Molecular, Pathogenic, and Antigenic Characterization of Emerging Avian Reovirus Variant Strains

By

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ABSTRACT

Avian reoviruses are recognized for their genetic variability. The emergence of pathogenic variant strains causes negative impacts to the poultry industry worldwide. The disease prevention is based on the use of live classical and inactivated autogenous vaccines, however, they have a limited effect against the circulating variants, so they require a frequent update. The current molecular classification methods use the partial S1 gene that encodes the σC protein, but different genotypes do not always correlate with pathogenicity and sometimes induce a deficient cross-protection. Therefore, investigation of other variable genes and correlations between pathogenicity and antigenicity of variant strains is crucial for a better classification method. The present investigation focuses on the molecular, pathogenic, and antigenic characterization of emerging avian reovirus variant strains. This research is divided into three chapters. In the first chapter, we did a molecular characterization based on the S1 gene of 85 avian reoviruses obtained from the California outbreak and we did a whole-genome characterization of a portion of them. The characterized reoviruses were categorized in six genotypic clusters. We detected a shift in cluster representation throughout time occurred. According to the whole genome analyses, our results suggested that the L3, M2 and S1 genes accounted for most of the variability of these viruses. In chapter two we investigated the pathogenicity within the same S1 genotypic cluster variants. The genomes of two variant strains and a classical strain belonging to the same S1 genotype one were compared. Additionally, these strains were used in a challenge experiment involving inoculated and horizontally exposed specific-pathogen-free chickens. The whole-genome sequence analysis of the three strains revealed nucleotide identity differences in the L3, M2, and S1 genes. In addition, the variant strains indicated nucleotide identities differences in

the S4 gene, despite having high homologies in all other genes. The challenge experiments showed that variant and classical strains replicated differently in tendons, hearts and duodena of the challenged and exposed chickens and caused dissimilar pathologic lesions and lymphoid depletion degrees in bursas and thymi of the challenged chickens. Because we consider that it is critical to associate genetic changes with the pathogenicity and antigenicity of variant strains in the selection of autogenous vaccines, in chapter three, we explored the association between the genetic and antigenic characteristics of 29 avian reoviruses, including conventional and variant strains using genetic and antigenic cartography. We detected high variation in terms of antigenicity which was not strongly correlated with the genetic composition of variable genes. The information generated in the present investigation contributes to the understanding of the epidemiology and pathobiology of avian reoviruses variant strains. In addition, it provides insights on how other genes besides S1 add variability in the diverse avian reovirus phenotypes.

To my beloved parents Maria and Richard, who encourage me every day to pursue my dreams no matter how big the obstacles are.

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INTRODUCTION AND OUTLINE

Avian Reoviruses (ARV) belong to the genus Orthoreovirus and are prevalent worldwide in chickens, turkeys and other bird species (1). They are ubiquitous in poultry farms worldwide. The ARV pathogenic strains cause endemic infections in chickens ranging from viral arthritis-tenosynovitis, pericarditis, immunosuppression (2), to enteric and respiratory disorders (1,3). The impact of ARV infections is represented by economic losses due to poor productive parameters, condemnations at processing and welfare issues in meat-type poultry. Avian reoviruses possesses a double-stranded and segmented ribonucleic acid (RNA) genome predisposing them to mutations, recombination and reassortment events that generate variant strains.

Since 2011, the poultry industry has been facing the consequences of the emergence these variants (4–6). The variant ARV strains have been linked to severe viral arthritis, tenosynovitis and pericarditis in meat type chickens. Current preventative strategies are based in the use of classic and autogenous vaccines that, occasionally, provide a limited protection. Therefore, it is critical to perform ARV molecular surveillance, understand better their pathogenicity and antigenicity that contribute to an accurate characterization , the selection of autogenous vaccines and elucidate the genetic bases for antigenicity. These were all goals of this Ph.D. project.

In the first chapter, we evaluated the genetic diversity of avian reoviruses associated with clinical outbreaks in broiler chickens by performing molecular characterization based on partial S1 gene sequences and whole genome characterization of selected isolates. We observed a high

divergence of the S1 gene represented by the distribution of the ARV into six distinct genotypic cluster and we observed a shift on genotype representation between the isolates obtained since 2015 to 2018 due to the evolutive pressure that vaccines added. Additionally, detected high variability in the L3 and M2 genes, which encode structural proteins that might play a role in the pathogenicity and antigenicity of ARV. Our results contributed to the epidemiology of avian reoviruses.

In the second chapter, we developed a challenge model to assess the pathogenicity, transmissibility and immunosuppression of selected ARV variants that belonged to the same σC genotype. We detected different pathologic outcomes regarding viral loads in tendons, hearts, and duodena and microscopic lesions in tendons of the inoculated chickens. We were also able to assess lymphoid depletion in bursas and in thymi of inoculated and exposed birds. A genomic analysis highlighted difference in L3, M2, S1 and S4 genes of the characterized viruses. Our results contributed to build associations between genomic composition and pathogenicity of avian reoviruses.

The third chapter focused on finding associations between the genetic and antigenic composition of ARV variants. We employed an antigenic and genetic cartography model to assess the variability of the L3, M2, S1 and S4 genes. Our results showed different levels of cross-neutralization between a selected antisera panel and selected avian reoviruses. The genetic characterization exposed noticeable diversity between the ARV genes. We found a positive correlation between genetic and antigenic profiles in the L3 and partial S1 genes. Our investigation provides the baseline for study the antigenic and genetic relatedness of avian reoviruses variant strains.

Each chapter of this dissertation is represented by a research paper. The first and second chapters have been published in Scientific reports (7) and Avian Diseases (8), respectively. The last chapter is in preparation for publication in peer-reviewed journal.

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CHAPTER 1

Genotypic Characterization of Emerging Avian Reovirus Genetic Variants in California

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SUMMARY

This study focuses on virus isolation of avian reoviruses from a tenosynovitis outbreak between September 2015 and June 2018, the molecular characterization of selected isolates based on partial S1 gene sequences, and the full genome characterization of seven isolates. A total of 265 reoviruses were detected and isolated, 83.3% from tendons and joints, 12.3% from the heart and 3.7% from intestines. Eighty five out of the 150 (56.6%) selected viruses for

sequencing and characterization were successfully detected, amplified and sequenced. The characterized reoviruses grouped in six distinct genotypic clusters (GC1 to GC6). The most represented clusters were GC1 (51.8%) and GC6 (24.7%), followed by GC2 (12.9%) and GC4 (7.2%), and less frequent GC5 (2.4%) and GC3 (1.2%). A shift on cluster representation throughout time occurred. A reduction of GC1 and an increase of GC6 classified strains was noticed. The highest homologies to S1133 reovirus strain were detected in GC1 (~77%) while GC2 to GC6 homologies ranged between 58.5 and 54.1%. Over time these homologies have been maintained. Seven selected isolates were full genome sequenced. Results indicated that the L3, S1 and M2 genes, coding for proteins located in the virus capsid accounted for most of the variability of these viruses. The information generated in the present study helps the understanding of the epidemiology of reoviruses in California. In addition, provides insights on how other genes that are not commonly studied add variability to the reovirus genome.

KEY WORDS: Avian reoviruses, characterization, molecular, whole genome, genotypes

INTRODUCTION

Avian reoviruses (ARV) are non-enveloped and possess a double-stranded, segmented ribonucleic acid (RNA) genome. They are members of the family Reoviridae, subfamily *Spinareovirinae* and genus *Orthoreovirus*. The ten different segments that have been identified and classified based on their electrophoretic mobility are: L1-L3, M1-M3 and S1-S4. The S1 segment encodes for three viral proteins, including the minor capsid protein σC (1). This particular protein plays a key role during early stages of infection, mediating the interaction between the virion and the host cell, and elicits type-specific neutralizing antibodies (2,3).

Amplification and sequencing analysis of the portion of the S1 gene that encodes the σC protein, has been commonly used as a genetic marker for the characterization and classification of ARV isolates (4-7). To date, five and six genotypes have been described based on Lu and Kant's classification, respectively (2,6).

Extreme variability is an inherent characteristic of ARV. This is based on their RNA nature and their segmented genome favoring mutations, recombination and reassortment events (8,9). Since 2011, the poultry industry worldwide has been facing the consequences of the emergence of ARV variants (6,7,10-12). These variants of ARV have been linked to severe viral arthritis, tenosynovitis and pericarditis mainly in vaccinated broiler chickens and their breeders (13, 14). The above mentioned pathological outcomes, in addition to subclinical presentations of the disease (10) cause severe economic losses to the poultry industry. The affected productive parameters on ARV diseases are represented by reduced weight gain, lack of flock uniformity, impaired feed conversion rates, increased condemnations in the processing plants and welfare issues related to lameness (15). In California, the reovirus tenosynovitis outbreak started in August 2015. It has affected broilers ranging from 14 to 47 days of age. Clinically, broiler flocks reported lameness due to deviation of legs either laterally or anteriorly, stunting and lack of uniformity. Most of the broilers had swollen hock joints with increased exudate that extended along the tendon sheath. Morbidity ranged between 0.3 to 15% and mortality ranged from 0.1 to 1%.

Despite the better understanding of the biology of the virus, their variability and the efforts of several groups across the U.S. (5,6,16, 17), Europe (14, 18) , Canada (19, 20) and China (10) in detecting and typing ARV variants, classical vaccine strains used for immunization of

commercial flocks, namely S1133, 1733 and 2408, have not changed since the 1970's. These strains have proven to be inefficient in controlling the infection, partly due to the RNA virus nature being prone to mutation and recombination events and generating variants that are partially or incompletely protected by antibodies generated by classical vaccine strains. The generation of variant strains that circumvent vaccine immunity, perpetuate the cycle of variability, and enhances the need for prompt detection, typing and autogenous vaccine formulation (5,18,21). The first step to generate control and prevention strategies against reoviruses is to be able to characterize the strains causing disease in the field and based on that characterization select virus strains to be included in autogenous vaccines.

This study focused on virus isolation of avian reoviruses between September 2015 and June 2018, the molecular characterization of selected reoviruses based on partial S1 gene sequences, and the full genome characterization of seven selected isolates. These detection and characterization efforts have generated a molecular surveillance system that can be used to assess the variability of reoviruses in the field and guide virus selection for vaccine production in the State of California.

MATERIALS AND METHODS

ARV isolation

Tendon, heart, joint, intestine, liver and pancreas from broiler chickens suspected of tenosynovitis and pericarditis were individually minced with a scalpel and homogenized in viral transport media (VTM) using a gentleMACS Octo dissociator (Milteny Biotech, Bergisch Gladbach, Germany). Homogenized samples were diluted in VTM to a concentration of 1:10 weight/volume

and syringe filtered through a 0.2-micron sterile filter. One millilitre of filtered homogenate was inoculated onto 70 -90% confluent chicken embryo liver (CEL) cells in 12.5 cm2 tissue culture flasks and incubated at 37^{o}C for 1 hour. The cells were rinsed with 2ml of Hank's balanced salt solution and 2.5 ml of 1% fetal bovine serum (FBS) maintenance medium was added to each flask. The flasks were kept in a 5% CO2 incubator at 37^{o}C for up to 5 days. The flasks were observed daily, compared to a negative control flask, for the development of characteristic cytopathic effect (CPE). Samples that showed evidence of reovirus-like CPE were submitted for RT-PCR confirmation of a conserved segment of the S4 gene (23). Samples with no visible CPE after 5 days were freeze and thawed 3 times and re-inoculated onto fresh CEL cells for a 2nd passage.

Reovirus molecular characterization

One hundred and fifty virus isolates obtained from broiler cases of tenosynovitis between September 2015 and June 2018 were selected for molecular characterization. The selection criteria involved clinical relevance, gross pathology and cytopathic effect in CEL. Isolates, confirmed as positive by RT-PCR of the S4 gene (437 bp) (23), by using the primers described by Bruhn et al., ARV_S4_P4 5'-GTGCGTGTTGGAGTTTC-3' and ARV_S4_P5 5'-ACAAAGCCAGCCATRAT-3' were submitted for a partial S1 gene (1,088 bp) RT-PCR using the primers described by Kant *et al*, P1 5'-AGTATTTGTGAGTACGATTG-3' and P4 5'-GGCGCCACACCTTAGGT-3' (2). Positive samples were sent for sequencing using Kant's forward and reverse primers to obtain a segment of 1,088 bp of the S1 gene (2) (Supplemental Table S1.1). Nucleotide sequences were transformed into amino acid sequences. Amino acid sequence

identities to the vaccine strain (S1133) were calculated from the effectively sequenced isolates, using Clustal Omega (27). Sequence alignments were crafted, using MEGA7 (28) including 49 reference sequences obtained from the available literature (2,6,9,11,20,22) and three sequences representing commercially available vaccines: S1133 (AF330703), 1733 (AF004857) and 2408 (AF204945). The 49 sequences obtained from the literature represented the previously described genotypic groups for ARV (Supplemental Table S1.2). A segment of 278 amino acids corresponding to position 678 to 1,512 of the full S1 gene of each ARV strain was used in the phylogenetic analysis. This analysis was performed using maximum likelihood method with 1,000 bootstrap replicates using MEGA7 (28). Phylogenetic trees were generated using FigTree (29) obtaining a visual representation of the genetic clusters.

Whole genome sequencing of ARV isolates

In order to determine the variability of the ARV genes, seven ARV isolates were selected, based on clinical signs severity in the field, tissue of isolation (tendon / joints and heart) and CPE in cells, and submitted to full genome sequencing: K1502030, T1502036, T1600137, T1600260, K1600402, K1600600 and K1600657. Extraction of RNA was completed from 100 □l of the isolate using Trizol (ThermoFisher, Waltham, MA). Deoxyribonucleic acid (DNA) was removed using the Turbo DNA-free kit, followed by rRNA depletion using the Terminator 5'-Phosphate-Dependent Exonuclease (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's instructions. After stopping the reaction by adding Ethylenediaminetetraacetic acid (EDTA) to a concentration of 5 mMol, it was cleaned up using the QIAamp Viral RNA Mini kit (Qiagen,

Valencia, CA) without the addition of carrier RNA. The elution volume was 30 μl. The rRNA contamination was evaluated by RNA pico chip using a 2100 Bioanalyzer (Agilent, Santa Clara, CA). The cDNA libraries were prepared using the NEB Next Ultra Directional RNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, MA). Sequencing was performed using Illumina HiSeq 3000 at the 100 bp paired end. Raw reads were aligned to the chicken genome (galGal5) using Tophat 2 (30) with default parameters. Contigs were built from the non-host reads and viral contigs were determined by using NCBI-BLAST with default parameters to find contigs with sequences matching GeneBank reovirus sequences. The non-host sequences were then aligned to the identified viral contigs using Tophat 2 to determine the number of viral reads. The obtained gene sequences were compared to the vaccine strain S1133 full genome available at GenBank (KF741756 to KF741765). Sequence homologies to S1133 were calculated. The seven full genome-sequenced ARV's, in addition to 28 chicken ARV whole genomes obtained from GenBank (Table 1.5), were aligned and phylogenetic trees were constructed for each of their genes using Clustal Omega (Cambridgeshire, U.K.).

Ethics statement

Tissue collections for virus isolation were conducted in accordance with procedural guidelines approved by the United States Department of Agriculture (USDA)(http://www.aphis.usda.gov/animal_health/lab_info_services/downloads/necropsyGuideline.pdf). Virus isolation and biological use was approved by the University of California, Davis Institutional Biosafety Committee (IBC) under Biological Use Authorization (BUA) approval #

R2109. Procedures involving animals were reviewed and authorized by the University of California Institutional Animal Care and Use Committee (IACUC) Approval # 19092.

RESULTS

Virus isolation

From September 2015 to June 2018, we received 265 commercial broiler cases where ARV was detected by Reverse transcriptase polymerase chain reaction (RT-PCR) followed by virus isolation and tenosynovitis was confirmed by histopathological findings. ARV isolates were obtained from tendons (78.9%), heart (12.3%), joints (4.4%), intestines (3.7%), liver (0.35%) and pancreas (0.35%).

Molecular characterization

One hundred and fifty isolates were selected for molecular characterization. The selection criteria involved clinical relevance, gross pathology and cytopathic effect in chicken embryo liver cells (CEL). Effective amplification of a 1,088 bp segment of the ARV S1 gene was accomplished in 85 out of the 150 selected reovirus isolates (56.6%) using RT-PCR. The obtained sequences were aligned and subsequently used to construct a phylogenetic tree, including 49 reference sequences representing the genotypic groups, 1 to 6, obtained from the available literature (2,6,9,11,20,22) (Figure 1.1). The typed viruses grouped in six different genotypic clusters (GC); two of these, GC1 and GC6, were predominant. In percentage, the distribution of the sequences by GC was as follows: GC1 (51.8%), GC2 (12.9%), GC3 (1.2%), GC4 (7.1%), GC5 (2.4%) and GC6

(24.7%) (Table 1.1). The percent amino acid homology of the S1 sequences to the reference ARV S1133 strain were: GC1 (77.0%), GC2 (58.5%), GC3 (58.0%), GC4 (53.5%), GC5 (53.1%) and GC6 (54.1) (Table 1.2). The percent homologies of the S1 sequences to S1133 by year from 2015 to 2018 showed consistency; GC1 was the group with the highest homology to S1133 (77.0%) while GC4, GC5 and GC6 the groups with the lowest homologies (53.1 to 54.1%). In order to assess the similarity of the S1 sequences within each cluster we calculated the average of the pairwise homologies between all S1 gene sequences in a cluster i.e. homology within GC. From high to low, these homologies were GC5 (97.8%), GC1 (96.4%), GC6 (94.8%), GC4 (77.3%) and GC2 (76.4%) (Table 1.2). A summary of the sequence distribution on the different genetic clusters and their homologies by year were summarized in Figure 1.2. A considerable reduction of sequences clustered in GC1 from 76.7% in 2016 to 9.1% in 2018, was followed by an increase in GC2, GC4 and GC6 from 6.7% in 2016 to 36.4%, 36.4% and 18.2%, respectively in 2018. GC3 was first identified in 2016 with 3.3% of the sequences disappearing in 2017. Finally, GC5 was first identified in 2017 with 4.8% of the sequences disappearing in 2018.

Whole genomes

Seven ARV isolates K1600600, K1600402, K1502030, K1600657, T1502036, T1600137 and T1600260, associated with severe clinical signs in the field, were selected for full genome studies. These viruses molecularly grouped into GC1, except K1600657 that grouped into GC4 under a partial S1 gene molecular characterization. The goal was to determine how variable were the genes that are not frequently used for avian reovirus molecular characterizations and how much each gene contributes to the reovirus variability. Information about the raw reads after NGS,

non-host reads, contigs, viral contigs, viral reads and % viral reads are summarized in Table 1.3. The percent identity of each of the genes of these viruses to ARV S1133 genes was calculated (Table 1.4). Considering average gene identities to S1133 among the seven sequences, the lowest detected identities were found in: L3 gene, coding for λC an inner and outer capsid protein (72.7%); S1 gene, coding for σC a minor capsid protein (77.9%); and finally M2 coding for μB an outer-capsid protein (79.0%). Additionally, phylogenetic trees were made to graphically compare the differences and clustering of the ARV's by gene. Figure 1.3 shows the trees prepared with all gene segments corresponding to the ARV genes analyzed. The California strains in all gene trees grouped distantly to the vaccine cluster defined by KF741758 (S1133) (Figure 1.3). California molecular variants T1600137, T1600260, K1600402 and K1600600 formed a sub-cluster. Two of the seven California ARV's: K1502030 and T1502036 form a different cluster, while K1600657 doesn't cluster with any of the CA viruses. The clustering pattern apply to all phylogenetic trees constructed except in M2, S2 and S4. The M2 gene AA sequences grouped in two defined clusters, one containing K1502030, K1600657 and T1502036 while the other contained K1600600, K1600402, T1600260 and T1600137. In S2 two distinct clusters were formed one containing K1502030, K1600657 and T1502036 and the other T1600137, T1600260, K1600402 and K1600600. In the case of S4, K1502030 and T1502036 clustered in different sub-clusters but close together in the phylogenetic tree.

DISCUSSION

Molecular surveillance is crucial to strategize control and prevention of endemic diseases. This is particularly important for reoviruses considering their segmented RNA genome and their

potential for variation (2,6,7,11,12). Since 2015, the isolation of reoviruses causing tenosynovitis in broiler chickens obtained from breeders vaccinated with live and inactivated conventional strains (S1133, 1733, 2408, etc.) has raised the concern of the existence of reovirus genetic variants in California.

The first step in surveillance is pathogen detection and isolation. Since diagnostic RT-PCR's focuses on a conserved segment of the ARV genome i.e. S4 (23) will not be able to differentiate variant strains. This is when virus culture / isolation and subsequent virus genomic characterization, from highly variable genes, becomes crucial in the surveillance strategy. In this project, in a period of 3 years, we were able to detect and isolate 265 reoviruses. The highest percentage of recovery was from tendons and joint tissues, or swabs (83.3%) followed by heart (12.3%) and intestines (3.7%), liver and pancreas provided lower virus recovery. Other studies have focused on tendons and joint tissues for their typing work, without reporting the isolation effectiveness in these tissues (6, 20).

The reovirus isolation method involves passages on CEL cells. Passaging RNA viruses in cells involve genetic changes as part of the adaptation of these viruses to a new cell culture or host (24). We assume that those changes in the studied reoviruses are minimum, since only one and at the most two passages in cells were performed for the obtention of cytopathic effect (CPE) and the subsequent virus isolation.

Sequencing analysis of a portion of the S1 gene has been commonly used as a genetic marker for the characterization and classification of ARV isolates (4). From the isolated reovirus strains, 150 were selected for partial S1 genotypic characterization based on clinical relevance, gross pathology and cytopathic effect in chicken embryo liver cells (CEL). Using the primers

described by Kant *et al* (2), only 85 out of 150 (56.6%) partial S1 genes were detected, amplified and sequenced. We attribute the lack of amplification of more than 30% of the isolates partially to the molecular divergence on ARV variant strains. Kant *et al*, using the same primers and viral isolates between 1980 to 2000, had a higher amplification success i.e. (28/40) 70% (2).

Partial S1 gene characterization methods have classified ARV strains into five 2,11 and/or six 6,12,20 genotypic clusters. Our research showed that reovirus strains isolated in California belong to all six distinct genotypic clusters (GC1 to GC6). These clusters were clearly defined and confirmed by the addition of reference sequences representing earlier ARV isolates and vaccine strains. The most predominant clusters were GC1 (51.8%) and GC6 (24.7%), followed by GC2 (12.9%), GC4 (7.1%) and with lower frequencies GC5 (2.4%) and GC3 (1.2%). Similar results were described in Europe by Kant. Most of the isolates associated with malabsorption syndrome belonged to clusters 1 and 4 and few in clusters 2 and 5. Most the tenosynovitis clusters belonged to cluster 4 and unclear cases to cluster 1 (2). Different molecular characterization results were reported by Lu *et al* (6) and Palomino *et al* (20). While Lu stated that most of their sequences clustered in GC2, followed by GC4 and GC1, Palomino affirmed that their most predominant sequences were from GC5, followed by GC4 and GC1. While this “cluster” nomenclature is used to compare the viruses detected in different parts of the country and the world, we need to take into consideration that fragment size, the number of sequences in the analysis, sequences selected as reference strains and the subjectivity of the analysis play a role in the formation of the clusters. Genetic variants detected in California follow the same S1 gene phylogenetic classification than the S1 sequences used as references. Some interesting points to consider are that older isolates from Taiwan group closer to vaccine strains S1133 and 1733. Isolates after

1996, group throughout the 6 genetic clusters and far from the subgroup of GC1 containing the vaccine strains. The exception are two Taiwanese strains from 1992 reported by Liu *et al* (9) that grouped in GC2 and GC6 . These results might be suggesting major genetic changes occurred starting in 1992 generating genetic variants from the "conventional vaccine types" of reovirus.

Between 2015 and 2018, the ARV isolates genotypic cluster representation in the State of California has shifted. A decrease on the representation of GC1 and an increase of GC6 classified strains has occurred (Table 1.1). Multiple factors might be influencing this relevant shift, including the use of autogenous vaccines. The use of certain GC's as antigen in autogenous vaccines might be important in driving the change in the representation of the different ARV genetic clusters causing disease in the field. While the most predominant strains of reovirus belonged to GC1 in 2016, autogenous vaccines with two GC1 and one GC5 variant isolates were prepared to be used in breeders that supply chickens to the state of California. Our hypothesis relies in the fact that inactivated non-homologous vaccines provide partial protection to the field challenge not eliminating viral shedding in the infected birds; allowing the selection of strains different from GC1 and/or GC5 altering the representation of ARV's in the environment. However, this explanation would not explain why GC4 or GC3 were not selected. We hypothesize that those genotypes were not selected due to their lack of fitness in the current environment being GC6 more fit than the rest of the genotypes. Traditionally, reported surveillance efforts do not discuss the variation of GC detections by year (2,6,20). Since these viruses are extremely variable, their predominant genotypes change throughout time. It is important to consider GC's predominance as a method of antigen selection for autogenous vaccine candidates.

In addition to calculating the GC frequencies temporally, we calculated homologies to a reference strain, in this case the commercial vaccine strain i.e. S1133. The advantage is to follow each of the cluster's variant variability and assess if there are major changes throughout time. We found that GC1 had the highest homology. Even though, GC1 is the group that encompasses the vaccine strains, the average homology of this group was 77%. The rest of the GCs had average homologies to S1133 between 58.5 and 53.1%, very distant from the viruses that are used in commercial live and inactivated vaccines (S1133, 1733 and 2408). These results might be explaining the lack of effectiveness of these vaccines in protecting commercial broilers. Based on the homologies over time, we see that each of the clusters have maintained their homologies to S1133 since 2016 (Figure 1.2).

The objective of performing whole genome sequencing on the seven selected isolates was to examine the variability of the different reoviruses by gene and evaluate the influence that each gene has on the whole virus variability. Sequencing results and the % identities of each of the viral genes with S1133, indicated that the L3, S1 and M2 genes, coding for proteins located in the virus capsid, were the genes that accounted for most of the variability of these reoviruses. The location of the proteins that they encode for, suggest a potential role in viral antigenicity and pathogenicity. In 2006, Su and collaborators described the sequence divergence of the M2 gene using the M-class genome segments of 12 distinct avian reovirus strains (25). They deduced that the M2 gene and μB protein showed the greatest level of sequence divergence, partially confirming our results. However, no correlation with antigenicity and pathogenicity was detected. These findings should be considered in future studies in order to associate these genes variability with antigenicity and pathogenicity. Hsu and collaborators demonstrated the

effectiveness of monoclonal antibodies in σC epitope recognition compared to S1133 polyclonal antibodies (26). In the future, if a clear association between genetics and antigenicity or pathogenicity is found, sequencing and characterization of these genes might generate a tool for a better and more comprehensive characterization. The information generated in the present study helps us understand the epidemiology of reoviruses in California. In addition, provides insights on how other genes that are not commonly studied add variability to the reovirus genome.

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Table 1.1. Sequence frequencies by genotypic cluster (GC) and year from 2015 to 2018, arithmetic sum and percentage of the total sequences by genotypic cluster.

Genotypic cluster	Total sequences by year				Sum	Total (%)
	2015	2016	2017	2018		
GC1	2	23	18	1	44	51.8
GC2	ND	2	5	4	11	12.9
GC3	ND	1	ND	ND	1	1.2
GC4	ND	2	2	2	6	7.1
GC5	ND	ND	2	ND	2	2.4
GC6	ND	2	15	4	21	24.7

ND = not detected

Table 1.2. Amino acid identities (%) between S1133 and the 85 ARV isolates based on sigma C protein by year.

Genotypic cluster	AA identity (%)				Average (%)	AA identity within GC (%)
	2015	2016	2017	2018		
GC1	77.9	77.0	76.8	76.9	77.0	96.4
GC2	ND	59.2	58.6	58.0	58.5	76.4
GC3	ND	58.0	ND	ND	58.0	ND
GC4	ND	53.7	52.6	53.8	53.5	77.3
GC5	ND	ND	53.1	ND	53.1	97.8
GC6	ND	54.2	54.2	53.9	54.1	94.8

ND = not detected

Table 1.3. Raw reads, non-host reads, contigs, viral contigs, viral reads and % viral reads obtained after processing the information obtained after the NGS.

Strain	Raw reads	Non-host reads	Contigs	Viral contigs	Viral reads	% viral reads
K1600600	17931054	446486	816	11	2578	0.01438%
K1600402	23077166	553129	802	11	5404	0.02342%
K1502030	21005141	557814	686	11	3712	0.01767%
K1600657	23693462	567956	958	11	3760	0.01587%
T1502036	21193861	534662	1008	11	4953	0.02337%
T1600137	22793555	537209	602	11	33228	0.14578%
T1600260	23297817	544087	1218	11	5933	0.02547%

Table 1.4. Amino acid sequence identities (%) between S1133 and each of the genes of seven whole genome sequences from selected ARV isolates.

Isolate ID	Viral segment	L 1	L 2	L 3	M1	M2	M3	S1	S2	S3	S4
	Encoded proteins	λA[a]	λB[a]	λC[b]	μA[a]	μB[c] μBN[c] μBC[c]	μNS[d] μNS C[d] μNS N[d]	σC[c] p10[d] p17[d]	σA[a]	σB[c]	σNS[d]
K1600600		88.1	90.1	73.0	89.3	74.7	81.2	81.5	89.9	85.1	81.4
K1600402		88.1	90.1	73.0	89.3	74.8	81.1	81.3	89.9	85.2	81.4
K1502030		88.7	83.8	72.2	87.2	84.7	87.4	80.8	91.6	84.9	80.0
K1600657		89.2	89.4	72.9	88.2	84.5	89.3	58.4	91.2	88.5	79.6
T1502036		88.7	83.7	72.2	87.1	84.7	87.5	80.7	91.3	85.0	81.4
T1600137		88.1	90.1	73.0	89.4	74.7	81.3	81.4	89.9	85.1	81.4
T1600260		88.1	90.1	72.9	89.4	74.7	81.2	81.3	89.9	85.2	81.4
	Average identity (%)	88.4	88.2	72.7	88.6	79.0	84.2	77.9	90.5	85.6	81.0

Superscripts indicate: a = Inner core, b= Inner capsid and outer capsid, c= Outer capsid, d= Non-structural

Table 1.5. Avian Reovirus (ARV) GenBank accession numbers by gene of 7 ARV isolates from California plus 26 field isolate and 2 vaccine sequences (S1133 and 2408) used as backbone for phylogenetic tree analysis. Country of isolation and authors are also reported.

#	Strain nomenclature	GenBank accession number of each segment										Country	Authors
		L1	L2	L3	M1	M2	M3	S1	S2	S3	S4		
1	Strain S1133	KF741756	KF741757	KF741758	KF741759	KF741760	KF741761	KF741762	KF741763	KF741764	KF741765	China	Teng et al., 2013
2	SD10-1	KP288857	KP288858	KP288859	KP288860	KP288861	KP288862	KP288863	KP288864	KP288865	KP288866	China	Chu, 2014
3	526	KF741696	KF741697	KF741698	KF741699	KF741700	KF741701	KF741702	KF741703	KF741704	KF741705	China	Teng et al., 2013
4	AVS-B	FR694191	FR694192	FR694193	FR694194	FR694195	FR694196	FR694197	FR694198	FR694199	FR694200	USA	Bányai et al., 2016
5	C78	KF741716	KF741717	KF741718	KF741719	KF741720	KF741721	KF741722	KF741723	KF741724	KF741725	China	Teng et al., 2012
6	GuangxiR1	KC183748	KC183749	KC183750	KC183751	KC183752	KC183743	KC183744	KC183745	KC183746	KC183747	China	Teng et al., 2012
7	GuangxiR2	KF741726	KF741727	KF741728	KF741729	KF741730	KF741731	KF741732	KF741733	KF741734	KF741735	China	Teng et al., 2012
8	GX/2010/1	KJ476699	KJ476700	KJ476701	KJ476702	KJ476703	KJ476704	KJ476705	KJ476706	KJ476707	KJ476708	China	Li et al., 2014
9	GX110058	KF741736	KF741737	KF741738	KF741739	KF741740	KF741741	KF741742	KF741743	KF741744	KF741745	China	Teng et al., 2013
10	GX110116	KF741746	KF741747	KF741748	KF741749	KF741750	KF741751	KF741752	KF741753	KF741754	KF741755	China	Teng et al., 2013
11	HB10-1	KP288827	KP288828	KP288829	KP288830	KP288831	KP288832	KP288833	KP288834	KP288835	KP288836	China	Chu, 2014
12	K738/14	MF686695	MF686696	MF686697	MF686698	MF686699	MF686700	*	MF686701	MF686702	MF686703	Korea	Noh et al., 2017
13	LN09-1	KP288837	KP288838	KP288839	KP288840	KP288841	KP288842	KP288843	KP288844	KP288845	KP288846	China	Chu, 2014
14	PA/05682/12	KM877325	KM877326	KM877327	KM877328	KM877329	KM877330	KM877331	KM877332	KM877333	KM877334	USA	Tang and Lu, 2014
15	PA/15511/13	KP731611	KP731612	KP731613	KP731614	KP731615	KP731616	KP731617	KP731618	KP731619	KP731620	USA	Lu et al., 2015
16	PA/01224A/14	KT428298	KT428299	KT428300	KT428301	KT428302	KT428303	KT428304	KT428305	KT428306	KT428307	USA	Lu and Tang, 2015
17	PA/01224B/14	KT428308	KT428309	KT428310	KT428311	KT428312	KT428313	KT428314	KT428315	KT428316	KT428317	USA	Lu and Tang, 2015

18	PA/27614/13	KU169288	KU169289	KU169290	KU169291	KU169292	KU169293	KU169294	KU169295	KU169296	KU169297	USA	Lu and Tang, 2015
19	SD09-1	KP288847	KP288848	KP288849	KP288850	KP288851	KP288852	KP288853	KP288854	KP288855	KP288856	China	Chu, 2014
20	2408	AY641742	*	AY652694	AY639613	AY635937	AY573907	AY436605[a], AY438594[b], AF204945[c]	*	AF208038	AF213468	USA	Liu et al., 2003
21	750505	DQ238093		AY652695	AY639615	AY635942	AY573909	AY395797[a], AY436608[b], AF204950[c]	AF294767	AF208035	AF213470	Taiwan	Liu et al., 2003
22	601SI	*	*	*	*	*	*	AY436599[a], AY438588[b], AF204947[c]	AF294769	AF208037	AF294773	Taiwan	Liu et al., 2003
23	916	AY641737	*	AY652701	*	*	*	AY436604[a], AY438593[b], AF297214[c]	AF294764	AY008383	AF294774	Taiwan	Liu et al., 2003
24	918	AY641738	*	AY652700	AY639617	AY635945	AY573911	AY436596[a], AY436610[b], AF297215[c]	AF294766	AF301473	AF294775	Taiwan	Liu et al., 2003
25	R2/TW	AY641744	*	*	*	*	*	AY436602[a], AY438591[b], AF297213[c]	AF294765	AF301472	AF294778	Taiwan	Liu et al., 2003
26	1017-1	AY641740	*	DQ238096	AY639611	AY635935	AY573905	AY436600[a], AY438589[b], AF297216[c]	AF294762	AF301474	AF294771	Taiwan	Liu et al., 2003
27	601G	AY641736	*	AY652699	AY639614	AY635941	AY573908	AY436597[a], AY436609[b], AF297217[c]	AF311322	AY008384	AY008385	Taiwan	Liu et al., 2003
28	T6	DQ238094	*	AY652698	AY639621	AY635936	AY573915	AY436598[a], AY438587[b], AF204948[c]	AF294768	AF208036	AF213469	Taiwan	Liu et al., 2003
29	K1600600	MK416133	MK416134	MK416135	MK416136	MK416137	MK416138	MK416139	MK416140	MK416141	MK416142	USA	Current research
30	K1600402	MK551735	MK551736	MK551737	MK551738	MK551739	MK551740	MK551741	MK551742	MK551743	MK551744	USA	Current research
31	T1600260	MK554704	MK554705	MK554706	MK554707	MK554708	MK554709	MK554710	MK554711	MK554712	MK554713	USA	Current research
32	T1600137	MK562467	MK562468	MK562469	MK562470	MK562471	MK562472	MK562473	MK562474	MK562475	MK562476	USA	Current research
33	K1502030	MK583321	MK583322	MK583323	MK583324	MK583325	MK583326	MK583327	MK583328	MK583329	MK583330	USA	Current research
34	K1600657	MK583331	MK583332	MK583333	MK583334	MK583335	MK583336	MK583337	MK583338	MK583339	MK583340	USA	Current research
35	T1502036	MK616643	MK616644	MK616645	MK616646	MK616647	MK616648	MK616649	MK616650	MK616651	MK616652	USA	Current research

a=P10; b= P17; c= Sigma C

*No information

Supplementary Table S1.1. List of GenBank accession numbers and supplementary information for the partial S1 sequences of the isolates present in the current research.

GenBank accession #	Isolation source	Year of isolation	Genotypic cluster partial S1 gene
MK246972	Heart	2016	1
MK246973	Heart	2016	1
MK246975	Tendon	2017	1
MK246978	Tendon	2017	1
MK246979	Intestine	2017	1
MK246980	Intestine	2017	1
MK246981	Heart	2015	1
MK246982	Heart	2016	1
MK246983	Joint swab	2016	1
MK246984	Heart	2016	1
MK246985	Tendon	2018	1
MK246986	Tendon	2016	1
MK246989	Joint swab	2016	1
MK246990	Tendon	2016	1
MK246991	Tendon	2016	1
MK246992	Joint swab	2016	1
MK246993	Tendon	2016	1
MK246995	Joint swab	2016	1
MK246997	Tendon	2016	1
MK246998	Tendon	2016	1
MK247002	Tendon	2017	1
MK247003	Tendon	2017	1
MK247009	Tendon	2015	1
MK247010	*	2016	1
MK247011	Joint swab	2016	1
MK247013	Tendon	2016	1
MK247014	Tendon	2016	1
MK247016	Tendon	2016	1
MK247017	Tendon	2016	1
MK247018	Heart	2016	1
MK247019	Tendon	2016	1
MK247022	Tendon	2016	1
MK247026	Tendon	2017	1
MK247027	Tendon	2017	1
MK247028	Tendon	2017	1
MK247029	Tendon	2017	1
MK247030	Intestine	2017	1

MK247031	Tendon	2017	1
MK247032	Tendon	2017	1
MK247033	Tendon	2017	1
MK247039	Tendon	2017	1
MK247046	Tendon	2017	1
MK247047	Tendon	2017	1
MK247053	Tendon	2017	1
MK246977	Tendon	2017	2
MK247007	Tendon	2018	2
MK247012	Heart	2016	2
MK247020	Tendon	2016	2
MK247023	Tendon	2009	2
MK247034	Tendon	2018	2
MK247035	Intestine	2017	2
MK247038	Tendon	2017	2
MK247050	Tendon	2018	2
MK247051	Tendon	2017	2
MK247052	Tendon	2017	2
MK247057	Tendon	2018	2
MK246988	Heart	2016	3
MK246974	Heart	2016	4
MK246987	Tendon	2016	4
MK246999	Heart	2017	4
MK247001	Pancreas	2017	4
MK247008	Tendon	2018	4
MK247056	Tendon	2018	4
MK247040	Tendon	2017	5
MK247054	Heart	2017	5
MK246976	Tendon	2017	6
MK246994	Tendon	2018	6
MK246996	Tendon	2016	6
MK247000	Tendon	2018	6
MK247004	Tendon	2017	6
MK247005	Tendon	2017	6
MK247006	Tendon	2017	6
MK247015	Tendon	2018	6
MK247021	Tendon	2016	6
MK247024	Tendon	2017	6
MK247025	Tendon	2017	6
MK247036	Tendon	2017	6
MK247037	Tendon	2017	6
MK247041	Tendon	2017	6
MK247042	Tendon	2017	6
MK247043	Tendon	2017	6

MK247044	Tendon	2017	6
MK247045	Tendon	2017	6
MK247048	Tendon	2017	6
MK247049	Tendon	2017	6
MK247055	Tendon	2018	6

*No information

Supplementary Table S1.2. List of GenBank accession numbers of the partial S1 sequences reference strains used for the phylogenetic tree classification.

GenBank Accession #	Country of isolation	Year of Isolation	Genotypic cluster partial S1 gene	Author
KR856980	USA	2014	2	Lu et al., 2015
KR856992	USA	2014	3	Lu et al., 2015
KR856994	USA	2014	4	Lu et al., 2015
KP727805	USA	2014	5	Lu et al., 2015
KP727783	USA	2012	6	Lu et al., 2015
KP727795	USA	2014	6	Lu et al., 2015
KJ803976	USA	2012	1	Sellers, 2016
KJ879692	USA	2012	1	Sellers, 2016
KJ879625	USA	2013	2	Sellers, 2016
KJ879682	USA	2013	2	Sellers, 2016
KJ879660	USA	2013	3	Sellers, 2016
KJ879667	USA	2013	3	Sellers, 2016
KJ803996	USA	2012	4	Sellers, 2016
KJ879644	USA	2013	4	Sellers, 2016
KJ803958	USA	2011	5	Sellers, 2016
KJ803959	USA	2012	5	Sellers, 2016
MG822695	Canada	2016	1	Palomino et al., 2018
MG822686	Canada	2014	1	Palomino et al., 2018
MG822669	Canada	2014	2	Palomino et al., 2018
MG822682	Canada	2014	2	Palomino et al., 2018
MG822679	Canada	2016	3	Palomino et al., 2018
MG822676	Canada	2016	3	Palomino et al., 2018
MG822678	Canada	2016	4	Palomino et al., 2018
MG822697	Canada	2015	4	Palomino et al., 2018
MG822690	Canada	2017	6	Palomino et al., 2018
MG822670	Canada	2017	5	Palomino et al., 2018
MG822703	Canada	2014	5	Palomino et al., 2018
L39002	Canada	1973	1	Shapouri et al., 1995

AF330703	*	*	1	Bodelon et al., 2001
AF004857	*	1997	1	Vakharia et al.,1997
AF204945	USA	1983	1	Liu et al., 2003
AF204950	Taiwan	1986	1	Liu et al., 2003
AF204947	Taiwan	1992	1	Liu et al., 2003
AF297214	Taiwan	1992	2	Liu et al., 2003
AF297215	Taiwan	1992	6	Liu et al., 2003
AF204948	Taiwan	1970	1	Liu et al., 2003
AF297213	Taiwan	1992	1	Liu et al., 2003
AF297216	Taiwan	1992	6	Liu et al., 2003
AF297217	Taiwan	1992	1	Liu et al., 2003
AF354224	Germany	1997	1	Kant et al., 2003
AF354226	Germany	1998	2	Kant et al., 2003
AF354227	Germany	1998	3	Kant et al., 2003
AF354221	Germany	1996	4	Kant et al., 2003
AF354230	Germany	1996	4	Kant et al., 2003
AF354219	Germany	1997	5	Kant et al., 2003
KX855920	Canada	*	2	Ayalew et al., 2017
KX855906	Canada	*	4	Ayalew et al., 2017
KX855918	Canada	*	5	Ayalew et al., 2017
KX855908	Canada	*	6	Ayalew et al., 2017

*No information

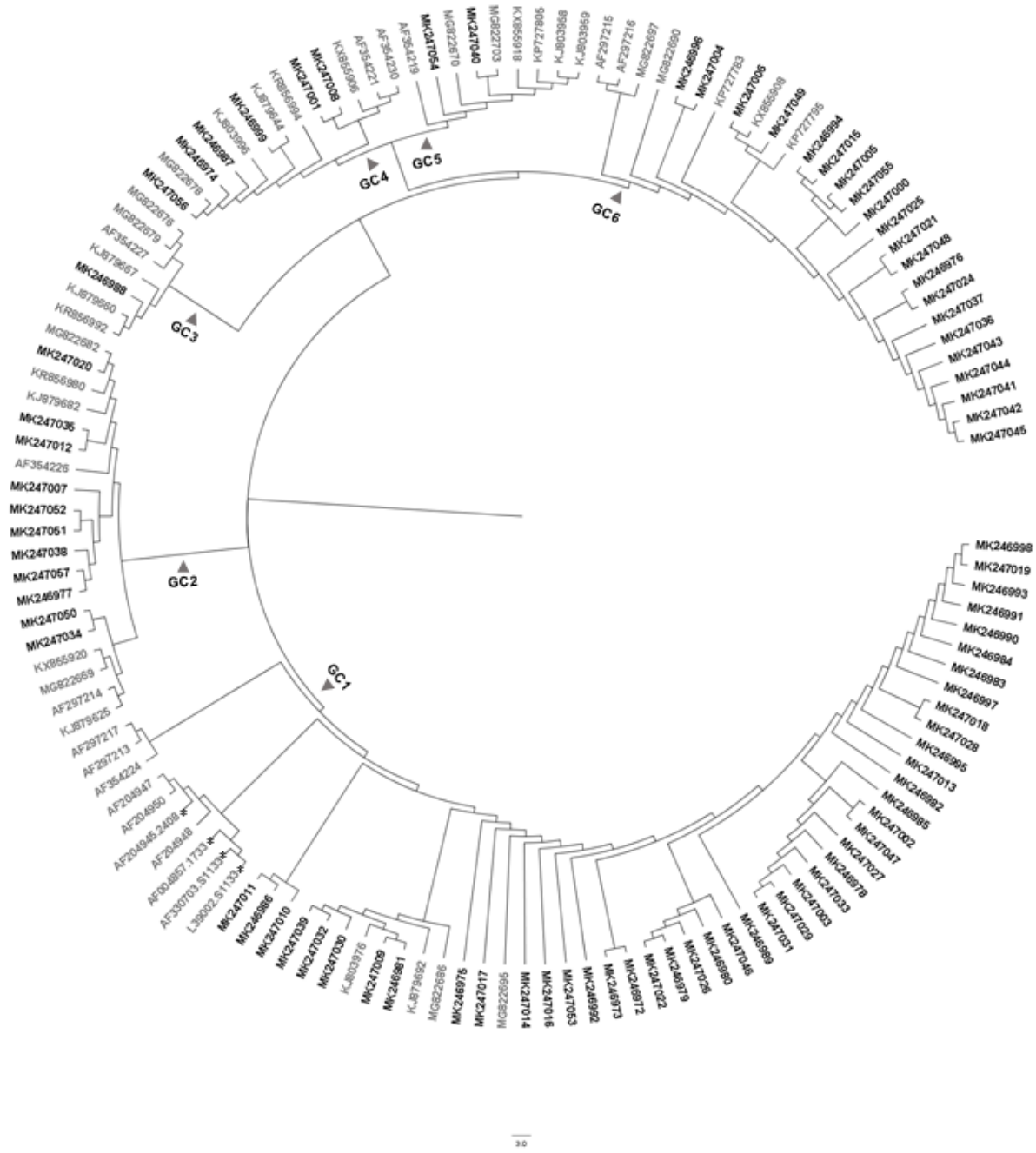

Figure 1.1. Phylogenetic tree depicting 85 ARV S1 sequences (278 AA). Sequences were obtained from reoviruses isolated from tenosynovitis cases in CA between 2015 and 2018. The reference sequences (gray) were obtained from GenBank. Outbreak sequences (Black) were grouped into six genotypic clusters (GC). Commercial vaccine strains were labeled by asterisks.

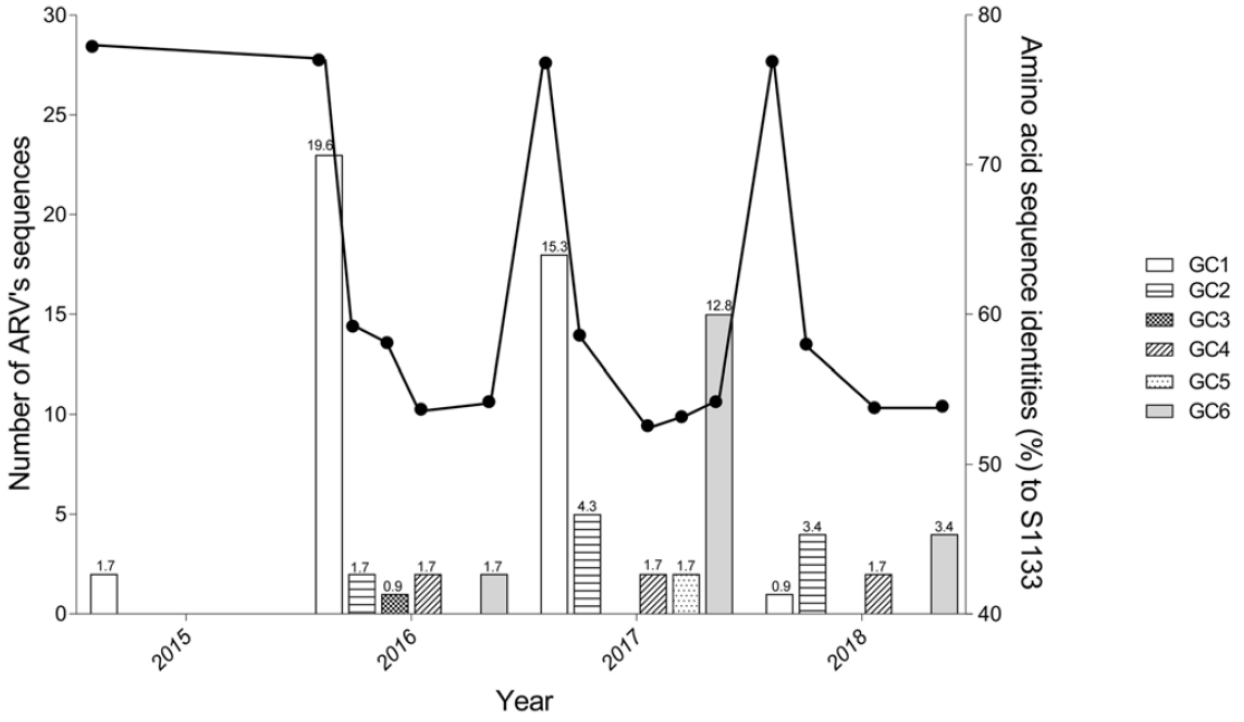

Figure 1.2. Frequencies and average homologies based on 85 ARV S1 sequences (278 AA) obtained from avian reovirus (ARV) isolates from tenosynovitis clinical cases. Bars are showing the isolate frequencies in each genotypic cluster (GC) per year. Numbers above bars represent the percentage (%) from the total samples. The bold line represents the average homology to S1133 based on the S1 sequences. GC 1 to 6 represents genotypic clusters 1 to 6.

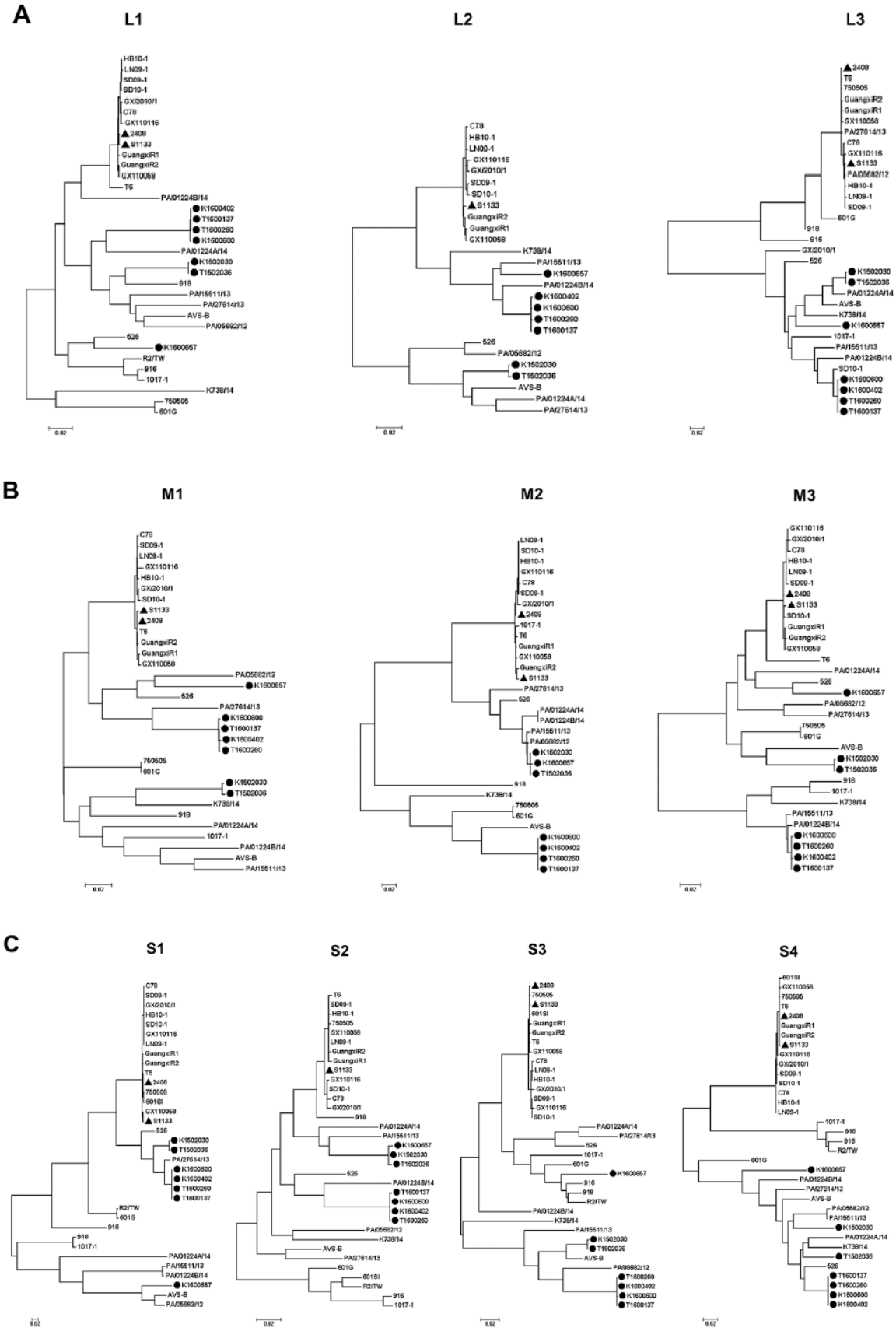
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Figure 1.3. Phylogenetic trees showing each gene of seven California ARV's isolates. Twenty-eight sequences were obtained from GenBank and used as reference strains except in S1 phylogeny where only 17 were used. Amino acid sequences were aligned and trimmed: Amino acid sequences were aligned and trimmed: L1 (1,291 AA); L2 (1,260 AA); L3 (1,218 AA); M1 (677 AA); M2 (677 AA); M3 (636 AA); S1 (514 AA); S2 (280 AA); S3 (367 AA); S4 (366 AA) phylogeny was performed using the Maximum Likelihood Method with 1,000 bootstrap replicates using MEGA 7. The commercial vaccine strains (S1133 and 2048) are labeled with a triangle. Black dots are showing the clustering of the California isolates.

CHAPTER 2

Avian Reoviruses of the Same Genotype Induce Different Pathology in Chickens

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SUMMARY

The emergence of avian reovirus variant strains has caused negative impacts to the poultry industry worldwide. Regardless of the efforts in molecular characterization and classification of these variants, there is limited information about their pathogenicity,

transmissibility, and immunosuppression in chickens. The genomes of two variant strains (A and B) and a classical S1133 strain (C) belonging to the same sigma C genotype 1 were compared. In addition, these strains were used in a challenge experiment to evaluate inoculated and indirectly exposed specific pathogen-free (SPF) chickens. The whole genome sequence analysis of the three strains revealed nucleotide identity differences in the L3, M2, and S1 genes. In addition, strains A and B showed homology differences in the S4 gene, despite having high homologies in all other genes. The in vivo challenge experiments showed that while variant A induced high viral loads in tendons, hearts and duodena of inoculated chickens, variant B induced high viral loads in indirectly exposed chickens. Likewise, histopathology reflected differences in the pathologic effects induced by these strains. For instance, the B and C strains induced more severe microscopic lesions compared with the A strain. Lymphoid depletion was more severe in bursas than in thymi, and inoculated birds were more affected than exposed birds. In conclusion, different pathological outcomes in chickens were observed depending on the strain and transmission route. This study provides insights onto the relationship between pathogenicity and genomic composition of ARVs.

KEY WORDS: avian reovirus, genetic variants, pericarditis, tenosynovitis, pathotyping, genotyping, histopathology

INTRODUCTION

Since its first isolation in chickens in 1954 (1), avian reoviruses (ARV) continue to cause negative impacts to the poultry industry worldwide. Avian reoviruses belong to the genus

Orthoreovirus in the Reoviridae family, possessing a segmented double-stranded ribonucleic acid (dsRNA) genome. These characteristics make ARV prone to mutations, recombination and re-assortments that generate molecular variants. The ten segments forming the ARV genome are classified into three large (L1, L2, L3), three medium (M1, M2, M3), and four small (S1, S2, S3, S4) genes according to their migration pattern in gel electrophoresis (2).

The S1 segment encodes for three viral proteins, including the outer capsid protein sigma C (σC). The viral S1 gene is the most variable, and the encoded structural protein σC plays a key role in mediating attachment to the host cells. In addition, σC elicits type-specific neutralizing antibodies (2,3). In 2003, Kant *et al* (4) reported the emergence of new strains and proposed their classification into 5 genotypic groups using partial S1 sequences from different parts of the world. The current molecular characterization uses a segment of the S1 gene to calculate nucleotide and amino acid distances and estimate their phylogenetic topology. As a criterion, strains that belong to the same genotypic linage are rooted together with a bootstrapping of at least 70% using 1,000 replicates (5–8). However, the nucleotide and amino acid identity can vary from 42 to 100% within a genotypic cluster. Therefore, certain ARV genotypic clusters are divided into sub-genotypes (5,7–11), which complicates the molecular classification of these viruses. To date, seven genotypes have been reported globally according to their S1 sequences (4,6–10,12). Conventionally, emerging strains that are antigenically distant from the reference strain of a genotype but not distant enough to form a new cluster are considered variants. Variant strains have shown changes in antigenicity and pathogenicity when compared to classical ARV strains (9,13–15). Although the molecular characterization of S1 helps understand the epidemiology of ARV variants, it does not provide sufficient information on their pathogenicity.

Whereas structural proteins other than σC have a proven role in antigenicity (16,17), their role in pathogenicity has not been thoroughly studied in chickens (18,19). Preliminary results from whole genome sequence comparisons have detected high variability in the L3 and M2 genes in addition to the S1 gene when variants were compared to classical ARV strains (8). Before the genotyping era, avian reoviruses were grouped according to their pathogenicity in chickens by challenge studies or antigenicity models using serum neutralization assays (20–23). Molecular characterization is currently used to classify reoviruses, even though no clear correlation between genotyping, pathogenicity and antigenicity has been found (4,13,24,25).

Avian reoviruses can cause subclinical or clinical disease in chickens, ranging from viral arthritis-tenosynovitis to respiratory infections, malabsorption syndrome (4,14,26), and cardiac disease (27). Although avian reoviruses can be vertically and horizontally transmitted (26,28,29), there is incomplete information on how effectively different variant strains can be transmitted compared with classical strains (30). Horizontal transmission usually occurs through the fecal-oral route. After the virus is ingested, replication occurs in the intestines and bursa of Fabricius. Subsequently, the virus is distributed to other organs and tissues through viremia (29,31). Although the pathogenicity of classical strains is well characterized (23,31–33), the pathobiology of variants remains unclear. Additionally, reports comparing the pathogenicity of different S1 genotypes have been published (10,16,27,34–37), but none of them compares strains within the same genotype (6,11). In addition, avian reovirus infections have been associated with immunosuppression (17,38–44). The most plausible mechanism by which ARVs induce immunosuppression is lymphoid depletion in organs of the immune system (35,45) and the induction of cell apoptosis (34,46–48).

This manuscript aims to compare the pathogenicity of avian reoviruses that were isolated from chickens with different clinical signs and that belong to the same genotype based on the current molecular characterization. We hypothesized that wild-type avian reoviruses differ from a classic vaccine strain in genes other than the S1, and therefore induce different pathological outcomes and transmission kinetics. For that, the whole genomes of the three isolates that belong to the σC genotypic cluster I were compared. In addition, the pathological outcomes induced by these viruses were characterized in specific-pathogen free (SPF) chickens, focusing on lesions in their tendons, hearts, thymi, and bursas of Fabricius. Lastly, we compared the horizontal transmission capabilities of the ARV strains using naive SPF chickens in direct contact with chickens challenged with ARV.

MATERIALS AND METHODS

Chickens

One hundred and ninety-two SPF chickens (Charles River, Catskill, NY) were hatched and raised in experimental facilities at the University of California, Davis. The birds were randomly allocated in eight biosafety level 2 (BSL2) rooms (n=24) under controlled temperature in a 12-hour light program. Chickens were raised on wood shavings, and food and water were provided ad libitum. All chickens were tagged with numbered and color-coded wing bands before starting the experiment.

Viruses and vaccines

Two avian reovirus field isolates (strains A and B) were molecularly classified as σC genotype I previously (8) and selected for this study. The third isolate (strain C) is an S1133 vaccine (ENTEROVAX®, Merck Animal Health. Omaha, NE, USA), which is the reference strain for genotype I ARVs. Although S1133 is a vaccine strain, this live attenuated vaccine can induce clinical signs when provided in higher doses than recommended. Based on the S1 gene, strains A and B share 99% identity between each other and less than 80% identity to strain C. Due to this low homology to strain C, strains A and B were classified as variants in this study. The A strain was isolated from two-week-old broilers presenting with necrotic myocarditis. The B strain originated from tendons from two-week-old broilers suffering from arthritis-tenosynovitis. Reoviruses were amplified and titrated in 7-day-old SPF embryonated eggs (Charles River, Catskill, NY) inoculated via yolk sac (49). These isolates were screened by RT-PCR for chicken astrovirus and rotavirus and by PCR for parvovirus and adenoviruses as previously described (50–52).

ARV whole genome sequencing and genomic analyses

Whole genomes of reovirus strains A, B and C were sequenced using a MinION (Oxford Nanopore Technologies, Oxford, UK). Total nucleic acid was extracted using the MagMax Pathogen RNA/DNA kit (Thermo Fisher Scientific, Carlsbad, CA). Total RNA was used as a template for double-stranded cDNA synthesis using the Maxima H Minus kit (Thermo Scientific, Carlsbad, CA) and random hexamers were used in the synthesis reaction. The double-stranded cDNA was purified using AMPure XP beads (Beckman Coulter, Indianapolis, IN). Libraries were prepared

using the Nanopore Ligation Sequencing Kit (Nanopore, Oxford, UK) as per the manufacturer's recommendations. Geneious Prime software version 2021.1.1 (Biomatters Ltd., San Diego, CA) was used to map sequencing reads to reference avian reovirus genomes (GenBank accession numbers KF741706, KP731611 and MK583331) using the MiniMap2 plugin (53). For each segmented gene, sequence alignments were generated using MEGA7 (54), and nucleotide sequences were transformed into amino acid sequences. Amino acid identities between segments were calculated using Clustal Omega (55). The whole genome segments of each ARV strain were concatenated and compared using the mVista Software (56).

Experimental design

This experiment consisted of four experimental groups (n=24), and each group was divided into two rooms and handled as replicates. At one day of age, half of the chickens in each room (n=6 per replicate) were randomly selected and inoculated subcutaneously (SC) in the right footpad with 100 µl of a suspension containing 1x105 50% embryo infective dose (EID)50 of the respective inoculum. The other half of chickens under each treatment (n=6 per replicate) remained non-inoculated and served as exposed birds to evaluate horizontal transmission. Phosphate buffered saline (PBS)-inoculated chickens were used as a negative control group (n=12). Body weight and right hock joint thickness were measured at 8, 15, 21 and 28 days post-challenge (DPC). Leg integrity scores were calculated by dividing the chicken body weight (g) by the hock joint thickness (mm). The lower the leg integrity scores, the more severe are the leg lesions. At 8, 15, 21, and 28 DPC, 12 inoculated and 12 exposed chickens per treatment were randomly selected, euthanized, and necropsied. Tendons (right foot digital flexor and

gastrocnemius), hearts, bursas, thymi, and sections of duodenum were collected at all time points and tested by RT-qPCR for ARV at 8 and 15 DPC. In addition, tendons, hearts, bursas and thymi were collected in formalin for histopathology. Microscopic lesions were evaluated and scored. All samples were processed and assessed blindly. Mortality was recorded daily and pathological findings in dead chickens were recorded. Experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal use guidelines (IACUC protocol #19092). The University of California Davis, School of Veterinary Medicine is an Association for Assessment and Accreditation of Laboratory Animal Care-accredited institution.

Viral RNA quantification. Organs were minced and homogenized individually using scissors and forceps. Briefly, 0.1 ml of tissue homogenate was collected in 1.5-ml tubes with 2.3 mm beads, 600 µl of lysis buffer, and processed in a bullet blender (Next Advance, Troy, New York). A mixture of 590 µl of RNAse-free water and 10 µl of proteinase K was added to the lysate, mixed, and incubated at 55oC for 10 minutes. Tubes were centrifuged for 3 minutes at 10,000 x g. The supernatant was collected and 500 µL was used for RNA extractions with the RNeasy Mini Kit on a QIAcube (QIAgen, Valencia, CA). RNA from duodenum sections was extracted with the QIAmp Viral RNA Mini Kit (QIAgen, Valencia, CA). RNA samples were denatured at 90oC for 10 minutes. A fragment of 100 bp of the M1 gene of ARV was amplified using a TaqMan RT-qPCR assay (57) with the One-Step RT-PCR Kit (QIAgen, Valencia, CA). Viral load was expressed as the total number of cycles in the RT-qPCR assay minus the quantification cycle (Cq) value (45-Cq).

Histopathology. Tissues were fixed in 10% neutral-buffered formalin and then embedded in paraffin, sectioned at 4 µm, and stained with hematoxylin and eosin (H&E) for histopathologic examination. Tendon and heart lesions were scored from 0 to 4 based on inflammatory and

lymphocytic infiltration in synovial joints or pericardia (0 = no lesions with less than 10 lymphocytes; 1 = mild lesions with 10 to 50 lymphocytes; 2 = moderate lesions with 50 to 100 lymphocytes, 3 = severe lesions with countless lymphocytes) (27,58). Lymphoid depletion was scored in bursas from 0 to 5 (0 = no lesions; 1 = minimal depletion; 2 = mild depletion; 3 = moderate depletion; 4 = marked depletion; and 5 = severe depletion). In thymi, lymphoid depletion was scored from 0 to 4 (0 = no lesions; 1 = minimal depletion; 2 = mild depletion; 3 = moderate depletion; 4 = severe depletion) (33,59,60).

Statistical design and analyses

Body weights, leg integrity scores, viral loads and histopathology were compared between inoculated and exposed groups separately at each time point using one- and two-way analyses of variance (ANOVA) followed by Tukey's multiple comparisons test. The statistical significances were calculated based on $P<0.05$ and a 95% confidence interval. Results were analyzed using Prism version 8.4.3 (GraphPad, La Jolla, CA)(61).

RESULTS

Concomitant viruses

The three isolates tested negative for astrovirus, rotavirus, parvovirus and adenoviruses by RT-PCR and PCR assays. No ambiguous reads were observed during the ARV assemblies, demonstrating that most likely these isolates were single genotypes that belong to genotype I based on the S1 gene.

Whole genome analyses

Considering whole genomes, the two ARV variant strains (A and B) had less than 85% nucleotide similarity compared to the classical vaccine strain (C). The most variable genes were L3, M2, and S1, with nucleotide identities of approximately 73, 75, and 79%, respectively (Figure 2.1, Table 2.1). Although the two ARV variant strains shared 99% similarity in their whole genomes, they only shared 89% nucleotide identity in the S4 gene (Table 2.1).

Body weights

Body weights in all inoculated groups were significantly lower compared with the negative control group at all time points ($P<0.05$). At 8 DPC, no body weight differences were observed between the groups inoculated with the A, B and C strains. Between 15 and 28 DPC, the body weights from chickens inoculated with the A strain were lower than the body weights of chickens inoculated with the B and C strains ($P<0.05$, Figure 2.2A). In horizontally exposed groups, chickens exposed to the B strain at 21 DPC and 28 DPC presented with body weights that were lower than those of control birds ($P<0.05$, Figure 2.2B).

Leg integrity scores

All inoculated groups presented with significantly lower scores compared with the negative control group at all time points. At 15, 21 and 28 DPC, the A strain-inoculated chicks showed lower leg integrity scores compared with the B and C strain-inoculated chickens ($P<0.05$, Figure 2.3A). No statistical differences in leg scores were detected between horizontally exposed groups at 8 and 15 DPC. However, chickens exposed to A and B strains showed lower leg scores

at 21 DPC (P<0.05). At 28 DPC, all ARV exposed groups showed scores lower than the negative control (Figure 2.3B).

Viral load

Viral RNA was not detected in the negative control groups in any tissues at any time point. At 8 DPC, viral loads in tendons of chickens inoculated with strains A and C were higher compared to strain B-inoculated chickens. Conversely, chickens horizontally exposed to strain B were the only group showing higher viral loads compared with the negative control group (P<0.05, Figure 4A). At 15 DPC, the C strain-inoculated chickens showed higher viral loads than chickens inoculated with strains A and B, while in horizontally exposed birds the B strain group showed higher viral load compared with A and C strain (P<0.05, Figure 2.4A). At 8 DPC, viral loads in hearts of chickens inoculated with A and C strains were higher than the viral load in birds inoculated with strain B, while birds horizontally exposed to strain B had statistically higher loads than all other groups (Figure 2.4B). At 15 DPC, viral load was higher in the hearts of chicks inoculated with the C strain compared with the A and B strains (P<0.05, Figure 2.4B), and only strain B-exposed birds showed viral load in hearts at an insignificantly low quantification (Figure 2.5B). Viral loads in duodenum sections at 8 DPC were higher in all inoculated groups compared to the negative control (P<0.05), and only the birds exposed to strain B showed viral loads higher than the negative control (Figure 2.4C). At 15 DPC, the only group that presented with viral load in the intestine was the B strain-exposed group (P<0.05) (Figure 2.4C).

Microscopic lesions in tendons and hearts

In tendons, the B and C strains caused mild to moderate lesions in inoculated chickens ($P<0.05$), while none of the strains caused significant lesions in horizontally exposed birds at 8 DPC (Figure 2.6A). At 28 DPC, both strains A and B caused mild to moderate lesions in the tendons of inoculated chickens, while the C strain caused severe tendinitis ($P<0.05$, Figure 2.5B). In the horizontally exposed groups, the B and the C strains caused mild to moderate lesions at 28 DPC, while the A strain exposure induced minimal tendon lesions (Figure 2.5B). Examples of the tendinitis scores are presented in Figure 2.5C.

The B and C strains induced mild to moderate lymphocyte infiltration in the hearts of inoculated chickens, while the A strain caused mild cardiac lesions at 8 DPC (Figure 2.6A). All ARV strains induced only mild lesions in the hearts of horizontally exposed chickens at 8 DPC (Figure 2.6A). Both variants A and B caused mild to moderate lesions in the hearts of inoculated chickens, while the C strain caused severe lesions in the hearts of inoculated birds at 28 DPC ($P<0.05$, Figure 2.6B). In the horizontally exposed groups, only the B strain caused mild to moderate heart lesions at 28 DPC (Figure 2.6B).

Lymphoid depletion in bursas and thymi

All ARV strains caused mild to marked lymphoid depletion in bursas of inoculated and exposed chickens between 8 and 28 DPC (Figure 2.7). The highest scores were observed at 8 DPC with decreasing scores at 15 and 21 DPC (data not shown). The A strain caused more severe lymphoid depletion compared with the B strain in horizontally exposed chickens at 8 DPC ($P<0.05$). All ARV strains triggered moderate lymphoid depletion in thymi of inoculated chickens

at 8 DPC ($P<0.05$, Figure 2.8A) and mild lymphoid depletion in inoculated and exposed chickens at 28 DPC ($P<0.05$, Figure 2.8B). Birds exposed to ARV strains at 8 DPC did not present thymic lesions that were statistically different from the unchallenged/unexposed controls (Figure 2.8A).

Mortality

Mortality was observed only in ARV-inoculated chickens at 6 and 7 DPC. The groups inoculated with A and B strains had 4% mortality (2/48) and the C strain inoculated group had a 16% mortality (8/48). The clinical and pathological findings in birds from all groups were dehydration, weight loss, food pad inflammation, tenosynovitis, and pericarditis. No mortality was observed in exposed chickens or in the negative control groups.

DISCUSSION

Although the ARV strains used in our study belong to the same genotype, they presented with a relatively low homology and induced slightly different pathological outcomes under lab settings. Strains A and B displayed less than 80% nucleotide similarity in the S1 gene compared to the classic C strain; therefore, they were considered variants in this study. The term "variant" is widely used for emergent ARV strains that are not closely related to the reference strains of the genotype they belong. However, there is no defined threshold of nucleotide or amino acid identity for an ARV strain to be considered a variant. For example, with infectious bronchitis virus (IBV), a variant is considered when a known genotype and field strains share amino acid similarities below 95% in their S1 gene (62). In ARV research, S1 nucleotide and amino acid identities within a phylogenetic group can be as low as 58% (5,7–11). For this reason, the

phylogenetic tree topology for ARV S1 is an important tool for the molecular characterization of ARVs.

The whole genome analysis of the A and B strains revealed minor differences; however, the genetic divergence between A and B was specifically in the S4 gene, where the nucleotide identity was only 89%. The S4 gene encodes for the non-structural σNS protein that has a central role in RNA packaging and replication (2,63,64). Changes in the σNS protein might increase or reduce the efficiency of viral packaging and/or replication, subsequently affecting viral pathogenicity (65). When comparing whole genomes of all three strains, the greatest differences between the variant strains (A and B) and the classic C strain were in the L3 and M2 genes, with a nucleotide divergence of 28 and 25%, respectively. The L3 gene encodes for λC, which is the protein that mediates the guanylyltransferase activity in the viral mRNA cap formation (2,66). Genomic studies have shown that variations in certain regions of λC can be found between ARV, and even though no pathogenic associations have been made to date, these variations might interfere with the capping activity of the protein and the viral replication cycle (8,67,68). The M2 gene encodes for the major outer capsid protein μB, which is related to the stability, binding affinity and membrane association capabilities, therefore it can affect the viral infectivity, replication, and produce apoptosis (69,70). In our experiment, we observed higher viral loads in tendons and hearts of chickens inoculated with the classic C strain at 15 DPC as well as more severe histopathologic lesions in tendons and hearts at 28 DPC, and these variations might be associated with the λC and μB proteins. However, targeted studies are required to fully understand the role of these proteins in viral replication and consequently disease outcome.

The clinical signs and lesions induced by all 3 ARV strains used in this experiment are typical and have been described previously (3), including for S1133 (strain C) (6,11,34,71–75). Remarkably, there was a significant difference in the timeline of onset of clinical signs and lesions, severity of the tissues affected, and viral loads. Reduced body weights were seen in all inoculated chickens at 15 DPC compared to unchallenged controls and remained low throughout the experiment. Similar outcomes have been reported in the literature, where body weight gain was significantly affected by different ARV challenges (32,74,76). Strain A induced the lowest mean body weight and the lowest leg integrity scores in inoculated chickens between 15 and 28 DPC. In this case, the leg integrity scores of birds challenged with strain A were determined by both the body weight and the hock joint thickness. Conversely, the B strain caused the lowest mean body weight in the indirectly exposed birds. The indirectly exposed chickens might have been infected through the oral-fecal route, with a likely lower and less uniform viral dose than the inoculated chickens, which could explain why the growth retardation was seen relatively late in exposed birds. The B strain also caused the highest growth retardation in challenged birds, which might be correlated with the high viral loads found in the intestines. Despite the arthrogenic origin of strain B, it seems that this strain could contribute to malabsorption, affecting growth.

The tissue of isolation has been suggested as being key to the understanding of ARV pathogenicity (33,77). However, the origin of the ARV isolate did not seem to be relevant in this experiment. For instance, the necrotic myocardium-origin strain A presented with the lowest microscopic lesion scores in the myocardium. Until now, there were no reports on the pathogenicity of ARV isolated from myocardium lesions, and the dynamics of these strains in the chicken host need to be further investigated.

In the inoculated groups, the histologic lesions in tendons and hearts caused by the C strain were observed earlier and persisted until the end of our trial, and these lesions were also associated with higher and persistent viral loads in these organs. We also observed high mortality in the C strain-inoculated birds at an early age that might be related to the high challenge dose and to an induced immunosuppression that might have led to opportunistic infections. The A strain showed similar replication dynamics to the C strain in inoculated birds at 8 DPC, inducing the lowest body weights; however, it did not produce major lesions in tendons and hearts. Analyzing the lesions in the intestines might have given us more evidence to support variant A's ability to induce malabsorption syndrome.

Although the infection occurred in all horizontally exposed groups as demonstrated with the histopathologic lesions, the B strain appears to be more efficiently transmitted compared to A and C strains. A and C strains showed no viral load in hearts and duodena and very low viral load in the tendons of horizontally exposed birds at 8 and 15 DPC compared to B. To detect ARV in horizontally exposed birds, the samples probably should have been collected before 8 DPC. Despite its arthrogenic origin, the B strain was more efficient in replicating in duodena and causing lesions in hearts of exposed chickens at 8 DPC. In addition, strain B was able to diligently be transmitted horizontally, even though the viral loads were lower than in the hearts and tendons of birds inoculated with A and C strains via footpad.

Immunosuppression has already been linked with ARV in the past (45,78,79). Here, we report that lymphoid depletion occurred in the presence of genetic variants. When the strains were parenterally inoculated in high doses, all of them caused moderated to severe lymphoid

depletion in thymi and bursas. However, in the horizontally exposed chickens, the A strain seems more prone to induce immunosuppression and lymphoid depletion, particularly in bursas.

In conclusion, we were able to demonstrate that isolates classified into the same genotypic cluster have different pathogenicity, and these differences might be associated with genes other than the S1. We demonstrated that ARV strains that are genotypically related can induce different disease dynamics in identical experimental settings. Since ARVs can replicate in various tissues, the origin of the virus isolate does not necessarily represent its preferred tissue tropism. It is very likely that genes other than the S1 play an important role in virus tropism, replication rate and disease outcome. For instance, the major difference between variant strains A and B was in the S4 gene, while differences between the variants and the classical strain were in genes L3 and M4. This research provides meaningful insights into the development of challenge experiments to associate the pathogenicity of ARVs to their genomic composition.

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Table 2.1. Percent nucleotide identities between ARV strains A, B and C for each viral gene and for the concatenated whole genome (WG) sequences.

Gene		L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	WG
Size (bp)		3,882	3,768	3,865	2,272	2,124	1,971	1,631	1,307	1,171	1,158	23,149
Comparison		Nucleotide identity (%)										
C	A	89.4	88.8	72.5	90.0	74.9	88.5	79.9	88.3	86.3	88.1	84.18
C	B	89.4	88.9	73.0	89.9	75.1	88.7	79.8	88.4	86.0	90.7	84.42
A	B	99.9	99.9	99.4	99.8	99.4	99.6	99.2	99.9	99.3	89.0	99.12

WG = whole genome

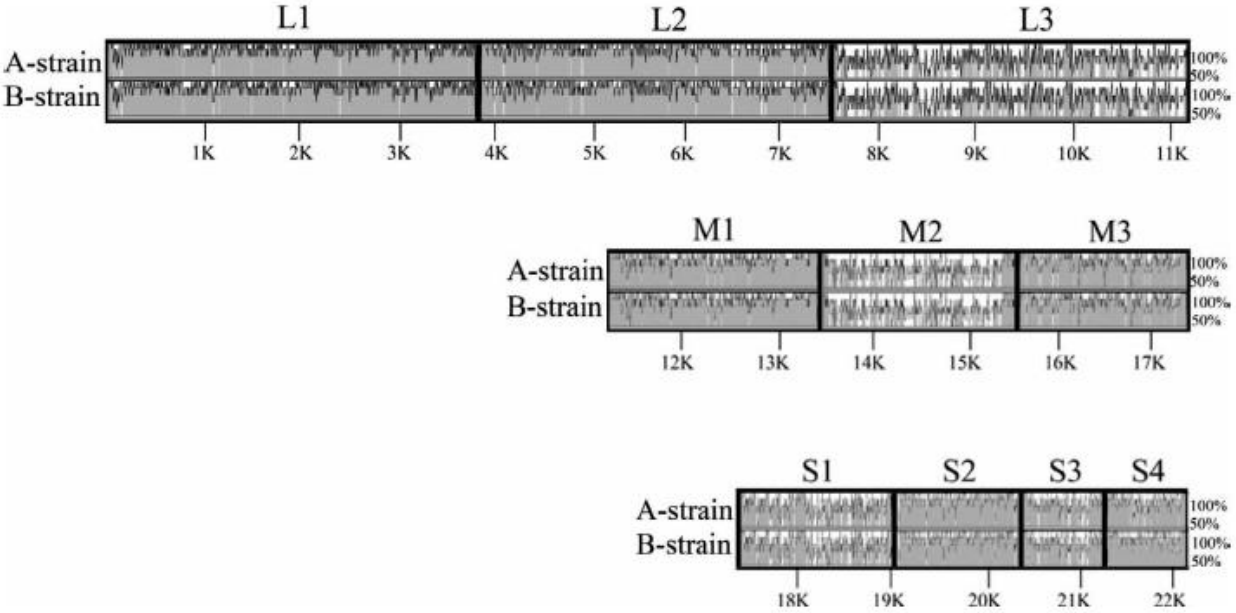

Figure 2.1. Alignment of concatenated whole-genome segments of ARV A and B strains compared with the S1133 vaccine (C strain). The gray-colored areas represent ≥90% of nucleotide identities; the white areas represent <90% nucleotide identities. The height of the contoured area is proportional to the genetic relatedness. The scale bar at the bottom shows the approximate length of the concatenated genome. The figure was created using the mVISTA software.

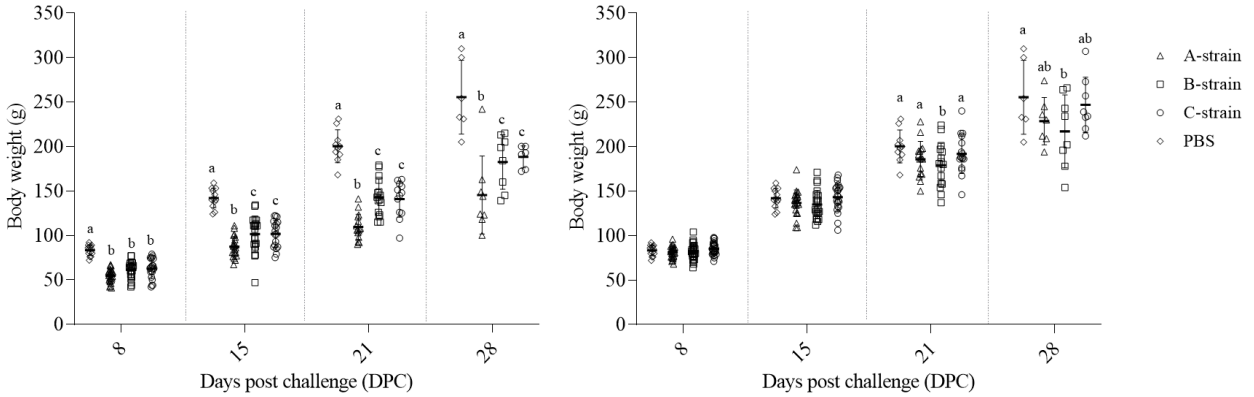

Figure 2.2. Body weights (g) at 8, 15, 21, and 28 days post-infection in chickens inoculated with (A) or exposed to (B) ARV strains A, B or C. The PBS group represents the negative controls. Lowercase letters represent significant differences between groups at each timepoint ($P < 0.05$).

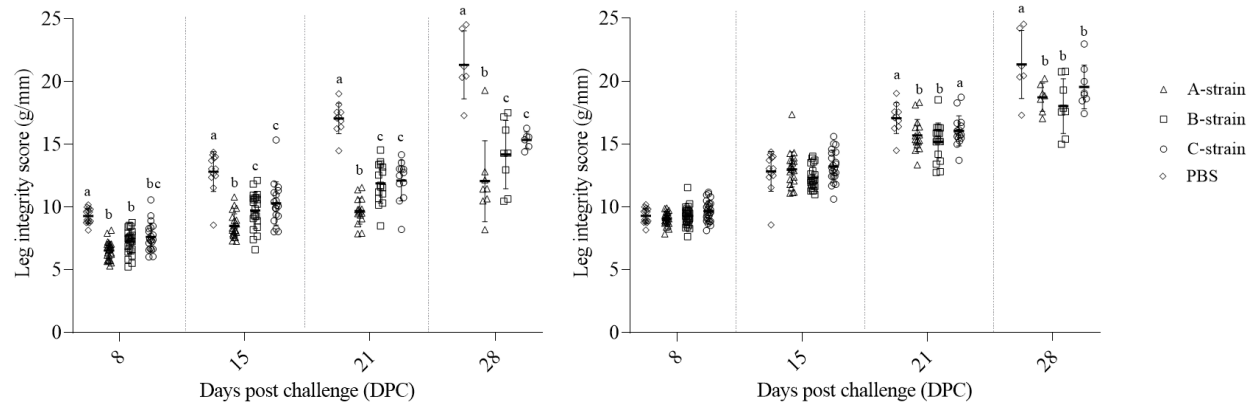

Figure 2.3. Leg integrity score (g/mm) at 8, 1, 21, and 28 days post-infection in chickens inoculated with (A) or exposed to (B) ARV strains A, B or C. The PBS group represents the negative controls. Lowercase letters represent significant differences between groups at each timepoint ($P < 0.05$).

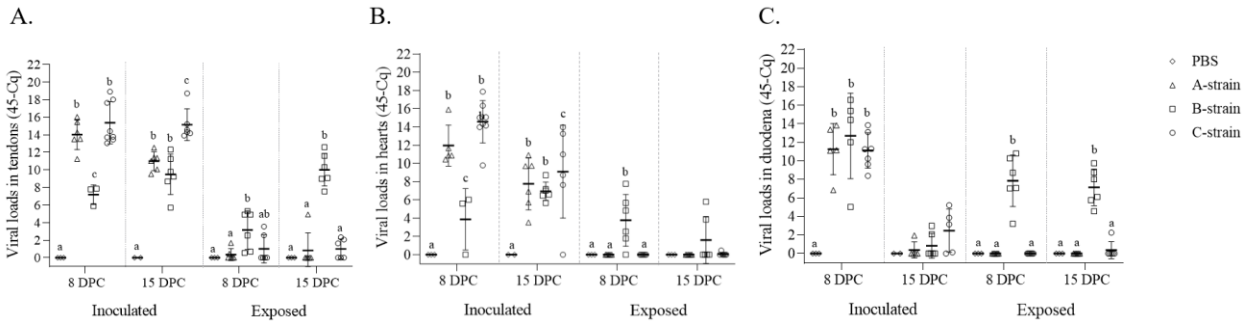

Figure 2.4. ARV viral loads (45 Cq) in tendons (A), hearts (B) and duodena (C) at 8 and 15 DPC in chickens inoculated with or exposed to avian reoviruses A, B or C strains. The PBS group represents the negative controls. Lowercase letters represent significant differences between groups at each timepoint ($P < 0.05$).

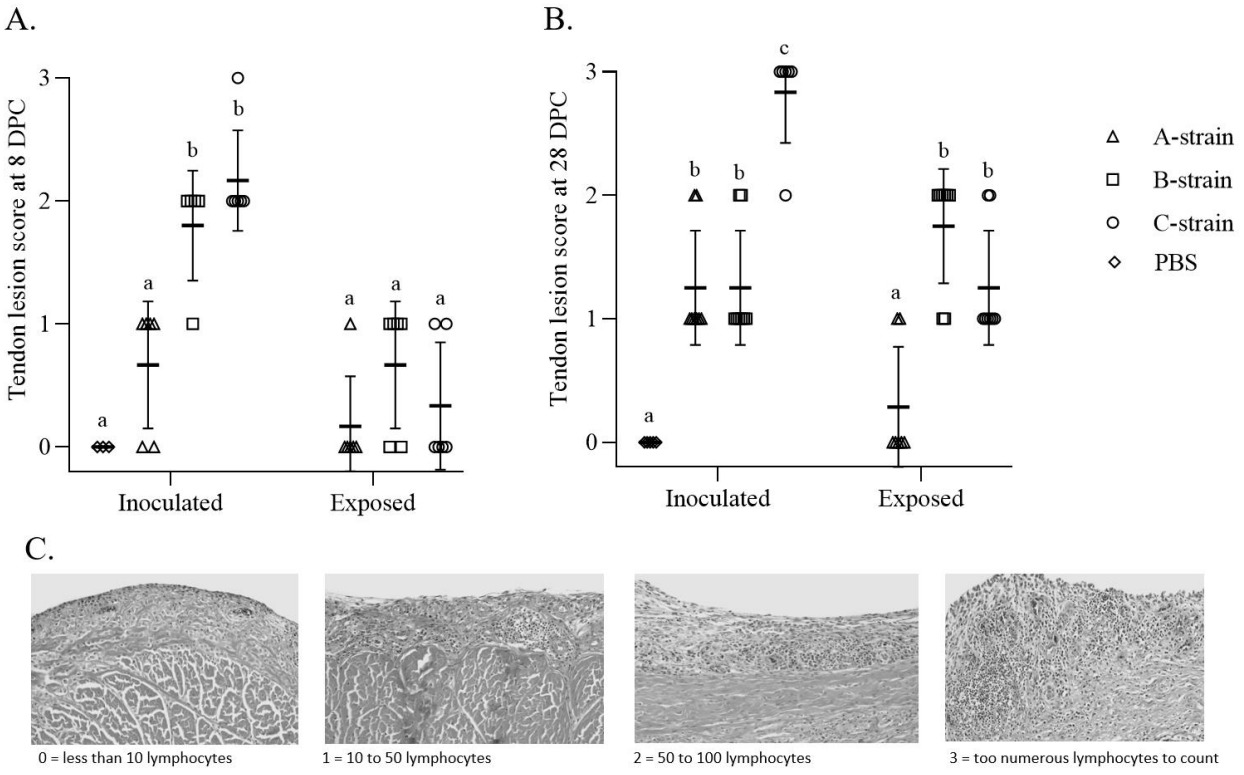

Figure 2.5. Microscopic tendon lesion scores in chickens inoculated with or horizontally exposed to ARV strains A, B and C at 8 (A) and 28 DPC (B). (C) Examples of histopathologic scores in tendons

(20X, H&E). The PBS group represents the negative controls. Lowercase letters represent significant differences between groups at each timepoint ($P < 0.05$).

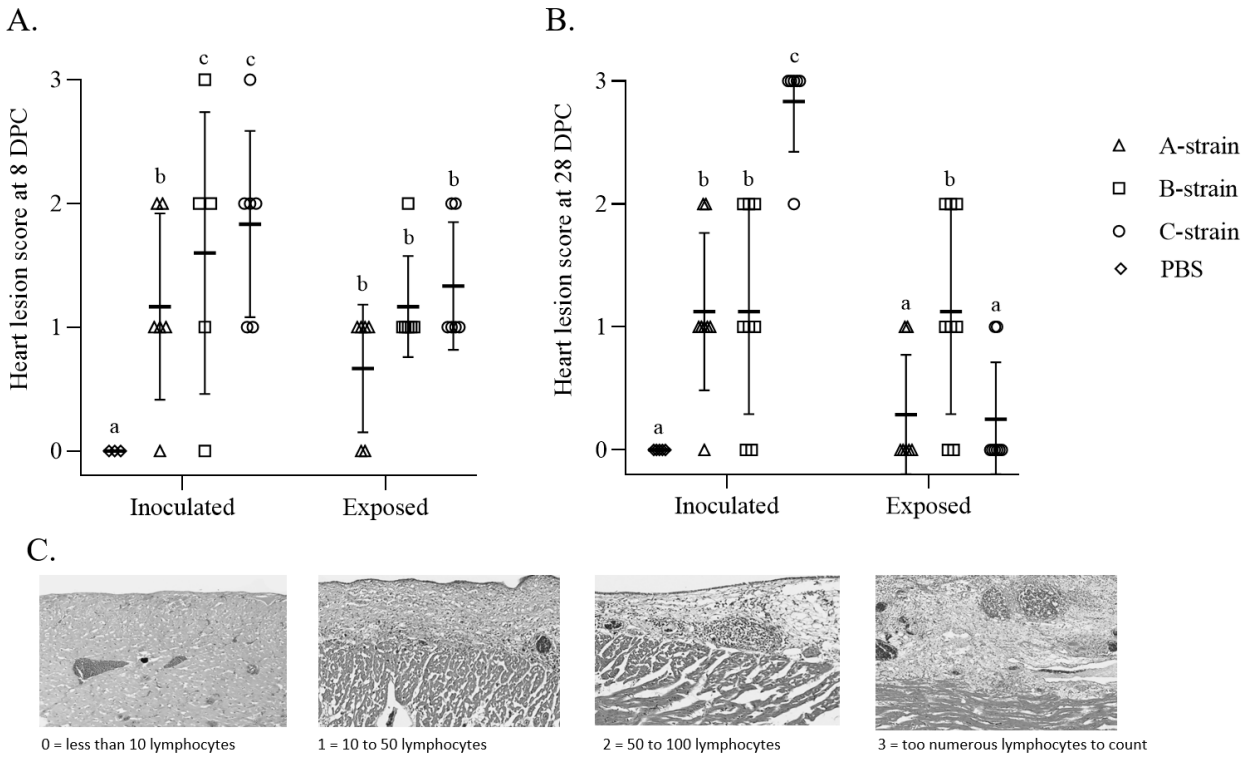

Figure 2.6. Microscopic heart lesion scores in chickens inoculated with or horizontally exposed to ARV strains A, B and C at 8 (A) and 28 DPC (B). Examples of histopathologic scores in hearts are shown in C (20X, H&E). The PBS group represents the negative controls. Lowercase letters represent significant differences between groups at each timepoint ($P < 0.05$).

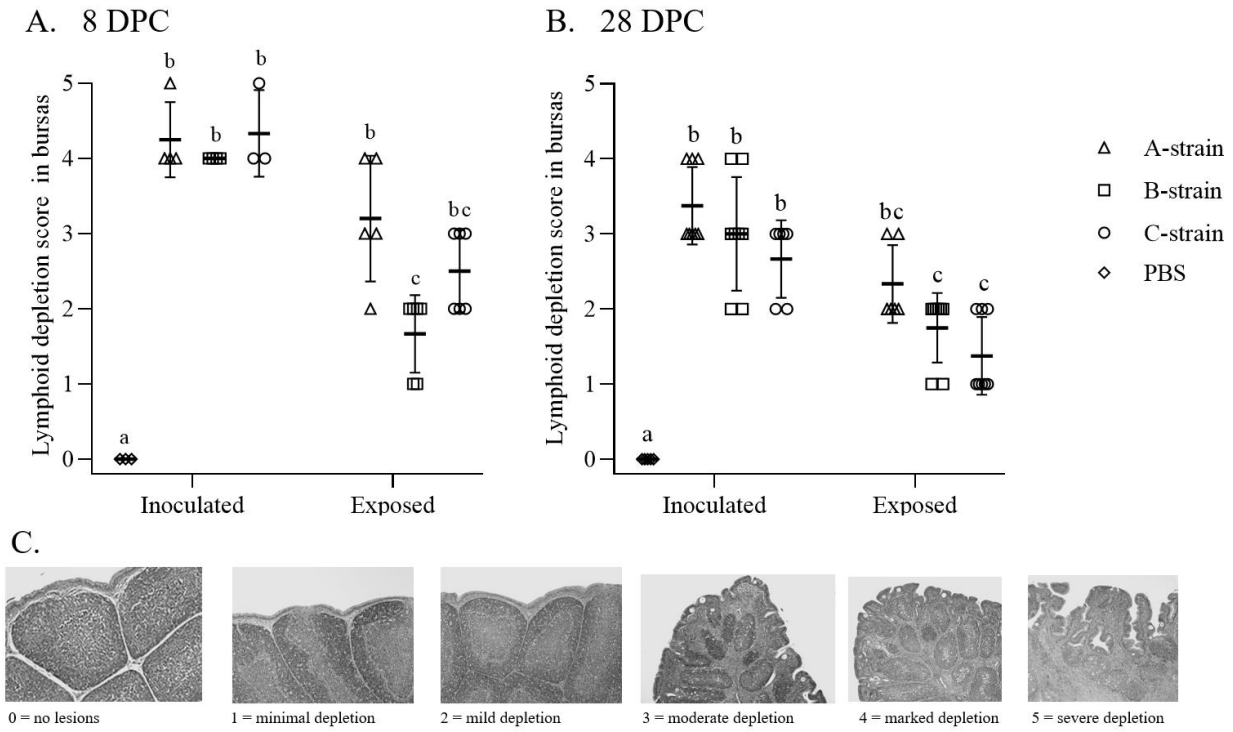

Figure 2.7. Bursal lymphoid depletion scores in chickens inoculated with or horizontally exposed to ARV strains A, B and C at 8 (A) and 28 DPC (B). Examples of histopathologic scores in bursas are shown in C (20X, H&E). The PBS group represents the negative controls. Lowercase letters represent significant differences between groups at each timepoint ($P < 0.05$).

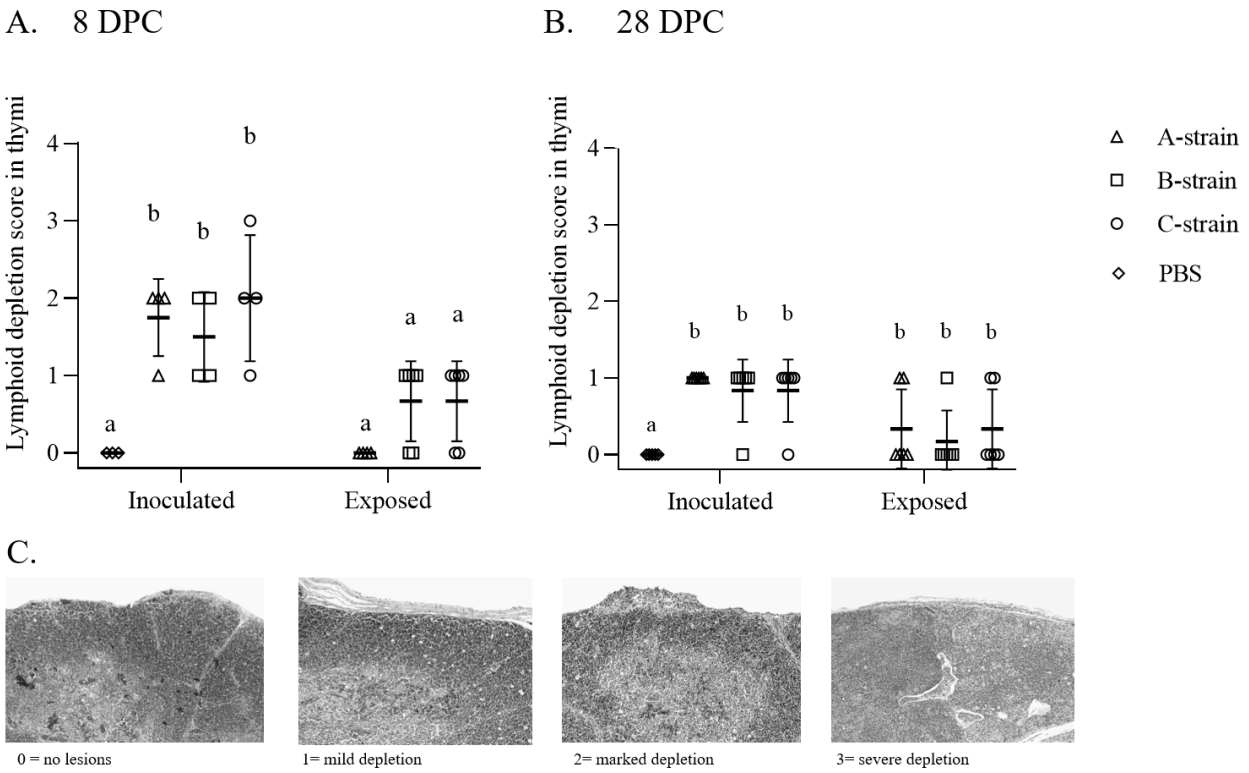

Figure 2.8. Thymic lymphoid depletion scores in chickens inoculated with or horizontally exposed to ARV strains A, B and C at 8 (A) and 28 DPC (B). (C) Examples of histopathologic scores in thymi are shown in C (20X, H&E). The PBS group represents the negative controls. Lowercase letters represent significant differences between groups at each timepoint ($P < 0.05$).

CHAPTER 3

Antigenic and Genetic Relatedness of Avian Reovirus Variants Based on the Cartography of Variable Genes

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SUMMARY

Avian reovirus genomes are in constant evolution, reason why it is important to associate genetic changes with the antigenicity of variant strains. This information is crucial to streamline the selection of candidate viruses to include in autogenous vaccines. Twenty-nine avian reoviruses, including conventional and variant strains, were plaque purified and used to produce hyperimmune sera. Cross-neutralization was performed, and the results were assessed by antigenic cartography. The antigenic distances between the isolates were calculated from the antigenic map. Additionally, variable genes (i.e. L3, M2, S1 and S4) of these strains were amplified

and sequenced. The amino acid substitutions were calculated and used in a genetic cartography, to obtain the genetic distances between ARV. Correlations between antigenic and genetic distances were obtained.

KEYWORDS: Avian reoviruses, antigenic cartography, cross neutralization, genetic characterization, variants.

INTRODUCTION

Avian reoviruses (ARV) cause endemic infections in chickens causing clinical signs such as viral arthritis-tenosynovitis, pericarditis, malabsorption (1–3) and immunosuppression (4–8), leading to economic and welfare concerns in poultry production. Reoviral disease prevention is based on the use of classical live vaccines and killed autogenous products in breeders. The success of the vaccination strategy depends on a good priming of the immune system with live vaccines and the use of killed products representing the ARV strains in the environment where chickens are raised. When the homology of field challenge and killed vaccines is low, the efficacy of the vaccination program is limited (2,9).

The ARV genome is composed of ten-segmented double-strand RNA which makes it prone to mutations, recombination and reassortment events that contribute to the emergence of molecular variants (1,9–12). Due to the ARV variability, it is imperative to perform epidemiological surveillance. One of the goals of this surveillance strategy is to evaluate and provide accurate field information that improves the selection of field viruses for autogenous vaccines production. The ARV epidemiological surveillance in the U.S. is based on molecular classification methods using a partial sequence of the S1 gene, the gene that encodes the σC

structural protein(14,1). This protein is responsible for cell attachment and elicits specific neutralizing antibodies (13,14). Currently isolated ARV strains show low nucleotide identities with classical live vaccine strains, i.e. S1133 and its derivatives. This is also one of the reasons why they are considered as genetic variants (1,15). These variants are grouped into different clusters according to their nucleotide sequences. To date, seven ARV genotypic clusters have been reported based on their σC phylogenetic topology (1,9–12,15,16). Nucleotide sequences within a single genotypic cluster or genotype can vary up to 60% adding significantly to the total variability (12). The current genotyping using partial S1 gene sequences has shown to be useful to understand the prevalence, epidemiology and distribution of the virus in poultry flocks (17) but does not provide a full picture of the antigenicity of circulating strains. While two strains can be in the same genotype using the S1 gene sequences, they might be classified into different serotype groups (18). In addition, a considerable amount on non-typable strains have been detected through serotyping (19), demonstrating the influence other genes have regarding virus antigenicity (20) and urges the investigation on robust reovirus classification methods to assess viral antigenicity.

Avian reovirus sequencing have provided information that indicates a significant variability in L3, M2, and S4 genes in addition to S1 (12,21–24). The L3 gene encodes for the λC protein which mediates the guanidyl transferase activity in the viral mRNA cap formation (14,25). Variability in regions of λC might cause interference in the capping activity affecting viral replication (26,27). The M2 gene encodes the major outer capsid protein μB. This protein undergoes post-transcriptional modifications cleaving the protein into μBN and μBC (14). This cleavage increases viral infectivity and replication rate (23,25,28). The S4 gene encodes for the

non-structural σNS protein, which has a central role in RNA packaging and replication (14,26,29). Changes in this protein might influence viral pathogenicity (27). Besides σC, other ARV proteins such as λB, λC, σA and σB have proven to have neutralizing activity (14,25,30–34).

Basic information on how ARV gene sequences correlate with antigenicity is crucial to develop a robust classification method and inform virus selection for vaccine development. Antigenic cartography is the process of mapping antigenically variable pathogens based on quantitative serological data to calculate antigenic distances between antigens (35). Genetic cartography creates maps based on the amino acid substitutions on antigenic proteins and compares the genetic distances between pathogens. A correlation of the outcomes of the two mapping strategies will allow us to associate antigenicity and gene sequencing. This strategy has been successfully used in the virus selection for vaccine production in influenza viruses (35,36). In addition, the concept is utilized to predict emerging strain fitness considering population immunity and viral evolution (35–37).

We hypothesize that a correlation of different ARV isolates antigenic and genetic characteristics could help us determine the genetic basis of antigenicity on ARV. An effective model that includes the aforementioned attributes can contribute to epidemiological surveillance and disease prevention by streamlining the selection of avian reovirus isolates to be included in autogenous vaccines.

MATERIALS AND METHODS

Viruses

A total of 28 ARV isolates obtained from clinical cases of arthritis/tenosynovitis during 2016 and 2020 were selected based on pathology, tissue of isolation and cytopathic effects (CPE) in cell cultures. All viruses were plaque purified and titrated representing single virus isolates as described by Rovozzo *et al* (38). In brief, CELc were inoculated with ten-fold dilutions of each ARV and covered with a 4% agar overlay. The CELc were incubated at 37^{o}C with 5% CO2 and examined daily. Single plaques showing CPE were collected and freeze/thawed three times. The final plaque purified stocks were replicated, titrated (38) and stored at -80^{o}C. Another ARV, the classic S1133 strain was obtained (Charles River, Catskill, NY) and used as a conventional reference. All the isolates were processed under the Biological Use Authorization (BUA) #R2109.

Chickens and embryonated eggs

Chicks (n= 40) were hatched from specific pathogen free (SPF) eggs, wing banded and used for hyperimmune sera obtention. Chickens were placed in Horsfall Bauer isolators, feed and water was provided ad-libitum, temperature and light were controlled. *The animal care and experimental procedures were performed in compliance to all Federal and institutional animal use guidelines* protocol #19092. *Polyclonal hyperimmune sera*

A total of eight polyclonal hyperimmune serum stocks were produced in SPF chickens. For each serum stock five chickens of three weeks of age were inoculated intraocularly with an ARV plaque purified isolate at a viral titer of 103 tissue culture infectious dose 50% (TCID50). At six, eight and ten weeks of age, booster inoculations were performed intramuscularly in the breast

muscle using inactivated homologous viruses. The virus titer before 4% formaldehyde inactivation was 103 TCID50. Each virus solution was mixed with Freund's incomplete adjuvant (Sigma Aldrich Burlington, MA) in a 1:1 volume to volume emulsion using an IKA homogenizer (IKA Works, Wilmington, NC). An enzyme-linked immunosorbent assay (ELISA) (IDEXX Laboratories, Westbrook, ME) was performed after two weeks of each booster. At 12 weeks of age, the chickens were bled to obtain sera. Individual serum samples in each group with a titer over 8,000 Geometric mean were pooled and stored at 4oC. A commercial S1133 polyclonal hyperimmune antiserum (Charles River, Catskill, NY) was utilized as reference.

Cross-neutralization assay

A virus neutralization (VN) test was performed for each serum and its homologous virus (39). Sera were diluted to 512 virus neutralizing units. A cross-neutralization assay was performed using 29 virus isolates and nine antisera following the beta method adapted from Gauger *et al* (39). In brief, polyclonal serum samples were filtered and heat-inactivated at 56^{o}C for one hour. Each serum was diluted two-fold, mixed with each ARV strain at 100 TCID50% per well and incubated at 37oC for an hour. After the incubation, the serum / virus mixtures were inoculated in confluent CEL cell monolayers in duplicate. The last row of each 96-well plate served as a negative control. Cell cultures were incubated at 37^{o}C with 5% CO2 and examined daily for 72 hours under a light inverted microscope for cytopathic effect. The neutralization index was calculated by the reciprocal number of the highest dilution at which the virus was inhibited.

Antigenic cartography

Data obtained from the cross-neutralization assay was used to calculate antigenic distances between avian reovirus strains and plotted spatially into a 2-dimension antigenic maps using the ACMACS software (available through https://acmacs-web.antigenic-cartography.org/) following the protocol by Smith et al., 2004 (35). Briefly, each viral neutralization titer was used to populate a table with the list of viruses and antisera panel. An algorithm based on a multidimensional scaling was used to generate cartesian coordinates that were used to create a map. Both axes, vertical and horizontal, represent antigenic distances, each neutralizing index value were plotted individually in the map (35,36). The antigenic distance between an antigen and an antiserum was used to determine the potential of that antiserum to neutralize the virus. Therefore, an antiserum with a high virus neutralizing titer will have a shorter distance.

Amplicon sequencing

A total of 29 avian reoviruses were selected for L3, M2, S1, and S4 amplicon sequencing. A two-step RT-PCR was used to obtain amplicons from the targeted genes using the primers described by Banyai *et al* (40). Due to the length of the amplicons L3 and M2, amplicons were subjected to a third-generation sequencing strategy (Oxford Nanopore Technologies, Oxford, UK). Briefly, 1 µg of double-stranded cDNA was purified using Agencourt AMPure XP beads (Beckman Coulter) and used as input to a library generated with the Ligation Sequencing Kit SQK-LSK109 (Oxford Nanopore Technologies (ONT), Oxford, UK). First, DNA was end-repaired using the NEBNext Ultra II DNA End Prep and Repair kit (New England Biolabs, Ipswich, MA), purified using AMPure XP beads in a ratio of 1:1 volume of beads per sample and eluted in 25 µL nuclease-

free water. Each sample was individually barcoded using the Native Barcoding Expansion (EXP-NBD104-114) (ONT). Barcodes were ligated using NEB Blunt/T4 Ligase Master Mix (New England Biolabs) by incubation at room temperature for 10 minutes. In addition, the sequencing adapters (AMII and ONT) were ligated to the DNA library using NEBNext Quick T4 DNA ligase (New England Biolabs) by incubation at room temperature for 10 minutes. The adapter-ligated DNA library was purified with AMPure XP beads in a ratio of 1:2 volume of beads per sample, followed by two washes with the short fragment buffer (ONT) and elution in 15 µL elution buffer (ONT). The library was loaded onto a Spot-On Flow Cell Flow Cell R9.4.1 (ONT) and run via MinKNOW software (ONT) for 24 hours. Base-called Fastq files containing "pass" reads (Q-score ≥8) were loaded into Geneious Prime software version 2021.1.1 (Biomatters Ltd., San Diego, CA) and mapped to reference avian reovirus L3 and M2 segments (AY652693, AY635934). De novo assembly was performed with the list of mapped reads and a consensus sequence was obtained for each amplicon.

Amplicons for S1 and S4 were obtained through a two-step RT-PCR using the primers described in 2003 by Kant *et al* (1). Partial S1 and whole S4 sequences were sent out for Sanger sequencing.

Genetic characterization

The nucleotide sequences of the L3, M2, partial S1 and S4 genes were translated into amino acid sequences in Geneious Prime software version 2021.1.1 (Biomatters Ltd., San Diego, CA). Multiple alignments and amino acid identities were calculated using Clustal Omega (41). Phylogenetic trees were generated using the Neighbor joining method with 1,000 bootstrap replicates. To classify our isolates based on the partial S1 gene, we included 42 reference

sequences from the genotypic clusters from 1 to 6, obtained from available literature (1,9,10,21). In addition, genetic distances between viruses were calculated based on the amino acid substitutions for each protein by the No. of pairwise differences method described by Kumar *et al* (42). Genetic distances were spatially plotted into a two-dimension genetic cartography plot using the ACMACS Software following the protocol by Horton and collaborators (43).

Correlation between antigenic ang genetic distances

Data collected was tested for normal distribution using Shapiro-Wilk and Levene's tests for homogeneity of variance. Correlation between antigenic and genetic distances was calculated by a linear regression model Pearson's correlation analysis with 95% confidence interval. Statistical analyses were done using GraphPad Prism 9 (44).

RESULTS

Cross-neutralization assay

The results of the cross-neutralization assay are displayed in Table 3.1. Each antiserum was able to neutralize their homologous virus. Different levels of cross neutralization were found through this method. For example, ARV #1 was neutralized by all the heterologous antisera, in contrast its homologous antiserum (S1) showed a poor neutralization for the other viruses. ARV #4 was strongly neutralized by the heterologous antisera S5, S6, S8 and partially by S3. ARV #11 and 23 were both neutralized by S6 and S7. ARV #5 and 10 were both neutralized by S9, and ARV # 2 was neutralized by S6.

Antigenic cartography

Antigenic maps were constructed in different dimensions (2D, 3D and 4D) to determine the best resolution of the cartography. Two dimensions map was chosen to help in the visualization of the data. Figure 3.1 displays a two-dimensions antigenic map representing the antigenic relationships between 29 ARVs and the panel was conformed by nine antisera. Each square on the grid represents one antigenic unit of distance (AU) on each side. A short distance between viruses and one antiserum represents a higher possibility of cross-neutralization conferred by the anti-sera.

All the ARVs studied were closely related to their homologous antiserum. All antisera panel is in the center of the map and some ARVs form clusters around them. Other ARVs (i.e. 19, 20, 21 and 28) are far from the virus clusters and antisera from the panel. As a reference ARV# 9 represents the classical strain S1133. In the map, S9 is closer to ARV# 3, 4, 5, 8, 22, 25, and 27 and far from ARV# 19, 21, 26, 28.

Genetic characterization and cartography

A total of four phylogenetic trees were prepared to illustrate the genetic relation between the amino acid sequences of ARV variable proteins (Figure 3.2). Complete amino acid sequences were used for λC, μB and σNS, while a portion was used for σC. The obtained phylogenetic trees illustrate the level of variability of these strains in all the genes selected for the analysis. The λC phylogeny shows considerable diversity on its encoding L3 gene. The tree shows three main clusters (Figure 3.2A). The first cluster comprises the reference S1133 and only two other ARVs i.e. 9 and 19. The second cluster looks relatively uniform compared with the third cluster that

shows two sub lineages. The amino acid identities in this group ranged between 72 to 99%. The µB phylogeny shows three main clusters (Figure 3.2B). In this comparison the classical S1133 strain groups with seven ARVs. Clusters one and three consisted of two sub lineages each. The amino acid identities in the µB phylogeny range between 73 to 99%. The σC phylogeny evidenced high variability (Figure 3.2C), here three main clusters are represented. The second and third clusters are divided in two sub groups and some of those in additional clusters. While the largest subcluster is the one containing our reference sequence i.e. S1133, there is a considerable diversity on it. The amino acid identities in the σC phylogeny range between 52 to 99%. In addition, when the 26 out of 29 ARVs of this study were plotted using a common backbone of sequences,they grouped into six genotypic clusters (Figure 3.3). The σNS phylogeny illustrates the diversity of this gene. While only one sequence is grouped in the second cluster, the other two clusters depict a considerable number of sequences and sub lineages (Figure 3.2D). The amino acid identities in the σNS phylogeny range between 63 to 99%. ARV #9 corresponds to the S1133 classic commercial isolate used in this investigation. All of the ARV #9 amino acid sequences were classified very close to the S1133 sequences retrieved from GenBank (Figure 3 A-D).

Based on the amino acid sequence substitution data, genetic maps were constructed for each of the above-mentioned genes (Figure 3.4). A slightly different distribution of the viral sequences is noticed in each of the investigated genes. Some viruses maintained its relation e.g. 7, 5 and 3 forming one cluster that was consistent between genes and 4 and 8 forming another cluster.

Correlations between antigenic and genetic distances

Correlations between antigenic and genetic distances among the studied viruses and their antisera per each of the reviewed genes was calculated. No correlations were observed between antigenic and genetic distances between the 29 avian reoviruses. A positive correlation was observed between the antigenic and genetic distances of λC (R= 0.31; P< 0.05) and σC (R= 0.47; P< 0.05) when the sequences of the 9 viruses and their antisera panel were assessed (Figure 3.5). No correlation, between antigenic and genetic distances, was visualized in μB or σNS amino acid sequences (data not shown).

DISCUSSION

To prevent and control avian reovirus outbreaks, it is crucial to strategize an adequate virus selection for autogenous vaccine selection. For this, it is important to understand the antigenicity of viral isolates. Currently, vaccine virus candidates are selected based on σC genotyping and while σC provides some antigenicity information, and this is not enough to elucidate the antigenic makeup of these viruses (18,32). Other proteins i.e. λB, λC, σA and σB have been described as potential antigenic determinants of ARVs (14,23,25,30–32). Since ARVs are highly variable viruses we assume that variable genes encoding for structural proteins might have an influence in antigenic determination (11,12,21,23,24). In this study we attempted to determine the genetic bases for antigenicity on ARVs. For that, we studied genetic and antigenic composition of ARV variant strains by using antigenic cartography. The information obtained from the genetic and antigenic makeup of these viruses was correlated to understand if any gene was providing viral antigenic capabilities.

In the cross-neutralization assay, we detected different degrees of cross neutralization between antisera and viruses, this has been previously described (17,18,45,46). Only one virus (ARV# 1) was neutralized by all the heterologous antiserum, but its homologous antiserum (S1) showed insufficient cross-neutralization. This result suggests the presence of a conserved feature in ARV antibodies that might be providing neutralization to that specific virus. Similarly, ARV# 4, was effectively neutralized by the antisera S5, S6 and S8. Interestingly, ARV# 4 was classified as σC genotype 1, in which antisera S8 was produced with an ARV from the same the σC genotype, while the antisera S5 and S6 were produced using viruses from the σC genotype 3 and 4. Additionally, the antiserum S6 revealed cross-protection against 7/28 heterologous ARV, all of them from diverse σC genotypes. These results indicate that ARV neutralizing antibodies are elicited by proteins that are not necessarily related to σC. The generation of hyperimmune sera is a tedious process requiring several immunizations. Some of the ARVs we used in this process were poorly immunogenic, that factor might be associated with potential mutations in the plaque assay and adaptation of certain ARV to the chicken embryo liver cell cultures. This can be crucial in the prevention of this disease since a robust immune response induced by vaccines are needed to provide protection against infection and viral clearance. One example are the ARV# 1 and ARV# 7 which produced the lowest Geometric mean titers (data not shown). The ARV# 1, was unable to neutralize all of the heterologous viruses in this study, however ARV# 7, neutralized only 3 out of 28 heterologous viruses. Interestingly both viruses were classified in the σC genotypic cluster 6 and they were grouped in the same cluster when were classified under λC.

Antigenic maps contribute with an optimized visualization and interpretation of tests like virus neutralization (35). It has been employed to study antigenic relations of rapidly evolving

viruses such as orthomyxoviruses (35,47), coronaviruses (48) and noroviruses (49). Additionally, this tool has been employed in the selection of vaccine candidates against influenza in humans. The antigenic map depicted in Figure 3.1 shows the antigenic relationship of selected ARVs and the antisera in our panel. The distribution of the different viruses does not correlate with the σC, λC or μB classification patterns shown in Figure 3.2. Virus clustering in the map shows complex antigenic relationships that might be accounting for immune interactions that consider antibodies elicited by different proteins. One example is antisera S6 and S7 which are closely surrounded by 7 out of the 28 heterologous viruses. These associations warranted further investigation to find the basis of their antigenic similarity and in practice to select successful vaccine candidates. Our map was constructed based on polyclonal antibodies and given that ARV has more than one antigenic determinant, the immunogenicity of multiple antigens can be complicated to analyze. Using monoclonal antibody might be a better way to determine accurate associations between antigens and neutralization.

Through phylogeny we were able to demonstrate high variability in the studied genes. A diverse cluster generation was seen, as reported in a previous study (12). M2, the coding gene for μB, possesses the largest sequence divergence of the M-class segments (22,31,50). Interestingly, O'Hara *et al* (20) reported that the μB protein affected the macrophage infection efficiency in two genetically different avian reoviruses (20). Another research described the variability of the L3 gene which encodes the mRNA capping enzyme, λC (23). S4 encode the non-structural σNS which is viral RNA-binding protein. This protein has a role in genome reassortment (26), crucial in the ARV variability. The above-mentioned genes / proteins have an important role in the virus functionality, and they denote high variability (50). These findings further suggest

their potential involvement in antigenicity. Additional research including a larger and more diverse dataset of viruses and antisera is needed to add robustness to these results.

Genetic maps display the ARV distributed in two-dimension model, according to their amino acid sequence substitution data. We can see clear clusters in these maps but is difficult to interpret their relevance.This is the reason why we utilized correlations between antigenic and genetic distances. Positive correlations between genetic and antigenic information were observed in λC and σC when we focused only on the data generated by the viruses that had homologous antisera. Because we expected to observe over 0.7 of correlation, the sample size was calculated and obtained by a convenience sampling approach. Nevertheless, the correlations obtained in this study were under 0.5, indicating the need of generating additional sequences and hyperimmune sera to add power to our studies. The lack of correlation between antigenic and genetic distance of other ARV might be related to other ARV genes that were not assessed in this investigation. Other ARV proteins (i.e. λB, σA and σB) , have the potential to alter ARV antigenicity (14, 30–34), nevertheless we decided to begging our research with the protein encoding genes (i.e. L3, M2, S1 and S4) because of their variability observed in our previous investigations (12,51). . The different sequencing methods (i.e. Sanger and Oxford Nanopore) in this investigation have dissimilar levels of accuracy and this should be considered in the obtained genetic results. Additionally, the other factors like the functional information that neutralization incorporates which accounts for protein folding and post translational modifications that are not considered in amino acid sequences. In addition, there might be other ARV proteins that were not considered here that might play other roles in immunogenicity (27,52).

This study has tried to effectively correlate antigenicity and amino acid sequences from different ARVs. While cartography proved to be a good method to visualize antigenicity and genetic differences, a bigger and more diverse set of samples need to be used to really prove the effectiveness of this tool and increase the power of the associations.

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Table 3.1. Homologous and heterologous virus neutralizing titers of avian reoviruses.

	Antiserum								
Virus	S1	S2	S3	S4	S5	S6	S7	S8	S9
1	**512**	512	512	512	512	512	512	512	32
2	*	**512**	*	*	*	512	*	*	*
3	*	*	**512**	*	*	*	*	*	*
4	16	*	128	**512**	512	512	*	512	32
5	*	*	*	*	**512**	*	*	*	512
6	*	*	*	*	*	**512**	*	*	32
7	*	*	*	*	*	128	**512**	*	*
8	*	*	*	*	*	128	*	**512**	32
9	*	*	32	*	*	*	*	*	**512**
10	*	*	32	32	64	*	*	32	512
11	*	32	*	*	32	512	512	64	*
12	32	*	*	*	*	*	64	*	*
13	*	*	32	*	*	*	*	*	*
14	32	32	*	32	*	32	32	*	32
15	32	32	32	32	*	*	*	*	*
16	32	*	32	*	*	*	*	*	*
17	*	*	*	*	64	*	*	*	*
18	*	*	*	*	*	64	*	*	*
19	*	*	*	*	*	*	*	*	*
20	*	*	*	*	*	*	*	*	*
21	*	*	*	*	*	*	*	*	*
22	*	*	*	*	*	*	*	*	32
23	*	32	32	64	*	512	256	*	64
24	*	*	*	*	*	*	*	*	*
25	*	*	*	*	32	32	*	*	32
26	*	*	*	*	*	*	*	*	*
27	*	32	32	32	64	64	32	32	*
28	*	*	*	*	*	*	*	*	*
29	*	32	32	32	*	*	*	*	*

* Indicates values below 16 virus neutralization units

Bolded numbers correspond to different viruses and their homologous antisera

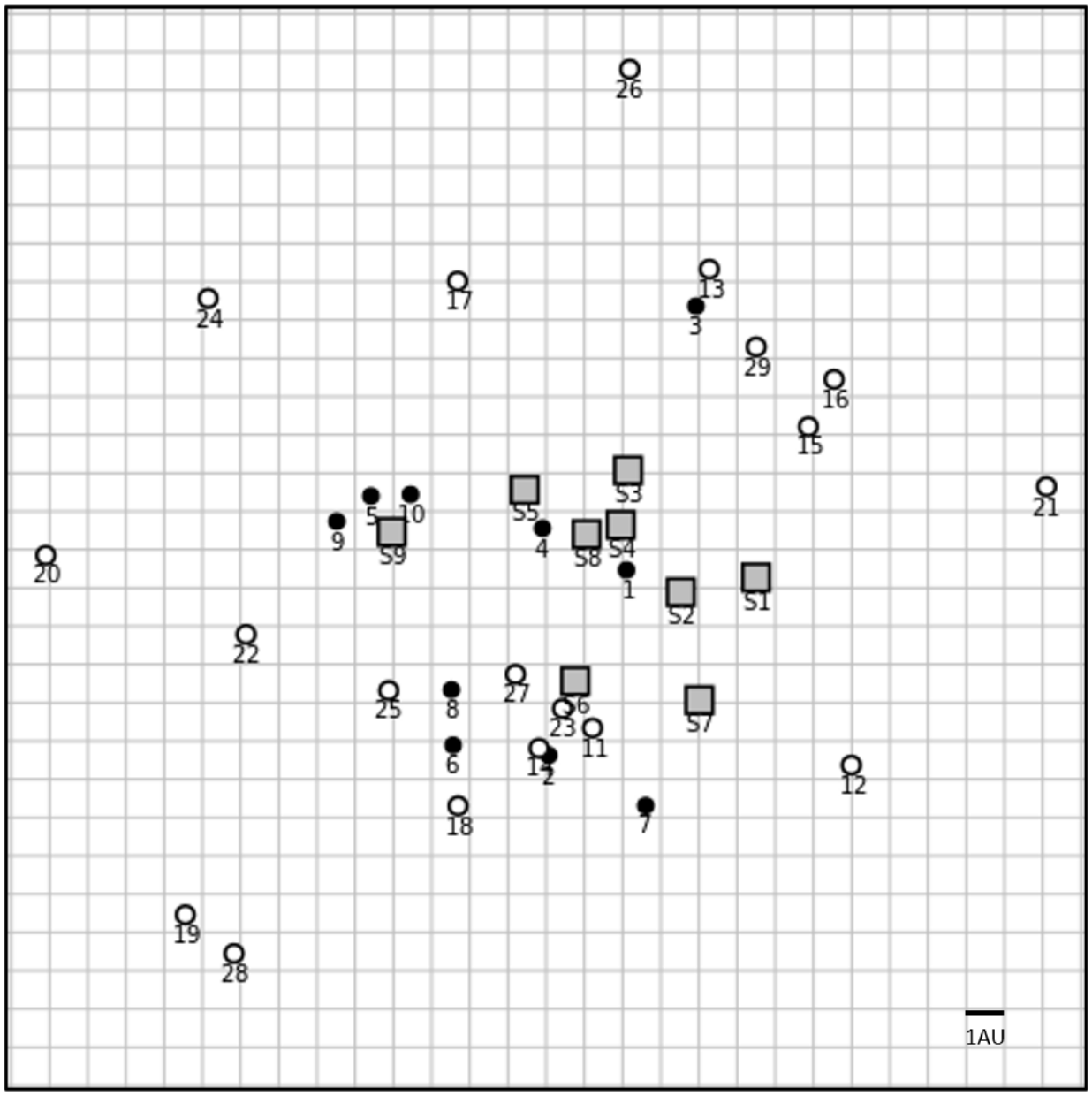

Figure 3.1. Antigenic cartography of 29 avian reoviruses and 9 antisera distributed in a grid where each side of a square represents one antigenic unit (AU) equivalent to a 2-fold dilution in antibody titer, calculated based on cross-neutralization indices. The black circles represent viruses with homologous antisera (gray squares). Empty circles represent viruses without homologous antisera. The closer the viruses are to the squares, the higher is the neutralization potential.

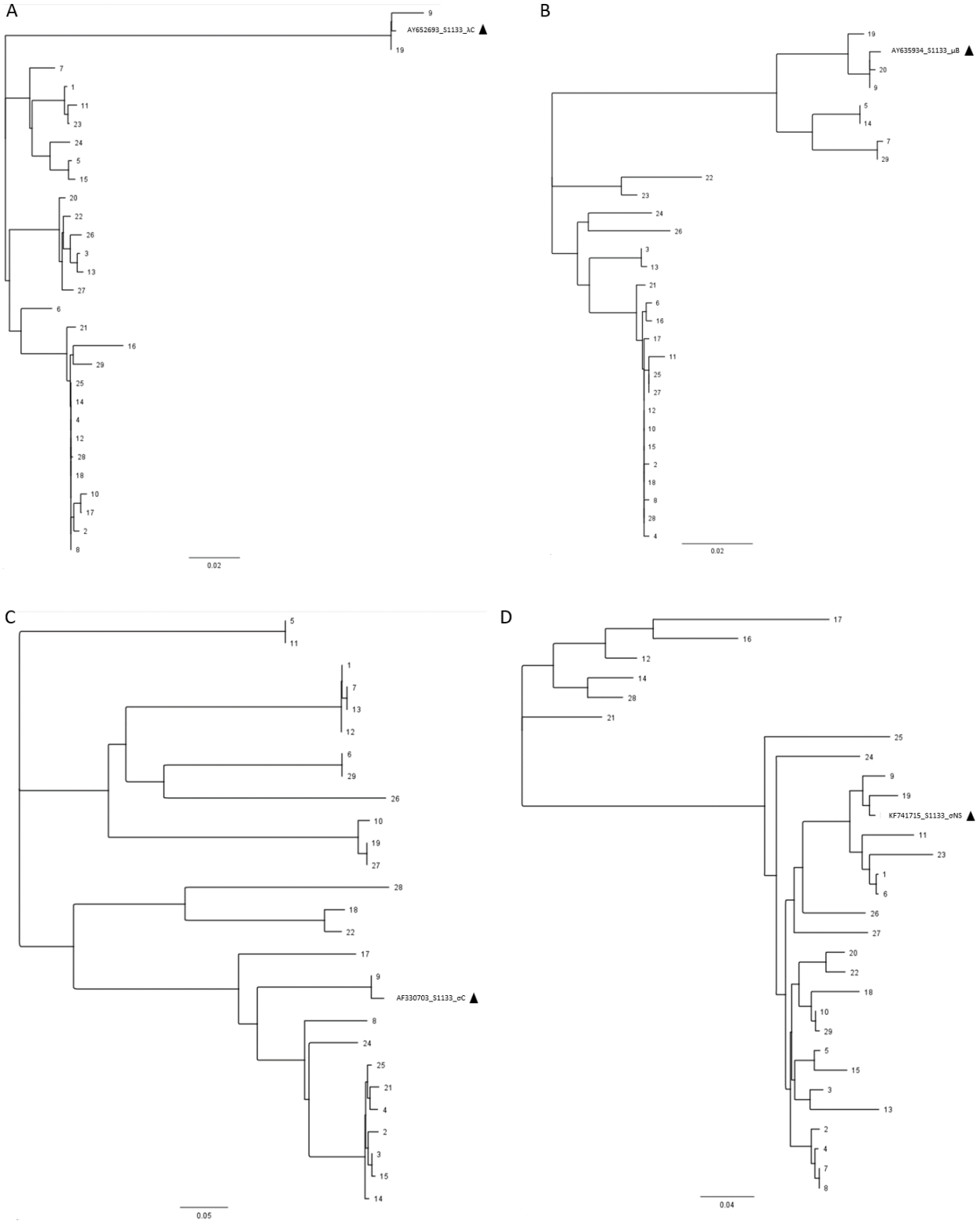
A
AY652693_S1133_λC
0.02
B
AY635934_S1133_μB
0.02
C
AF330703_S1133_σC
0.05
D
KF741715_S1133_σNS
0.04

Figure 3.2. Phylogenetic trees showing ARV amino acid sequences. A. λC= lambda C protein (1,285 amino acids), B. µB= mu B protein (676 amino acids), C. σC= sigma C protein (293 amino acids), D. σNS= sigma NS protein (359 amino acids). Phylogeny was performed using the Neighbor joining method in Geneious prime software. The reference strain S1133 is labelled with a black. triangle.

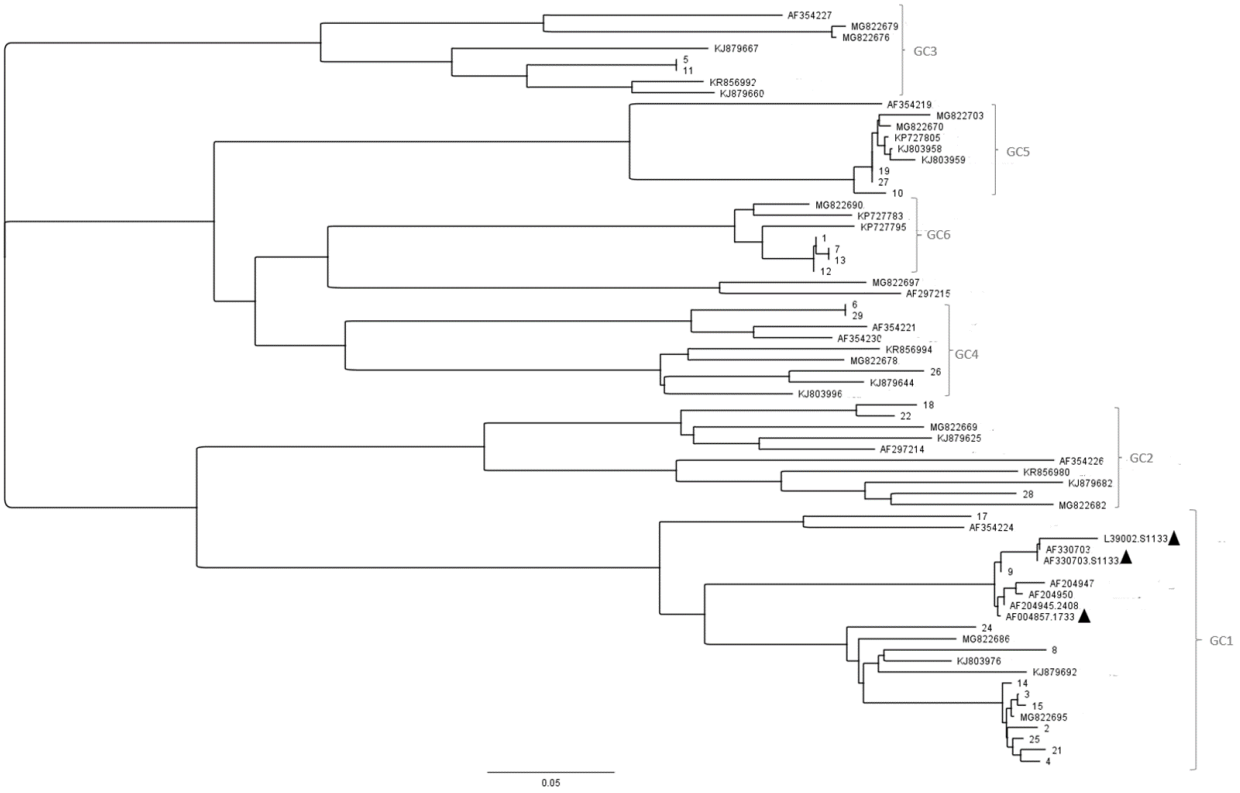

Figure 3.3. E. Phylogenetic tree depicting showing 26 ARV aligned σC protein sequences (293 amino acids). Reference sequences (GenBank codes) were used as a backbone. Genotypic clusters (GC) are represented by parentheses. Phylogeny was performed using the Neighbor joining method using Geneious prime software. The reference classic strains S1133 and 1733 are labelled with black triangles.

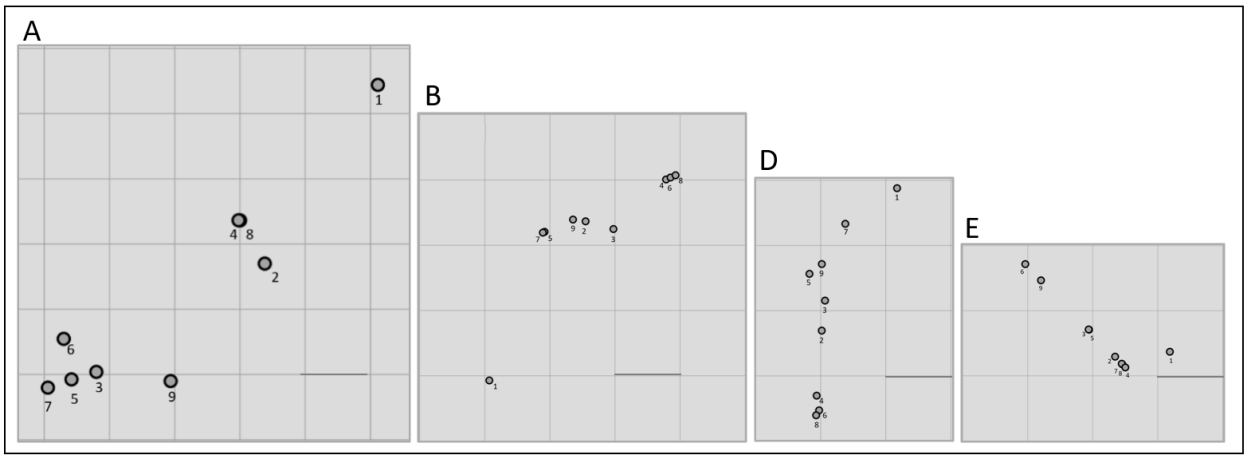

Figure 3.4. Genetic cartography. A. λC= lambda C amino acid sequence, B. µB= mu B amino acid sequence, C. σC= sigma C amino acid sequence, D. σNS= sigma NS amino acid sequence. The amino acid sequences are arranged relative to each other using multidimensional scaling and target distances obtained from the number of amino acid substitutions between sequences. Each scale bar corresponds to one genetic distance (GD) calculated by the model.

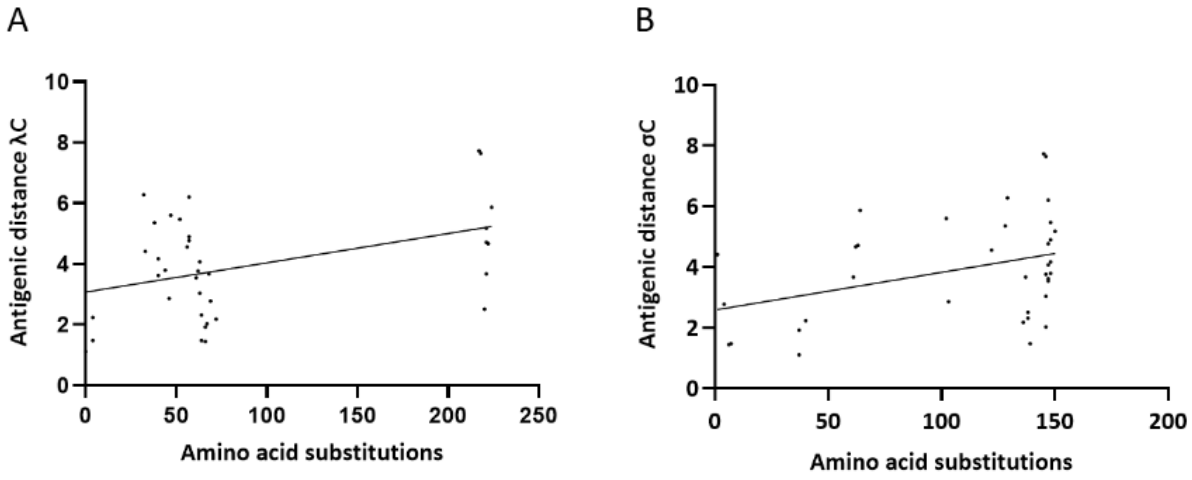

Figure 3.5. Comparison between antigenic and genetic distances between ARV structural proteins. A. λC= lambda C protein, B. σC= sigma C protein. The antigenic distances were retrieved from the antigenic map of nine ARV strains. The genetic distances between proteins are represented by the amino acid substitutions in λC and σC respectively. The line represents a linear regression model for λC ($R= 0.31$; $P< 0.05$) and σC ($R= 0.47$; $P< 0.05$).

FINAL CONCLUSIONS

The experiments within this dissertation contribute to a better understanding of the pathobiology of avian reovirus variants and provide insights with respect to the association between its genetic, pathogenic and antigenic properties.

At the beginning of this investigation, we hypothesized that by performing molecular surveillance we would be able to identify and classify avian reovirus variants that were causing outbreaks in California between 2015 and 2020. We not only identified variants but also detected important specific genes that concentrate the viral variability besides S1. Additionally, we were able to witness a shift of genotype through the years that is most likely driven by the preventative measures taken i.e. vaccination.

In the second set of experiments our results suggested that viruses classified in the same genotypic group differed in replication rates, pathogenicity and transmissibility of avian reoviruses, while immunosuppression was one of the outcomes of the infection with these variants.

Finally, through a pilot study we were able to detect associations between genetic and antigenic characteristics of pathogenic avian reoviruses variants. These associations might be meaningful in future studies to provide insights into new molecular classification methods that connected with cross-protection information would provide an improved vaccine development and autogenous vaccine selection.

In conclusion, our results suggest that avian reoviruses are genetically, pathogenically, and antigenically diverse and their variability depends on other genes besides S1. Therefore, a better characterization method that consider all the variable genes and the connections between

genotype and phenotype are necessary to improve the prevention and control of avian reovirus evolving strains.