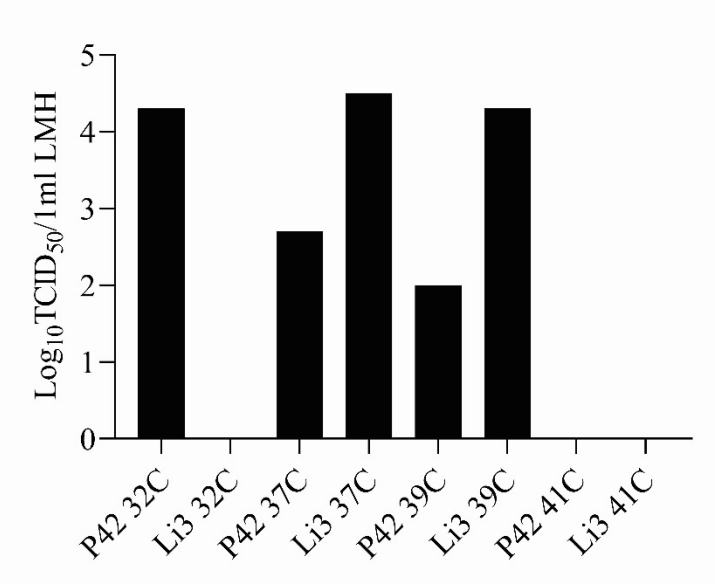

Fig. 3.1. Culture and titration of the P42 and Li3 viruses at different temperatures. Titers of the P42 and Li3 viruses after dilution to 10^2TCID$_{50}$/0.1ml and culture at 32°C, 37°C, 39°C, and 41°C are plotted on the y-axis. Titrations were performed on pre-formed monolayers of LMH cells and were incubated at the corresponding culture temperature for 96 hours. Viruses, propagation temperatures, and titration temperatures are listed on the x-axis. Log10TCID50/1ml LMH Reed-Muench titers are on the y-axis. Bars represent the titers for the P42 and Li4 viruses.

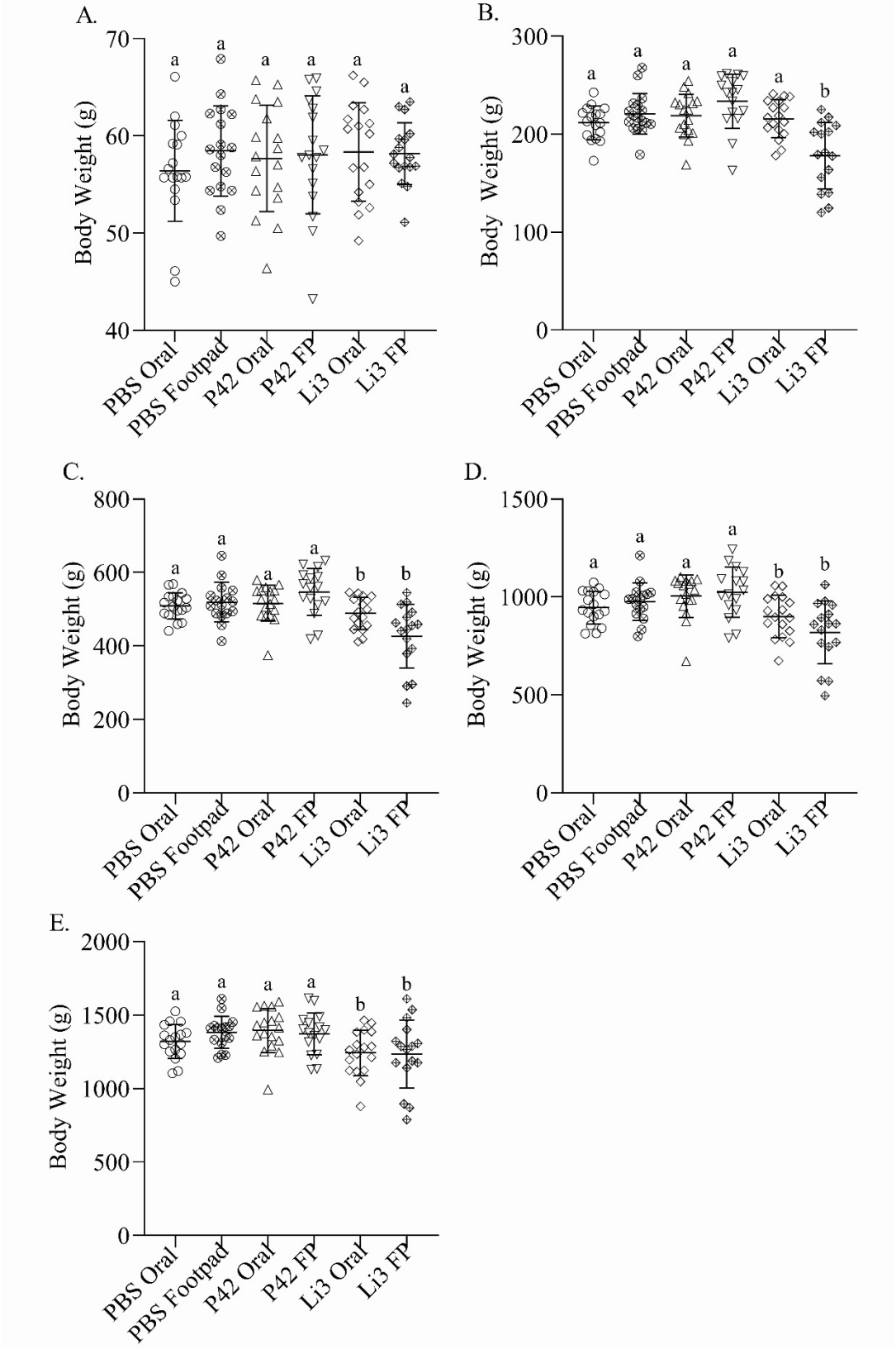
A.
Body Weight (g)
70
60
50
40
a
a
a
a
a
a
PBS Oral
PBS Footpad
P42 Oral
P42 FP
Li3 Oral
Li3 FP
B.
Body Weight (g)
300
200
100
0
a
a
a
a
a
b
PBS Oral
PBS Footpad
P42 Oral
P42 FP
Li3 Oral
Li3 FP
C.
Body Weight (g)
800
600
400
200
0
a
a
a
a
b
b
PBS Oral
PBS Footpad
P42 Oral
P42 FP
Li3 Oral
Li3 FP
D.
Body Weight (g)
1500
1000
500
0
a
a
a
a
b
b
PBS Oral
PBS Footpad
P42 Oral
P42 FP
Li3 Oral
Li3 FP
E.
Body Weight (g)
2000
1500
1000
500
0
a
a
a
a
b
b
PBS Oral
PBS Footpad
P42 Oral
P42 FP
Li3 Oral
Li3 FP

Fig. 3.2. Body weight in grams for each treatment group at day-of-age (3.2A), day 7 (3.2B), day 14 (3.2C), day 21 (3.2D), and day 28 (3.2E). Statistical analyses were performed by multiple one way ANOVA (GraphPad Prism®). Treatment groups with common scripts above scatterplots do not have significantly different mean body weights ($P < 0.05$).

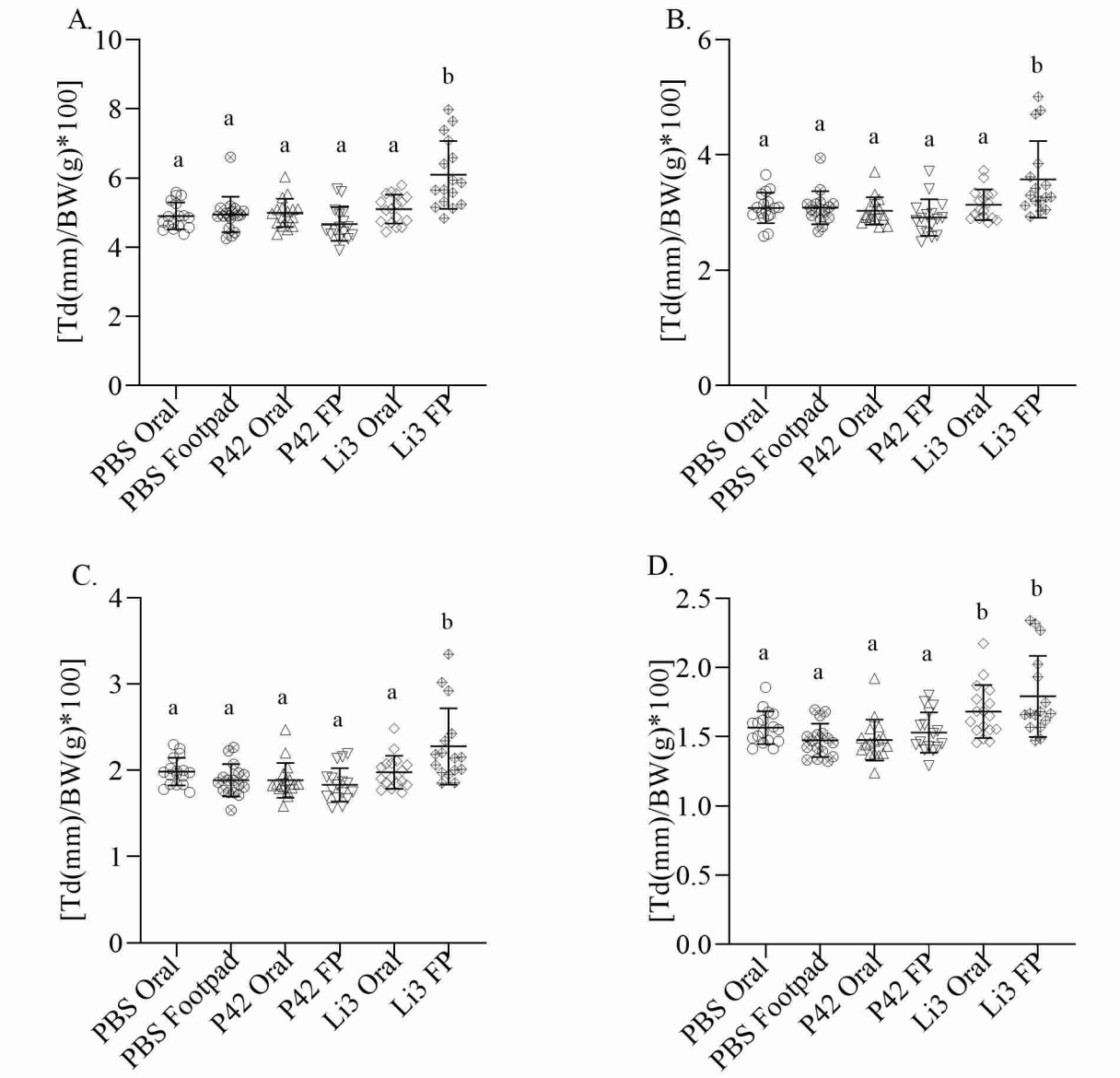

Fig. 3.3. Digital flexor tendon measurements standardized by body weight. Measurements were taken on day 7 (3.3A), day 14 (3.3B), day 21 (3.3C), and day 28 (3.3D). Statistical analyses were performed by Multiple One-Way ANOVA (GraphPad Prism®). Treatment groups with common scripts above scatterplots do not have significantly different tendon swelling ($P < 0.05$).

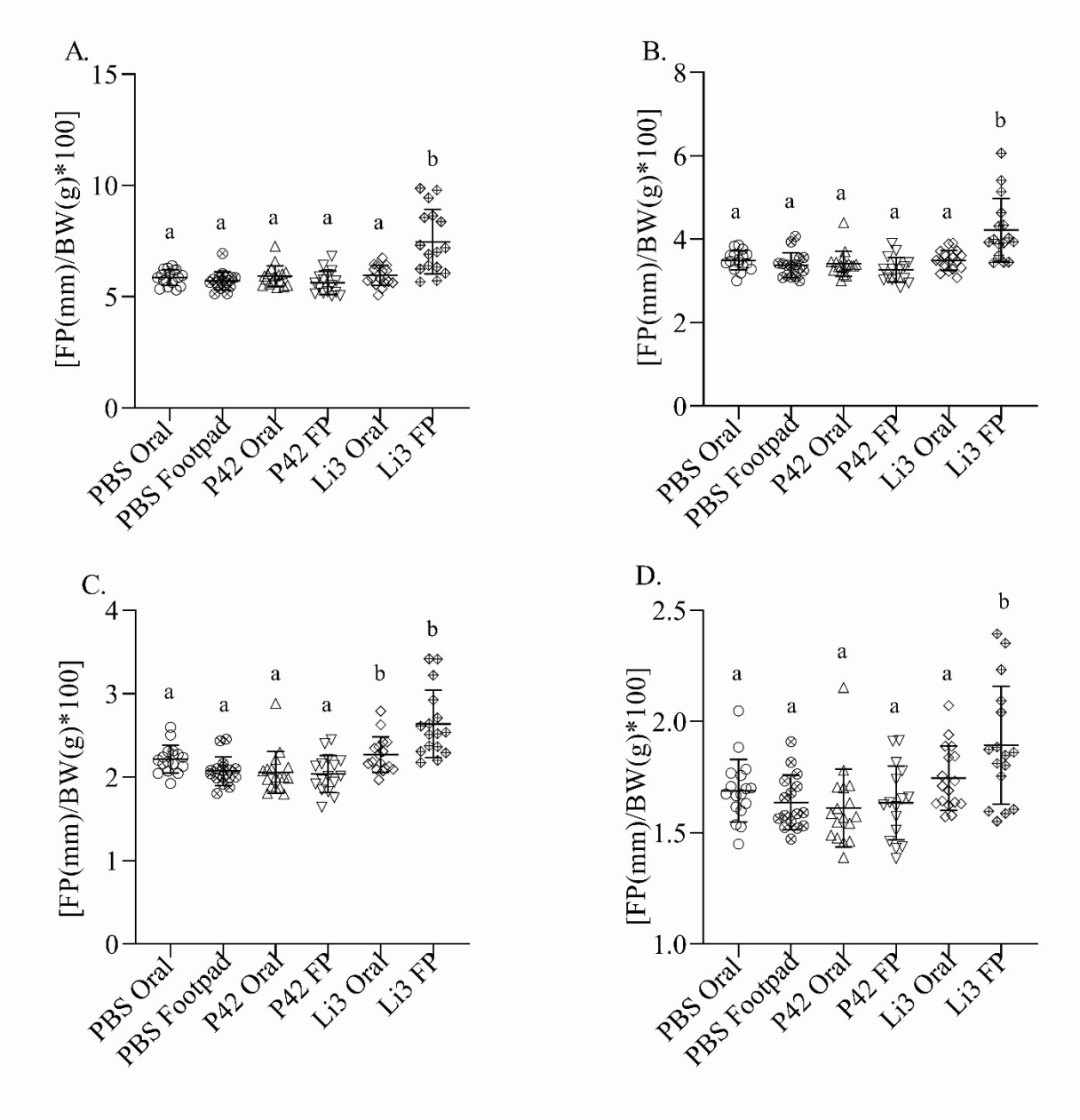

Fig. 3.4. Footpad measurements standardized by body weight. Measurements were taken on day 7 (3.4A), day 14 (3.4B), day 21 (3.4C), and day 28 (3.4D). Statistical analyses were performed using Multiple One-Way ANOVAs (GraphPad Prism®). Treatment groups with common scripts above scatterplots do not have significantly different footpad swelling ($P < 0.05$).

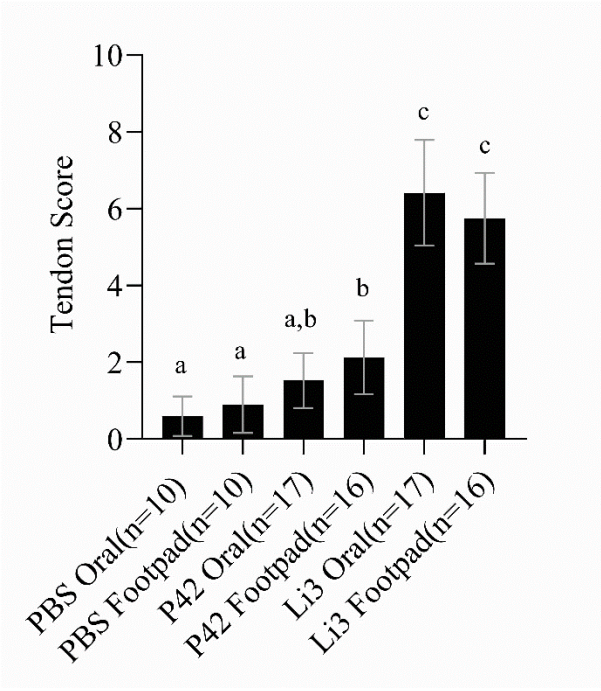

Fig. 3.5. Microscopic tendon scoring based on the sum of the synovium, subsynovium, and additional features scores (Sharafeldin *et al*. 2014). Synovium scoring: 0 = normal single squamous epithelium, 1 = single layer of hypertrophied synoviocytes, 2 = 2 to 4 layers of hyperplastic synoviocytes, and 3 = more than 4 layers of hyperplastic lymphocytes. Subsynovium scoring: 0 = less than 10 lymphocytes, 1 = 10 to 50 lymphocytes, 2 = 50 to 100 lymphocytes, and 3 = too numerous to count. Additional features: 1 = lymphoid follicles, fibroplasia, or dilated subsynovial blood vessels. Statistical analyses were performed using the Mann-Whitney U test (GraphPad Prism®). Treatment groups with different scripts above bars indicate a significant difference in median tendon scores between groups ($P < 0.05$).

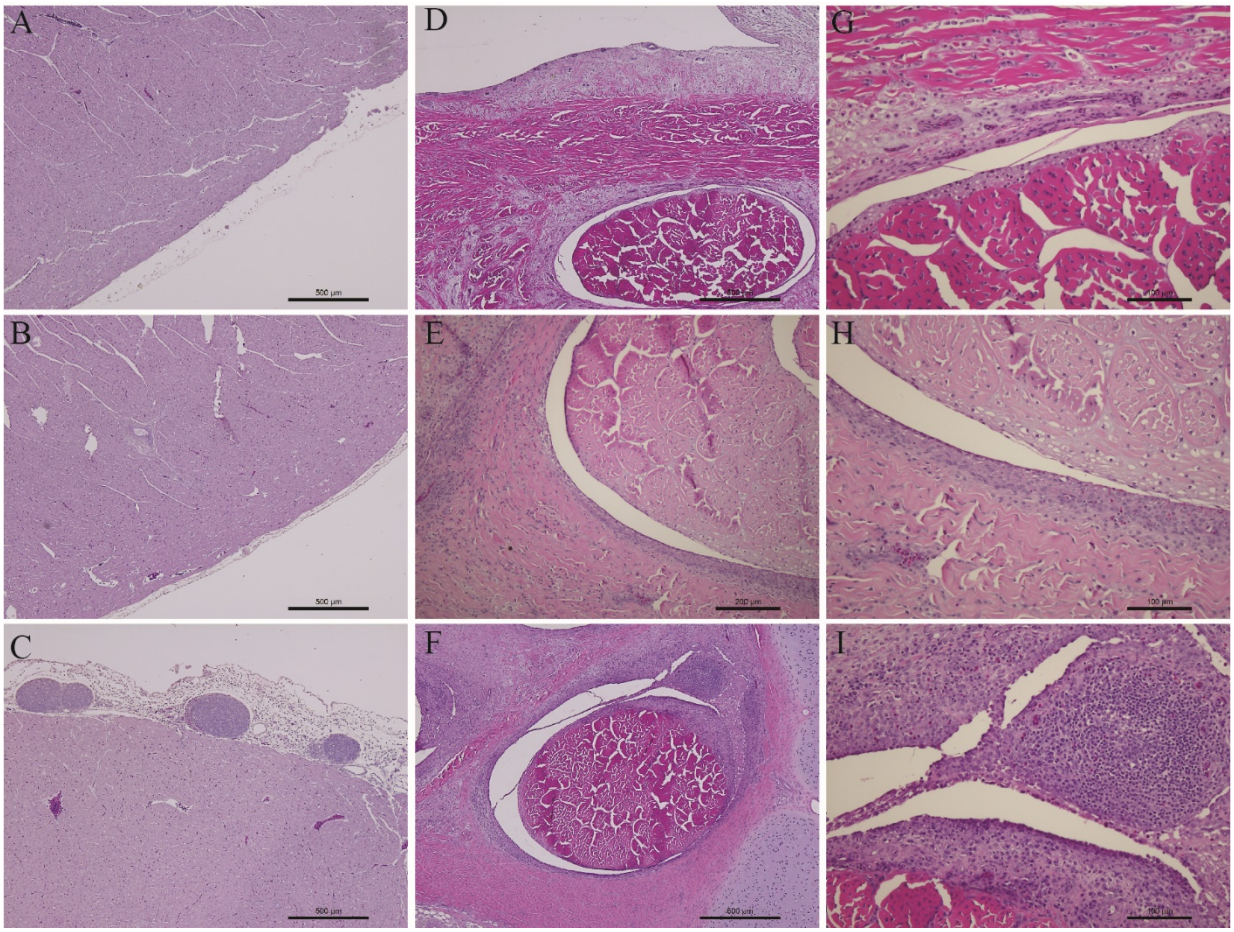

Fig. 3.6. Hematoxylin and Eosin stained hearts and tendons from birds footpad inoculated with PBS, P42, or Li3: (A) heart from PBS footpad group, (B) heart from P42 footpad group, (C) heart from Li3 footpad group with multiple germinal centers on the epicardial surface, (D and G) tendon from PBS footpad group, (E and H) tendon from P42 footpad group with synovial cell hyperplasia gradient and (F and I) tendon from Li3 group with germinal centers and synovial cell hyperplasia and lymphocytes, respectively.

CHAPTER 4

HIGH-RESOLUTION LINEAR EPITOPE MAPPING OF B-CELL RESPONSES FOLLOWING IMMUNIZATION WITH AVIAN REOVIRUS S1133 AND TWO VARIANT REOVIRUSES[A]

[A]Dawe, W.H., Linnemann, E.G., and Sellers, H.S. To be submitted to *Avian Diseases.*

SUMMARY

Avian reoviruses are causative agents of tenosynovitis and viral arthritis. Commercial reovirus vaccines do not protect against disease caused by emerging variants associated with tenosynovitis and lameness. Custom made inactivated reovirus vaccines are commonly utilized to help protect against disease. Neutralizing epitopes located on the σC protein have not been clearly defined. In this study, polyclonal chicken sera from birds vaccinated with a commercial S1133 live attenuated/inactivated vaccine, the genotype 1/117816, or the genotype 5/94826 were used for B-cell linear epitope mapping of the σC protein. SPF chickens were vaccinated twice with either: 1) live and live 2) inactivated and inactivated 3) live and inactivated. Epitope mapping was performed on individual serum samples from each group using S1133, 117816, and 94826 σC sequences translated into linear 15 amino acid peptides with an overlap of 14 peptides. Multiple proposed epitopes and single peptide interactions were identified. Birds vaccinated with only live or inactivated 117816 or 94826 revealed fewer B-cell epitopes to σC compared to birds vaccinated with both live and inactivated 117816 or 94826. In contrast, birds vaccinated with only live or inactivated S1133 had more B-cell epitopes than birds vaccinated with both live and inactivated S1133. Vaccination with a combination of live and inactivated viruses resulted in more complex B-cell responses to the outer-capsid domains of σC for 117816, 94826 and S1133. The identification of antibody binding sites within σC following vaccination contributes to understanding why current commercial vaccines do not protect against field challenge with variant reoviruses.

Key words: avian reovirus, S1133, tenosynovitis, epitope, B-cell, vaccine

Abbreviations: ARV = avian reovirus; BPL = β-Propiolactone; CELiC = chicken embryo liver cells; GT = genotype; MAS = malabsorption syndrome; RSS = runting/stunting syndrome

INTRODUCTION

Avian reoviruses (ARVs) are ubiquitous in commercial poultry and can be isolated from healthy flocks. ARVs are associated with diseases such as malabsorption syndrome (MAS), runting/stunting syndrome (RSS), and immunosuppression (1-4). ARV is the causative agent of viral arthritis/tenosynovitis and contributes to disease-related economic losses in chickens and turkeys (5-7). Reoviruses vary in pathogenicity and have been associated with clinical signs such as swollen hocks and tendons, lameness, ruptured tendons, myocarditis, poor flock uniformity, and mortality (8-10).

ARVs can be horizontally transmitted through the fecal-to-oral route following contact with infected birds, post-hatch exposure, or contact with contaminated litter (9, 11, 12). Vertical transmission occurs when broiler-breeders become infected during lay and shed virus to broiler progeny through the eggs (13, 14). Broilers infected at an earlier age are more susceptible to developing clinical signs later in life (15, 16). Historically, the use of commercial live-attenuated and inactivated vaccines provided good protection against disease through the vaccination of broiler-breeders and the subsequent transfer of maternal antibodies to progeny (17). Despite the continued use of these vaccines, there has been an increasing incidence of viral arthritis/tenosynovitis in commercial poultry from 2010 to 2019 in many countries, suggesting that current vaccines do not completely protect against new field viruses (18-21).

Avian reoviruses are members of the *Reoviridae* family and belong to the genus *Orthoreovirus*. Avian reoviruses are small, non-enveloped, icosahedral particles with a diameter 70nm or less and are resistant to inactivation by treatment with chloroform, environmental pH 3.0 for 30 minutes, or incubation at 56°C for one hour (14, 22-25). The avian reovirus genome consists of 10 segments of dsRNA. There are three large (L1, L2, L3), three medium (M1, M2, M3), and

four small (S1, S2, S3, S4) genome segments (26). The genome codes for 10 structural proteins and 4 non-structural proteins (23, 27, 28). The S1 genome segment is tricistronic, having three out-of-phase, partially overlapping reading frames, and codes for the structural protein σC and the non-structural proteins p10 and p17 (28). σC is a minor outer capsid protein that exists as a homotrimer, binds to host cells, and stimulates the production of neutralizing antibodies (29, 30). Genome reassortment of co-circulating strains and point mutations within the S1 gene contribute to escape from neutralization by vaccine-induced antibodies and the emergence of antigenic variants (31-34)

Despite the use of commercial vaccines, widespread isolation of variant reoviruses from clinical cases of tenosynovitis has increased in many countries since 2010 (18-21, 35-37). Six genotypes have been reported based on σC amino acid sequences, with the commercial vaccines only representing a small sub-group within Genotype 1 (GT1) (18). A seventh genotype was recently identified from clinical case submissions at the University of Georgia Poultry Diagnostic and Research Center (PDRC) (personal communication). The outer-capsid domains of σC are identified as: the α-helical (amino acids 117-154), hinge (amino acids 155-159), triple β-spiral (amino acids 160-191), and β-barrel head (amino acids 192-326) domains (38).

Vaccination programs vary among companies, and in recent years, custom made inactivated reovirus vaccines have been included in an attempt to protect against field challenge with antigenic variants (20, 39). Previously, a linear σC B-cell epitope was identified using recombinant σC and monoclonal antibodies, but the location of the epitope was in the inner-capsid domain of σC (40). Another prior study identified an epitope in an outer capsid domain of σC for S1133, but was shown to be absent in variants (35).

In this study, linear σC B-cell epitopes for the S1133 vaccine strain, a GT1 variant 117816, and a genotype 5 (GT5) variant 94826 were identified to evaluate the molecular basis behind the antigenic differences between vaccine and variant viruses.

MATERIALS AND METHODS

Viruses. A commercial modified live S1133 tenosynovitis vaccine (Boehringer-Ingelheim, Gainesville, GA) and two field isolates Ck/USA/TX/117816/Tendon/2017 (117816) and Ck/USA/GA/94826/Tendon/2012 (94826) were used in this study for epitope mapping. Using methods previously described, RT-PCR of the S1 gene third open reading frame σC protein coding region was performed for each of the viruses and the products were sequenced (41). The σC-deduced amino acid sequence identities from the three viruses used in this study were compared. The S1133 GT1 modified live tenosynovitis vaccine (Merial, Gainesville, GA) was used in this study because it is the primary commercially available live vaccine and epitope mapping has not been previously performed. Reovirus isolate 117816, a GT1 variant field isolate shares a 79.1% amino acid identity to the S1133 vaccine, and 94826, a GT5 variant field isolate with a 49.4% amino acid identity to the S1133 vaccine. The 94826 and 117816 viruses share a 50.3% amino acid identity. In addition, the reoviruses used in this study were determined to be serologically unrelated to S1133 following 2-way cross neutralization tests in primary chicken embryo liver cells (42). An additional 14 GT1 and 9 GT5 isolates submitted to the Poultry Diagnostic and Research Center from clinical cases of disease were included in the analysis of the epitope mapping data (Table 4.1). Multiple alignments were performed by Clustal Omega using Geneious Prime (version 2020.1.2 for Windows, Biomatters, Auckland, New Zealand) and phylogenetic analysis was performed by the neighbor-joining method with 1000 bootstrap replicates using MEGA X

(https://www.megasoftware.net/) (Fig. 4.1) (41). Reference sequences with GenBank accession numbers are listed in Appendix A, Table 1.

Vaccines. Field isolates 117816 and 94826 were propagated and plaque purified in specific pathogen free (SPF) primary chicken embryo liver cells (CELiC). Isolate 94826 was further propagated in SPF embryos via chorioallantoic membrane (CAM) inoculation, and the 140th CAM passage was used in this study. Viruses were used as live preparations or inactivated water-in-oil adjuvanted emulsions. Commercial S1133 modified live vaccine was administered at full dose by subcutaneous injection with 0.2ml in the back of the neck. Live 117816 and 94826 viruses were titrated in CELiC, diluted to 10^4TCID$_{50}$/0.2ml, and administered by the same route and volume as the commercial vaccine. Inactivated vaccinations were prepared by incubation of 6ml of the commercial vaccine or propagated field isolate with 0.2% β-Propiolactone (BPL) at 4°C overnight on a tube rotator. Following inactivation, dialysis with 1L of PBS at 4°C was performed three times over the course of 24 hours to remove the BPL. Emulsification of inactivated virus with Montanide ISA 70 VG (Seppic, Fairfield, NJ) was performed at a 30/70 (v/v) ratio following the manufacturer's directions. Inactivated vaccines were administered by intramuscular injection of 0.5mL into the breast muscle.

Chickens. Four-week-old SPF chickens (Charles River Laboratories, North Franklin, CT) were housed in negative pressure isolation units, with five birds per unit, and given access to water and feed *ad libitum* for the duration of the experiment. The animals were maintained and reared following good animal husbandry practices. All protocols for the animal experiments were approved by the University of Georgia Institutional Animal Care and Use Committee (IACUC AUP A2013-09-022-Y1-A0).

Birds were vaccinated at four and six week-of-age with either a series of live, inactivated, or live (at four weeks-of-age) then inactivated (at six weeks-of-age) virus (Table 4.2). Serum was collected from individual birds at four, six, eight, and ten weeks-of-age. For each treatment group, three individual serum samples were selected for epitope peptide mapping based on antibody reactivity by reovirus ELISA (IDEXX® Laboratories, Inc., Westbrook, ME USA), virus neutralization assays, and western blot. Serum from antibody negative birds at four weeks-of-age was used for a negative control. Collection timepoints and serology results are listed in Table 4.3.

Peptide microarray

The peptide mapping and analysis was performed by PEPperPRINT (Heidelberg, Germany) using serum samples and the respective σC protein sequences submitted by our lab. In short, S1133, 117816, and 94826 σC capsid protein sequences were extended by neutral GSGSGSG linkers at the C and N terminus to avoid truncated peptides. The protein sequences were then translated into 15 amino acid peptides onto separate microarray chips with a peptide-peptide overlap of 14 amino acids. Each of the microarray copies consisted of 326 different peptides printed in duplicate, for a total of 652 peptide spots. The microarray chips were framed by 78 spots of YPYDVPDYAG HA control peptides. Peptide microarray chips were stored at 4°C until use.

Pre-staining of each microarray chip was performed with goat anti-chicken IgG (H+L) DyLight™680 (1:5000) secondary antibody and mouse monoclonal anti-HA (12CA5) DyLight™800 (1:2000) control antibody to identify background interactions that may interfere with the assay results. Microarray chips were blocked with Rockland blocking buffer MB-070 for 30 minutes before each assay. After each assay, the microarray chips were given three, one-minute washes with phosphate buffered saline (PBS) at pH 7.4 with 0.05% Tween 20. Individual

polyclonal serum samples from vaccinated birds were reacted with the peptide chips corresponding to the virus strains used for immunization at 1:1000, 1:500, 1:300, and 1:100 dilutions. Incubation with the chicken serum samples was carried out for 16 hours at 4°C with shaking at 140 rotations per minute. Each microarray's HA peptide frame was simultaneously stained with mouse monoclonal anti-HA (12CA5) DyLight™800 (1:2000) to confirm internal assay quality and integrity of the microarray.

Staining with secondary antibody was performed and images were captured using a LI-COR Odyssey® Imaging System with a scanning offset of 0.65mm, a resolution of 21μm, and scanning intensities of 7/7 (red = 700nm/green = 800nm). Quantification of spot intensities was carried out using a PepSlide® analyzer. An intensity map was generated based on the averaged median foreground intensities and interactions to analyze signal-to-noise ratios and spot intensities. Intensity plots and maps were combined with visual inspections of the microarray scans to identify linear B-cell epitopes and single peptides recognized by antibodies in the chicken serum. The results were recorded in tables, as sequence annotations, and by highlighting residues on the σC protein surface crystal structure using the PyMOL Molecular Graphics System (version 2.3.2 for Windows, Schrödinger, LLC, New York, NY). The σC crystal structure used for modeling epitopes was PDB 2VRS (43).

RESULTS

Peptide microarrays. B-cell epitopes were identified in the outer-capsid domains of S1133, 117816, and 94826 σC proteins (Table 4.4). Single peptide interactions and complete lists of epitopes identified across all domains for S1133, 117816, and 94826 are listed in Appendix A, Tables 2, 3, and 4, respectively. Fluorescence intensity plots for S1133, 117816, and 94826 microarrays provide an overview of serum reactivity, but do not distinguish single peptide

interactions from epitope-like spot patterns (Fig. 4.2, 4.3, and 4.4). For birds that received two S1133 live vaccinations, one epitope was identified in the α-helical domain (^{120}TVDGNST^{126}) and two epitopes were identified in the β-barrel head domain (^{220}AHCHGRR^{226} and ^{239}VTSNVVLLTFD^{249}) (Fig. 4.5A). Five epitopes in the β-barrel head domain (^{236}NLTVTSNVVLLTF^{248}, ^{249}DLSDITHIP^{257}, ^{273}SFPVDVSFTR^{282}, ^{287}HAYQAYGVY^{295}, and ^{292}YGVYSSSRVFTITF^{305}) were identified for birds that received only inactivated S1133 vaccinations (Fig. 4.5B). One epitope in the β-triple spiral was identified from birds that received S1133 live and inactivated vaccinations (^{162}FSPPLSVADGV^{172}) (Fig. 4.5C).

Three 117816 epitopes were identified in the α-helical, hinge, β-triple spiral, and β-barrel head domains (^{124}NSTIIDNL^{131}, ^{147}SRVKSLESTSGHEL^{160}, and ^{206}GTNGSSDNIDM^{216}) from birds that had received two live vaccinations (Fig. 4.6A). Birds vaccinated twice with the inactivated variant revealed five epitopes in the hinge, β-triple spiral, and β-barrel head domains (^{157}GHELSFSPPL^{166}, ^{168}VTDGVVSLNMDPY^{180}, ^{222}CHGRRTDYIMS^{232}, ^{254}TSLPPDLSRLIPSA^{267}, and ^{265}PSAGFQVASFPVDV^{278}) (Fig. 4.6B). A total of twelve epitopes were identified within the α-helical, hinge, β-triple spiral, and globular head regions in birds receiving both live followed inactivated 117816 variant vaccinations (^{128}IDNLKGDVSSNSLA^{141}, ^{132}KGDVSSNSLAI^{142}, ^{147}SRVKSLESTSGHEL^{160}, ^{160}LSFSPPLS^{167}, ^{168}VTDGVVSLNMDPY^{180}, ^{205}RGTNGSSDNIDM^{216}, ^{217}NVNAHCHGRRTD^{228}, ^{258}PDLSRLIPSAGFQ^{270}, ^{270}QVASFPVDVSFT^{281}, ^{292}YGVYSSSRVFTI^{303}, ^{302}TITFPTGGDGPANI^{315}, and ^{305}FPTGGDGPANIRF^{317}) (Fig. 4.6C).

Eleven epitopes ranging in distribution from the α-helical to the β-barrel head domains (^{126}GEVGNLKTS^{134}, ^{166}LKLEAGTVSLEVDP^{179}, ^{172}TVSLEVDPYFCSV^{184}, ^{213}SIDMDVNAHSHGSR^{226}, ^{239}VTTSPATLVFE^{249}, ^{243}PATLVFE^{249}, ^{258}SDLSRLIPCYGF^{269}, ^{266}CYGFQQATFPVDIS^{279}, ^{268}GFQQATF^{274}, ^{269}FQQATFPVDISFQR^{282}, and ^{286}SHTYQVYGTYTSSR^{299}) were identified in birds administered two immunizations with the live 94826 virus (Fig. 4.7A). Six epitopes were identified from birds vaccinated twice with the inactivated 94826 virus and had a distribution ranging from the α-helical to the β-barrel head domains (^{120}EVSVLSGEV^{128}, ^{159}TLSFADP^{165}, ^{168}LEAGTVSLEV^{177}, ^{250}LDRIAALPSDL^{260}, ^{266}CYGFQQATFPVDIS^{279}, and ^{293}GTYTSSRVF^{301}) (Fig. 4.7B). Six epitopes were identified in the 94826 live/inactivated treatment groups and were contained in the β-triple spiral and β-barrel head domains (^{167}KLEAGTVSLEV^{177}, ^{239}VTTSPATLVFE^{249}, ^{250}LDRIAALPSDLSRL^{263}, ^{266}CYGFQQATFPVD^{277}, ^{278}ISFQRDGVSHTYQV^{291}, and ^{292}YGTYTSSRVF^{301}) (Fig. 4.7C). The negative control serum from bird 4-1 reacted with the 94826 microarray chip, but the spot pattern morphology was blurry and communications with PEPperPRINT verified this sample was negative.

Multiple alignments. An alignment of S1133, 117816, and 94826 was generated and multiple substitutions were identified in 117816 and 94826 compared to S1133 and located within positions identified as antibody binding sites for S1133 (Fig. 4.8). The S1133 GT1 vaccine had a 79.1% amino acid similarity to the 117816 GT1 variant and a 49.4% amino acid similarity to the 94826 GT5 variant. When compared to the S1133 GT1 vaccine, amino acid substitutions were identified to a greater extent in the C-terminal β-barrel head of the 94826 GT5 variant than the 117816 GT1 variant. A total of 9 substitutions were present in the 117816 GT1 variant within antibody binding sites compared to those identified for S1133. The 94826 GT5 variant had 35 substitutions within

antibody binding sites compared to those identified for S1133. Residues 287-311 of the 117816 GT1 variant were identical to the S1133 GT1 vaccine, but the 94826 GT5 variant had 9 substitutions in this region compared to S1133. Antibody binding sites in the N-terminal domain of the β-triple spiral (residues 162-172) were identified for all three viruses in birds that received a series of live and inactivated immunizations. Within this antibody binding site, Thr-169 (polar, uncharged) was present in 117816, while Ala-169 (hydrophobic) was present in S1133. Glu-169 (charged) was present in 94826, and was accompanied by six additional different residues compared to S1133 and 117816 at the S1133 antibody-binding site 162-172.

In this study, 14 of the GT1 isolates for which full length σC sequence was obtained were phylogenetically divided into three GT1 subgroups. The S1133 GT1 vaccine shared a 78.8-80% identity to the 13 GT1 variants that belong to two variant subgroups. The 117816 GT1 variant belongs to a subgroup distinct from S1133 and shared a 97.5-99.4% identity to 11 other isolates in this subgroup. The most conserved substitution in the GT1 117816 subgroup within an antibody binding site identified for 117816 was at position 159. Glu-159 (charged) in 117816 was substituted by Gly-159 (hydrophobic) in 9 of the GT1 variants that grouped with 117816. A GT1 subgroup distinct from S1133 and 117816, composed of three isolates, shared a 91.4-91.9% identity to 117816 and had 9 conserved substitutions compared to 117816 within antibody binding sites identified for 117816. These conserved substitutions were also present in 4 positions in S1133 when compared to 117816.

For GT5, 10 of the GT5 isolates for which full length σC sequence was obtained were phylogenetically divided into three subgroups. The S1133 GT1 vaccine shared a 47.5-49.7% identity to these GT1 variants. The 94826 GT5 variant belonged to a GT5 subgroup composed of 5 other GT5 variant isolates and shared a 97.1-100% identity with these isolates. There were three

conserved substitutions within antibody binding sites identified for 94826 for four isolates in this subgroup: Val-121 (hydrophobic), Ala-254 (hydrophobic), and Tyr-267 (polar, uncharged) in 94826 were substituted for Ala-121 (hydrophobic), Val-254 (hydrophobic), and His-267 (basic, charged) in these four isolates. A GT5 subgroup distinct from 94826 consisted of three Colombian GT5 variants that shared an 81% identity to 94826, and had 20 conserved substitutions within antibody binding sites identified for 94826. Out of these 20 conserved substitutions within 94826 antibody binding sites, 9 were also present in the GT5 variant 131930 that shared an 81.9% identity to the Colombian GT5 variant subgroup and an 81.6% identity to the GT5 variant 94826. Among the GT5 variants, the most common substitution within an antibody binding site identified for 94826 was His-267 (basic, charged) in the place of Tyr-267 (polar, uncharged). This substitution was present in 8 of the GT5 variants closely examined in this study.

DISCUSSION

Linear epitopes and single peptide interactions identified as the sites responsible for host cell receptor binding in mammalian reoviruses were located in areas of the globular head and β-triple spiral (44-46). For the 117816 and 94826 viruses, more epitopes were identified in the receptor binding domain in birds that received both live and inactivated vaccinations, supporting vaccination with live vaccines followed by vaccination with inactivated vaccines. Since the isolation of the GT5 94826 virus in 2012 and the GT1 117816 virus in 2014, the incidence of clinical disease has remained high, providing data to support generation of new vaccines with contemporary reovirus strains.

The S1133 GT1 vaccine and 117816 GT1 variant were found to be serologically unrelated despite a sigma C sequence similarity of 80%. The phylogenetic analysis performed in this study combined with the epitope mapping results for S1133 and 117816 identified amino acid

substitutions in GT1 variant isolates within antibody binding sites for S1133 and 117816 and is likely the reason that antibodies generated by S1133 do not neutralize variant genotype 1 reoviruses. The substitutions within antibody binding sites were identified to the greatest extent when GT1 variants were compared to the classic S1133 GT1 vaccine.

In the GT1 variant subgroup containing 117816, there was only one highly conserved substitution in variants within this subgroup compared to 117816, and this substitution was identified in an antibody binding site for 117816.

Similar to the case for GT1, the GT5 variants utilized in the analysis of the GT5 variant 94826 epitope mapping results had conserved amino acid substitutions present in antibody binding sites identified for 94826. Substitutions within 94826 antibody binding sites were identified in isolates within the GT5 94826 subgroup, but were more prevalent in GT5 isolates outside of this subgroup. For 94826 and the other GT5 variants used in this study, multiple conserved antigenic motifs that contain cystine, leucine, or valine substitutions were identified while absent from S1133, suggesting these motifs may be highly antigenic components contributing to the lack of protection provided by S1133 vaccine-induced antibodies against 94826 and other GT5 ARVs (47).

Overall, the intensity of the antibody response measured by the peptide microarrays varied between S1133, 117816, and 94826. The live viruses utilized for immunizing the chickens varied in the level of attenuation and this may have contributed to the overall antibody response and binding to σC. Specifically, the S1133 vaccine used in this study was highly attenuated and produced lower levels of antibodies compared to the 117816 and 94826 viruses. The peptide microarray reactivity for 94826 negative control bird 4-1 was not considered significant in the analysis performed by PEPperPRINT, but does correlate with reactivity observed in immunized

birds, and thus provides reason for caution and the need for validation when interpreting the results of this study. A similar occurrence was documented in a prior study using PEPperPRINT peptide microarrays and suggested non-specific background binding as a possible explanation for the occurrence (48).

In a study published in 2013, Yin *et al*. used a pepscan-mAb epitope mapping approach and identified the S1133 σC linear B-cell epitope ^{45}ELLHRSISDI^{54} (40). In our study, we identified the epitopes ^{33}IYERLTNLEASTEL^{46} and ^{51}ISDISTTVSNISA^{63}, correlating with this prior finding. Because the location of these epitopes was in the inner-capsid domain of σC, it is unlikely there is a significance of these findings in regards to neutralizing antibodies. In 2010, Goldenberg *et al.* reported the successful use of antigenic prediction tools, bioinformatics, and structural analysis methods that lead to the identification of the S1133 epitope ^{254}THIPSDL^{260}, and found that this epitope was unique to the GT1 subgroup and was not conserved in variants included in their study (35). This correlates with the epitope-like spot pattern ^{249}DLSDITHIP^{257} and single peptide interactions ^{243}VVLTFDLSDITHIP^{257} and ^{256}IPSDLARLVPSAGFQ^{270} our study identified in birds that were vaccinated twice with inactivated S1133.

While our study identified many linear B-cell epitopes for three antigenically dissimilar ARVs, further work is needed to identify the neutralizing epitopes and the potential role of conformational epitopes in protective B-cell responses. The generation of specific peptide immune serum and virus neutralization assays would identify epitopes associated with the induction of neutralizing antibodies. Such an experiment would have the potential to identify epitopes that are responsible for cross-neutralization between different serotypes and could also be applied to custom vaccine design by facilitating the selection of isolates to optimize protection against relevant field challenge viruses. Identification of the antibody binding sites on σC that allow these

viruses to escape vaccine-induced protection enhances diagnostic evaluations and vaccination strategies by targeting specific regions of σC. The antigenic diversity of contemporary, pathogenic ARVs highlights the utility of inactivated custom-made vaccines currently in use in the United States and warrants the development of novel live-attenuated vaccines to further protect against disease and economic loss caused by ARV variants.

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TABLES

Table 4.1. Avian reovirus σC amino acid sequences used for epitope mapping and analysis.

Genotype[A]	Isolate	Sequence ID	Accession number
1	S1133	S1133 Vaccine Strain	AF330703
1	104190	Ck/USA/GA/104190/Tendon/2014	KM282091
1	107174	Ck/USA/AR/107174/Tendon/2014	MT127436
1	115940	Ck/USA/TX/115940/Tendon/2016	MT127437
1	117816	Ck/USA/TX/117816/Tendon/2017	MT127438
1	120651	Ck/USA/TX/120651/Tendon/2017	MT127439
1	125607	Ck/USA/MS/125607/Tendon/2018	MT127440
1	125785	Ck/USA/MO/125785/Tendon/2018	MT127441
1	126109	Ck/USA/IN/126109/Tendon/2018	MT127442
1	126197	Ck/USA/MO/126197/Tendon/2018	MT127443
1	126198	Ck/USA/MO/126198/Tendon/2018	MT127444
1	126201	Ck/USA/MO/126201/Tendon/2018	MT127445
1	126783p1	Ck/USA/TN/126783/Tendon/p1/2018	MT127459
1	126908p2	Ck/USA/MO/126908/Tendon/p2/2018	MT127458
1	129204	Ck/USA/GA/129204/Tendon/2019	MT127446
1	132042	Ck/USA/VA/132042/Tendon/2019	MT127455
5	94826	Ck/USA/GA/94826/Tendon/2012	KJ803967.1
5	126783p2	Ck/USA/TN/126783/Tendon/p2/2018	MT127456
5	131089	Ck/USA/MO/131089/Tendon/2019	MT127447
5	131307	Ck/USA/MO/131307/Tendon/2019	MT127448
5	131328	Ck/USA/GA/131328/Tendon/2019	MT127449
5	131416	Ck/Colombia/131416/FTAcard/2019	MT127450
5	131417	Ck/Colombia/131417/FTAcard/2019	MT127451
5	131418	Ck/Colombia/131418/FTAcard/2019	MT127452
5	131495	Ck/USA/TN/131495/Tendon/2019	MT127453
5	131930	Ck/USA/GA/131930/Tendon/2019	MT127454

[A]Genotype determined by Clustal Omega multiple alignment of σC protein sequences and the neighbor-joining tree building method using 1,000 bootstrap replicates.

Table 4.2. Treatment groups and schedule of vaccinations.

	Age at time of vaccination and vaccine administered	
Treatment group	Four weeks-of-age	Six weeks-of-age
S1133-1	S1133 Live[A]	S1133 Live
S1133-2	S1133 Inactivated[B]	S1133 Inactivated
S1133-3	S1133 Live	S1133 Inactivated
117816-1	117816 Live[C]	117816 Live
117816-2	117816 Inactivated[D]	117816 Inactivated
117816-3	117816 Live	117816 Inactivated
94826-1	94826 Live[E]	94826 Live
94826-2	94826 Inactivated[F]	94826 Inactivated
94826-3	94826 Live	94826 Inactivated

[A]S1133 commercially available modified live tenosynovitis vaccine (Merial, Gainesville, GA) administered as a full dose, subcutaneously into the back of the neck (S1133 Live).
[B]S1133 inactivated virus administered intramuscularly in the breast (S1133 Inactivated).
[C]117816 live virus administered subcutaneously in the back of the neck (117816 Live).
[D]117816 inactivated virus administered intramuscularly in the breast (117816 Inactivated).
[E]94826 live virus administered subcutaneously in the back of the neck (94826 Live).
[F]94826 inactivated virus administered intramuscularly in the breast (94826 Inactivated).

Table 4.3. Sera collection timepoints and serology.

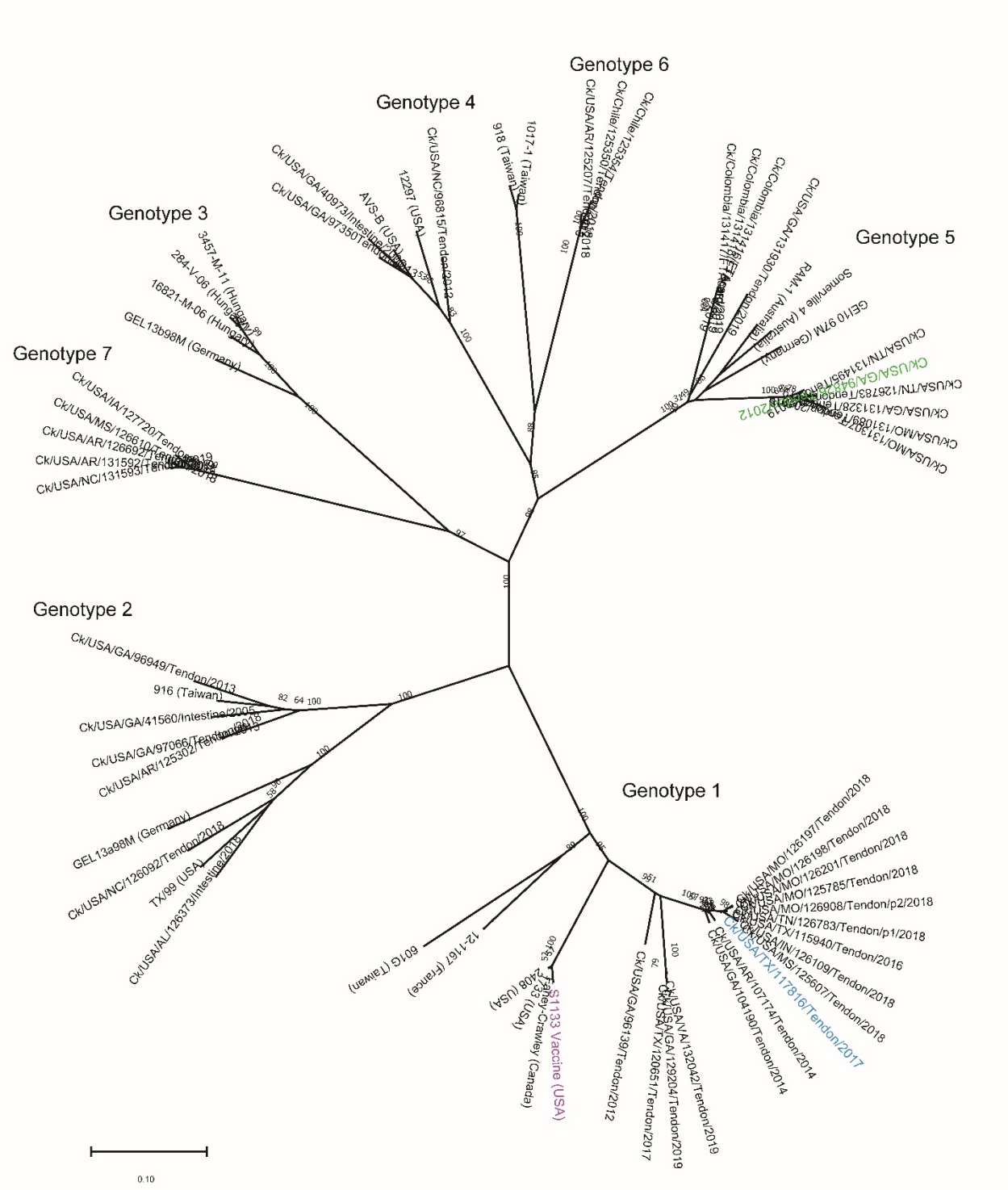
Virus	Bird ID	Treatment	DPV[A]	ELISA[B]	VN[C]
S1133	1-3	Live+Live	28	2359	8
S1133	1-2	Live+Live	28	2066	256
S1133	1-1	Live	14	198	1
S1133	2-3	Inact.+Inact.	28	770	128
S1133	2-2	Inact.+Inact.	28	668	32
S1133	2-1	Inact.+Inact.	28	979	16
S1133	3-3	Live+Inact.	28	5486	64
S1133	3-2	Live+Inact.	28	3990	256
S1133	3-1	Live+Inact.	28	12162	128
S1133	4-1	None	-	0	1
117816	1-3	Live+Live	28	1412	512
117816	1-2	Live+Live	28	3379	512
117816	1-1	Live+Live	28	3597	256
117816	2-3	Inact.+Inact.	28	4168	1024
117816	2-2	Inact.+Inact.	28	5289	256
117816	2-1	Inact.+Inact.	28	7624	1024
117816	3-3	Live+Inact.	28	3552	1024
117816	3-2	Live+Inact.	28	1520	1024
117816	3-1	Live+Inact.	28	2576	1024
117816	4-1	None	-	0	1
94826	1-3	Live+Live	21	2082	1
94826	1-2	Live+Live	21	2229	32
94826	1-1	Live+Live	21	2251	8
94826	2-3	Inact.+Inact.	21	13744	256
94826	2-2	Inact.+Inact.	21	4804	512
94826	2-1	Inact.+Inact.	21	7057	128
94826	3-3	Live+Inact.	21	1473	512
94826	3-2	Live+Inact.	21	5763	256
94826	3-1	Live+Inact.	21	5421	256
94826	4-1	None	-	113	1

[A]Days post vaccination (DPV) when serum was collected. Serum from bird 4-1 was collected from a four-week-old bird that was not vaccinated.
[B] IDEXX® Reovirus ELISA titer.
[C] Corresponding S1133, 117816 or 94826 virus neutralization (VN) titer.

Table 4.4. Proposed outer-capsid B-cell epitopes.

Virus	Bird ID	Peptide(s)
S1133	1-3	^{220}AHCHGRR^{226}, ^{239}VTSNVVLLTFD^{249}
S1133	1-2	^{120}TVDGNST^{126}
S1133	1-1	^{120}TVDGNST^{126}
S1133	2-3	^{249}DLSDITHIP^{257}, ^{273}SFPVDVSFTR^{282}, ^{287}HAYQAYGVY^{295}, ^{292}YGVYSSSRVFTITF^{305}
S1133	2-2	None
S1133	2-1	^{236}NLTVTSNVVLLTF^{248}
S1133	3-3	None
S1133	3-2	None
S1133	3-1	^{162}FSPPLSVADGV^{172}
S1133	4-1	None
117816	1-3	^{206}GTNGSSDNIDM^{216}
117816	1-2	None
117816	1-1	^{124}NSTIIDNL^{131}, ^{147}SRVKSLESTSGHEL^{160}
117816	2-3	^{157}GHELSFSPPL^{166}, ^{168}VTDGVVSLNMDPY^{180}, ^{222}CHGRRTDYIMS^{232}, ^{254}TSLPPDLSRLIPSA^{267}, ^{265}PSAGFQVASFPVDV^{278}
117816	2-2	None
117816	2-1	^{169}TDGVVSLNMDP^{179}, ^{254}TSLPPDLSRLIPS^{266}, ^{267}AGFQVASFPVDV^{278}
117816	3-3	^{132}KGDVSSNSLAI^{142}, ^{169}TDGVVSLNMDPY^{180}, ^{258}PDLSRLIPSAGFQ^{270}, ^{270}QVASFPVDVSFT^{281}, ^{292}YGVYSSSRVFTI^{303}, ^{302}TITFPTGGDGPANI^{315}
117816	3-2	^{147}SRVKSLESTSGHEL^{160}, ^{168}VTDGVVSLNMDPY^{180}, ^{205}RGTNGSSDNIDM^{216}
117816	3-1	^{128}IDNLKGDVSSNSLA^{141}, ^{160}LSFSPPLS^{167}, ^{169}TDGVVSLNMDPY^{180}, ^{206}GTNGSSDNIDM^{216}, ^{217}NVNAHCHGRRTD^{228}, ^{305}FPTGGDGPANIRF^{317}
117816	4-1	None
94826	1-3	^{167}KLEAGTVSLEVDP^{179}, ^{243}PATLVFE^{249}, ^{266}CYGFQQATFPVDIS^{279}
94826	1-2	^{172}TVSLEVDPYFCSV^{184}, ^{213}SIDMDVNAHSHGSR^{226}, ^{269}FQQATFPVDISFQR^{282}, ^{286}SHTYQVYGTYTSSR^{299}
94826	1-1	^{126}GEVGNLKTS^{134}, ^{166}LKLEAGTVSLEVD^{178}, ^{239}VTTSPATLVFE^{249}, ^{258}SDLSRLIPCYGF^{269}, ^{268}GFQQATF^{274}
94826	2-3	^{159}TLSFADP^{165}, ^{168}LEAGTVSLEV^{177}, ^{250}LDRIAALPSDL^{260}, ^{266}CYGFQQATFPVDIS^{279}
94826	2-2	^{120}EVSVLSGEV^{128}, ^{293}GTYTSSRVF^{301}
94826	2-1	^{160}LSFADP^{165}
94826	3-3	^{239}VTTSPATLV^{247}, ^{250}LDRIAALPSDLSRL^{263}, ^{278}ISFQRDGVSHTYQV^{291}, ^{292}YGTYTSSRVF^{301}
94826	3-2	^{240}TTSPATLV^{247}, ^{266}CYGFQQATFPVD^{277}
94826	3-1	^{167}KLEAGTVSLEV^{177}, ^{239}VTTSPATLVFE^{249}
94826	4-1	None

FIGURES


Genotype 6
Genotype 4
Genotype 3
Genotype 5
Genotype 7
Genotype 2
Genotype 1
Ck/USA/GA/40973/Intestine/2005
Ck/USA/GA/97350Tendon/2013
AVS-B (USA)
12297 (USA)
Ck/USA/NC/96815/Tendon/2012
918 (Taiwan)
1017-1 (Taiwan)
Ck/USA/AR/125207/Tendon/2018
Ck/Chile/125350/Tendon/2018
Ck/Chile/125354/Tendon/2018
Ck/Colombia/131411/Tendon/2018
Ck/Colombia/131413/Tendon/2018
Ck/USA/GA/131930/Tendon/2018
PAM-1 (Australia)
Somerville 4 (Australia)
GEI/10 9/M (Germany)
3457-M-11 (Hungary)
284-V-06 (Hungary)
16821-M-06 (Hungary)
GEL13b98M (Germany)
Ck/USA/IA/127720/Tendon/2019
Ck/USA/MS/126610/Tendon/2019
Ck/USA/AR/126692/Tendon/2019
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Ck/USA/MO/131307/Tendon/2018
Ck/USA/MO/131089/Tendon/2018
Ck/USA/GA/96949/Tendon/2013
916 (Taiwan)
Ck/USA/GA/41560/Intestine/2005
Ck/USA/GA/97066/Tendon/2013
Ck/USA/AR/125302/Tendon/2018
GEL13a98M (Germany)
Ck/USA/NC/126092/Tendon/2018
TX/99 (USA)
Ck/USA/AL/126373/Intestine/2018
Ck/USA/MO/126197/Tendon/2018
Ck/USA/MO/126198/Tendon/2018
Ck/USA/MO/126201/Tendon/2018
Ck/USA/MO/125785/Tendon/2018
Ck/USA/MO/126908/Tendon/p2/2018
Ck/USA/TN/126783/Tendon/p1/2018
Ck/USA/TX/115940/Tendon/2016
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Ck/USA/VA/132042/Tendon/2019
Ck/USA/GA/96139/Tendon/2012
Ck/USA/TX/120651/Tendon/2017
S1133 Vaccine (USA)
Fabey-Crawley (Canada)
J408 (USA)
12-1167 (France)
601G (Taiwan)
0.10

Fig. 4.1. Multiple alignments of the of avian reovirus σC sequence were performed using Clustal Omega 1.2.2 (http://www.clustal.org/omega/) in Geneious Prime 2020.2.4 (https://www.geneious.com/) followed by phylogenetic analysis using the neighbor-joining method with 1000 bootstrap replicates using MEGA X (https://www.megasoftware.net/). Accession numbers for the taxa included in the analysis can be found in Table 1 for the genotype 1 and genotype 5 isolates sequenced for this study, and Appendix A, Table 1 for the remaining isolates. The S1133 vaccine strain, 117816 variant, and 94826 variant are highlighted respectively in purple, blue, and green.

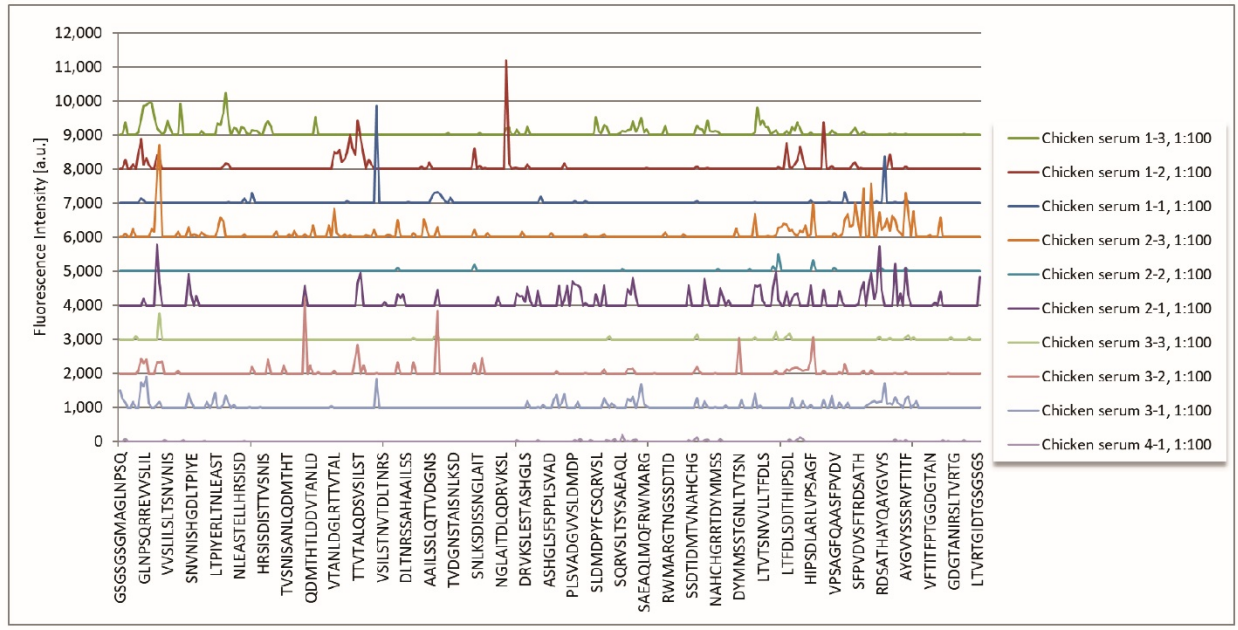

Fig. 4.2. Comparison of all S1133 chicken sera assayed against S1133 σC peptide microarrays. Fluorescence signal intensity baselines on the y-axis are leveled. Birds 1-1, 1-2, and 1-3 are from treatment group one. Birds 2-1, 2-2, and 2-3 are from treatment group two. Birds 3-1, 3-2, and 3-3 are from treatment group three. Bird 4-1 is a non-vaccinated negative control.

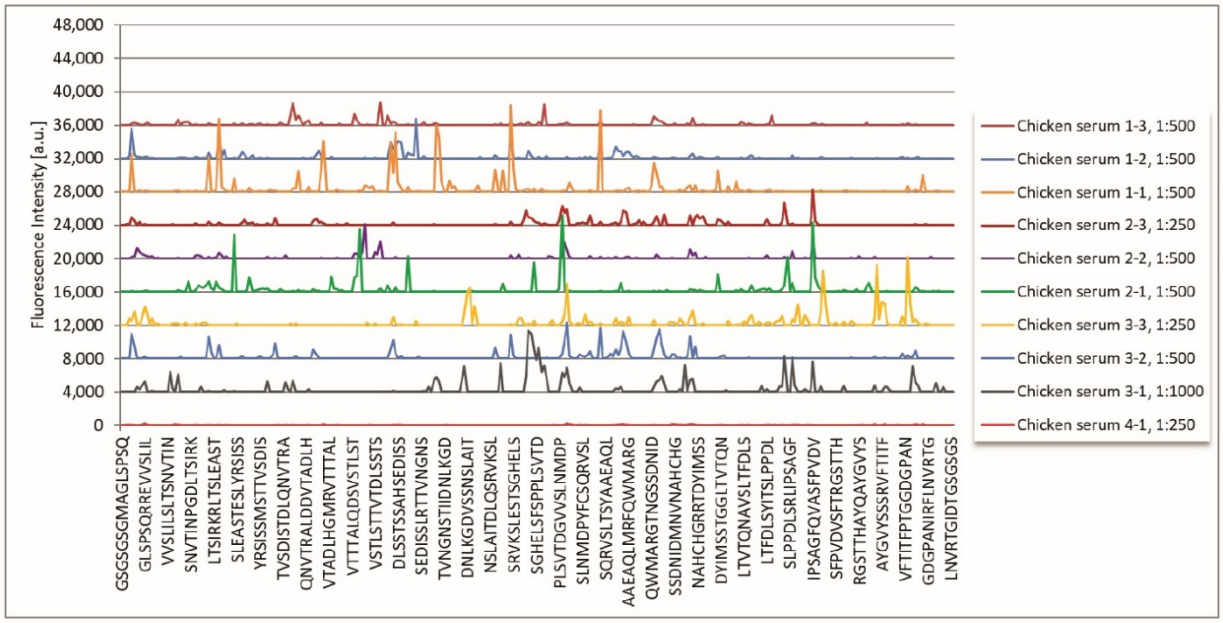

Fig. 4.3. Comparison of all 117816 chicken sera assayed against 117816 σC peptide microarrays. Fluorescence signal intensity baselines on the y-axis are leveled. Birds 1-1, 1-2, and 1-3 are from treatment group one. Birds 2-1, 2-2, and 2-3 are from treatment group two. Birds 3-1, 3-2, and 3-3 are from treatment group three. Bird 4-1 is a non-vaccinated negative control.

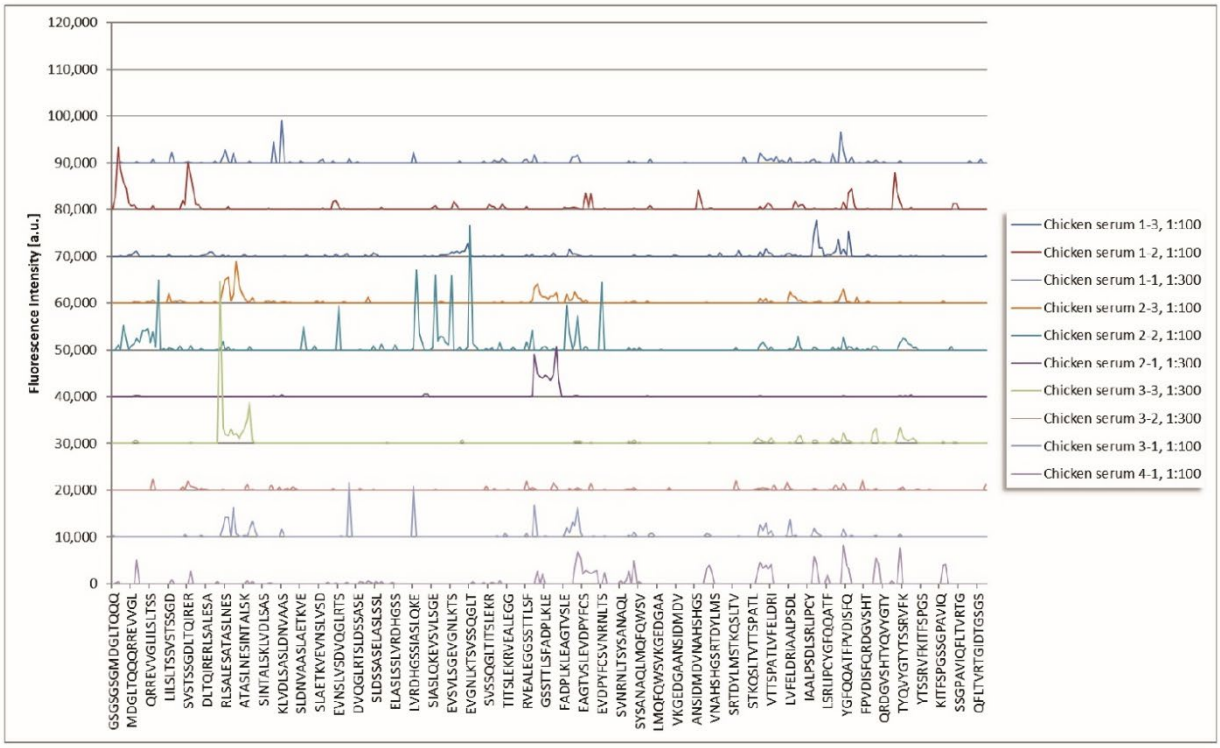

Fig. 4.4. Comparison of all 94826 chicken sera assayed against 94826 σC peptide microarrays. Fluorescence signal intensity baselines on the y-axis are leveled. Birds 1-1, 1-2, and 1-3 are from treatment group one. Birds 2-1, 2-2, and 2-3 are from treatment group two. Birds 3-1, 3-2, and 3-3 are from treatment group three. Bird 4-1 is a non-vaccinated negative control.

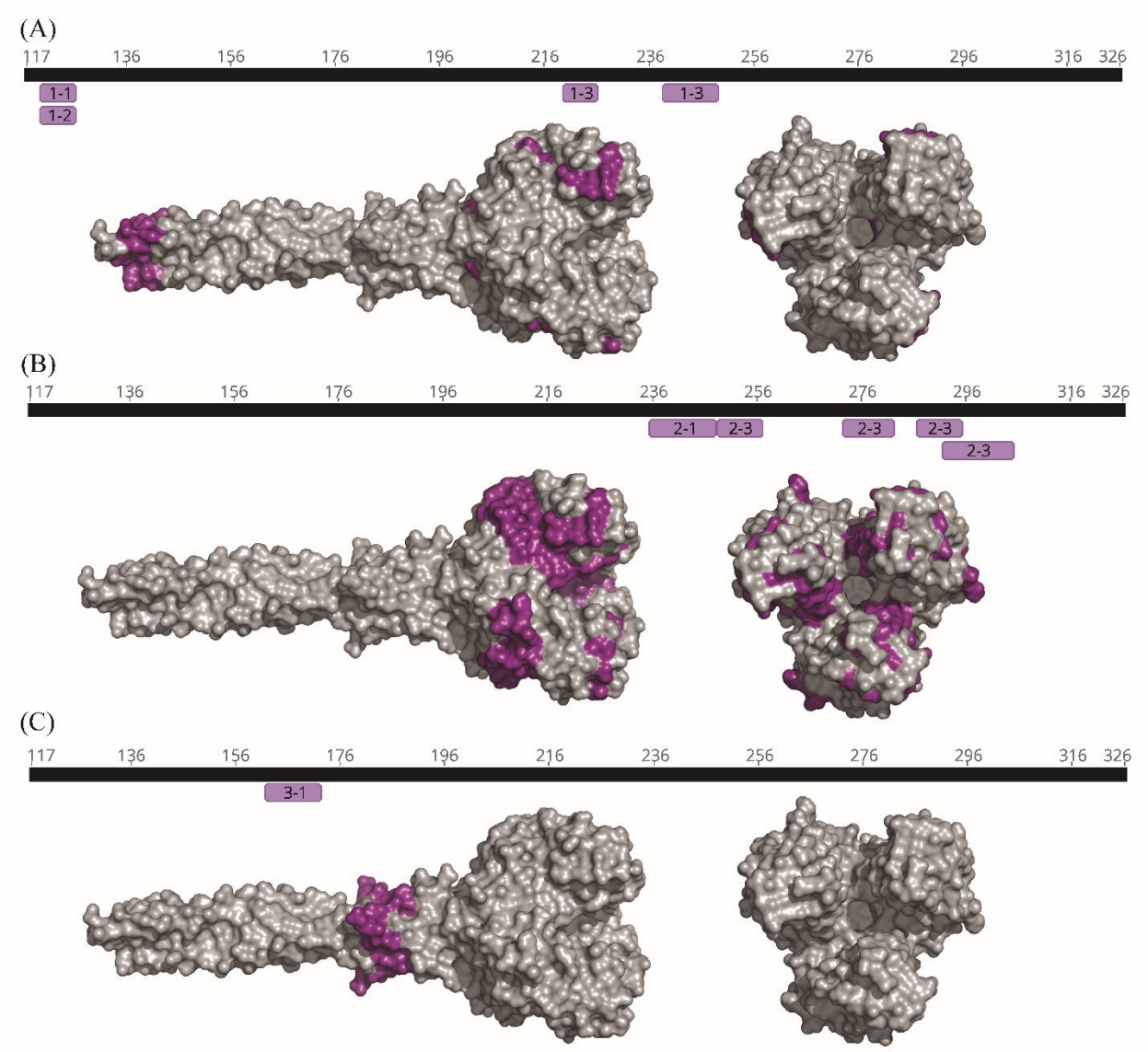

Fig. 4.5. S1133 B-cell epitopes highlighted on the surface of the σC crystal structure PDB 2VRS in purple. Birds vaccinated with live S1133 at four and six weeks-of-age (A), birds vaccinated with inactivated S1133 at four and six weeks-of-age (B) and birds vaccinated with live S1133 at four weeks-of-age and inactivated S1133 at six weeks-of-age (C).

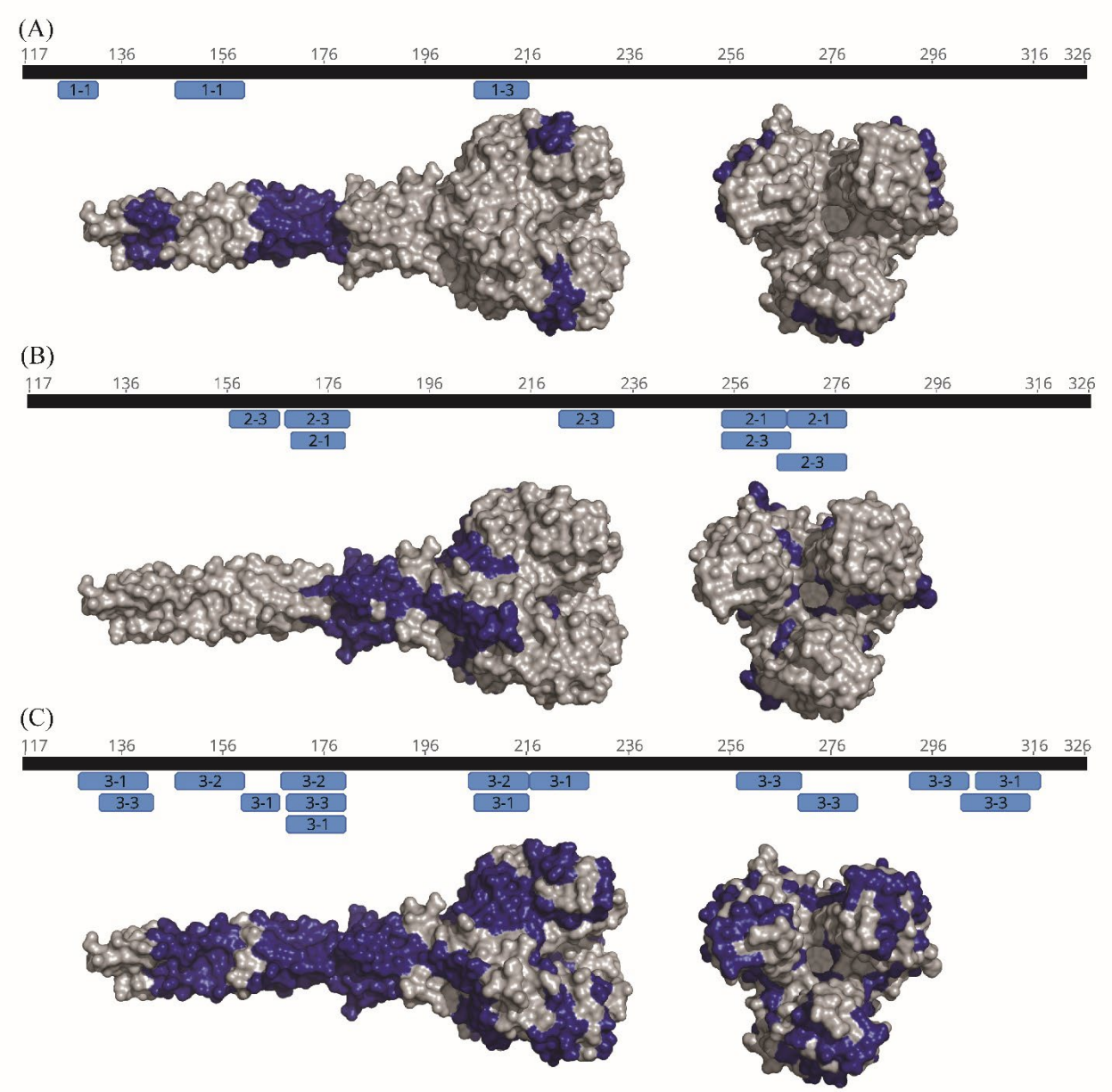

Fig. 4.6. 117816 B-cell epitopes highlighted on the surface of the σC crystal structure PDB 2VRS in purple. Birds vaccinated with live 117816 at four and six weeks-of-age (A), birds vaccinated with inactivated 117816 at four and six weeks-of-age (6B) and birds vaccinated with live 117816 at four weeks-of-age and inactivated 117816 at six weeks-of-age (C).

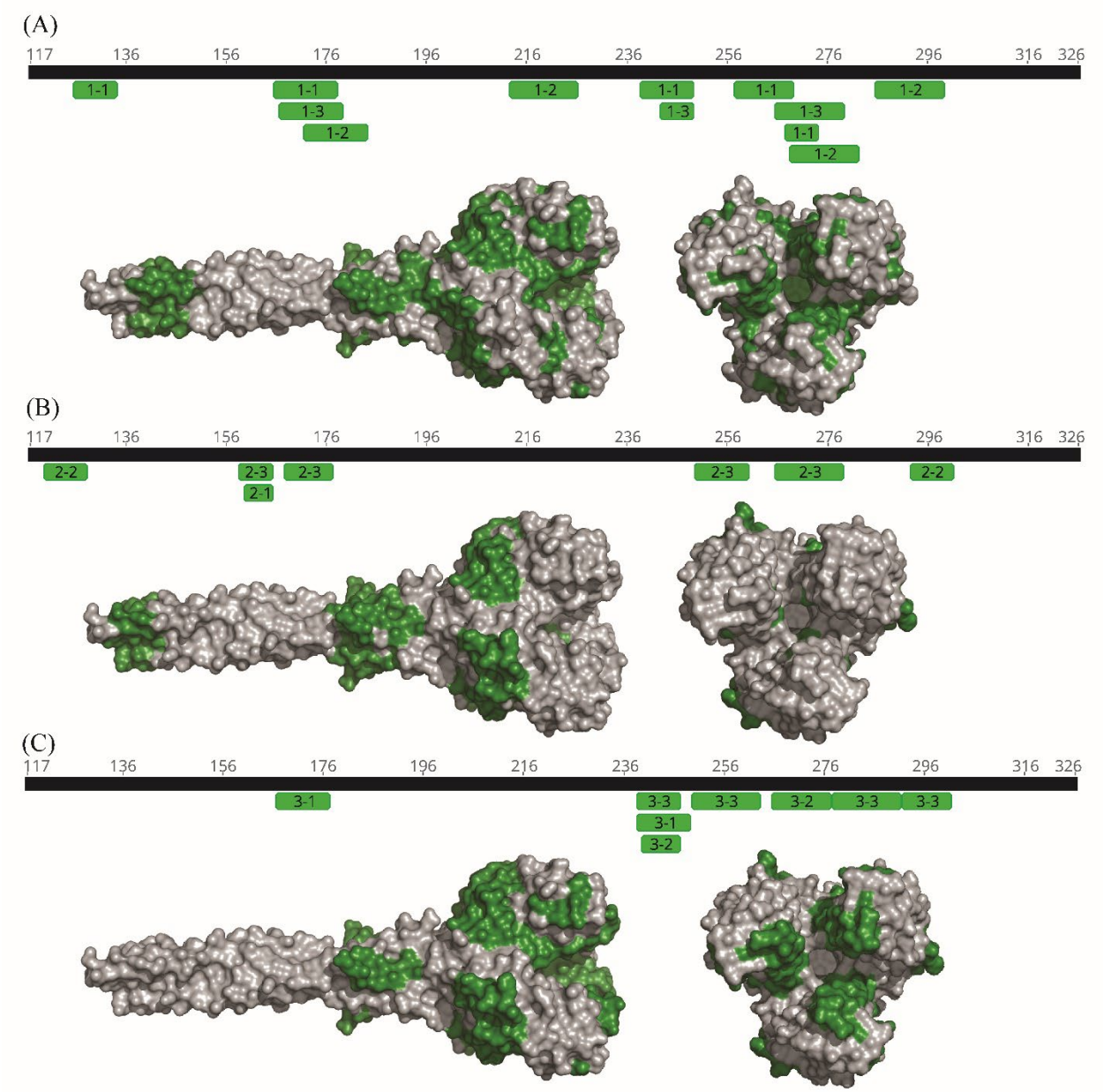

Fig. 4.7. 94826 B-cell epitopes highlighted on the surface of the σC crystal structure PDB 2VRS in purple. Birds vaccinated with live 94826 at four and six weeks-of-age (A), birds vaccinated with inactivated 94826 at four and six weeks-of-age (B) and birds vaccinated with live 94826 at four weeks-of-age and inactivated 94826 at six weeks-of-age (C).

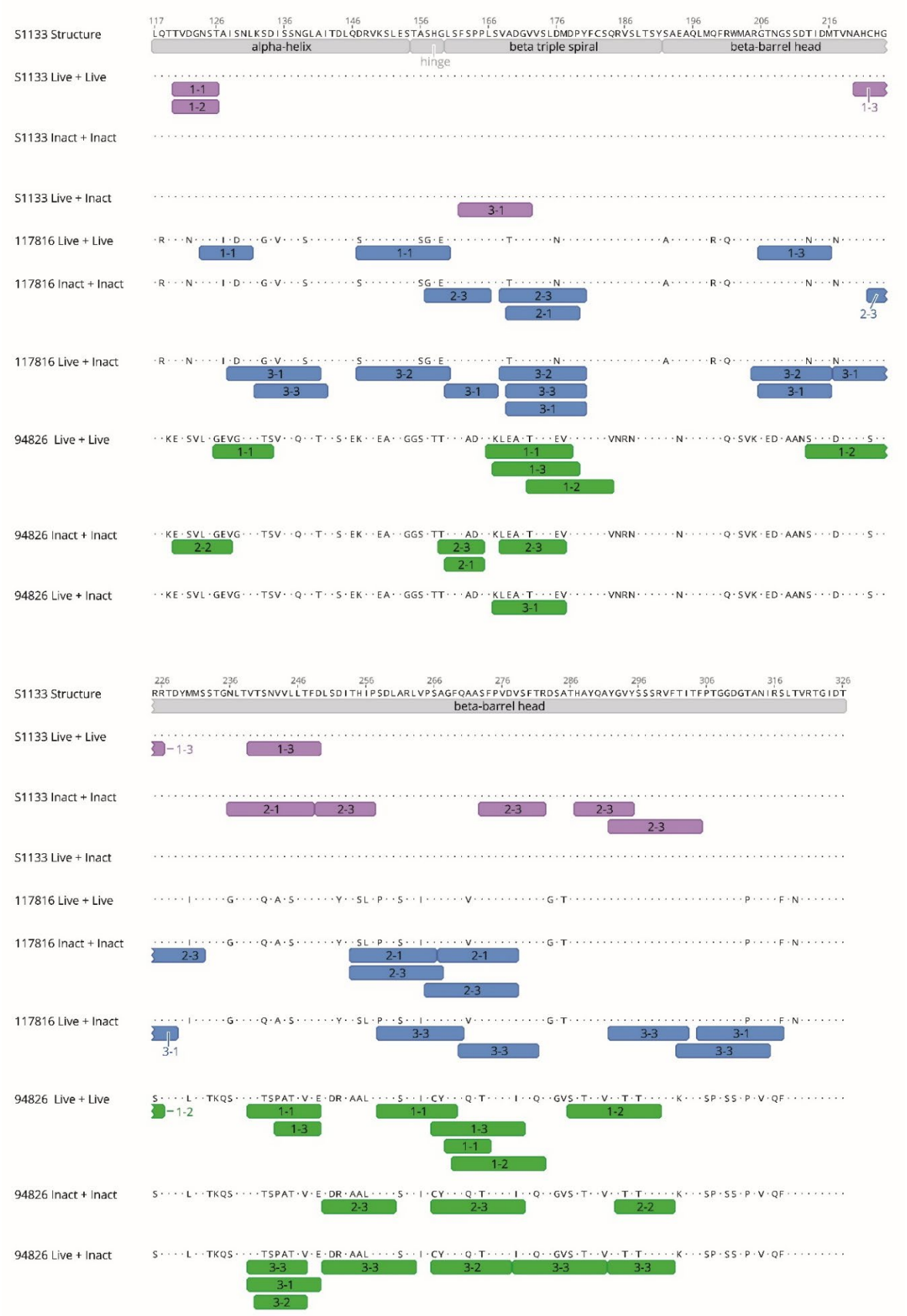
S1133 Structure
LQTTVDGNSTAISNLKSDISSNGLAITDLQDRVKSLESTASHGLSFSPPLSVADGVVSLDMDPYFCSQRVSLTSYSAEAQLMQFRWMARGTNGSSDTIDMTVNAHCHG
alpha-helix
hinge
beta triple spiral
beta-barrel head
S1133 Live + Live
S1133 Inact + Inact
S1133 Live + Inact
117816 Live + Live
117816 Inact + Inact
117816 Live + Inact
94826 Live + Live
94826 Inact + Inact
94826 Live + Inact
S1133 Structure
RRTDYMMSSTGNLTVTSNVVLLTFDLSDITHIPSDLARLVPSAGFQAASFPVDVSFTRDSATHAYQAYGVYSSSRVFTITFPTGGDGTANIRSLTVRTGIDT
beta-barrel head
S1133 Live + Live
S1133 Inact + Inact
S1133 Live + Inact
117816 Live + Live
117816 Inact + Inact
117816 Live + Inact
94826 Live + Live
94826 Inact + Inact
94826 Live + Inact

Fig. 4.8. Multiple alignment of S1133, 117816, and 94826 using S1133 with structural annotations as a reference sequence for the comparison of epitope mapping results between treatment groups and viruses. Sequence positions with a (.) in the place of an amino acid single letter identifier represent residues that are identical to the S1133 reference sequence. Purple bars represent S1133 epitopes, blue bars represent 117816 epitopes, and green bars represent 94826 epitopes. Each bar representing an epitope is labeled with a bird identifier.

APPENDIX A

Table 1. Figure 4.1 taxa utilized in multiple alignments of reovirus σC amino acid sequences, subsequent phylogenetic analyses, and GenBank accession numbers.

Genotype	Isolate	GenBank accession number
1	S1133	AF330703
1	Fahey-Crawley	DQ868789
1	1733	AF004857
1	2408	AF204945
1	601G	AF297217
1	12-1167	HE985301
1	Ck/USA/GA/96139/Tendon/2012	KJ803990
1	Ck/USA/GA/104190/Tendon/2014	KM282091
1	Ck/USA/AR/107174/Tendon/2014	MT127436
1	Ck/USA/TX/115940/Tendon/2016	MT127437
1	Ck/USA/TX/117816/Tendon/2017	MT127438
1	Ck/USA/TX/120651/Tendon/2017	MT127439
1	Ck/USA/MS/125607/Tendon/2018	MT127440
1	Ck/USA/MO/125785/Tendon/2018	MT127441
1	Ck/USA/IN/126109/Tendon/2018	MT127442
1	Ck/USA/MO/126197/Tendon/2018	MT127443
1	Ck/USA/MO/126198/Tendon/2018	MT127444
1	Ck/USA/MO/126201/Tendon/2018	MT127445
1	Ck/USA/TN/126783/Tendon/p1/2018	MT127459
1	Ck/USA/MO/126908/Tendon/p2/2018	MT127458
1	Ck/USA/GA/129204/Tendon/2019	MT127446
1	Ck/USA/VA/132042/Tendon/2019	MT127455
2	GEL13a98M	AF354226
2	916	AF297214
2	TX/99	DQ996602
2	Ck/USA/GA/41560/Intestine/2005	DQ872798
2	Ck/USA/GA/96949/Tendon/2013	KJ879625
2	Ck/USA/GA/97066/Tendon/2013	KJ879635
2	Ck/USA/AR/125302/Tendon/2018	
2	Ck/USA/NC/126092/Tendon/2018	
2	Ck/USA/AL/126373/Intestine/2018	
3	284-V-06	APD15383
3	3457-M-11	KX398288
3	16821-M-06	KX398308
3	GEL13b98M	AF354227
4	918	AF297215
4	1017-1	AF297216

4	12297	JX983600
4	AVS-B	NC_015132
4	Ck/USA/GA/40973/Intestine/2005	DQ872797
4	Ck/USA/NC/96815/Tendon/2012	KJ803996
4	Ck/USA/GA/97350Tendon/2013	KJ879644
5	GEI10 97M	AF354219
5	RAM-1	L38502
5	Somerville 4	L07069
5	Ck/USA/GA/94826/Tendon/2012	KJ803967.1
5	Ck/USA/TN/126783/Tendon/p2/2018	MT127456
5	Ck/USA/MO/131089/Tendon/2019	MT127447
5	Ck/USA/MO/131307/Tendon/2019	MT127448
5	Ck/USA/GA/131328/Tendon/2019	MT127449
5	Ck/Colombia/131416/FTAcard/2019	MT127450
5	Ck/Colombia/131417/FTAcard/2019	MT127451
5	Ck/Colombia/131418/FTAcard/2019	MT127452
5	Ck/USA/TN/131495/Tendon/2019	MT127453
5	Ck/USA/GA/131930/Tendon/2019	MT127454
6	Ck/USA/AR/125207/Tendon/2018	
6	Ck/Chile/125350/Tendon/2018	
6	Ck/Chile/125354/Tendon/2018	
7	Ck/USA/IA/127720/Tendon/2018	
7	Ck/USA/MS/126610/Tendon/2018	
7	Ck/USA/AR/126692/Tendon/2018	
7	Ck/USA/AR/131592/Tendon/2019	
7	Ck/USA/NC/131593/Tendon/2019	

Table 2. S1133 linear B-cell epitopes and single peptide interactions.

Bird ID	Peptide microarray serum antibody reaction type and peptides identified	
	Epitope-like spot patterns	Single peptide interactions
1-1	^{4}LNPSQRREVVSLI^{16},^{120}TVDGNST^{126}	^{91}DSVSILSTNVTDLTN^{105}, ^{283}DSATHAYQAYGVYSS^{297}
1-2	^{9}RREVVSLI^{16}, ^{84}TTVTAL^{89}, ^{120}TVDGNST^{126}	^{128}ISNLKSDISSNGLAI^{142}, ^{140}LAITDLQDRVKSLES^{154}, ^{260}LARLVPSAGFQAASF^{274},
1-3	^{9}RREVVSLIL^{17}, ^{51}ISDISTTVSNISA^{63}, ^{220}AHCHGRR^{226}, ^{239}VTSNVVLLTFD^{249}	^{17}LSLTSNVNISHGDLT^{31}
2-1	^{9}RREVVSLILSLTSN^{22}, ^{85}TVTALQDSVSILST^{98},^{236}NLTVTSNVVLLTF^{248}	None
2-2	^{3}GLNPSQRREV^{12}	^{128}ISNLKSDISSNGLAI^{142}, ^{243}VVLLTFDLSDITHIP^{257},
2-3	^{9}RREVVSLILSLT^{20}, ^{33}IYERLTNLEASTEL^{46}, ^{249}DLSDITHIP^{257}, ^{273}SFPVDVSFTR^{282}, ^{287}HAYQAYGVY^{295}, ^{292}YGVYSSSRVFTITF^{305}	^{256}IPSDLARLVPSAGFQ^{270}
3-1	^{1}MAGLNPSQ^{8}, ^{5}NPSQRREVVSLI^{16}, ^{162}FSPPLSVADGV^{172}	^{91}DSVSILSTNVTDLTN^{105}
3-2	^{4}LNPSQRREVVSLI^{16}, ^{9}REVVSLILSLTSN^{22}	^{64}NLQDMTHTLDDVTAN^{78}, ^{114}LSSLQTTVDGNSTAI^{128}, ^{228}DYMMSSTGNLTVTSN^{242}, ^{256}IPSDLARLVPSAGFQ^{270},
3-3	None	^{9}RREVVSLILSLTSNV^{23}

Table 3. 117816 linear B-cell epitopes and single peptide interactions.

Bird ID	Peptide microarray serum antibody reaction type and peptides identified	
	Epitope-like spot patterns	Single peptide interactions
1-1	^{35}KRLTSLEASTE^{45}, ^{73}DDVTADLHGMRVTT^{86}, ^{102}DLSSTSSAHSED^{113}, ^{124}NSTIIDNL^{131}, ^{147}SRVKSLESTSGHEL^{160}	^{1}MAGLSPSQRREV^{12}, ^{28}GDLTSIRKRLTSLEA^{42}, ^{181}FCSQRVSLTSYAAEA^{195}
1-2	^{103}LSSTSSAHSED^{113}, ^{109}AHSEDISSLRTT^{120}	^{1}MAGLSPSQRREV^{12}, ^{32}SIRKRLTSLEASTES^{46}, ^{146}QSRVKSLESTSGHEL^{160}
1-3	^{66}QNVTRALDDV^{75}, ^{87}TALQDSVSTLSTT^{99}, ^{206}GTNGSSDNIDM^{216}	^{17}LSLTSNVTINPGDLT^{31}, ^{95}TLSTTVTDLSSTSSA^{109}, ^{159}ELSFSPPLSVTDGVV^{173}
2-1	^{31}TSIRKRLT^{38}, ^{80}HGMRVTTTALQ^{90}, ^{87}TALQDSVSTLSTT^{99}, ^{169}TDGVVSLNMDP^{179}, ^{254}TSLPPDLSRLIPS^{266}, ^{267}AGFQVASFPVDV^{278}	^{39}SLEASTESLYRSI^{51}, ^{106}TSSAHSEDISSLRTT^{120}, ^{155}TSGHELSFSPPLSVT^{169}
2-2	^{6}PSQRREVVS^{14}, ^{89}LQDSVSTLSTT^{99}, ^{95}TLSTTVTDLSSTS^{107}	^{264}IPSAGFQVASFPVDV^{278}
2-3	^{157}GHELSFSPPL^{166}, ^{168}VTDGVVSLNMDPY^{180}, ^{222}CHGRRTDYIMS^{232}, ^{254}TSLPPDLSRLIPSA^{267}, ^{265}PSAGFQVASFPVDV^{278}	None
3-1	^{14}SLILSLTSNVTINP^{27}, ^{128}IDNLKGDVSSNSLA^{141}, ^{160}LSFSPPLS^{167}, ^{169}TDGVVSLNMDPY^{180}, ^{206}GTNGSSDNIDM^{216}, ^{217}NVNAHCHGRRTD^{228}, ^{305}FPTGGDGPANIRF^{317}	^{16}ILSLTSNVTINPGDL^{30}, ^{142}ITDLQSRVKSLESTS^{156}, ^{253}ITSLPPDLSRLIPSA^{267}, ^{256}LPPDLSRLIPSAGFQ^{270}, ^{264}IPSAGFQVASFPVDV^{278}
3-2	^{1}MAGLSPSQRREV^{12}, ^{101}TDLSSTSSAHSED^{113}, ^{147}SRVKSLESTSGHEL^{160}, ^{168}VTDGVVSLNMDPY^{180}, ^{205}RGTNGSSDNIDM^{216}	^{28}GDLTSIRKRLTSLEA^{42}, ^{32}SIRKRLTSLEASTES^{46}, ^{54}MSTTVSDISTDLQNV^{68}, ^{181}FCSQRVSLTSYAAEA^{195}, ^{216}MNVNAHCHGRRTDYI^{230}
3-3	^{4}LSPSQRREVVSLI^{16}, ^{101}TDLSSTSSAHSEDI^{114}, ^{132}KGDVSSNSLAI^{142}, ^{169}TDGVVSLNMDPY^{180}, ^{258}PDLSRLIPSAGFQ^{270}, ^{270}QVASFPVDVSFT^{281}, ^{292}YGVYSSSRVFTI^{303}, ^{302}TITFPTGGDGPANI^{315}	None

Table 4. 94826 linear B-cell epitopes and single peptide interactions.

Group/bird ID	Peptide microarray serum antibody reaction type and peptides identified	
	Epitope-like spot patterns	Single peptide interactions
1-1	^{126}GEVGNLKTS^{134}, ^{166}LKLEAGTVSLEVD^{178}, ^{239}VTTSPATLVFE^{249}, ^{258}SDLSRLIPCYGF^{269}, ^{268}GFQQATF^{274}	None
1-2	^{1}MDGLTQQQR^{9}, ^{24}STSSGDLTQIRER^{36}, ^{78}EVNSLVSDVQGLR^{90}, ^{172}TVSLEVDPYFCSV^{184}, ^{213}SIDMDVNAHSHGSR^{226}, ^{269}FQQATFPVDISFQR^{282}, ^{286}SHTYQVYGTYTSSR^{299}	None
1-3	^{36}RLSALESATASLNES^{50}, ^{167}KLEAGTVSLEVDP^{179}, ^{243}PATLVFE^{249}, ^{266}CYGFQQATFPVDIS^{279}	^{16}ILSLTSSVSTSSGDL^{30}, ^{39}ALESATASLNESINT^{53}, ^{54}ALSKLVDLSASLDNV^{68}, ^{57}KLVDLSASLDNVAAS^{71}, ^{106}LVRDHGSSIASLQKE^{120}
2-1	^{160}LSFADP^{165}	None
2-2	^{1}MDGLTQQQRREV^{12}, ^{11}EVVGLIL^{17}, ^{109}DHGSSIASLQKEV^{121}, ^{120}EVSVLSGEV^{128}, ^{293}GTYTSSRVF^{301}	^{65}LDNVAASLAETKVEV^{79}, ^{78}EVNSLVSDVQGLRTS^{92}, ^{127}EVGNLKTSVSSQGLT^{141}, ^{176}EVDPYFCSVNRNLTS^{190}
2-3	^{43}ATASLNE^{49}, ^{159}TLSFADP^{165}, ^{168}LEAGTVSLEV^{177}, ^{250}LDRIAALPSDL^{260}, ^{266}CYGFQQATFPVDIS^{279}	^{15}LILSLTSSVSTSSGD^{29}, ^{89}LRTSLDSSASELASL^{103}
3-1	^{39}ALESATASLNE^{49}, ^{47}LNESINTALSKLV^{59}, ^{167}KLEAGTVSLEV^{177}, ^{239}VTTSPATLVFE^{249}	^{82}LVSDVQGLRTSLDSS^{96}, ^{106}LVRDHGSSIASLQKE^{120}, ^{151}ALEGGSSTTLSFADP^{165}, ^{246}LVFELDRIAALPSDL^{260}
3-2	^{30}LTQIRER^{36}, ^{240}TTSPATLV^{247}, ^{266}CYGFQQATFPVD^{277}	^{9}RREVVGLILSLTSSV^{23}, ^{44}TASLNESINTALSKL^{58}, ^{53}TALSKLVDLSASLDN^{67}, ^{148}RVEALEGGSSTTLSF^{162}, ^{172}TVSLEVDPYFCSVNR^{186}, ^{226}RTDYLMSTKQSLTVT^{240}, ^{273}TFPVDISFQRDGVSH^{287}, ^{319}TVRTGIDT^{326}GSGSGSG
3-3	^{45}ASLN^{48}, ^{239}VTTSPATLV^{247}, ^{250}LDRIAALPSDLSRL^{263}, ^{278}ISFQRDGVSHTYQV^{291}, ^{292}YGTYTSSRVF^{301}	None

CHAPTER 5

CONCLUSION

ARV is ubiquitous in poultry and causes economic losses to the commercial broiler, layer, and turkey industries worldwide. Control by vaccination was historically effective and dates back to the 1970's. In the U.S., commercially available ARV vaccines are all antigenically similar to the S1133 virus isolated by van der Heide in Connecticut in 1975. Since 2010, the emergence of variant ARVs antigenically and genetically distinct from the commercially available vaccines have been problematic for the industry. To date, 7 genotypes have been reported based on phylogenetic analysis of the σC protein. The genome of ARV consists of segmented dsRNA and the emergence of variants is attributed to reassortment events, accumulation of point mutations, and selective pressures from vaccination. In addition, the control of transmission is challenging because ARV is resistant to low pH, heat, and solvents.

Despite the increased incidence of viral arthritis/tenosynovitis in recent years, the use of commercially available vaccines derived from the 45-year-old S1133 isolate is still common practice. Vaccination programs vary widely between companies and many include the use of autogenous inactivated ARV vaccines containing one or more isolates. The use of custom-made vaccines is permitted under a limited-license for production and complicates control efforts after the license expires. ARV vaccination programs in the U.S. aim to: 1) generate antibody responses in broiler-breeders to protect these birds from infection to limit losses due to disease in these flocks 2) prevent vertical transmission to progeny broilers and 3) providing maternal antibodies to progeny broilers to protect them from becoming infected at a young age when they are most

susceptible. To achieve a long-lasting antibody response, broiler-breeders are usually given multiple live-attenuated vaccinations beginning at day-of-hatch and periodically throughout the first several weeks of life. Before the birds come into production, they are vaccinated with commercially available inactivated vaccines, and custom-made vaccines, if they are being used. We carried out an investigation to evaluate the reovirus antibody response in chickens following vaccination with a combination of commercial S1133 live attenuated/inactivated and a bivalent GT5 CMV. The results of the study provided evidence to support the common belief that homologous live and inactivated vaccines elicit a higher neutralizing antibody compared to heterologous combinations. This investigation also concluded the antibody response to vaccination with the GT5 CMV was substantially improved when used in conjunction with a commercially available live-attenuated vaccine. Despite the antigenic dissimilarity between GT5 and the commercial live-attenuated vaccines, this work supports the continued use of classic vaccines to improve antibody responses to custom-made vaccines.

Passaging of the S1133 virus in embryos and cell culture for attenuation was historically effective, but required years of passaging to produce safe, highly attenuated vaccine strains. S1133 modified-live vaccines were attenuated by passaging the S1133 isolate 235 times in SPF embryos, followed by 65 passages in chicken embryo fibroblast (CEF) cells at 32°C, and an additional 35 passages in CEF cells at 37°C. The use of cold adaptation in the attenuation process of S1133 captured our interest and we carried out a series of experiments to evaluate the potential for the rapid attenuation of variant reoviruses for use as antigenically relevant vaccines. We used the GT1 variant 117816 that was isolated from a clinical case of disease and antigenically distinct from S1133. We were able to successfully propagate this virus in an immortal cell line at 32°C. Following 42 passages at the reduced temperature, the replication dynamics of the virus indicated

the emergence of a cold-adapted phenotype. The cold-adapted virus was less pathogenic in day-old broiler chicks compared to the parent virus isolate, providing evidence this method has the potential for use as a more rapid means of attenuating variants. Day old chicks were used for initial evaluation of pathogenicity of parent and cold-passaged viruses, but evaluation of the pathogenicity of these viruses in older birds would provide information about the potential utility of the cold-passaged virus as a vaccine in older birds. The cold-passaged virus has not been evaluated in older birds or tested for safety, immunogenicity, or efficacy, so these are the next logical steps in evaluating the utility of the cold-passaging process. An in vivo back-passage study to evaluate reversion to virulence would also aid in further evaluating the phenotypic stability and safety of the cold-passaged virus.

In order to better understand the antigenic similarities and differences between the commercially available classic vaccines and variant ARVs, we carried out a study to identify linear B-cell epitopes in the S1133 GT1 vaccine, 117816 GT1 variant, and 94826 GT5 variant. Epitopes were identified in areas of the globular head and beta-triple spiral domains of σC that have been shown in mammalian reoviruses to be the sites responsible host cell receptor binding. We also observed that B-cell responses to σC were more complex when birds were vaccinated with a series of live and inactivated variant viruses compared to birds that received a series of only live or inactivated variant viruses. This finding further supports benefit from the common structure of ARV vaccination programs where birds receive live vaccinations early in life, then inactivated vaccinations prior to the onset of lay, and suggests a live-inactivated dose-series results in B-cell responses with optimal specificity and duration. We identified many linear B-cell epitopes for three antigenically distinct ARVs, but further work is needed to clarify which of the identified epitopes are associated with the induction of neutralizing antibodies. Future studies should also

investigate the serologic relationships by two-way cross neutralizations between the variants we performed epitope mapping on and isolates within their respective genotypes and genotype subgroups to better understand the impact of amino acid substitutions present in variants in antibody binding sites identified for the 117816 GT1 variant and the 94826 GT5 variant.

This work has examined antibody responses to current vaccination strategies that include variants in custom made vaccines, identified the utility of a rapid method for attenuation of variants that could be used to create novel live vaccines based on clinically relevant isolates, and identified the antibody binding sites on σC for the classic S1133 vaccine, a GT1 variant, and a GT5 variant. The results of this work will be used to improve ARV control efforts, thereby reducing economic and animal welfare impacts caused by ARV infections in commercial poultry.