DIAGNOSIS, PATHOGENESIS AND CONTROL OF CHICKEN AND TURKEY ARTHRITIS REOVIRUSES

A THESIS
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

RAHUL KUMAR

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Advisors: Drs. Sagar M. Goyal and Sunil K. Mor

October 2021

Acknowledgements

"As we express our gratitude, we must never forget that the highest appreciation is not to utter words, but to live by them." The journey of writing a thesis and earning a doctorate is long and arduous, and the work certainly is not done singlehandedly. Its completion is due in large part to all the special people who challenged, braced, and traversed with me along the way. I am tremendously fortunate to have Drs. Sagar Goyal and Sunil Mor as my advisors who complemented each other wonderfully well in grooming and mentoring me. In addition to being great mentors, they are fantastic human beings. They always provided refreshing insight, critical questions and wonderful suggestions that were exhilarating. I feel lucky to have advisors like them. I am extremely grateful for their help and guidance through different phases of my study. I shall ever remain grateful to them for inculcating positive attitude in me.

My deepest gratitude is for my advisory committee members Drs. Robert Porter, Carol Cardona, Sally Noll and Avishai Lublin. Thank you all for your great scientific advice, constant encouragement and providing constructive suggestions for successful completion of my research work. Thank you for making my advisory committee meetings a great learning experience to which I always looked forward to.

Special mention for you Dr. Robert Porter, you are a gem of a person. In addition to being a great pathologist, you are an outstanding mentor who taught me some of the finest things about pathology. I will always be indebted for your lessons to live a happy and cheerful life and will cherish the memories of working with you for the rest of my life.

This thesis has become a reality with the kind support and help of many individuals. I would like to extend my sincere thanks to all of them who have contributed in numerous ways with special mention to Drs. Pawan, Vikash, Tamer, Soumesh, Nirmala, Venkat and Nader. I express my feelings of gratitude to all of you. I am thankful to Dr. Devi Patnayak for his guidance, support and kind words of wisdom. I would also like to thank Wendy and Lotus for their help and for the special bond we built over the last three years. Special thanks to my friends in graduate school namely, Dora, Kruthika, Shiv, Alex, Anders, Markus, Zelmar, Catalina and Sam. Thank you all for making me feel like having a family in the US.

I thank Dr. Jerry Torrison, Director of Minnesota Veterinary Diagnostic Laboratory for his encouragement and positive vibes with his ever-smiling face. I would also like to thank and mention the help from Lisa, Sarah, Susan and Brenda for making the official processes of graduate studies a smooth experience. I am extremely thankful to the families of Drs. Goyal, Mor, Vikash and Hemant for welcoming me like an integral member.

Last but not the least, I am writing this for you Mom with tears in my eyes and your memories in my heart. I would never have been able to achieve anything without your blessings. Words can never translate my love for you. I want to mention here that my dad is my hero from whom I learnt the fighting spirit for life. Mom and Dad, I must be a dear son of God; that's why I was born to you. I want to thank my brother, who is like a father figure to me, my sisters and my wife Priyanka for their love, trust and endearing support.

Finally, thank you God for giving me this opportunity to meet all the persons mentioned above and always showing me the light.

This thesis is dedicated to my parents and my wife Priyanka

For always being there for me in all ups and downs

Abstract

Avian reoviruses are the causative agent of arthritis/tenosynovitis in broilers and turkeys. Recently, variants of chicken arthritis reovirus (CARV) and turkey arthritis reovirus (TARV) have occurred worldwide leading to vaccine failures, causing huge economic loses, and increasing animal welfare concerns. Whole genome sequencing of 35 and 14 isolates of CARV from the US and Germany, respectively, indicated significant divergence in these strains. In fact, US strains had more divergence among themselves than the German strains. The US strains clustered in six previously established genotype clusters (GCs) while the German strains clustered in only four of the six GCs. Strains from both countries were highly divergent from the currently used vaccine strains. Based on distinct evolutionary trees of each gene, we discovered that all genes evolve in an independent manner and contribute to the evolutionary process. The S1 (σC) genome segment showed noticeably higher divergence followed by M2 (μB) and L3 (λC) genes. Congruent topologies of these isolates indicated frequent genetic re-assortment among multiple co-circulating variants. We believe that the genetic variability among CARVs is due to a combination of evolutionary mechanisms involving multiple cocirculating lineages and genetic reassortments.

We developed a recombinant pichinde virus-vectored vaccine (rPICV-CARV) that expresses the sigma C (SC) and sigma B (SB) antigenic proteins of CARVs. Several combinations of monovalent and bivalent rPICV-CARV vaccines were developed carrying codon-optimized SC and SB genes from two divergent CARV strains. The S1 and S3 genes and antigens were found to be expressed in virus-infected cells via reverse

transcriptase polymerase chain reaction (RT-PCR) and direct fluorescent antibody (DFA) technique, respectively. We conducted an *in vivo* study to determine safety and efficacy of two vaccine formulations against a wide spectrum of virus challenges. The vaccinated birds produced serum-neutralizing antibodies, which were responsible for early clearing of the virus from the host, inhibited virus replication in intestine and tendons, and decreased fecal shedding of the virus relative to non-vaccinated controls. The vaccine is a promising candidate that needs to be further evaluated in breeders. The survival of bivalent codon optimized rPICV-CARV vaccine was studied in poultry litter and water at room temperature (approx. 25°C). In spiked samples, it was found that the vaccine virus survived for approximately six hours in litter and drinking water.

Reoviruses have been isolated from three different disease syndromes in turkeys, e.g., turkey enteric reovirus (TERV) from cases of turkey enteritis, turkey arthritis reovirus (TARV) from cases of tenosynovitis/arthritis in turkeys, and turkey hepatitis reovirus (THRV) from cases of hepatitis in turkeys. The comparative pathogenesis of these viruses, and correlation with their genetic make-up (if any), is not known. All nine viruses were found to be enterotropic; the virus gene copy number in the intestine reached a peak at 5 dpi followed by a sharp decline at 7 dpi. All viruses caused significant decrease in body weight gain of birds compared to the negative control group. Both TARV and THRV strains replicated in tendons and produced histologic lesions consistent with tenosynovitis. Hepatic lesions were produced by THRV only and the virus was re-isolated from liver and spleen of inoculated birds thereby fulfilling Koch's postulates.

We then conducted a study to determine the age at which turkey poults become susceptible to infection with TARV. All turkeys were susceptible to TARV infection at all ages studied. However, virus replication was more pronounced in the intestine and gastrocnemius tendons of turkeys at 2-weeks of age or less. Additionally, turkeys at all ages of TARV challenge developed typical lesions of lymphoplasmacytic tenosynovitis, shed TARV in feces, and transmitted TARV to sentinels. The sentinels, in turn, also showed virus replication in their intestines and tendons leading to histological lesions of arthritis/tenosynovitis. These findings indicate that turkeys at the age of 28 days or less are susceptible to infection with TARV following oral challenge. It was also found that TARV-infected birds could transmit the infection to naïve sentinel turkeys of the same age.

We also created a recombinant live pichinde virus-vectored bivalent subunit vaccine that expresses Sigma C and Sigma B proteins of TARV SKM121. The efficacy of this vaccine was tested against both homologous (TARV SKM121) and heterologous (TARV O'Neil) virus challenges. Immunized poults produced serum-neutralizing antibodies that neutralized both viruses. The body weights of vaccinated and non-vaccinated birds were similar indicating no adverse effect of the vaccine on feed efficiency. Comparison of virus gene copy numbers in intestine and histologic lesion scores in tendons of vaccinated and non-vaccinated birds showed a decrease in the replication of challenge viruses in the intestines and tendons of vaccinated birds. These results indicate the potential usefulness of this vaccine. In addition, the vaccine virus was found to be transmissible horizontally to non-vaccinated pen mates of vaccinated birds inducing serum neutralizing antibodies.

Table of Contents

List of Tables

List of Figures

CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES

INTRODUCTION

1.1. Avian reoviruses: Avian reoviruses (ARVs) are one of the 15 members of the genus Orthoreovirus in the family *Reoviridae*. An estimated 85-90% of ARVs are not known to be pathogenic while the known pathogenic ARVs cause lameness, distressed immune system and infection of liver, heart and intestine. These viruses can be readily isolated from the tissues or organs of the affected birds (Rosenberger et al., 1997; Jones, 2008) and from the gastrointestinal and respiratory tracts of clinically healthy birds (Rosenberger et al., 1989). The clinical disease is dependent upon the host's age and immune status, virus pathotype and route of exposure (oral, respiratory, footpad, or subcutaneous) (Davis et al., 2012; Dandar et al., 2013; Jones, 2013; Troxler et al., 2013; Egana et al., 2019).

The ARVs may be transmitted either vertically or horizontally and are usually associated with clinical presentation of low mortality but high morbidity. Economic losses in the form of increased mortality, viral arthritis/tenosynovitis and general lack of performance due to diminished weight gains, poor feed efficiency, uneven growth rate, non-uniformity of the flock, and reduced marketability owing to downgraded carcass quality at slaughter (Dobson et al, 1992; Jones et al, 1994; Rosenberger et al., 1997). Infection of breeder flocks prior to or during egg production causes increased mortality, decreased egg production, decreased hatchability, and lameness, all of which contribute to the increased costs of production (Rosenberger et al., 1997).

1.2. Chicken reovirus: Chicken reovirus (CRV) is ubiquitous in commercial poultry/broilers. Arthritis/tenosynovitis caused by chicken arthritis reovirus (CARV) is an

economically important disease of meat type chickens, egg layers and breeders (Hemzani et al, 1996; Jones 2000). The pathogenesis of the disease is well established; day-old chicks are most susceptible and most likely to be infected. Vertical and horizontal (via the fecal-oral route) transmission of the virus may occur from infected breeders to their progeny. Infection through the broken skin of footpad has also been reported (Al Afaleq and Jones, 1989; van der Heide and Kalbac, 1975). Pathogenic strains of CARV cause lameness in chickens, mainly at 5–7 weeks of age in heavy breeds, as a result of inflammation in the digital flexor and tarsometatarsal extensor tendons leading to swelling of the hock and shank (Wilcox et al., 1985; Jones 2008; Sellers et al., 2016). Histologically, there is a lymphoplasmacytic tenosynovitis with synoviocyte hyperplasia. Recently, variant CARVs have been isolated from cases of tenosynovitis in 2.5 to 8-week-old commercial broilers in Europe and North America (Rosenberger et al., 2013; Sellers et al., 2013; Troxler et al., 2013). Viral arthritis/tenosynovitis has been recognized and reported from virtually all major poultry-producing countries and the last decade has witnessed a surge in the incidence of reovirus- associated arthritis/tenosynovitis.

Chicken reovirus has also been associated with enteric disease in chickens named variously as runting-stunting/malabsorption syndrome (RSS/MAS), brittle bone disease, and helicopter disease in young broilers. However, several studies have suggested that CRV probably plays a secondary rather than a primary role in these conditions rather than a primary one (Dutta and Pomeroy, 1969; Goodwin et al, 1993). These conditions are collectively characterized by delayed growth, non-uniformity of the flock, lethargy, and diarrhea (Veen et al, 2017). Histologically, there is denudation of intestinal villi in the small intestine.

Chickens are most susceptible to reovirus infection at day-old of age and attain age-related resistance around two weeks of age. Because of this 2-week duration of susceptibility, vaccination strategies are needed that can provide protection to day-old chicks via maternally derived antibodies. Commercial vaccines containing attenuated strains of the virus (S1133, 1733, 2408, and 2177) are commonly used in the U.S and around the world (Van der Heide et al, 1983; Rosenberger et al, 1989). Breeder flocks are vaccinated with one of the live vaccines followed by an inactivated vaccine before egg production (Heide 1986; Wood et al, 1986). Autogenous vaccines are also being used when the prevalent virus is a variant strain than the strain used in the commercial vaccine (Samberg and Meroj 1996; Hemzani et al, 1996). Heide (1983) and Giambrone et al, (1992) recommended subcutaneous or coarse spray application of attenuated vaccines. Published literature suggests that the CARV genome is continuously changing by the mechanisms already known for segmented RNA viruses (Domingo, 1997; Trites et al, 2011), thereby creating genetic and antigenic variants that may be more pathogenic. Consequently, commercially available CARV vaccines are not able to provide adequate protection against newly emerging and variant field strains leading to outbreaks of disease in vaccinated poultry flocks across the world including the US (Chénier et al., 2014; Tang and Lu, 2015a, 2015b; Lu et al., 2015; Sellers et al., 2016; Egana et al., 2019).

1.3. Turkey reovirus: Turkey reoviruses (TRVs) can cause enteritis, myocarditis, hepatitis and arthritis in turkeys. The reovirus associated with enteritis in turkeys was first reported by Simmons *et al* (1972) and is now called turkey enteric reovirus (TERV). The

TERVs have been associated with poult enteritis complex (PEC), which includes poult enteritis syndrome (PES) and light turkey syndrome (LTS) (Pantin-Jackwood et al., 2008; Jindal et al., 2010; Mor et al., 2013a). Turkeys affected by this disease complex are diarrheic, have frothy intestinal contents, and exhibits non-uniform and diminished growth in the flock. In severe cases, runting, immune dysfunction, and increased morbidity (up to 100%) and mortality have been reported. Other etiological agents involved in turkey enteritis are enteropathogenic *Escherichia coli*, turkey coronavirus (Guy et al, 2000) and turkey astrovirus (Koci, and Schulz-Cherry. 2002).

The association of reovirus with arthritis/lameness clinical cases in turkeys was reported in the early 1980s (Levisohn et al, 1980; Page et al, 1982) and then again in 2011 (Mor et al. 2013). Myocarditis and hepatitis associated with TRVs have also been reported (Shivaprasad et al, 2009). The arbitrary nomenclature of TRVs is solely based on the tissue from which the virus is isolated tentatively naming them as TERV (turkey enteric reovirus), TARV (turkey arthritis reovirus), and THRV (turkey hepatitis reovirus), respectively.

In 2010-2011, TARV-associated lameness and tenosynovitis dramatically re-emerged in the upper Midwest. Between 35 and 70% of turkeys in flocks experiencing TARV-associated clinical disease were affected, resulting in substantial economic losses (Mor et al, 2013). Sharafeldin et al (2014) successfully reproduced the disease experimentally by oral inoculation of commercial turkey poults with TARV. The increased incidence of variant TARVs in the US during the last decade is thought to be due to reassortment events happening in the segmented dsRNA reoviral genome (Trites et

al, 2011). The variant TARVs circulating in the turkey flocks are very divergent, hence the cases of lameness in turkeys continues to increase.

In 2019, young turkey poults with a history of spiking mortality were submitted to the University of Minnesota Veterinary Diagnostic laboratory for disease diagnosis. The average age of dead poults was 15 days, which were dull and depressed before death. On necropsy, the affected birds had hepatosplenomegaly with white necrotic foci extensively dispersed in the hepatic and splenic parenchyma. Histopathology revealed islands of hepatic necrosis with infiltration of lymphocytes. Interestingly, reovirus was isolated from these cases. Similarly, reovirus isolation from the liver of two-week old turkey poults have been reported previously (Van der Heide et al, 1980)

There is currently no commercial vaccine available against TARV infection. Some turkey producers have adopted a strategy of using autogenous vaccines. In this approach, the prevalent TARV strain circulating is attenuated and used in the same flock. The breeders are being vaccinated with multiple doses at different time points before egg production to provide maternally derived antibodies to the chicks. This method of vaccination was successful for some time, but it has its own shortcomings. Firstly, the emergence of divergent and variant TARVs pushes the need to frequently identify, isolate, and update of the autogenous vaccines with the newly emergent strains. Second, the production of autogenous vaccines is itself a cumbersome and time-consuming process. Therefore, the pressing need is to formulate a live and vectored vaccine against TARVs.

1.4. Virus Variants:

Reoviruses are non-enveloped, 70-80 nm (diameter) viruses with icosahedral symmetry (King et al. 2012). The viral genome has 10 segments of double stranded RNA (dsRNA) packaged into a double-shelled capsid. The genome segments are three large/L-class (L1, L2 and L3), three medium/M-class (M1, M2, M3), and four small/S-class (S1, S2, S3, S4) segments based on their electrophoretic mobility in polyacrylamide gel (Benavente and Martínez-Costas 2007). The L-class and M-class genome segments express three primary translation proteins each viz. λA (core shell), λB (core RdRp), λC (core turret) and μA (core NTPase), μB (outer shell), μNS (NS factory), respectively (Benavente and Martínez-Costas 2007). The S-class segments express four (σC (outer fiber), σA (core clamp), σB (outer clamp), σNS (NS RNAb)) proteins (Benavente and Martinez-Costas 2007). Eight of these reoviral proteins are structural and two are non-structural. In addition, there are two additional nonstructural proteins encoded by the first two cistrons of the tricistronic S1 gene, namely the p10 (NS-FAST) and p17 (NS other) proteins. The sigma C (σC) protein encoded by the third and largest open reading frame of the S1 gene (Benavente and Martínez-Costas, 2007) is a minor component of the outer capsid of the virion and serves as the cell attachment protein.

Sequence data of CARV and TARV suggests that the reovirus genome is continuously changing, and all gene segments evolve resulting in the multitude of genetic and antigenic variants (Mor et al., 2014; Ayalew et al., 2020). Having a segmented genome, segmental reassortment and exchange of genes can introduce genotype and pathotype variations in ARVs (Liu et al., 2003; Joklik et al., 1995). Recent studies based on the phylogenetic analysis of immunogenic σC protein have also indicated the

existence of many genotypic variants, which are classified into distinct genotype clusters within CARV (Egana et al., 2019; Ayalew et al., 2020; Carli et al., 2020). Commercially available CARV vaccines or autogenous TARV vaccines do not provide adequate protection against newly emerging and variant field strains leading to outbreaks in vaccinated poultry flocks across the world including the US (Sharafeldin et al., 2014, Tang et al., 2015; Sellers et al., 2016; Egana et al., 2019). It is likely that the nature of reovirus evolution contributes to the inability of v=current vaccines strategies to provide adequate protection.

1.5. Pichinde Virus:

Pichinde virus (PICV) is a non-pathogenic virus in the family *Arenaviridae*. It was first isolated from rice rats (*Oryzomys albigularis*) in the Pichinde valley of Columbia, South America (Trapido and Sanmartin, 1971), and is not known to be pathogenic to humans, animals or birds. Arenaviruses have an enveloped bi-segmented (L and S segments) negative-sense RNA that encodes for four genes. The L segment encodes for an RNA-dependent RNA polymerase L and a ring-finger motif containing the Z protein. The S segment encodes for the nucleoprotein, NP and the glycoprotein GPC. Pichinde virus targets macrophages and dendritic cells (antigen presenting cells/APCs) in the initial phase of infection and has developed different strategies to evade host innate immune recognition (Meyer and Ly, 2016), thereby making it a potential vaccine vector (Emonet et al., 2009; Flatz et al., 2010; Popkin et al., 2011; Ortiz-Riano et al., 2013).

Using reverse genetics techniques, a tri-segmented recombinant Pichinde virus

(triPICV) vector has been developed (Dhanwani et al., 2016). The rP18tri-based viruses are attenuated *in vitro* and *in vivo* and can effectively induce strong T cell and humoral immune responses with limited anti-vector neutralizing antibodies (Dhanwani et al., 2016). Therefore, this novel rP18tri vector exhibits optimum features of safety, immunogenicity, robust antigen-encoding capacity, selective targeting of antigen-presenting cells, and the lack of a strong anti-vector immunity.

The four aims of this thesis are listed below.

1) To study the genetic variation and evolutionary dynamics of CARVs circulating in Germany and the US and to use this genetic information to select appropriate strains for inclusion in the vaccine.

2) To use well characterized CARVs for the development of live vectored vaccine that contains immunogenic proteins of CARV (Sigma C and Sigma B) and evaluate the safety, efficacy, genetic stability, and environmental survivability of the vaccine.

3) To study the comparative pathogenesis, age susceptibility and lateral transmission of turkey reoviruses.

4) To test the safety and efficacy of Pichinde virus vectored vaccine against turkey reovirus challenge.

CHAPTER 2

REVIEW OF LITERATURE

2.1. The family *Reoviridae*

The family *Reoviridae* is the largest and one of the most complex of the eight recognized double-stranded RNA (dsRNA) virus families. Reoviruses infect a wide range of hosts e.g., insects, mammals, aves, reptiles, amphibians, fishes, invertebrates, and plants etc. (Attoui *et al*., 2012). Family *Reoviridae* is further classified into two subfamilies, namely *Spinareovirinae* and *Sedoreovirinae* based on their structure and 15 genera (Attoui *et al*., 2012). Subfamily *Spinareovirinae* have "turreted" viruses which have 12 spikes or turrets on the viral core arranged with icosahedral symmetry (Attoui *et al*., 2012). Subfamily *Sedoreovirinae* represents "non-turreted" viruses that have a 3-capsid core structure with a smooth surface (Attoui *et al*., 2012). Nine genera are included in the subfamily *Spinareovirinae,* e.g., *Aquareovirus* (aquatic species, Attoui et al., 2002)*, Coltivirus* (Colorado tick fever in human, Attoui et al., 1998)*, Cypovirus* (insects, Yang et al., 2012)*, Dinovernavirus* (unknown host, Attoui et al., 2005)*, Fijivirus* (plants, Isogai et al., 1998)*, Idnoreovirus* (insects, King et al., 2012)*, Mycoreovirus* (fungi, Suzuki et al., 2004)*, Orthoreovirus* (mammals and birds, Duncan, 1999)*,* and *Oryzavirus* (rice, Miyazaki et al., 2008). Subfamily *Sedoreovirinae* consists of six genera namely *Orbivirus* (arthropod-borne virus, Belhouchet et al., 2011)*, Rotavirus* (diarrhea in humans and animals, Martella et al., 2010)*, Seadornavirus* (arthropod borne viruses infecting humans and animals, Jaafar et al., 2005)*, Phytoreovirus* (plants, Lu et al., 1998)*, Cardoreovirus* (crabs, Attoui et al., 2002) and *Mimoreovirus* (environmental marine water virus, King et al., 2012)).

The genus *Orthoreovirus*

Member of genus *Orthoreovirus* infects mammals, birds and reptiles by respiratory or fecal-oral routes. They are non-enveloped viruses with a well-defined icosahedral turreted outer capsid. The *Orthoreovirus* genus is divided into two subgroups, fusogenic and non-fusogenic orthoreoviruses. Fusogenic orthroeviruses can induce cell to cell fusion of infected cells which results in syncytia (Duncan, 1999; Benavente and Martinez-Costas, 2007). The fusogenic Orthoreoviruses include avian orthoreovirus (ARV) and three atypical fusogenic mammalian orthoreoviruses namely Nelson Bay orthoreovirus (NBV), Baboon orthoreovirus (BRV), and Reptilian orthoreovirus (RRV) (Attoui *et al*., 2012). Non-fusogenic orthoreoviruses includes other mammalian reoviruses (MRVs). The six existing orthoreovirus species are primarily defined on their genomic and antigenic properties, host range and percent nucleotide and amino acid identity between cognate genome segments (Duncan, 1999). Non-fusogenic orthreoviruses form a distinct genetic clade from fusogenic orthoreoviruses (Day, 2009; Duncan and Sullivan, 1998).

As a group, avian reoviruses (ARVs) have structural and molecular similarities but they are antigenically much more heterogenous than MRVs. Unlike the non-fusogenic MRVs, ARVs lack the capacity to agglutinate red blood cells (Ni and Ramig 1993) but form syncytia in infected cell cultures. Avian reoviruses are a biologically, pathologically and molecularly diverse group of viruses (Spackman *et al.*, 2005a). Additionally, ARVs have diverse host range and can infect almost all avian species causing tenosynovitis, runting and stunting syndrome, respiratory infection, hepatitis, myocarditis, nervous infection and immunosuppression (Robertson and Wilcox, 1986).

2.2. History avian reovirus

REO in reovirus is an acronym for Respiratory Enteric Orphan virus. Originally, it was named an "orphan" virus because it was not known to be associated with any known disease (Chua et al. 2008). Mammalian reovirus was discovered in the early 1950s after its isolation from the respiratory and gastrointestinal tracts of sick and healthy individuals (White and Fenner 1994). Very little information was available about ARV until the serendipity by Olson et al. (1959) while studying *Mycoplasma synoviae.* Similar signs and lesions of synovitis in broiler chickens caused by an unknown agent were observed as reported in *Mycoplasma synoviae infection*. However, this agent was insensitive to chlortetracycline and furazolidone and serologically unrelated to *Mycoplasma gallisepticum* and *M. synoviae* and displayed an age-related susceptibility to young chicks (Kerr and Olson, 1964). These findings suggested that the agent was of viral origin and hence was named as "viral arthritis agent" (Olson and Kerr, 1966). This viral arthritis agent was further studied and characterized and found to be a member of the genus *Orthoreovirus* by Petek et al. (1967). In 1972, electron microscopy studies demonstrated that the virus had similar virion structure, size, and location in an infected cells as that of MRVs (van der Heide and Page, 1980) which confirmed that the "viral arthritis agent" was an avian origin reovirus.

Subsequent studies revealed that viral arthritis is not the only clinical syndrome caused by ARVs. The "Fahey-Crawley virus" was first isolated from the respiratory tract of chickens affected with chronic respiratory disease (Fahey and Crawley, 1954). This virus not only had a similar molecular structure but also had similar antigenicity with arthritis ARVs (Olson and Weiss, 1972; Walker et al., 1972). Olson and Solomon (1968)

reported the first clinical case of viral arthritis in US chickens. Subsequent serological and pathological studies established that the virus is ubiquitous in poultry and most of the ARV strains causes asymptomatic infection (Van der Heide, 2000; Jones, 2013).

The ARVs are associated with several disease presentations in breeders and commercial poultry but arthritis/tenosynovitis is the most important ARV disease in chickens. These diseases associated with ARVs include enteritis with cloacal pasting (Dutta and Pomeroy, 1969), inclusion body hepatitis (McFerran et al., 1976), myocarditis (Bains et al., 1974), spiking mortality because of liver and kidney lesions in young chickens (Bagust and Westbury, 1975), and runting-stunting syndrome (RSS; also called malabsorption syndrome) (Goodwin et al., 1993a, 1993b; Page et al., 1982a, 1982b; Vertommen et al., 1980), osteoporosis i.e., brittle bone disease and femoral head necrosis in broiler chickens (Van der Heide et al.,1981) and myocarditis and hepatitis (Van der Heide, 2000). ARVs have been recognized as an important pathogen and research target for disease prevention.

ARV infections have been reported in chicken, turkeys and other avian species from various poultry producing countries around the world. Historically, ARVs have been isolated from turkeys (Lozano et al. 1989; Simmons et al. 1972), geese (Hlinak et al. 1998; Palya et al. 2003), Pekin ducks (Jones and Guneratne 1984), mallard and Muscovy ducks (Gaudry et al. 1972; Kuntz-Simon et al. 2002; McFerran et al. 1976), pheasants, pigeons (Vindevogel et al. 1982), quails and psittacine birds (Conzo et al. 2001). Avian reovirus have been reported from wild birds as well (Hlinak et al. 1998; Hollmen et al. 2002; Huhtamo et al. 2007; McFerran et al. 1976; Sakai et al. 2008; Sanchez-Cordon et al. 2002).

Avian reoviruses are relatively resistant to environmental conditions, chemical and physical agents and survive in the environment for long, which makes the notion of maintaining an ARV-free flock impossible (Mor et al., 2015a, 2015b). Anti-reoviral antibodies in breeder flocks does not prevent the vertical transmission of virus to progeny. Hence, universal strategy adopted for the prevention and control of ARV infection is vaccination with an inactivated virus in breeders followed by live vaccine in progeny (Sellers, 2017) in addition to strict biosecurity, good management practices e.g., minimizing entry into the barn, practicing all-in-all-out system, effective cleaning and disinfection of the barn, and good record keeping. Commercially available vaccines are based on viral arthritis strain S1133 and enteritis strains 1733, 2408 and 2177, all belonging to genotype 1 based on Sigma C gene sequences (Sellers, 2017). However, these vaccines are inefficient in protecting flocks due to emergence of variant ARVs (Egaña et al., 2019; Ayalew et al., 2020; De Carli et al., 2020; Torre et al., 2021). Because ARVs are resistant to many common disinfectants, effective cleaning and disinfection requires a careful planning and selection of barn-cleaning products for the control of ARV infection in the flock (Meulemanns and Halen, 1982; Mor et al., 2015a).

Avian reovirus of domestic turkeys (*Meleagris gallopavo*)

Avian reoviruses had been isolated from apparently normal as well as clinically sick turkeys (França *et al.*, 2010; Gershowitz and Wooley, 1973; McFerran *et al.*, 1976; Simmons *et al.*, 1972; van der Heide *et al.*, 1980; Wooley and Gratzek, 1969). The turkey was more resistant than the chicken to ARV related disease problems (Al-Afaleq and Jones, 1989; Al-Afaleq *et al.*, 1989; Al-Afaleq and Jones, 1991; Glass *et al.*, 1973). Clinical disease in domestic turkeys was reported to be consistent with that in chickens,

e.g., viral arthritis/tenosynovitis (Mor *et al.*, 2013; Page *et al.*, 1982a; Sharafeldin *et al.*, 2014), sudden death, infectious enteritis with occasional high mortality in turkey poults (Gershowitz and Wooley, 1973; McFerran *et al.*, 1976; Saif *et al.*, 1985), decreased weight gain (Spackman *et al.*, 2005a), age-dependent moderate to severe atrophy of the bursa of Fabricius causing immunosuppression (Day *et al.*, 2008; Spackman *et al.*, 2005a), and myocarditis (França *et al.*, 2010; Shivaprasad *et al.*, 2009). Nersessian *et al*., (1985b) noted that turkeys inoculated with a turkey enteric reovirus (TERV) had a viremia 7 dpi with virus distributed in most organs by 3-7 dpi and virus recovered from tendons at 3-7 dpi and 28 dpi. Sharafeldin *et al*., (2014) fulfilled Koch's postulates by reisolating turkey arthritis reovirus (TARV) from experimentally inoculated birds establishing the causal relationship between reovirus and tenosynovitis in turkeys. After initial reports of TARVs reported in 1980s (Levisohn et al., 1980; Page et al. 1982), turkey viral arthritis was not reported in the published literature until Mor et al., (2013b) isolated TARV from 12 to 18-week-old tom turkeys and partially characterized based on S4 genome segment. Mor et al., (2014a, 2014b, 2015) isolated and described the comparative molecular characterization of TARVs with turkey enteric reoviruses (TERV) and CARV based on the L-, M- and S-class segments of reoviral genome, Seven TARV strains were isolated and genetically characterized from clinical cases of arthritis in turkeys from Pennsylvania, USA (Tang *et al.*, 2015).

Avian reoviruses were implicated in several multifactorial enteric syndromes in young turkeys, and the milder form was characterized by diarrhea and poor weight gain. Based on clinical presentation TERVs have been associated with poult enteritis and mortality syndrome (PEMS), poult enteritis complex (PEC), poult enteritis syndrome

(PES), light turkey syndrome (LTS) (Barnes et al., 2000; Jindal et al., 2009, 2010a; Mor et al., 2011, 2013a; Pantin-Jackwood et al., 2007; 2008; Woolcock et al., 2008). When the disease was associated with high mortality in the affected flocks it was called poult enteritis complex (PEC) (Spackman *et al.*, 2005a), and an even more severe form was documented as poult enteritis mortality syndrome (PEMS) (Day *et al.*, 2008). PEMS is highly infectious in young turkeys and is characterized by diarrhea, increased feed consumption, increased time-to market, runting, stunting, decreased weight gain, immune dysfunction, and mortality (Barnes *et al.*, 2000; Day *et al.*, 2008). In addition to reoviruses, numerous viruses, including turkey coronavirus and turkey astrovirus type 2, have been associated with PEC and PEMS and have been the cause of substantial economic losses to the turkey industry (Spackman *et al.*, 2005b). Heggen-Peay *et al.* (2002) demonstrated that reovirus alone could induce some of the clinical signs associated with PEMS, including the intestinal lesions and suppression of bursal and hepatic growth and development. The TERVs have been characterized as genetically distinct group in the *Reoviridae* family based on their S1, S3 and S4 genome segments (Day et al., 2007, Jindal et al, 2010, Pantin-Jackwood et al., 2008).

Avian reovirus of ducks and geese

Avian reoviruses were isolated from Muscovy Ducks (*Cairina moschata*) in South Africa, France, Israel and Hungary (Heffels-Redmann *et al.*, 1992; Malkinson *et al.*, 1981; Palya *et al.*, 2003), commonly affecting young Muscovy 2-4 weeks of age. The clinical signs included general malaise, diarrhea, respiratory signs, stunted growth, (Heffels-Redmann *et al.*, 1992; Malkinson *et al.*, 1981) and microscopic, multifocal hepatic, splenic and renal necrosis (Malkinson *et al.*, 1981). Liu *et al*. (2011) and Chen *et*

al. (2012) reported the isolation of a highly virulent duck reovirus (DRV) from Pekin ducklings (*Anas* platyrhynchos) showing hepatosplenomegaly with spiking mortality. McFerran *et al*. (1976) isolated a DRV from feces of mallard ducks in chick kidney and chicken embryo liver cell cultures.

A goose reovirus (GRV) related to DRV was isolated from young geese showing lesions of splenitis, hepatitis, epicarditis, arthritis, and tenosynovitis at necropsy (Palya *et al.*, 2003). Avian reovirus antibodies were reported in wild bean geese (*Anser fabalis*) and white-fronted geese (*Anser albifrons*) by Hlinak *et al*. (1998).

Avian reoviruses of wild birds

Many avian species in different orders were reported to be infected by avian reoviruses e.g., Anseriformes, Charadriiformes, Columbiformes, Falconiformes, Galliformes, Gruiformes, Passeriformes, Piciformes, and Psittaciformes (Gough *et al.*, 1988; Jones and Guneratne, 1984; Robertson and Wilcox, 1986; Takehara *et al.*, 1989) in addition to serologic evidence of ARV infections in Sphenisciformes (Karesh *et al.*, 1999). The ARVs are likely to have much wider host range as only a limited number of studies have been conducted in free-ranging birds (Hollmén and Docherty, 2007).

The first recoded isolation of reovirus from pigeons with diarrhea (*Columba spp.*) was by Mcferran *et al*., (1976). This pigeon reovirus isolate shared a common antigen with chicken reoviruses suggesting cross species transmission.

Avian reoviruses isolation was reported from imported psittacine birds (Meulemans *et al.*, 1983), with frequent outbreaks over the years (van den Brand *et al.*, 2007). Rigby *et al*. (1981) isolated reovirus from psittacine and passerine birds affected with enteritis. Meulemans *et al*., (1983) isolated reovirus from 15/28 batches of dead

imported psittacine birds affected with enteritis, hepatitis and splenitis. A reovirus associated outbreak with high mortality was reported in Italy among imported African grey parrots (*Psittacus erithacus erithacus*) and Australian king parrots (*Alisterus scapularis*) (Conzo *et al.*, 2001). Senne *et al*. (1983) described an asymptomatic reovirus infection in quarantined psittacine birds.

Avian reoviruses were isolated from live young African grey parrots showing depression, drooping plumage, loss of appetite, diarrhea and respiratory symptoms (Sánchez-Cordón *et al.*, 2002) as well as from dead young African grey parrots having disseminated necrotizing hepatopathy (Wilson *et al.*, 1985). Reovirus infection in psittaciformes produced a high mortality in all age groups and affected parakeets (van den Brand *et al.*, 2007). The most susceptible species for reovirus infection includes the young ones of African grey parrots (*Psittacus erithacus, Psittacus erithacus, Psittacus erithacus timneth*), and cockatoo (*Cacatua alba*) (Spenser, 1991).

Reoviruses were previously thought to be mildly pathogenic for budgerigars (*Melopsittacus undulates*) until Pennycott (2004) reported high mortality in affected birds with hepatosplenomegaly.

Reovirus was isolated from droppings of a clinically normal wedge-tail eagle *(Aquila andax)* by Jones and Guneratne (1984). An orthoreovirus was isolated from dead brown-eared bulbul (*Hypsipetes amaurotis*) with hemorrhages in intestine (Ogasawara *et al.*, 2015).

In corvid species, reovirus was isolated in southern Finland from a diseased wild hooded crow (*Corvus corone cornix*) having neurological signs like abnormal flying with incoordination, abnormal postures, cramps, and paralysis (Huhtamo *et al.*, 2007).

Intracytoplasmic reovirus-like particles in the spleen and duodenum of carrion crows (*Corvus corone*) were reported by Mast *et al*. (2006). Meteyer *et al*. (2009) reported reovirus associated fatal hemorrhagic and necrotizing enteritis in American crows (*Corvus brachyrhynchos*). Crow mortality associated with this syndrome have been identified in eastern Canada since 2004 (Campbell *et al.*, 2004; Campbell *et al.*, 2008; Stone, 2008; Meteyer *et al*., 2009).

In 2011, reoviruses were isolated from the intestinal contents of dead underweight Black capped chickadees (*Poecile atricapillus*) with watery and yellowish intestinal content and no significant lesions in Minnesota (Mor *et al.*, 2014). Lawson *et al.* (2015) reported reovirus isolation from a dead free-living magpie (*Pica pica*) with hepatic and splenic necrosis.

2.3. Molecular and physicochemical properties of avian reovirus

Molecular properties

The morphology of ARVs has been described by electron microscopy in chickens (Zhang *et al.*, 2005), turkeys (Mor *et al.*, 2013; Nersessian *et al.*, 1985a; Simmons *et al.*, 1972), Pekin ducks (Liu *et al.*, 2011), geese (Palya *et al.*, 2003), quails (Ritter *et al.*, 1986), psittaciformes (van den Brand *et al.*, 2007), black-capped chickadees (Mor *et al.*, 2014), magpie (*Pica pica*) (Lawson *et al.*, 2015) and brown-eared bulbul (*Hypsipetes amaurotis*) (Ogasawara *et al*., 2015). Avian reovirus lacks a lipid envelop but has double layer icosahedral outer capsid with as evidenced by transmission electron microscopy (Simmons *et al.*, 1972; Zhang *et al.*, 2005) of 70-80 nm in diameter (Lawson *et al.*, 2015; Liu *et al.*, 2011; Mor *et al.*, 2013; Ogasawara *et al*., 2015; van den Brand *et al.*, 2007)

encasing 10 segments of dsRNA categorized into three groups designated as large (L), medium (M) and small (S) segments based on their size and electrophoretic mobility (Benavente and Martınez-Costas, 2007; Varela and Benavente, 1994) (Table 1). There are three segments in L class (L1, L2 and L3) and M class (M1, M2 and M3) each and four segments in S class (S1, S2, S3 and S4) (Benavente, and Martinez-Costas, 2007). The overall genome size is 23,492 base pairs (bp) for strain 138, 23,493 bp for strain 176 (Xu and Coombs, 2009), 23,494 bp for strain AVS-B isolate (Banyai *et al*., 2011) and 23,593 bp for strain Pycno-1 (Ogasawara *et al*., 2015).

Nucleotide sequences of ARV S1133 were described by Martinez-Costas *et al.* (1995) where sequence analysis revealed that all genome segments (except S1 segment) encode only one primary translation product. The 10 reoviral genome segments have 12 open reading frames (ORFs), which encode for 12 primary translation products, among which S1 is a tricistronic segments, which express three protein products. Out of 12 viral proteins, eight structural proteins are incorporated into the newly formed virions while rest of the four proteins are nonstructural which are not found in the mature progeny viral particles, but only expressed during virus replication (Martinez-Costas et al., 1997; Varela and Benavente, 1994). The ARV proteins encoded L, M, and S class genes are designated as lambda (λ), mu (The S1 (σC) genome segment showed noticeably higher divergence followed by M2 and L3 genes.), and sigma (σ) proteins, respectively. Eight of the 10 structural proteins in ARV virions are the primary translation products from their corresponding encoded genome segments: λA, λB, λC, μA, μB, σA, σB, and σC while μBN and μBC are the other two structural proteins formed as post-translational cleavage products of the N-terminus and C-terminus of their precursor μB, respectively (Varela et

al., 1996). The M3 and S4 segments express two major nonstructural proteins named with their own group designation and "NS", namely μNS and σNS. The two additional nonstructural proteins were encoded by first two cistrons of the tricistronic S1 gene translating to p10 and p17 (Bodelón *et al.*, 2001; Shmulevitz *et al.*, 2002). Avian reovirus genes, encoded proteins, their functions, and the location in the virion are explained in Table 1.1

Table 2.1. Avian orthoreovirus genes, encoded proteins and their functions

Genome segment (bp)	**Encoded viral protein (aa)**	**ORF position**	**Location in virus structure**	**Functions**
L1 (3958)	λA (1293)	21-3899	Inner core	• A major core protein that forms the inner core shell (Guardado-Calvo *et al.*, 2008) • Serves as a scaffold during the early stages of viral morphogenesis (Benavente and Martínez-Costas, 2007)
L2 (3829)	λB (1259)	14-3790	Inner core	• Viral RNA-dependent RNA polymerase (RdRp) enzyme (Putative transcriptase) (Xu and

				Coombs, 2008) • Allow template RNAs, nucleotides and divalent cations to access the interior catalytic site (McDonald *et al.*, 2009)
L3 (3907)	λC (1285)	13-3867	Turret	• As a pentamer forms the turrets that extend from inner core through outer capsid (core turret protein) (Martínez-Costas *et al.*, 1997; Zhang *et al.*, 2005) • Guanylyltransferase/ viral capping enzyme that catalyzes the addition of a 5' cap on extruded viral mRNA (Martinez-Costas *et al.*, 1995)
M1 (228)3	μA (732)	13-2208	Inner core	• The co-factor for the enzyme RdRp (Benavente and Martínez-Costas, 2007)

M2 (2158)	μB (676) (μBN and μBC originated by post translational cleavage of precursor μB, μBC is further cleaved)	30-2050	Outer capsid	• A major outer capsid protein that forms outer capsid with σB (Martínez-Costas *et al.*, 1997; Zhang *et al.*, 2005) • Penetration (virus entry to cytoplasm) (O'Hara *et al.*, 2001) • Transcriptase activation (O'Hara *et al.*, 2001) • Cleavage and removal of μB associated with the endosomal membrane (O'Hara *et al.*, 2001) • Interaction and conformational changes in the capsid required for delivery of the transcriptionally active core particle to the cytoplasm (O'Hara *et al.*, 2001)
M3	μNS	25-1929	Nonstructural	• Forms viral factory

(1996)	(635) (Nonstructural Conserved protein) (Produce smaller μNS isoforms μNSC and μNSN)			scaffold (matrix) in infected cells and plays an important role in the early steps of viral morphogenesis by temporally and selectively controlling the recruitment of specific viral proteins to viral factories (Brandariz-Nuñez *et al.*, 2010; Tourís-Otero *et al.*, 2004) • Mediate association of σNS and λA to inclusions during morphogenesis (Brandariz-Nuñez *et al.*, 2010; Tourís-Otero *et al.*, 2004)
S1 (1643)	P10 (98)	25-318	Nonstructural	• Fusion associated small transmembrane (FAST) protein responsible for

				fusion of host cells and syncytium formation (Barry and Duncan, 2009; Bodelón *et al*., 2001; Shmulevitz and Duncan, 2000) • A viroporin (Bodelón *et al.*, 2002; Wu *et al.*, 2016) • Increases plasma membrane permeability (Bodelón *et al.*, 2002) • Induces apoptosis of host cell (Salsman *et al.*, 2005; Wu *et al.*, 2016)
	P17 (146) Membrane associated non-structural protein	293-730	Nonstructural	• Causes host cell protein translation shutoff through activation of p53-dependent pathway (Chulu *et al.*, 2010) • Cell cycle arrest (Chiu *et al.*, 2016; Chulu *et al.*, 2010)

				• A shuttle protein that moves between nucleus and cytoplasm continuously making it available to participate in cellular nuclear processes such as host cell translation, cell cycle and autophagosome formation benefiting virus replication (Chi *et al.*, 2013; Costas *et al.*, 2005; Huang *et al.*, 2015; Ji *et al.*, 2009; Liu *et al.*, 2005)
	σC (326) A minor component of outer capsid The most variable	630-1607	Outer capsid	• An elongated homotrimer responsible for virus attachment to the host cell through its C-terminal globular domain while anchored in the λC pentamer via its N-terminus to protrude

	protein			from the turret tops (Grande *et al.*, 2000; Grande *et al.*, 2002; Guardado-Calvo *et al.*, 2009; Martínez-Costas *et al.*, 1997; Shapouri *et al.*, 1996) • Determines tissue tropism and contribute to host restriction (Bodelón *et al.*, 2001). • Carries serological specificity. Induce type-specific neutralizing antibodies in virus infection and protective antibodies after vaccination (Lin *et al.*, 2006; Shapouri *et al.*, 1996; Shih *et al.*, 2004; Wickramasinghe *et al.*, 1993). • Induces apoptosis of the

				host cell (Shih *et al.*, 2004). • A viroporin
S2 (1324)	σA (416) Highly conserved major inner capsid protein	16-1263	Inner core	• Stabilizes λA shell while seated on top of λA and act as a bridge between the inner core and the outer capsid (Xu *et al.*, 2004). • dsRNA binding in a sequence independent manner (Martínez-Costas *et al.*, 2003; Yin *et al.*, 2000) • Anti-interferon activity by preventing the activation of the dsRNA dependent protein kinase (PKR) by competing for dsRNA (Gonzalez-Lopez *et al.*, 2003; Martínez-Costas *et al.*, 2000) • Displays nucleoside

				triphosphate phosphohydrolase (NTPase) activity (Yin *et al.*, 2002) generating energy used for transcription and replication of the viral genome.
S3 (1202)	σB (367)	31-1131	Outer capsid	• Major outer capsid protein that forms outer capsid with μB (Martinez-Costas *et al.*, 1995; Varela *et al.*, 1996). • Induces antibodies with broadly specific (Group-specific) neutralizing activity (Wickramasinghe *et al.*, 1993)
S4 (1192)	σNS (367) Nonstructural	24-1124	Nonstructural	• Binds to ssRNA in a nucleotide sequence non-specific manner in

	(highly conserved)			morphogenesis (Touris-Otero *et al.*, 2005; Yin and Lee, 1998). • Recruited into inclusion bodies by μNS protein soon after synthesis (Benavente and Martínez-Costas, 2007). • RNA packaging and replication (Benavente and Martínez-Costas, 2007).

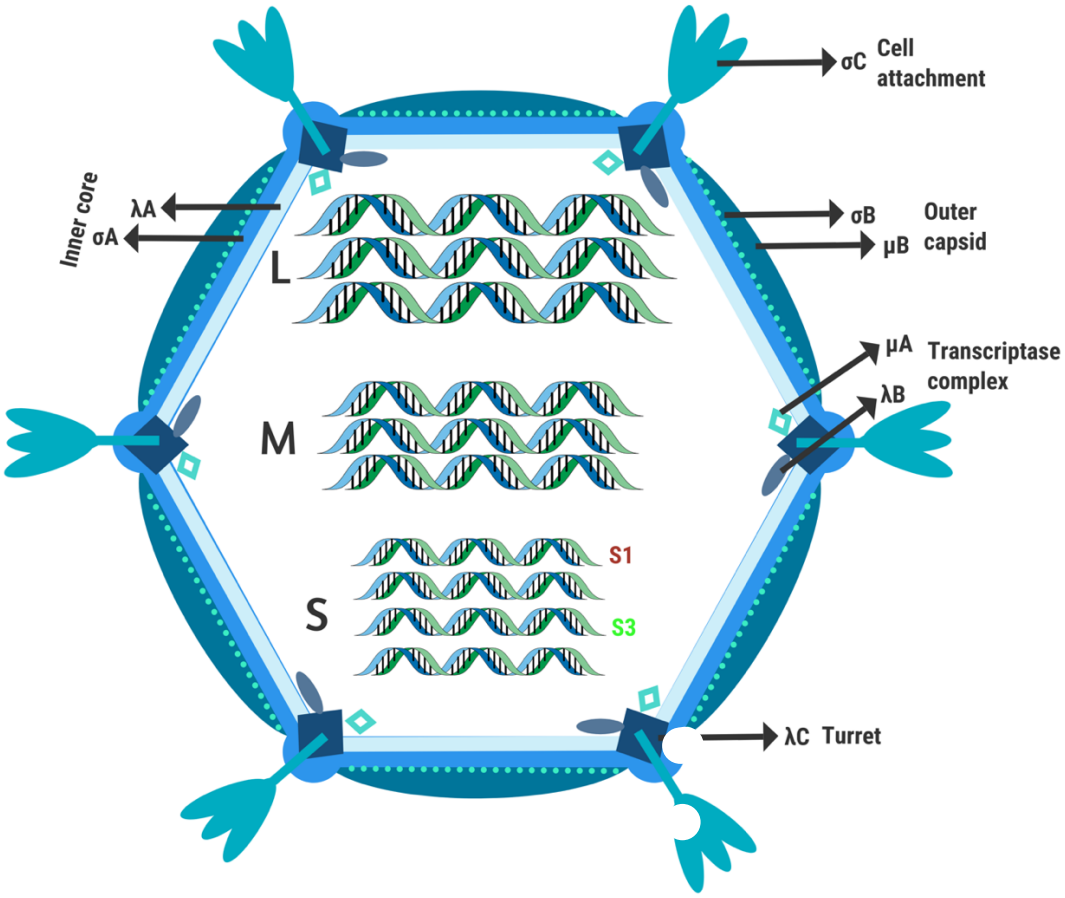


Figure 2.1. Structure of an ARV virion prepared by the author of this thesis inspired by the work of Benavente and Martinez-Costas (2007). The virion consists of an inner core (viral proteins λA, σA); an outer shell (viral proteins μB, σB); turrets that extend from the inner core into the outer core (viral protein λC), and the cell-attachment protein σC, which projects from the turrets.

Physicochemical properties

Avian reoviruses are non-enveloped viruses thereby making them resistant to adverse conditions. They are stable in a wide spectrum of pH, resistant to heat and disinfectants commonly used in poultry houses (Meulemanns and Halen, 1982). The ARVs have been reported to be resistant to lipid solvents (Quinn and Markey, 2003) but are sensitivity to 2% chloramine, phenol, mercury bichloride (Deshmukh and Pomeroy, 1969b), 100%

ethanol, tincture of iodine (Petek *et al.*, 1967), 70% ethanol, 0.5% organic iodine, 5% hydrogen peroxide (Hollmén and Docherty, 2007) and a complex disinfectant containing formalydehyde, glutaraldehyde, and alkyl dimethyl benzyl ammonium chloride (Meulemans and Halen, 1982). Reoviruses (chicken and turkey reoviruses) have been reported to be inactivated within 10 minutes by using oxidizing agents, aldehyde, and phenolic groups of disinfectants (Mor et al., 2015a). On the other hand, chicken, and turkey reoviruses have been inactivated using quaternary ammonium compounds and aldehyde group of disinfectants within 2-5 minutes (Mor et al., 2015a).

Historically, ARVS were considered to be resistant to trypsin, however later studies reported trypsin sensitivity of some strains (Al-Afaleq and Jones, 1991; Drastini *et al.*, 1994; Jones *et al.*, 1994). Trypsin sensitivity of some strains leads to their poor replication in intestine following oral exposure, resulting into limited dissemination to other tissues (Swayne and Glisson, 2013). Physicochemical analysis of ARVs revealed that they have identical composition of both A/U and C/G ratios and the genome segments are resistant to single-strand-specific nucleases (Nibert and Schiff, 2001).

The studies on survivability of reoviruses in poultry barn material showed that ARVs can survive up to 10 days on feathers, wood shavings, glass, rubber, and galvanized metal but can survive up to 10 weeks in water (Savage and Jones, 2003). Chicken and turkey reoviruses were reported to survive in autoclaved dechlorinated water for 9-13 weeks and for 2 weeks in non-autoclaved water (Mor et al., 2015b). Chicken and turkey reoviruses can survive in autoclaved litter for 6-8 weeks and for 6-8 days in non-autoclaved litter (Mor et al., 2015b). In a recent study, we observed that ARV infection can be transmitted horizontally to non-inoculated pen mates through litter and water

contaminated with the virus shed in the feces of infected birds (Kumar et al., 2021, submitted). The survivability of pathogenic ARVs in water poses a potential threat because if drinking water system is contaminated, it can remain a source of infection for up to 10 weeks, which is long enough to infect the next flock raised in the same barn. Reoviruses lose their polypeptide capsid when incubated at 40ºC (Grande and Benavente, 2000), resulting in release of core virus particles. The internal body temperature of aves is 39.5ºC, playing a positive role in uncoating and penetration of the virus in endosomes and intestinal epithelial cells, respectively (Benavente and Martínez-Costas, 2007). Reoviruses are less stable in salt free neutral or acidic pH buffer and lose their infectivity (Benavente and Martínez-Costas, 2007). They also lose their infectivity when subjected to repeated freeze and thaw cycles (Benavente and Martínez-Costas, 2007).

2.4. Genetic diversity in reoviruses

The ARVs with dsRNA genome have slight advantage of stability over ssRNA viruses. However, the absence of proofreading and post replicative error correction mechanisms in their RNA polymerases creates higher chances of mismatching during virus replication leading to higher mutation rates and rapid evolution (Steinhauer and Holland, 1987). Avian reoviruses are reported to have a high degree of antigenic heterogeneity compared to the MRVs (Meanger *et al.*, 1995; Shapouri *et al.*, 1996). Genomic research in the last decade on ARVs has contributed ample genetic information about their continuous and rapid evolution. Recent sequence data from Asia (Liu et al., 2003; Zhong et al., 2016), North America (Ayalew et al., 2017; Palamino-Tapia et al., 2018; Egaña et al., 2019; Ayalew et al., 2020), South America (Carli et al., 2020; Torre et al., 2021), Europe

(Troxler et al., 2013; Farkas et al., 2016) suggest that all gene segments participate in evolution and creation of genetic and antigenic variants of CARVs. The highly pathogenic variant CARVs causes vaccination failure leading to disease outbreaks in vaccinated flocks across the world including the US (Chénier et al., 2014; Tang and Lu, 2015a, 2015b; Lu et al., 2015; Sellers et al., 2017; Egana et al., 2019). Most of the genetic studies on ARVs were conducted on σC-encoding gene (and σC protein) which have higher divergence and is the antigenic determinant inducing type specific neutralizing antibodies (Liu et al., 2003). All the phylogenetic studies on nucleotide and amino acid sequence analysis of σC protein have classified ARVs into distinct genotypes (Kant et al., 2003; Egana et al., 2019; Ayalew et al., 2020; Carli et al., 2020) which seem to differ from vaccine strains. However, meta-analytically there is no identical pattern suggesting ARV genome segments may evolve in an independent manner (Hsu et al., 2006; Liu et al., 2003). Phylogenetic analyses of reovirus sequences from different species indicated that non-fusogenic MRV, ARV, Nelson Bay virus, and Baboon reovirus are four distinct species. These four species were clustered in three separate clades, in which ARV and Nelson Bay virus were clustered in the one clade while MRV and Baboon reovirus were clustered in two separate clades (Duncan, 1999). Avian reovirus in genus *Orthoreovirus* includes a variety of reovirus sub-species from different avian species having limited nucleotide identity and amino acid sequence similarity. Researchers suggested that non-chicken origin reoviruses, especially turkey reovirus should be considered in a separate subgroup and duck and goose reovirus should be considered in a separate subgroup (Banyai et al., 2005; Day et al., 2007; Kapczynski et al., 2002; Sellers et al., 2004; Mor et al., 2013).

The high genetic variability in σC protein and moderate to low genetic variability in proteins encoded by other gene segments is due to antigenic drift and reassortment events (Liu et al., 2003; Shen et al., 2007; Su et al., 2006; Duncan and Sullivan, 1998) under strong immunological selection due to vaccine pressure and host's immunity. Antigenic drift happens because of cumulative accumulation of mutations in the gene regions encoding antibody-binding sites resulting into formation of new variants that cannot be completely neutralized (Donnelly et al., 1995).

On the other hand, reassortment is common among segmented dsRNA viruses such as influenza virus, rotavirus and reovirus. Genetic reassortment usually happens with exchange of gene segments of two avian reovirus strains infecting the same host/cell producing novel progeny virion (reassortant) containing mixed genetic information of parental strains (Farkas et al., 2016; Liu et al., 2003; Zhou et al., 1999). The properties of the novel progeny virion will depend on which segments are inherited from which parent and the functional behavior of each group of gene segments and their protein products. In Reoviridae, reassortment from mixed infections is limited to species level. Reassortment in viruses provides advantages in mutation and evolution. Studying the recombinant (reassorted) viruses is very helpful for determining the functionality and phenotypic characteristics of genome segments. It can also reveal important information such as the evolution patterns and genetic lineage of a particular ARV strain (Liu et al., 2003). Phylogenetic studies also provided evidence of frequent reassortments among the circulating lineages that are responsible for the topological variation and segmental incongruence in the ARV genome segments. Most importantly, all studies concluded that genomic diversity is not associated with the serotypes or pathotypes of the ARV isolates,

but the time and geographic location of the isolation has more determinative influence on ARV genetic divergence (Hsu et al., 2005; Kant et al., 2003; Liu et al., 2003).

2.5. Pathogenesis of avian reovirus

Avian orthoreovirus was first isolated from chickens affected with chronic respiratory disease (Fahey and Crawley, 1954). It was originally named as "Fahey-Crawley agains" which was later characterized as an orthoreovirus (Petek *et al.*, 1967). Subsequent studies have isolated reovirus from a variety of clinical diseases in chickens e.g., arthritis/tenosynovitis (Glass *et al.*, 1973; Jones *et al.*, 1975), runting-stunting syndrome/malabsorption syndrome (Page *et al.*, 1982b; Robertson *et al.*, 1984; van der Heide *et al.*, 1981), cloacal pasting (Deshmukh and Pomeroy, 1969a), hydropericardium (Bains *et al.*, 1974; Jones, 1976; Spradbrow and Bains, 1974), myocarditis and pericarditis (Mustaffa-Babjee *et al.*, 1973), and hepatitis (Mandelli *et al.*, 1978). While reoviruses have been conclusively, but not exclusively, associated with arthritis/ tenosynovitis in chickens (van der Heide, 1977; van der Heide, 2000), they are also associated with an array of diseases including gastroenteritis and cloacal pasting (Deshmukh and Pomeroy, 1969a), myocarditis and pericarditis (Mustaffa-Babjee *et al.*, 1973), respiratory disease (Fahey and Crawley, 1954), feathering abnormalities (Helicopter disease), hepatitis (Mandelli *et al.*, 1978), hydropericardium (Bains *et al.*, 1974; Jones, 1976; Spradbrow and Bains, 1974), rupture of gastrocnemius tendon (Jones *et al.*, 1975), non-uniform and stunted growth (Murphy *et al.*, 1999), reduced feed conversion, immunosuppression and atrophy of bursa of Fabricius (Montgomery *et al.*, 1986a), thymic atrophy (Hollmén and Docherty, 2007) and sudden death (Huhtamo *et al.*,

2007) in chickens. Clinical disease in turkeys has been reported to be like chickens including viral arthritis (Page et al. 1982; Mor et al., 2013) and poult enteritis (Reynolds et al. 1987; Jindal et al., 2009, 2010a; Mor et al., 2011, 2013a), Myocarditis (Pantin-Jackwood et al., 2007) and hepatitis (Farnca et al., 2010; Shivaprasad et al., 2009). The ARVs from chickens and turkeys are closely related, however, a group of ARV that are genetically distinct from chicken origin reoviruses have been collected from commercial turkey flocks with enteric disease (Sellers et al. 2004). The infection can be subclinical in poultry, but pathogenic strains can cause low mortality (2-10%) and high morbidity (5-50 %) resulting in significant economic losses (Glass *et al.*, 1973; Olson and Solomon, 1968; Perelman et al., 2019; Sedghi et al., 2013).

Reoviral pathogenesis starts with viral entry into the host by adsorbing on the target intestinal and respiratory cells followed by virus replication in the mucosa and viremia leading to dissemination of virus causing damage to host tissues (Ellis *et al.*, 1983; Joklik, 1983; Jones *et al.*, 1989; Menendez *et al.*, 1975b; Ni and Kemp, 1995). After surviving the proteolytic enzymes and bile salts in the gastrointestinal tract of the host, the virus enters the epithelial cells of gastric mucosa causing local or systemic infection leading to viremia and spread of virus to different predilection sites and causing tissue damage in spleen as it is among the first tissues infected during viremia (Pantin-Jackwood *et al.*, 2007). Intestine and bursa are primary entry and replication sites for reoviruses in chickens (Jones *et al.*, 1989; Kibenge *et al.*, 1985; Pantin-Jackwood *et al.*, 2007) and turkeys (Pantin-Jackwood *et al.*, 2007). The intestinal tract is also a principal route of excretion of the virus (Jones *et al.*, 1989).

Fecal-oral route is the natural route of ARV infection in chickens and turkeys (Jones and Onunkwo, 1978; Sahu and Olson, 1975; Sharafeldin et al., 2014, 2015). The incubation period depends upon the virus pathotype, age of host, and route of exposure (Robertson and Wilcox, 1986; van der Heide, 1977). Incubation period was reported to be approximately 4 days for footpad inoculation, 1-30 days for intravenous inoculations, and approximately 13 days for contact exposure (Olson, 1959). Virus infection by footpad inoculation resulted in more severe disease and slower growth rates than the oral route of inoculation (Jones and Kibenge, 1984).

Different viral factors and host factors affect the pathogenicity and outcome of reovirus infection in poultry (Kibenge and Wilcox, 1983). Viral factors include the virulence of the strain (Gouvea and Schnitzer, 1982a; Jones and Guneratne, 1984), the dose (Gouvea and Schnitzer, 1982a), the route of infection and the tissue tropism. Host factors include the breed (Jones and Kibenge, 1984), the age at infection (Jones and Georgiou, 1984) and the immune status of the affected individuals.

Virus factors: virus strain, dose, and the route of infection

The pathogenicity of ARV infection may vary from mild to severe infection (Kibenge et al., 1983; Takase *et al.*, 1984a). Severity of disease depends on the viral strain (Glass *et al.*, 1973; Jones and Kibenge, 1984), strain heterogeneity (Gouvea and Schnitzer, 1982a) and the dose (Kibenge et al., 1983). The most pathogenic strain can kill day-old chicks (Gouvea and Schnitzer, 1982a; Hieronymus *et al.*, 1983b; Takase *et al.*, 1984b). The route of infection determines the incubation period severity of disease (Glass *et al.*, 1973; Sahu *et al.*, 1979). Chicks are more susceptible to respiratory route of infection than the oral route (Montgomery *et al*., 1986b). Olson and Khan, (1972) reported that Fahey-

Crawley virus was capable of inducing tenosynovitis in chickens infected via respiratory route.

Host factors: age and breed

Age related susceptibility of chickens to reovirus infection was reported where day old chicks are more susceptible to infection (Jones and Georgiou, 1984; Kerr and Olson, 1964). Infection in 1–7-day old chicks caused more severe disease with higher mortality than birds infected at 2 weeks or older age (Jones and Georgiou, 1984; Mustaffa-Babjee *et al.*, 1973; Roessler and Rosenberger, 1989; Subramanyam and Pomeroy, 1960). Age related susceptibility to infection was related to the ability of the birds to evoke an effective immune response (Gouvea and Schnitzer, 1982a). Higher resistance to infection in older birds may be associated with maturation of humoral and maturation of the T-cell mediated immunity (Roessler and Rosenberger 1989). Additionally, the susceptibility of one-day-old chicks to oral infection by S1133 strain was reported to be related to the immune status of their dams (Wood *et al.*, 1986).

Severity of reovirus infection is also related to breed of bird (Glass *et al.*, 1973; Jones and Kibenge, 1984) where tenosynovitis was observed as primarily a disease of broiler birds (Jones and Onunkwo, 1978) and layers are much less affected (Schwartz *et al*., 1976). Additionally, tenosynovitis was observed more commonly in heavy breeds than lighter breeds (Jones and Kibenge, 1984). The reason for more susceptibility of heavy broiler breeds to tenosynovitis may be due to their higher weight gain and rapid growth rate leading to physical changes to the weight- bearing leg tendons predisposing them to mechanical damage due to infection (Kibenge and Wilcox, 1983). Additionally,

broiler tendons have more fibrous connective tissue and lower tensile strength than lighter breeds leading to increased susceptibility of broiler breeds (Walsum, 1977).

Almost 80% of avian reoviruses are non-pathogenic and causes innocuous infection (Jones, 2008). Although, pathogenic strains exist and has been reported to cause different diseases affecting different body systems as reviewed below.

Arthritis/ Tenosynovitis

Arthritis was first recognized as an important cause of leg weakness in poultry by Olson (1959). Arthritis of chickens with a definite viral etiology was first described in the USA by Olson and Kerr (1966) and the etiology was identified as an orthoreovirus by electron microscopy (Walker *et al.*, 1972). A similar condition called tenosynovitis was reported by Dalton and Henry (1967) in the United Kingdom. The term avian tenosynovitis was originally used for lameness caused by *Mycoplasma synoviae* as an inflammation of the tendons and tendon sheaths (Dalton and Henry 1967), whereas term viral arthritis was used for reovirus associated disease (Olson, 1973). Later, both terms were used to describe the orthoreovirus-associated lameness (Kibenge and Wilcox, 1983), but the true arthritic lesions were present only in the late stages of the reoviral arthritis (Kerr and Olson, 1969). Jones *et al*. (1975) isolated an arthrotropic reovirus from broiler chickens in Britain, and Jones and Onunkwo (1978) experimentally reproduced the disease in light breed chickens. Although many pathogens such as adenoviruses (MacKenzie and Bains, 1976), *Staphylococcus aureus* (Johnson, 1972; MacDonald *et al.*, 1978; MacKenzie and Bains, 1976), *Mycoplasma synoviae* (Kerr and Olson, 1969) and *Mycoplasma iowae* (Dobson and Glisson, 1992) were commonly isolated from chickens having tenosynovitis lesions, ARVs were considered the primary etiology (van der Heide, 1977). MacKenzie

and Bains (1976) suggested *Staphylococcus aureus* was a secondary pathogen that exacerbated the primary avian reovirus lesion, and similarly other bacteria were more likely to be secondary opportunistic pathogens possibly following initial avian reovirus induced tendon damage. However, bacteria were not present in all clinical outbreaks of tenosynovitis (Kibenge *et al.*, 1982a).

The clinical signs following oral inoculation of reovirus in day-old SPF light hybrid chicks were depression and lameness (Kibenge et al., 1983), prostration (Jones and Georgiou, 1984) and anorexia at 2 dpi (Tang *et al.*, 1987b), but return to normal by 8 dpi (Tang *et al.*, 1987b). There was unilateral swelling of hock joints on the plantar aspect by 3-4 weeks post inoculation (wpi) (Jones and Georgiou, 1984). Acute infection causes mortality, poor growth, decreased feed conversion efficiency and carcass condemnation (Schwartz *et al.*, 1976). Mortality of chicks began at 4 dpi (Al-Afaleq and Jones, 1991; Tang *et al.*, 1987b) and continued until 10 dpi (Kibenge and Dhillon, 1987). The body weight gains were significantly lower at 5 wpi (Kibenge and Dhillon, 1987). Affected birds are usually reluctant to move to feed and water and become emaciated (Kibenge and Wilcox, 1983). In field conditions, reovirus associated lameness is generally observed at 7 weeks of age or later (Jones and Onunkwo, 1978), even though the affected birds were infected at a very young age or through egg transmission (van Loon *et al.*, 2001).

The gross lesions of reoviral arthritis were mainly confined to the hock joints characterized by inflammation of the hock joints and the gastrocnemius tendons (Benavente and Martínez-Costas, 2007; Rhyan and Spraker, 2010). Acute lesions were characterized by inflammation in the joints progressing to pannus formation, erosion of

underlying cartilage, and ultimately fibrosis (Stott, 1999). A yellowish-brown gelatinous exudate between tendons of swollen legs, thickening and fusion of tendons, and pitted erosions of the articular cartilage of the hock joints were observed by 12 wpi (Jones and Kibenge, 1984; Jones and Georgiou, 1985). The most prominent findings in the disease were swelling of the tibiotarsal-tarsometatarsal region and extensive swelling of the digital flexor and metatarsal extensor tendons. Inflammatory lesion often proceeds to a chronic hardening and fusion of the tendon sheaths (Jones and Georgiou, 1984; Stott, 1999). Consequently, adhesions in the tendons, synovial sheath and skin, renders the tendons partially nonfunctional (Johnson, 1972). Tenosynovitis may lead to rupture of the gastrocnemius tendons causing hemorrhage (Jones and Georgiou 1984; Johnson and van der Heide 1971; Johnson 1972).

Histologic lesions of acute viral arthritis include edema, coagulation necrosis, and perivascular infiltration of lymphocytes and macrophages. Thickening of the tendon sheath was caused by reticular cell proliferation, synovial cell hyperplasia and hypertrophy, infiltrates of heterophils and macrophages, and periostitis. Synovial cavities were filled with sloughed synovial and inflammatory cells (Stott, 1999). Loose connective tissue surrounding the sheath was replaced by granulomatous inflammation and fibrous connective tissue. Granulomatous inflammation infiltrated into the tendons causing them to adhere firmly to their surrounding sheath (Johnson, 1972). The chronic disease was characterized by formation of villi on synovial membranes, increase in fibrous connective tissue, and infiltration or proliferation of reticular cells, lymphocytes, macrophages, and plasma cells (Stott, 1999). Olson and Weiss (1972) described the histopathology of birds infected with Fahey-Crawley agent via the footpad. Extensive

fibrosis and the presence of numerous lymphoid follicles in the digital flexor tendon sheaths was observed 43 days PI. There were hypertrophy and hyperplasia of the synovial lining cells and a diffuse infiltrate of lymphocytes, plasma cells, macrophages, and a few heterophils. Clumps of heterophils and desquamated synovial cells were occasionally present in the synovial spaces. Chronic inflammatory changes were evident in the articular cartilage by replacement of the cartilage by connective tissue (pannus) that was grossly evident as pitting of the articular surface (Gouvea and Schnitzer, 1982a). At 7.5 weeks PI, van der Heide *et al*. (1974) observed chronic fibrosis of the tendon sheaths with fibrous connective tissue invading and replacing the normal architecture of the tendon, resulting in ankylosis and immobility. At 33 weeks PI, mononuclear cell infiltration was still present in the sheaths and tendons. Heterophils were still prominent in some areas and large lymphoid foci were occasionally seen at the tendon periphery (Jones and Onunkwo, 1978). Fibroplasia had also occurred in the tendon sheaths. According to Islam *et al*. (1990), infection with arthrotropic ARVs provided evidence of an autoimmune reaction. The ability of avian reovirus to establish persistent infections may be due to the joint and tendon acting as sequestered sites protecting the virus from elimination by the immune system (Jones and Georgiou, 1985).

Respiratory disease

Avian reovirus was originally isolated from chickens with chronic respiratory disease (Fahey and Crawley, 1954). Fahey reported the isolation of identical viruses from ducks (Fahey, 1955) and turkeys (Fahey, 1956) having chronic respiratory disease with infectious sinusitis and suggested a common etiology in other avian species. Subramanyam and Pomeroy (1960) experimentally reproduced respiratory infection with

the Fahey and Crawley virus. Avian reovirus has been isolated from a laying flock concurrently infected with infectious bronchitis virus (McFerran *et al.*, 1971). Hieronymus *et al.* (1983b) noted some ARV infected birds had air sacculitis and pulmonary congestion.

Enteric disease

Avian reoviruses have been isolated from chicks with severe cloacal pasting (Deshmukh and Pomeroy, 1969a; Dutta and Pomeroy, 1967), digestive tract of chickens with malabsorption (Hieronymus *et al.*, 1983; Page *et al.*, 1982b), stunted, pale broilers with reduced digestion, depressed weight gain and feed conversion, and nutritional deficiencies (Giambrone *et al.*, 1992), broiler flocks with high mortality and signs of malabsorption (van Loon *et al.*, 2001), turkeys with depression, anorexia, 30% mortality (Simmons *et al.*, 1972) or infectious enteritis (Gershowitz and Wooley, 1973), and quails experiencing severe enteritis (Guy *et al.*, 1987; Ritter *et al.*, 1986).

The RSS/MAS is characterized by a high prevalence of birds with stunted growth, leg weakness, markedly decreased weight, poor feed conversion, high condemnation rates at slaughter and retarded feathering at 2 - 5 weeks of age (Bracewell and Wyeth, 1981; Kouwenhoven *et al.*, 1978b; Page *et al.*, 1982b; Page, 1983), poor pigmentation of the shanks (Page, 1983), enlargement of the proventriculus and a decrease in the size of the ventriculus (Page *et al.*, 1982b), diarrhea (Page, 1983; Vertommen *et al.*, 1980), orange to yellowish mucus in the feces (Clark *et al.*, 1990), significant gross and microscopic pancreatic damage (Davis *et al.*, 2013), femoral head fractures, and osteoporosis (van der Heide *et al.*, 1981). Elevated feed conversions and decreased body weights were constant clinical features of the syndrome, and there was a considerable

amount of undigested feed in the fecal material, relating to decrease feed conversion efficiency and economic loss (Page *et al.*, 1982b).

At necropsy, the most prominent lesions were proventriculitis and catarrhal enteritis (Page *et al.*, 1982b) which consequently impaired digestion of feed and could have resulted in decreased absorption and deficiency of these vitamins and minerals (Bracewell and Wyeth, 1981; Page, 1983). The relationship between avian reoviruses and arthritis/tenosynovitis was well established, the causative role was less clear in RSS/MAS (Kouwenhoven *et al.*, 1988; van der Heide *et al.*, 1981). Reovirus has been most frequently isolated from affected birds and implicated as the etiology (Songserm *et al.*, 2002). However, experimental reproduction of the disease from isolated viruses was inconsistent. Using infectivity trials with reovirus, several authors were able to reproduce some clinical signs and lesions of RSS/MAS, but not all (Hieronymus *et al.*, 1983a; Page *et al.*, 1982b; van der Heide *et al.*, 1981; van Loon *et al.*, 2001), while others were unable to produce any of the clinical or pathological features (Guy *et al.*, 1988; McNulty *et al.*, 1984). In a later study, the disease was experimentally reproduced by oral or subcutaneous inoculation of day-old commercial broilers and SPF chicks using enteric reovirus strains (ERS), and the authors subsequently postulated that ERS played a role in RSS/MAS even though it is not considered the only cause (van Loon *et al.*, 2001). Microscopically, enteritis of small intestinal in chicks associated with intralesional reovirus supported the etiology (Goodwin *et al*. 1993b). The microscopic lesions consisted of mild villus atrophy and crypt hypertrophy with mild to marked multifocal distention of crypts filled inflammatory cells and sloughing of necrotic epithelial cells. There were increased numbers of a mixed population of inflammatory cells in the lamina

propria immediately surrounding crypts including macrophages, lymphocytes and heterophils. In field cases, there is cystic dilation of the crypts of Lieberkuhn, necrosis of crypt epithelial cells, deposition of cellular debris within the crypts, apparent loss of crypts in a high proportion of birds in the first week (Reece and Frazier, 1990; Smart *et al.*, 1988) and vacuolar degeneration and sloughing of enterocytes in the small intestine (Songserm *et al.*, 2003).

Immunosuppression

Numerous authors have described the immunosuppressive activity of reovirus on the avian immune system. Kerr and Olson (1969) noted lymphoid cell degeneration in the bursa of Fabricius of birds infected with a tenosynovitis as early as 7 dpi. Montgomery *et al*. (1985) demonstrated the ability of reovirus to cause transient alterations in bursal and splenic weights, and many authors have discussed reoviral related bursal atrophy (Montgomery *et al.*, 1986a; Ni and Kemp, 1995; Page *et al.*, 1982b), hemorrhages, congestion and necrosis (Hieronymus *et al.*, 1983b; Tang *et al.*, 1987b). Roessler and Rosenberger (1989) noted avian reovirus infection causes cell damage *in vivo* in several organs including bursa of Fabricius, thymus and spleen characterized by lymphoid depletion. Chenier *et al*., (2014) observed a generalized depletion of lymphocytes and lymphocytolysis in lymphoid organs associated with reovirus infection. Immunosuppression results in a poor response to vaccinations and predisposes the host to infection with other pathogens, which might account for the diversity of syndromes associated with avian reoviruses (Montgomery *et al.*, 1986a). Therefore, avian reovirus induced immunosuppression in chickens has been documented to cause either a

depressed humoral immune response to other pathogens (Montgomery *et al.*, 1985; Springer *et al.*, 1983) or as diminished cellular immune response (Hill *et al.*, 1989).

2.6. Laboratory diagnosis of avian reovirus

Diagnosis of ARV diseases is difficult because they are clinically indistinguishable from several other common disease conditions such as adenovirus infection and bacterial and mycoplasmal synovitis (Stott, 1999). Therefore, laboratory diagnostic tests for rapid detection of reovirus are needed for early diagnosis to prevent spread of the problem and avoid economic losses.

Virus isolation

Virus isolation and identification in cell cultures, serological methods and histopathology were the most common traditional approaches for diagnosis of avian reovirus diseases (Robertson and Wilcox, 1986). Although virus isolation and identification in cell culture is a gold standard for reovirus detection. The procedure is laborious and time consuming (Caterina et al., 2004; Meanger et al., 1995; van der Heide et al., 1976; Wood et al., 1986), usually taking more than 7 days and need SPF embryonated eggs to prepare sensitive primary cell cultures (Zhang et al., 2006). Different culture systems available are primary chicken cell cultures of embryo, lung, kidney, liver, macrophages, and testicle but primary embryo liver cells are preferred for reovirus isolation (Back and Nagaraja, 1996; Gouvea and Schnitzer. 1982). Chicken embryo fibroblasts are suitable for reovirus culture after virus adaptation, but the virus often requires adaptation (Barta et al., 1982; Guneratne et al., 1982; Jones et al., 1975). Reovirus infection of chicken-origin cell cultures causes formation of syncytia and sloughing of the monolayer after 24-48

hours. Eosinophilic or basophilic intracytoplasmic inclusions are observed in infected cells (Robertson and Wilcox, 1986). Other cells lines used for reovirus isolation and culture are Vero (Sahu and Olson, 1975), BHK 21/13, 1TT, feline kidney (CRFK), Georgia bovine kidney (GBK), rabbit kidney (RK), porcine kidney (PK) (Barta et al., 1982), a Japanese quail fibrosarcoma cell line (QT35) (Cowen and Braune, 1988). Samples intended for virus isolation can be stored at 4 °C in transport medium for several days or at -20 °C or -70 °C for longer periods (Hollmén and Docherty, 2007).

Immunodiagnostics

A variety of immunodiagnostic methods have been developed for the identification of avian reoviruses or antibodies against them including the agar-gel precipitation test (Olson and Weiss, 1972), plaque neutralization test (Ide and Dewitt, 1979), direct immunofluorescence staining technique (Jones and Onunkwo, 1978), indirect immunofluorescence assay (Ide, 1982), microtiter serum neutralization test (Robertson and Wilcox, 1984), immunoperoxidase technique using avidin-biotin-peroxidase complex (ABC) (Tang and Fletcher, 1987), virus neutralization test (Giambrone and Solano, 1988), western blotting (Endo-Munoz, 1990) and many enzyme-linked immunosorbent assay (ELISA) techniques (Chen *et al.*, 2004; Hsu *et al.*, 2006; Islam and Jones, 1988; Xie *et al.*, 2010; Yang *et al.*, 2010; Zhang *et al.*, 2007).

Molecular diagnostics

Molecular methods for detecting enteric viruses offer several advantages over traditional methods (Pantin-Jackwood *et al.*, 2008). Thus, the detection of viral RNA from clinical samples by conventional reverse transcriptase polymerase chain reaction (RT-PCR) remains the first choice in early diagnosis (Zhang *et al.*, 2006) and has been commonly

used to detect avian reoviruses in clinical samples. One main advantage of conventional RT-PCR over real-time RT-PCR is that the identity of the amplicons (viruses) can be confirmed and further characterized by sequencing them for accurate identification. Molecular approaches to identification of avian reoviruses in clinical samples have been described by several authors. These include an *in situ* hybridization (ISH) technique using a digoxigenin (DIG)-labeled complementary DNA (cDNA) probe (Liu and Giambrone (1997), dot blot hybridization assay using a radio-labelled cDNA probe (Yin & Lee, 1998), conventional RT-PCR (Bruhn *et al.*, 2005; Lee *et al.*, 1998; Liu *et al.*, 1999a; Liu *et al.*, 2004; Xie *et al.*, 1997), and conventional RT-PCR combined with restriction fragment length polymorphism (RFLP) (Lee *et al.*, 1998; Liu *et al.*, 1999a). Liu *et al*. (1999b) developed an *in situ* hybridization (ISH) technique and an *in situ* RT-PCR to detect avian reovirus in formalin-fixed, paraffin-embedded chicken tissues and demonstrated that the latter was more sensitive and accurate because avian reovirus often result in latent infections that cannot be identified by ISH.

2.7. Vectored viruses as vaccines

In an era of molecular biology, genetic engineering and reverse genetics systems have allowed us to use only the relevant genes and their immunogenic proteins for inducing neutralizing antibodies produced by an expression system. Recombinant live viral vaccine vectors are being noticed for use as vaccine vectors over traditional vaccine strategies. Live viral vaccine vectors can replicate and induce pronounced immunity against the antigens of interest without using irritable adjuvants.

Several options of such viral vectors are available to use as vaccine vectors. Some attenuated poultry vaccine viruses e.g., Newcastle disease virus (NDV) (Nakaya et al., 2001; Park et al., 2006; Chellappa et alk., 2017; Dey et al., 2017), fowl pox virus (FPV) (Swayne et al., 2000; Boyle and Couper, 1988; Bubolt et al., 2006), adenovirus (Eterradossi et al., 2004), herpesvirus of turkeys (HVT) (Li et al., 2011; Darteil et al., 1995), infectious laryngotracheitis virus (ILTV) (Veits et al., 2003; Luschow et al., 2001) and Marek's disease virus (MDV) (Tsukamoto et al., 1999; Sakaguchi et al., 1998) have been genetically modified in order to use them as vaccine vectors for developing combinations of live attenuated vaccines for controlling the diseases of poultry.

Different efficient recombinant vaccines have been developed in the past for several viruses, including vaccines for hepatitis B (McAleer et al., 1984) and for papillomavirus (Valentino K, Poronsky, 2015) for humans, as well as infectious bursal disease (IBD) (Pitcovski et al., 2003) and egg drop syndrome (Fingerut et al., 2003) for chickens and hemorrhagic enteritis virus (Pitcovski et al., 2005) and turkey arthritis reovirus (Kumar et al., 2021) for turkeys.

2.8. Pichinde virus as a vector

Live virus vaccine vectors have many advantages over traditional vaccine strategies because they can replicate inside the vaccinated host and can induce strong immunity against the antigenic gene inserted into the vector. Many viral vectors e.g., adenoviruses, poxviruses, new castle disease viruses and alphaviruses etc. are in preclinical or clinical phase of development (Choi and Chang, 2013). These pathogenic virus vectors have an

inherent risk of reversal to its wild type. Scientists all around the world are searching for safe and immunogenic viral vectors.

Pichinde virus (PICV) belongs to Arenaviruses which are enveloped viruses having bi-segmented negative strand RNA encoding four genes. The L segment encodes for the RNA-dependent RNA polymerase L and the ring-finger motif containing Z protein. The S segment encodes for the nucleoprotein NP and the glycoprotein GPC. These viruses target the antigen-presenting cells (i.e., macrophages and dendritic cells) early in the infection and have developed different strategies to evade host innate immune recognition (Meyer and Ly, 2016). Unlike other viruses of arenavirus family, PICV are not known to cause any disease in humans or animals (Dhanwani et al., 2015). The PICV was isolated from rice rat (Oryzomys albigularis) from south Columbia, South America (McLay et al., 2014). Therefore, this viral vector can overcome a major challenge of preexisting vector immunity that often limits the use of viral vectors as vaccines. Recently developed recombinant pichinde virus vector (rPICV) belongs to the family of recombinant arenavirus which is potentially safe does not compromise the immunogenicity (Emonet et al., 2009; Dhanwani et al., 2015). The rPICV have been proved to be safe and efficiently deliver different foreign antigens of highly pathogenic avian influenza (HA and NA, Dhanwani et al., 2015) and turkey arthritis reovirus (Sigma C and Sigma B, Kumar et al., 2021). Additionally, this viral vector was proved ideal for use in a prime-boost vaccination strategy (Dhanwani et al., 2015; Kumar et al., 2021). The rPICV induces very low anti-PICV immunity in vaccinated animals because of highly glycosylated envelop glycoprotein which dampens the development of neutralizing antibodies against rPICV (Sommerstein et al., 2015).

CHAPTER 3

CHARACTERIZATION OF GENETIC VARIATION IN CHICKEN ARTHRITIS REOVIRUS FROM THE UNITED STATES

The material in this chapter has been prepared for publication:
Kumar R, Vasquez JM, Farnoushi Y, Singh VK, Mor SK, Kulkarni AB, Porter RE, Lublin A, and Goyal SM. 2021. Characterization of Genetic variation in Chicken Arthritis Reovirus from the United States. Transbound. Emerg. Dis.

Introduction

Avian reoviruses (ARVs) are important pathogens of poultry and are responsible for considerable economic losses due to lameness, poor growth, non-uniform flock and sometimes death (Pitcovski and Goyal, 2020). Avian orthoreovirus can infect various avian species, including chickens, turkeys, pheasants, ducks, geese, and other domestic poultry (Pitcovski and Goyal, 2020). Reoviruses infecting chicken are named as chicken reovirus (CRV), which generally affects young broilers and is transmitted horizontally through the oral-fecal route, broken skin and vertically from breeders to progeny (Mansour et al. 2018). The associated diseases are viral arthritis, runting-stunting syndrome (RSS), myocarditis, hepatitis, respiratory disease, and even central nervous system infections (Banyai et al. 2011; Crespo and Shivaprasad, 2011; Davis et al., 2013; Jones, 2013). Chicken arthritis reovirus (CARV) infects the hock joints of 4-7-week-old broilers leading to edema of footpad and tibiotarsus-tarsometatarsal joint. In severe cases, joint infection results in rupture of gastrocnemius and digital flexor tendons. The affected birds feel pain and are either reluctant to walk or walk with the support of their wings (wing walkers) leading to poor and non-uniform growth, secondary infections, mortality, and downgrading of the carcasses at processing plants (Jones, 2013).

The ARVs are taxonomically classified under genus *Orthoreovirus*, subfamily *Spinareovirinae*, and family *Reoviridae* (Attoui et al., 2012). Reoviruses are non-enveloped, 70-80 nm in diameter with icosahedral symmetry (King et al. 2012). The viral genome has 10 segments of double stranded RNA (dsRNA) ranging in size between 1 and 4 kb packaged into a double-shelled capsid. The genome segments are 3 large/L-class

(L1, L2 and L3), 3 medium/M-class (M1, M2, M3), and 4 small/S-class (S1, S2, S3, S4) segments based on their electrophoretic mobility in polyacrylamide gel (Benavente and Martínez-Costas 2007). The L-class and M-class genome segments express three primary translation proteins each viz. λA (core shell), λB (core RdRp), λC (core turret) and μA (core NTPase), μB (outer shell), μNS (NS factory), respectively (Benavente and Martínez-Costas 2007). The S-class segments express four (σC (outer fiber), σA (core clamp), σB (outer clamp), σNS (NS RNAb)) proteins (Benavente and Martinez-Costas 2007). Eight of these proteins are structural and two are non-structural in addition to two additional nonstructural proteins encoded by the first two cistrons of the tricistronic S1 gene, namely p10 (NS-FAST) and p17 (NS other) proteins. Sigma C (σC) protein encoded by the third and largest open reading frame of S1 gene (Benavente and Martínez-Costas, 2007) is a minor component of the outer capsid of the virion and serves as the cell attachment protein having type specific neutralizing epitopes (Wickramasinghe *et al.*, 1993).

Next-generation sequencing (NGS) is a high-throughput sequencing method that generates millions of sequencing reads (Schuster, 2008; Marston et al., 2013) directly from viral RNA thereby offering new perspectives in whole genome sequencing (WGS) studies (Zhu et al., 2013). Recent sequence data suggests that the CARV genome is continuously changing, and all gene segments participates in evolution thereby resulting in the creation of genetic and antigenic variants. Consequently, commercially available CARV vaccines are not able to provide adequate protection against newly emerging and variant field strains leading to outbreaks of disease in vaccinated poultry flocks across the world including the US (Chénier et al., 2014; Tang and Lu, 2015a, 2015b; Lu et al., 2015;

Sellers et al., 2017; Egana et al., 2019). Recent studies based on the phylogenetic analysis of immunogenic σC protein have also indicated the existence of many genotypic variants, which are classified into distinct genotype clusters within CARV (Egana et al., 2019; Ayalew et al., 2020; Carli et al., 2020). Having a segmented genome, segmental reassortment and exchange of genes can introduce genotype and pathotype variations in CARVs (Liu et al., 2003).

The present study was designed to perform NGS of virus isolates from two different states in the US; to analyze the nucleotide (nt) diversity of each virus at the WGS level including phylogenetic incongruences and reassortment events between genome segments from different genotypic clusters. The results of this study will help us understand the pattern and evolutionary mechanism of arthrogenic ARVs and will guide us in the selection of candidate viruses for the development of safe and effective broad-spectrum vaccines.

Materials and Methods

Source of samples. The CARV isolates used in the present study were obtained from routine diagnostic cases of arthritic chickens submitted to the Georgia Poultry Laboratory Network (GPLN) and University of Minnesota Veterinary Diagnostic Laboratory (UMVDL). The background information on these isolates is provided in Table 3.1.

Isolates from Georgia: The viruses were isolated by inoculation of homogenized samples in chicken embryo kidney cells (CEK). The virus-infected cells were incubated at 37°C under 5% CO_2 and examined daily for the development of reovirus specific cytopathic effects i.e., syncytia formation, formation of multinucleated giant cells and

sloughing of monolayer. Once the CPE involved 80-90% of the monolayer (usually within 48 hours of inoculation), the cell cultures were frozen and thawed three times followed by centrifugation at 6000xg for 10 min. The pellet was re-suspended in infected cell culture fluid, subjected to three freeze-thaw cycles with vigorous vortexing followed by centrifugation at 8000xg for 1 minute. The supernatants were aliquoted in small amounts and stored at −80°C until used. A total of 22 CARV strains were isolated from swollen hock joints of chickens affected with viral arthritis. In addition, five chicken enteric reovirus (CERV) strains were isolated from liver, pancreas and small intestine of chicken affected with RSS.

Isolates from Minnesota: The viruses were isolated by inoculation of homogenized tendon samples in embryonated chicken eggs by the yolk sack route. The infected yolk sac material on passage 1 was inoculated in Japanese quail fibrosarcoma cells (QT-35). The virus-infected cells were incubated at 37°C under 5% CO_2 and examined daily for the development of CPE. Immunostaining by fluorescent antibody test (FAT) using an ARV-conjugated antibody (NVSL, Ames, IA, USA) demonstrated that cells infected with these isolated viruses had detectable reovirus specific fluorescent signals. All samples showing positive CPE were harvested as described above. A total of 13 CARV strains were isolated from swollen hock joints of chickens affected with viral arthritis

Illumina sequencing, full-length viral genome assembly and sequence analysis

The supernatant collected from the above two sources were submitted to Molecular development laboratory at UMVDL for whole genome sequencing. The isolated were filtered using 0.45 µm filter and 200 µl of the filtrate was used for RNA extraction using a QIAamp MinElute Virus Spin (QIAGEN, Valencia, CA, USA) following

manufacturer's instructions with a change that linear acrylamide was used instead of carrier RNA. Library preparation from total RNA was done using the SMARTer Stranded Total RNA-Seq – Pico Mammalian Kit v2 (Takara Bio, Mountain View, CA USA) following manufacturer's protocol. Indexed libraries were then normalized and pooled for sequencing. Libraries were sequenced on an Illumina MiSeq 250bp paired-end cycle at University of Minnesota Genomic Centre (UMGC) to obtain raw NGS reads.

Viral genome assembly and Bioinformatics analysis

The raw fastq files obtained from UMGC were analyzed at UMVDL using in-house bioinformatics pipeline. In short, trimming was done to remove Illumina adapters using Trimmomatic (v 0.39, with a minimum quality score of 20 (Bolger et al., 2014). Then, bowtie2 (v 2.4.4) was used to remove host contamination (Langmead et al., 2012) and unmapped reads were used for assembly with SPAdes (v3.15.2) with k-mer values of 21, 31, 41, 51, 61, and 71 and the --careful option (Prjibelski et al., 2020). Extracted contigs were analyzed using BLASTx at NCBI to determine taxonomy. Contigs belonging to *Reoviridae* were subjected to ORFs prediction using Vgas tool with default parameters (Zhang et al., 2019).

Sequence dataset compilation for molecular characterization and whole-genome alignment

Molecular characterization and genotype clustering of our study isolates was determined based on σC gene sequences. For this purpose, a dataset was created of σC gene sequences of all worldwide ARVs available in GenBank (https://www.ncbi.nlm.nih.gov/) (till March 2021) under taxon ID 38170 (Avian orthoreovirus). Reference sequences with ambiguity in information like location and year of isolation were not included in our

analysis. Similarly, segment wise (L1-L3, M1-M3 and S2-S4) dataset was created for maximum likelihood (ML) phylogenetic analysis of each gene segment. Segment wise sequences of this study with respective dataset were aligned with Muscle (Madeira et al., 2016) and visually inspected in AliView (Larsson, 2014), where short sequences were excluded. Pairwise distance analysis of nucleotide sequences was performed on Geneious Prime® 2020.0.5 (Biomatters Ltd., Auckland, New Zealand) (Drummond et al., 2011). The visual analysis of entire genome alignment of the nucleotide (nt) sequences of 35 CARV isolates generated in this study with the reference isolate S1133 was carried out on the mVISTA online platform (http://genome.lbl.gov/vista/mvista/submit.shtml).

Maximum likelihood (ML) phylogenetic and reassortment analysis

The ML phylogenetic trees for each gene segment were created and inferred on IQ-TREE web server (Trifinopoulos et al., 2016) with an inbuilt ModelFinder option. The branch supports were calculated using ultrafast bootstrap approximation (UFBoot) with 1000 replicates (Nguyen et al., 2011). Trees were visualized and edited in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Our isolates were assigned genotypic clusters according to tree topology and the clustering of previously classified reference strains (Kant et al., 2003; Liu et al., 2003; Egana et al., 2019; Ayalew et al., 2020; Carli et al., 2020; Torre et al. 2021). Genetic reassortment events were observed and inferred by analyzing the incongruent topologies of the study isolates among the phylogenetic trees of different gene segments.

Results

Virus isolation: Virus isolation, RT-PCR and histopathological examination (hock joint) samples from affected birds confirmed the presence of reovirus and tenosynovitis caused by CARVs. Reovirus specific cytopathic effects (syncytium formation and detachment of cells) were observed in cell culture for reovirus-positive samples. Embryonated eggs infected with sample homogenates caused mortality, subcutaneous hemorrhages and stunting of the embryo. The presence of CARV in infected cell culture supernatants was further confirmed by FAT and RT-PCR.

Whole-genome sequence comparison and molecular characterization

The comparative whole genome analysis of our 35 isolates showed high nt divergence and genetic heterogeneity among chicken reoviruses circulating in the US. In comparison to the vaccine strain S1133, the study isolates showed 9% to 21% genome wide nt divergence, whereas the study isolates showed approximately 24% genome wide nt divergence among themselves. Based on the mVISTA alignment of concatenated nt sequences of our isolates with the reference vaccine strains S1133, segment wise differences in divergence pattern with the vaccine strain and among themselves were observed (Fig. 3.1). Isolate 12/161 showed >90% nt identity with the vaccine strain S1133 in all the gene segments except highly divergent M2 (66% nt identity) segment and moderately divergent S4 (83% nt identity) segment. Other isolates in the GC1 showed localized divergence in the L3 (27-28%) and M2 (15-26%) segments. The S1 segment (more precisely Sigma C gene) of other isolates in GC1 are highly conserved showing close ancestral relationship with the vaccine strains. Isolates from other GCs

showed segment wide high nt identity in other gene segments except L3 (27-28%) and S1 (21-46%) segments (Fig. 3.1).

Molecular characterization based on Sigma C gene

Our isolates showed marked divergence and were clustered in all six well supported different GCs based on the ML phylogenetic tree of σC gene segment (Fig. 3.2). Eighteen study isolates were clustered in GC1 where only one of the study isolates was clustered with the vaccine strains in GC1.1 while rest (17) of them were clustered in GC1.2. In GC2, one of the study isolates was clustered in GC2.1 and four were clustered in GC2.2. The GC 3 did not form any sub-lusters and three isolates were clustered in it. Two of the study isolates were clustered in GC4 with one isolate in GC4.1 and GC4.2 each. Six of the study isolates were clustered in GC5. The least expressed GC in this study was GC6 clustering only one of the study isolates.

GC1 is subdivided into 3 subclusters but the study isolates were clustered in two GCs. One of the study isolates (CARV/USA/GA/2012/161) was clustered in well supported GC1.1 (100% bootstrap) with vaccine strains (99% nt identity) and 17 in the other well supported GC1.2 (100% bootstrap) with isolates from Canada, Israel and the US having 78%-100% nt identity among themselves and were divergent form the vaccine strain having only 78-79% nt identity. Interestingly, our MN isolates from 2012 (CARV/MN/2012/48558-48562) formed one monophyletic cluster (100 % nt identity) and our MN isolates from 2017 (CARV/MN/2017/15345, CARV/MN/2017/16861, CARV/MN/2017/18850, CARV/MN/2017/20572 and CARV/MN/2017/26946) formed another monophyletic cluster (100 % nt identity). Our GA isolates from 2012 (CARV/GA/2012/111-112) are clustered together (100% nt identity) and have 100% nt

identity with MN isolates from 2012 (CARV/MN/2012/48558-48562). Our GA isolates from 2013 (CARV/GA/2013/016, CARV/GA/2013/022 and CARV/GA/2013/033) have 99-100% nt identity with each other and were clustered with reference sequences from Georgia, California, Missouri and Pennsylvania of the US.

GC2 formed three subclusters clustering five of the study isolates. One of the study sequences was clustered in well supported subcluster GC2.1 (100% bootstrap) with strains from Canada and the US having 59% nt identity with vaccine strain. Rest of the four sequences were clustered in well supported subcluster GC2.2 (100%) with strains from Canada and the US having 81%-100% nt identity with each other, 69-70% nt identity with isolate in GC2.1 and 59-60% nt identity with the vaccine strain.

GC3 is a well-supported cluster (100% bootstrap) having three of our sequences clustered with sequences from Brazil, Canada, Germany, Israel and the US. Our sequences have 85-88% nt identity with each other and 57% nt identity with the vaccine strain.

GC4 formed two well supported subclusters (100% bootstrap) clustering one of our isolates in GC4.1 with sequences from the US and one of our isolates in GC4.2 with sequences from Canada, Germany and Netherlands. The study sequences have 69% nt identity with each other and 54-55% nt identity with the vaccine strain.

GC5 clustered six of our study sequences with sequences from Brazil, Canada, Chile, China and the US having 98-100% nt identity with each other and 54% nt identity with the vaccine strain.

GC6 is the cluster recently evolved in the US, but it was the least expressed cluster in this study clustering only one of our sequences with sequences from Canada and the US in a well-supported cluster (100% bootstrap) having 56% nt identity with the vaccine strain. With **S2 segment** (Fig 3.3A), our sequences were clustered in two clusters and none of our isolates was closely clustered with the vaccine strains. Cluster 1 have 29 of our study sequences with sequences from Canada and the US having 89-100% nt identity among themselves and 90-93% nt identity with the vaccine strain. Isolate CARV/GA/2005/727 had 100% nt identity with isolate CARV/GA/2014/196 and CARV/GA/2014/179 while isolate CARV/GA/2005/815 had 95% nt identity with isolates from GA isolated in 2013 (030, 033, 037 and 042) (04 non-synonymous nt substitutions). Cluster 2 had our six sequences with strains from Brazil, Canada, China, Hungary and the US having 89-100% nt identity among themselves and 91-93% nt identity with vaccine strain. In **S3 segment** (Fig 3.3A), all our study sequences were clustered in one cluster. Three of our sequences were clustered close to vaccine cluster and strains from Canada, China, Russia, Taiwan and the US having 88-95% nt identity with the vaccine strain while one of our sequences (CARV/USA/GA/161) is in the vaccine cluster having 95% nt identity with the vaccine strain. Isolate CARV/GA/2005/727 had 100% nt identity with isolate CARV/GA/2014/196 and CARV/GA/2014/179 while isolate CARV/GA/2005/815 had 98% nt identity with isolates from GA isolated in 2012 (275), 2013 (023, 030, 037 and 042) and 2014 (947) (all synonymous nt substitutions). The **S4 segment** (Fig 3.3A) of our isolates was more divergent clustering in four cluster. Cluster 1 had our 15 sequences with strains from Canada, South Korea and the US having 91-100% nt identity among themselves and 80-82% nt identity with the vaccine strain. Cluster 2 had our 16

sequences with strains from Canada, Hungary and the US having 90-100% nt identity among themselves and 79-81% nt identity with the vaccine strain. Isolate CARV/GA/2005/727 had 100% nt identity with isolate CARV/GA/2014/196 and CARV/GA/2014/179 while isolate CARV/GA/2005/815 had 95% nt identity with CARV/GA/2013/030 with 52/65 non-synonymous. Three of our sequences (cluster 5) were clustered near the vaccine cluster having 94-100% nt identity among themselves and 81-82% nt identity with the vaccine strain. In all the S class segments, turkey reovirus sequences formed a separate cluster and another separate cluster was formed by ducks and goose reovirus (Fig. 3.3A).

M class gene segments: In ML tree of **M1 segment** (Fig 3.3B), sequences formed two clusters where all of our sequences were clustered in cluster 1 formed by sequences from Canada, Taiwan and the US. Our sequences in cluster 1 had 79-100% nt identity with each other and 81-100% nt identity with the vaccine strain. Isolate CARV/GA/2005/727 had 100% nt identity with isolate CARV/GA/2014/196 and CARV/GA/2014/179 while isolate CARV/GA/2005/815 had 93% nt identity with isolates CARV/GA/2005/727, CARV/GA/2014/196 and CARV/GA/2014/179 having 162 nt substitutions of which majority (146) are synonymous substitutions. With the **M2 segment** (Fig 3.3B), our sequences were clustered into three well supported clusters (cluster 2, 3 and 5) and were very divergent from each other. None of our study sequences were clustered in the vaccine cluster. Cluster 2 had clustered our 18 sequences with strains from Canada, China and the US having 90-100% nt identity among themselves and 83-85% nt identity with the vaccine strain. Isolate CARV/GA/2005/727 had 100% nt identity with isolate CARV/GA/2014/196 and CARV/GA/2014/179 while isolate CARV/GA/2005/815 was

very divergent (77% nt identity) and was clustered in cluster 3. Cluster 3 have clustered our 15 sequences with chicken reovirus strains from Canada, China, South Korea and the US and turkey reovirus sequences from Hungary and the US and have 80-100% nt identity among themselves and 74-75% nt identity with the vaccine strain. The two big clusters (cluster 2 and 3) have 74-78% nt identity with each other. Interestingly, one of our isolates (CARV/USA/GA/161) was very divergent and was clustered in a well-supported cluster with strains from Hungary and the US and have 66-67% nt identity with our other sequences and 66% nt identity with the vaccine strain. The **M3 segment** (Fig 3.3B) was more conserved where our sequences were clustered in two clusters. Twenty-four of our study sequences were clustered in a cluster 2 with strains from Canada, China, Hungary and the US having 98-100% nt identity among themselves and 81-82% nt identity with the vaccine strain. Isolate CARV/GA/2005/727 had 100% nt identity with isolate CARV/GA/2014/196 and CARV/GA/2014/179 while isolate CARV/GA/2005/815 was very divergent (80% nt identity) and was clustered in cluster 1. Eleven of our study sequences were clustered close to the vaccine cluster having 85-100% nt identity with each other and 87-100% nt identity with the vaccine strain. One of our isolates (CARV/USA/GA/161) is clustered closely with the vaccine cluster having 100% nt identity with the vaccine strain. Reoviruses from turkeys and ducks formed one separate cluster each in M segment except M2 segment where more than one clusters of turkey and duck reoviruses were observed (Fig. 3.3B).

L class gene segments: Based on the ML phylogenetic tree of **L1 segment** (Fig 3.3C), our sequences were clustered in four well supported clusters having isolates from Canada, Hungary and the US. Only one of our sequences (CARV/USA/GA/161) in

cluster 1 was clustered with the vaccine cluster having 100% nt identity with vaccine strain. Cluster 1 formed a monophyletic subcluster with isolates from 2012 (CARV/MN/2012/48558-48562, CARV/GA/2012/111, 112 and 211) having 100% nt identity with each other and 91-92% nt identity with the vaccine sequences. Cluster 2 clustered 18 sequences having 89-100% nt identity among themselves and 88-89% nt identity with the vaccine strain. Isolate CARV/GA/2005/727 had 100% nt identity with isolate CARV/GA/2014/196 while isolate CARV/GA/2005/815 had 94.7% nt identity with isolate CARV/GA/2014/179 with 207 nt substitutions of which majority are non-synonymous substitutions. Two of the study isolates were clustered in cluster 3 (94% nt identity) with sequences from Canada, China and Taiwan. One of the study isolates (CARV/GA/2015/010) is very divergent having 78-79% nt identity with rest of the study isolates and 78% nt identity with the vaccine strain. The ML tree of **L2 segment** (Fig 3.3C) has all our sequences clustered in two well supported clusters. Cluster 1 have our 16 sequences with sequences from Canada, China, Hungary, South Korea and the US having 86%-100% nt identity with each other and 89-99% nt identity with the vaccine strain. One of our sequences (CARV/USA/GA/161) was closely related to the vaccine sequence having 99% nt identity. Cluster 2 clustered our 19 sequences with strains from China, Canada, Hungary and the US having 87-100% nt identity with each other and 83-84% nt identity with the vaccine strains. Isolate CARV/GA/2005/727 had 100% nt identity with isolate CARV/GA/2014/196 and CARV/GA/2014/179 while isolate CARV/GA/2005/815 had 93% nt identity with isolates CARV/GA/2005/727, CARV/GA/2014/196 and CARV/GA/2014/179 with 277 nt substitutions of which majority are non-synonymous substitutions. The L2 segment of our sequences was quite

conserved with >83% nt identity among themselves. The ML tree of **L3 segment** (Fig 3.3C) of our sequences suggest more convergence clustering 34 of our sequences in one cluster with sequences from Canada and the US having 88-100% nt identity with each other. Isolate CARV/GA/2005/727 had 100% nt identity with isolate CARV/GA/2014/196 and CARV/GA/2014/179 while isolate CARV/GA/2005/815 had 97% nt identity with isolates CARV/GA/2005/727, CARV/GA/2014/196 and CARV/GA/2014/179 with 126 nt substitutions of which majority (111) are synonymous substitutions. One of our sequences (CARV/USA/GA/161) were clustered with the vaccine clusters with 99% nt identity with vaccine strain. In all the L class segments, turkey reovirus sequences formed a separate cluster and another separate cluster was formed by ducks and goose reovirus (Fig. 3.3C).

Reassortment analysis based on phylogenetic incongruence

The study sequences were aligned with the vaccine strain S1133 by the mVISTA method which showed that our isolates are divergent form the vaccine strain on segmental basis (Fig 3.1). Segments L1, L2, M1, M3, S2 and S3 are more conserved and have higher nt identity with vaccine strain while L3, M2, S1 and S4 segments are more divergent from the vaccine strain. This pattern of segmental divergence was followed across the different GCs indicating segmental reassortment events. To understand this phenomenon, we analyzed the segment wise ML phylogenetic trees for clustering and incongruent topologies of our isolates (Fig. 3.4). Isolate CARV/USA/GA/2012/211 is always clustered in the same cluster except M1, M2, S1, S2 and S3 segment. It was clustered separately with CARV/USA/GA/2012/112 in M2 segment and with CARV/USA/GA/2012/111 in S1 segment. All three isolates viz.

CARV/USA/GA/2012/111, CARV/USA/GA/2012/112 and CARV/USA/GA/2012/211 are clustered together in the S2 segment separately from their original cluster. Isolate CARV/USA/GA/2012/161 is always clustered with the vaccine strain except in M2 and S2 segments. Isolate CARV/USA/GA/2014/179 is always clustered with CARV/USA/GA/2005/727 and CARV/USA/GA/2014/196 except in the L1 segments. Isolates CARV/USA/GA/2013/030 and CARV/USA/GA/2012/355 are clustered together only in L1, L3 and S4 segments. Isolates that are color shaded maintained the phylogenetic congruent clusters in different gene segments (Fig. 3.4). Isolates CARV/USA/GA/2013/023 and CARV/USA/GA/2012/355 have congruent topologies in L2, M2, S1, S2 and S3 segments whereas CARV/USA/GA/2013/023 was clustered with CARV/USA/MN/2020/013669 in L1 and S1 segments. Isolates CARV/USA/MN/2017/18850 and CARV/USA/MN/2017/20572 are reassortants based on their topologies in L2 and M3 segments.

The heat map (Supplementary table 1) based on distance analysis shows that most of our isolates had highest % nt identity with isolates within the same cluster (WIC) having some exceptions with higher in between cluster (IBC) nt identity in each GC. All the isolates in GC1 have highest % nt identity with other isolates from GC1 except 2012/161 (highest WIC % nt identity only in L1, L2, L3 and M1 segments), 2013-033 (Highest % nt identity in L1, L2 L3, M1, M2 and S3 segments), 2005/815 (highest WIC % nt identity in L3 and S1 segments), 2015/031 (highest IBC % nt identity in M1, M2, S3 and S4), 2017/18850 (highest IBC % nt identity in L1) and 2017/20572 (highest IBC % nt identity in S3). The GC2 showed more varied pattern in highest WIC and IBC % nt identity, where highest WIC % nt identity was observed in 20/013669 (all segments

except L2 and S3), 2012/355 (all segments except L1, M3, S3 and S4), 2013/023 (all segments except L3, M1, M3 and S3), 2013/033 (S1 and S3 segments) and 2013/037 and 2013/042 (all segments except M2). Isolates in GC3, highest IBC % nt identity was observed in all the segments of 2012/148, 2014/947 and 2015/010 (except S1 segments). In GC4, isolates 2012/275 and 2011/40107 have highest IBC % nt identity in all the segments except S1 segment, All the isolates in GC5 have highest WIC % nt identity in all the gene segments with few exceptions viz. 2013/218 has highest WIC % nt identity only in M2 M3 and S1 segments; 2012/211 had highest IBC % nt identity in all segments except M1, S1and S2 segment; 2014/179 has highest IBC % nt identity in L1 and L2 segments; 2017/12859 has highest WIC % nt identity in S1 segment only. Isolates 2012/48558-62 in GC1 showed 100 % IBC nt identity mostly with isolates 2012/211 in GC5 (in L1 and L2 segments) and vice versa in L1, L2 and L3 segments. Isolate 2012/275 in GC4 had >95% IBC nt identity in all the segments except L2, S1, S2 and S4 segments. Isolate 2013/030 in GC6 had >95% nt identity with isolates from other GCs (IBC) in all the segments except M1 and S1 segments.

Discussion

Emerging CARV variants have resulted into increased incidences of reovirus associate arthritis/tenosynovitis outbreaks causing huge economic losses to the poultry industry worldwide (Troxler et al., 2013; Lu et al., 2015; Sellers, 2017; Souza et al., 2018; Chen et al., 2019; Ayalew et al., 2020). Vaccination and biosecurity measures were adopted to curb this infection, but everything looks inefficient due to emergence of pathogenic variants (Sellers, 2017; Palomino-Tapia et al. 2018; Egaña-Labrin et al. 2019; Zhang et

al. 2019). High rate of mutations in reoviral genome due to lack of efficient exonuclease proofreading activity of RNA polymerase in RNA viruses (Steinhauer and Holland, 1987; Domingo, 1997) giving them inherent property of causing genetic variations (Lu, et al., 2015; Sellers, 2017; Ayalew et al., 2017) and reassortment of viral gene segments are responsible for formation of variant CARVs (Tang et al. 2015; Farkas et al., 2016; McDonald et al. 2016; Ayalew et al. 2020). In lieu of the above facts, the present study focused on studying and characterizing evolutionary divergence in variant CARVs from the US.

Cell culture grown virus isolates (P2-P3) were submitted for WGS by illumina sequencing. Multiple passage of RNA viruses in cell culture causes genetic changes in the viral genome (Ghetas et al., 2015). Considering this fact, we have selected the P2/P3 of most of our isolates for NGS, eliminating or minimizing the possibility of cell culture passage induced genetic changes in the viral genome.

Sigma C (σC), σB and μB are the highly variable genes in the CARV genome (Su et al. 2006) where σC is the most variable and most studied gene for genotypic classification and evolution of avian reoviruses (Ayalew et al., 2020; Carli et al., 2020). Based on σC gene, CARVs have been classified into well-established genotype clusters (GCs) (Schnitzer, 1985; Kant et al., 2003; Guardado et al., 2005). Based on the ML phylogenetic tree of σC gene sequences of the dataset used in this study, the US isolates from this study were clustered in all established six well supported genotype clusters GC1 to GC6) (Egãna-Labrin et al., 2019; Ayalew et al., 2020). Virus isolates from the outbreaks of tenosynovitis/arthritis in the US and Canada are from all six GCs (Lu et al. 2015; Ayalew et al. 2017; Palomino-Tapia et al. 2018; Egãna-Labrin et al. 2019; Ayalew

et al., 2020). Although, variant CARVs are highly divergent but the degree of pathogenicity is not related to the genetic divergence (Ayalew et al., 2020). On closer examination of each GC, GC1 is further subdivided into three subclusters viz. GC1.1 (vaccine subcluster), GC1.2 and GC1.3. Similarly, GC2 (GC2.1, GC2.2 and GC2.3), GC4 (GC4.1, GC4.2 and GC4.3), and GC6 (GC6.1 and GC6.2) formed 3, 3, and 2 subclusters, respectively. Our isolates were clustered in all six well established GCs. However, the concatenated whole-genome sequences created a different phylogenetic clustering pattern signifying a phylogenetically incongruent isolate topology. The most expressed GC in our study was GC1 followed by GC5 and GC2 clustering 18, 06 and 05 of our isolates, respectively. In previous studies, the most expressed GC were GC1 and GC6 (Egãna-Labirin et al., 2019) and GC4 and GC5 (Palomino-Tapia et al, 2018). The GC3, GC4 and GC6 were the least expressed clusters in this study clustering three, two and one of our isolates in each, respectively. Similar observations were reported by Palomino-Tapia et al., (2018) but Egãna-Labirin et al., (2019) reported different observations. The predominance of GCs varies with time and geographical location as reported previously (Lu et al. 2015; Ayalew et al. 2017; Sellers, 2017; Palomino-Tapia et al. 2018). A shift in the predominance of GCs from GC1 to GC6 with time is reported by Egãna et al., (2019) although we did not observe such shift. Thorough genetic studies are desired to explore the predominant GCs for considering the strains or their antigenic segments to include into the autogenous or vectored vaccines. Autogenous vaccines are being used by some commercial produces which may be one of the contributing factors influencing this shift. Autogenous vaccines use strains from the predominant GCs in the

respective area which may derive the change in the representation of variant GCs leading to vaccination failure (Sellers, 2017).

Only one of our isolates was clustered in the vaccine subcluster (GC1.1) having 99% nt identity with the vaccine strains but the rest of the 17 isolates were clustered distantly in the GC1.2 and are highly divergent form the vaccine strain. Similarly, our isolates clustered in GC2 to GC6 were very divergent from the vaccine strain and have high in between cluster nt divergence. The high divergence of our isolates from the vaccine strain explains the reason for vaccine failure as reported previously (Lu et al. 2015; Ayalew et al. 2017; Palomino-Tapia et al. 2018; Egãna-Labrin et al. 2019; Ayalew et al., 2020). The high within cluster nt divergence of our isolates among themselves suggests that autogenous vaccines may not protect against the virus challenge from the same GC. Although GC1 has higher nt identity with the vaccine strains than the other GCs but are still up to 22% divergent from the vaccine strains as reported by Zhang et al. (2019). Similarly, 50% genetic diversity was reported in chicken reovirus from Israel (Goldenberg et al., 2010) and turkey reovirus from the US (Day et al., 2007). Genetic diversity explains the accelerated evolution and appearance of new GCs in reovirus. Palomino et al. (2018) reported low cross protection by commercial or autogenous vaccines prepared from prevalent field strains from the same cluster having low identity. Studies on other RNA viruses have reported low cross protection when the amino acid difference between the challenge and vaccine viruses exceeds 5% (Cavanagh, 2007).

Structure modelling of σC protein and antigenicity prediction studies by Ayalew et al., (2017) and Torre et al., (2021) have reported a difference in the secondary structure of the predicted antigenic epitopes between viruses in different GCs because of amino

acid substitutions. Multiple variant strains are reported to cocirculate at the same time, so CARV induced arthritis/tenosynovitis cannot be controlled by using strains from one or two GCs (Perelman et al., 2019). Therefore, objective and WGS based characterization of CARV variants should be considered for selection of candidate viruses in designing effective vaccine and mitigation strategies. Till date, 07 whole genome sequences from California (Egaña-Labrin et al. 2019), 02 from Pennsylvania (Tang et al. 2015) and 12 from Canada (Ayalew et al., 2020) are only available for reference. Here, this study contributes 35 whole genomes of CARVs isolated from two different states (Georgia and Minnesota) of the US between 2005 to 2020 which will make the dataset more robust for future WGS studies. We focused on evaluation of divergence of each gene segment from the vaccine strain S1133 on the whole genome sequence basis. Also, the contribution of each gene segment and the possible reassortment of gene segments was evaluated. Based on our WGS results, L3, M2 and the S1 segments were most variable and showed maximum divergence from the vaccine strains. Our observations corraborate the observations reported by Egana et al., (2019) but slightly different from the observation of Ayalew et al., (2020) where M3 segment was more divergent than M2 segment in addition to the S1 segment. The possible reason for the divergence of L3, M2 and S1segments is their translated proteins that are the component of the outer capsid which face the maximum selection pressure and pressure from the host immune system. Additionally, the genetic divergence of these segments can be correlated with the potential role they play in the antigenic variability and pathogenicity (Sellers, 2017) of the variant reoviruses which need to be further studied to establish this relationship. Additionally, the S4 segment showed moderate divergence from the vaccine strain

suggesting its possible role in divergence of CARV. The S4 gene segment translates σNS protein which is a nonstructural RNA-binding protein that accumulates in viral factories of avian reovirus-infected cells playing key roles in RNA packaging and replication (Benavente and Martínez-Costas, 2007). The clustering of our sequences with strains from different countries in phylogenetic trees of different gene segments suggest widespread global intermixing of variant CARVs due to trade of live birds, poultry products, migratory birds or by other means. Since ARVs are resistant to common disinfectants and can survive in the environment for longer periods (Mor et al.,2015), might be helping in cross country introductions. Similar geographical mixing between Canada and the US strains was reported by Ayalew et al., (2020).

Our enteric isolates from 2005 were having high nt identity with the arthrotropic isolates from 2012 to 2014. One of the enteric isolate CARV/GA/2005/727 was highly similar and was always clustered with CARV/GA/2014/179 and CARV/GA/2014/196. This nt identity pattern between pre 2011 and post 2011 isolates may be because of reversion/mutation of arthrotropic reovirus strains circulating in the US between 2012-14 back to their previous enteric strains of 2005 but maintaining their arthrotropic tendency.

Another important mechanism of evolution and divergence of segmented RNA viruses is reassortment of genome segments (McDonald et al., 2016; Ayalew et al. 2020). We observed extensive reassortment events in this study from the incongruent topologies in phylogenetic trees of different gene segments which is further supported by the highest segmental nucleotide identity between isolates of different GCs (Supplementary table 1)). Our results strongly support that segmental reassortment events play a key role in the rapid evolution and genetic diversity of CARVs. Also, segments other than σC gene also

contribute to the evolution, hence should be given due attention and studied extensively. Our contribution of 35 WGS to the GenBank will help future investigations in the whole genome characterization of ARVs. Co-circulation of multiple CARV variants (from different GCs) in the same farm at the same time may be responsible for the re-assortment. Our results suggest that isolates 2017/18850 and 2017/20572 are reassortants based on their incongruent topologies in L2 and L3 segments. Therefore, there is high probability that evolution through exchange of gene segments of viruses from different GCs is an important phenomenon in avian reoviruses. However, no reassortment events were found by Egana-Labirin et al., (2019) may be because six of the seven viral genomes analyzed were from the same GC which would miss important information from other GCs. Complex phylogenetic patterns observed in this study indicate a mutual beneficial relationship among divergent CARVs where timely exchange of gene segments provides the needed variability to overcome the vaccination and host immunity and hence these variant viruses continue to circulate in poultry flocks. Additionally, our isolates in GC1 showed most phylogenetic congruence suggesting limited segmental exchange. This is probably due to the reason that the GC1 is the vaccine cluster, and all the segments have high nt identity with the respective segments of the vaccine strain and possibly face least vaccination pressure or the evolutionary pressure exerted by the host immune response. Also, reassortment may be beneficial as it may increase the survivability and capacity of virus to replicate in different host (McDonald et a., 2016).

In conclusion, this study addresses the molecular characterization, divergence and evolutionary pattern of CARVs circulating in the US. Our results give new insights into the contribution of different gene segments in the evolution and formation of variant

CARVs in addition to Sigma C gene segment. Previous studies have classified CARVs into different lineages based on the Sigma C gene, but other segments did not follow the identical pattern suggesting that other gene segments evolve in an independent manner. Additionally, this study will also guide researchers to consider other immunogenic gene segments to include in the future sub-unit vaccines to be developed and formulating control strategies for future outbreaks.

Acknowledgments: This study was supported by a grant from Binational Agricultural Research Fund (BARD), Netaji Subhash Indian Council of Agricultural Research international fellowship and Pomeroy Legacy scholarship from the University of Minnesota.

Table 3.1. List of reovirus isolates from the US used in this study

S. No	Case id	State	Year of isolation	Sample	cells (Passage)	Disease	GC
1	727	GA	2005	SI[a]	CEF (P3)	RSS[c]	5
2	815	GA	2005	SI[a]	CEF (P2)	RSS	1.2
3	111	GA[d]	2012	Tendon	CEF (P3)	VA[b]	1.2
4	112	GA	2012	Tendon	CEF (P2)	VA	1.2
5	148	GA	2012	Tendon	CEF (P1)	VA	3
6	161	GA	2012	Liver	CEF (P3)	RSS	1.1
7	211	GA	2012	Tendon	CEF (P2)	VA	5
8	275	GA	2012	SI	CEF (P2)	RSS	4.1
9	355	GA	2012	Pancreas	CEF (P2)	RSS	2.3
10	16	GA	2013	Tendon	CEF (P2)	VA	1.2
11	22	GA	2013	Tendon	CEF (P2)	VA	1.2
12	23	GA	2013	Tendon	CEF (P2)	VA	2.3
13	30	GA	2013	Tendon	CEF (P2)	VA	6
14	33	GA	2013	Tendon	CEF (P2)	VA	1.2
15	37	GA	2013	Tendon	CEF (P2)	VA	2.3
16	42	GA	2013	Tendon	CEF (P2)	VA	2.3
17	218	GA	2013	Tendon	CEF (P2)	VA	5
18	179	GA	2014	Tendon	CEF (P2)	VA	5
19	196	GA	2014	Tendon	CEF (P2)	VA	5
20	31	GA	2015	Tendon	CEF (P2)	VA	1.2
21	10	GA	2015	Tendon	CEF (P2)	VA	3
22	947	GA	2014	Tendon	CEF (P2)	VA	3
23	40107	MN[e]	2011	Tendon	QT-35 (P2)	VA	4.2
24	48558	MN	2012	Tendon	QT-35 (P2)	VA	1.2
25	48559	MN	2012	Tendon	QT-35 (P2)	VA	1.2
26	48560	MN	2012	Tendon	QT-35 (P2)	VA	1.2

27	48561	MN	2012	Tendon	QT-35 (P2)	VA	1.2
28	48562	MN	2012	Tendon	QT-35 (P2)	VA	1.2
29	12859	MN	2017	Tendon	QT-35 (P2)	VA	5
30	15345	MN	2017	Tendon	QT-35 (P2)	VA	1.2
31	16861	MN	2017	Tendon	QT-35 (P2)	VA	1.2
32	18850	MN	2017	Tendon	QT-35 (P2)	VA	1.2
33	20572	MN	2017	Tendon	QT-35 (P2)	VA	1.2
34	26946	MN	2017	Tendon	QT-35 (P2)	VA	1.2
35	13669	MN	2020	Tendon	QT-35 (P2)	VA	2.1

[a] small intestine, [b] viral arthritis, [c] runting stunting syndrome, [d] Gerogia, [e] Minnesota

Fig 3.1. Results of the mVISTA analysis of the concatenated genome sequences of the 35 studied US avian orthoreovirus strains in comparison to the reference vaccine strain S1133. Areas shaded with pink and white indicate >90%, and <90% nt sequence identities, respectively. Scale bar: The approximate length of each gene.

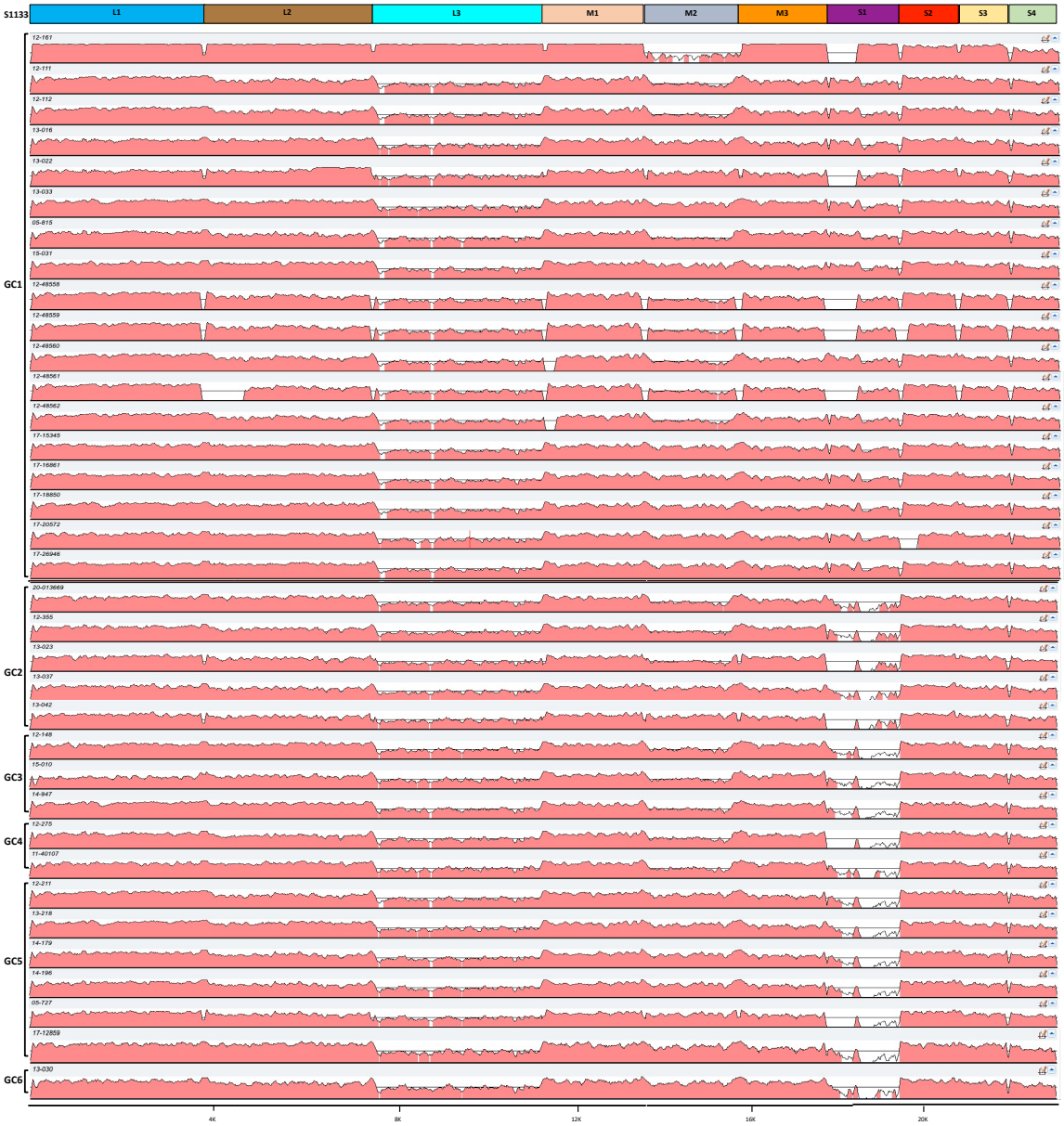


Fig 3.2. Maximum Likelihood (ML) phylogenetic tree of 690 ARV strains based on the σC sequence variability. Color codes of branches represent genotype cluster classification (GC1-Red; GC2-Green; GC3-Pink; GC4-Blue; GC5-Brown; GC6-Orange) and sub-clusters are identified by the name.

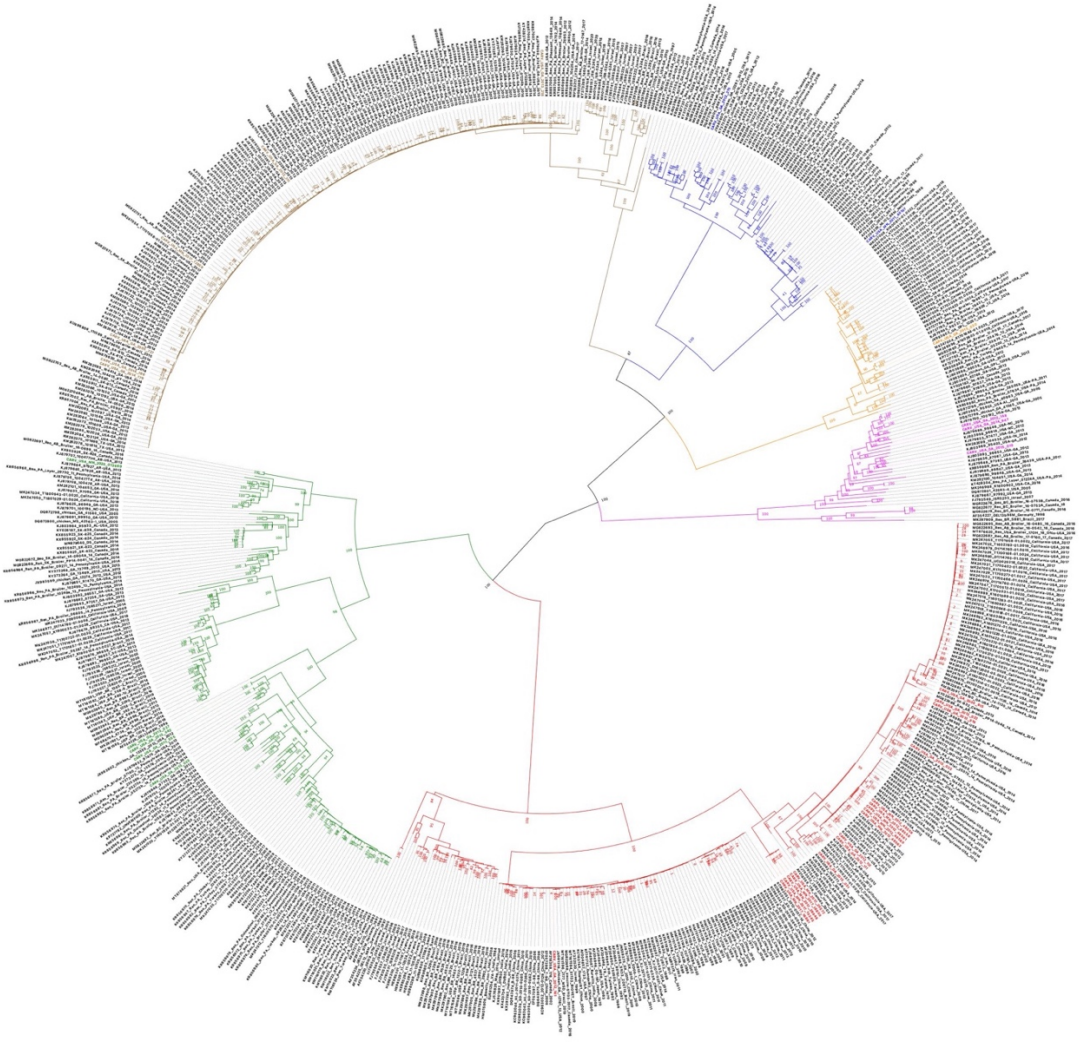
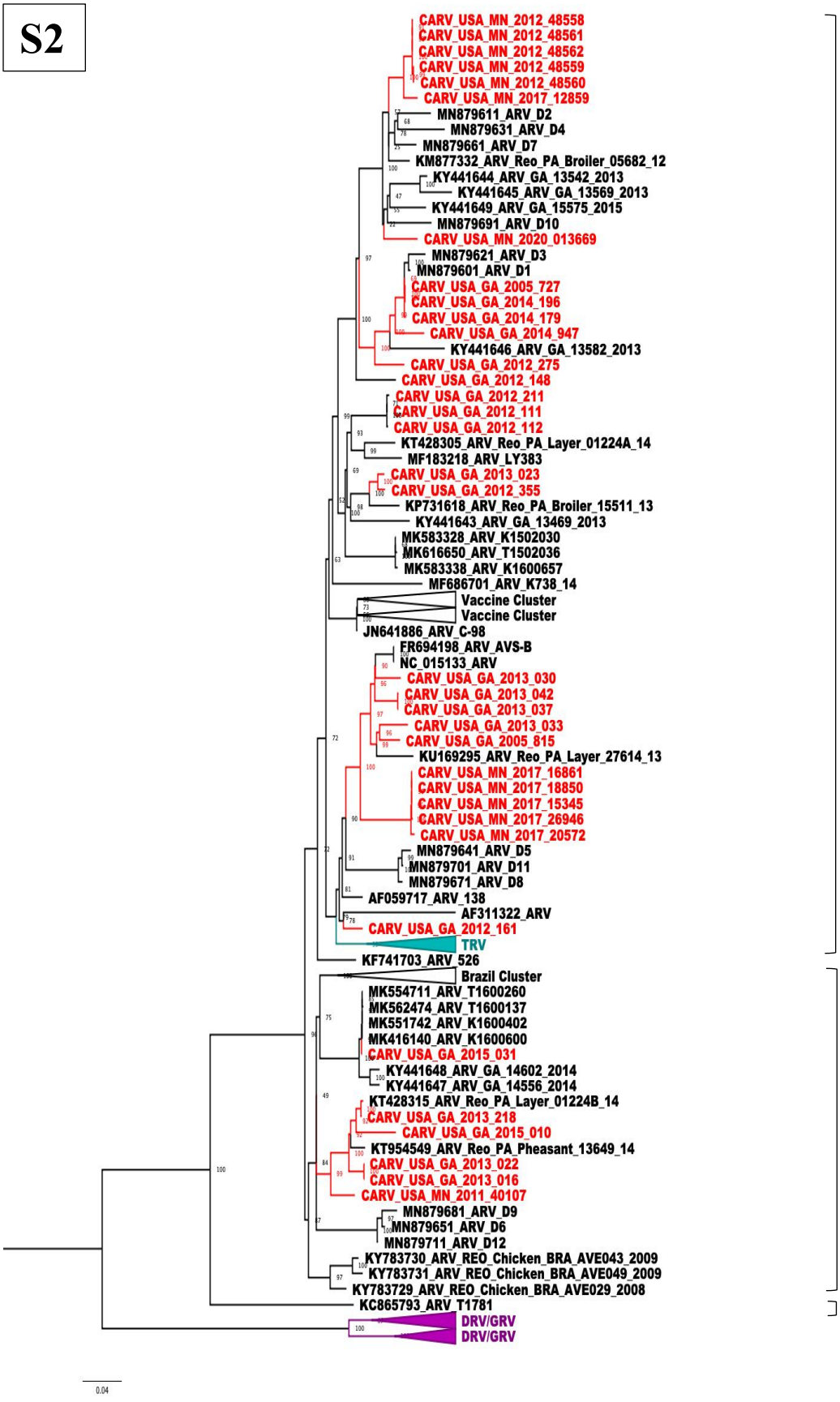
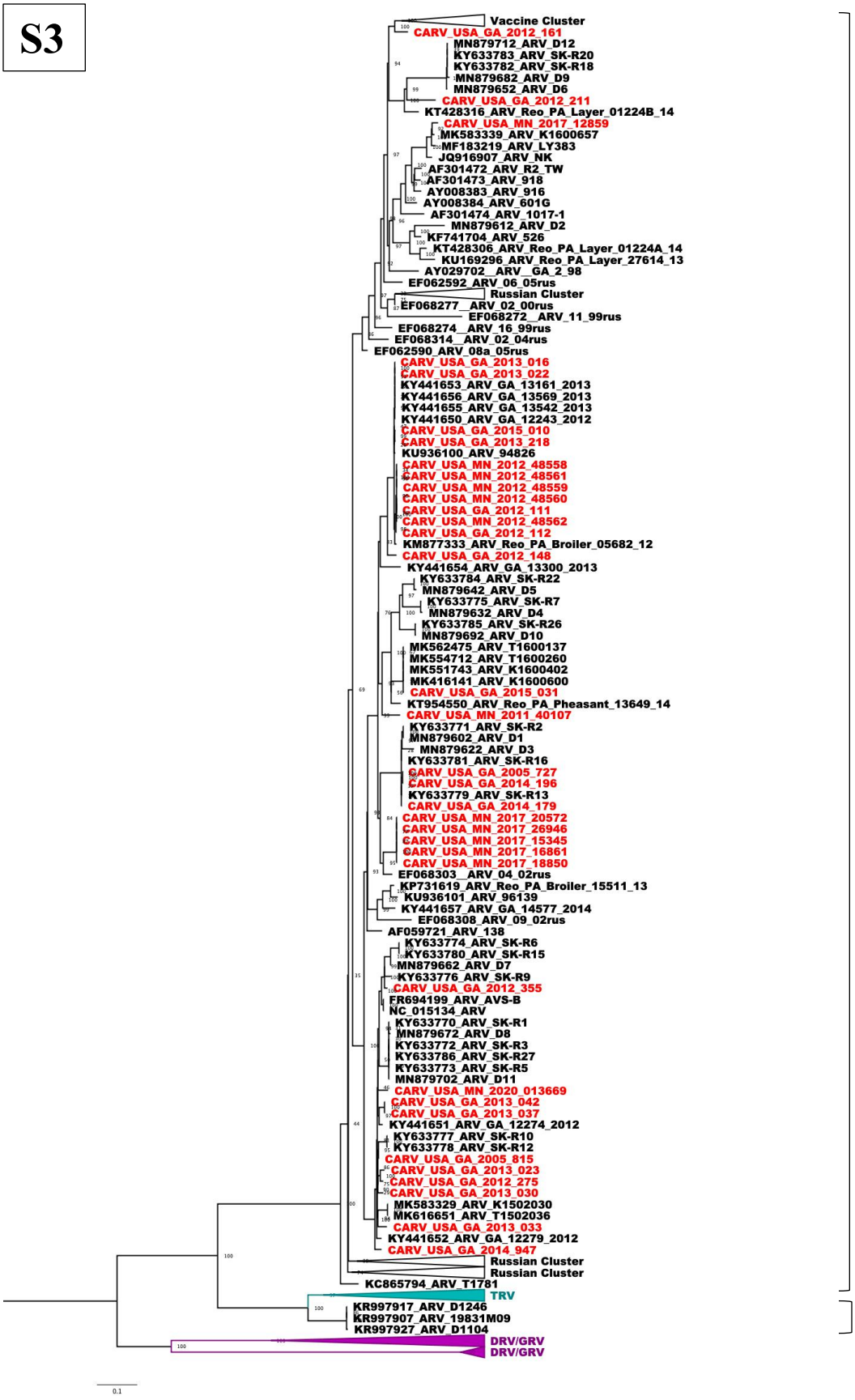


Fig 3.3A. Nucleotide sequence-based ML phylogenetic trees showing the clustering of CARVs based on the S2, S3 and S4 gene segments of viruses available from GenBank. Phylogenetic calculations were carried out using the maximum-likelihood method applying the best-fit models calculated for each gene. The scale bar is proportional to the genetic distance.

S2


CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48561
CARV_USA_MN_2012_48562
CARV_USA_MN_2012_48559
CARV_USA_MN_2012_48560
CARV_USA_MN_2017_12859
MN879611_ARV_D2
MN879631_ARV_D4
MN879661_ARV_D7
KM877332_ARV_Reo_PA_Broiler_05682_12
KY441644_ARV_GA_13542_2013
KY441645_ARV_GA_13569_2013
KY441649_ARV_GA_15575_2015
MN879691_ARV_D10
CARV_USA_MN_2020_013669
MN879621_ARV_D3
MN879601_ARV_D1
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
CARV_USA_GA_2014_179
CARV_USA_GA_2014_947
KY441646_ARV_GA_13582_2013
CARV_USA_GA_2012_275
CARV_USA_GA_2012_148
CARV_USA_GA_2012_211
CARV_USA_GA_2012_111
CARV_USA_GA_2012_112
KT428305_ARV_Reo_PA_Layer_01224A_14
MF183218_ARV_LY383
CARV_USA_GA_2013_023
CARV_USA_GA_2012_355
KP731618_ARV_Reo_PA_Broiler_15511_13
KY441643_ARV_GA_13469_2013
MK583328_ARV_K1502030
MK616650_ARV_T1502036
MK583338_ARV_K1600657
MF686701_ARV_K738_14
Vaccine Cluster
Vaccine Cluster
JN641886_ARV_C-98
FR694198_ARV_AVS-B
NC_015133_ARV
CARV_USA_GA_2013_030
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
CARV_USA_GA_2013_033
CARV_USA_GA_2005_815
KU169295_ARV_Reo_PA_Layer_27614_13
CARV_USA_MN_2017_16861
CARV_USA_MN_2017_18850
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_20572
MN879641_ARV_D5
MN879701_ARV_D11
MN879671_ARV_D8
AF059717_ARV_138
AF311322_ARV
CARV_USA_GA_2012_161
TRV
KF741703_ARV_526
Brazil Cluster
MK554711_ARV_T1600260
MK562474_ARV_T1600137
MK551742_ARV_K1600402
MK416140_ARV_K1600600
CARV_USA_GA_2015_031
KY441648_ARV_GA_14602_2014
KY441647_ARV_GA_14556_2014
KT428315_ARV_Reo_PA_Layer_01224B_14
CARV_USA_GA_2013_218
CARV_USA_GA_2015_010
KT954549_ARV_Reo_PA_Pheasant_13649_14
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
CARV_USA_MN_2011_40107
MN879681_ARV_D9
MN879651_ARV_D6
MN879711_ARV_D12
KY783730_ARV_REO_Chicken_BRA_AVE043_2009
KY783731_ARV_REO_Chicken_BRA_AVE049_2009
KY783729_ARV_REO_Chicken_BRA_AVE029_2008
KC865793_ARV_T1781
DRV/GRV
DRV/GRV
0.04
Cluster 1
Cluster 2
Cluster 3

S3


Vaccine Cluster
CARV_USA_GA_2012_161
MN879712_ARV_D12
KY633783_ARV_SK-R20
KY633782_ARV_SK-R18
MN879682_ARV_D9
MN879652_ARV_D6
CARV_USA_GA_2012_211
KT428316_ARV_Reo_PA_Layer_01224B_14
CARV_USA_MN_2017_12859
MK583339_ARV_K1600657
MF183219_ARV_LY383
JQ916907_ARV_NK
AF301472_ARV_R2_TW
AF301473_ARV_918
AY008383_ARV_916
AY008384_ARV_601G
AF301474_ARV_1017-1
MN879612_ARV_D2
KF741704_ARV_526
KT428306_ARV_Reo_PA_Layer_01224A_14
KU169296_ARV_Reo_PA_Layer_27614_13
AY029702__ARV__GA_2_98
EF062592_ARV_06_05rus
Russian Cluster
EF068277__ARV_02_00rus
EF068272__ARV_11_99rus
EF068274__ARV_16_99rus
EF068314__ARV_02_04rus
EF062590_ARV_08a_05rus
CARV_USA_GA_2013_016
CARV_USA_GA_2013_022
KY441653_ARV_GA_13161_2013
KY441656_ARV_GA_13569_2013
KY441655_ARV_GA_13542_2013
KY441650_ARV_GA_12243_2012
CARV_USA_GA_2015_010
CARV_USA_GA_2013_218
KU936100_ARV_94826
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48561
CARV_USA_MN_2012_48559
CARV_USA_MN_2012_48560
CARV_USA_GA_2012_111
CARV_USA_MN_2012_48562
CARV_USA_GA_2012_112
KM877333_ARV_Reo_PA_Broiler_05682_12
CARV_USA_GA_2012_148
KY441654_ARV_GA_13300_2013
KY633784_ARV_SK-R22
MN879642_ARV_D5
KY633775_ARV_SK-R7
MN879632_ARV_D4
KY633785_ARV_SK-R26
MN879692_ARV_D10
MK562475_ARV_T1600137
MK554712_ARV_T1600260
MK551743_ARV_K1600402
MK416141_ARV_K1600600
CARV_USA_GA_2015_031
KT954550_ARV_Reo_PA_Pheasant_13649_14
CARV_USA_MN_2011_40107
KY633771_ARV_SK-R2
MN879602_ARV_D1
MN879622_ARV_D3
KY633781_ARV_SK-R16
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
KY633779_ARV_SK-R13
CARV_USA_GA_2014_179
CARV_USA_MN_2017_20572
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_16861
CARV_USA_MN_2017_18850
EF068303__ARV_04_02rus
KP731619_ARV_Reo_PA_Broiler_15511_13
KU936101_ARV_96139
KY441657_ARV_GA_14577_2014
EF068308_ARV_09_02rus
AF059721_ARV_138
KY633774_ARV_SK-R6
KY633780_ARV_SK-R15
MN879662_ARV_D7
KY633776_ARV_SK-R9
CARV_USA_GA_2012_355
FR694199_ARV_AVS-B
NC_015134_ARV
KY633770_ARV_SK-R1
MN879672_ARV_D8
KY633772_ARV_SK-R3
KY633786_ARV_SK-R27
KY633773_ARV_SK-R5
MN879702_ARV_D11
CARV_USA_MN_2020_013669
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
KY441651_ARV_GA_12274_2012
KY633777_ARV_SK-R10
KY633778_ARV_SK-R12
CARV_USA_GA_2005_815
CARV_USA_GA_2013_023
CARV_USA_GA_2012_275
CARV_USA_GA_2013_030
MK583329_ARV_K1502030
MK616651_ARV_T1502036
CARV_USA_GA_2013_033
KY441652_ARV_GA_12279_2012
CARV_USA_GA_2014_947
Russian Cluster
Russian Cluster
KC865794_ARV_T1781
TRV
KR997917_ARV_D1246
KR997907_ARV_19831M09
KR997927_ARV_D1104
DRV/GRV
DRV/GRV
0.1
Cluster 1
Cluster 2

S4

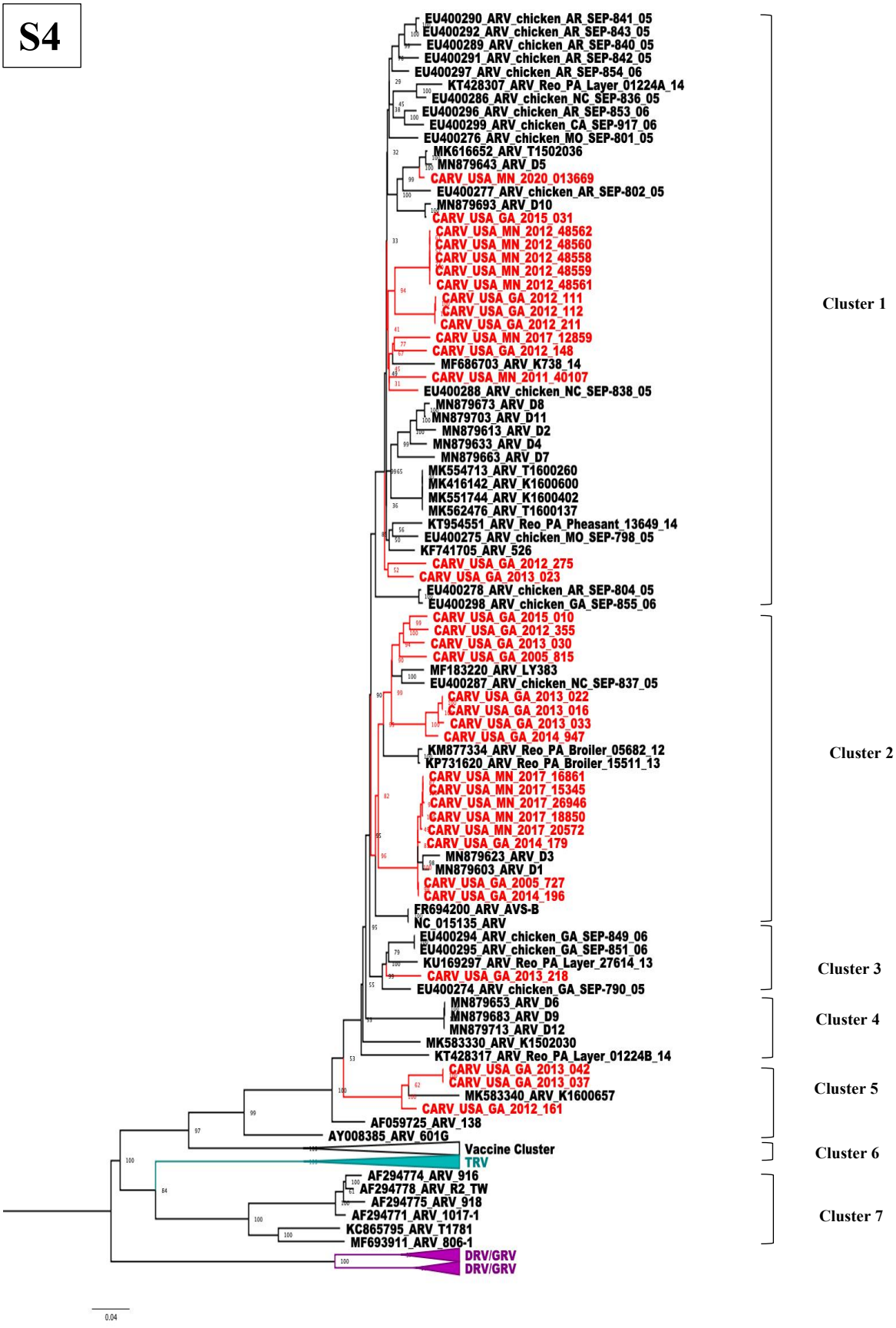
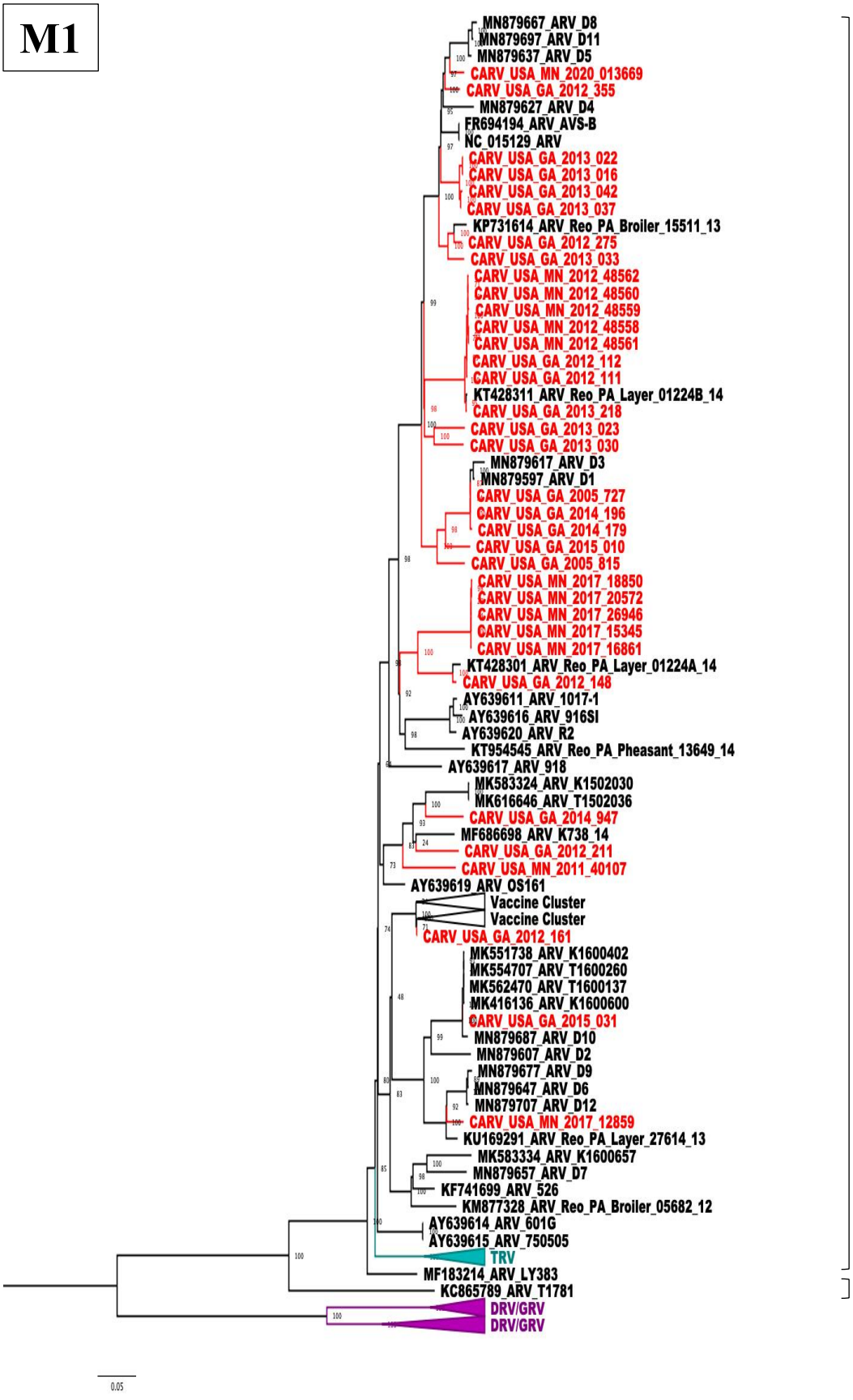
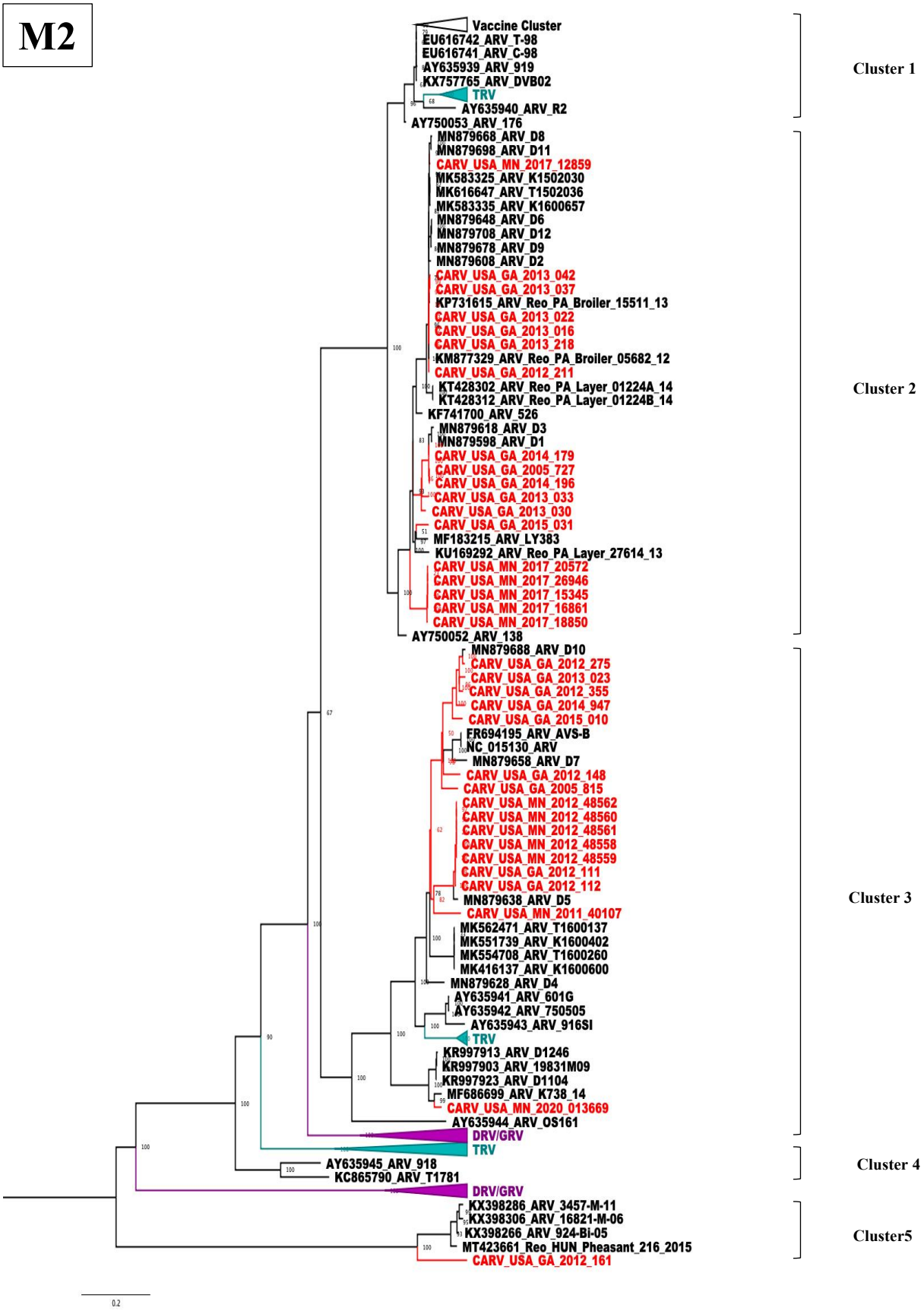

EU400290_ARV_chicken_AR_SEP-841_05
EU400292_ARV_chicken_AR_SEP-843_05
EU400289_ARV_chicken_AR_SEP-840_05
EU400291_ARV_chicken_AR_SEP-842_05
EU400297_ARV_chicken_AR_SEP-854_06
KT428307_ARV_Reo_PA_Layer_01224A_14
EU400286_ARV_chicken_NC_SEP-836_05
EU400296_ARV_chicken_AR_SEP-853_06
EU400299_ARV_chicken_CA_SEP-917_06
EU400276_ARV_chicken_MO_SEP-801_05
MK616652_ARV_T1502036
MN879643_ARV_D5
CARV_USA_MN_2020_013669
EU400277_ARV_chicken_AR_SEP-802_05
MN879693_ARV_D10
CARV_USA_GA_2015_031
CARV_USA_MN_2012_48562
CARV_USA_MN_2012_48560
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48559
CARV_USA_MN_2012_48561
CARV_USA_GA_2012_111
CARV_USA_GA_2012_112
CARV_USA_GA_2012_211
CARV_USA_MN_2017_12859
CARV_USA_GA_2012_148
MF686703_ARV_K738_14
CARV_USA_MN_2011_40107
EU400288_ARV_chicken_NC_SEP-838_05
MN879673_ARV_D8
MN879703_ARV_D11
MN879613_ARV_D2
MN879633_ARV_D4
MN879663_ARV_D7
MK554713_ARV_T1600260
MK416142_ARV_K1600600
MK551744_ARV_K1600402
MK562476_ARV_T1600137
KT954551_ARV_Reo_PA_Pheasant_13649_14
EU400275_ARV_chicken_MO_SEP-798_05
KF741705_ARV_526
CARV_USA_GA_2012_275
CARV_USA_GA_2013_023
EU400278_ARV_chicken_AR_SEP-804_05
EU400298_ARV_chicken_GA_SEP-855_06
CARV_USA_GA_2015_010
CARV_USA_GA_2012_355
CARV_USA_GA_2013_030
CARV_USA_GA_2005_815
MF183220_ARV_LY383
EU400287_ARV_chicken_NC_SEP-837_05
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
CARV_USA_GA_2013_033
CARV_USA_GA_2014_947
KM877334_ARV_Reo_PA_Broiler_05682_12
KP731620_ARV_Reo_PA_Broiler_15511_13
CARV_USA_MN_2017_16861
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_18850
CARV_USA_MN_2017_20572
CARV_USA_GA_2014_179
MN879623_ARV_D3
MN879603_ARV_D1
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
FR694200_ARV_AVS-B
NC_015135_ARV
EU400294_ARV_chicken_GA_SEP-849_06
EU400295_ARV_chicken_GA_SEP-851_06
KU169297_ARV_Reo_PA_Layer_27614_13
CARV_USA_GA_2013_218
EU400274_ARV_chicken_GA_SEP-790_05
MN879653_ARV_D6
MN879683_ARV_D9
MN879713_ARV_D12
MK583330_ARV_K1502030
KT428317_ARV_Reo_PA_Layer_01224B_14
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
MK583340_ARV_K1600657
CARV_USA_GA_2012_161
AF059725_ARV_138
AY008385_ARV_601G
Vaccine Cluster
TRV
AF294774_ARV_916
AF294778_ARV_R2_TW
AF294775_ARV_918
AF294771_ARV_1017-1
KC865795_ARV_T1781
MF693911_ARV_806-1
DRV/GRV
DRV/GRV
0.04
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7

Fig 3.3B. Nucleotide sequence-based ML phylogenetic trees showing the clustering of CARVs based on the M1, M2 andM3 gene segments of viruses available from GenBank. Phylogenetic calculations were carried out using the maximum-likelihood method applying the best-fit models calculated for each gene. The scale bar is proportional to the genetic distance.

M1


MN879667_ARV_D8
MN879697_ARV_D11
MN879637_ARV_D5
CARV_USA_MN_2020_013669
CARV_USA_GA_2012_355
MN879627_ARV_D4
FR694194_ARV_AVS-B
NC_015129_ARV
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
KP731614_ARV_Reo_PA_Broiler_15511_13
CARV_USA_GA_2012_275
CARV_USA_GA_2013_033
CARV_USA_MN_2012_48562
CARV_USA_MN_2012_48560
CARV_USA_MN_2012_48559
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48561
CARV_USA_GA_2012_112
CARV_USA_GA_2012_111
KT428311_ARV_Reo_PA_Layer_01224B_14
CARV_USA_GA_2013_218
CARV_USA_GA_2013_023
CARV_USA_GA_2013_030
MN879617_ARV_D3
MN879597_ARV_D1
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
CARV_USA_GA_2014_179
CARV_USA_GA_2015_010
CARV_USA_GA_2005_815
CARV_USA_MN_2017_18850
CARV_USA_MN_2017_20572
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_16861
KT428301_ARV_Reo_PA_Layer_01224A_14
CARV_USA_GA_2012_148
AY639611_ARV_1017-1
AY639616_ARV_916SI
AY639620_ARV_R2
KT954545_ARV_Reo_PA_Pheasant_13649_14
AY639617_ARV_918
MK583324_ARV_K1502030
MK616646_ARV_T1502036
CARV_USA_GA_2014_947
MF686698_ARV_K738_14
CARV_USA_GA_2012_211
CARV_USA_MN_2011_40107
AY639619_ARV_OS161
Vaccine Cluster
Vaccine Cluster
CARV_USA_GA_2012_161
MK551738_ARV_K1600402
MK554707_ARV_T1600260
MK562470_ARV_T1600137
MK416136_ARV_K1600600
CARV_USA_GA_2015_031
MN879687_ARV_D10
MN879607_ARV_D2
MN879677_ARV_D9
MN879647_ARV_D6
MN879707_ARV_D12
CARV_USA_MN_2017_12859
KU169291_ARV_Reo_PA_Layer_27614_13
MK583334_ARV_K1600657
MN879657_ARV_D7
KF741699_ARV_526
KM877328_ARV_Reo_PA_Broiler_05682_12
AY639614_ARV_601G
AY639615_ARV_750505
TRV
MF183214_ARV_LY383
KC865789_ARV_T1781
DRV/GRV
DRV/GRV
0.05
Cluster 1
Cluster 2


M2
Vaccine Cluster
EU616742_ARV_T-98
EU616741_ARV_C-98
AY635939_ARV_919
KX757765_ARV_DVB02
TRV
AY635940_ARV_R2
AY750053_ARV_176
MN879668_ARV_D8
MN879698_ARV_D11
CARV_USA_MN_2017_12859
MK583325_ARV_K1502030
MK616647_ARV_T1502036
MK583335_ARV_K1600657
MN879648_ARV_D6
MN879708_ARV_D12
MN879678_ARV_D9
MN879608_ARV_D2
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
KP731615_ARV_Reo_PA_Broiler_15511_13
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
CARV_USA_GA_2013_218
KM877329_ARV_Reo_PA_Broiler_05682_12
CARV_USA_GA_2012_211
KT428302_ARV_Reo_PA_Layer_01224A_14
KT428312_ARV_Reo_PA_Layer_01224B_14
KF741700_ARV_526
MN879618_ARV_D3
MN879598_ARV_D1
CARV_USA_GA_2014_179
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
CARV_USA_GA_2013_033
CARV_USA_GA_2013_030
CARV_USA_GA_2015_031
MF183215_ARV_LY383
KU169292_ARV_Reo_PA_Layer_27614_13
CARV_USA_MN_2017_20572
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_16861
CARV_USA_MN_2017_18850
AY750052_ARV_138
MN879688_ARV_D10
CARV_USA_GA_2012_275
CARV_USA_GA_2013_023
CARV_USA_GA_2012_355
CARV_USA_GA_2014_947
CARV_USA_GA_2015_010
FR694195_ARV_AVS-B
NC_015130_ARV
MN879658_ARV_D7
CARV_USA_GA_2012_148
CARV_USA_GA_2005_815
CARV_USA_MN_2012_48562
CARV_USA_MN_2012_48560
CARV_USA_MN_2012_48561
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48559
CARV_USA_GA_2012_111
CARV_USA_GA_2012_112
MN879638_ARV_D5
CARV_USA_MN_2011_40107
MK562471_ARV_T1600137
MK551739_ARV_K1600402
MK554708_ARV_T1600260
MK416137_ARV_K1600600
MN879628_ARV_D4
AY635941_ARV_601G
AY635942_ARV_750505
AY635943_ARV_916SI
TRV
KR997913_ARV_D1246
KR997903_ARV_19831M09
KR997923_ARV_D1104
MF686699_ARV_K738_14
CARV_USA_MN_2020_013669
AY635944_ARV_OS161
DRV/GRV
TRV
AY635945_ARV_918
KC865790_ARV_T1781
DRV/GRV
KX398286_ARV_3457-M-11
KX398306_ARV_16821-M-06
KX398266_ARV_924-Bi-05
MT423661_Reo_HUN_Pheasant_216_2015
CARV_USA_GA_2012_161
0.2
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster5

M3

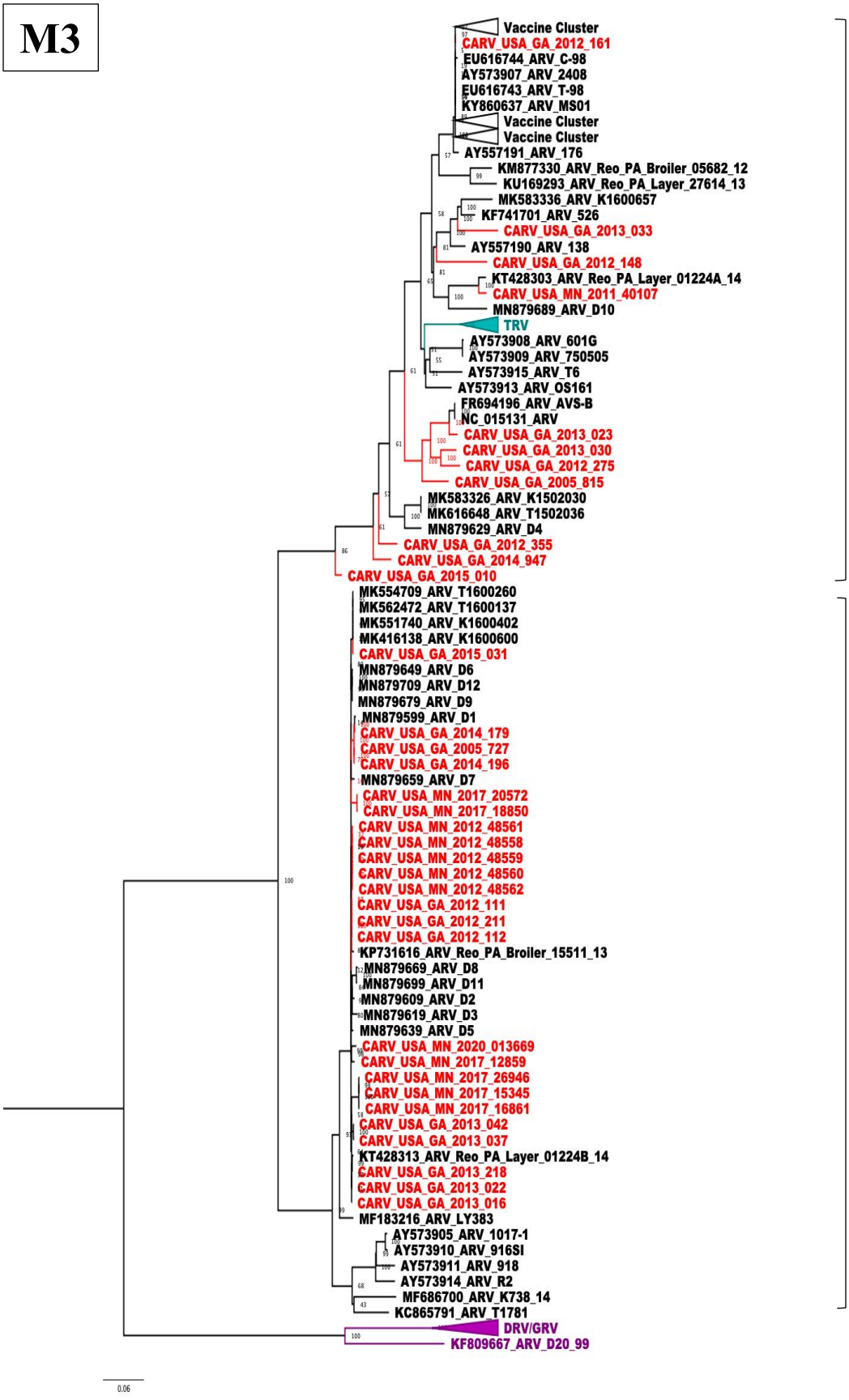
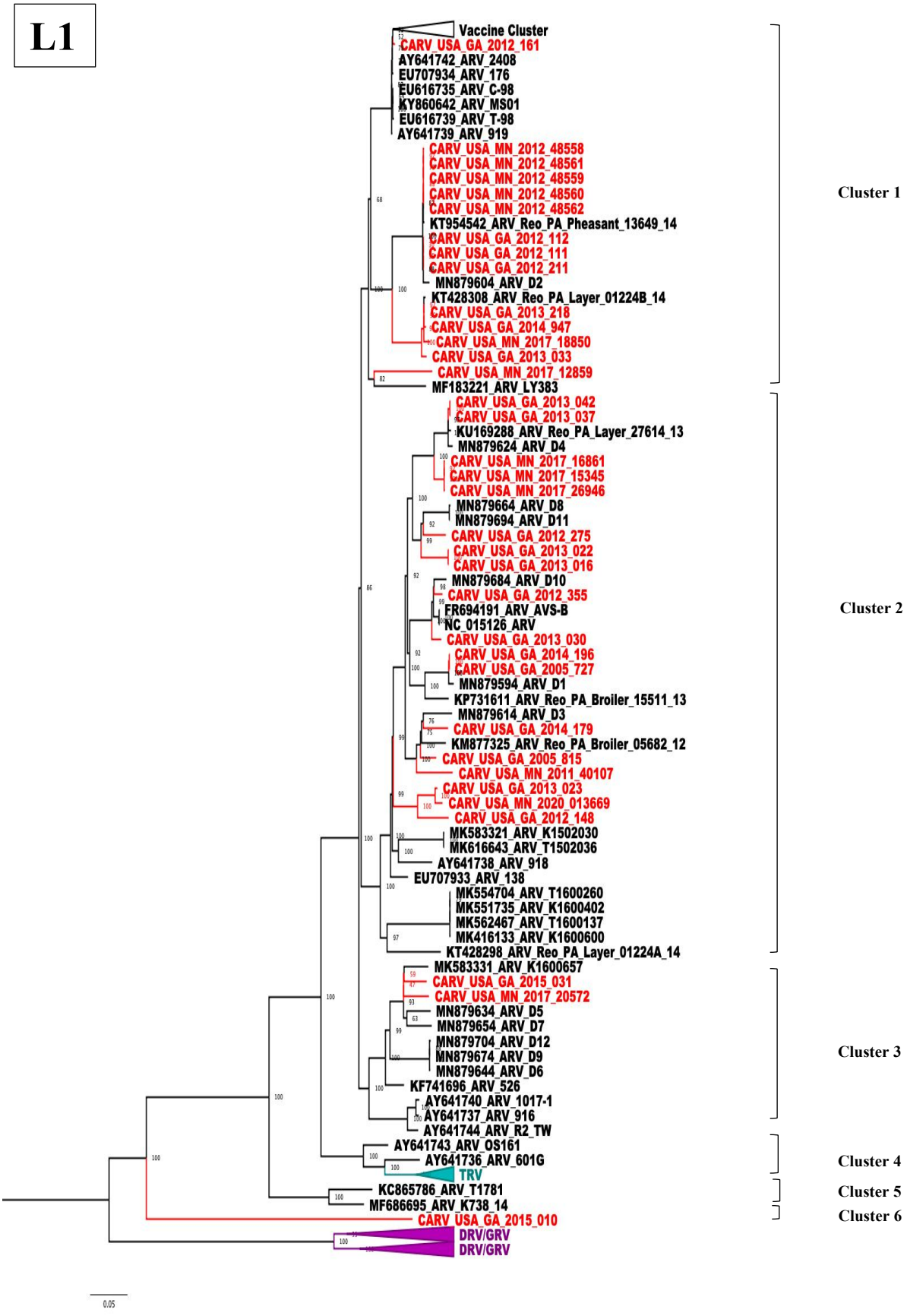
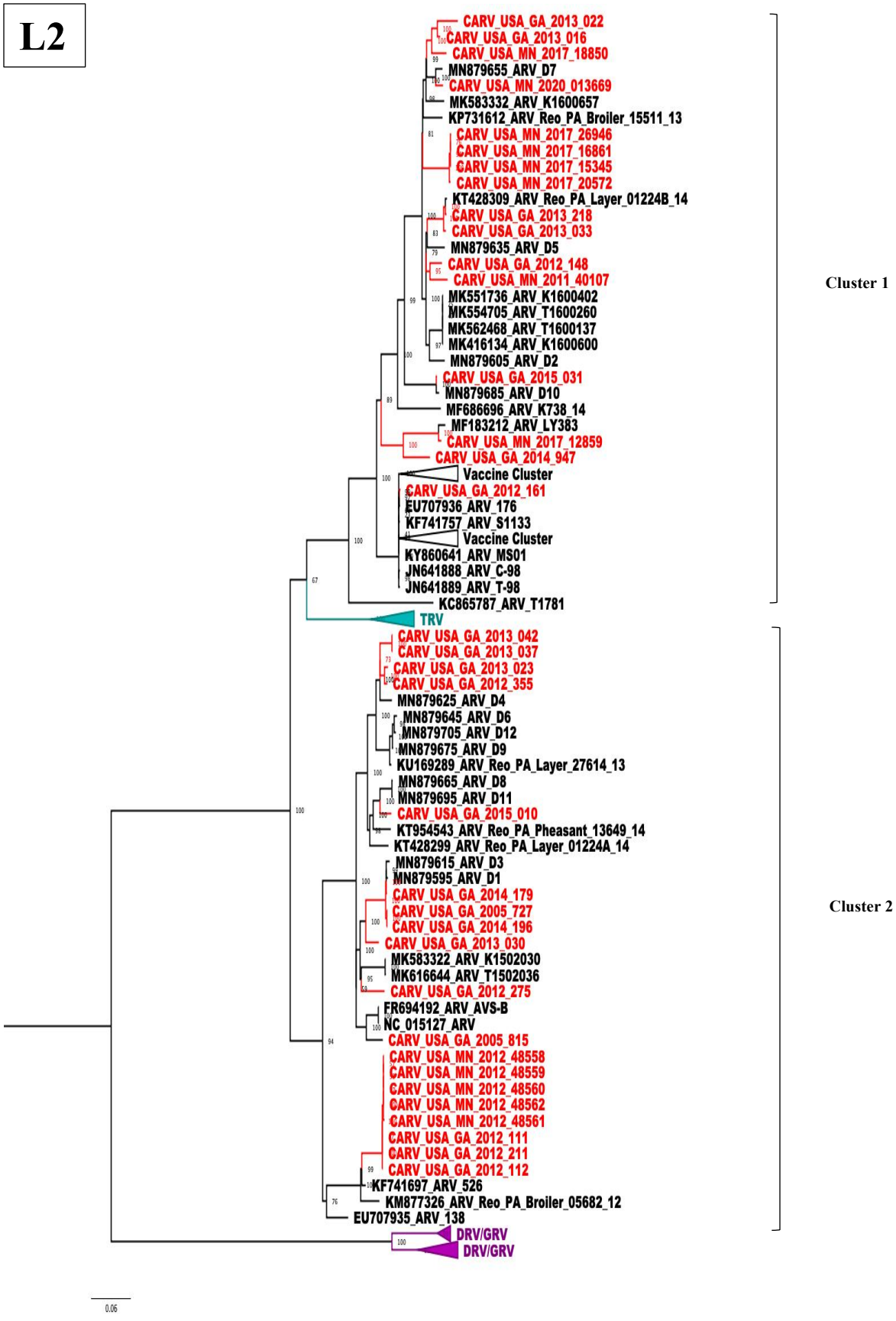

Vaccine Cluster
CARV_USA_GA_2012_161
EU616744_ARV_C-98
AY573907_ARV_2408
EU616743_ARV_T-98
KY860637_ARV_MS01
Vaccine Cluster
Vaccine Cluster
AY557191_ARV_176
KM877330_ARV_Reo_PA_Broiler_05682_12
KU169293_ARV_Reo_PA_Layer_27614_13
MK583336_ARV_K1600657
KF741701_ARV_526
CARV_USA_GA_2013_033
AY557190_ARV_138
CARV_USA_GA_2012_148
KT428303_ARV_Reo_PA_Layer_01224A_14
CARV_USA_MN_2011_40107
MN879689_ARV_D10
TRV
AY573908_ARV_601G
AY573909_ARV_750505
AY573915_ARV_T6
AY573913_ARV_OS161
FR694196_ARV_AVS-B
NC_015131_ARV
CARV_USA_GA_2013_023
CARV_USA_GA_2013_030
CARV_USA_GA_2012_275
CARV_USA_GA_2005_815
MK583326_ARV_K1502030
MK616648_ARV_T1502036
MN879629_ARV_D4
CARV_USA_GA_2012_355
CARV_USA_GA_2014_947
CARV_USA_GA_2015_010
MK554709_ARV_T1600260
MK562472_ARV_T1600137
MK551740_ARV_K1600402
MK416138_ARV_K1600600
CARV_USA_GA_2015_031
MN879649_ARV_D6
MN879709_ARV_D12
MN879679_ARV_D9
MN879599_ARV_D1
CARV_USA_GA_2014_179
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
MN879659_ARV_D7
CARV_USA_MN_2017_20572
CARV_USA_MN_2017_18850
CARV_USA_MN_2012_48561
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48559
CARV_USA_MN_2012_48560
CARV_USA_MN_2012_48562
CARV_USA_GA_2012_111
CARV_USA_GA_2012_211
CARV_USA_GA_2012_112
KP731616_ARV_Reo_PA_Broiler_15511_13
MN879669_ARV_D8
MN879699_ARV_D11
MN879609_ARV_D2
MN879619_ARV_D3
MN879639_ARV_D5
CARV_USA_MN_2020_013669
CARV_USA_MN_2017_12859
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_16861
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
KT428313_ARV_Reo_PA_Layer_01224B_14
CARV_USA_GA_2013_218
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
MF183216_ARV_LY383
AY573905_ARV_1017-1
AY573910_ARV_916SI
AY573911_ARV_918
AY573914_ARV_R2
MF686700_ARV_K738_14
KC865791_ARV_T1781
DRV/GRV
KF809667_ARV_D20_99
0.06
Cluster 1
Cluster 2

Fig 3.3C. Nucleotide sequence-based ML phylogenetic trees showing the clustering of CARVs based on the L1, L2 and L3 gene segments of viruses available from GenBank. Phylogenetic calculations were carried out using the maximum-likelihood method applying the best-fit models calculated for each gene. The scale bar is proportional to the genetic distance.

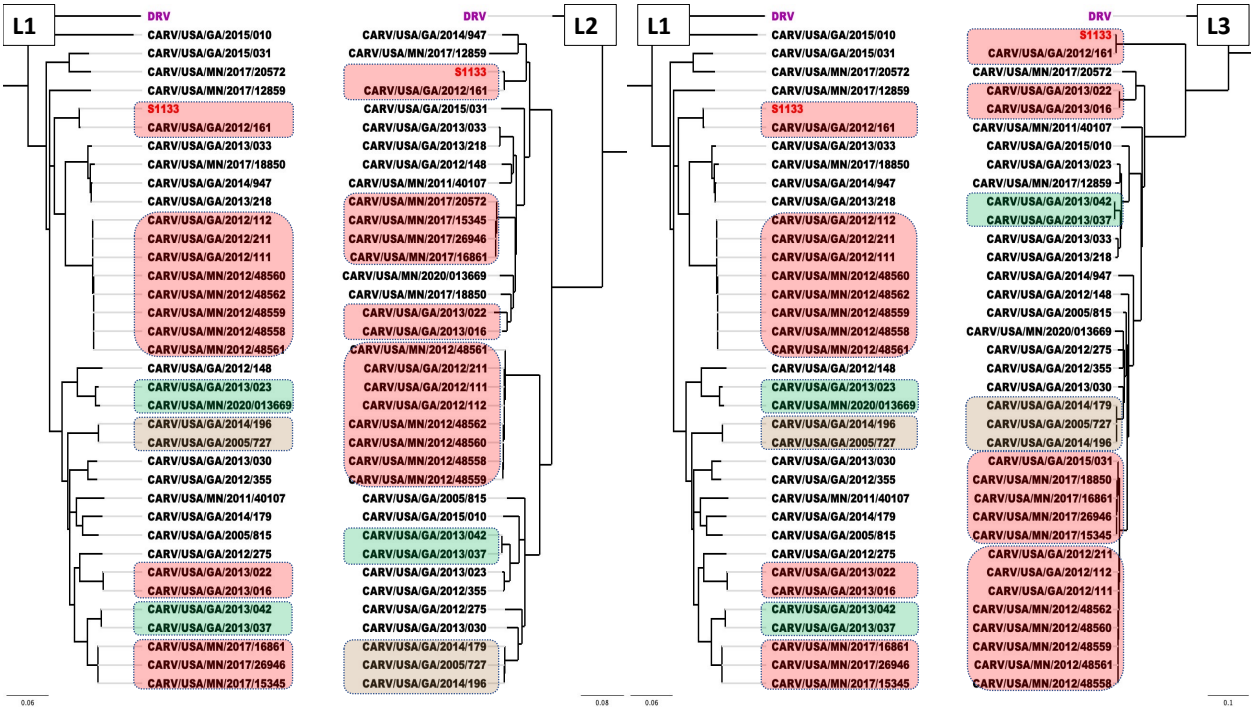

L1
Vaccine Cluster
CARV_USA_GA_2012_161
AY641742_ARV_2408
EU707934_ARV_176
EU616735_ARV_C-98
KY860642_ARV_MS01
EU616739_ARV_T-98
AY641739_ARV_919
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48561
CARV_USA_MN_2012_48559
CARV_USA_MN_2012_48560
CARV_USA_MN_2012_48562
KT954542_ARV_Reo_PA_Pheasant_13649_14
CARV_USA_GA_2012_112
CARV_USA_GA_2012_111
CARV_USA_GA_2012_211
MN879604_ARV_D2
KT428308_ARV_Reo_PA_Layer_01224B_14
CARV_USA_GA_2013_218
CARV_USA_GA_2014_947
CARV_USA_MN_2017_18850
CARV_USA_GA_2013_033
CARV_USA_MN_2017_12859
MF183221_ARV_LY383
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
KU169288_ARV_Reo_PA_Layer_27614_13
MN879624_ARV_D4
CARV_USA_MN_2017_16861
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_26946
MN879664_ARV_D8
MN879694_ARV_D11
CARV_USA_GA_2012_275
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
MN879684_ARV_D10
CARV_USA_GA_2012_355
FR694191_ARV_AVS-B
NC_015126_ARV
CARV_USA_GA_2013_030
CARV_USA_GA_2014_196
CARV_USA_GA_2005_727
MN879594_ARV_D1
KP731611_ARV_Reo_PA_Broiler_15511_13
MN879614_ARV_D3
CARV_USA_GA_2014_179
KM877325_ARV_Reo_PA_Broiler_05682_12
CARV_USA_GA_2005_815
CARV_USA_MN_2011_40107
CARV_USA_GA_2013_023
CARV_USA_MN_2020_013669
CARV_USA_GA_2012_148
MK583321_ARV_K1502030
MK616643_ARV_T1502036
AY641738_ARV_918
EU707933_ARV_138
MK554704_ARV_T1600260
MK551735_ARV_K1600402
MK562467_ARV_T1600137
MK416133_ARV_K1600600
KT428298_ARV_Reo_PA_Layer_01224A_14
MK583331_ARV_K1600657
CARV_USA_GA_2015_031
CARV_USA_MN_2017_20572
MN879634_ARV_D5
MN879654_ARV_D7
MN879704_ARV_D12
MN879674_ARV_D9
MN879644_ARV_D6
KF741696_ARV_526
AY641740_ARV_1017-1
AY641737_ARV_916
AY641744_ARV_R2_TW
AY641743_ARV_OS161
AY641736_ARV_601G
TRV
KC865786_ARV_T1781
MF686695_ARV_K738_14
CARV_USA_GA_2015_010
DRV/GRV
DRV/GRV
0.05
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6

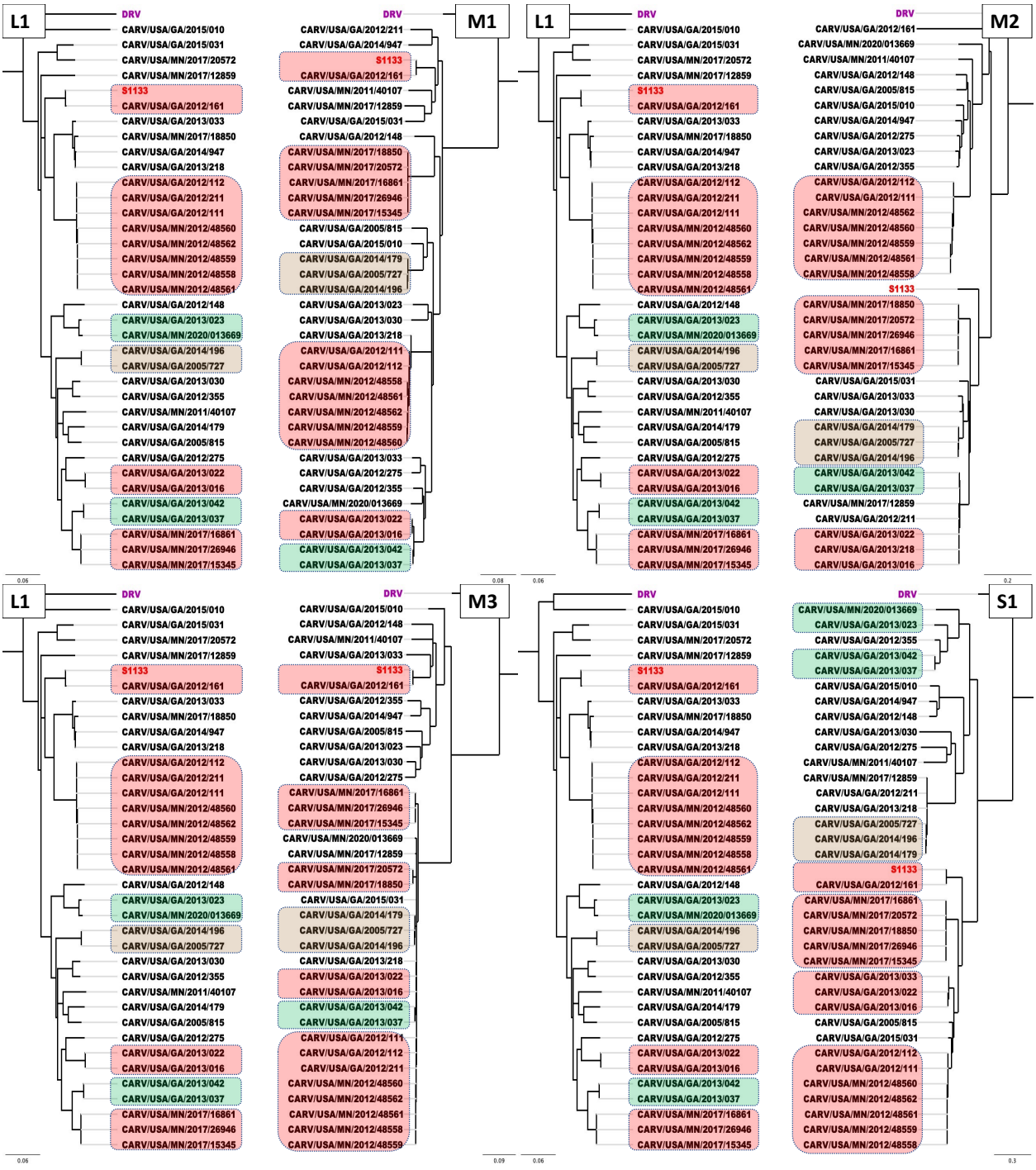

L2
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
CARV_USA_MN_2017_18850
MN879655_ARV_D7
CARV_USA_MN_2020_013669
MK583332_ARV_K1600657
KP731612_ARV_Reo_PA_Broiler_15511_13
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_16861
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_20572
KT428309_ARV_Reo_PA_Layer_01224B_14
CARV_USA_GA_2013_218
CARV_USA_GA_2013_033
MN879635_ARV_D5
CARV_USA_GA_2012_148
CARV_USA_MN_2011_40107
MK551736_ARV_K1600402
MK554705_ARV_T1600260
MK562468_ARV_T1600137
MK416134_ARV_K1600600
MN879605_ARV_D2
CARV_USA_GA_2015_031
MN879685_ARV_D10
MF686696_ARV_K738_14
MF183212_ARV_LY383
CARV_USA_MN_2017_12859
CARV_USA_GA_2014_947
Vaccine Cluster
CARV_USA_GA_2012_161
EU707936_ARV_176
KF741757_ARV_S1133
Vaccine Cluster
KY860641_ARV_MS01
JN641888_ARV_C-98
JN641889_ARV_T-98
KC865787_ARV_T1781
TRV
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
CARV_USA_GA_2013_023
CARV_USA_GA_2012_355
MN879625_ARV_D4
MN879645_ARV_D6
MN879705_ARV_D12
MN879675_ARV_D9
KU169289_ARV_Reo_PA_Layer_27614_13
MN879665_ARV_D8
MN879695_ARV_D11
CARV_USA_GA_2015_010
KT954543_ARV_Reo_PA_Pheasant_13649_14
KT428299_ARV_Reo_PA_Layer_01224A_14
MN879615_ARV_D3
MN879595_ARV_D1
CARV_USA_GA_2014_179
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
CARV_USA_GA_2013_030
MK583322_ARV_K1502030
MK616644_ARV_T1502036
CARV_USA_GA_2012_275
FR694192_ARV_AVS-B
NC_015127_ARV
CARV_USA_GA_2005_815
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48559
CARV_USA_MN_2012_48560
CARV_USA_MN_2012_48562
CARV_USA_MN_2012_48561
CARV_USA_GA_2012_111
CARV_USA_GA_2012_211
CARV_USA_GA_2012_112
KF741697_ARV_526
KM877326_ARV_Reo_PA_Broiler_05682_12
EU707935_ARV_138
DRV/GRV
DRV/GRV
0.06
Cluster 1
Cluster 2

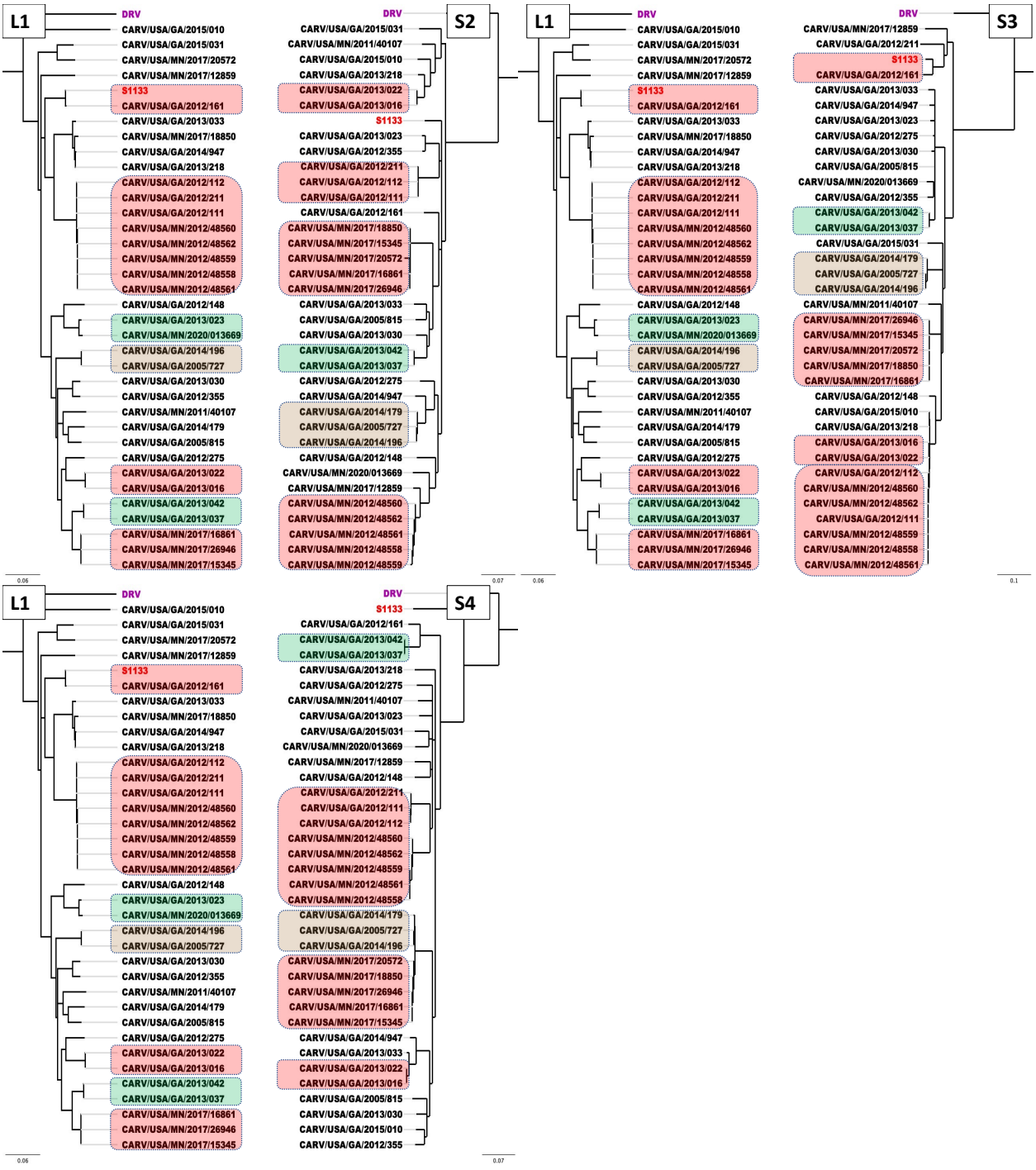

L3
CARV_USA_MN_2012_48561
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48562
CARV_USA_MN_2012_48560
CARV_USA_MN_2012_48559
CARV_USA_GA_2012_111
CARV_USA_GA_2012_112
KP731613_ARV_Reo_PA_Broiler_15511_13
CARV_USA_GA_2012_211
MN879656_ARV_D7
MK551737_ARV_K1600402
MK562469_ARV_T1600137
MK554706_ARV_T1600260
MK416135_ARV_K1600600
CARV_USA_GA_2015_031
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_16861
CARV_USA_MN_2017_18850
MN879616_ARV_D3
MN879596_ARV_D1
CARV_USA_GA_2014_179
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
CARV_USA_GA_2013_030
CARV_USA_MN_2020_013669
CARV_USA_GA_2012_355
CARV_USA_GA_2012_275
KT954544_ARV_Reo_PA_Pheasant_13649_14
CARV_USA_GA_2005_815
KU169290_ARV_Reo_PA_Layer_27614_13
KT428300_ARV_Reo_PA_Layer_01224A_14
CARV_USA_GA_2012_148
CARV_USA_GA_2014_947
KT428310_ARV_Reo_PA_Layer_01224B_14
CARV_USA_GA_2013_218
CARV_USA_GA_2015_010
CARV_USA_GA_2013_033
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
MN879666_ARV_D8
MN879696_ARV_D11
MN879636_ARV_D5
CARV_USA_MN_2017_12859
CARV_USA_GA_2013_023
MK583323_ARV_K1502030
MK616645_ARV_T1502036
FR694193_ARV_AVS-B
NC_015128_ARV
MF686697_ARV_K738_14
MN879646_ARV_D6
MN879706_ARV_D12
MN879676_ARV_D9
MN879626_ARV_D4
MN879686_ARV_D10
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
MK583333_ARV_K1600657
CARV_USA_MN_2017_20572
EU707937_ARV_138
MN879606_ARV_D2
CARV_USA_MN_2011_40107
KM877327_ARV_Reo_PA_Broiler_05682_12
KF741698_ARV_526
TRV
AY652696_ARV_OS161
CARV_USA_GA_2012_161
AY652698_ARV_T6
AY652694_ARV_2408
Vaccine Cluster
Vaccine Cluster
EU616737_ARV_C-98
EU616738_ARV_T-98
Vaccine Cluster
KY860640_ARV_MS01
Vaccine Cluster
AY652699_ARV_601G
AY652700_ARV_918
MF183213_ARV_LY383
KC865788_ARV_T1781
AY652701_ARV_916
DRV/GRV
DRV/GRV
0.07
Cluster 1
Cluster 2

Fig 3.4. Topologies of segmental phylogenetic trees of the genome (nt) of each CARV isolate. The trees were built using nucleotide sequences by ML method with best fit distance models.

Supplementary Table 1. The comparative heatmap of the highest nt identity of each segment of virus isolates in between cluster (IBC) and virus isolates within cluster group (WIC).
* This is an excel file




L1
DRV
CARV/USA/GA/2015/010
CARV/USA/GA/2015/031
CARV/USA/MN/2017/20572
CARV/USA/MN/2017/12859
S1133
CARV/USA/GA/2012/161
CARV/USA/GA/2013/033
CARV/USA/MN/2017/18850
CARV/USA/GA/2014/947
CARV/USA/GA/2013/218
CARV/USA/GA/2012/112
CARV/USA/GA/2012/211
CARV/USA/GA/2012/111
CARV/USA/MN/2012/48560
CARV/USA/MN/2012/48562
CARV/USA/MN/2012/48559
CARV/USA/MN/2012/48558
CARV/USA/MN/2012/48561
CARV/USA/GA/2012/148
CARV/USA/GA/2013/023
CARV/USA/MN/2020/013669
CARV/USA/GA/2014/196
CARV/USA/GA/2005/727
CARV/USA/GA/2013/030
CARV/USA/GA/2012/355
CARV/USA/MN/2011/40107
CARV/USA/GA/2014/179
CARV/USA/GA/2005/815
CARV/USA/GA/2012/275
CARV/USA/GA/2013/022
CARV/USA/GA/2013/016
CARV/USA/GA/2013/042
CARV/USA/GA/2013/037
CARV/USA/MN/2017/16861
CARV/USA/MN/2017/26946
CARV/USA/MN/2017/15345
0.06
M1
DRV
CARV/USA/GA/2012/211
CARV/USA/GA/2014/947
S1133
CARV/USA/GA/2012/161
CARV/USA/MN/2011/40107
CARV/USA/MN/2017/12859
CARV/USA/GA/2015/031
CARV/USA/GA/2012/148
CARV/USA/MN/2017/18850
CARV/USA/MN/2017/20572
CARV/USA/MN/2017/16861
CARV/USA/MN/2017/26946
CARV/USA/MN/2017/15345
CARV/USA/GA/2005/815
CARV/USA/GA/2015/010
CARV/USA/GA/2014/179
CARV/USA/GA/2005/727
CARV/USA/GA/2014/196
CARV/USA/GA/2013/023
CARV/USA/GA/2013/030
CARV/USA/GA/2013/218
CARV/USA/GA/2012/111
CARV/USA/GA/2012/112
CARV/USA/MN/2012/48558
CARV/USA/MN/2012/48561
CARV/USA/MN/2012/48562
CARV/USA/MN/2012/48559
CARV/USA/MN/2012/48560
CARV/USA/GA/2013/033
CARV/USA/GA/2012/275
CARV/USA/GA/2012/355
CARV/USA/MN/2020/013669
CARV/USA/GA/2013/022
CARV/USA/GA/2013/016
CARV/USA/GA/2013/042
CARV/USA/GA/2013/037
0.08
L1
DRV
CARV/USA/GA/2015/010
CARV/USA/GA/2015/031
CARV/USA/MN/2017/20572
CARV/USA/MN/2017/12859
S1133
CARV/USA/GA/2012/161
CARV/USA/GA/2013/033
CARV/USA/MN/2017/18850
CARV/USA/GA/2014/947
CARV/USA/GA/2013/218
CARV/USA/GA/2012/112
CARV/USA/GA/2012/211
CARV/USA/GA/2012/111
CARV/USA/MN/2012/48560
CARV/USA/MN/2012/48562
CARV/USA/MN/2012/48559
CARV/USA/MN/2012/48558
CARV/USA/MN/2012/48561
CARV/USA/GA/2012/148
CARV/USA/GA/2013/023
CARV/USA/MN/2020/013669
CARV/USA/GA/2014/196
CARV/USA/GA/2005/727
CARV/USA/GA/2013/030
CARV/USA/GA/2012/355
CARV/USA/MN/2011/40107
CARV/USA/GA/2014/179
CARV/USA/GA/2005/815
CARV/USA/GA/2012/275
CARV/USA/GA/2013/022
CARV/USA/GA/2013/016
CARV/USA/GA/2013/042
CARV/USA/GA/2013/037
CARV/USA/MN/2017/16861
CARV/USA/MN/2017/26946
CARV/USA/MN/2017/15345
0.06
M2
DRV
CARV/USA/GA/2012/161
CARV/USA/MN/2020/013669
CARV/USA/MN/2011/40107
CARV/USA/GA/2012/148
CARV/USA/GA/2005/815
CARV/USA/GA/2015/010
CARV/USA/GA/2014/947
CARV/USA/GA/2012/275
CARV/USA/GA/2013/023
CARV/USA/GA/2012/355
CARV/USA/GA/2012/112
CARV/USA/GA/2012/111
CARV/USA/MN/2012/48562
CARV/USA/MN/2012/48560
CARV/USA/MN/2012/48559
CARV/USA/MN/2012/48561
CARV/USA/MN/2012/48558
S1133
CARV/USA/MN/2017/18850
CARV/USA/MN/2017/20572
CARV/USA/MN/2017/26946
CARV/USA/MN/2017/16861
CARV/USA/MN/2017/15345
CARV/USA/GA/2015/031
CARV/USA/GA/2013/033
CARV/USA/GA/2013/030
CARV/USA/GA/2014/179
CARV/USA/GA/2005/727
CARV/USA/GA/2014/196
CARV/USA/GA/2013/042
CARV/USA/GA/2013/037
CARV/USA/MN/2017/12859
CARV/USA/GA/2012/211
CARV/USA/GA/2013/022
CARV/USA/GA/2013/218
CARV/USA/GA/2013/016
0.06
L1
DRV
CARV/USA/GA/2015/010
CARV/USA/GA/2015/031
CARV/USA/MN/2017/20572
CARV/USA/MN/2017/12859
S1133
CARV/USA/GA/2012/161
CARV/USA/GA/2013/033
CARV/USA/MN/2017/18850
CARV/USA/GA/2014/947
CARV/USA/GA/2013/218
CARV/USA/GA/2012/112
CARV/USA/GA/2012/211
CARV/USA/GA/2012/111
CARV/USA/MN/2012/48560
CARV/USA/MN/2012/48562
CARV/USA/MN/2012/48559
CARV/USA/MN/2012/48558
CARV/USA/MN/2012/48561
CARV/USA/GA/2012/148
CARV/USA/GA/2013/023
CARV/USA/MN/2020/013669
CARV/USA/GA/2014/196
CARV/USA/GA/2005/727
CARV/USA/GA/2013/030
CARV/USA/GA/2012/355
CARV/USA/MN/2011/40107
CARV/USA/GA/2014/179
CARV/USA/GA/2005/815
CARV/USA/GA/2012/275
CARV/USA/GA/2013/022
CARV/USA/GA/2013/016
CARV/USA/GA/2013/042
CARV/USA/GA/2013/037
CARV/USA/MN/2017/16861
CARV/USA/MN/2017/26946
CARV/USA/MN/2017/15345
0.06
M3
DRV
CARV/USA/GA/2015/010
CARV/USA/GA/2012/148
CARV/USA/MN/2011/40107
CARV/USA/GA/2013/033
S1133
CARV/USA/GA/2012/161
CARV/USA/GA/2012/355
CARV/USA/GA/2014/947
CARV/USA/GA/2005/815
CARV/USA/GA/2013/023
CARV/USA/GA/2013/030
CARV/USA/GA/2012/275
CARV/USA/MN/2017/16861
CARV/USA/MN/2017/26946
CARV/USA/MN/2017/15345
CARV/USA/MN/2020/013669
CARV/USA/MN/2017/12859
CARV/USA/MN/2017/20572
CARV/USA/MN/2017/18850
CARV/USA/GA/2015/031
CARV/USA/GA/2014/179
CARV/USA/GA/2005/727
CARV/USA/GA/2014/196
CARV/USA/GA/2013/218
CARV/USA/GA/2013/022
CARV/USA/GA/2013/016
CARV/USA/GA/2013/042
CARV/USA/GA/2013/037
CARV/USA/GA/2012/111
CARV/USA/GA/2012/112
CARV/USA/GA/2012/211
CARV/USA/MN/2012/48560
CARV/USA/MN/2012/48562
CARV/USA/MN/2012/48561
CARV/USA/MN/2012/48558
CARV/USA/MN/2012/48559
0.09
0.06
S1
DRV
CARV/USA/MN/2020/013669
CARV/USA/GA/2013/023
CARV/USA/GA/2012/355
CARV/USA/GA/2013/042
CARV/USA/GA/2013/037
CARV/USA/GA/2015/010
CARV/USA/GA/2014/947
CARV/USA/GA/2012/148
CARV/USA/GA/2013/030
CARV/USA/GA/2012/275
CARV/USA/MN/2011/40107
CARV/USA/MN/2017/12859
CARV/USA/GA/2012/211
CARV/USA/GA/2013/218
CARV/USA/GA/2005/727
CARV/USA/GA/2014/196
CARV/USA/GA/2014/179
S1133
CARV/USA/GA/2012/161
CARV/USA/MN/2017/16861
CARV/USA/MN/2017/20572
CARV/USA/MN/2017/18850
CARV/USA/MN/2017/26946
CARV/USA/MN/2017/15345
CARV/USA/GA/2013/033
CARV/USA/GA/2013/022
CARV/USA/GA/2013/016
CARV/USA/GA/2005/815
CARV/USA/GA/2015/031
CARV/USA/GA/2012/112
CARV/USA/GA/2012/111
CARV/USA/MN/2012/48560
CARV/USA/MN/2012/48562
CARV/USA/MN/2012/48561
CARV/USA/MN/2012/48559
CARV/USA/MN/2012/48558
0.2
0.3


L1
DRV
CARV/USA/GA/2015/010
CARV/USA/GA/2015/031
CARV/USA/MN/2017/20572
CARV/USA/MN/2017/12859
S1133
CARV/USA/GA/2012/161
CARV/USA/GA/2013/033
CARV/USA/MN/2017/18850
CARV/USA/GA/2014/947
CARV/USA/GA/2013/218
CARV/USA/GA/2012/112
CARV/USA/GA/2012/211
CARV/USA/GA/2012/111
CARV/USA/MN/2012/48560
CARV/USA/MN/2012/48562
CARV/USA/MN/2012/48559
CARV/USA/MN/2012/48558
CARV/USA/MN/2012/48561
CARV/USA/GA/2012/148
CARV/USA/GA/2013/023
CARV/USA/MN/2020/013669
CARV/USA/GA/2014/196
CARV/USA/GA/2005/727
CARV/USA/GA/2013/030
CARV/USA/GA/2012/355
CARV/USA/MN/2011/40107
CARV/USA/GA/2014/179
CARV/USA/GA/2005/815
CARV/USA/GA/2012/275
CARV/USA/GA/2013/022
CARV/USA/GA/2013/016
CARV/USA/GA/2013/042
CARV/USA/GA/2013/037
CARV/USA/MN/2017/16861
CARV/USA/MN/2017/26946
CARV/USA/MN/2017/15345
0.06
S2
DRV
CARV/USA/GA/2015/031
CARV/USA/MN/2011/40107
CARV/USA/GA/2015/010
CARV/USA/GA/2013/218
CARV/USA/GA/2013/022
CARV/USA/GA/2013/016
S1133
CARV/USA/GA/2013/023
CARV/USA/GA/2012/355
CARV/USA/GA/2012/211
CARV/USA/GA/2012/112
CARV/USA/GA/2012/111
CARV/USA/GA/2012/161
CARV/USA/MN/2017/18850
CARV/USA/MN/2017/15345
CARV/USA/MN/2017/20572
CARV/USA/MN/2017/16861
CARV/USA/MN/2017/26946
CARV/USA/GA/2013/033
CARV/USA/GA/2005/815
CARV/USA/GA/2013/030
CARV/USA/GA/2013/042
CARV/USA/GA/2013/037
CARV/USA/GA/2012/275
CARV/USA/GA/2014/947
CARV/USA/GA/2014/179
CARV/USA/GA/2005/727
CARV/USA/GA/2014/196
CARV/USA/GA/2012/148
CARV/USA/MN/2020/013669
CARV/USA/MN/2017/12859
CARV/USA/MN/2012/48560
CARV/USA/MN/2012/48562
CARV/USA/MN/2012/48561
CARV/USA/MN/2012/48558
CARV/USA/MN/2012/48559
0.07
L1
DRV
CARV/USA/GA/2015/010
CARV/USA/GA/2015/031
CARV/USA/MN/2017/20572
CARV/USA/MN/2017/12859
S1133
CARV/USA/GA/2012/161
CARV/USA/GA/2013/033
CARV/USA/MN/2017/18850
CARV/USA/GA/2014/947
CARV/USA/GA/2013/218
CARV/USA/GA/2012/112
CARV/USA/GA/2012/211
CARV/USA/GA/2012/111
CARV/USA/MN/2012/48560
CARV/USA/MN/2012/48562
CARV/USA/MN/2012/48559
CARV/USA/MN/2012/48558
CARV/USA/MN/2012/48561
CARV/USA/GA/2012/148
CARV/USA/GA/2013/023
CARV/USA/MN/2020/013669
CARV/USA/GA/2014/196
CARV/USA/GA/2005/727
CARV/USA/GA/2013/030
CARV/USA/GA/2012/355
CARV/USA/MN/2011/40107
CARV/USA/GA/2014/179
CARV/USA/GA/2005/815
CARV/USA/GA/2012/275
CARV/USA/GA/2013/022
CARV/USA/GA/2013/016
CARV/USA/GA/2013/042
CARV/USA/GA/2013/037
CARV/USA/MN/2017/16861
CARV/USA/MN/2017/26946
CARV/USA/MN/2017/15345
0.06
S3
DRV
CARV/USA/MN/2017/12859
CARV/USA/GA/2012/211
S1133
CARV/USA/GA/2012/161
CARV/USA/GA/2013/033
CARV/USA/GA/2014/947
CARV/USA/GA/2013/023
CARV/USA/GA/2012/275
CARV/USA/GA/2013/030
CARV/USA/GA/2005/815
CARV/USA/MN/2020/013669
CARV/USA/GA/2012/355
CARV/USA/GA/2013/042
CARV/USA/GA/2013/037
CARV/USA/GA/2015/031
CARV/USA/GA/2014/179
CARV/USA/GA/2005/727
CARV/USA/GA/2014/196
CARV/USA/MN/2011/40107
CARV/USA/MN/2017/26946
CARV/USA/MN/2017/15345
CARV/USA/MN/2017/20572
CARV/USA/MN/2017/18850
CARV/USA/MN/2017/16861
CARV/USA/GA/2012/148
CARV/USA/GA/2015/010
CARV/USA/GA/2013/218
CARV/USA/GA/2013/016
CARV/USA/GA/2013/022
CARV/USA/GA/2012/112
CARV/USA/MN/2012/48560
CARV/USA/MN/2012/48562
CARV/USA/GA/2012/111
CARV/USA/MN/2012/48559
CARV/USA/MN/2012/48558
CARV/USA/MN/2012/48561
0.1
L1
DRV
CARV/USA/GA/2015/010
CARV/USA/GA/2015/031
CARV/USA/MN/2017/20572
CARV/USA/MN/2017/12859
S1133
CARV/USA/GA/2012/161
CARV/USA/GA/2013/033
CARV/USA/MN/2017/18850
CARV/USA/GA/2014/947
CARV/USA/GA/2013/218
CARV/USA/GA/2012/112
CARV/USA/GA/2012/211
CARV/USA/GA/2012/111
CARV/USA/MN/2012/48560
CARV/USA/MN/2012/48562
CARV/USA/MN/2012/48559
CARV/USA/MN/2012/48558
CARV/USA/MN/2012/48561
CARV/USA/GA/2012/148
CARV/USA/GA/2013/023
CARV/USA/MN/2020/013669
CARV/USA/GA/2014/196
CARV/USA/GA/2005/727
CARV/USA/GA/2013/030
CARV/USA/GA/2012/355
CARV/USA/MN/2011/40107
CARV/USA/GA/2014/179
CARV/USA/GA/2005/815
CARV/USA/GA/2012/275
CARV/USA/GA/2013/022
CARV/USA/GA/2013/016
CARV/USA/GA/2013/042
CARV/USA/GA/2013/037
CARV/USA/MN/2017/16861
CARV/USA/MN/2017/26946
CARV/USA/MN/2017/15345
0.06
S4
DRV
S1133
CARV/USA/GA/2012/161
CARV/USA/GA/2013/042
CARV/USA/GA/2013/037
CARV/USA/GA/2013/218
CARV/USA/GA/2012/275
CARV/USA/MN/2011/40107
CARV/USA/GA/2013/023
CARV/USA/GA/2015/031
CARV/USA/MN/2020/013669
CARV/USA/MN/2017/12859
CARV/USA/GA/2012/148
CARV/USA/GA/2012/211
CARV/USA/GA/2012/111
CARV/USA/GA/2012/112
CARV/USA/MN/2012/48560
CARV/USA/MN/2012/48562
CARV/USA/MN/2012/48559
CARV/USA/MN/2012/48561
CARV/USA/MN/2012/48558
CARV/USA/GA/2014/179
CARV/USA/GA/2005/727
CARV/USA/GA/2014/196
CARV/USA/MN/2017/20572
CARV/USA/MN/2017/18850
CARV/USA/MN/2017/26946
CARV/USA/MN/2017/16861
CARV/USA/MN/2017/15345
CARV/USA/GA/2014/947
CARV/USA/GA/2013/033
CARV/USA/GA/2013/022
CARV/USA/GA/2013/016
CARV/USA/GA/2005/815
CARV/USA/GA/2013/030
CARV/USA/GA/2015/010
CARV/USA/GA/2012/355
0.07

CHAPTER 4

MOLECULAR CHARACTERIZATION AND GENOMIC DIVERSITY OF CHICKEN ARTHRITIS REOVIRUSES IN GERMANY

The material in this chapter has been prepared for publication:
Kumar R, Singh VK, Vasquez JM, Mor SK, Porter RE, and Goyal SM. 2021. Molecular characterization and genomic diversity of chicken arthritis reoviruses in Germany. Sci Rep.

Introduction

Arthrotropic avian reovirus, tentatively named as chicken arthritis reovirus (CARV) causes tenosynovitis in chicken resulting in lameness characterized by uni- or bilateral swelling of the hock joint. The infected flock shows poor growth, lack of uniformity, and death in severe cases (Gouvea and Schnitzer 1982; Liu et al. 2003). Meat type chicken including the broiler and breeders are mostly affected raising economic and welfare concerns (Davis et al., 2013; Jones, 2013; Liu et al., 2013; Troxler et al., 2013; Lu et al., 2015; Sellers 2017; Palomino et al., 2018). Incidence and frequency of CARV associated arthritis/tenosynovitis have increased in the last decade, which is believed to be due to the emergence of new variants (Carli et al., 2020).

The CARVs are classified in the genus *Orthoreovirus* of family *Reoviridae*. They are non-enveloped viruses of 70–80 nm in diameter having icosahedral symmetry with a double-layered outer capsid (Spandidos & Graham, 1976). The viral genome consists of 10 segments of double stranded RNA (dsRNA) classified as large (L1, L2, and L3), medium (M1, M2, and M3), and small (S1, S2, S3, and S4) segments based on their electrophoretic mobility on polyacrylamide gel (Spandidos and Graham 1976). All the gene segments encode a single protein except the S1 segment (tri-cistronic, having three open reading frames) enabling the virus to encode eight structural and four non-structural proteins. Viral inner core is made up of five highly conserved proteins (λ A, λ B, λ C, μ A, and σ A), while the outer capsid consists of three more variable proteins (μ B, σ B, and σ C) (Benavente et al., 2007). The σC and σB proteins contain specific epitopes, which induce the production of type-specific and group-specific neutralizing antibodies, respectively (Wickramasinghe et al., 1993).

Sigma C (σC) protein encoded by the S1 segment is the most variable protein (Guardado-Calvo et al. 2005) and most studied for classification of CARVs into five or six genotypic clusters (GCs) (Kant et al. 2003; Egana et al., 2019; Ayalew et al., 2020). Dutch and German avian reoviruses from healthy and unidentified cases, malabsorption syndrome (MAS) and tenosynovitis cases isolated between 1980 and 2000 were studied and classified into GC1, GC2, GC4 and GC5, where most MAS isolates studied group in GC1 and GC4 while tenosynovitis isolates studied in group GC4 (Kant et al., 2003). Similarly, French CARV isolates belong to GC1 as reported by Troxler et al. (2013). Although the whole genome sequence data available from Europe is limited, but recent whole genome sequencing (WGS) studies in other parts of the world suggest CARVs genome is continuously changing due to accumulation of point mutation and reassortment of cognate genome segments (Farkas et al., 2016; Ayalew et al., 2020). These mechanisms are thought to be the driving force for the emergence of genetic and antigenic variants of CARVs leading to vaccination failures (Tang and Lu, 2015a, 2015b; Farkas et al., 2016; Egana et al., 2019). Additionally, gene segments other than σC gene were rarely taken into account for genetic characterization of CARVs.

Paucity of avian reovirus sequence data from Germany/Europe is a hurdle in the molecular epidemiological studies for surveillance and control of reovirus infection in Germany/Europe. In this study, we performed the whole genome sequencing (WGS) of 14 CARV isolates circulating in Germany for genetic characterization, molecular phylogeny and to assess the re-assortment events undergoing between different gene segments. Results of this study will help in understanding the variant reoviruses

circulating in Germany and devise appropriate control strategies and selection of candidate vaccine virus strains.

Materials and Methods

Virus isolation

Pooled tissue samples from cecal tonsils, kidney, leg joints and tendons and pooled swabs from trachea and cloaca were collected from 2–5-week-old broilers from different geographical locations in Germany and sent to AniCon Labor GmbH, Hoeltinghausen, Germany (Table 4.1). The presence of CARV was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) followed by virus isolation in primary chicken embryo liver (CEL) cells. Briefly, a 10% tissue homogenate was inoculated in CEL cells, followed by incubation at 37°C with 5% CO_2. The cells were checked daily for reovirus specific cytopathic effects (CPE) viz. syncytia formation and monolayer detachment. One to two passages were given until CPEs were evident in 75% of the cells. The cell culture flasks were then subjected to 3 freeze thaw cycles followed by virus harvest. The harvested virus isolates were then kept frozen at -80°C for further use.

Illumina sequencing, full-length viral genome assembly and sequence analysis

Fourteen reovirus isolates were sent to our lab on FTA cards for NGS. Viral RNA extraction from the FTA cards was carried out using a QIAamp MinElute Virus Spin (QIAGEN, Valencia, CA, USA) following manufacturer's instructions. The total RNA was submitted to the University of Minnesota Genomics Center (UMGC) for quantification, cDNA synthesis and library preparation using SMARTer Stranded Total RNA-Seq – Pico Mammalian Kit v2 (Takara Bio, Mountain View, CA, USA) following

manufacturer's protocol. Libraries were sequenced on an Illumina MiSeq v2 250 paired-end cycle. The fastq files were received from UMGC for analyses using in-house bioinformatics pipeline of our lab for QC check, trimming, *de novo* and reference-based assemblies.

Viral genome assembly and annotation

Raw FASTQ files were trimmed to remove Illumina adapters using Trimmomatic (v 0.39, https://github.com/usadellab/Trimmomatic) with a minimum quality score of 20. Then, bowtie2 (v 2.4.4, https://github.com/BenLangmead/bowtie2) was used to remove host contamination and unmapped reads were used for assembly with SPAdes (v3.15.2, https://github.com/ablab/spades) with k-mer values of 21, 31, 41, 51, 61, and 71 and the options --careful. Extracted contigs were analyzed using BLASTx at NCBI to determine taxonomy. Contigs belonging to *orthoreoviridae* were subjected to ORFs prediction using Vgas; a tool for viral genome annotation (Zhang et al., 2019) with default parameters.

Genetic characterization and whole genome nucleotide alignment

For molecular characterization and genotype clustering, S1 gene (σC) segment sequences of the study isolates (Table 1) and all the sequences available in GenBank (https://www.ncbi.nlm.nih.gov/) (till March 2021) under taxon ID 38170 (Avian orthoreovirus) were downloaded. Similarly, the dataset for other segments (L1-L3, M1-M3 and S2-S4) was also downloaded for segment wise phylogenetic analyses. Sequence alignments were constructed separately for all the segments using MUSCLE v3.8.3 (Edgar, 2004) incorporated in AliView v1.26 (Larsson, 2014), where short sequences less than 75% of the respective gene segments were excluded . The software Geneious

Prime® 2020.0.5 (Biomatters Ltd., Auckland, New Zealand) was used for nucleotide sequence translation, pairwise distance analysis, percent nucleotide (nt) identities and amino acid (aa) similarities (Drummond et al., 2011). To evaluate the whole genome wide diversity, the nucleotide (nt) sequences of 14 CARV isolates (Table 1) generated in this study were concatenated and aligned with the reference vaccine strain (S1133) by the mVISTA method (http://genome.lbl.gov/vista/mvista/submit.shtml).

Maximum likelihood phylogenetic analysis and reassortment analysis

The phylogenetic trees were inferred by the maximum likelihood method using IQ-TREE web server (Trifinopoulos et al., 2016) with a substitution model selection option (ModelFinder implemented in IQ-TREE) and branch supports were calculated using ultrafast bootstrap approximation (UFBoot) with 1000 replicates (Nguyen et al., 2014). Finally, trees were visualized and edited in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). The genotypic clustering was determined according to tree topology and the clustering of previously classified reference strains (Egana et al., 2019; Ayalew et al., 2020; Torre et al. 2021). The genetic reassortment events were inferred by analyzing the incongruent topologies among the segment wise phylogenetic trees.

Results

Virus isolation: Histopathological examination (hock joint), virus isolation (VI) and RT-PCR of tendons and other samples from affected birds confirmed the presence of reovirus and CARV associated tenosynovitis. When the samples were inoculated in CEL cells, the reovirus-positive samples demonstrated reovirus specific cytopathic effects (syncytium

formation and detachment of cells) within 72 hours of incubation. The presence of CARV in infected cell culture supernatant was further confirmed by RT-PCR.

Genetic characterization based on concatenated whole genome

The concatenated whole genome alignment and comparative genome wide analysis of 14 CARV isolates of the present study showed high genetic divergence (up to 20%) among themselves. The study isolates showed (27-29%) genome wide nt divergence from the vaccine strain S1133 (Fig. 1). Based on the mVISTA analysis the L2, M1, M3, S2, S3 and S4 segments of all the study isolates showed segment wide nt identity with vaccine strain suggesting close ancestral relationship. On the other hand, L1, L3 and M2 segments showed localized divergence from the vaccine strain and having 79-84%, 87-90% and 67-85% nt identity with the vaccine strain, respectively. The S1 segment was very divergent having localized regions of nt identity (56-76 %) with the vaccine strain. The S1 segments of isolates 15/378 and 17/368 showed highest nt identity (76%) with the vaccine strain.

Molecular characterization based on genotype clustering

Based on the ML phylogenetic tree of σC gene of S1 segment, our isolates were clustered into four of the six well supported divergent genotypic clusters (GCs) viz GC1 (n=02), GC2 (n=03), GC4 (n=03) and GC6 (n=06) (Fig 4.2A). However, a different pattern of phylogenetic clustering was observed with concatenated whole-genome sequences (Fig 2B). Isolates clustered in GC1 and GC2 were clustered in subclusters GC1.3 and GC2.3, respectively. Isolates clustered in GC4 were clustered in two subclusters viz. GC4.1 (n=01) and GC4.2 (n=02) and six of our study isolates were clustered in GC6 (Table 4.1).

With phylogenetic analysis of GC1, it is evident that our sequences converge into the well-supported subcluster 1.3 (100% bootstrap) (Fig. 2A), which is formed by sequences form China, France, Germany, Taiwan, Israel and Brazil and are not closely related to vaccine sequences (S1133, 1733 and 2408). The study sequences have 97% of nt identity with each other and 86-88% nt identity with German sequences from 1997-98. Interestingly, our isolates are not closely related to the previously published German sequences but are closely related with Brazilian and French sequences having 96-97% and 95% nt identity, respectively. The study isolates have 79% nt identity with the vaccine strain S1133.

GC2 is comprised of three subclusters where the study isolates are clustered in the subcluster GC2.3 (Fig. 4.2A) formed by sequences from Brazil, Israel, South Korea and the US having 93-94% nt identity. Interestingly, the study isolates are closely related to strains from Israel and Brazil. The study isolates in GC2.3 have 93-99% nt identity with each other and 60% nt identity with the vaccine strains (S1133).

GC4 formed 3 subclusters viz. 4.1, 4.2 and 4.3. One of the study isolates was clustered in GC4.1 (97% bootstrap) clustering strains from Canada, Netherlands, Germany and the US. Our study isolate was closely related having 90-95% nt identity with strains from Netherlands and Germany and 58% nt identity with the vaccine strain S1133. The subcluster GC4.2 clustered two of the highly similar study isolates in a monophyletic cluster (100% bootstrap) having 95% nt identity with each other and 59% nt identity with the vaccine strain S1133.

GC6 is the recently evolved and is the most expressed cluster in this study clustering six of the study isolates in GC6 (100% bootstrap) with isolates from the US. The study

isolates are clustered in a monophyletic cluster having 99-100% nt identity with each other and 59% nt identity with the vaccine strain (S1133).

With the **S2 segment**, only one of our isolates (CARV/Germany/347/2017) was clustered with the vaccine strains having 91% nt identity with the vaccine strains (Fig. 4.3A). The rest 13 of the study isolates formed two monophyletic clusters having 2 (cluster 3) and 11 (cluster 2) of the study isolates with 95% and 97-100% within cluster nt identity, respectively. The study isolates in these two clusters (cluster 2 and 3) have 82-83% nt identity with the vaccine strain (S1133). The **S3 segment** formed 2 clusters where our isolates were clustered in cluster 1 forming 3 different monophyletic subclusters of eight, two and four of the study isolates. None of the study isolates clustered closely with the vaccine strains (Fig. 4.3). The 3 monophyletic subclusters formed by our isolates have 93%, 98-100% and 98-99% within subcluster nt identity and 86% nt identity with the vaccine strain (S1133), respectively. The **S4 segment** of our isolates is more conserved, forming one monophyletic cluster with the strains from Brazil, Hungary and Taiwan with two subclusters (Fig. 4.3). The two subclusters have 98-100% and 93-100% within subcluster nt identity, respectively and 78-80% nt identity with the vaccine strain (S1133). In S2, S3 and S4 gene segments, turkey reovirus sequences formed a separate cluster and another separate cluster was formed by reovirus from ducks and goose (Fig. 4.3A).

M class gene segments: In the ML tree of M1 segment our isolates formed two monophyletic clusters (Fig. 4.3B). Cluster 2 has big monophyletic cluster having ten of our isolates and 4 isolates were clustered with a Hungarian strain forming a subcluster having 86-98% within subcluster nt identity and the other subcluster have our 05 of the

study isolates having 100% nt identity. This monophyletic cluster 2 has 79-81% nt identity with the vaccine strain (S1133). The other monophyletic cluster is formed by our 4 study isolates having 92-94% within cluster nt identity and 85% nt identity with the vaccine strain (S1133) in cluster 1. Our isolates in the two monophyletic clusters have 80-81% nt identity between them. The **M2 segment** showed more diversity where one our isolates (CARV/Germany/2017/348) is very divergent from the other isolates and has 66-74% nt identity with other study isolates and 65% nt sequence identity with the vaccine strain (S1133). The rest of the study isolates were clustered in three separate clusters 2, 3 and 4 (Fig. 4.3B). Cluster 1 with 9 of the study isolates have 93-100% within cluster nt identity and 84% nt identity with the vaccine strain (S1133), cluster 4 with 03 of our study isolates are clustered with Hungarian and Taiwanese strains having 94-98% within cluster nt identity and 74% nt identity with the vaccine strain (S1133). One of our study isolates in cluster 3 is clustered with reference strains from Hungary, South Korea and Taiwan having 93-100% within subcluster nt identity and 76% nt identity with the vaccine strain (S1133). The two separate clusters of our isolates have 73-74% in between cluster nt identity. The **M3 segment** was more conserved where one of our study isolates was clustered closely with strains from Korea and Hungary in cluster 2 separated from the subcluster formed by 13 of the study isolates (Fig. 4.3B) having 95-100% within cluster and 87-88% between subclusters nt identity. The study isolates showed 79-81% nt identity with the vaccine strain (S1133) in the M3 segment. Reoviruses from turkeys and ducks formed one separate cluster each in M segment except M2 segment where more than one clusters of turkey and duck reoviruses were observed (Fig. 4.3B). Additionally,

two turkey reoviruses from Minnesota (US) were clustered with chicken reoviruses suggesting interspecies reassortment of M2 segments.

L class gene segments: Based on the ML phylogenetic tree of **L1 segment**, 13 of the study isolates are clustered in a monophyletic cluster 6 and one isolate is clustered in cluster 5 with reference strains from South Korea and Hungary (Fig. 4.3C). The cluster formed by German isolates is subdivided into two subclusters having 11 of the study isolates in one subcluster and two study isolates clustered in the other subcluster with a US strain. Cluster 6 has nt sequence identity of 87-100% whereas, within subclusters nt sequence identity is 95-100% and 92%, respectively. One of our study isolates is clustered with strains from Korea and Hungary having nt sequence identity of 86-91%. The monophyletic cluster 6 of our study isolates has 79-80% nt sequence identity with the vaccine strain (S1133) whereas our study isolates clustered with Korean and Hungarian strain showed 84% nt sequence identity with the vaccine strain (S1133). The ML tree of **L2 segment** (Fig. 4.3C) has 12 of our study isolates forming a monophyletic cluster in cluster 1 having 96-100% nt sequence identity with each other and 87-88% nt sequence identity with the vaccine strain (S1133). One of our study isolates (CARV/Germany/326/2018) is clustered in the vaccine cluster having 90% nt sequence identity with the vaccine strain (S1133) while one isolate (CARV/Germany/325/2016) was clustered with a Hungarian strain having 85% nt identity with the Hungarian strain and 87% nt identity with the vaccine strain (S1133). With the **L3 segment**, 11 of our study isolates are clustered with strains from Taiwan and Hungary in cluster 2 (Fig. 4.3C) having 90-100% nt identity with each other and 82% nt identity with the vaccine strain (S1133). Three of our study isolates are clustered in cluster 1 with strains from the US,

Canada, China, Korea and Taiwan having 88% nt identity with each other and 73% nt identity with the vaccine strain (S1133). In all the L class segments, turkey reovirus sequences formed a separate cluster and another separate cluster was formed by reovirus from ducks and goose (Fig. 4.3C).

Reassortment analysis based on phylogenetic incongruence

As evidenced with the mVISTA whole genome analysis, the relationship of our study isolates with the reference genome varied with each gene segment. To get the objective picture of genetic diversity due to segmental reassortment events, we analyzed the of segment wise phylogenetic trees based on clustering and incongruent topology of the isolates (Fig. 4.4). The study isolates showed multiple re-assortments. The isolates (CARV/Germany/) 2016/311 and 2017/368 had distant relationship with each other clustering separately in S1 segments. However, they are closely related w.r.t L1, L2, M1, M2, M3 and S4 segments. Similarly, the isolates 2017/367 and 2017/368 are clustered separately in S1 segment but are closely clustered in L1, L2, L3, S3 and S4 segments. Additionally, 18/326 and 18/372 are also closely clustered in L3, M1, M2, M3 and S2 segments but distantly related in L1, L2, S1, S3 and S4 segments. The isolate 2015/378 showed incongruent topologies in L2, M1, M2, M3, S2, S3 and S4 gene segments while it is clustered with the vaccine strain in the L1 and S1 segments. Five of the study isolates (2015/349, 2015/367, 2016/321, 2016/327 and 2016/328) are always clustered together in all the gene segments.

All the isolates in different GCs had highest within cluster (WIC) nt identity with the other isolates of the same GC in all the segments, whereas the highest in between cluster (IBC) nt identity varies according to the genome segments with the isolates of

other GCs (Supplementary table 2). In summary, the L1 segment of GC2 had highest IBC nt identity with only one GC (GC1) whereas, the L1 segment of GC1, GC4 and GC6 had highest IBC nt identity with more than one GCs. In L2 segment, GC1 and GC6 (except 16/328) has highest IBC nt identity with GC4 whereas GC2 and GC4 had highest IBC nt identity with more than one GCs. The L3 segment had highest IBC nt identity of GC6 with GC4 only but the other GCs had highest IBC nt identity with more than one GCs. The M1 segment of the study isolates of GC1 and GC4 showed highest IBC nt identity with each other. The highest IBC nt identity in M2 segment of GC4 and GC6 was with GC1 (except GC6-18/372 with GC2). The M3 segment showed GC wide more homology based on IBC nt identity. The S1 segment was more divergent showing only 61%-67% IBC nt identity. The S2 (84-98%), S3 (93-99%) and S4 (93-99%) segments showed more homology with higher WIC nt identity. The segment wise within cluster and in between cluster nt identity is summarized in supplementary table 2.

Discussion

Emerging CARV variants have caused significant economic losses to the poultry industry in the last decade worldwide. Therefore, genetic studies on variant CARVs need urgent emphasis for studying their evolutionary divergence and pathogenicity (Lu et al., 2015). This is supported by the fact that reoviruses have segmented RNA genome which has an inherent potential for causing genetic variations (Lu, et al., 2015; Gallardo, et al., 2017; Sellers, 2017; Ayalew et al., 2017). The availability of genetic data on variant CARVs from Europe is limited compared to the abundant genetic data from other countries (Yi and Lu, 2015). Hence, molecular surveillance based on WGS analysis of

CARVs is crucial in formulating strategies for prevention and control of reovirus infection. Here we studied CARV strains from Germany providing new insights into the current scenario of evolutionary characteristics of avian reovirus in Germany/Europe.

Genetic changes are evident in RNA viruses when passed multiple times in cell culture (Ghetas et al., 2015). In this study, reoviruses showed CPE in the first (to the most second) passage, so we expect minimal genetic changes in our isolates due to virus isolation and sequences should represent the variants circulating in the field.

Sigma C gene of S1 segment is the most variable region in the CARV genome and is the most widely studied genome segment of ARVs to classify reovirus isolates into different genotypic clusters (Schnitzer, 1985; Kant et al., 2003; Guardado et al., 2005). Based on the ML phylogenetic tree of Sigma C gene, the study isolates are clustered into four (GC1, GC2, GC4 and GC6) of the well-established six genotypic clusters (GC1 to GC6) as previously described (Egana et al., 2019; Ayalew et al., 2020) (Fig. 1) where GC1 is the vaccine cluster clustering the vaccine strains. Two of the study isolates are clustered in the GC1 (GC1.3). Three of the study isolates are clustered in GC4 (01 in GC4.1 and 02 in GC4.2) and GC2 (GC2.3) each. The most recently evolved GC6 clustered 06 of our isolates in GC6. The most predominant GC in this study is the recently evolved GC6 (46%) which is similar to the results reported by Zhang et al. (2019) but different from the studies by Lu et al., (2015), Palomino et al., (2018) and Egana et al. (2019) which reported the predominant GCs as GC2, GC4 and GC1, respectively. Our results are different from the previous results of Kant et al. (2003) and Troxler et al. (2013) where they reported that most of the Dutch and German isolates were clustered in GC1 and GC4 while the French isolates were clustered in GC1. Our

results suggests that there is a shift in the predominance of GCs over the years from 1980-2013 to 2015-2018 but no such time related difference could be established by Kant et al. (2003) between 1980-2000. Similar shift in the predominance from GC1 to GC6 between 2015 to 2018 was observed in CARVs circulating in the state of California in the US (Egaña et al., 2019). The predominance of GCs varies with time and geographical location as reported previously (Lu et al. 2015; Ayalew et al. 2017; Sellers, 2017; Palomino-Tapia et al. 2018). Similar pattern in the shift of predominant GCs suggests that CARVs are rapidly evolving all around the world and variant strains from GC6 are prevalent and circulating in the poultry flocks. Extensive use of autogenous vaccines using strains from the predominant GCs may be one of the contributing factors influencing this shift leading to vaccination failure. The predominant GCs should be studied more thoroughly to consider the strains or their antigenic segments to include into the vectored vaccines.

The study isolates in GC1 have the nt sequence identity (79%) higher than isolates in the other GCs (58-62%) but it is still low as reported by Zhang et al. (2019). Similarly, 50% genetic diversity was reported in chicken reovirus from Israel (Goldenberg et al., 2010) and turkey reovirus from the US (Day et al., 2007). High genetic diversity explains the accelerated evolution and appearance of new GCs responsible for ineffectiveness of the commercially available vaccines against the German variants. Studies in other RNA viruses have reported low cross protection when the aa difference between the challenge and vaccine viruses exceeds 5% (Cavanagh, 2007). Palomino et al. (2018) reported low cross protection by commercial or autogenous vaccines prepared from prevalent field strains from the same cluster having low identity.

With WGS data, we evaluated the divergence of each gene segment from the vaccine strains, the contribution of each gene segment in the formation of reovirus variants and the possible segmental reassortment events undergoing in the reovirus variants. Our WGS results show that L3, M2 and the S1 segments are most variable and showed maximum divergence from the vaccine strains as observed by Egana et al., (2019). The probable reason behind the divergence of these segments is that they code for the outer capsid proteins of the reovirus and face the maximum selection pressure. The genetic divergence of these segments can be correlated with the potential role they play in the antigenic variability and increased pathogenicity (Sellers, 2017) of the variant reoviruses which need to be further studied to establish this relationship. The clustering of German sequences with strains from different countries in phylogenetic trees of different gene segments suggest widespread global intermixing of variant CARVs due to trade of live birds, poultry products, migratory birds or by other means (Ayalew et al., 2020). Since ARVs are resistant to common disinfectants and can survive in the environment for longer periods, might be helping in cross country introductions (Mor et al.,2015). Similar phenomenon between Canada and the US was reported by Ayalew et al., (2020).

Another important mechanism by which segmented RNA viruses evolve is by the re-assortment of genome segments (McDonald et al., 2016; Ayalew et al. 2020). Extensive re-assortment events were observed in this study from the incongruent topologies in phylogenetic trees of different segments which is further supported by the highest segmental nucleotide identity between isolates. These results give strong evidence that segmental re-assortment events contribute to the rapid evolution and

genetic diversity of CARVs. Also, segments other than σC gene also contribute to the evolution, hence should be given due attention. The re-assortment phenomenon might be due to co-circulation of multiple CARV variants from different GCs. Co-circulation of CARV variants in the same farm at the same time may be responsible for the re-assortment as multiple variants have been reported in previous outbreaks (Lu et al. 2015; Ayalew et al. 2017; Palomino-Tapia et al. 2018; Egana-Labrin et al. 2019). Therefore, there is high probability co-infection and evolution through exchange of gene segments of viruses from different GCs. Tang et al., (2015) have reported presence of natural co-infection of two variant CARVs. However, no re-assortment events were found by Egana-Labirin et al., (2019). It might be because six of the seven viral genomes analyzed were from the same GC which would miss important information from other GCs. Additionally, our isolates in GC6 showed most phylogenetic congruence suggesting limited segmental exchange. This is probably due to the reason that the GC6 has recently evolved and may be still in the process of its establishment. Also, reassortment may be beneficial as it may increase the survivability and capacity of virus to replicate in different host (McDonald et a., 2016).

In conclusion, this study addresses the molecular characterization, divergence and evolutionary pattern of CARVs in Germany/Europe. This study also provides new insights into the contribution of gene segments other than Sigma C in the evolution and formation of variant CARVs. Previous studies have classified CARVs into different lineages based on the Sigma C gene, but other segments did not follow the identical pattern which suggests that different CARV genome segments may evolve in an independent manner. To understand the evolutionary dynamics of CARVs circulating in

Germany/ Europe, there is a need to fill the gap in the available sequence data from year 2000 to 2021 and continuous molecular epidemiological and reovirus surveillance studies. In this study, we provided some important information about CARVs circulating in Germany during 2015-2018. Additionally, this study will also guide researchers to consider other immunogenic gene segments to include in the future sub-unit vaccines to be developed.

Acknowledgments: RK acknowledges the financial assistance in the form of Netaji Subhash International Fellowship from the Indian Council of Agricultural Research, India, Pomeroy Legacy scholarship and MNDRIVE fellowship from the University of Minnesota to conduct this Ph.D. research project.

Table 4.1. List of reovirus isolates from Germany used in this study

S. No.	Case ID	Year of Isolation	GC	Disease	Samples	Cells culture
1.	378	2015	1.3	VA[a]	Tendons	CEL (P2)
2.	368	2017	1.3	VA	Tendons	CEL (P2)
3.	326	2018	2.3	VA	Tendons	CEL (P2)
4.	367	2017	2.3	VA	Tendons	CEL (P2)
5.	325	2016	2.3	VA	Tendons	CEL (P2)
6.	311	2016	4.1	VA	Tendons	CEL (P2)
7.	348	2017	4.2	VA	Tendons	CEL (P2)
8.	347	2017	4.2	VA	Tendons	CEL (P2)
9.	328	2016	6.1	RSS[b]	Cecal tonsils	CEL (P2)
10.	321	2016	6.1	NA[c]	Kidneys	CEL (P2)
11.	367	2015	6.1	VA	Tendons	CEL (P2)
12.	349	2015	6.1	RSS	Cecal tonsils	CEL (P2)
13.	327	2016	6.1	VA	Tendons	CEL (P2)
14.	372	2018	6.1	RSS	Trachea	CEL (P2)

[a] viral arthritis, [b] runting stunting syndrome, [c] Information not available

Fig 4.1. Results of the mVISTA analysis of the concatenated genome sequences of the 35 studied US avian orthoreovirus strains in comparison to the reference vaccine strain S1133. Areas shaded with pink and white indicate >90%, and <90% nt sequence identities, respectively. Scale bar: approximate length of each gene

Supplementary Table 2. The comparative heatmap of the highest nt identity of each segment of virus isolates in between cluster (IBC) and virus isolates within cluster group (WIC).
* This is an excel file

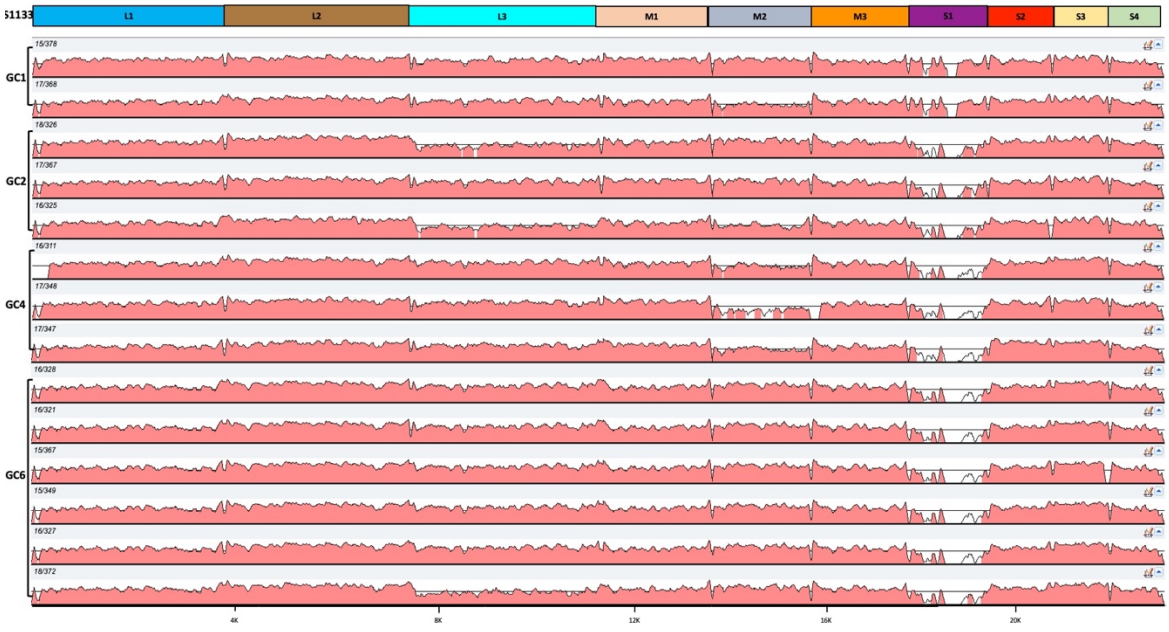
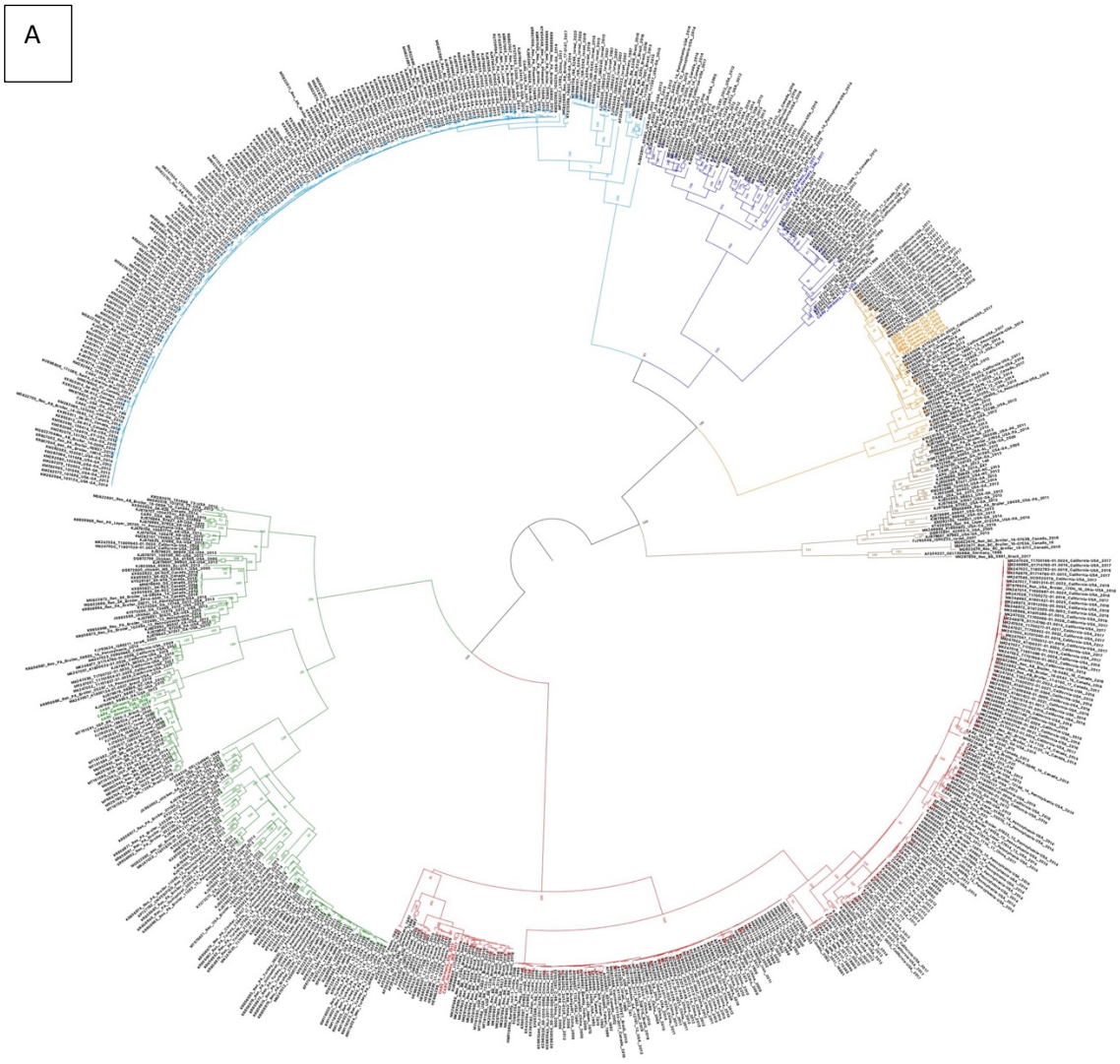


Fig 4.2. (A) Maximum Likelihood (ML) phylogenetic tree of 690 ARV strains based on the σC sequence variability. Color codes of branches represent genotype cluster classification (GC1-Red; GC2-Green; GC3-Pink; GC4-Blue; GC5-Brown; GC6-Orange) and sub-clusters are identified by the name. (B) ML phylogenetic tree constructed based on the concatenated full-genome sequence of the fourteen CARV isolates with vaccine strain S1133.



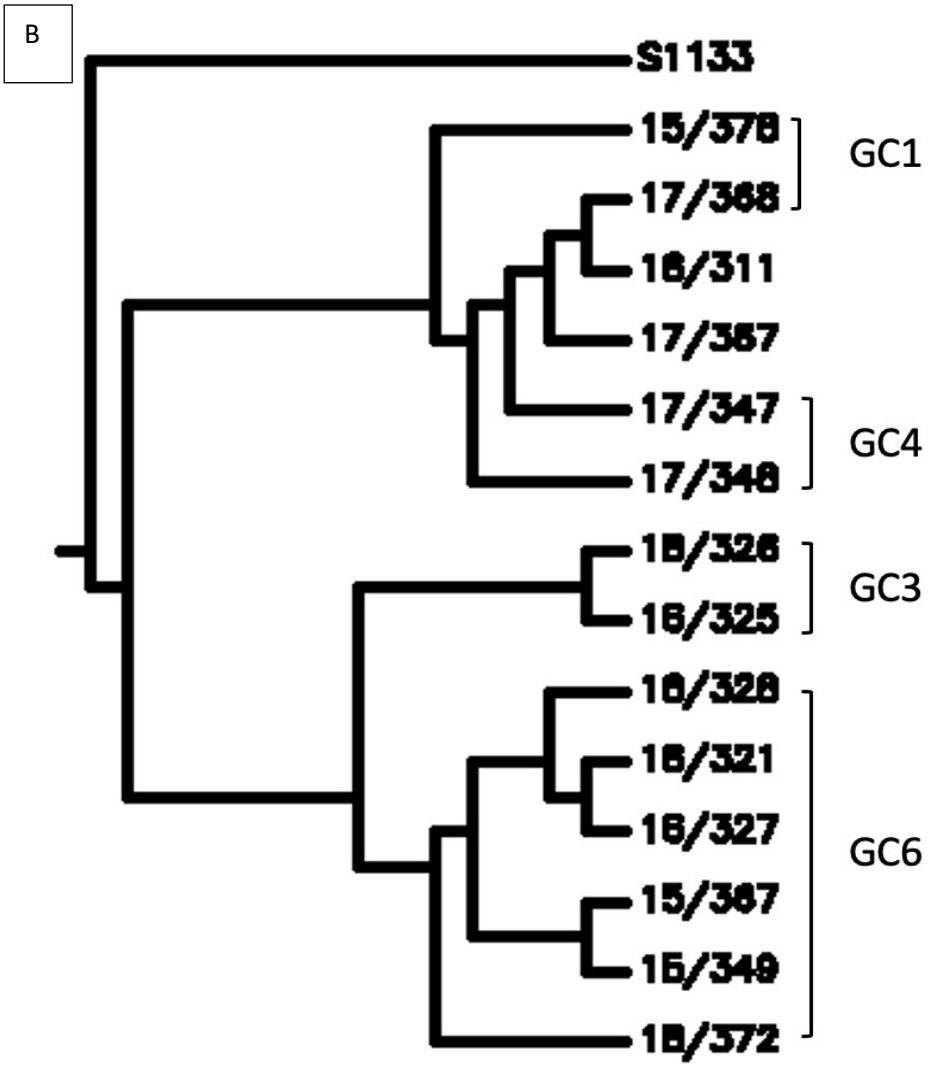
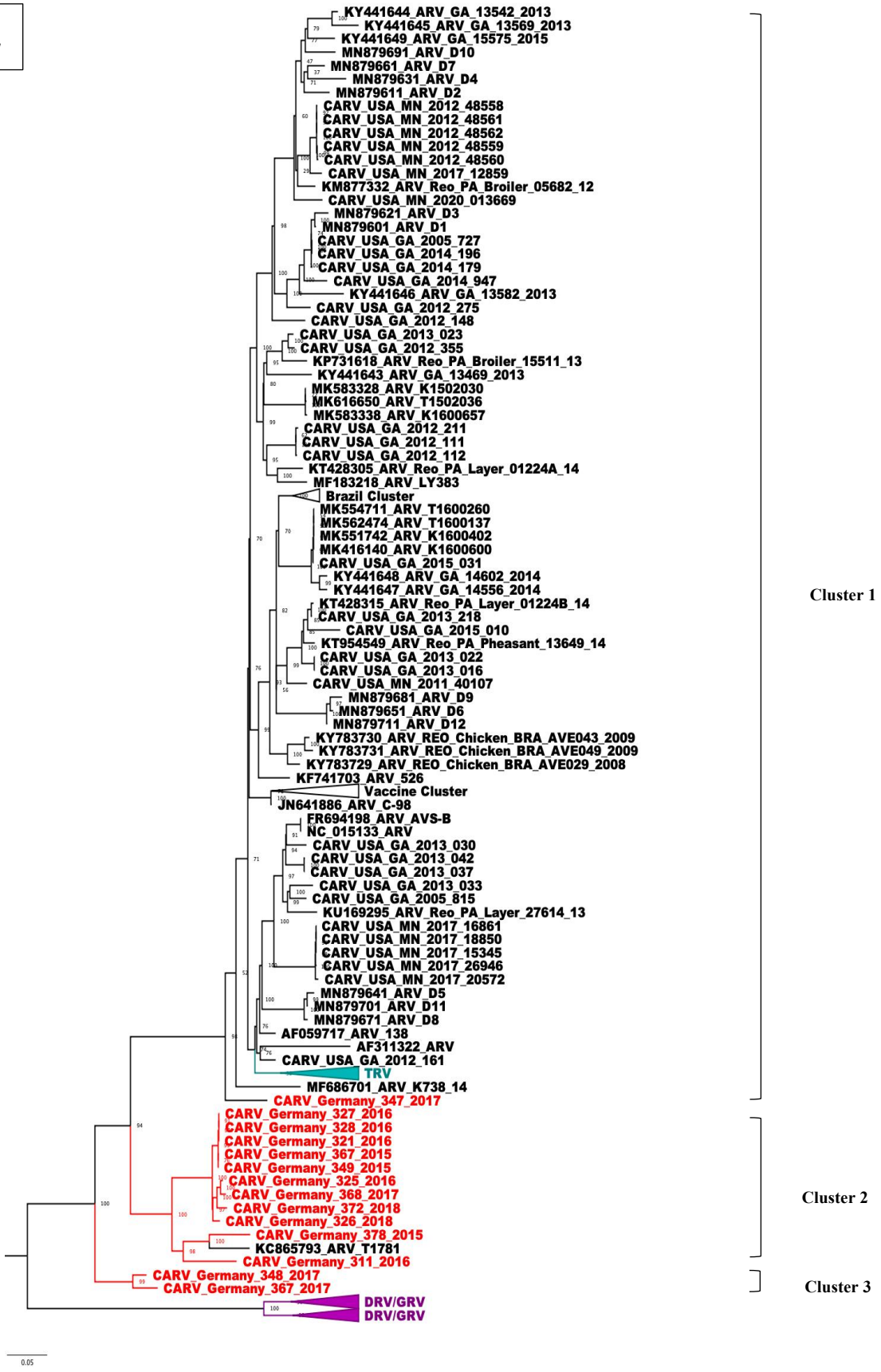
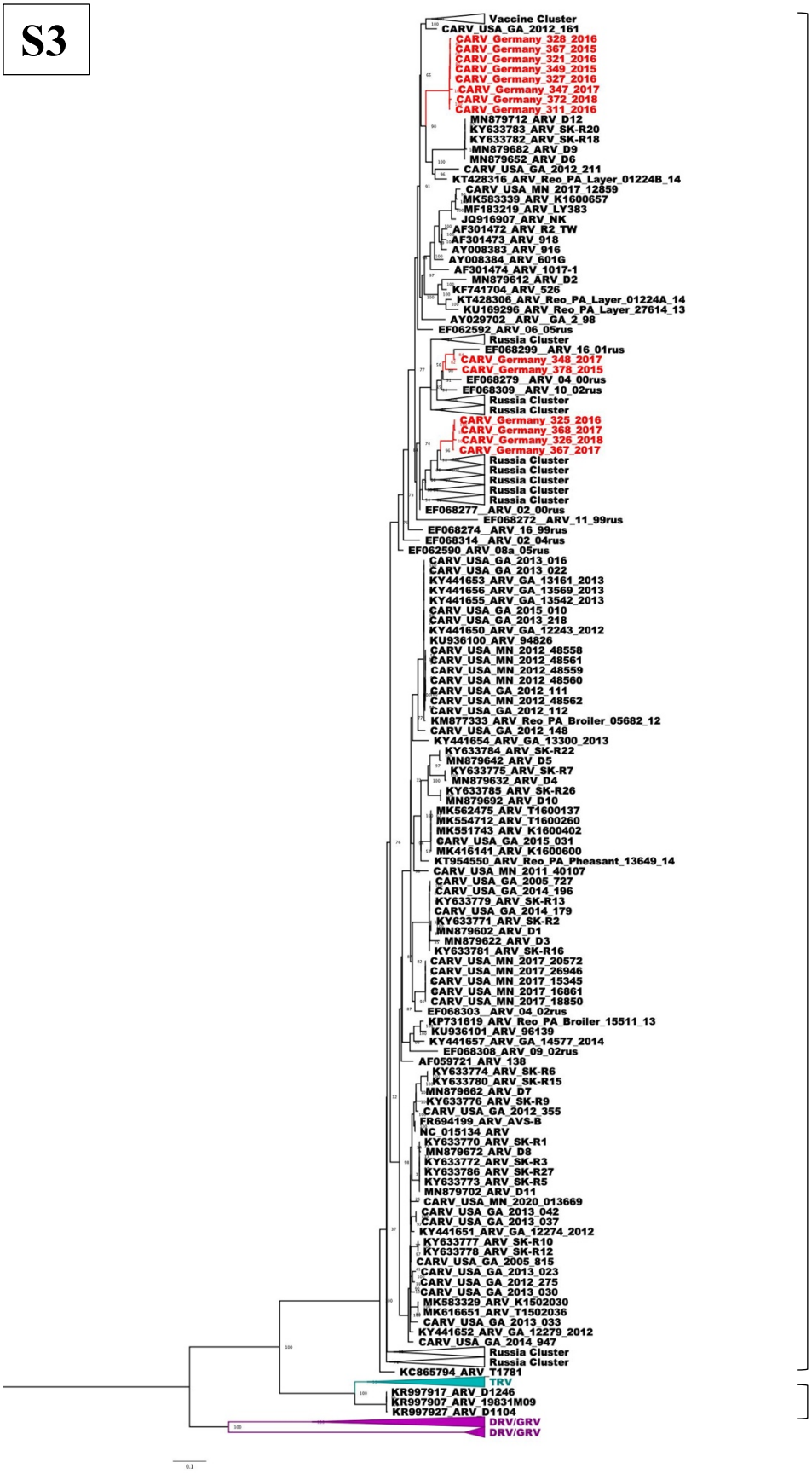

B
S1133
15/378
17/368
16/311
17/367
17/347
17/348
16/326
16/325
16/328
16/321
16/327
15/367
15/349
16/372
GC1
GC4
GC3
GC6

Fig 4.3A. Nucleotide sequence-based ML phylogenetic trees showing the clustering of CARVs based on the S2, S3 and S4 gene segments of viruses available from GenBank. Phylogenetic calculations were carried out using the maximum-likelihood method applying the best-fit models calculated for each gene. The scale bar is proportional to the genetic distance.

S2


KY441644_ARV_GA_13542_2013
KY441645_ARV_GA_13569_2013
KY441649_ARV_GA_15575_2015
MN879691_ARV_D10
MN879661_ARV_D7
MN879631_ARV_D4
MN879611_ARV_D2
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48561
CARV_USA_MN_2012_48562
CARV_USA_MN_2012_48559
CARV_USA_MN_2012_48560
CARV_USA_MN_2017_12859
KM877332_ARV_Reo_PA_Broiler_05682_12
CARV_USA_MN_2020_013669
MN879621_ARV_D3
MN879601_ARV_D1
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
CARV_USA_GA_2014_179
CARV_USA_GA_2014_947
KY441646_ARV_GA_13582_2013
CARV_USA_GA_2012_275
CARV_USA_GA_2012_148
CARV_USA_GA_2013_023
CARV_USA_GA_2012_355
KP731618_ARV_Reo_PA_Broiler_15511_13
KY441643_ARV_GA_13469_2013
MK583328_ARV_K1502030
MK616650_ARV_T1502036
MK583338_ARV_K1600657
CARV_USA_GA_2012_211
CARV_USA_GA_2012_111
CARV_USA_GA_2012_112
KT428305_ARV_Reo_PA_Layer_01224A_14
MF183218_ARV_LY383
Brazil Cluster
MK554711_ARV_T1600260
MK562474_ARV_T1600137
MK551742_ARV_K1600402
MK416140_ARV_K1600600
CARV_USA_GA_2015_031
KY441648_ARV_GA_14602_2014
KY441647_ARV_GA_14556_2014
KT428315_ARV_Reo_PA_Layer_01224B_14
CARV_USA_GA_2013_218
CARV_USA_GA_2015_010
KT954549_ARV_Reo_PA_Pheasant_13649_14
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
CARV_USA_MN_2011_40107
MN879681_ARV_D9
MN879651_ARV_D6
MN879711_ARV_D12
KY783730_ARV_REO_Chicken_BRA_AVE043_2009
KY783731_ARV_REO_Chicken_BRA_AVE049_2009
KY783729_ARV_REO_Chicken_BRA_AVE029_2008
KF741703_ARV_526
Vaccine Cluster
JN641886_ARV_C-98
FR694198_ARV_AVS-B
NC_015133_ARV
CARV_USA_GA_2013_030
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
CARV_USA_GA_2013_033
CARV_USA_GA_2005_815
KU169295_ARV_Reo_PA_Layer_27614_13
CARV_USA_MN_2017_16861
CARV_USA_MN_2017_18850
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_20572
MN879641_ARV_D5
MN879701_ARV_D11
MN879671_ARV_D8
AF059717_ARV_138
AF311322_ARV
CARV_USA_GA_2012_161
TRV
MF686701_ARV_K738_14
CARV_Germany_347_2017
CARV_Germany_327_2016
CARV_Germany_328_2016
CARV_Germany_321_2016
CARV_Germany_367_2015
CARV_Germany_349_2015
CARV_Germany_325_2016
CARV_Germany_368_2017
CARV_Germany_372_2018
CARV_Germany_326_2018
CARV_Germany_378_2015
KC865793_ARV_T1781
CARV_Germany_311_2016
CARV_Germany_348_2017
CARV_Germany_367_2017
DRV/GRV
DRV/GRV
0.05
Cluster 1
Cluster 2
Cluster 3

S3


Vaccine Cluster
CARV_USA_GA_2012_161
CARV_Germany_328_2016
CARV_Germany_367_2015
CARV_Germany_321_2016
CARV_Germany_349_2015
CARV_Germany_327_2016
CARV_Germany_347_2017
CARV_Germany_372_2018
CARV_Germany_311_2016
MN879712_ARV_D12
KY633783_ARV_SK-R20
KY633782_ARV_SK-R18
MN879682_ARV_D9
MN879652_ARV_D6
CARV_USA_GA_2012_211
KT428316_ARV_Reo_PA_Layer_01224B_14
CARV_USA_MN_2017_12859
MK583339_ARV_K1600657
MF183219_ARV_LY383
JQ916907_ARV_NK
AF301472_ARV_R2_TW
AF301473_ARV_918
AY008383_ARV_916
AY008384_ARV_601G
AF301474_ARV_1017-1
MN879612_ARV_D2
KF741704_ARV_526
KT428306_ARV_Reo_PA_Layer_01224A_14
KU169296_ARV_Reo_PA_Layer_27614_13
AY029702__ARV__GA_2_98
EF062592_ARV_06_05rus
Russia Cluster
EF068299__ARV_16_01rus
CARV_Germany_348_2017
CARV_Germany_378_2015
EF068279__ARV_04_00rus
EF068309__ARV_10_02rus
Russia Cluster
Russia Cluster
CARV_Germany_325_2016
CARV_Germany_368_2017
CARV_Germany_326_2018
CARV_Germany_367_2017
Russia Cluster
Russia Cluster
Russia Cluster
Russia Cluster
Russia Cluster
EF068277__ARV_02_00rus
EF068272__ARV_11_99rus
EF068274__ARV_16_99rus
EF068314__ARV_02_04rus
EF062590_ARV_08a_05rus
CARV_USA_GA_2013_016
CARV_USA_GA_2013_022
KY441653_ARV_GA_13161_2013
KY441656_ARV_GA_13569_2013
KY441655_ARV_GA_13542_2013
CARV_USA_GA_2015_010
CARV_USA_GA_2013_218
KY441650_ARV_GA_12243_2012
KU936100_ARV_94826
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48561
CARV_USA_MN_2012_48559
CARV_USA_MN_2012_48560
CARV_USA_GA_2012_111
CARV_USA_MN_2012_48562
CARV_USA_GA_2012_112
KM877333_ARV_Reo_PA_Broiler_05682_12
CARV_USA_GA_2012_148
KY441654_ARV_GA_13300_2013
KY633784_ARV_SK-R22
MN879642_ARV_D5
KY633775_ARV_SK-R7
MN879632_ARV_D4
KY633785_ARV_SK-R26
MN879692_ARV_D10
MK562475_ARV_T1600137
MK554712_ARV_T1600260
MK551743_ARV_K1600402
CARV_USA_GA_2015_031
MK416141_ARV_K1600600
KT954550_ARV_Reo_PA_Pheasant_13649_14
CARV_USA_MN_2011_40107
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
KY633779_ARV_SK-R13
CARV_USA_GA_2014_179
KY633771_ARV_SK-R2
MN879602_ARV_D1
MN879622_ARV_D3
KY633781_ARV_SK-R16
CARV_USA_MN_2017_20572
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_16861
CARV_USA_MN_2017_18850
EF068303__ARV_04_02rus
KP731619_ARV_Reo_PA_Broiler_15511_13
KU936101_ARV_96139
KY441657_ARV_GA_14577_2014
EF068308_ARV_09_02rus
AF059721_ARV_138
KY633774_ARV_SK-R6
KY633780_ARV_SK-R15
MN879662_ARV_D7
KY633776_ARV_SK-R9
CARV_USA_GA_2012_355
FR694199_ARV_AVS-B
NC_015134_ARV
KY633770_ARV_SK-R1
MN879672_ARV_D8
KY633772_ARV_SK-R3
KY633786_ARV_SK-R27
KY633773_ARV_SK-R5
MN879702_ARV_D11
CARV_USA_MN_2020_013669
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
KY441651_ARV_GA_12274_2012
KY633777_ARV_SK-R10
KY633778_ARV_SK-R12
CARV_USA_GA_2005_815
CARV_USA_GA_2013_023
CARV_USA_GA_2012_275
CARV_USA_GA_2013_030
MK583329_ARV_K1502030
MK616651_ARV_T1502036
CARV_USA_GA_2013_033
KY441652_ARV_GA_12279_2012
CARV_USA_GA_2014_947
Russia Cluster
Russia Cluster
KC865794_ARV_T1781
TRV
KR997917_ARV_D1246
KR997907_ARV_19831M09
KR997927_ARV_D1104
DRV/GRV
DRV/GRV
0.1
Cluster 1
Cluster 2

S4

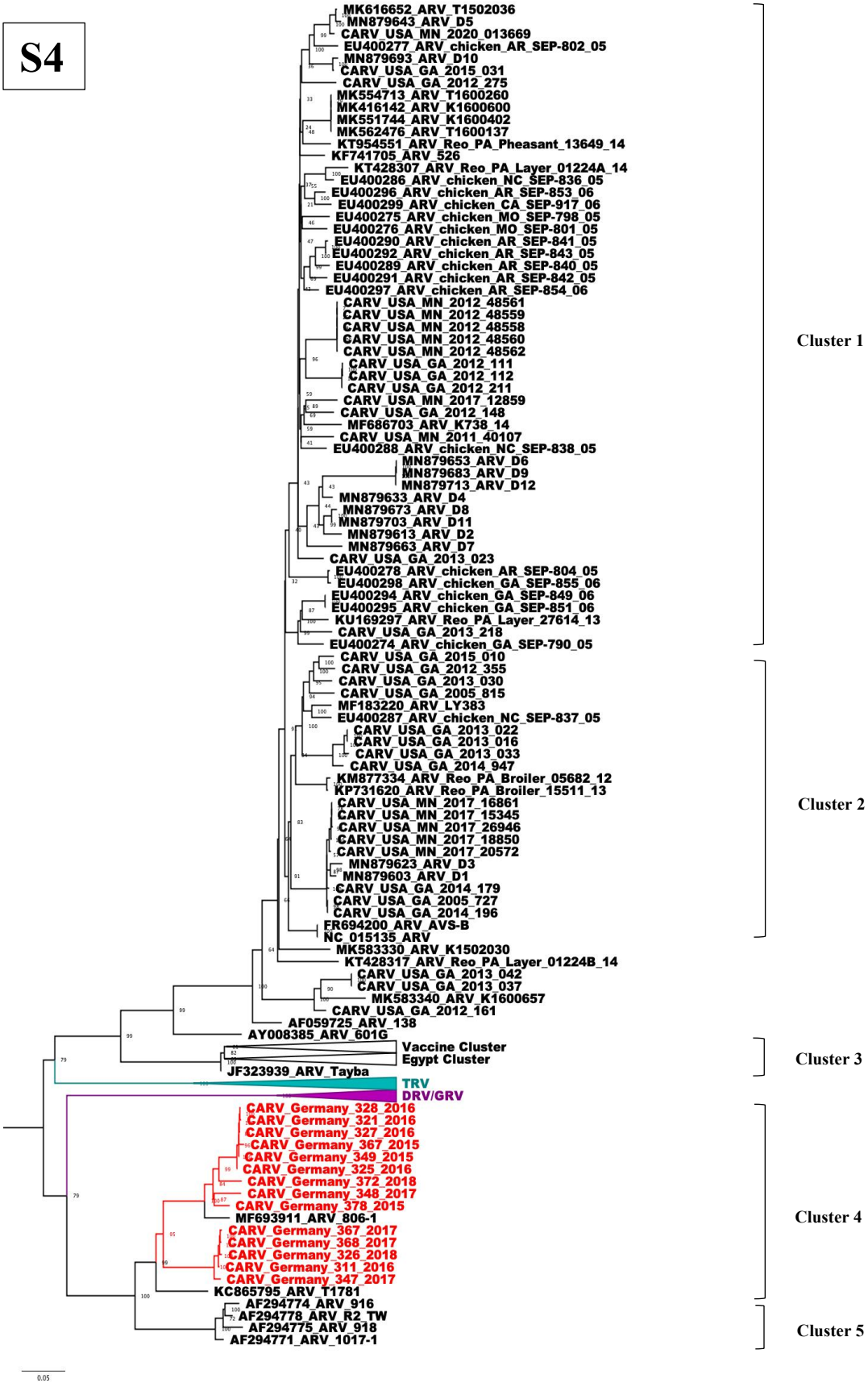
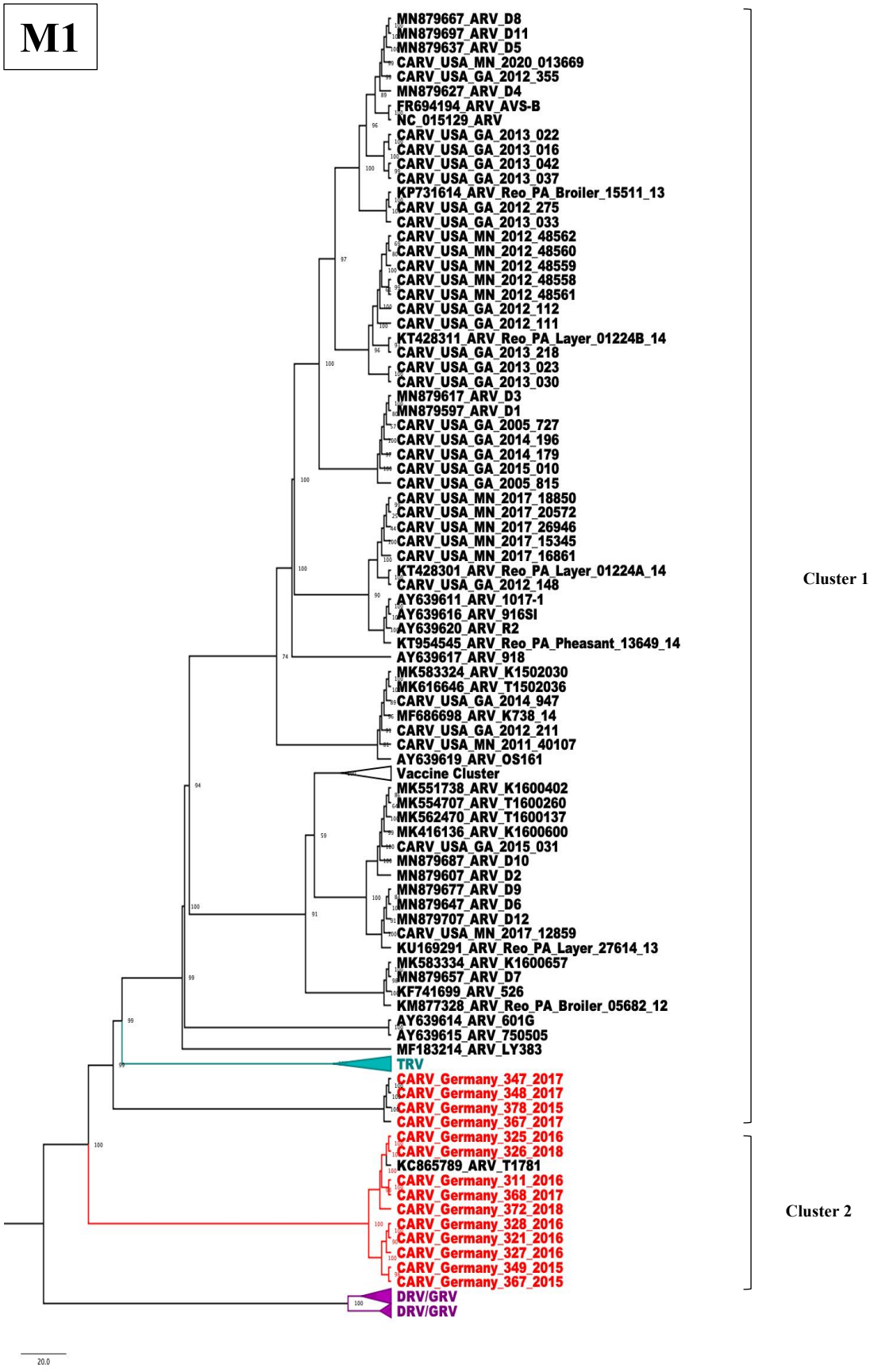
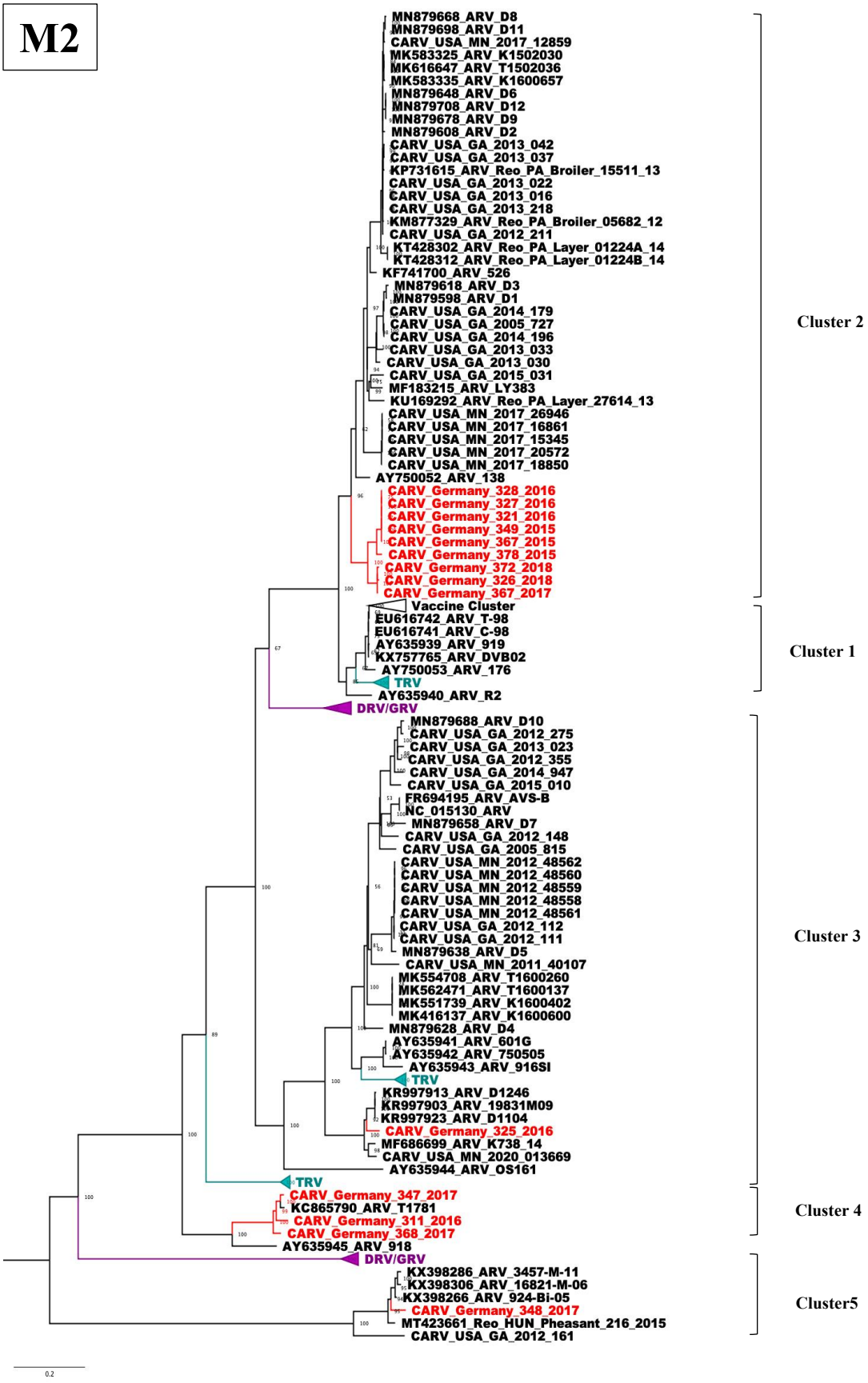

MK616652_ARV_T1502036
MN879643_ARV_D5
CARV_USA_MN_2020_013669
EU400277_ARV_chicken_AR_SEP-802_05
MN879693_ARV_D10
CARV_USA_GA_2015_031
CARV_USA_GA_2012_275
MK554713_ARV_T1600260
MK416142_ARV_K1600600
MK551744_ARV_K1600402
MK562476_ARV_T1600137
KT954551_ARV_Reo_PA_Pheasant_13649_14
KF741705_ARV_526
KT428307_ARV_Reo_PA_Layer_01224A_14
EU400286_ARV_chicken_NC_SEP-836_05
EU400296_ARV_chicken_AR_SEP-853_06
EU400299_ARV_chicken_CA_SEP-917_06
EU400275_ARV_chicken_MO_SEP-798_05
EU400276_ARV_chicken_MO_SEP-801_05
EU400290_ARV_chicken_AR_SEP-841_05
EU400292_ARV_chicken_AR_SEP-843_05
EU400289_ARV_chicken_AR_SEP-840_05
EU400291_ARV_chicken_AR_SEP-842_05
EU400297_ARV_chicken_AR_SEP-854_06
CARV_USA_MN_2012_48561
CARV_USA_MN_2012_48559
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48560
CARV_USA_MN_2012_48562
CARV_USA_GA_2012_111
CARV_USA_GA_2012_112
CARV_USA_GA_2012_211
CARV_USA_MN_2017_12859
CARV_USA_GA_2012_148
MF686703_ARV_K738_14
CARV_USA_MN_2011_40107
EU400288_ARV_chicken_NC_SEP-838_05
MN879653_ARV_D6
MN879683_ARV_D9
MN879713_ARV_D12
MN879633_ARV_D4
MN879673_ARV_D8
MN879703_ARV_D11
MN879613_ARV_D2
MN879663_ARV_D7
CARV_USA_GA_2013_023
EU400278_ARV_chicken_AR_SEP-804_05
EU400298_ARV_chicken_GA_SEP-855_06
EU400294_ARV_chicken_GA_SEP-849_06
EU400295_ARV_chicken_GA_SEP-851_06
KU169297_ARV_Reo_PA_Layer_27614_13
CARV_USA_GA_2013_218
EU400274_ARV_chicken_GA_SEP-790_05
CARV_USA_GA_2015_010
CARV_USA_GA_2012_355
CARV_USA_GA_2013_030
CARV_USA_GA_2005_815
MF183220_ARV_LY383
EU400287_ARV_chicken_NC_SEP-837_05
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
CARV_USA_GA_2013_033
CARV_USA_GA_2014_947
KM877334_ARV_Reo_PA_Broiler_05682_12
KP731620_ARV_Reo_PA_Broiler_15511_13
CARV_USA_MN_2017_16861
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_18850
CARV_USA_MN_2017_20572
MN879623_ARV_D3
MN879603_ARV_D1
CARV_USA_GA_2014_179
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
FR694200_ARV_AVS-B
NC_015135_ARV
MK583330_ARV_K1502030
KT428317_ARV_Reo_PA_Layer_01224B_14
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
MK583340_ARV_K1600657
CARV_USA_GA_2012_161
AF059725_ARV_138
AY008385_ARV_601G
Vaccine Cluster
Egypt Cluster
JF323939_ARV_Tayba
TRV
DRV/GRV
CARV_Germany_328_2016
CARV_Germany_321_2016
CARV_Germany_327_2016
CARV_Germany_367_2015
CARV_Germany_349_2015
CARV_Germany_325_2016
CARV_Germany_372_2018
CARV_Germany_348_2017
CARV_Germany_378_2015
MF693911_ARV_806-1
CARV_Germany_367_2017
CARV_Germany_368_2017
CARV_Germany_326_2018
CARV_Germany_311_2016
CARV_Germany_347_2017
KC865795_ARV_T1781
AF294774_ARV_916
AF294778_ARV_R2_TW
AF294775_ARV_918
AF294771_ARV_1017-1
0.05
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5

Fig 4.3B. Nucleotide sequence-based ML phylogenetic trees showing the clustering of CARVs based on the M1, M2 andM3 gene segments of viruses available from GenBank. Phylogenetic calculations were carried out using the maximum-likelihood method applying the best-fit models calculated for each gene. The scale bar is proportional to the genetic distance.


M1
MN879667_ARV_D8
MN879697_ARV_D11
MN879637_ARV_D5
CARV_USA_MN_2020_013669
CARV_USA_GA_2012_355
MN879627_ARV_D4
FR694194_ARV_AVS-B
NC_015129_ARV
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
KP731614_ARV_Reo_PA_Broiler_15511_13
CARV_USA_GA_2012_275
CARV_USA_GA_2013_033
CARV_USA_MN_2012_48562
CARV_USA_MN_2012_48560
CARV_USA_MN_2012_48559
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48561
CARV_USA_GA_2012_112
CARV_USA_GA_2012_111
KT428311_ARV_Reo_PA_Layer_01224B_14
CARV_USA_GA_2013_218
CARV_USA_GA_2013_023
CARV_USA_GA_2013_030
MN879617_ARV_D3
MN879597_ARV_D1
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
CARV_USA_GA_2014_179
CARV_USA_GA_2015_010
CARV_USA_GA_2005_815
CARV_USA_MN_2017_18850
CARV_USA_MN_2017_20572
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_16861
KT428301_ARV_Reo_PA_Layer_01224A_14
CARV_USA_GA_2012_148
AY639611_ARV_1017-1
AY639616_ARV_916SI
AY639620_ARV_R2
KT954545_ARV_Reo_PA_Pheasant_13649_14
AY639617_ARV_918
MK583324_ARV_K1502030
MK616646_ARV_T1502036
CARV_USA_GA_2014_947
MF686698_ARV_K738_14
CARV_USA_GA_2012_211
CARV_USA_MN_2011_40107
AY639619_ARV_OS161
Vaccine Cluster
MK551738_ARV_K1600402
MK554707_ARV_T1600260
MK562470_ARV_T1600137
MK416136_ARV_K1600600
CARV_USA_GA_2015_031
MN879687_ARV_D10
MN879607_ARV_D2
MN879677_ARV_D9
MN879647_ARV_D6
MN879707_ARV_D12
CARV_USA_MN_2017_12859
KU169291_ARV_Reo_PA_Layer_27614_13
MK583334_ARV_K1600657
MN879657_ARV_D7
KF741699_ARV_526
KM877328_ARV_Reo_PA_Broiler_05682_12
AY639614_ARV_601G
AY639615_ARV_750505
MF183214_ARV_LY383
TRV
CARV_Germany_347_2017
CARV_Germany_348_2017
CARV_Germany_378_2015
CARV_Germany_367_2017
CARV_Germany_325_2016
CARV_Germany_326_2018
KC865789_ARV_T1781
CARV_Germany_311_2016
CARV_Germany_368_2017
CARV_Germany_372_2018
CARV_Germany_328_2016
CARV_Germany_321_2016
CARV_Germany_327_2016
CARV_Germany_349_2015
CARV_Germany_367_2015
DRV/GRV
DRV/GRV
Cluster 1
Cluster 2
20.0


M2
MN879668_ARV_D8
MN879698_ARV_D11
CARV_USA_MN_2017_12859
MK583325_ARV_K1502030
MK616647_ARV_T1502036
MK583335_ARV_K1600657
MN879648_ARV_D6
MN879708_ARV_D12
MN879678_ARV_D9
MN879608_ARV_D2
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
KP731615_ARV_Reo_PA_Broiler_15511_13
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
CARV_USA_GA_2013_218
KM877329_ARV_Reo_PA_Broiler_05682_12
CARV_USA_GA_2012_211
KT428302_ARV_Reo_PA_Layer_01224A_14
KT428312_ARV_Reo_PA_Layer_01224B_14
KF741700_ARV_526
MN879618_ARV_D3
MN879598_ARV_D1
CARV_USA_GA_2014_179
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
CARV_USA_GA_2013_033
CARV_USA_GA_2013_030
CARV_USA_GA_2015_031
MF183215_ARV_LY383
KU169292_ARV_Reo_PA_Layer_27614_13
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_16861
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_20572
CARV_USA_MN_2017_18850
AY750052_ARV_138
CARV_Germany_328_2016
CARV_Germany_327_2016
CARV_Germany_321_2016
CARV_Germany_349_2015
CARV_Germany_367_2015
CARV_Germany_378_2015
CARV_Germany_372_2018
CARV_Germany_326_2018
CARV_Germany_367_2017
Vaccine Cluster
EU616742_ARV_T-98
EU616741_ARV_C-98
AY635939_ARV_919
KX757765_ARV_DVB02
AY750053_ARV_176
TRV
AY635940_ARV_R2
DRV/GRV
MN879688_ARV_D10
CARV_USA_GA_2012_275
CARV_USA_GA_2013_023
CARV_USA_GA_2012_355
CARV_USA_GA_2014_947
CARV_USA_GA_2015_010
FR694195_ARV_AVS-B
NC_015130_ARV
MN879658_ARV_D7
CARV_USA_GA_2012_148
CARV_USA_GA_2005_815
CARV_USA_MN_2012_48562
CARV_USA_MN_2012_48560
CARV_USA_MN_2012_48559
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48561
CARV_USA_GA_2012_112
CARV_USA_GA_2012_111
MN879638_ARV_D5
CARV_USA_MN_2011_40107
MK554708_ARV_T1600260
MK562471_ARV_T1600137
MK551739_ARV_K1600402
MK416137_ARV_K1600600
MN879628_ARV_D4
AY635941_ARV_601G
AY635942_ARV_750505
AY635943_ARV_916SI
TRV
KR997913_ARV_D1246
KR997903_ARV_19831M09
KR997923_ARV_D1104
CARV_Germany_325_2016
MF686699_ARV_K738_14
CARV_USA_MN_2020_013669
AY635944_ARV_OS161
TRV
CARV_Germany_347_2017
KC865790_ARV_T1781
CARV_Germany_311_2016
CARV_Germany_368_2017
AY635945_ARV_918
DRV/GRV
KX398286_ARV_3457-M-11
KX398306_ARV_16821-M-06
KX398266_ARV_924-Bi-05
CARV_Germany_348_2017
MT423661_Reo_HUN_Pheasant_216_2015
CARV_USA_GA_2012_161
0.2
Cluster 2
Cluster 1
Cluster 3
Cluster 4
Cluster5

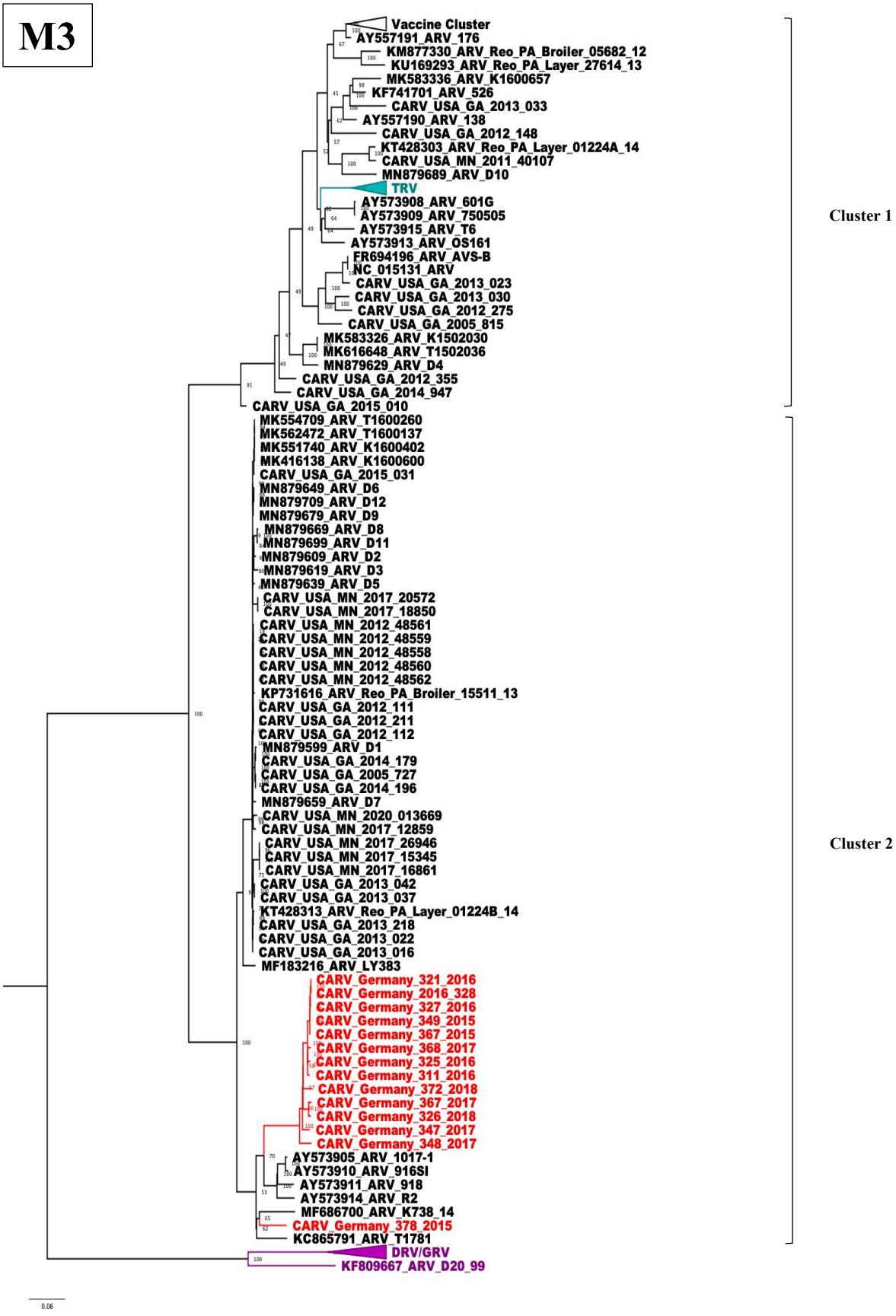
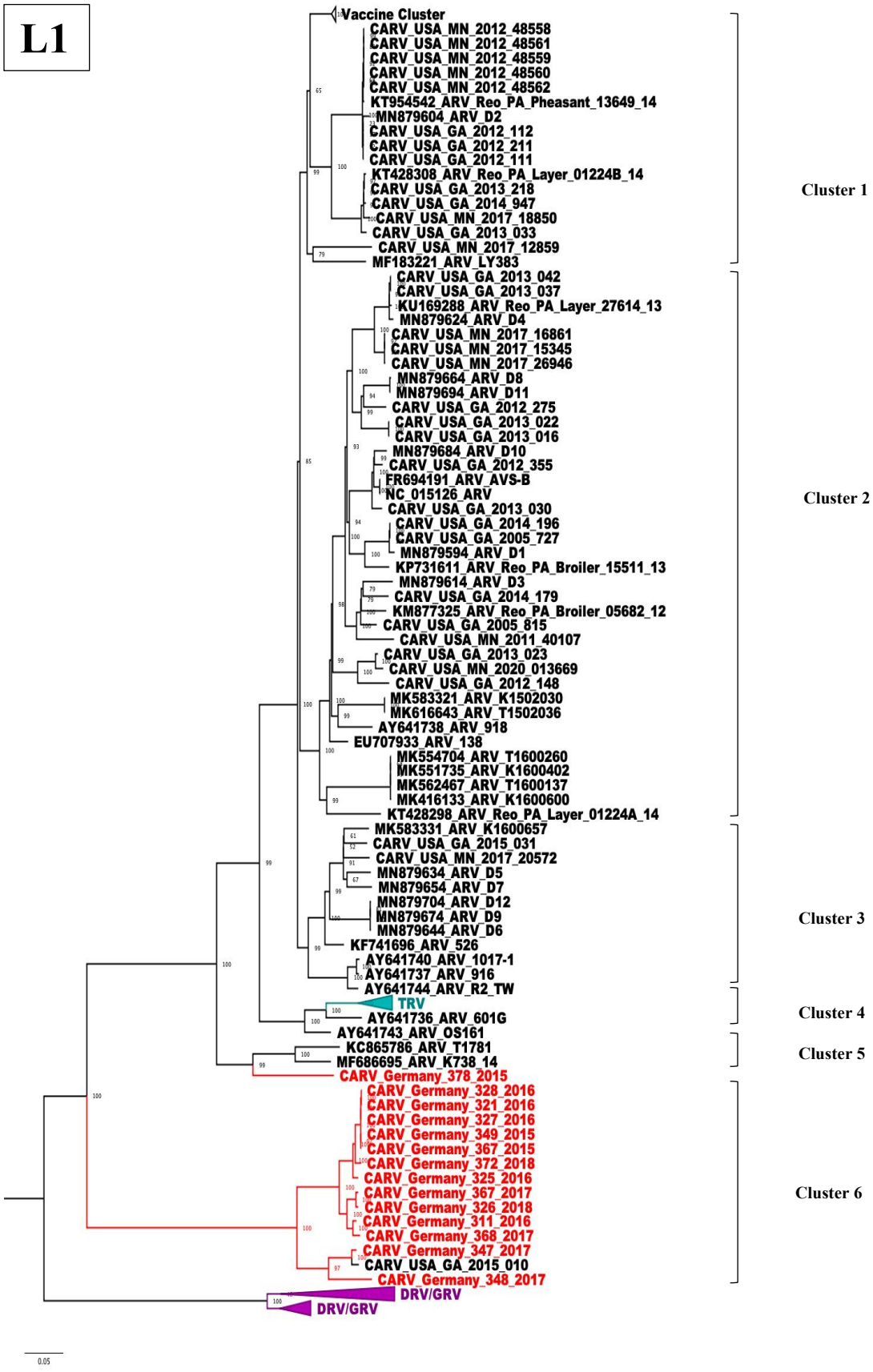
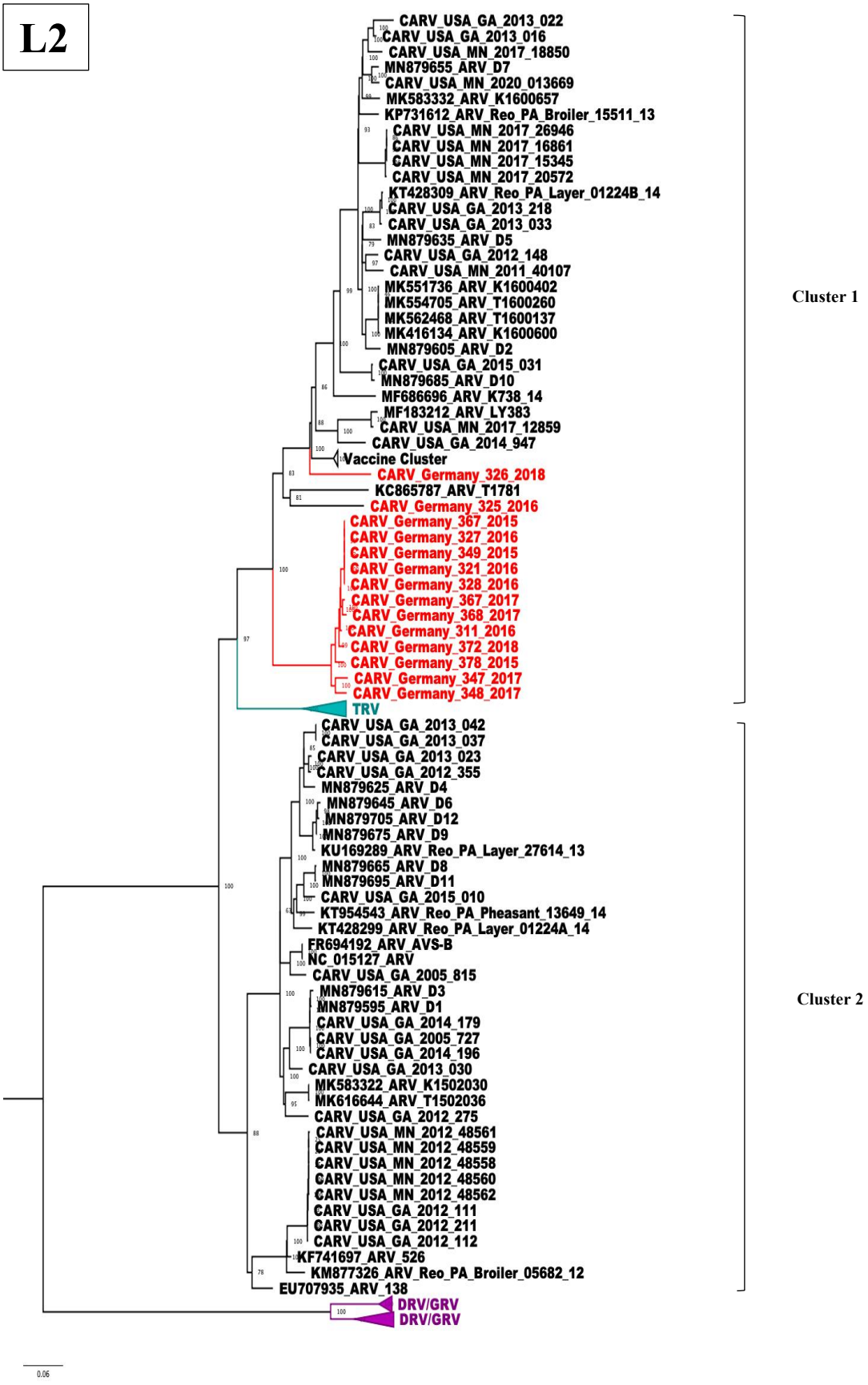

M3
Vaccine Cluster
AY557191_ARV_176
KM877330_ARV_Reo_PA_Broiler_05682_12
KU169293_ARV_Reo_PA_Layer_27614_13
MK583336_ARV_K1600657
KF741701_ARV_526
CARV_USA_GA_2013_033
AY557190_ARV_138
CARV_USA_GA_2012_148
KT428303_ARV_Reo_PA_Layer_01224A_14
CARV_USA_MN_2011_40107
MN879689_ARV_D10
TRV
AY573908_ARV_601G
AY573909_ARV_750505
AY573915_ARV_T6
AY573913_ARV_OS161
FR694196_ARV_AVS-B
NC_015131_ARV
CARV_USA_GA_2013_023
CARV_USA_GA_2013_030
CARV_USA_GA_2012_275
CARV_USA_GA_2005_815
MK583326_ARV_K1502030
MK616648_ARV_T1502036
MN879629_ARV_D4
CARV_USA_GA_2012_355
CARV_USA_GA_2014_947
CARV_USA_GA_2015_010
MK554709_ARV_T1600260
MK562472_ARV_T1600137
MK551740_ARV_K1600402
MK416138_ARV_K1600600
CARV_USA_GA_2015_031
MN879649_ARV_D6
MN879709_ARV_D12
MN879679_ARV_D9
MN879669_ARV_D8
MN879699_ARV_D11
MN879609_ARV_D2
MN879619_ARV_D3
MN879639_ARV_D5
CARV_USA_MN_2017_20572
CARV_USA_MN_2017_18850
CARV_USA_MN_2012_48561
CARV_USA_MN_2012_48559
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48560
CARV_USA_MN_2012_48562
KP731616_ARV_Reo_PA_Broiler_15511_13
CARV_USA_GA_2012_111
CARV_USA_GA_2012_211
CARV_USA_GA_2012_112
MN879599_ARV_D1
CARV_USA_GA_2014_179
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
MN879659_ARV_D7
CARV_USA_MN_2020_013669
CARV_USA_MN_2017_12859
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_16861
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
KT428313_ARV_Reo_PA_Layer_01224B_14
CARV_USA_GA_2013_218
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
MF183216_ARV_LY383
CARV_Germany_321_2016
CARV_Germany_2016_328
CARV_Germany_327_2016
CARV_Germany_349_2015
CARV_Germany_367_2015
CARV_Germany_368_2017
CARV_Germany_325_2016
CARV_Germany_311_2016
CARV_Germany_372_2018
CARV_Germany_367_2017
CARV_Germany_326_2018
CARV_Germany_347_2017
CARV_Germany_348_2017
AY573905_ARV_1017-1
AY573910_ARV_916SI
AY573911_ARV_918
AY573914_ARV_R2
MF686700_ARV_K738_14
CARV_Germany_378_2015
KC865791_ARV_T1781
DRV/GRV
KF809667_ARV_D20_99
0.06
Cluster 1
Cluster 2

Fig 4.3C. Nucleotide sequence-based ML phylogenetic trees showing the clustering of CARVs based on the L1, L2 and L3 gene segments of viruses available from GenBank. Phylogenetic calculations were carried out using the maximum-likelihood method applying the best-fit models calculated for each gene. The scale bar is proportional to the genetic distance.


L1
Vaccine Cluster
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48561
CARV_USA_MN_2012_48559
CARV_USA_MN_2012_48560
CARV_USA_MN_2012_48562
KT954542_ARV_Reo_PA_Pheasant_13649_14
MN879604_ARV_D2
CARV_USA_GA_2012_112
CARV_USA_GA_2012_211
CARV_USA_GA_2012_111
KT428308_ARV_Reo_PA_Layer_01224B_14
CARV_USA_GA_2013_218
CARV_USA_GA_2014_947
CARV_USA_MN_2017_18850
CARV_USA_GA_2013_033
CARV_USA_MN_2017_12859
MF183221_ARV_LY383
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
KU169288_ARV_Reo_PA_Layer_27614_13
MN879624_ARV_D4
CARV_USA_MN_2017_16861
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_26946
MN879664_ARV_D8
MN879694_ARV_D11
CARV_USA_GA_2012_275
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
MN879684_ARV_D10
CARV_USA_GA_2012_355
FR694191_ARV_AVS-B
NC_015126_ARV
CARV_USA_GA_2013_030
CARV_USA_GA_2014_196
CARV_USA_GA_2005_727
MN879594_ARV_D1
KP731611_ARV_Reo_PA_Broiler_15511_13
MN879614_ARV_D3
CARV_USA_GA_2014_179
KM877325_ARV_Reo_PA_Broiler_05682_12
CARV_USA_GA_2005_815
CARV_USA_MN_2011_40107
CARV_USA_GA_2013_023
CARV_USA_MN_2020_013669
CARV_USA_GA_2012_148
MK583321_ARV_K1502030
MK616643_ARV_T1502036
AY641738_ARV_918
EU707933_ARV_138
MK554704_ARV_T1600260
MK551735_ARV_K1600402
MK562467_ARV_T1600137
MK416133_ARV_K1600600
KT428298_ARV_Reo_PA_Layer_01224A_14
MK583331_ARV_K1600657
CARV_USA_GA_2015_031
CARV_USA_MN_2017_20572
MN879634_ARV_D5
MN879654_ARV_D7
MN879704_ARV_D12
MN879674_ARV_D9
MN879644_ARV_D6
KF741696_ARV_526
AY641740_ARV_1017-1
AY641737_ARV_916
AY641744_ARV_R2_TW
TRV
AY641736_ARV_601G
AY641743_ARV_OS161
KC865786_ARV_T1781
MF686695_ARV_K738_14
CARV_Germany_378_2015
CARV_Germany_328_2016
CARV_Germany_321_2016
CARV_Germany_327_2016
CARV_Germany_349_2015
CARV_Germany_367_2015
CARV_Germany_372_2018
CARV_Germany_325_2016
CARV_Germany_367_2017
CARV_Germany_326_2018
CARV_Germany_311_2016
CARV_Germany_368_2017
CARV_Germany_347_2017
CARV_USA_GA_2015_010
CARV_Germany_348_2017
DRV/GRV
DRV/GRV
0.05
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6


L2
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
CARV_USA_MN_2017_18850
MN879655_ARV_D7
CARV_USA_MN_2020_013669
MK583332_ARV_K1600657
KP731612_ARV_Reo_PA_Broiler_15511_13
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_16861
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_20572
KT428309_ARV_Reo_PA_Layer_01224B_14
CARV_USA_GA_2013_218
CARV_USA_GA_2013_033
MN879635_ARV_D5
CARV_USA_GA_2012_148
CARV_USA_MN_2011_40107
MK551736_ARV_K1600402
MK554705_ARV_T1600260
MK562468_ARV_T1600137
MK416134_ARV_K1600600
MN879605_ARV_D2
CARV_USA_GA_2015_031
MN879685_ARV_D10
MF686696_ARV_K738_14
MF183212_ARV_LY383
CARV_USA_MN_2017_12859
CARV_USA_GA_2014_947
Vaccine Cluster
CARV_Germany_326_2018
KC865787_ARV_T1781
CARV_Germany_325_2016
CARV_Germany_367_2015
CARV_Germany_327_2016
CARV_Germany_349_2015
CARV_Germany_321_2016
CARV_Germany_328_2016
CARV_Germany_367_2017
CARV_Germany_368_2017
CARV_Germany_311_2016
CARV_Germany_372_2018
CARV_Germany_378_2015
CARV_Germany_347_2017
CARV_Germany_348_2017
TRV
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
CARV_USA_GA_2013_023
CARV_USA_GA_2012_355
MN879625_ARV_D4
MN879645_ARV_D6
MN879705_ARV_D12
MN879675_ARV_D9
KU169289_ARV_Reo_PA_Layer_27614_13
MN879665_ARV_D8
MN879695_ARV_D11
CARV_USA_GA_2015_010
KT954543_ARV_Reo_PA_Pheasant_13649_14
KT428299_ARV_Reo_PA_Layer_01224A_14
FR694192_ARV_AVS-B
NC_015127_ARV
CARV_USA_GA_2005_815
MN879615_ARV_D3
MN879595_ARV_D1
CARV_USA_GA_2014_179
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
CARV_USA_GA_2013_030
MK583322_ARV_K1502030
MK616644_ARV_T1502036
CARV_USA_GA_2012_275
CARV_USA_MN_2012_48561
CARV_USA_MN_2012_48559
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48560
CARV_USA_MN_2012_48562
CARV_USA_GA_2012_111
CARV_USA_GA_2012_211
CARV_USA_GA_2012_112
KF741697_ARV_526
KM877326_ARV_Reo_PA_Broiler_05682_12
EU707935_ARV_138
DRV/GRV
DRV/GRV
0.06
Cluster 1
Cluster 2

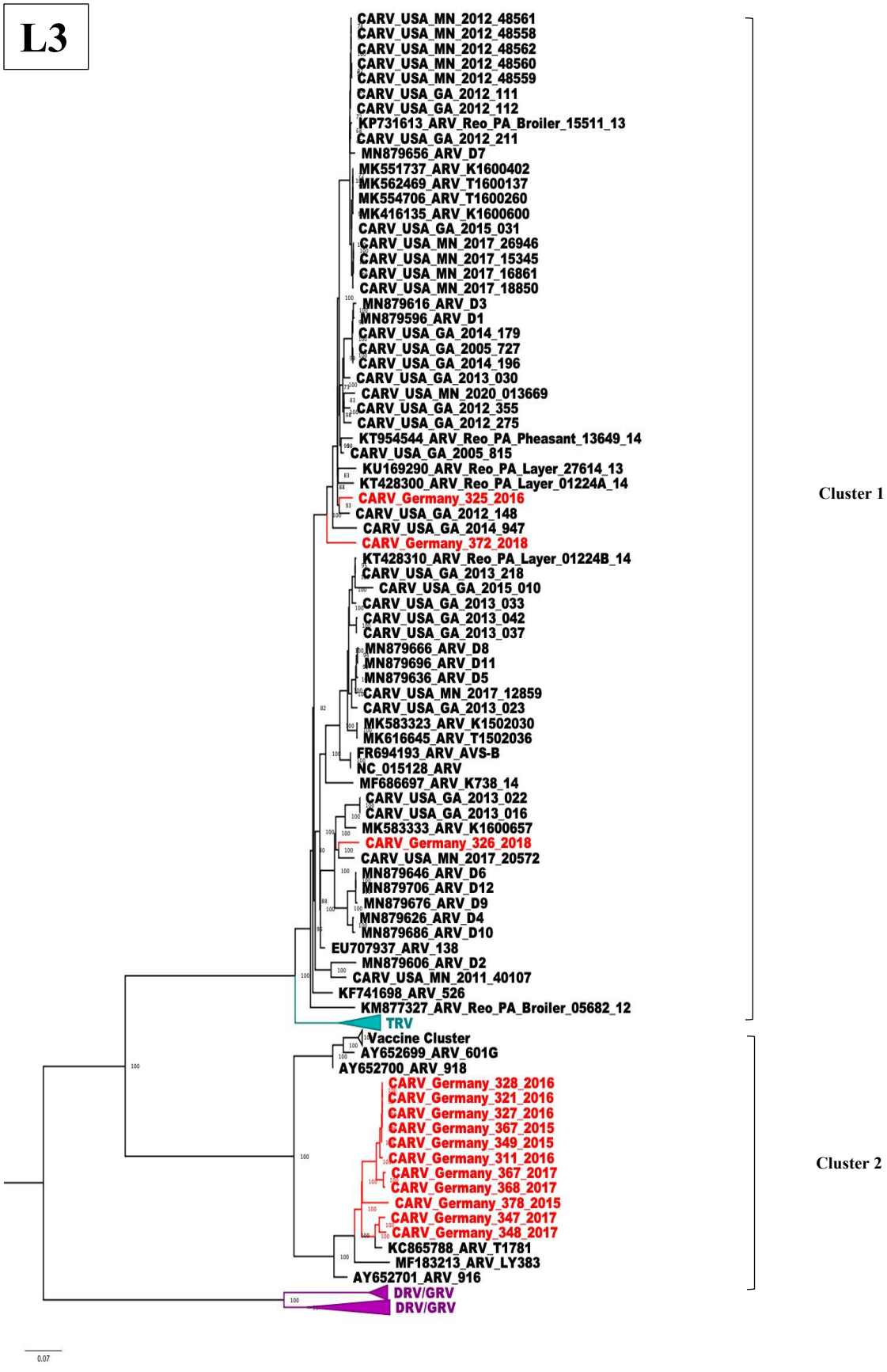
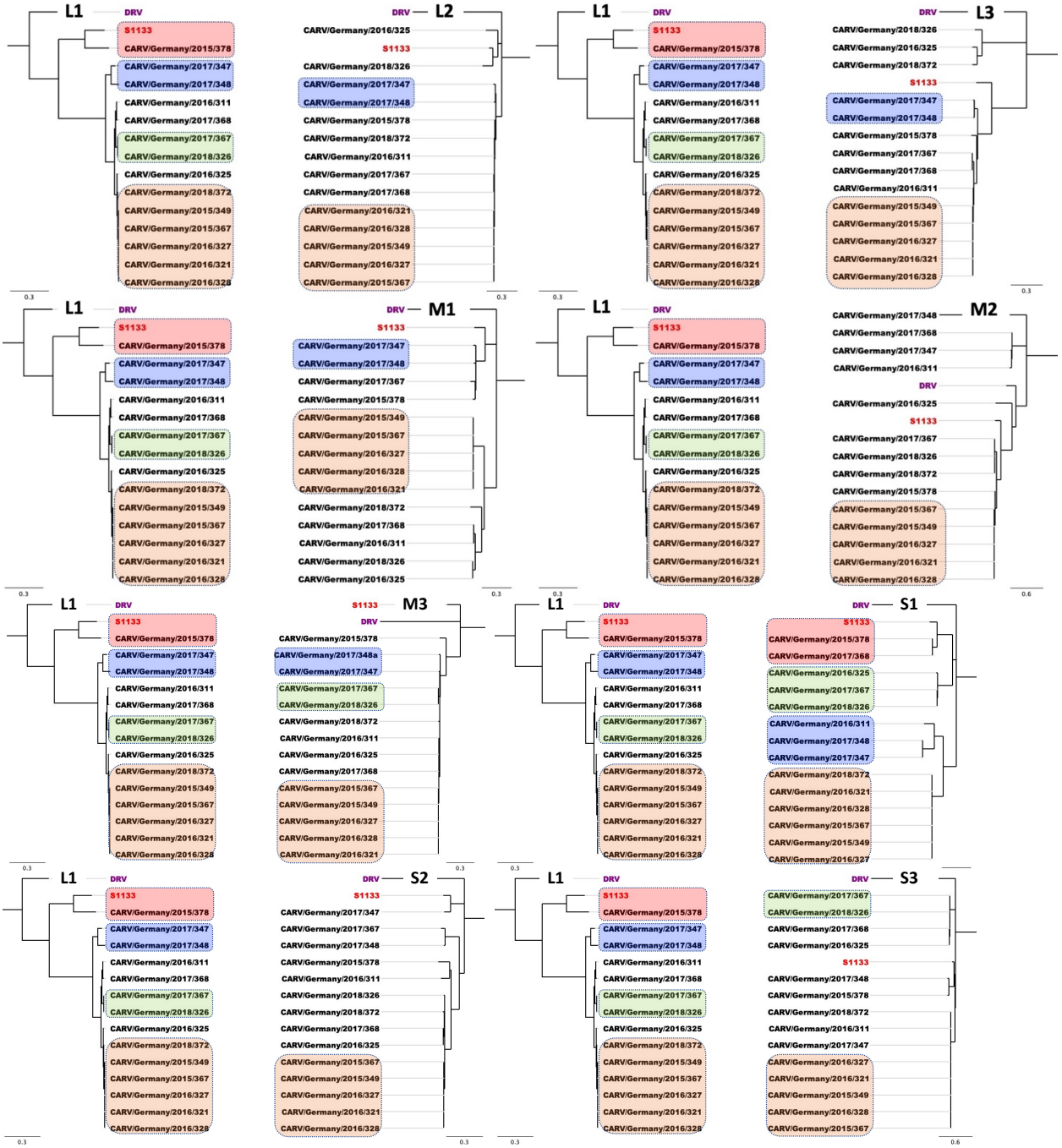
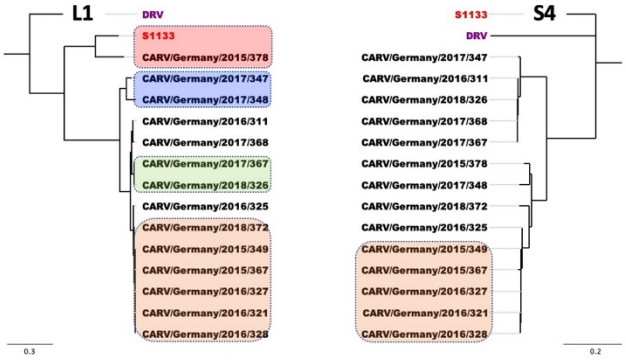

L3
CARV_USA_MN_2012_48561
CARV_USA_MN_2012_48558
CARV_USA_MN_2012_48562
CARV_USA_MN_2012_48560
CARV_USA_MN_2012_48559
CARV_USA_GA_2012_111
CARV_USA_GA_2012_112
KP731613_ARV_Reo_PA_Broiler_15511_13
CARV_USA_GA_2012_211
MN879656_ARV_D7
MK551737_ARV_K1600402
MK562469_ARV_T1600137
MK554706_ARV_T1600260
MK416135_ARV_K1600600
CARV_USA_GA_2015_031
CARV_USA_MN_2017_26946
CARV_USA_MN_2017_15345
CARV_USA_MN_2017_16861
CARV_USA_MN_2017_18850
MN879616_ARV_D3
MN879596_ARV_D1
CARV_USA_GA_2014_179
CARV_USA_GA_2005_727
CARV_USA_GA_2014_196
CARV_USA_GA_2013_030
CARV_USA_MN_2020_013669
CARV_USA_GA_2012_355
CARV_USA_GA_2012_275
KT954544_ARV_Reo_PA_Pheasant_13649_14
CARV_USA_GA_2005_815
KU169290_ARV_Reo_PA_Layer_27614_13
KT428300_ARV_Reo_PA_Layer_01224A_14
CARV_Germany_325_2016
CARV_USA_GA_2012_148
CARV_USA_GA_2014_947
CARV_Germany_372_2018
KT428310_ARV_Reo_PA_Layer_01224B_14
CARV_USA_GA_2013_218
CARV_USA_GA_2015_010
CARV_USA_GA_2013_033
CARV_USA_GA_2013_042
CARV_USA_GA_2013_037
MN879666_ARV_D8
MN879696_ARV_D11
MN879636_ARV_D5
CARV_USA_MN_2017_12859
CARV_USA_GA_2013_023
MK583323_ARV_K1502030
MK616645_ARV_T1502036
FR694193_ARV_AVS-B
NC_015128_ARV
MF686697_ARV_K738_14
CARV_USA_GA_2013_022
CARV_USA_GA_2013_016
MK583333_ARV_K1600657
CARV_Germany_326_2018
CARV_USA_MN_2017_20572
MN879646_ARV_D6
MN879706_ARV_D12
MN879676_ARV_D9
MN879626_ARV_D4
MN879686_ARV_D10
EU707937_ARV_138
MN879606_ARV_D2
CARV_USA_MN_2011_40107
KF741698_ARV_526
KM877327_ARV_Reo_PA_Broiler_05682_12
TRV
Vaccine Cluster
AY652699_ARV_601G
AY652700_ARV_918
CARV_Germany_328_2016
CARV_Germany_321_2016
CARV_Germany_327_2016
CARV_Germany_367_2015
CARV_Germany_349_2015
CARV_Germany_311_2016
CARV_Germany_367_2017
CARV_Germany_368_2017
CARV_Germany_378_2015
CARV_Germany_347_2017
CARV_Germany_348_2017
KC865788_ARV_T1781
MF183213_ARV_LY383
AY652701_ARV_916
DRV/GRV
DRV/GRV
0.07
Cluster 1
Cluster 2

Fig 4.4. Topologies of segmental phylogenetic trees of each gene (nt) segment of CARV isolates. The trees were built using nucleotide sequences by ML method with best fit distance models.




L1
DRV
S1133
CARV/Germany/2015/378
CARV/Germany/2017/347
CARV/Germany/2017/348
CARV/Germany/2016/311
CARV/Germany/2017/368
CARV/Germany/2017/367
CARV/Germany/2018/326
CARV/Germany/2016/325
CARV/Germany/2018/372
CARV/Germany/2015/349
CARV/Germany/2015/367
CARV/Germany/2016/327
CARV/Germany/2016/321
CARV/Germany/2016/328
0.3
S1133
S4
DRV
CARV/Germany/2017/347
CARV/Germany/2016/311
CARV/Germany/2018/326
CARV/Germany/2017/368
CARV/Germany/2017/367
CARV/Germany/2015/378
CARV/Germany/2017/348
CARV/Germany/2018/372
CARV/Germany/2016/325
CARV/Germany/2015/349
CARV/Germany/2015/367
CARV/Germany/2016/327
CARV/Germany/2016/321
CARV/Germany/2016/328
0.2

CHAPTER 5

DEVELOPMENT AND EVALUATION OF THE IMMUNOGENICITY AND EFFICACY OF A NOVEL PICHINDE VIRUS-VECTORED CHICKEN ARTHRITIS REOVIRUS VACCINE

The material in this chapter has been prepared for publication:
Kumar R, Porter RE, Mor SK, Liang Y, Ly H and Goyal SM. 2021.Development and evaluation of the immunogenicity and efficacy of a novel pichinde virus-vectored chicken arthritis reovirus vaccine. Vaccines.

Introduction

Chicken reoviruses belong to genus *Orthoreovirus* of the family *Reoviridae* (Attoui et al., 2012), and are associated with a variety of diseases. Viral arthritis (VA) of chickens is the most economically important reoviral disease (Pitcovski and Goyal, 2020). Viral arthritis is usually seen in 4–8-week-old broilers, characterized by unilateral or bilateral leg lameness, swelling of the shank and hock, and rupture of gastrocnemius tendons in severe cases. Affected chickens walk with stilted gait and drag their wings to support the body (wing walkers). The infection can be subclinical with 5-50 % morbidity and 2-10% mortality (Sedghi et al., 2013; Perelman et al., 2019). Histologically, VA is characterized by lymphoplasmacytic tenosynovitis with synoviocyte hyperplasia followed by fibrosis and calcification (Porter, 2018). Broiler breeders, layer breeders and meat-type broilers are mostly affected in addition to duck, geese, turkeys, and wild birds etc. (Sellers, 2017; Pitcovski and Goyal, 2020).

The severity of VA in a flock depends on pathogenicity of the reovirus strain, age at infection and immune status of the birds. Chicks that are vertically infected at hatch can have early appearance of signs with severe disease, while birds infected after 2 weeks of age may develop subclinical or mild VA (Davis et al., 2013; Jones, 2013).

Avian reoviruses are the members of genus *Orthoreovirus* of family *Reoviridae.* The viral genome has 10 double-stranded RNA segments packed inside a double-shelled capsid. The viral genome segments are classified as L class (L1-L3), M class (M1-M3) and S class (S1-S4) (Spandidos and Graham, 1976) based on their migration patterns in poly acrylamide gel electrophoresis. The reoviral genome has 12 open reading frames (ORFs) encoding eight structural and four nonstructural proteins with their functions

reviewed in detail by Benavente and Martinez-Costas (2007). All gene segments are mono-cistronic except S1 segment (tri-cistronic). The third ORF of S1 segment translates to an outer capsid cell attachment Sigma C (SC) protein (Schnitzer et al., 1982; Martinez-Costas et al., 1997; Grande et al., 2002) and is the most divergent reoviral protein. The SC protein is the main immunogenic surface protein, inducing type- and broad-specific neutralizing antibodies (Martinez- Costas et al., 1997). The Sigma B (SB) protein of S3 segment is a major outer capsid protein, inducing group-specific neutralizing antibodies (Wickramasinghe et al., 1993). The SC and SB proteins have been used in subunit vaccines against avian reovirus infection (Wu et al., 2005; Lin et al., 2008; Bi et al., 2016; Goldenberg et al., 2016).

Genetic and antigenic variants of reoviruses have emerged in the last decade. These mutations have occurred worldwide and have resulted in frequent outbreaks that affected the welfare of chickens and resulted in economic losses to poultry production (Sellers, 2017; Perelman et al., 2019; Pitcovski and Goyal, 2020). Segmental reassortment and mutations in the viral genome cause genetic diversity in ARVs (Lu et al., 2015). Mutations in gene segments, (mainly the S1 segment encoding Sigma C protein and the S3 segment encoding the Sigma B protein), may be exacerbated by vaccination as well as and the host immune response (Grande et al., 2002; Liu et al., 2013; Sellers, 2017).

The primary control method for VA in chicks is vaccination of breeder hens, which then passively transfer antibody through eggs to the hatchlings (Gharaibeh et al., 2008). The commercially available live attenuated and inactivated vaccines (e.g., S1133, 1733, 2408, 2177) are based on a single serotype of reovirus strains (van der Heide et al.,

1983; Goldenberg et al., 2010), and are not protective against variant ARVs (Goldenberg et al., 2016; Perelman et al., 2019).

Molecular characterization of SC protein classified the circulating ARVs into six genotypes (Egaña et al., 2019; Carli et al., 2020). The SC and SB proteins induce type and group specific neutralizing antibodies, respectively (Benavente and Martínez-Costas 2007; Pitcovski and Goyal, 2020), making these proteins suitable candidates for a recombinant subunit vaccine. Recombinant subunit vaccines for ARV have been developed using SC alone or in combination with SB proteins (Wu et al., 2005; Lin et al., 2008; Goldberg et al., 2016) using different expression systems (Theophilos et al., 1995; Wu et al., 2005; Huang et al., 2006; Lin et al., 2008; Jung et al., 2014). In this study, molecular characterization and sequence analysis of SC and SB genes of CARVs ciruslating in the US (studied in Chapter 3) was done to select CARV strains and immunogenic genes for the development of vaccines.

With the advent of reverse genetics, recombinant live viral vaccine vectors are growing in favor over traditional vaccine strategies. Live viral vaccine vectors can replicate and induce pronounced immunity against the antigens of interest without using irritable adjuvants. The recombinant Pichinde virus vector (rPICV) can carry two foreign genes where green fluorescent protein (GFP) can be used as one of the foreign genes to mark virus-infected cells in cell culture (Dhanwani et al., 2015). Our lab recently developed an rPICV-based turkey arthritis reovirus (TARV) vaccine to deliver SC and SB proteins and was shown to be safe and efficacious (Kumar et al., 2021). In similar fashion, this current study describes the development of a rPICV-CARV vaccine to carry and deliver CARV genes into the host. The present study describes the development of

different combinations of monovalent and bivalent rPICV-CARV vaccines. Two vaccine formulations were tested for safety and efficacy against virus challenge.

Materials and Methods

Ethics statement: We confirmed that the present study (protocol id 2005-38169A) was carried out in accordance with the guidelines and recommendations of the Institutional animal care and use committee (IACUC) and research animal resources (RAR), University of Minnesota. The birds were treated humanely, and all procedures were taken under minimum suffering conditions. Chicks were monitored every 12 hours over a period of the study for health and signs of disease. A humane endpoint was used in the study in case any overt clinical signs or reovirus specific clinical signs, mortality or gross lesions were observed.

Selection of candidate CARV strain

The appropriate variant strains to be included in the live virus vectored vaccine were selected out of twenty CARV strains available in our laboratory. The isolates of choice had to represent the divergent and recently evolved genotypes; therefore, maximum likelihood (ML) phylogenetic analysis of S1/SC and S3/SB gene segments was performed to determine the genotype and clustering of CARV isolates. The obtained phylogenetic tree (Figure 5.1) revealed the six genotype clusters (GC) (based on SC gene segment) already described for ARV (Carli et al., 2020) and demonstrated that our CARV isolates were classified in GC1 (n=11), GC2 (n=2) GC4 (n=1) GC5 (n=2) and GC6 (n=4). Similarly, strain selection was done based on clustering of isolates with S3 segment.

Cell and Viruses: Japanese quail fibrosarcoma (QT-35) and Baby hamster kidney (BHK-21) cells were grown in Dulbecco's modified Eagle's medium (DMEM with high glucose) (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) and 50 μg/mL penicillin–streptomycin (Sigma-Aldrich). Vero cells and BSRT7-5 cells (BHK-21 cells stably expressing T7 RNA polymerase) were grown in Eagle's minimal essential medium (MEM) (Sigma-Aldrich) supplemented with 10% FBS, 1 μg/mL gentamicin (Invitrogen-Life Technologies) and 50 μg/mL penicillin–streptomycin. Vaccine viruses (rPICV-CARV) were plaque purified and amplified in BHK-21 cells, and the infectious vaccine virus titer was determined by a plaque assay in Vero cells as described previously (Lan et al., 2009). The original titers of different rPICV-CARV were summarized in table 2. Three chicken reovirus isolates (CARV-22, CAR-196 and CARV-30) were grown and titrated in QT-35 cells. The 50% tissue culture infective dose ($TCID_{50}$) was calculated by the Reed and Muench (1938) method.

Pichinde Virus Plasmids

Three plasmids were used: (i) pP18S1-GPC/MCS (S1/1614), which encodes the glycoprotein GPC and a multiple cloning site (MCS) to clone the gene of interest; (ii) pP18S2-MCS/NP (S2/1615), which encodes the nucleoprotein NP and an MCS; (iii) pP18L plasmid (L plasmid,), which expresses the full-length antigenomic strand of the rP18L segment under the control of the T7 promoter and does not contain any specific site to clone foreign genes (Dhanwani et al., 2015).

Preparation of Vectors and Gene Inserts

The S1 (SC) and S3 (SB) ORF sequences each of selected CARV strains (CARV-22 and CAR-196) were codon-optimized for expression in mammalian cells, commercially

custom-synthesized, and cloned into a pUC vector (Twist Biosciences). The primary sequences of these genes are provided in Supplementary Materials (Figure S1). The codon optimized genes and the plasmids of the PICV (S1/1614 and S2/1615) were restriction enzyme-digested (NheI and KpnI, NEB) and gel-purified using the QIAquick gel extraction kit (Qiagen). The codon-optimized versions of ORFs were extracted from the pUC vector via restriction double digestion.

Cloning and Transfection

The SC and SB genes of CARV-22 and CARV-196 were ligated in the MCS region of plasmids S1/1614 and S2/1615, in different combinations as described previously (Kumar et al., 2020). Briefly, the SC and SB inserts were cloned into the plasmids using 5 U/µL of T4 DNA ligase (Thermo Fisher Scientific). The ligation reaction mix was used to transform competent bacterial cells (DH5α) followed by selection using ampicillin antibiotic. All plasmids (plasmid L, S1/1614, S2/1615, and recombinant plasmids S1-SB/SC and/or S2-SC/SB) were isolated using the plasmid midi prep kit (Sigma-Aldrich). Recombinant plasmids were PCR-confirmed for reovirus genes and sequence confirmed for correct orientation and reading frame. The recombinant viruses were recovered by transfecting BSRT7-5 (BHK-T7 cells) cells with three plasmids: L, S1-SC or SB and/or S2-SB or SC within various combinations (Table 1) using Lipofectamine™ 3000 transfection reagent (Thermo Fisher Scientific) following the manufacturer's instruction with minor modifications. Briefly, BSRT7-5 cells were grown in six-well plates to 80% confluency. Four hours before transfection, the cells were washed, and fresh antibiotic free medium was added. For transfection, 8 µL of P3000 reagent, 2 µg of L plasmid and 1 µg each of S1/1614 and S2/1615 plasmids were diluted in 250 µL of Opti-MEM

(Invitrogen-Life Technologies) and incubated for 15 min at room temperature. In another tube, 10 µL of lipofectamine was diluted in 250 µL of Opti-MEM. Both mixtures were combined, followed by incubation at room temperature for 20 min to prepare DNA–lipid complexes. The cells were transfected with the resultant mixture, and MEM was changed after 4 h to remove the toxic lipofectamine. At 48, 72, and 96 h post transfection, cell supernatants were collected and stored at -80 °C. Different monovalent and bivalent vector viruses were generated as detailed in Table 1. The resultant viral recovery was confirmed by observing the green fluorescence of GFP in inoculated cell culture. The rescued virus was then grown in BHK-21 cells, and the GFP green fluorescence was observed. The expression of reovirus genes by the recombinant PICV vaccine virus was verified by RT-PCR.

Detection of Reovirus Antigenic Proteins

The SC and SB protein expression by monovalent and bivalent rPICV-CARV vaccines was determined by direct fluorescent antibody assay (DFA). The rPICV-CARV vaccine viruses were inoculated in BHK-21 cells and incubated at 37 ºC in a CO_2 incubator. At 96 hours post infection (hpi), cells were harvested, plated on a 12-chamber slide and air dried for 2 hours in a laminar flow hood. Cells were then fixed for 2 hours in acetone followed by overlaying of Fluorescein isothiocyanate (FITC-conjugated anti-avian reovirus antibodies (National Veterinary Services Laboratory). The slide was then incubated at 37 ºC for two hours followed by counterstaining with 0.1% Evan's blue biological stain (EBBS). Slide was then mounted and examined under a fluorescent microscope to observe apple green fluorescence indicative of expression of avian reovirus proteins by the vaccine viruses.

Vaccination and challenge Experiment design: Two vaccine formulations were evaluated in this study; vaccine 1 (V1) is a cocktail of two bivalent vaccines (CARV-22SC/SB and CARV-196SC/SB) and vaccine 2 (V2) is a cocktail of two monovalent vaccines (CARV-22SC and CARV-196SB). The cocktails were prepared by mixing equal volumes of individual vaccines immediately before vaccination of birds. Day-old SPF chicks (n=270) were purchased from VALO BioMedia North America LLC Adel, IA. Ten chicks were euthanized on the day of arrival to collect blood for serum, intestine, and tendon samples. Serum samples were tested by ELISA and meconium, intestine and tendon samples were tested by real time RT-PCR (rRT-PCR) to ensure that the chicks are free from reovirus infection and antibodies. Day-old chicks were randomly divided into 12 groups namely non-vaccinated and non-challenged negative control (NV-NCh), vaccine 1-non challenged control (V1-NCh, V1 control), vaccine 2-non challenged control (V2-NCh, V2 control), vaccine 1 challenged with CARV-22 (V1-Ch22), vaccine 1 challenged with CARV-196 (V1-Ch196), vaccine 1 challenged with CARV-30 (V1-Ch30), vaccine 2 challenged with CARV-22 (V2-Ch22), vaccine 2 challenged with CARV-196 (V2-Ch196), vaccine 2 challenged with CARV-30 (V2-Ch30), CARV-22 positive control (Ch22), CARV-196 positive control (Ch196) and CARV-30 positive control (Ch30) and housed in 12 different air-filtered isolators. Food and water were supplied *ad libitum*. The detailed experimental plan is shown in table 3. Briefly, chicks were vaccinated with primary dose of respective rPICV-CARV vaccine (V1 and V2) (0.2 ml, $5x10^7$ PFU/ml) by oral route at 1 days of age (doa). Chicks were boosted intranasally with 0.2 ml ($5x10^7$ PFU/ml) of respective rPICV-CARV vaccines (V1 and V2) at 8 doa. All the groups (except NC-NCh, V1-Nch and V2-NCh) were challenged orally with

0.2ml (10^5 $TCID_{50}$/ml) of CARV-22, CARV-196 and CARV-30 in respective groups at 13 doa. Groups NV-NCh, V1-NCh and V2-NCh were sham inoculated with 0.2 ml of virus free culture media (MEM). The birds were examined daily for any abnormal clinical signs or mortality. Birds displaying signs of severe illness were euthanized according to the IACUC and research animal resources (RAR) guidelines. Five birds from NC-NCh, V1-Nch and V2-NCh groups were euthanized and used for blood collection at 8doa before booster vaccination. Five birds from NC-NCh, V1-Nch and V2-NCh groups were euthanized and used for blood collection at 13doa before virus challenge. Five birds from each group were euthanized and used for blood collection at each euthanasia time point viz. 18, 23 and 33 doa. Body weights of the euthanized birds were noted before sample collection. At necropsy, gross lesions were noted followed by collection of heart, liver, intestine (ileo-cecum) and hock joint with gastrocnemius and digital flexor tendons for real time RT-PCR (rRT-PCR) and histopathology.

ELISA and serum neutralization test (SNT): Blood serum was tested for anti-CARV antibody using a commercial ELISA available at the Veterinary Diagnostic Laboratory, University of Minnesota (UMVDL) (https://www.vdl.umn.edu/node/14381). The sera were also tested for serum neutralization antibodies against virus strains CARV-22, CARV-196 and CARV-30 to analyze the spectrum of virus-neutralizing capability of antibodies produced by the vaccinated birds. The SN test was performed by Aviserve Inc., Newark, DE. Briefly, heat inactivated serum samples were 4-fold serially diluted in a 96 well plate, and 25 µl of reovirus preparations (100 $TCID_{50}$) was added to all wells except negative control wells. Subsequently, the virus-sera mixture was incubated at 37 °C for 1 hour before adding onto freshly seeded primary hepatocellular carcinoma

epithelial cells from a male leghorn chicken (LMH) (5×10^5 cell/well) with 10% fetal calf serum and incubated for 4-5 days. Virus-infected and uninfected cells were used as positive and negative controls, respectively. Virus controls, cell controls, and serum controls were included on each plate. The plates were observed daily for the appearance of reovirus specific cytopathic effects (CPE) e.g., cell swelling, syncytia formation, detachment from monolayer. Medium was removed on appearance of CPE and the cells were stained with a 1% crystal violet prepared in 10% buffered formalin for 2–3 min, followed by washing with warm tap water. Plates were air dried and antibody titers were recorded as the reciprocal of the highest dilution of serum that inhibited virus induced CPE in at least 50% of the cell monolayer.

Processing of tissue samples: Individual heart, liver and intestinal (ileocecum) samples (1 gram) were homogenized in Hanks' balanced salt solution (HBSS) for 1-2 min using a Stomacher (Model 80, Seward, Ltd.) to prepare a 10% suspension. Individual tendon samples (1 gram) were homogenized in phosphate buffered saline (PBS) for 2 cycles of 4 minute each in Geno/Grinder tubes using Geno/Grinder (SPEX Sample Prep 2010 Geno/Grinder®, Thomas Scientific). Tissue homogenates were centrifuged at 1800 xg for 10 min at 4^0C. The supernatant was decanted and frozen at -80 ^{0}C until tested by rRT-PCR.

Nucleic acid extraction: Nucleic acids (RNA) were extracted from 50 µL of each intestinal and tendon sample homogenate. Nucleic acid extraction was conducted using a MagMAX™ Pathogen RNA/DNA Kit (Thermo Fisher Scientific) on a Kingfisher-Flex instrument (Thermo Fisher Scientific) following manufacturer's instructions. Nucleic acids were eluted in 90 µL of elution buffer.

Virus gene copy number: Virus gene copy numbers in intestine and tendon samples were estimated using a universal avian reovirus rRT-PCR available at the Veterinary Diagnostic Laboratory, University of Minnesota (https://www.vdl.umn.edu/node/15341). A standard curve was constructed using ten-fold serial dilutions of TARV-positive RNA included with each 96-well plate. The gene copy numbers were calculated in intestine and tendon samples collected at different time points and were subjected to appropriate statistical analysis.

Histopathology: Soft tissues were fixed in formalin, trimmed, processed, and stained with hematoxylin and eosin (H&E). Formalin-fixed hock joints were decalcified in EDTA prior to processing for histopathological examination. Lesions in the gastrocnemius tendons were scored using a previously described histologic lesion scoring system (Sharafeldin et al., 2014).

Statistical analysis: Pairwise comparisons of body weights of birds in different groups were done using t-test. A non-parametric Kruskal Wallis test followed by pairwise Wilcoxon rank sum test with continuity correction and "Benjamini & Hochberg (BH)" as p value adjustment method was used to test the significance of difference in serum neutralizing antibody titers, virus gene copy (in intestine and tendons), and histologic lesion scores in gastrocnemius tendons. To neutralize the skewness, variability and non-normal distribution of data and small sample size, natural log of virus gene copy numbers in intestine and tendons was taken. Statistical significance was determined at p value < 0.05. Statistical analysis was done in R (R Core Team, 2017) and figures were produced using the package ggplot2 (Wickham, 2009).

Results

Cloning of Reovirus Genes into PICV Plasmids

The codon optimized SC and SB genes were cloned into S1 (1614) and S2 (1615) plasmids, in different combinations (Table 1). Restriction enzyme double digestion confirmed the presence of reovirus genes in rPICV plasmids (Figure 5.2). Sanger sequencing confirmed the absence of unintended mutations in the cloned viral gene, as well as their correct reading frame and correct orientation in the vector backbones (data not shown).

Plasmid Transfection and Virus Rescue

Viable recombinant rPICVs were successfully rescued following transfection of BSRT7-5 cells with the three plasmids in various combinations, as shown in Table 1. The GFP expression was observed at 48–72 h post transfection in cells transfected with at least one GFP-containing plasmid (Figure 5.3A) (all monovalent vaccines in Table 5.1). The GFP expressing foci increased in size over the time course of transfection. The supernatants were collected from transfected BSRT7-5 cells and were used to infect BHK21 cells. Strong GFP expression was detected in infected BHK21 cells at 24–48 hpi using fluorescent microscopy (Figure 5.3B), indicating the rescue of viable rPICV-CARV vaccines viruses. At every rescue attempt, we obtained infectious viruses at 48–72 h post transfection. As expected, the bivalent rPICV-CARV vaccines carrying two CARV genes on both PICV plasmids did not produce any green fluorescence (Figure 5.3C).

Recombinant PICVs Expressing Reovirus Antigens

Strong GFP expression by infected BHK21 cells indicated the successful rescue of

rPICV-CRAV vaccines. The supernatant from infected BHK21 cells (passages P1, P2, and P3) was used to detect reovirus genes by RT-PCR. The results confirmed the presence of both viral genes in bivalent rPICV-CARV vaccines and either SC or SB gene in the monovalent vaccine viruses. The RT-PCR amplification indicated CARV antigen expression at the messenger RNA (mRNA) level, where the supernatant harvested at 48 h post infection showed significantly higher amplification than the supernatant harvested at 24 h post infection (Figure 5.4). To verify the expression of reovirus antigenic proteins (SC and SB) by the recombinant PICVs, we infected BHK21 cells with transfection supernatant and then, at 96 hpi, conducted a direct fluorescence assay (DFA) using polyclonal FITC-conjugated anti-avian reovirus antibodies. The rPICV-CARVs grown on BHK-21 showed varying degrees of fluorescence (Figure 5.5). The monovalent and bivalent rPICV-CARV vaccines that contained SC and/or SB showed fluorescence in BHK-21 cells (Figure 5.5A–D). Although we did not quantify the amount of fluorescence, PICVs containing CARV-196 gene segments showed a remarkably higher degree of fluorescence, particularly the bivalent rPICV-CARV vaccine (Figure 5.4B). No or non-specific fluorescence was observed in negative controls (cells that contained rescued PICV without any CARV segment) (Figure 5.5D).

Clinical disease and gross lesions: At all euthanasia time-points, no reovirus specific clinical signs, mortality or gross lesions were observed in any of the vaccinated and/or virus inoculated birds.

Body weight: at 18 doa (5dpi), the mean BW of birds in V1-NCh group was significantly lower than the NC-NCh and V2-NCh groups. The mean BW of birds in vaccine 2 groups challenged with V2-Ch22, V2-Ch196 and V2-Ch30 was significantly higher than the

mean BW of birds in V1-Ch22, V1-Ch196 and V1-Ch30 and Ch-22, Ch-196 and Ch-30, respectively (Figure 5.6A). A similar pattern was observed at 23 doa (10dpi) and 33 doa (20dpi) where the mean BW of birds in V2-NCh group was significantly higher than the mean BW of NV-NCh and V1-NCh groups. The mean BW of birds in V2-Ch22, V2-Ch196 and V2-Ch30 was significantly higher than the mean BW of birds in V1-Ch22, V1-Ch196 and V1-Ch30 and Ch-22, Ch-196 and Ch-30, respectively (Figure 5.6B and C).

Serology: ELISA and serum neutralization antibody titers: Sera from the vaccinated birds showed zero ELISA antibody titer and hence negative results, whereas based on our experience, the sera from experimentally infected birds at < 2 weeks of age usually show an ELISA antibody titer of 771 to 2716 at 5 weeks of age (data not shown). Negligible to very low mean serum neutralization antibody titers against CARV-22, CARV-196 and CARV-30 were observed in sera from NV-NCh birds. Mean SN antibody titers in V1 and V2 groups against CARV-22 or CARV-196 after primary dose ranged from 32 to 64 and 16 to 64 against CARV-30. After booster vaccination, mean SN antibody titers in V1 and V2 groups ranged from 64 to 256 against CARV-22 or CARV-196 and 32 to 128 against CARV-30 (Figure 5.7A, B & C). In both vaccine groups, the mean SN antibody titers were significantly higher against CARV-22 and CARV-196 after the booster vaccination, but not against CARV-30 (Figure 5.7A, B & C). After booster vaccination, the mean SN Ab titer (against all the three challenge viruses) in the V2 group was numerically, but not significantly higher than that of the V1 group. During the entire course of the study, the mean SN antibody titers against all three challenge viruses in birds vaccinated with V1

and V2 were consistently higher in the V2 groups than the V1 groups (Figure 5.7D, E & F).

Virus gene copy number in intestine: At 18 doa (5dpi, Figure 5.8A), mean virus gene copy numbers in groups V1-Ch22 and V2-Ch22 were significantly lower than those of the Ch22 group. The mean virus gene copy number in V2-Ch22 was numerically, but not significantly, lower than the mean of the V1-Ch22 group. The mean virus gene copy number in group V2-Ch196 was significantly, lower than Ch-196 group whereas the mean virus gene copy number in V1-Ch196 was numerically but not significantly, lower than the mean of the Ch-196 group. The mean virus gene copy number in V1-Ch30 and V2-Ch30 was significantly lower than the mean of the Ch30 group. At 23 doa (10dpi, Figure 5.8B), mean virus gene copy numbers in Ch22 and Ch30 groups were numerically, but not significantly, lower than means of the respective groups vaccinated with V1 and V2, whereas the mean virus gene copy number in Ch-196 was higher than that of V2-Ch196, but lower than the mean of the V1-Ch196 group. At 33 doa (20dpi), no virus replication was observed in any of the vaccinated and/or virus challenge positive control groups (data not shown).

Virus gene copy number in tendons: At 18 doa (5dpi, data not shown), no virus replication and gene copy numbers were observed in tendons of any of the vaccinated and/or virus challenge groups. At 23 doa (10dpi, Figure 5.9A), minimal to no virus replication was observed in groups challenged with CARV-22. Mean virus gene copy numbers in V1-Ch196 and V2-Ch196 were numerically, but not significantly, lower than Ch196 group. Groups vaccinated and/or challenged with CARV-30 showed minimum virus replication and inconsistency in virus gene copy number. At 33 doa (20dpi, Figure

5.9B), minimal to no virus replication was observed in groups challenged with CARV-22 and CARV-30, whereas mean virus gene copy numbers in V1-Ch196 and V2-Ch196 were numerically, but not significantly higher than Ch-196 group.

Histopathologic lesion scores in gastrocnemius tendons: The histologic tendon lesions consisted of hypertrophy and hyperplasia of synoviocytes progressing to lymphoplasmacytic tenosynovitis and mild fibroplasia at a later stage (Figure 5.10A & B). At 18 doa (5dpi, data not shown), mean tendon inflammation scores were very low and of the range of NV-NCh, V1-NCh and V2-NCh groups and did not differ significantly in all groups. At 23 doa (10dpi, Figure 5.11A), histologic lesion scores in the V1-Ch22 and V2-Ch22 were numerically, but not significantly, lower than the Ch22 group. Mean inflammation scores in the V2-Ch196 group were numerically, but not significantly lower than V1-Ch196 and Ch196 groups. At 33 doa (20dpi, Figure 5.11B), histologic lesions became severe, and scores reached up to 36 (Ch196 group). Histologic scores were very low in V1-Ch22, V2-Ch22 and Ch22 groups. The mean inflammation score in the V2-Ch196 group was numerically, but not significantly, lower than means of the V1-Ch196 and Ch-196 groups. Similarly, the mean inflammation score in the V2-Ch30 group was lower than the means of the V1-Ch30 and Ch30 groups.

Discussion

With recent advancements in molecular biology, genetic engineering and reverse genetics technology have made it possible to use only relevant genes and their immunogenic proteins carried by recombinant viral vectors for immunization. This advancement is an alternative to the use of attenuated/inactivated/killed whole virus vaccines. Recombinant

live virus subunit vaccines have many advantages over conventional vaccines as discussed by Goldenberg et al., (2016) and Sellers (2017).

The effectiveness of recombinant vaccines in controlling viral infection has been proven for infectious bursal disease (IBD) (Pitcovski et al., 2003), egg drop syndrome (EDS) (Fingerut et al., 2003), avian influenza (AI) (Dhanwani et al., 2015) for chickens and hemorrhagic enteritis (HE) (Pitcovski et al., 2005) and reoviral arthritis (TARV) (Kumar et al., 2021) for turkeys. The PICV-based TARV vaccine showed a similar phenomenon of protection as observed in this study, where the recombinant vaccine induced neutralizing antibodies against challenge viruses (Kumar et al., 2021).

Variant ARVs are circulating and affecting the poultry industry worldwide. Antigenic variation, especially in the cell attachment and outer capsid proteins of ARVs of emerging genotypes, is the cause of vaccination failure (Sellers, 2017; Ayalew et al., 2017; Palomino-Tapia et al., 2018; Egána et al., 2019). Vaccines containing a strain from one genotype are ineffective against other genotypes (Vasserman et al., 2004; Goldenberg et al., 2010; Lu et al., 2015; Goldenberg et al., 2016; Preleman et al., 2019).

In the present study, two strains were selected as vaccine candidates based on their clustering pattern of SC and SB gene sequences. One strain (CARV-22) was selected from GC1 (vaccine cluster) to check the immunogenic efficiency of its SC and SB genes having high genetic similarity with other vaccine strains. The other strain (CARV-196) was selected from GC5, which is a new highly pathogenic genotypic cluster (Sellers, 2017; Ayalew et al., 2017; Egána et al., 2019) to check the immunogenic potential of its SC and SB genes. A third strain (CARV-30) was selected from GC6 (recently evolved GC) as a challenge virus to check the spectrum of protection provided

by our vaccines. Codon optimized SC and SB gene sequences of CARV-22 and CARV-196 were used based on our previous experience with PICV-TARV vaccine, where greater expression of TARV antigenic proteins and a strong immune response were observed both in vitro and in vivo (Kumar et al., 2020). We successfully recovered rPICV with CARV gene inserts (SC and/or SB) in the PICV plasmids.

The recovered and plaque-purified monovalent and bivalent vaccine viruses grew well in BHK-21 cells as evidenced by the expression of GFP in monovalent vaccines with one of the GFP genes, in addition to CARV genes. Bivalent vaccines did not show green fluorescence of GFP because both the recombinant plasmids carried CARV genes but no GFP gene. Similar observations were reported in rPICV- based AI and TARV vaccines (Dhanwani et al., 2015; Kumar et al., 2020). We assume that the bivalent vaccine virus transfection and virus rescue was successful because all the transfections and rescue were performed simultaneously under the same conditions. Previous studies have reported various subunit vaccines expressing SC and SB proteins against chicken and duck reoviruses (Wu et al., 2005; Lin et al., 2008; Bi et al., 2016; Goldenberg et al., 2016). Subunit vaccines against infectious bursal disease (Pitcovski et al., 2003) and adenovirus infection (Fingerut et al., 2003; Pitcovski et al., 2005) have previously been proved to be efficacious.

The gene inserts were confirmed in rPICV-CARV vaccines by RT-PCR and successfully tested for the expression of CARV antigenic proteins by DFA. The RT-PCR and DFA confirmation suggests that the rPICV vaccine stably carries CARV genes and successfully expresses the CARV antigenic genes. Based on the DFA results, bivalent rPICV-CARV vaccine showed higher apple green fluorescence than the monovalent

vaccines. Similar observation was reported in PICV-TARV vaccine by Kumar et al., (2020). Although, fluorescence was not quantified but the subjective observations suggest that bivalent vaccines have two antigenic genes that may be producing more antigenic proteins than the monovalent vaccines.

Two vaccine formulations were tested in this study; V1 is a cocktail of two bivalent vaccines (CARV-22SC/SB and CARV-196SC/SB) and vaccine 2 (V2) is cocktail of two monovalent vaccines (CARV-22SC and CARV-196SB). The idea behind using these formulations was to determine their effect on bird health and to test their safety and efficacy in protection against a wide spectrum of pathogenic viruses. Day-old chicks were primed orally with the respective vaccines and boosted intranasally (IN) at 8 doa. Oral prime and IN boost strategy was adopted to target oral and coarse spray potential of our vaccines which was effective in our PICV-TARV vaccine (Kumar et al., 2020). Chicks were primed and boosted at 1 and 8 doa, respectively, to elicit immune response in young birds when they are most prone to reovirus infection (Jones and Guneratne, 1984; Roessler and Rosenberger, 1989). Vaccine regimes against reovirus infection target breeder flocks to provide maternally derived antibodies to young chicks or directly vaccinating young chicks with live vaccines (Jones, 2000).

The ELISA antibody detection by commercial ELSA showed negative results in sera from vaccinated birds of our study and sera collected from experimentally infected birds showed ELISA antibody titer of 771 to 2716. The reason for this type of results is the whole virus commercial ELISA used in this study is incapable of detecting antibodies produced by subunit vaccines. A subunit ELISA would have been more sensitive to detect the subunit vaccine antibodies as reported previously (Shien et al., 2000; Liu et al.,

2002; Lin et al., 2006). An ELISA using SC and/or SB proteins as the target antigen shows a good correlation between ELISA and SNT (Yang et al., 2010) and would be more effective in detecting neutralizing antibodies (Yang et al., 2010; Lublin et al., 2011). Therefore, we plan to develop a subunit antigen ELISA and use it in our future vaccine studies. Chicks vaccinated with both the vaccine formulations (V1 and V2) produced serum neutralizing antibodies against the candidate strains (CARV-22 and CARV-196) as well as the heterologous challenge virus (CARV-30). Antibodies produced against the SC protein showed successful neutralization in cell culture (Lin et al., 2008; Jung et al., 2014). The SN antibody titer after booster dose (13 doa) (64-256) was significantly higher than that after the primary dose (8 doa) (32-64) against CARV-22 and CARV-196 challenge viruses. The SN antibody titer against CRV-30 followed the same pattern where the antibody titer (16-64) after prime dose (8 doa) was numerically, but not significantly, lower (16 -128) than the booster dose (13 doa). The mean SN antibody titer in the V2 group is numerically, but not significantly, higher than V1 group after prime as well as booster dose against all the challenge viruses (CARV-22, CARV-196 and CARV-30). The dynamics of SN antibody titers changed from 8 doa to 33 doa, where the antibody titers increased from 8 doa to 23 doa and then reached a plateau at 33 doa. The antibody titers might have changed if the study would have been extended for more time and the gap between prime and boost dose increased. Similar dose timing recommendations were given for rPICV-based AI and TARV vaccines (Dhanwani et al., 2015; Kumar et al., 2020) because rPICV-based vaccines need approximately three weeks to form memory cells for provoking better immune response in the immunized host (personal communication with Ly and Liang lab). The age at prime-boost (01 and 08

doa) and age at virus challenge (13 doa) was decided based on the age of chicken (<2 weeks of age) that is most susceptible to reovirus infection (Jones, 1984; Davis et al., 2013; Troxler et al., 2013; Niu et al., 2017; Perelman et al., 2019). Chicks >2 weeks of age show increasing resistance to reovirus infection (Kerr and Olson, 1969; Jones and Georgiou, 1984; Roessler and Rosenberger, 1989).

At all euthanasia time points (18, 23 and 23 doa), V1 had a deleterious health effect on birds, causing decreased weight gain compared to the corresponding V2 groups, suggesting that V2 is safer than V1. The reason for this effect of V1 cannot be ascertained at this point but it may be because of additional requirements by two bivalent vaccine viruses from the host for immunomodulation or may be due to antigenic shock to the chicks caused by bivalent vaccine viruses. Additionally, the body weight gain of all virus challenge groups (Ch22, Ch196 and Ch30) and V1 groups (V1-Ch22, V1-Ch196 and V1-Ch30) was significantly lower than the respective vaccinated groups (V2-Ch22, V2-Ch196 and V2-Ch30). The groups vaccinated with V1 (V1-Ch22, V1-Ch196 and V1-Ch30) showed inconsistent body weight gains relative to the respective virus challenge groups (Ch22, Ch196 and Ch30). These results suggest that V2 can reduce the intestinal replication of all three challenge viruses thereby minimizing any adverse effects on body weight. Additionally, the dose and duration between prime and boost should be modified to further evaluate both vaccine formulations.

At 18 doa (5dpi), the virus gene copy numbers in intestine showed significant decrease in virus replication in the V1 (V1-Ch22 and V1-Ch30) and V2 groups (V2-Ch22, V2-Ch196 and V2-Ch30) than the respective virus challenge groups (Ch22, Ch196 and Ch30). The virus gene copy number in the V1-Ch196 group was numerically, but not

significantly, lower than the Ch196 group, which may be due to reduced efficacy of V1 against a highly pathogenicCh196 virus. The significant decrease in virus replication in the vaccinated groups is likely due the production of neutralizing antibodies in the vaccinated birds. Immune protection against ARVs is mainly provided by humoral antibodies (Kibenge et al., 1987; Sellers, 2017). Type specific and broad specific neutralizing antibodies produced by SC and SB antigenic proteins inhibit virus attachment and cause lysis of virus and virus infected cells (van Loon et al., 2003). Additionally, the vaccines have inhibited replication of all the challenge viruses from GC1, GC5 and GC6 suggesting the spectrum of protection provided by these vaccines. This may be due to the induction of a spectrum of type and broad specific neutralizing antibodies for viruses from GC1 and GC5 eliciting strong mucosal immunity (Lin et al., 2012) and hence providing a wide spectrum of protection. Protection to ARV infection due to humoral immune response is dependent upon serotype and antigenic homogeneity, virulence of the virus, host age and levels of maternal derived antibodies (Rau et al., 1980; Takase et al., 1996). The mean virus gene copy numbers in intestine at later euthanasia time points were numerically, but not significantly, reduced (except V1-Ch196) in all the groups and there were no differences between the vaccinated and virus challenge groups. This may be attributed to the production of antiviral cytokines 7 dpi which are associated with a decrease in replication and eventual elimination of virus (Sharafeldin et al., 2015).

In tendons, at 18 doa (5dpi), no virus replication or very low and incidental virus replication was observed in all groups. At 23 doa (10dpi), virus replication was observed in V1 and V2 groups respective to Ch196 and Ch30 and incidental replication in some

birds of respective Ch22 groups, but no significant difference was observed in virus gene copy numbers. At 33 doa (20dpi), low virus replication and gene copy numbers were observed only in V1-Ch196, V2-Ch196 and Ch196 groups, but no significant differences were observed among the groups. The reason for very low Ch22 virus replication in tendons may be because Ch22 belongs to vaccine cluster containing mild to low pathogenic strains.

Histologic lesions observed in tendons over time were infiltration of lymphocytes and plasma cells in the tendon sheath and formation of lymphoid aggregates with progressive fibroplasia. At 18 doa (5dpi) histologic lesion scores were very low with incidental high scores in V2-Ch22 and V1-Ch196 groups. At 23 doa, (10dpi), histologic lesion scores in V1-Ch22 and V2-Ch22 were numerically, but not significantly, lower than Ch22 group. The mean tendon inflammation score in Ch196 was significantly higher than V2-Ch196 ($p<0.01$). The mean histologic lesion score in the Ch196 group was nearly equal to that of the V1-Ch196, indicating that the V1 was not protective against Ch196. At 33 doa (20dpi), mean histology lesion scores in Ch196, V1-Ch196, V2-Ch196 groups were not significantly different. The gastrocnemius tendon inflammation described in reoviral arthritis is also associated with increased level of IL-6 and IFN-γ cytokine, suggesting that f these cytokines play a role in the development of tenosynovitis (Sharafeldin et al., 2015b). The joints and tendons act as sequestered sites, thus preventing virus elimination by the immune system once the virus reaches the tendons (Jones and Georgiou, 1985).

The present study demonstrated that immunization with live rPICV-CARV vaccines provide immunity to chicks by early clearing of the virus out of the host,

inhibiting viral replication in intestine thereby decreasing fecal shedding of the virus. The cocktail of monovalent codon optimized rPICV-CARV vaccine (V2) as well as cocktail of bivalent codon optimized vaccine (V2) has a potential for vaccination against reoviral arthritis in chickens; however, additional studies are necessary to optimize vaccination timing and dose. Future evaluation of the vaccine is planned to study the immunogenicity of these vaccine in breeders. In this context, it is worth studying whether our vaccines would interfere with other chicken vaccines. We also need to develop and improve immunization protocols with defining the dose, route, and number of boosts in breeders for use in commercial farms. However, this study has provided a useful foundation by showing that the rPICV-CARV vaccine could control and prevent reovirus infection in chicken.

Additionally, our rPICV-CARV vaccines proposes other advantages over conventional vaccines e.g. modification of the subunit vaccine to new genetic variants is relatively rapid by cloning well characterized genes (Sigma C, Sigma B and Mu B) of new variants in rPICV with wide spectrum of potential immunogenicity against the variant strains; only gene segments of variant strains are used in the vaccine eliminating the possibility of generation of variant CARVs or reversal to virulent and pathogenic virus; the process is controlled, repeatable and avoid welfare concerns of using live embryos. This study provides promising potential of our vaccine for prevention and control of reovirus infection. Lastly, vaccines are successful when implemented with strict biosecurity and best management practices for the prevention and control of reovirus infection in chickens.

Acknowledgments: This study was supported by a grant from Binational Agricultural Research Fund (BARD). RK acknowledges the financial assistance in the form of Netaji Subhash International Fellowship from the Indian Council of Agricultural Research, India, Pomeroy Legacy scholarship and MNDRIVE fellowship from the University of Minnesota from to conduct this Ph.D. research project.

Table 5.1. Recombinant Pichinde virus (rPICV) plasmids used to generate PICV-based chicken arthritis reovirus (CARV) vaccines.

Sr. No.	Strain of CARV	Type of vaccine	Insert in plasmid 1*	Insert in plasmid 2*
1	022	Monovalent	GFP[a]	SC
2		Monovalent	SB	GFP
3		Bivalent-homologous	SB	SC
4	196	Monovalent	GFP	SB
5		Monovalent	SC	GFP
6		Bivalent-homologous	SC	SB
7	022 and 196	Bivalent-heterologous	SC-022	SC-196
8	022 and 196	Bivalent-heterologous	SB-022	SB-196
9	None	Control	GFP	GFP

[a]GFP= Green Fluorescence Protein. CARV-022, CARV-196 are strains of chicken arthritis reoviruses whose SC and SB genes were inserted into PICV plasmids. Plasmid 1* is pP18S1-GPC/MCS (1614); Plasmid 2* is pP18S2-MCS /NP (1615). SC is sigma C gene, SB is sigma B gene of CARV isolates.

Table 5.2. Monovalent and bivalent rPICV-CARV vaccines generated with their antigen combinations and vaccine titers.

S. No.	Vaccines	Antigen combination*	Vaccine titer
1	Bivalent	CARV-22 SB/SC	$7X10^6$ PFU/ mL
2	Bivalent	CARV-196 SB/SC	$2.5X10^6$ PFU/ mL
3	Bivalent	CARV-196 SC/CARV-22 SC	$1X10^6$ PFU/ mL
4	Bivalent	CARV-22 SB/CARV-196 SB	$2.5X10^6$ PFU/ mL
5	Monovalent	CARV-22 SC	$7X10^5$ PFU/mL
6	Monovalent	CARV-22-SB	$5X10^5$ PFU/ mL
7	Monovalent	CARV-196-SC	$7X10^5$ PFU/mL
8	Monovalent	CARV-196-SB	$7X10^4$ PFU/mL

*SC is sigma C gene, SB is sigma B gene of CARV-22 and CARV-196 strains.

Table 5.3. Summary of *in* vivo experimental design for evaluating efficacy of rPICV-CARV vaccine against a spectrum of virus challenge

Group No.	Group Name	Vaccination on indicated days of age	Challenge on indicated days of age	Euthanasia on indicated days of age	No. of birds
1	NV-NCh	None	-	8-13-18-23-33	25
2	V1-NCh	1 and 8	-	8-18-23-33	25
3	V2-NCh	1 and 8	-	8-18-23-33	25
4	V1-Ch22	1 and 8	13	13-18-23-33	25
5	V1-Ch196	1 and 8	13	18-23-33	20
6	V1-Ch30	1 and 8	13	18-23-33	20
7	V2-Ch22	1 and 8	13	13-18-23-33	25
8	V2-Ch196	1 and 8	13	18-23-33	20
9	V2- Ch30	1 and 8	13	18-23-33	20
10	Ch22	None	13	18-23-33	20
11	Ch196	None	13	18-23-33	20
12	Ch Ch30	None	13	18-23-33	20

Figure 5.1. Nucleotide sequence-based ML phylogenetic trees showing the clustering of CARVs based on the Sigma C and Sigma B protein coding genes of viruses. Phylogenetic calculations were carried out using the maximum-likelihood method applying the best-fit models calculated for each gene.

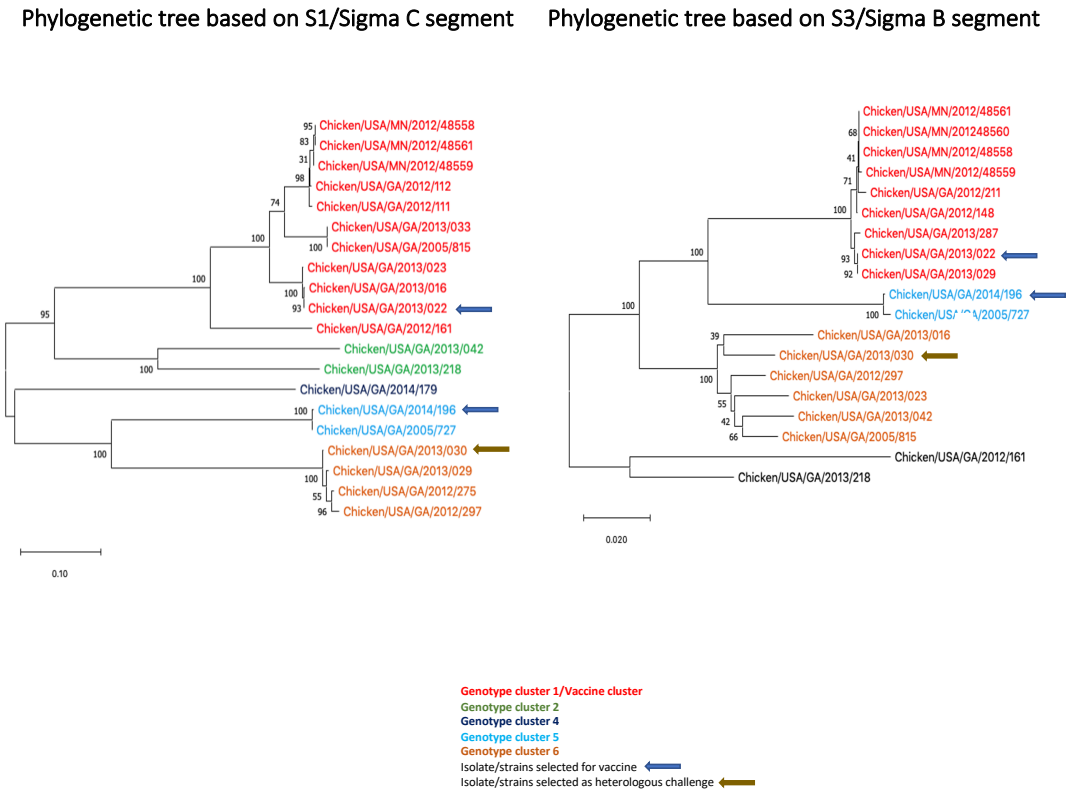


Figure 5.2. Confirmation of chicken arthritis reovirus genes (S1 and S3) in the rPICV vector by restriction enzyme (RE) double digestion. Restriction enzyme (RE) double digestion confirms the presence of S1 and S3 genes of CARVs in rPICV plasmids. Lanes 1, 2, 5 and 6: RE double digestion of recombinant pP18S2-S1/NP plasmid yielding plasmid backbone and codon-optimized insert of 1031 bp of S1 (SC) of CARV-22 and CARV-196; Lanes 3, 4, 7, and 8: RE double digestion of recombinant pP18S1-GPC/S3 plasmid yielding plasmid backbone and codon-optimized insert of 1157 bp of S3 (SB) of CARV-22 and CARV-196; M: Marker.

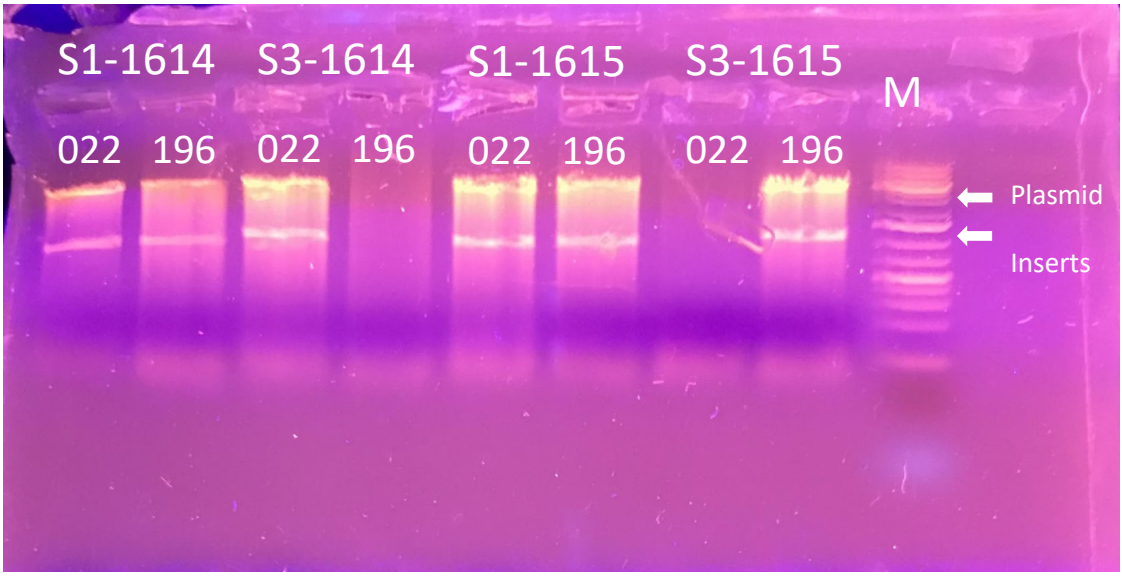


Figure 5.3. Transfection and infection of cells with plasmids and rPICV-based CARV vaccine viruses, respectively. (A) GFP expression in BSRT7-5 cells was observed under a fluorescence microscope following transfection of cells with pP18S1-GPC/GFP and pP18S2- S1/NP having a codon-optimized S1 gene insert, as shown in Table 1, to generate a rPICV-based CARV vaccine number 5. (B) GFP expression in BHK-21 cells infected with supernatant of monovalent rPICV- CARV vaccine number 5 at 96 hours post infection. (C) BSRT7-5 cells transfected with pP18S1-GPC/S3 and pP18S2- S1/NP having codon-optimized S1 and S3 gene inserts to generate a bivalent rPICV-CARV vaccine (recombinant vector virus number 6 of Table 1) that lacks the green fluorescence.

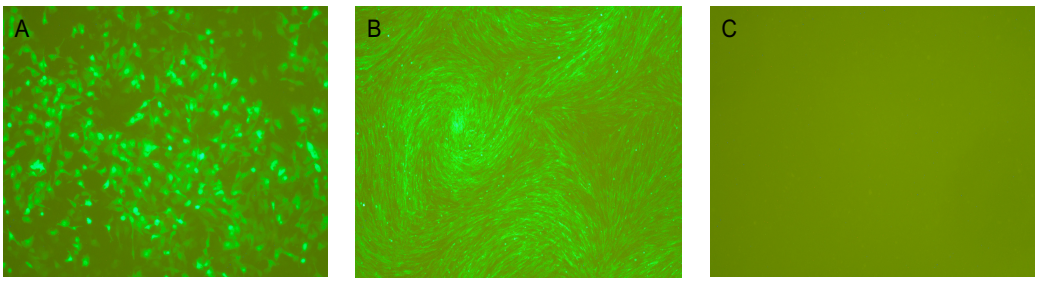


Figure 5.4. RT-PCR assay indicating the expression of chicken reovirus antigens at the messenger RNA (mRNA) level by recombinant rPICV-CARV vaccines. Lanes: M, marker; A, rPICV carrying only GFP gene; B, rPICV-CARV carrying SC gene; C, recombinant PICV carrying SB gene; D, rPICV-CARV carrying SC and SB genes (after 24 h of infection); E, rPICV-CARV carrying SC and SB genes (after 48 h of infection).

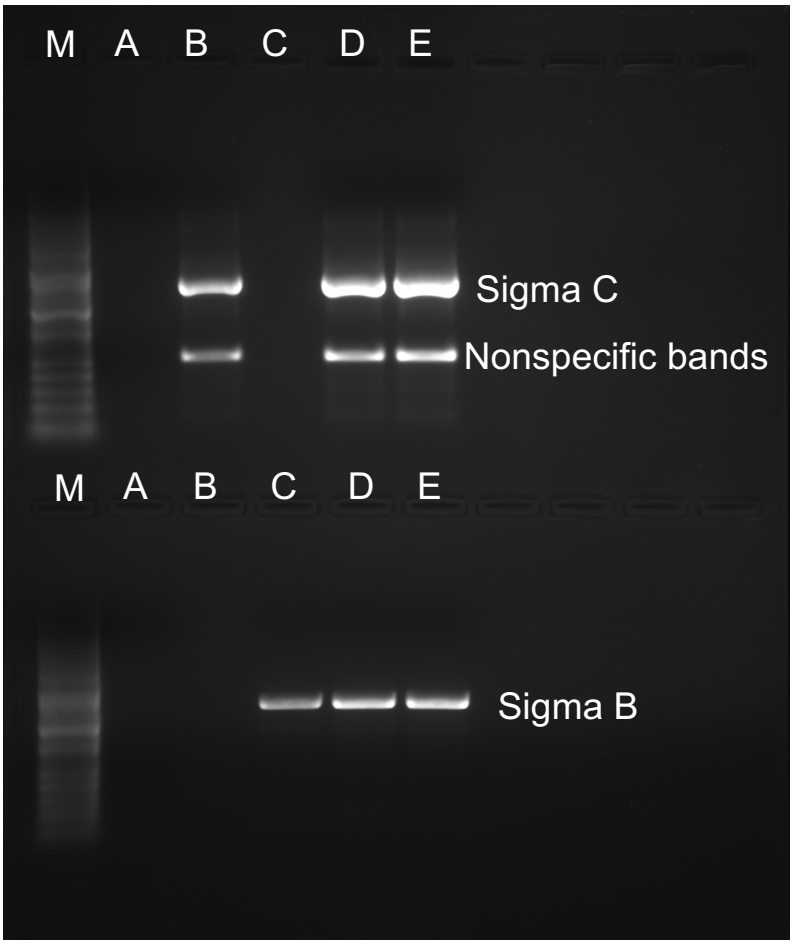


Figure 5.5. The rPICV-CARV vaccines expressing chicken arthritis reovirus antigens. Apple green fluorescence in BHK-21 cells (green fluorescent cells) based on direct fluorescent antibody test indicates CARV protein expression. (A) CARV wild-type virus control; (B) rPICV-CARV bivalent vaccine; (C) rPICV-CARV monovalent vaccine; (D) negative control.

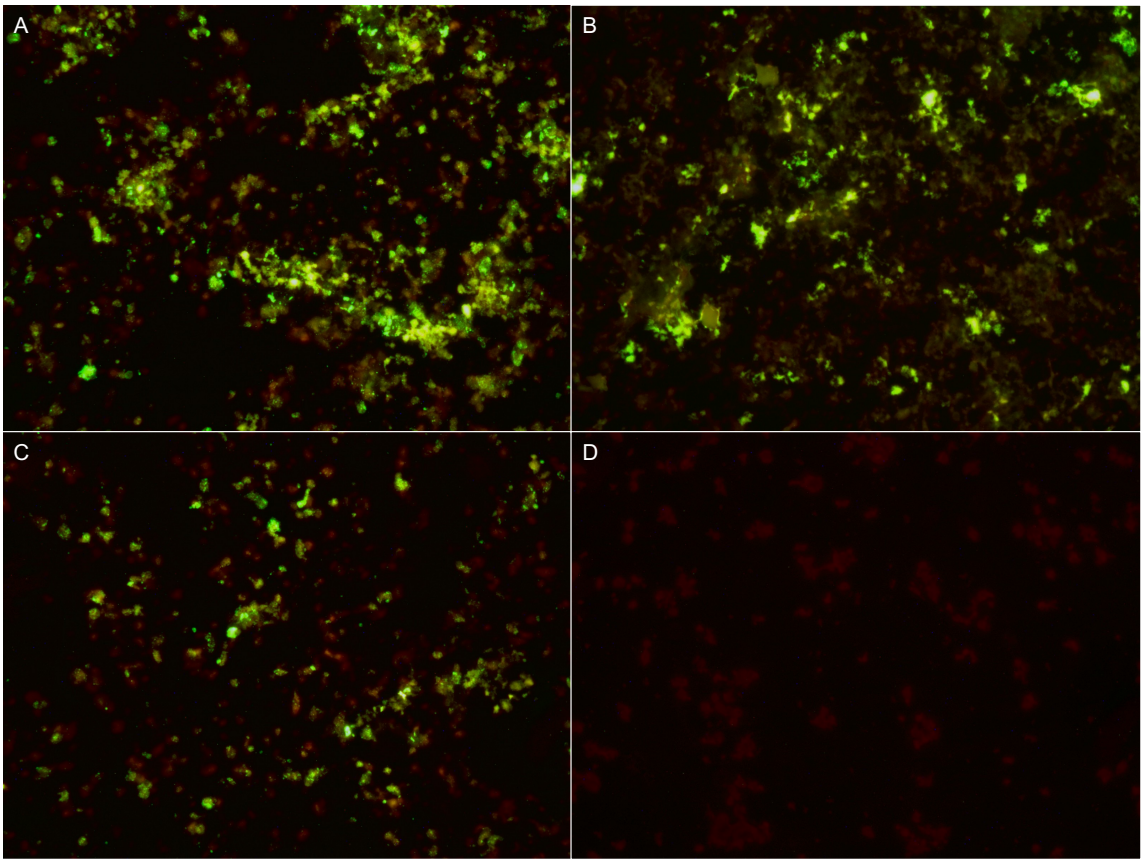
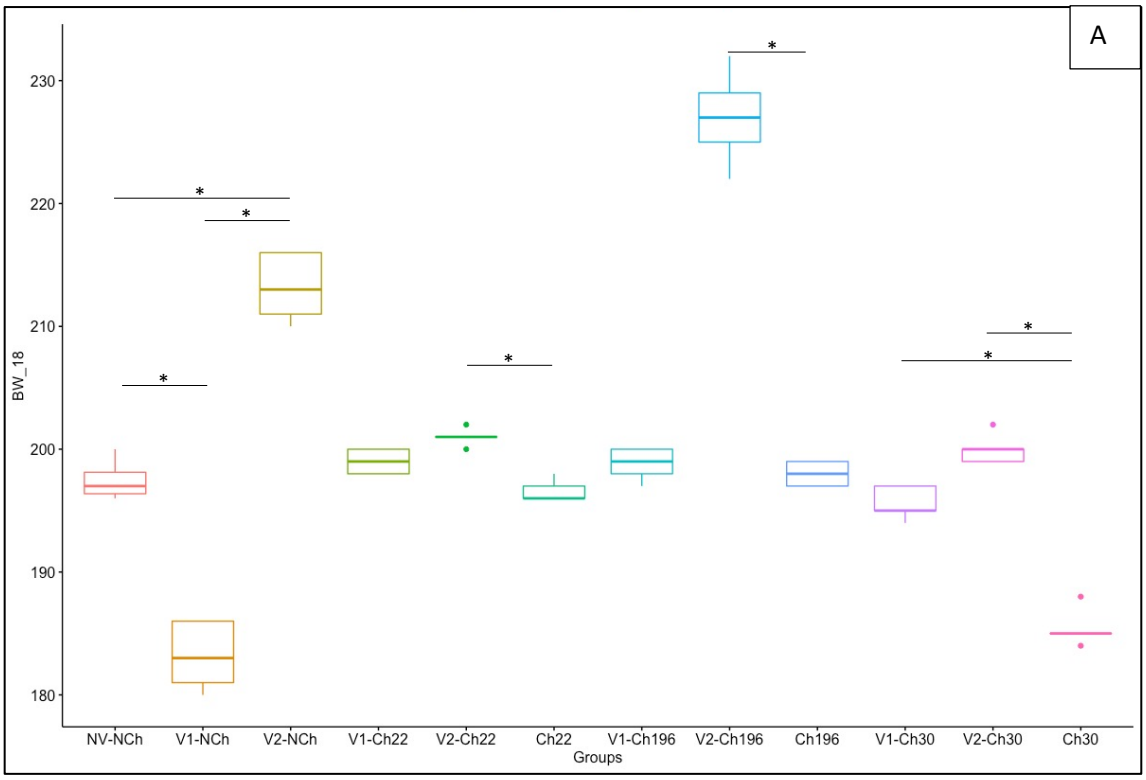
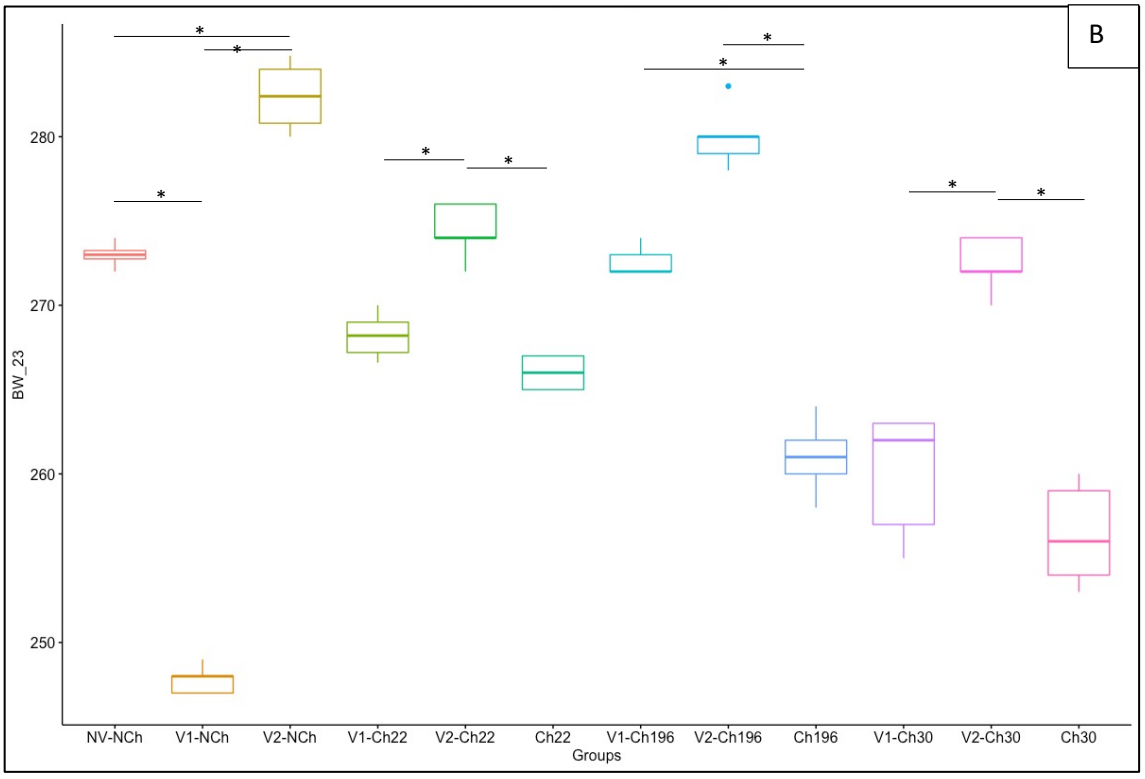


Figure 5.6 A) BW at 18 doa; **B**) BW at 22 doa; **C**) BW at 33 doa. Bar line with * between box plots have significant difference at p<0.05.





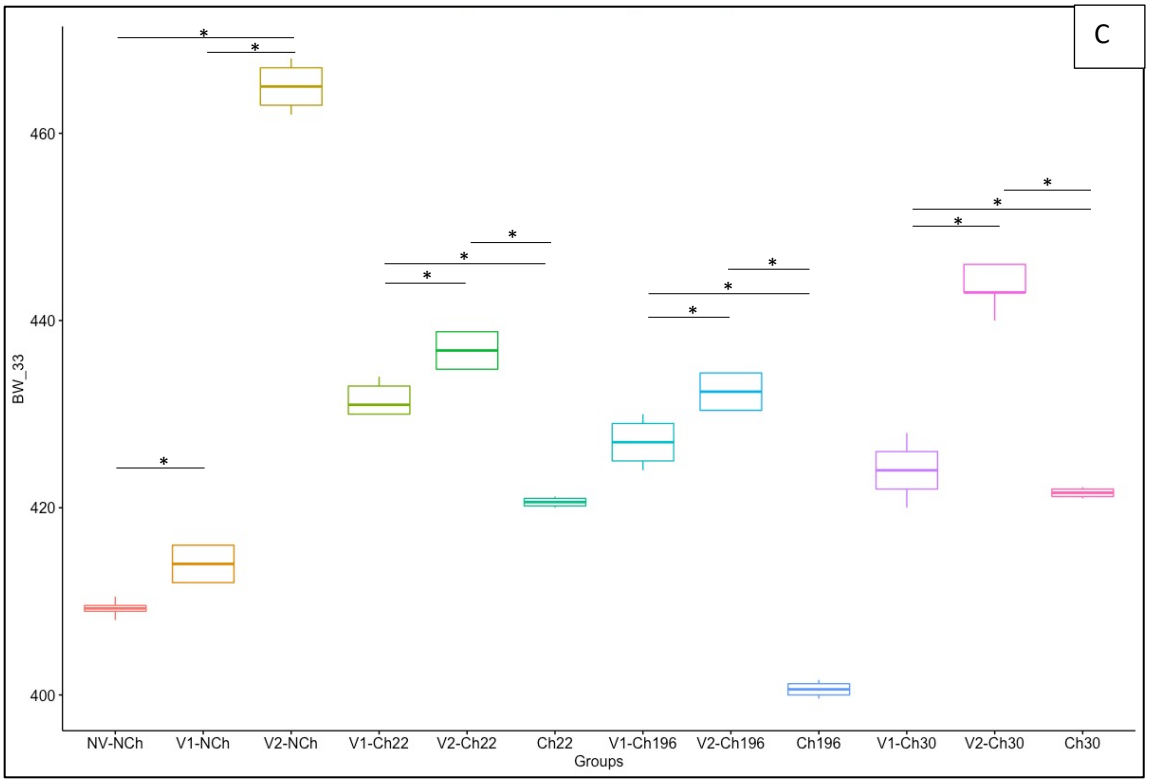
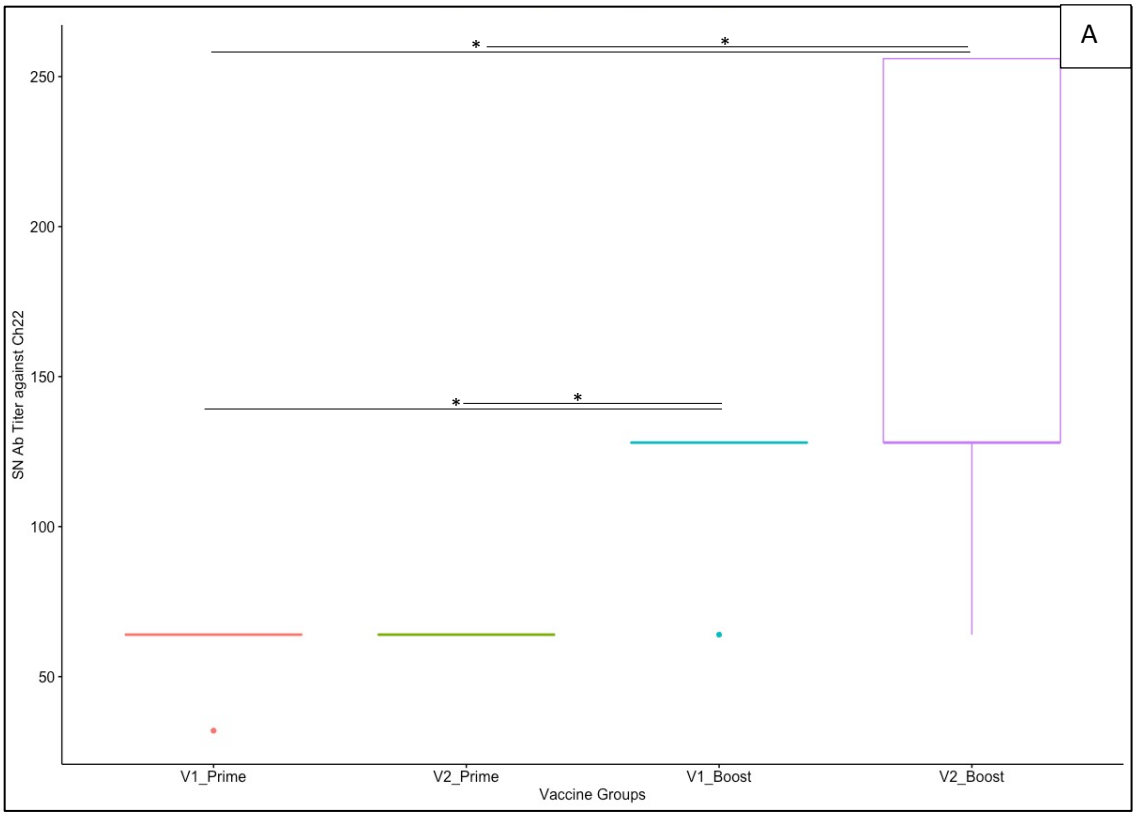
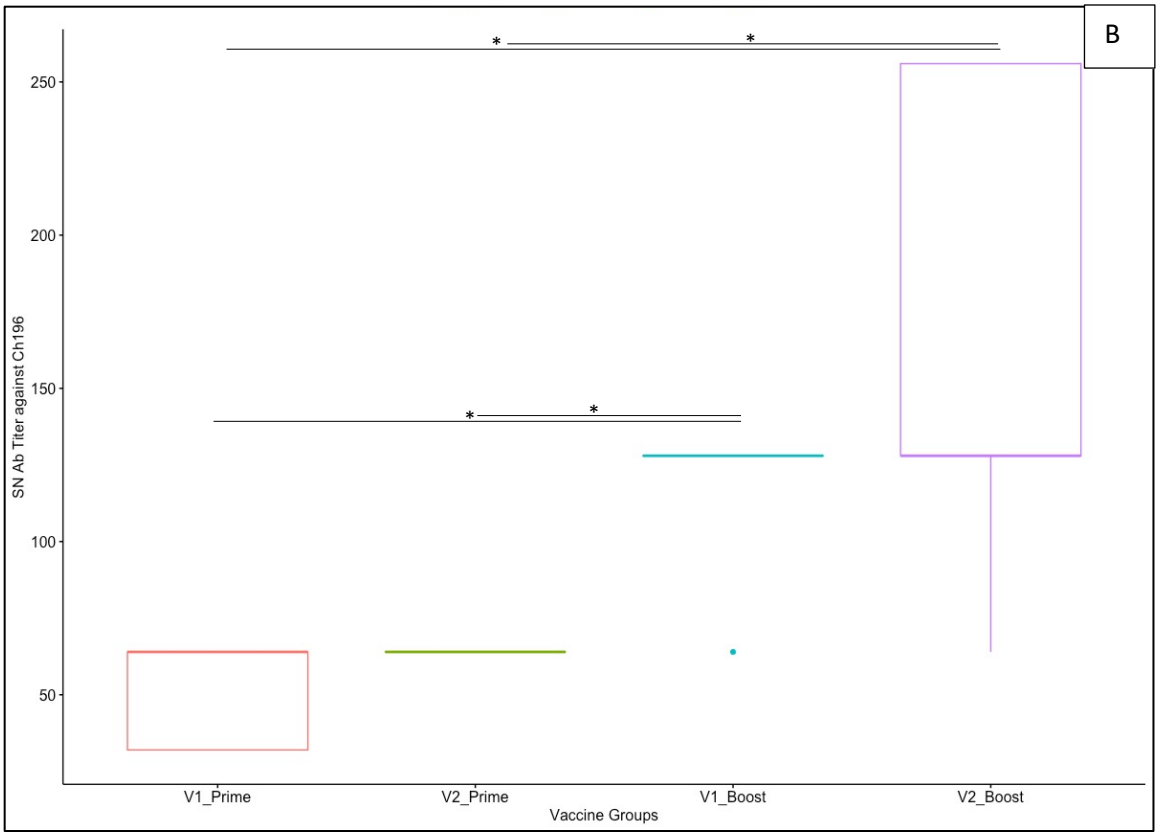
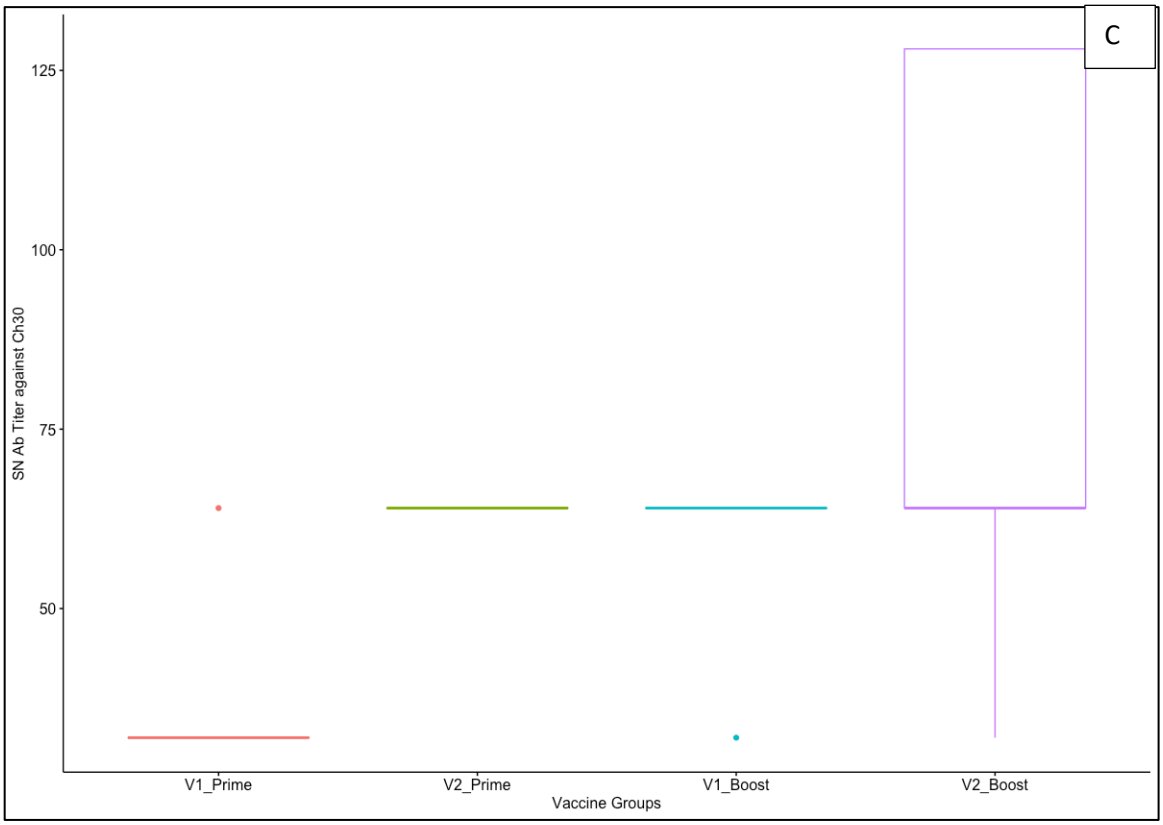
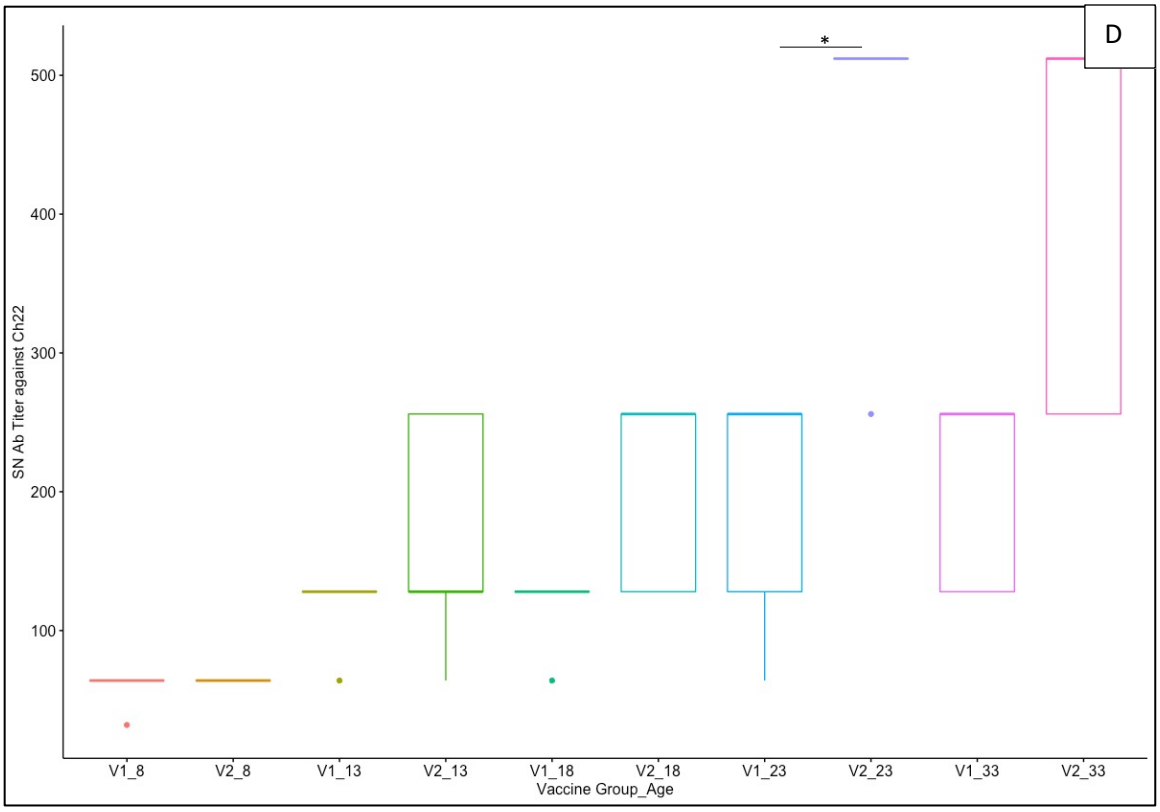

C
BW_33
460
440
420
400
NV-NCh
V1-NCh
V2-NCh
V1-Ch22
V2-Ch22
Ch22
V1-Ch196
V2-Ch196
Ch196
V1-Ch30
V2-Ch30
Ch30
Groups

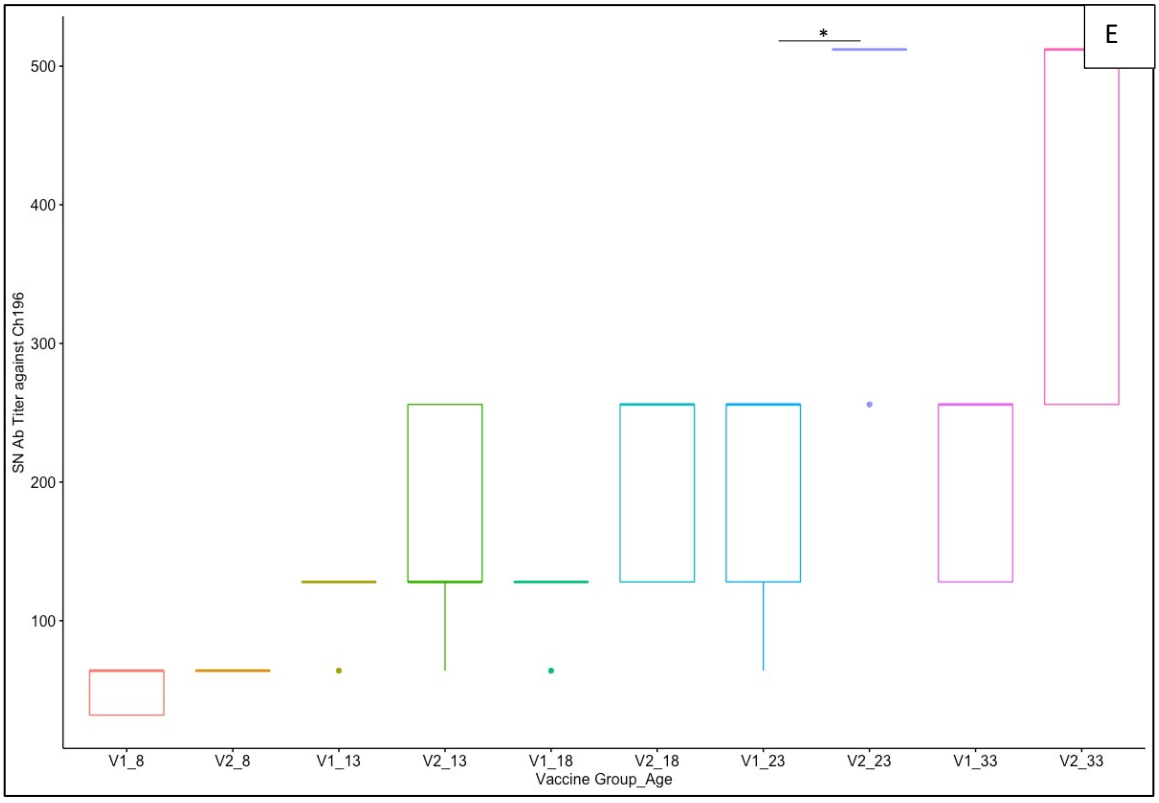
Figure 5.7 A) SN Ab titer against Ch22; **B**) SN Ab titer against Ch196; **C**) SN Ab titer against Ch30. **D**) SN Ab titer at different time points against Ch22; **E**) SN Ab titer at different time points against Ch196; **F**) SN Ab titer at different time points against Ch30. Bar line with * between box plots have significant difference at $p<0.05$.




B
SN Ab Titer against Ch196
250
200
150
100
50
*
*
*
*
V1_Prime
V2_Prime
V1_Boost
V2_Boost
Vaccine Groups


C
SN Ab Titer against Ch30
125
100
75
50
V1_Prime
V2_Prime
V1_Boost
V2_Boost
Vaccine Groups


D
*
500
400
300
200
100
SN Ab Titer against Ch22
V1_8
V2_8
V1_13
V2_13
V1_18
V2_18
V1_23
V2_23
V1_33
V2_33
Vaccine Group_Age


E
*
500
400
300
200
100
SN Ab Titer against Ch196
V1_8
V2_8
V1_13
V2_13
V1_18
V2_18
V1_23
V2_23
V1_33
V2_33
Vaccine Group_Age

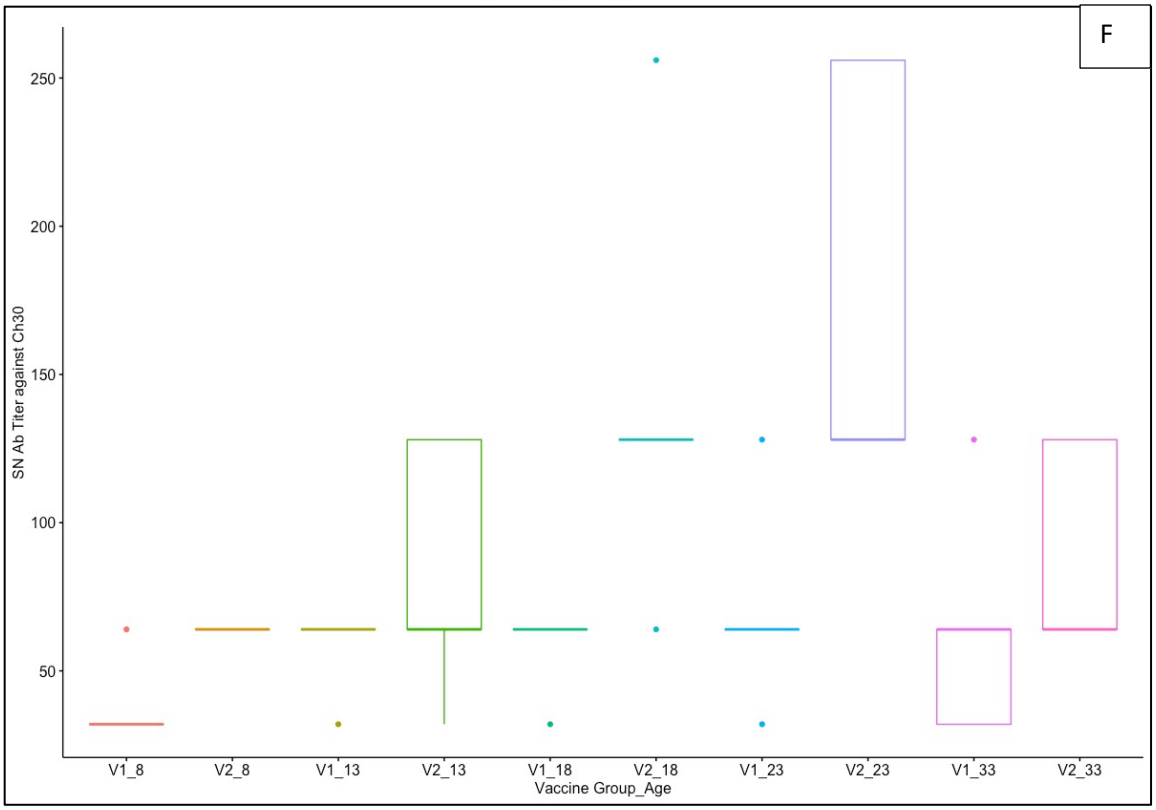
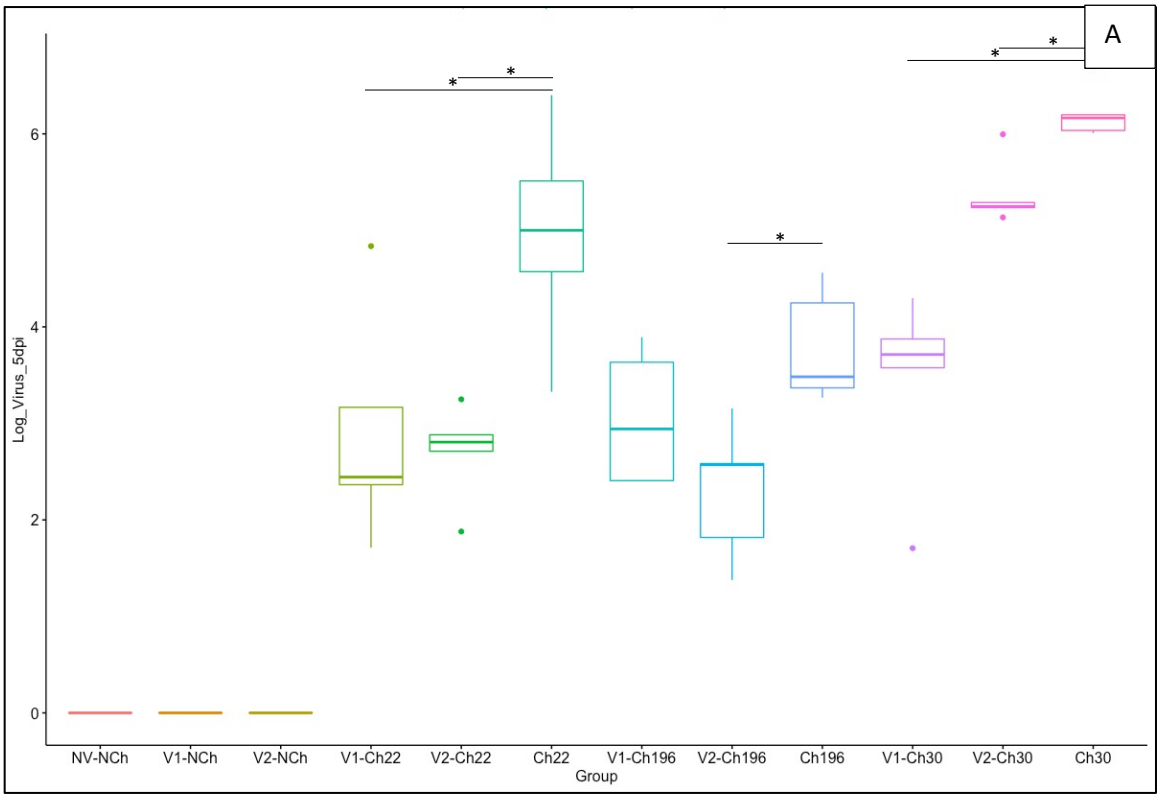

F
SN Ab Titer against Ch30
250
200
150
100
50
V1_8
V2_8
V1_13
V2_13
V1_18
V2_18
V1_23
V2_23
V1_33
V2_33
Vaccine Group_Age

Figure 5.8 Mean virus gene copy numbers **A**) Intestine at 18 doa (5dpi); **B**) Intestine at 23 doa (10dpi); Bar line with * between box plots have significant difference at $p<0.05$.



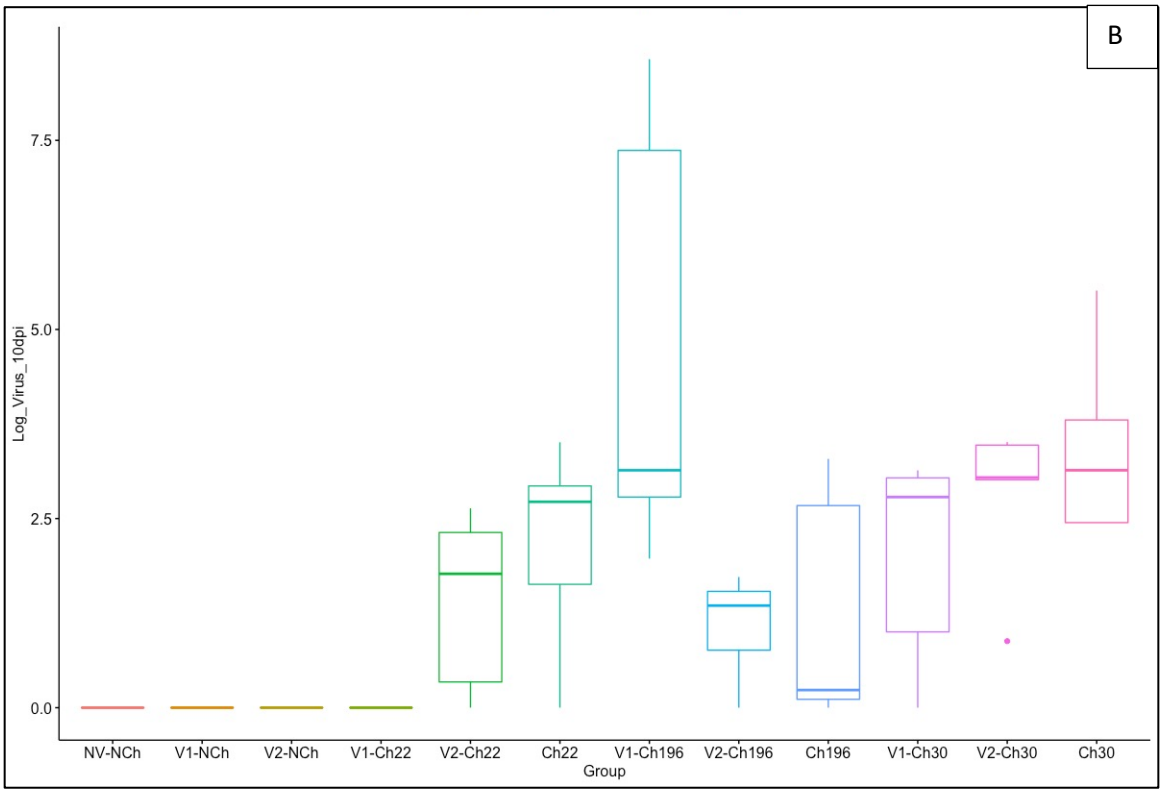


Figure 5.9 Mean virus gene copy numbers **A**) Tendons at 23 doa (10dpi); **B**) Intestine at 33 doa (20dpi); Bar line with * between box plots have significant difference at $p<0.05$.

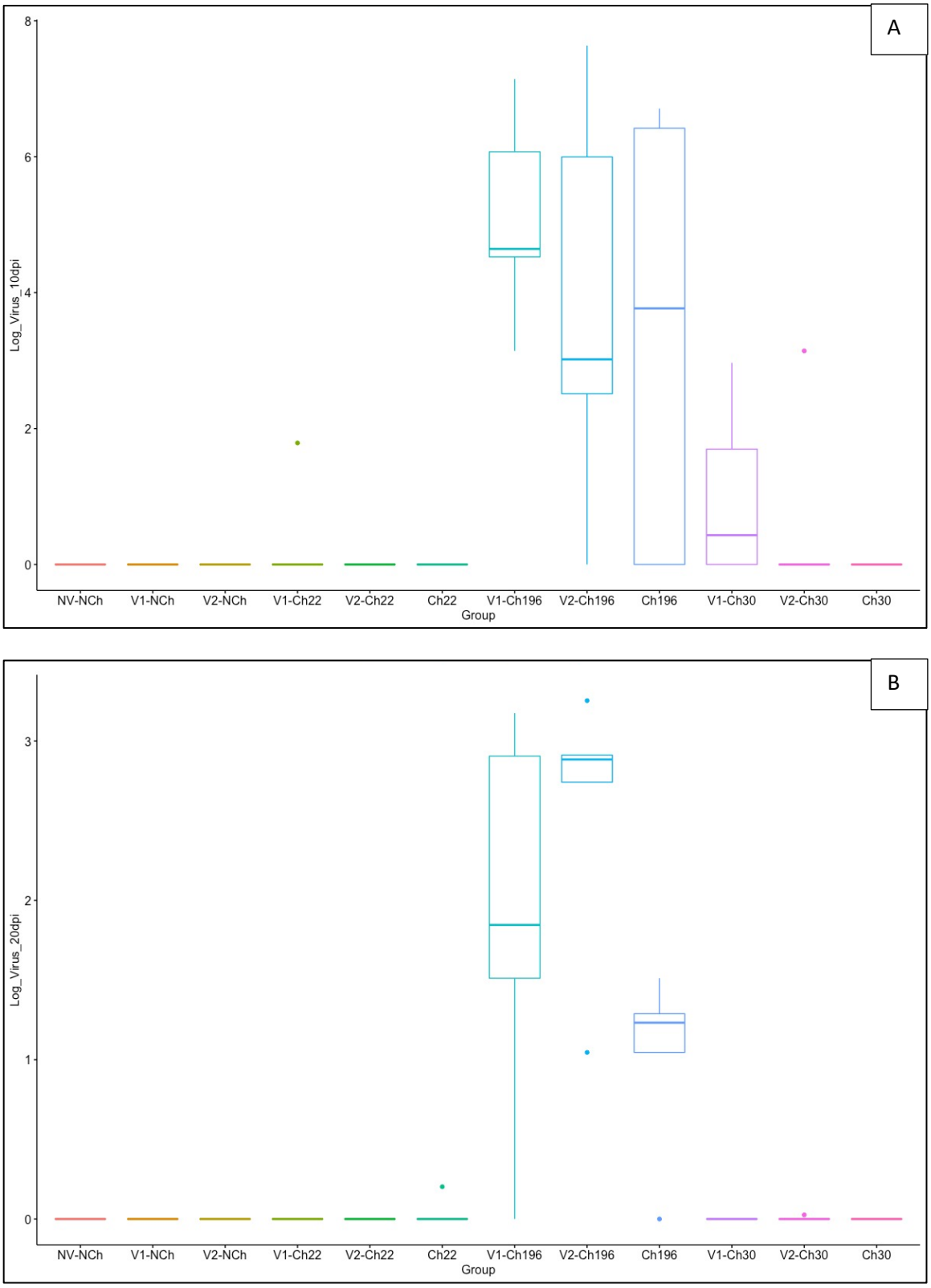


Figure 5.10 Histologic lesions in the gastrocnemius tendon of chicks infected with reovirus were characterized by lymphoplasmacytic infiltrates in the synovial sheath, hypertrophy and hyperplasia of synoviocytes and later mild fibroplasia A) **10X**; B) Higher magnification of A showing details **20X**, **H&E**.

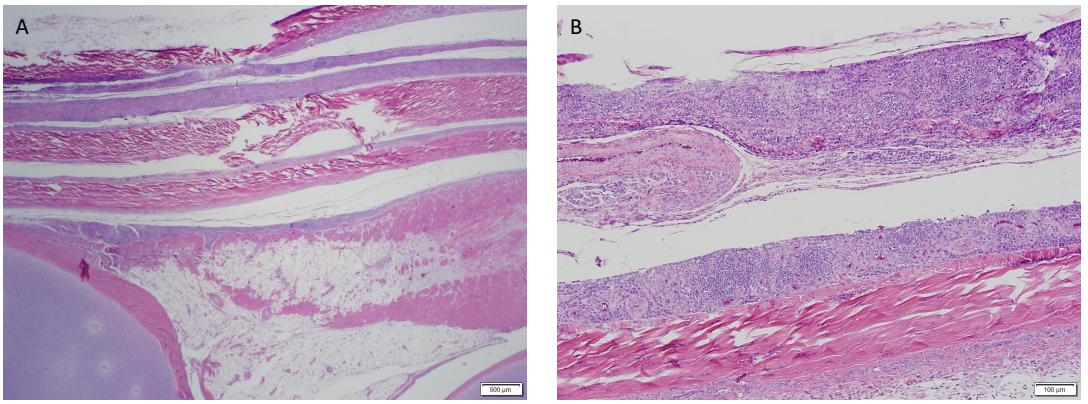
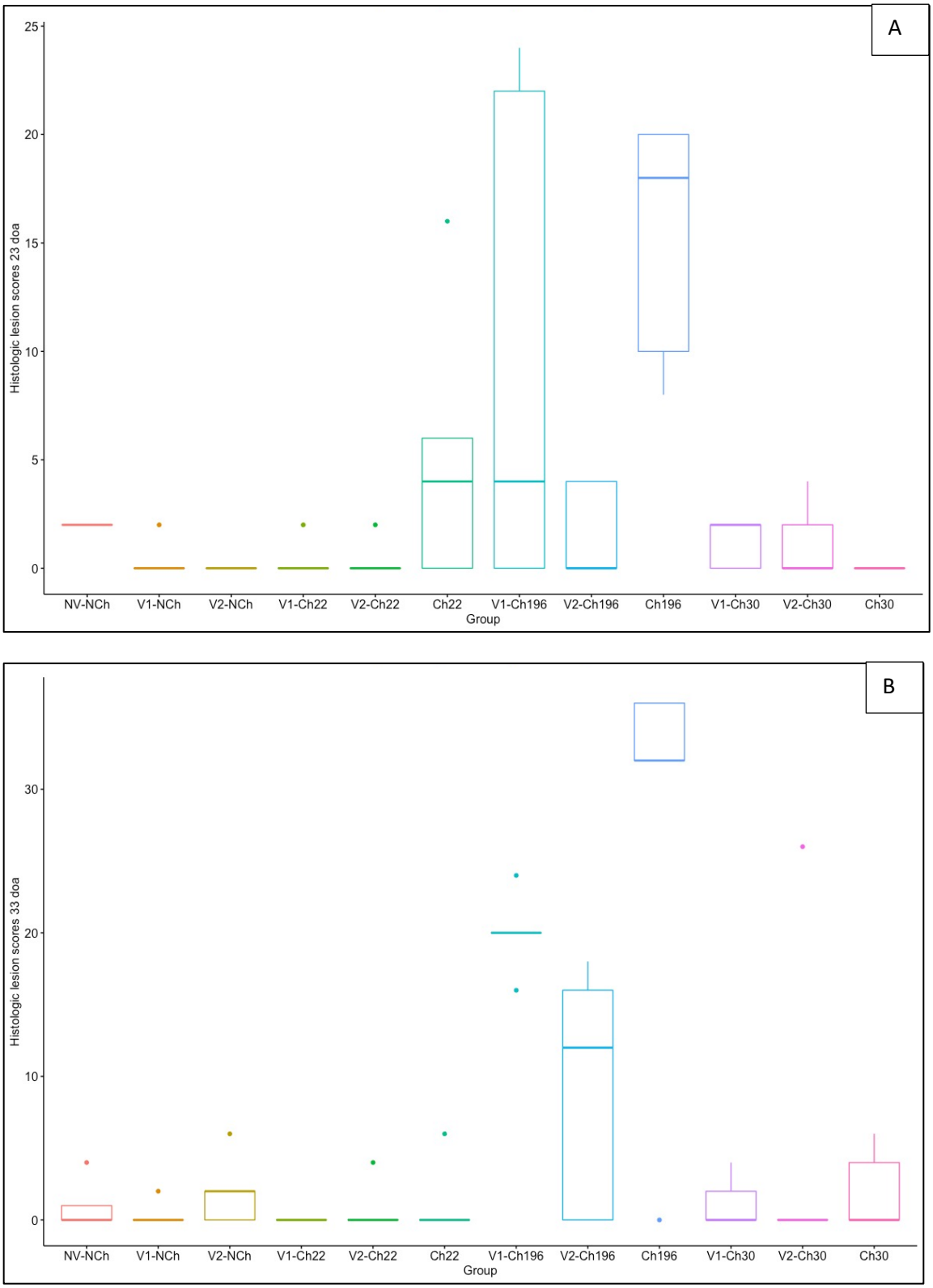


Figure 5.11 Histologic lesion scores in tendons at **A**) 23 doa; **B**) 33 doa. Statistics showing significant difference at $p<0.05$



CHAPTER 6

SURVIVAL OF RECOMBINANT LIVE VIRUS-VECTORED CHICKEN ARTHRITIS REOVIRUS VACCINE IN POULTRY LITTER AND DRINKING WATER

The material in this chapter has been prepared for publication:
Kumar R, Mor SK, Porter RE and Goyal SM. 2021. Survival of recombinant live virus-vectored chicken arthritis reovirus vaccine in poultry litter and drinking water. Avian Dis.

Introduction

Pichinde virus (PICV) is an enveloped RNA virus of the family *Arenaviridae.* The PICV genome has four ambisense genes on two genomic RNA segments namely L (large) and S (small) segments (Buchmeier et al., 2007). The L segment encodes Z protein which is a small RING domain-containing matrix protein that regulates virus budding, viral RNA synthesis, and host immune suppression (Fehling et al., 2012; Xing et al., 2015). The L protein is also encoded by L segment which is the RNA-dependent RNA polymerase (RdRp) required for viral RNA synthesis (Salvato et al., 1989). The S segment encodes for glycoprotein (GPC) and nucleoprotein (NP). The GPC is poattranslationally cleaved into stable signal peptide (SSP), G1 (receptor binding), and G2 (transmembrane) proteins (Burri et al., 2012). The NP protein encapsidates viral RNA and is responsible for viral RNA synthesis and host immune response (Lee et al., 2000; Pinschewer et al., 2003; Martínez-Sobrido et al., 2006; Qi et al., 2010; McLay et al., 2013; Huang et al., 2015). The PICV is a non-pathogenic virus with low seroprevalence, isolated from rice rats (*Oryzomys albigularis*) in the Pichinde valley of Columbia, South America (Trapido and Sanmartin, 1971).

Arenaviruses are considered a potential vaccine vector because dendritic cells (DCs) and macrophages are the target cells early in the infection (Emonet et al., 2009; Flatz et al., 2010; Popkin et al., 2011; Ortiz-Riano et al., 2013), Emonet et al., (2009) and Dhanwani et al., (2015) designed viral vectors based on two arenaviruses namely lymphocytic choriomeningitis virus (LCMV) and Pichinde virus (PICV) using reverse genetics technology. The recombinant PICV (rPICV) is a tri-segmented vaccine vector that successfully carries and expresses two foreign genes in addition to its own genes

while one of the foreign genes could be the green fluorescent protein (GFP) that can be used to mark virus-infected cells in cell culture (Dhanwani et al., 2015; Kumar et al., 2021).

Chicken arthritis reovirus (CARV) infects hock joints of 4 to 6-week-old broilers causing uni- or bilateral arthritis/tenosynovitis. The affected birds show lameness with poor growth and uniformity, secondary infections, mortality, and carcass downgrades at the processing plant (Jones, 2013). The CARV belongs to genus *Orthoreovirus*, subfamily *Spinareovirinae*, and family *Reoviridae* (Attoui et al., 2011). The non-enveloped virus is 70-80 nm in diameter (King et al. 2012). The viral genome has 10 segments of double stranded RNA (dsRNA). The genome segments are classified as large (L1, L2 and L3), medium (M1, M2, M3), and small (S1, S2, S3, S4) based on their electrophoretic mobility (Benavente and Martínez-Costas 2007). Sigma C (SC) protein encoded by the S1 gene segment (Benavente and Martínez-Costas, 2007) is an outer capsid cell attachment immunogenic surface protein containing type- and broad-specific neutralizing epitopes (Martinez- Costas et al., 1997; Grande et al., 2002). Sigma B (SB) protein of S3 segment is an outer capsid protein that contains a group-specific neutralizing epitope (Wickramasinghe et al., 1993).

Breeders are vaccinated with attenuated and inactivated vaccines to control CARV-associated lameness in chickens (van der Heide *et al.*, 1976; Wood *et al.*, 1986; Takase et al., 1996; De Herdt et al., 2016; Pitcovski and Goyal, 2020). However, emergence of variant CARVs causes vaccination failure and increased incidences of outbreaks (Troxler et al., 2013; Egaña et al., 2019; Torre et al., 2021)

We developed a bivalent codon-optimized recombinant live pichinde virus-vectored CARV vaccine (rPICV-CARV), which expresses SC and SB proteins. A similar rPICV based turkey arthritis reovirus vaccine (rPICV-TARV) developed in our lab was demonstrated to transmit horizontally to non-vaccinated pen mates providing immunity against TARV infection (unpublished). The choice of vaccination in the poultry industry is based on economics and ease of mass vaccination. Mass vaccination can often be achieved by administration of a live vaccine orally via drinking water or intranasally via coarse spray.

Virus vaccines are temperature sensitive that need cold chain for their viability and to maintain their efficacy. Recombinant live virus vectored subunit vaccine uses recombinant virus vectors prepared by manipulating the genome of various viruses. Herpes virus and adenovirus based viral vectors shows decreased production and transducibility at higher temperature (Wechuck et al., 2002; Howard et al., 2017). Similarly, the environmental survivability of lentivirus and adenovirus based viral vectors is shorter than their wild type viruses (Reuter et al., 2012).

The present study demonstrates that rPICV based reovirus vaccines could be administered safely via drinking water. This study was designed to determine the survival of this live vaccine in poultry litter and drinking water.

Materials and Methods

Growth of vaccine virus and a CARV strain: Vaccine virus (rPICV-CARV) and rPICV-GFP (without reovirus insert) vector having green fluorescence protein as foreign gene to mark virus-infected cells in cell culture (rPICV-GFP) were grown in BHK-21

cells. The rPICV-GFP was used as a positive control by observing GFP fluorescence in BHK-21 cells. The original titers of rPICV-CARV and rPICV-GFP were $5x10^6$ and $3x10^7$, respectively. An isolate of CARV grown in QT-35 cells was used as a wild virus positive control for fluorescence antibody test **(**FAT).

Fluorescence antibody test

Samples of eluted rPICV-CARV from water and litter were inoculated in BHK-21 cells and incubated in 5% CO_2 at 37 °C. After 96 hours post infection (hpi), the cell culture media was decanted and cells were washed with PBS, scraped, plated on 12-chamber glass slides, and air dried for 2 h in laminar flow. Cells were then fixed in acetone for 2 h followed by the addition of polyclonal fluorescein isothiocyanate (FITC)-conjugated anti-avian reovirus antibodies (National Veterinary Services Laboratory, Ames, IA, USA) and incubated at 37 °C for 2 h. Cells were then counterstained using 0.1% Evan's blue biological stain (EBBS). The slides were then mounted and examined under a fluorescent microscope to observe apple green fluorescence, which was indicative of avian reovirus protein expression by the rPICV-CARV vaccine virus.

Virus titration

The viable viruses were then titrated to determine quantitative change in virus titers at different time points. For this purpose, the water and litter samples were titrated in Vero cells by the plaque-forming unit (PFU) method (Lan et al., 2009). Briefly, 10-fold serial dilutions of samples were prepared in phosphate buffer saline (PBS). All dilutions were inoculated in monolayers of Vero cells in 6-well plates in triplicate using two wells per dilution followed by incubation in 5% CO_2 at 37°C for one hour with rocking the plates every 10 minutes for virus adsorption. After one hour, virus dilutions were aspirated and

3 ml of 20% mixture of 2% molten agar and complete MEM was overlayed and allowed to cool for 15 minutes before incubating the plates for 4 days in 5% CO_2 at 37°C. On day 5, 2 ml of 20% molten agar in complete MEM with neutral red dye (0.33%) was added as second overlay and allowed to cool for 15 minutes before putting the plates back into the incubator. The plaques were counted on the following day as PFU/ml.

Experimental design

Survival in Drinking Water: Two experiments were performed to determine the viability of rPICV-CARV at room temperature. In experiment 1, two sets of twelve aliquots of dechlorinated and autoclaved water (4 ml) were placed in 5ml sterile polystyrene tubes. The aliquots of water in set 1 and set 2 tubes were spiked with 200 µL of rPICV-CARV and PICV-GFP, respectively. After proper mixing, the tubes were stored at room temperature (25°C). A pair of aliquots (one from each of rPICV-CARV and PICV-GFP) was removed and stored at -80°C at 0, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96 and 120 hours. In experiment 2, non-autoclaved water from drinkers of a poultry farm was used to simulate field conditions. The experimental design was the same as that for autoclaved water. Both experiments were done in triplicate. The aliquots were thawed inside laminar flow, gently mixed and filtered. The filtrate was used to infect BHK-21 cells in a 24 well plate for FAT and Vero cells in a six well plate for titration. The viability of rPICV-CARV and rPICV-GFP was determined by FAT and green fluorescence of GFP, respectively under fluorescent microscope.

Survival in Poultry Litter: Litter samples from five different chicken farms were collected, mixed, and used as a single pool in two experiments. In experiment 1, a portion

of the litter pool was autoclaved and two sets of twelve aliquots of 2g were placed in sterile tubes. Pairs of aliquots were spiked with 200 μL of PICV-CARV and PICV-GFP, respectively. After mixing, the spiked aliquots were stored at room temperature. In experiment 2, non-autoclaved litter was used but the experimental design was the same. Pairs of aliquots were removed at 0, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96 and 120 hours and the surviving virus was eluted using 10 ml of PBS per tube. After vortexing, the samples were centrifuged at 2,500 xg for 10 min at 4°C. The eluted virus from both experiments was stored at -80°C until used for FAT and titrated in cell cultures. Both experiments were done in triplicate.

Results

Survival of vaccine virus in water: Both rPICV-CARV and rPICV-GFP survived in autoclaved dechlorinated water for six hrs as evidenced by apple green fluorescence in rPICV-CARV infected BHK-21 cells by FAT and GFP green fluorescence in BHK-21 cells shown by rPICV-GFP (Fig 6.1A and 1B). No virus growth was observed in BHK-21 cells after six hrs. The titers of rPICV-CARV and rPICV-GFP decreased from 0 to 6 hrs collection time points (Fig 6.2A). In non-autoclaved water, both viruses (rPICV-CARV and rPICV-GFP) survived for up to six hrs as evidenced by FAT and GFP green fluorescence (Fig 6.1C and 1D), but the titers of both viruses decreased from 0 to 6 hrs (Fig 6.2B).

Survival of vaccine virus in litter: Both PICV-CARV and PICV-GFP survived in autoclaved litter for six hrs. Apple green fluorescence was observed in rPICV-CARV infected BHK-21 cells by FAT and GFP green fluorescence in BHK-21 cells shown by rPICV-GFP (Fig 6.3A and 3B). The titers of PICV-CARV and PICV-GFP decreased

from 0 to 6 hrs (Fig 6.4A). In non-autoclaved litter, PICV-CARV and PICV-GFP survived up to 6 hrs as evidenced by FAT and GFP green fluorescence (Fig 6.3C and 3D). Titers of both viruses decreased from 0 to 6 hrs (Fig 6.4B).

Discussion

Live virus vectored subunit vaccines are safe, effective, and easy to update. They have many advantages over the conventional vaccines, but they need cold chain to maintain their efficacy. Liver virus vectored vaccines have a potential to transmit horizontally through fecal shedding of the vaccine virus. Coarse spray vaccination and vaccination in water is a common practice in poultry production, but high temperature of the poultry barn and the microbiome of the litter material are the potential hurdles in achieving the desired results. In the present study, a rPICV vector was used as a vehicle to carry two gene segments (SC and SB) of CARV (rPICV-CARV vaccine). The vaccine virus survived in autoclaved and non-autoclaved water for only six hrs with a small decrease in titer for up to 4 hrs but a precipitous drop in titer after 4 hrs. There is no information about the survivability of PICV in environment but rPICV was observed to survive at room temperature for 4 hrs (Personal communication with Dr. Ly). On the other hand, Savage and Jones (2003) have reported that some CARV strains survived for at least 10 weeks in drinking water. Mor et al. (2015) reported that CARV and TARV (turkey reovirus) survived for 9-13 weeks in autoclaved water and for 10 to 14 days in non-autoclaved water with a gradual decrease in viral titers over time. In the present study, rPICV-CARV vaccine virus survived for only 6 hrs in autoclaved and non-autoclaved litter. The wild type CARVs can survive for in autoclaved litter for 6 to 8 wks and in non-

autoclaved litter for 6 to 8 days (Mor et al., 2015). Wild type lentivirus and adenovirus can survive for months (Valtierra, 2008) but their modified viral vectors showed shorter survival on animal caging and bedding soiled with urine and feces of laboratory animals (Reuter et al., 2012). There was no evidence of viable rPICV-CARV vaccine virus in our study after 8 hrs in autoclaved and non-autoclaved water and litter samples. Perhaps the reason for same survival time of our vaccine virus in both water and litter samples is because of its short survivability of 6 hrs which can barely be affected by other factors related to litter and water contents at room temperature which needs to be studied in future studies.

The recombinant viral vectors are temperature sensitive and need cold chain to maintain the efficacy of vaccines. This is not surprising because various viruses differ in their capability to survive in the environment and so of the recombinant vectors developed by manipulating their genome. Previous studies have reported the temperature sensitivity and decreased production level of herpes simplex virus-based vectors (Kaptein et al., 1997; Kotani et al., 1994; Lee et al., 1996; Wechuck et al., 2002) and decreased transducibility of Adeno-associated virus vectors at higher temperature (Howard et al., 2017). Because veterinary vaccines are generally stored under non optimal conditions and delivered to remote locations, there is a need to develop thermostabilized viral vectors (Pellicia et al., 2016; Baron et al., 2018) to achieve efficient vaccination.

The results of the present study are important because our findings suggest that the rPICV-CARV vaccine can remain viable to be used for mass vaccination of poultry flocks when administered in drinking water or aerosol spray. Vaccination of poultry by drinking water and aerosol is both effective and economic while maintaining efficacy of

recombinant viral-vectored vaccines (Draper and Heeney, 2010) as aerosol spray vaccination is used for infectious bronchitis (IB), New castle disease (NCD) and infectious laryngotracheitis (ILT) viruses on poultry. The titer of the rPICV-CARV vaccine was stable for up to 4 hrs in both water and litter and decreased thereafter. When vaccinating poultry through drinking water, it is common practice to withhold water from the birds for 1-2 hours so that the water spiked with the vaccine virus is entirely consumed by the birds before there is a drop in live virus titer.

In an experimental study with rPICV-TARV vaccine, we observed horizontal transmition of vaccine virus to unvaccinated pen mates providing immunity against TARV infection (unpublished). The qualitative consequences of horizontal transmission of live virus vaccines are easily anticipated as increased herd immunity after an appropriate level of direct vaccination, whereas quantitative consequences are less apparent. The lateral transmission of live virus-vectored vaccines was not given due attention because it is difficult to measure and generally unknown except for oral polio vaccine in humans (Burns et al., 2014; Bull et al., 2018) and for rabies in wildlife (Stading et al., 2017; Nuismer and Bull, 2020).

Recombinant virus-vectored vaccines are self-replicating vaccines in which the vector is a harmless virus whose genome is engineered to carry one or more foreign antigenic genes from a pathogen of interest (Bull et al., 2018). The purpose is to elicit immunity against the pathogen's antigen where the vector merely provides the means to amplify the antigenic genes within the host. In contrast to the attenuated vaccines, recombinant virus vectored vaccines are intrinsically safe and incapable of reverting to a pathogenic wild-type virus because they carry only a small part of the pathogen's

genome. Additionally, rPICV-CARV vaccine does not have zoonotic potential because the vector has low seroprevalence in human and other animals, it has been attenuated in cell culture and mice and induce low levels of anti-vector immunity because of heavy glycosylation of its genes (Dhanwani et al., 2015).

In conclusion, rPICV-CARV vaccine can transmit horizontally because of its survival in water and litter with ample titer and is capable of inciting immune response in non-vaccinated sentinel pen mates. Additionally, the vaccine is safe and does not have a zoonotic potential for other animals.

Figure 6.1. The rPICV-CARV vaccine expressing chicken arthritis reovirus antigens (apple green fluorescent cells) and rPICV-GFP showing green fluorescence of GFP at 6 hrs. A) rPICV-CARV in autoclaved water, B) rPICV-GFP in autoclaved water, C) rPICV-CARV in Non-autoclaved water, D) rPICV-GFP in Non-autoclaved water.

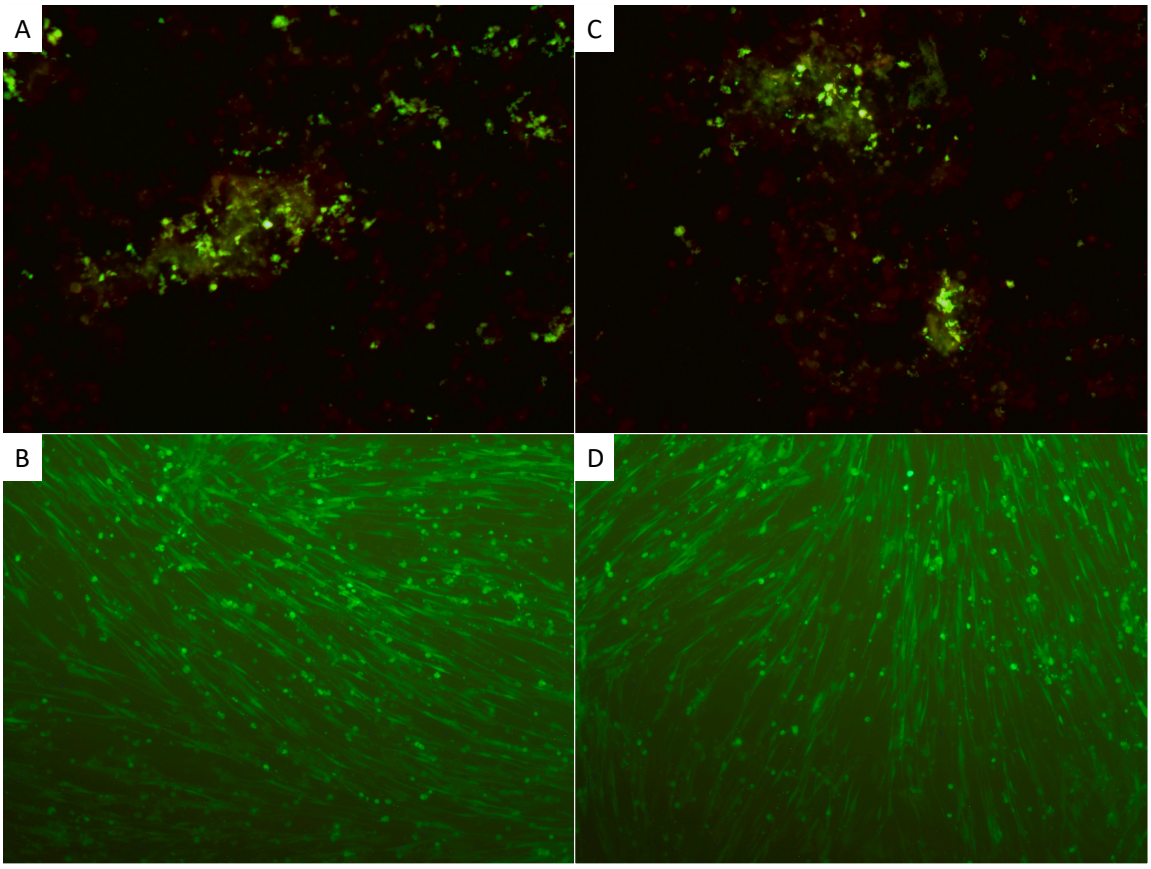
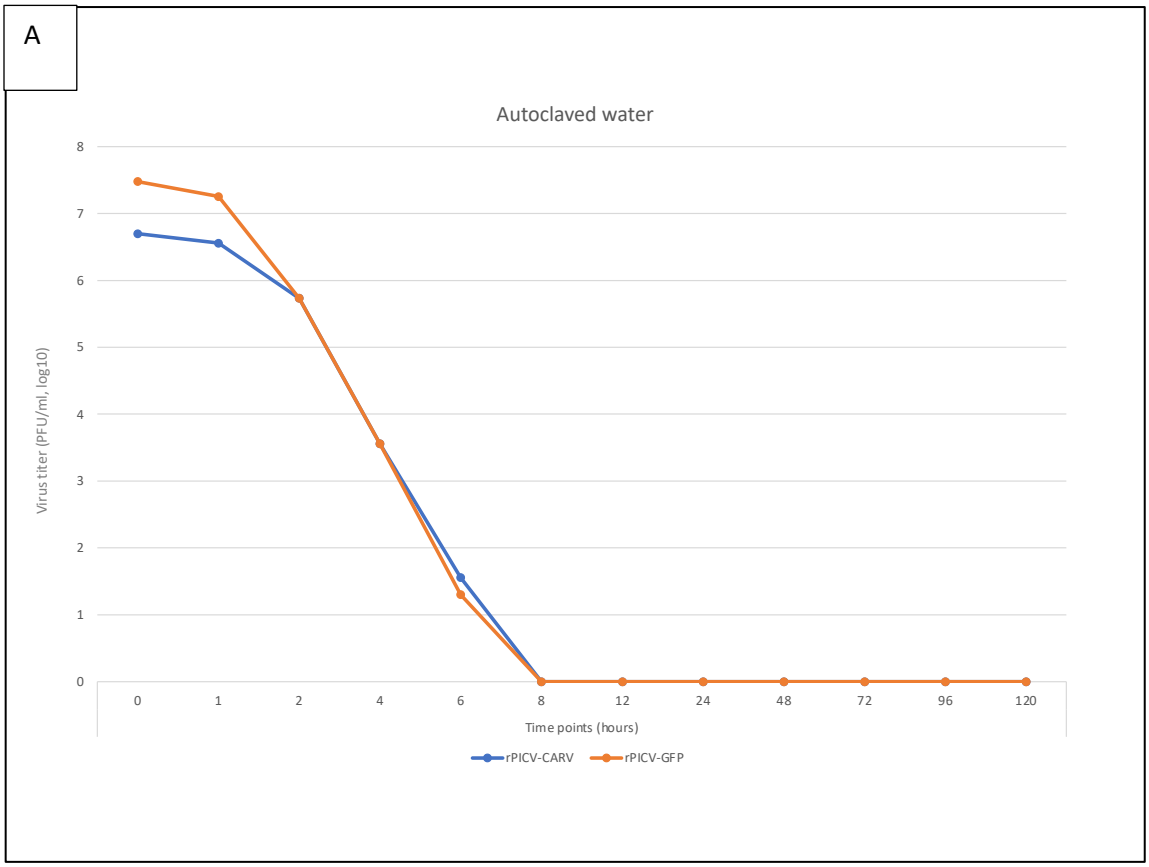


Figure 6.2. Vaccine virus titer (PFU/ml, log_{10}) of rPICV-CARV and rPICV-GFP A) in autoclaved water and B) non-autoclaved water.



B

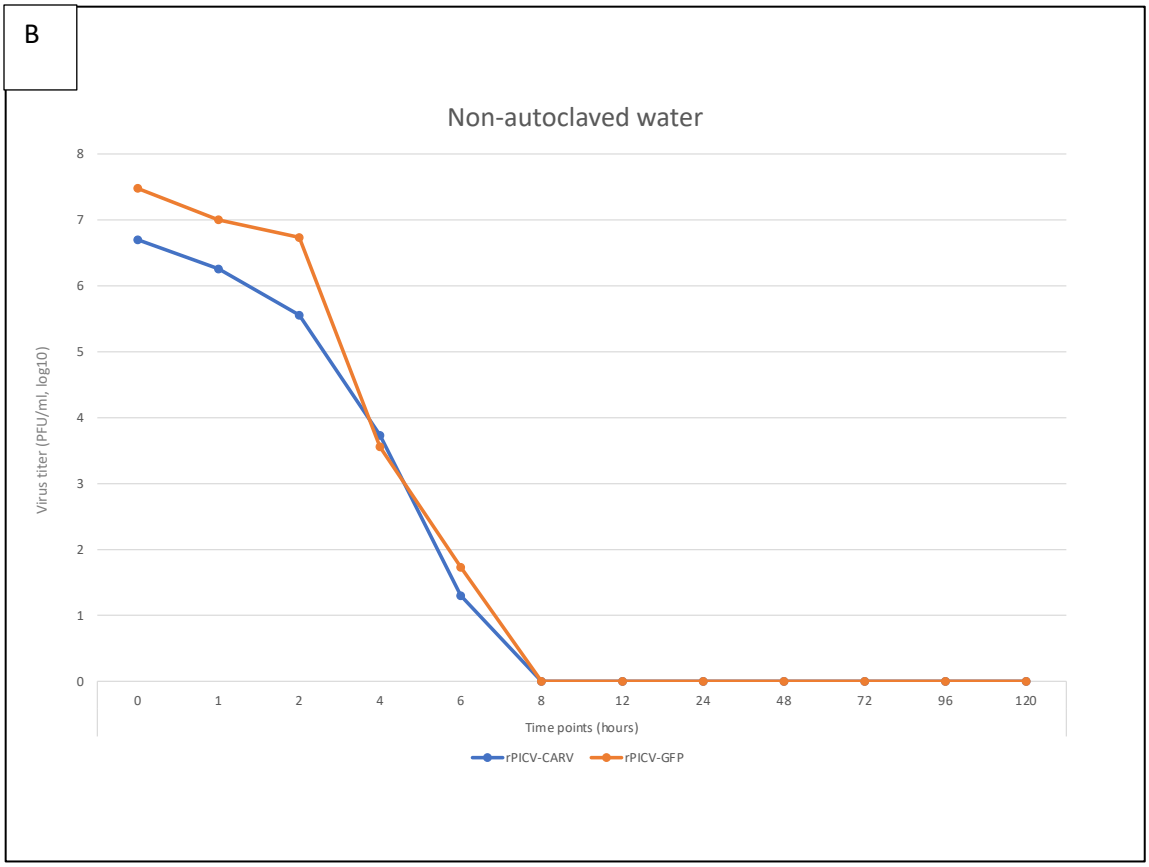
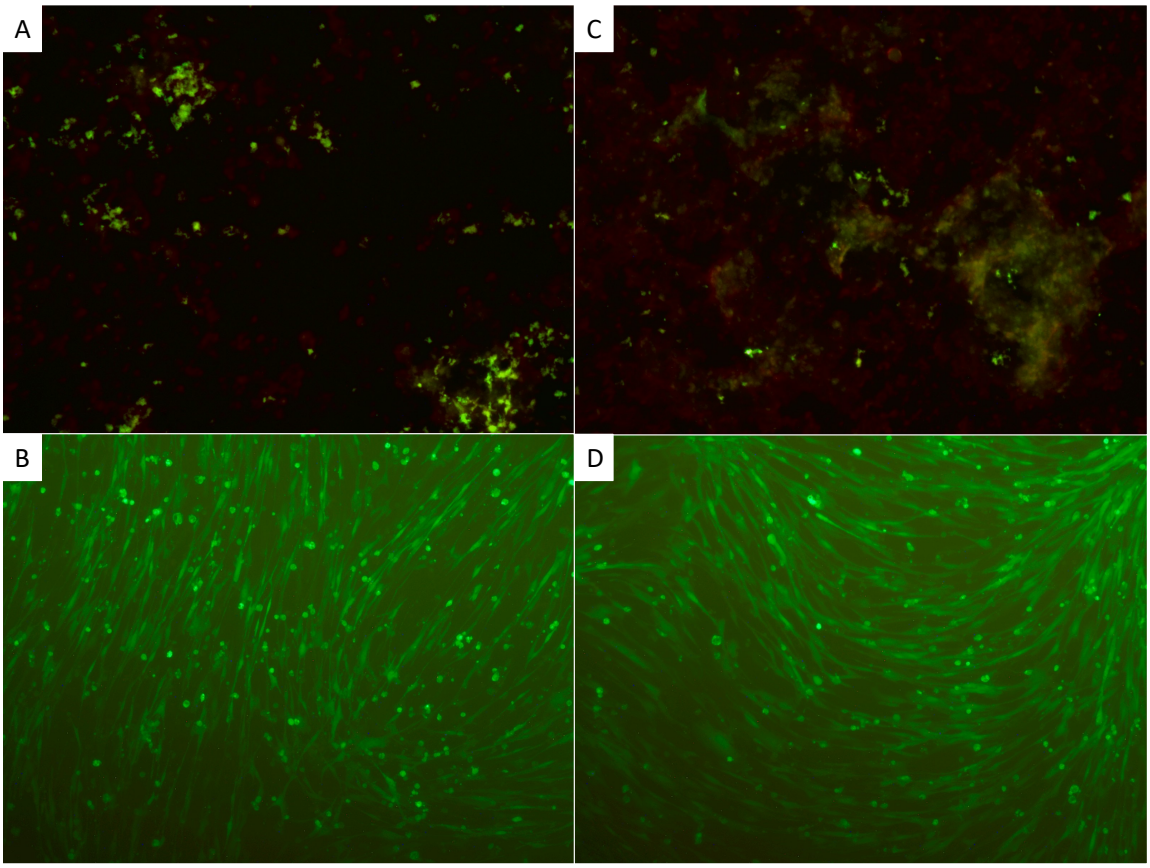

Non-autoclaved water
Virus titer (PFU/ml, log10)
0
1
2
3
4
5
6
7
8
0
1
2
4
6
8
12
24
48
72
96
120
Time points (hours)
rPICV-CARV
rPICV-GFP

Figure 6.3. The rPICV-CARV vaccine expressing chicken arthritis reovirus antigens (apple green fluorescent cells) and rPICV-GFP showing green fluorescence of GFP at 6 hrs. A) rPICV-CARV in autoclaved litter, B) rPICV-GFP in autoclaved litter, C) rPICV-CARV in Non-autoclaved litter, D) rPICV-GFP in Non-autoclaved litter. E) Positive control wild type chicken arthritis reovirus expressing chicken arthritis reovirus antigens. Fluorescence in BHK-21 cells (apple green fluorescent cells) based on direct fluorescent antibody test indicates CARV protein expression.



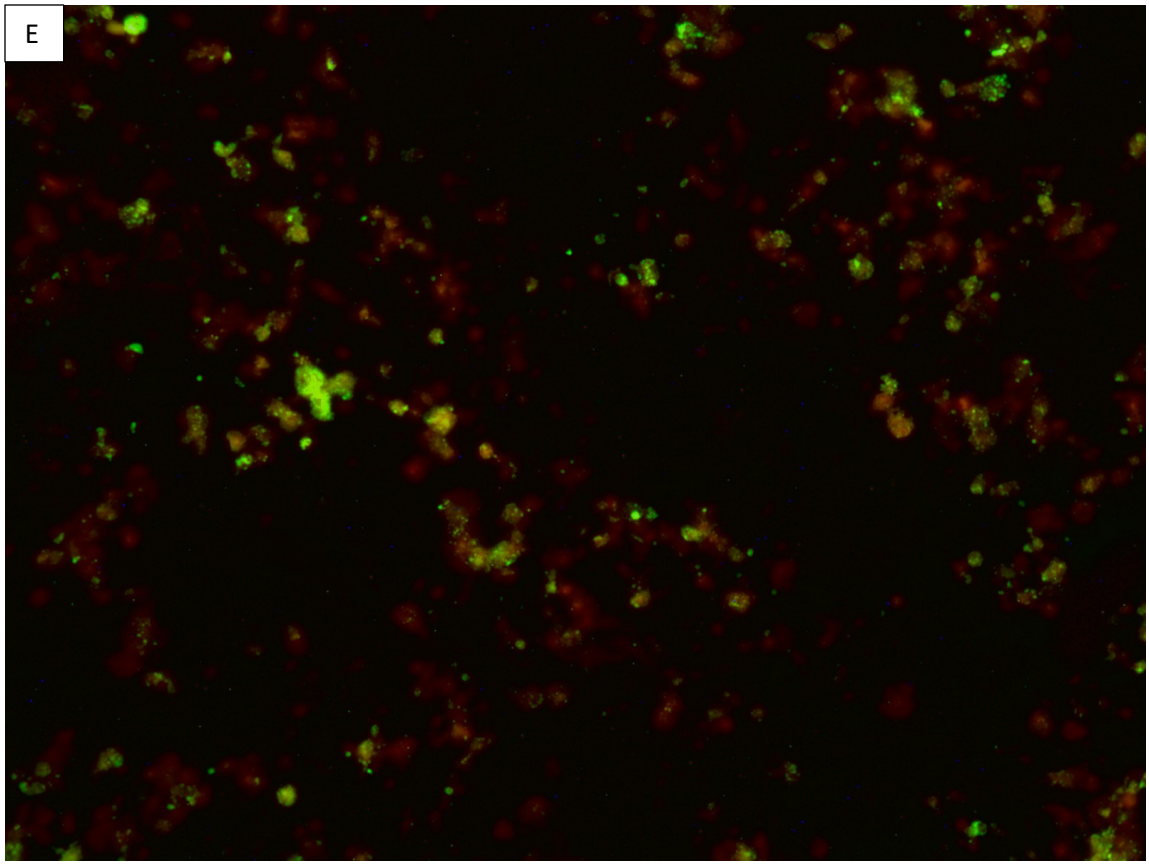
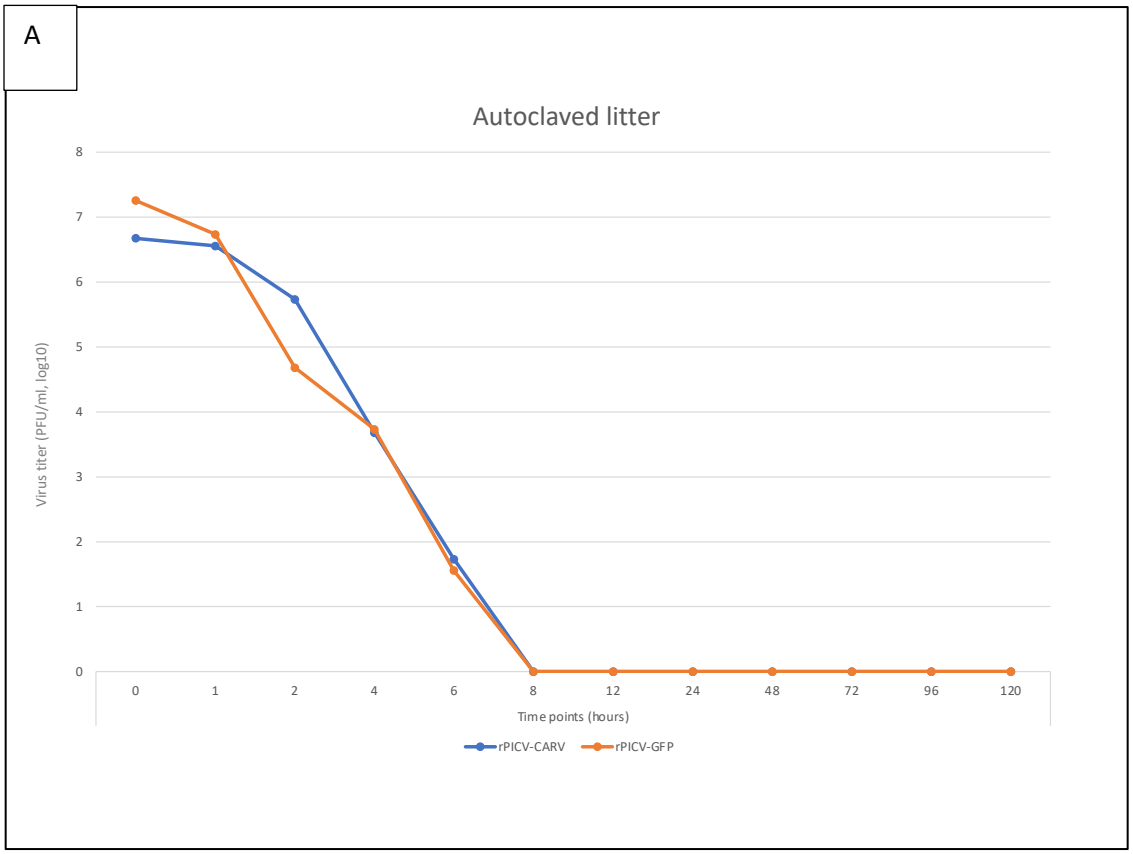
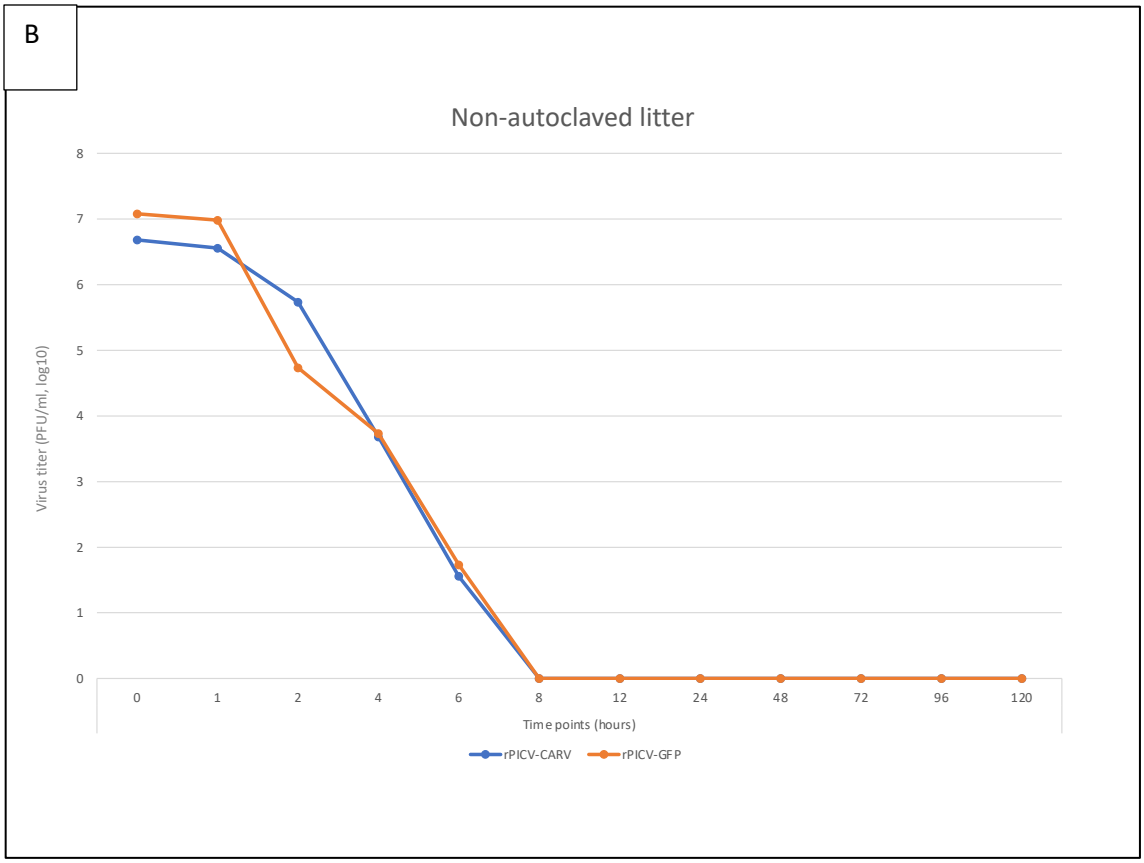

E

Figure 6.4. Vaccine virus titer (PFU/ml, log_{10}) of rPICV-CARV and rPICV-GFP a) autoclaved litter and b) non-autoclaved litter.



B


Non-autoclaved litter
Virus titer (PFU/ml, log10)
8
7
6
5
4
3
2
1
0
0
1
2
4
6
8
12
24
48
72
96
120
Time points (hours)
rPICV-CARV
rPICV-GFP

CHAPTER 7

COMPARATIVE PATHOGENESIS OF TURKEY REOVIRUSES

This material in this chapter has been submitted for publication in Avian Pathology as:
Rahul Kumar, Tamer A. Sharafeldin, Nader M. Sobhy, Sagar M. Goyal, Robert E. Porter, Sunil K. Mor. Comparative Pathogenesis of Turkey Reoviruses.

Introduction

Turkey reoviruses have been known for decades to be associated with poult enteritis complex often in association with other enteric viruses (Jindal *et al*., 2009; *Jindal et al*., 2010; Spackman *et al*., 2010; Mor *et al*., 2013a). In 1980s, two reports described the isolation of turkey reovirus from lame turkeys (Levisohn *et al*., 1980; *Page et al*., 1982), after which no more field cases were observed until 2011 when turkey reovirus was isolated from tendons of 13-week-old turkeys with lameness (Mor *et al*., 2013b). The isolated viruses were tentatively called turkey arthritis reovirus (TARV) while those isolated from cases of enteritis were labeled as turkey enteric reovirus (TERV). Inoculation of TARV isolate in 1-week-old turkey poults produced tenosynovitis and lameness, while TERV did not (Sharafeldin *et al*., 2014; Sharafeldin *et al*., 2015a). Lameness was attributed to decrease in the tensile strength and elasticity modulus of leg tendons (Sharafeldin *et al*., 2016). Both TARV and TERV replicated in the gastrointestinal tract while the former affected tendons and triggered significant innate antiviral (interferons alpha and beta) and T-helper-1 responses (Sharafeldin *et al*., 2015b).

Recently in 2019, we received several cases of 1-7-week-old turkeys with mortality approaching 5%. The dead birds exhibited multifocal hepatic and splenic necrosis. Reoviruses isolated from these cases have been tentatively named as turkey hepatitis reovirus (THRV). Molecular characterization of turkey reoviruses in our laboratory indicates that TARV has close homology with TERV, which suggests that TARVs may be a mutant of TERV (Mor *et al*., 2014). We further assume that THRV (reovirus associated with hepatic lesions in turkeys) may be a mutant of either TARV or TERV. This study was undertaken to compare the pathogenesis of turkey reoviruses

associated with enteritis, arthritis, and hepatitis and to establish Koch's postulates for THRVs.

Materials and Methods

Viruses: Nine different turkey reoviruses (2 THRVs, 5 TARVs and 2 TERVs) isolated from diseased birds were selected based on age of the affected birds, year of isolation, geographical location, severity of clinical disease (morbidity/mortality), gross and histologic lesions and molecular characterization (Table 7.1). In this study, we used TARV-O'Neil strain as a positive control. TARV1 used in the present study was isolated in 2019 from a 16-week-old turkey showing severe clinical signs, gross and histologic lesions of arthritis. TARV2 was isolated from gastrocnemius tendons of a 4-week-old bird showing osteodystrophy and chondrodystrophy but no tenosynovitis. Isolate TARV3 has unique sequence because it had some insertions and deletions in its S1 gene sequence. The TERV strains used (TERV1 and TERV2) in this study were isolated before 2010. We believe that these two TERV strains may be true enteric pathogens since reovirus-associated lameness was not reported until 2011. All viruses were propagated and titrated on Japanese quail fibrosarcoma (QT-35) cells. The method of Reed and Muench (1938) was used to calculate 50% tissue culture infective dose ($TCID_{50}$) of each virus.

Experimental design: One-day-old turkey poults (n=290) were divided into 10 groups of 29 poults each and were placed in 10 different air-filtered isolators. Food and water were supplied *ad libitum*. At one week of age, birds in groups 1-9 were inoculated with 0.3 ml of respective virus (~10^5 $TCID_{50}$/ml) via the oral route. Birds in group10 (sham

inoculated control) were inoculated with virus-free DMEM. Birds were examined daily for overt clinical signs or mortality. Birds displaying signs of severe illness were euthanized according to the Institutional Animal Care and Use Committee (IACUC) protocol and guidelines from Research Animal Resources (RAR) at the University of Minnesota. Four birds from each group were euthanized at 3-, 5-, 7-, 14-, 21-, and 28-days post inoculation (dpi). At necropsy, gross lesions were noted followed by sample collection (duodenum, jejunum, ileocecum, liver, spleen, heart, gastrocnemius tendon and bursa of Fabricius) for virus isolation, real time RT-PCR, and histopathology. Body weights were noted at euthanasia before removing tissues from 14 dpi onwards.

Sample processing: For virus isolation and real time RT-PCR (RT-qPCR), a 10% suspension was prepared by homogenizing all tissue samples individually in Hanks' balanced salt solution (HBSS) for 1-2 min using a Stomacher (Model 80, Seward, Ltd., UK). Tissue homogenates were centrifuged at 1800 xg for 10 min at 4^0C. The supernatant was decanted and frozen at -80^0C until used.

Virus gene copy number: Universal avian reovirus real time RT-PCR available at the Veterinary Diagnostic Laboratory, University of Minnesota (MVDL (https://www.vdl.umn.edu/node/15341) was used to calculate virus gene copy number in collected tissues. The gene copy number was calculated using a standard curve of ten-fold dilutions of TARV-positive RNA included in all 96-well plates. The gene copy numbers of different tissues at different times were calculated and subjected to statistical analysis as described below.

Virus isolation: Monolayers of QT-35 cells prepared in 24-well tissue culture plates were used for virus isolation. After removing cell culture fluid, the monolayers were

washed thrice with sterile PBS following which 0.5 ml of supernatant from each sample was inoculated duplicate. The plates were incubated at 37^0C under 5% CO_2 for 1 hour for virus adsorption followed by addition of maintenance medium (DMEM with 2% FBS and antibiotics) and incubation at 37^0C under 5% CO_2. The monolayers were examined daily for a period of 5–7 days for the appearance of virus-induced cytopathic effects (CPE). Two to three blind passages were given before calling a sample negative. Cell culture fluids were collected and subjected to RNA extraction and RT-qPCR as mentioned above.

Immunofluorescence: To confirm that observed CPE was induced by reovirus, the inoculated QT-35 cells were subjected to immunofluorescence. Briefly, the cell culture medium was removed from 24-well plates and the cells were scraped in 0.2 ml of PBS. The scraped cells were placed in a 12-well slide and air dried. The cells were then fixed in acetone for 2 hours followed by the addition of fluorescent tagged anti-ARV (avian reovirus) antibody (ID No. 680 VDL 9501, NVSL, Ames, IA, USA). The slide was placed in a humidified chamber at 37^0C for 2 hours, rinsed gently thrice with PBS, and counter stained with Evans blue for 5-7 min. After mounting in 50% glycerol (pH 8.4), a cover slip was applied, and the slide was examined under a fluorescent microscope for the appearance of apple-green color (positive for reovirus).

Histopathology: Formalin-fixed soft tissues were trimmed, processed, and stained with hematoxylin and eosin (H&E) stain. Formalin-fixed hock joints were decalcified prior to processing for histopathological examination. Lesions in the gastrocnemius tendons were scored using a previously described histologic lesion scoring system (Sharafeldin *et al*., 2014).

Statistics: Two-way ANOVA and t-test were used to test the significance of variation in virus gene copy numbers in different tissues at different time points. A non-parametric Kruskal Wallis test followed by Mann Whitney U test was used to test the significance of difference in gastrocnemius tendon lesion scores. Statistical software NCSS2020 (https://www.ncss.com/) was used to conduct statistical analysis. The statistical significance was determined at p value < 0.05.

Ethical statement

The following experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of College of Veterinary Medicine, University of Minnesota, USA.

Results

Body Weight: At 14 dpi, there was no statistically significant difference in the body weights of birds in any of the 10 groups. However, at 21 dpi, birds in the sham-inoculated group had statistically significant higher body weights as compared to the nine virus-inoculated groups. At 28 dpi, birds in the sham-inoculated group had statistically significant higher body weights than in groups inoculated with THRV1, THRV2, TARV1, TARV2, TARV3 and TERV2 but not with TARV4, TARV-O'Neil and TERV1 (Fig 7.1).

Clinical disease and gross lesions: At all euthanasia time-points, no reovirus specific clinical signs, mortality or gross lesions were observed in birds from any of the virus-inoculated groups except THRV2-inoculated group in which one bird died at 9, 13 and 14 dpi each after showing mild weakness and lethargy. Another bird showing signs of severe

clinical illness at 13 dpi was euthanized. At necropsy, these birds showed hepatosplenomegaly (n=2) or splenomegaly (n=2) with multifocal white necrotic foci in the hepatic parenchyma (Fig 7.2A). At 21 dpi, some birds in this group showed splenomegaly with mottling (n=2) and frothy cecal contents (n=3) (Fig 7.2B).

Virus gene copy number: The virus gene copy number in the duodenum, jejunum and ileocecum of THRV2-inoculated group peaked at 7 dpi while in the other eight groups this peak was seen at 5 dpi, which declined remarkably by 7 dpi. There were numerical but statistically non-significant differences among groups at various ages (Fig 7.3A-C). Viral gene copy numbers in bursa of Fabricius were not as high as those in intestinal segments. In bursa, the virus gene copy numbers in THRV2 and TARV3 groups reached a peak at 5 dpi and were significantly higher than the other groups (Fig 7.3D). In spleen, significantly higher virus gene copy numbers were seen in THRV2, TARV4 and TARV-O'Neil groups at 5 dpi than in other groups and at other time points (Fig 7.4A). In liver, THRV1, THRV2, TARV4 and TARV-O'Neil showed higher copy numbers than other groups at 5 dpi. Peak virus gene copy numbers reached at 7 dpi for THRV2 and at 14 dpi for THRV1 (Fig 7.4B). In tendons, THRV1, THRV2, TARV1, TARV4, and TARV-O'Neil groups had higher virus gene copy numbers than other groups at 14, 21, and 28 dpi. Surprisingly, TERV2 also had a high virus gene copy number in tendons at 21 dpi (Fig 7.5A).

Histopathology of gastrocnemius tendons: Initial tendon lesions consisted of hypertrophy and hyperplasia of synoviocytes progressing to formation of villous-like structures infiltrated with lymphoplasmacytic cells and later progressing to fibroplasia (Fig 7.6A). Some sections showed prominent lymphoid aggregates in villous proliferation

of synovium (villonodular hyperplasia) (Fig 7.6B). Histologic lesion scores started at 7 dpi and increased at 14, 21 and 28 dpi in the THRV2 and all TARV groups, whereas THRV1 showed tendon lesions at 14 dpi. Birds inoculated with TARV1, TARV4, and TARV-O'Neil showed consistently high tendon lesion scores at 7, 14, 21, and 28 dpi while TARV3 had relatively lower tendon lesion scores at 7, 21, and 28 dpi. TARV2 had tendon lesion scores only at 7 dpi. Of the two TERVs inoculated, only TERV2 showed tendon lesion scores at 21 and 28 dpi, but these scores were lower than those of most of the TARV and THRV inoculated groups (Fig 7.5B).

Histopathology of liver: No significant hepatic lesions were seen in any group except the THRV2 group. The birds in this group had extensive hepatic lesions at different time points. At 5 dpi, 3 of 4 dead/euthanized birds had mild to moderate multifocal hepatocellular necrosis with infiltration of mononuclear cells (Fig 7.6C). At 7 and 14 dpi, 3 of 4 and 2 of 4 birds, respectively, had moderate to severe hepatocellular necrosis. The virus was re-isolated from the liver and spleen tissue samples when inoculated in QT-35 cells and confirmed by immunofluorescence (Fig 7.6E) and RT-qPCR.

Histopathology of heart: Only the TARV-O'Neil group showed consistent histologic lesions in heart at different time points. Two of the 4 birds had mild epicarditis (1-2 small epicardial lymphoid aggregation) at 5 dpi while 3 of 4 birds showed moderate (3-4 small epicardial lymphoid aggregates) to severe (more than 4 epicardial lymphoid aggregates, focal extensive lymphoid infiltration, or circumferential lymphoid infiltration) epicarditis at 14 dpi. At 21 and 28 dpi, 3 of 4 and 2 of 4 birds, respectively, had mild epicarditis (Fig 7.6D).

Histopathology of other organs: No significant microscopic lesions were observed in the intestinal segments and spleen of any of the virus-inoculated groups. All organs of the sham- inoculated control groups showed normal histoarchitecture.

Discussion

Turkey reovirus (TRV) is known for years to cause either enteritis or inapparent infections in turkeys. Since 2011, it has emerged as a causative agent of tenosynovitis leading to lameness in turkeys. Recently, we have isolated TRV from several cases of turkey hepatitis. The reoviruses causing these diseases conditions are tentatively named as TERV, TARV, and THRV, respectively. This study was undertaken to compare the pathogenesis and tissue tropism of these viruses and to establish Koch's postulates for THRVs. We selected certain unique reoviral isolates for this pathogenesis study. Previously, we have conducted studies on the pathogenesis of TARV by oral inoculation of TARV-O'Neil strain in turkey poults (Sharafeldin *et al*., 2014; Sharafeldin *et al*., 2015a).

All three types of viruses showed very high replication rates and gene copy numbers in the intestine, which is not surprising since turkey reoviruses are generally enterotropic in nature. Significantly lower body weights in virus-inoculated birds were seen at 21 dpi and 28 dpi but not at 14 dpi (Figure 1). This is in agreement with Ngunjiri, *et al*. (2019) who observed that the difference in body weight of the virus-inoculated groups was non-significant at very young age (<2 weeks). The weight loss due to TERV and TARV has also been reported in several previously published reports (Spackman *et al*., 2005; Pantin-Jackwood *et al*., 2007; Sharafeldin *et al*., 2015a; Ngunjiri *et al*., 2019).

The depression in the body weight by turkey reoviruses can be due to intrinsic factors of the strains and time post infection (Spackman *et al*., 2005; Ngunjiri, *et al*. 2019). Although the viruses replicated in the intestinal segments and had an impact on body weight, they did not produce any significant histologic lesions. Previous studies have reported that TARVs and TERVs produce mild to severe enteritis with lymphocytic or heterophilic infiltration but not consistently (Spackman *et al*., 2005; Day *et al*., 2008; Sharafeldin *et al*., 2014). This may suggest that the virus can infect and replicate in the enterocytes but does not cause tissue damage in the absence of some other factors such as co-infection with other pathogens. Future studies are indicated to clarify this issue.

The gene copy numbers of TARV and TERV in intestinal segments reached a peak at 5 dpi followed by a sharp decline at 7 dpi (Figure 3). This is in agreement with our previous study in which this decline was attributed to an elevation in antiviral interferon-alpha (IFN-A) and IFN-B at 7 dpi (Sharafeldin *et al*., 2015b). In contrast, the replication of THRV2 in the intestines reached a peak at 7 dpi indicating that the predilection and pathogenic potential of THRV2 may be different than those of TARV and TERV. Future studies are indicated to investigate the ability of THRV to escape or delay the immune response and cause disease.

In liver and spleen, the replication of THRV1, THRV2, TARV4 and O'Neil was observed at 5 dpi, which reached a peak at 7 dpi and decreased thereafter. This observation further confirmed our previous results that these viruses cause viremia after initial replication in intestine and then are disseminated in different tissues (Sharafeldin *et al*., 2015b). The replication of only these four viruses in liver and spleen may be due to

intrinsic viral characteristics and molecular or genetic factors that need further investigation.

Intestine and bursa are the primary sites of reovirus entry and replication, and spleen is the first tissue infected after initial replication and viremia (Pantin-Jackwood *et al*., 2007). Unlike chicken reovirus, liver has not been shown to be the site of replication for turkey reoviruses (Pantin-Jackwood *et al*., 2007; Sharafeldin *et al*., 2015b). This is probably the first study reporting the replication of turkey reovirus in the liver indicating that these viruses are still evolving and changing in terms of their pathogenicity and tissue tropism. Thus, TRVs started out as being enteric pathogens causing mild to severe enteritis but later evolved to be arthrotropic (causing arthritis/tenosynovitis) and most recently to being hepatotropic (causing hepatitis).

The heart lesions (epicarditis) caused by TARV-O'Neil strain in this study is in contrast to previous results from our laboratory (Sharafeldin *et al*., 2014; Sharafeldin *et al*., 2015a). However, reovirus-induced heart lesions in turkeys have been reported previously (Spackman *et al*., 2005; Pantin-Jackwood *et al*., 2007; Shivaprasad *et al*., 2009; Davis *et al*., 2012). Our recent diagnostic findings indicate an increased lymphocytic myocarditis/epicarditis in TARV-positive turkey flocks (unpublished). It is worth mentioning that none of the other turkey reoviruses used in this study induced myocarditis/epicarditis. These findings indicate that the ability of inducing myocarditis/epicarditis varies among different turkey reovirus. Virus replication and gene copies were low in heart of birds from all the virus-inoculated groups (data not shown).

Virus replication in tendons started at 7 dpi, reached a peak at 21 dpi, and declined at 28 dpi. Previous studies have also reported high rates of reovirus detection as

early as 7 dpi in tendons of commercial turkeys (Sharafeldin *et al*., 2015b; Ngunjiri *et al*., 2019). In tendons, appreciable viral gene copy numbers were observed in THRV1, THRV2, TARV1, TARV4 and TARV-O'Neil groups. It is interesting to note that both strains of THRV migrated and replicated in tendons. This finding correlates with the field situation where some flocks show hepatitis and mortality at a young age (2-5 weeks of age) followed by clinical lameness at 8-10 weeks of age. We believe that TARVs have mutated from TERVs that are ubiquitously present in turkeys, and the same progression may hold true for THRVs. Rapid genetic and antigenic divergence, vaccination pressure, and inherent characteristics of reovirus to undergo reassortment and recombination might have resulted in the evolution of a different pathotype of TARV in the form of THRV. Further studies on genotyping and pathotyping are indicated.

Recently isolated TARV1 (in 2019) showed high replication in intestine but low replication in tendons as compared to TARV-O'Neil (positive control). However, the histologic lesion score in tendons was as high as that of TARV-O'Neil, which suggests that TARV1 is also highly pathogenic. On the other hand, TARV2 isolated from a case of chondrodysplasia in 4-week-old birds showed ample replication in intestine and bursa, but no replication and histologic lesion score in tendons (except at 7 dpi). This may suggest that this isolate either does not cause tenosynovitis or may do so at a higher dose or under conditions of stress or co-infection.

Two isolates (TARV3 and TERV1) had insertion and detection in their S1 gene sequence and were divergent from previously reported turkey reoviruses. However, they were similar to an enteric strain (NC/SEP-R44/03) based on S1 gene sequence, which is known to cause moderate to severe bursal atrophy (Day *et al*., 2008). Although TARV3

showed higher replication and gene copy numbers in intestine and bursa, it showed low replication in tendons and caused minimal to mild tenosynovitis as compared to other TARV strains. Although S1 gene sequence of TERV1 was similar to that of TARV3, it showed different replication and tissue tropism. This is in contrast with duck reovirus strain HN5d, which has 18 amino acid deletion is sigma C (S1 gene), but was highly pathogenic in ducklings causing severe hemorrhage and necrosis of liver with 20-30% mortality (Zheng *et al*., 2016)

Interestingly, both THRV strains produced severe histologic lesions in tendons but had consistently high viral gene copy numbers in liver, indicating their predilection for liver. It is important to note that THRV1 did not produce clinical disease and hepatic lesions while THRV2 did although both viruses were originally isolated from cases of hepatitis. The variation between THRV1 and THRV2 (and others) should be a focus of future studies to elaborate factors responsible for this variation.

Even TERV2 replicated in tendons at 21 dpi and showed histological lesions of tenosynovitis. However, the tendon lesion scores produced by TERV2 were significantly lower than those produced by TARVs. Histologic lesion scores above zero were observed in birds inoculated with enteric reovirus strain MN1 by Ngunjiri *et al*. (2019) but were lower than the TARV-inoculated birds. High tenosynovitis lesion scores generally do not manifest into clinical lameness or gross pathological lesions, but we do not know if this virus would have produced frank lameness in older turkeys if the inoculated birds were kept for a longer duration. One enteric virus-inoculated bird developed severe valgus and lameness suggesting enteric reovirus can cause arthritis in turkeys under stress conditions (Ngunjiri *et al*., 2019). Intrinsic characters of reovirus strains are responsible for different

pathogenesis (Gouvea and Schnitzer, 1982) and the amount of infectious dose may contribute to the pathotypic differences (Sharafeldin *et al*., 2014; Ngunjiri *et al*., 2019). Differential pathogenicity due to titer of turkey reoviruses used in this study is ruled out by inoculating uniform inoculum of all the virus strains. This strongly proves that the observed pathogenicity is due to inherent and intrinsic viral characters. Additionally, it is possible that this 2008 TERV isolate constituted an evolutionary linkage with TARV that emerged in 2010.

In summary, this study demonstrated the pathogenicity of various turkey reoviruses when inoculated via the oral route. This study, in conjunction with previous studies, confirmed that there are two main groups of avian reoviruses: highly pathogenic and low pathogenic in chicken and turkeys (Jones *et al*., 2984; Spackman *et al*., 2005; Sharafeldin *et al*., 2014; Ngunjiri *et al*., 2019). Reassortment may be a reason for low or high virulence. Ni and Kemp (1995) infected one-day-old broilers with two virus strains that differed greatly in virulence (mild strain 883 and virulent strain 176). A reassortant (R44) strain having Sl segment from strain 176 and the remaining backbone from strain 883 was also used. Virulent strain 176 replicated efficiently, spread to many tissues, and caused lesions. However, replication efficiency of the mild strain 883 was low with no or mild lesions. On the other hand, the reassortant strain R44 behaved similar to the mild strain 883 in chickens. Hence, there is possibility to determine pathotypes of reoviruses based on genomic characterization if a complete genomic database of old and new strains in a particular farm is available. We conclude that all turkey reoviruses are enterotropic since they showed a very high replication rate in the intestines. We further believe that turkey reoviruses follow a generalized pathogenicity affecting different organs according

to their predilection, the basis for which is not currently known. Only THRVs produced hepatic lesions and the virus was re-isolated from the liver and spleen of inoculated birds fulfilling Koch's postulates.

Table 7.1. Details of virus isolates used in the comparative pathogenesis of turkey reovirus study

Group No.	Virus[a]	Year of isolation	Disease	Passage No.	Age (weeks)	State
1	**THRV-1**	2019	Hepatitis	P2	2	MN
2	**THRV-2**	2019	Hepatitis	P2	3	AR
3	**TARV-1**	2019	Arthritis	P3	16	VA
4	**TARV-2**	2017	Arthritis	P3	4	SD
5	**TARV-3**	2017	Arthritis	P3	11	WI
6	**TARV-4**	2017	Arthritis	P4	19	WI
7	**TARV O'Neil**	2011	Arthritis	P4	18	MN
8	**TERV-1**	2007	Enteritis	P1	4	MN
9	**TERV-2**	2008	Enteritis	P1	3	MN
10	**DMEM (Sham Control)**	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]

a THRV=turkey hepatitis reovirus; TARV=turkey arthritis reovirus; TERV=turkey enteric reovirus

b NA=Not applicable,

Figure 7.1: Average body weight (kg) in different groups at 2-, 3-, and 4-weeks post inoculation. Groups with different letters at a time point indicate statistically significant difference ($p<0.05$).

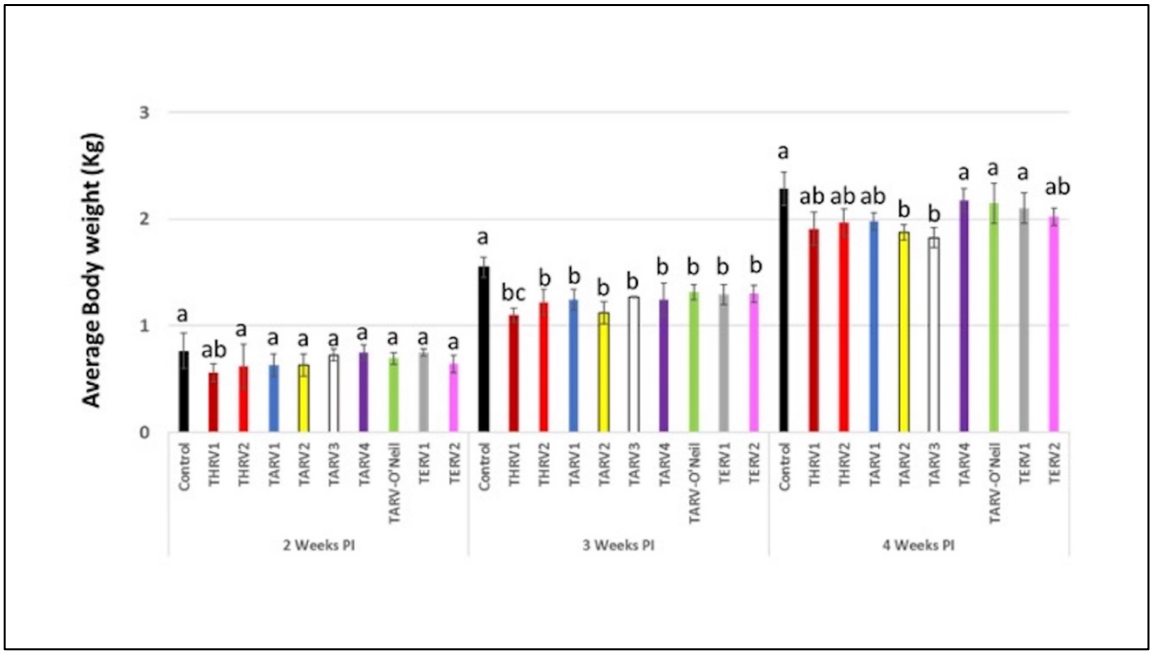


Figure 7.2: Gross lesions of THRV-infected turkeys: **(A)** Pinpoint necrotic foci in liver. **(B)** Frothy cecal contents.

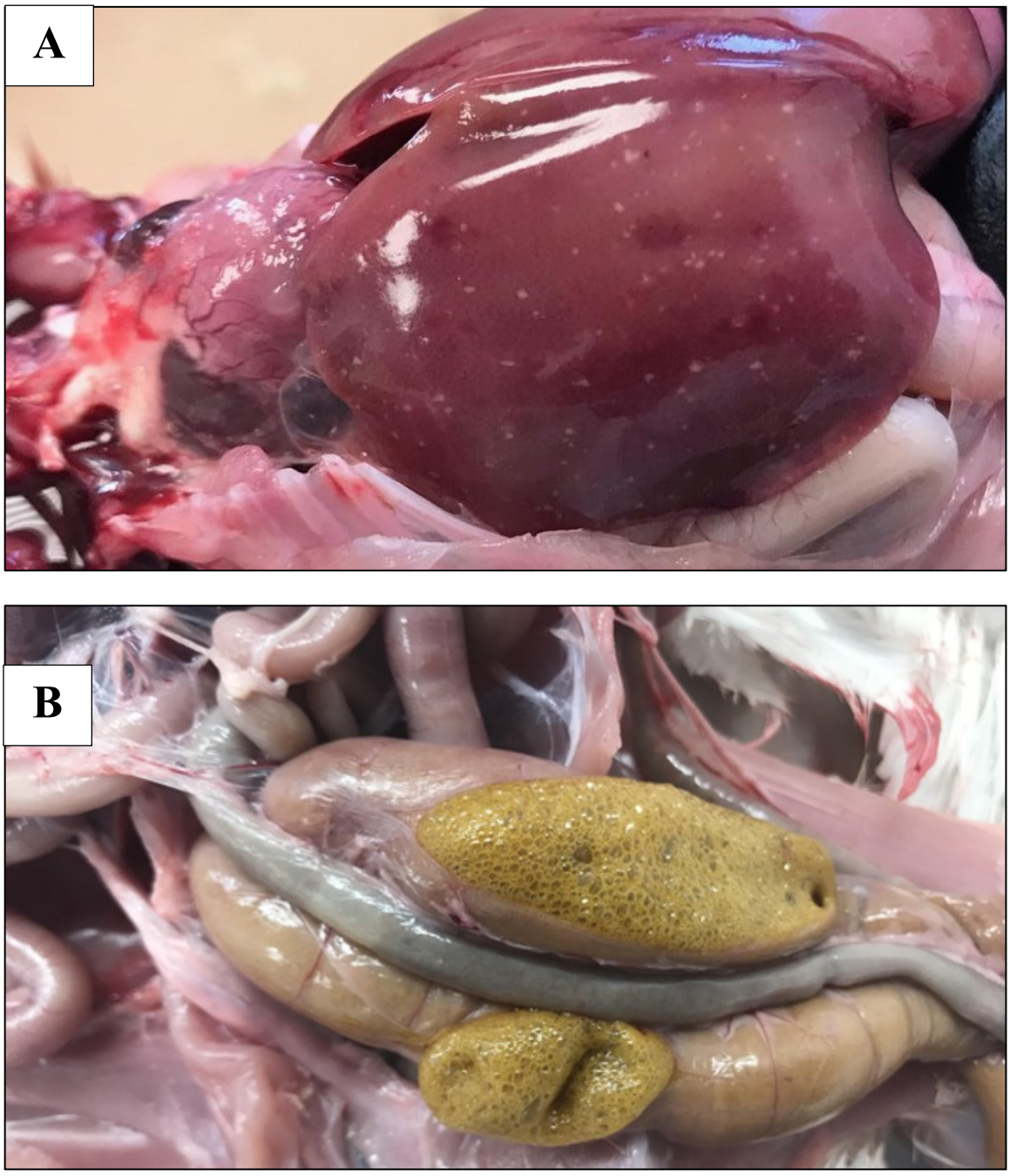
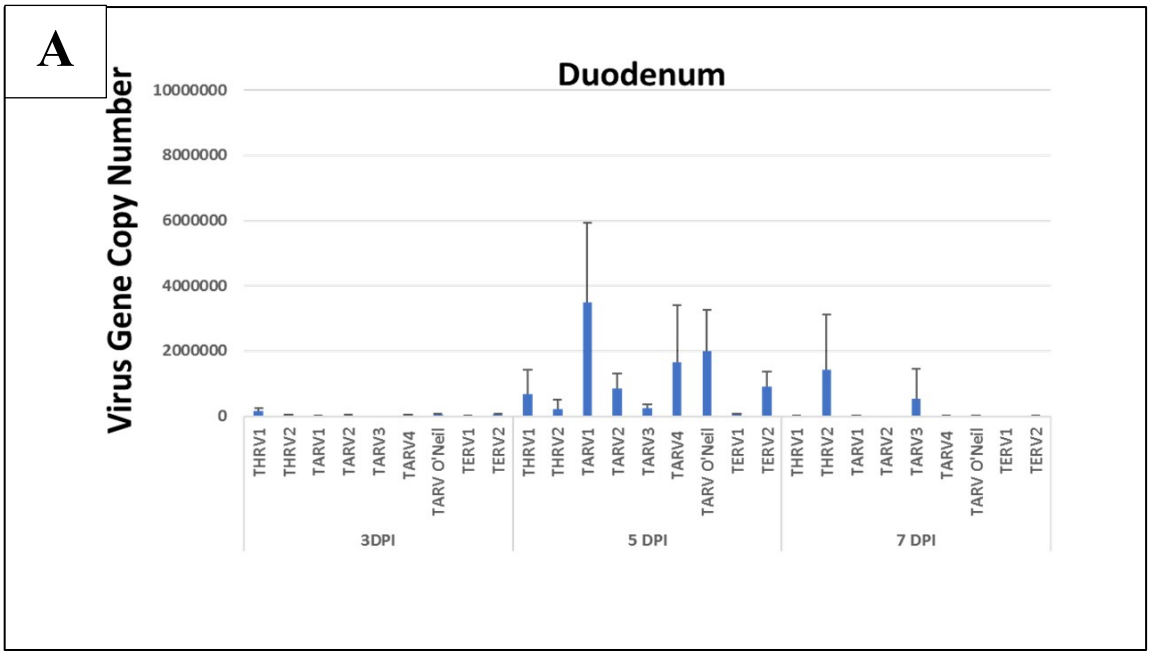
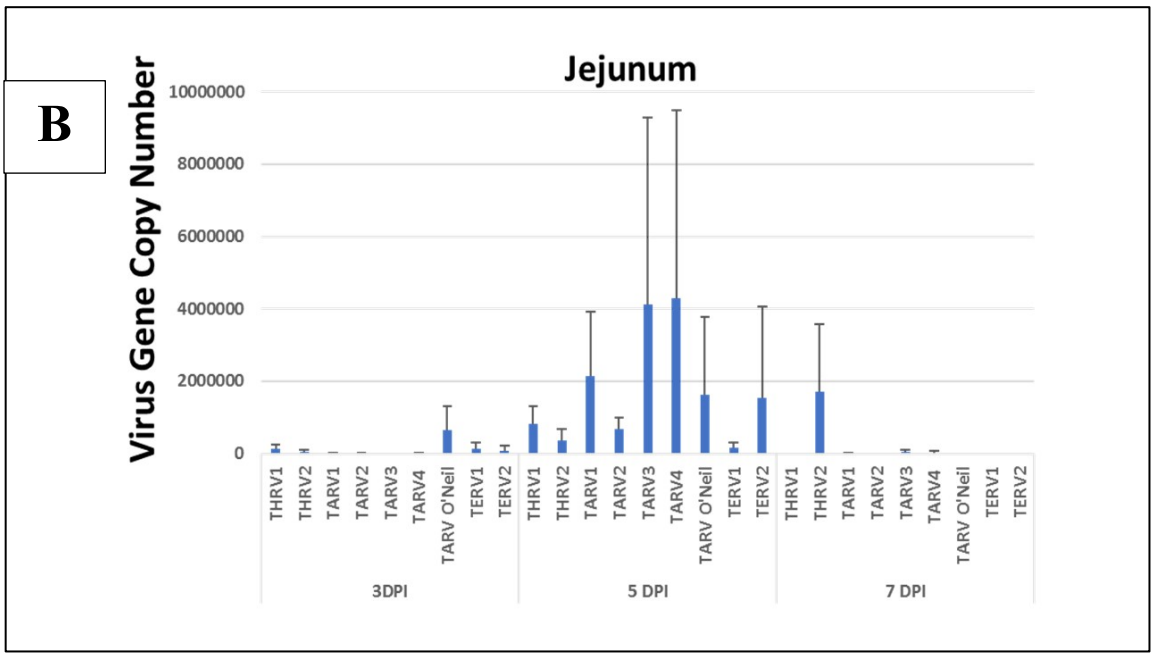
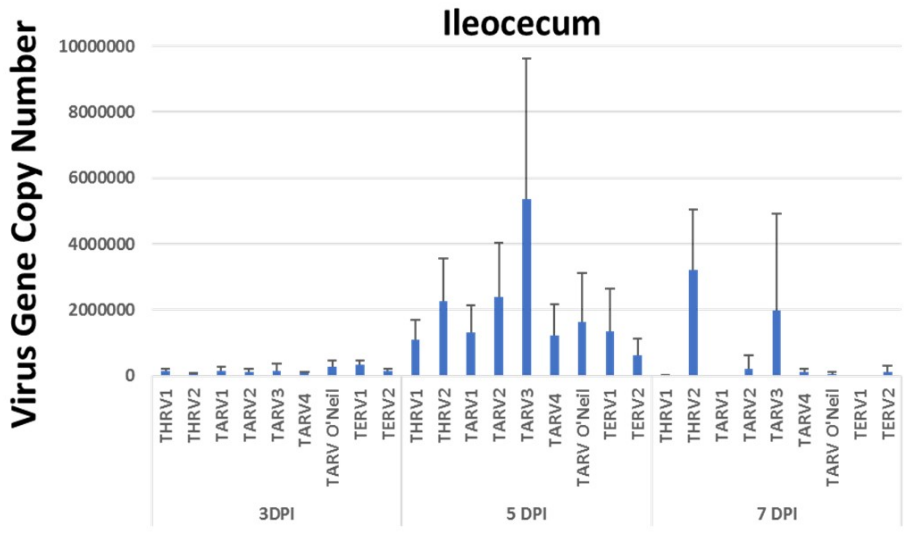


Figure 7.3: Virus gene copy number in (A) duodenum, (B) jejunum, (C) ileocecum and (D) bursa of Fabricius at 3-, 5-, and 7-days post inoculation.





C


Ileocecum
Virus Gene Copy Number
10000000
8000000
6000000
4000000
2000000
0
THRV1
THRV2
TARV1
TARV2
TARV3
TARV4
TARV O'Neil
TERV1
TERV2
3DPI
5 DPI
7 DPI

D

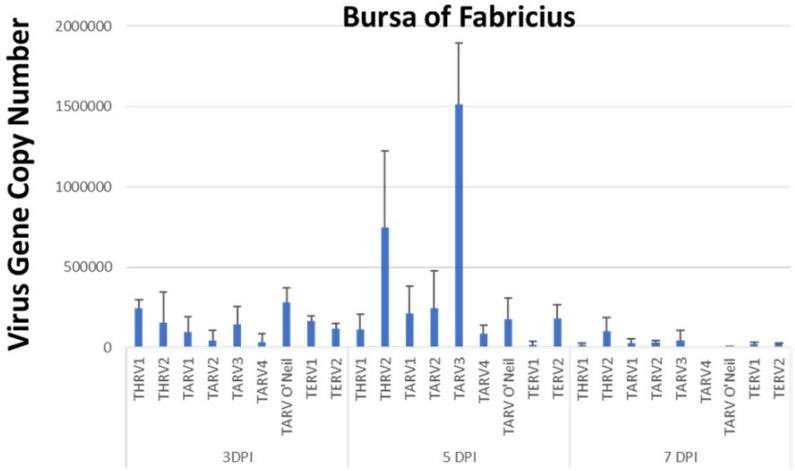
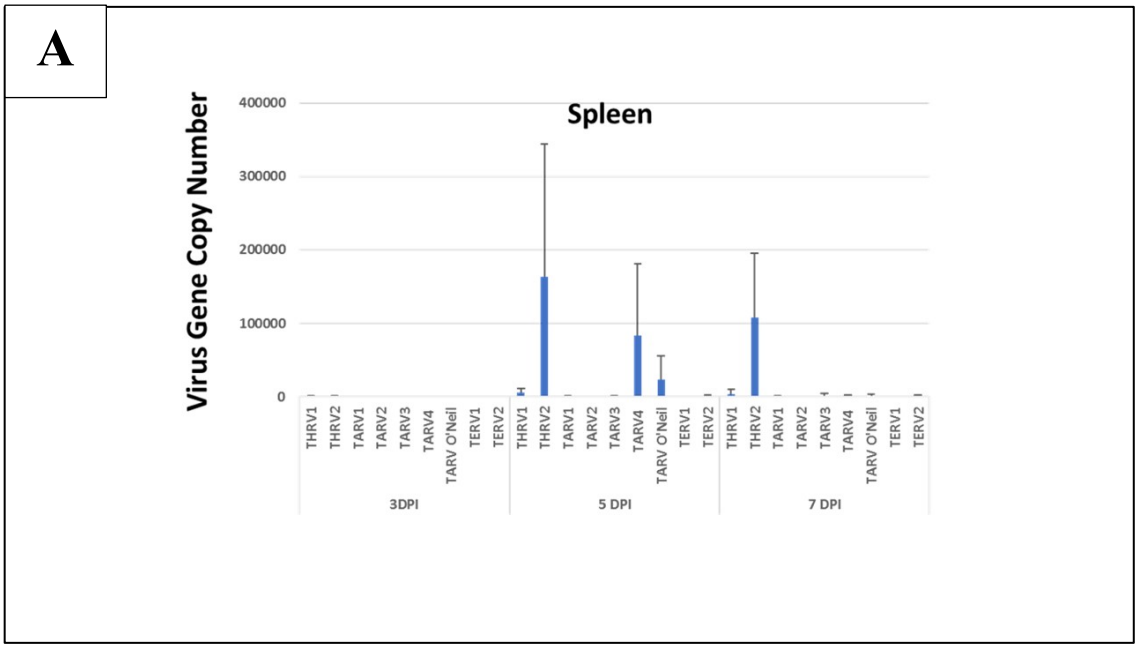

Bursa of Fabricius
Virus Gene Copy Number
2000000
1500000
1000000
500000
0
THRV1
THRV2
TARV1
TARV2
TARV3
TARV4
TARV O'Neil
TERV1
TERV2
3DPI
5 DPI
7 DPI

Figure 7.4: Virus gene copy number in (A) spleen at 3-, 5- and 7- days post infection and (B) liver at 5-, 7-, and 14-days post inoculation and in tendons at 14-, 21-, and 28-days post inoculation.



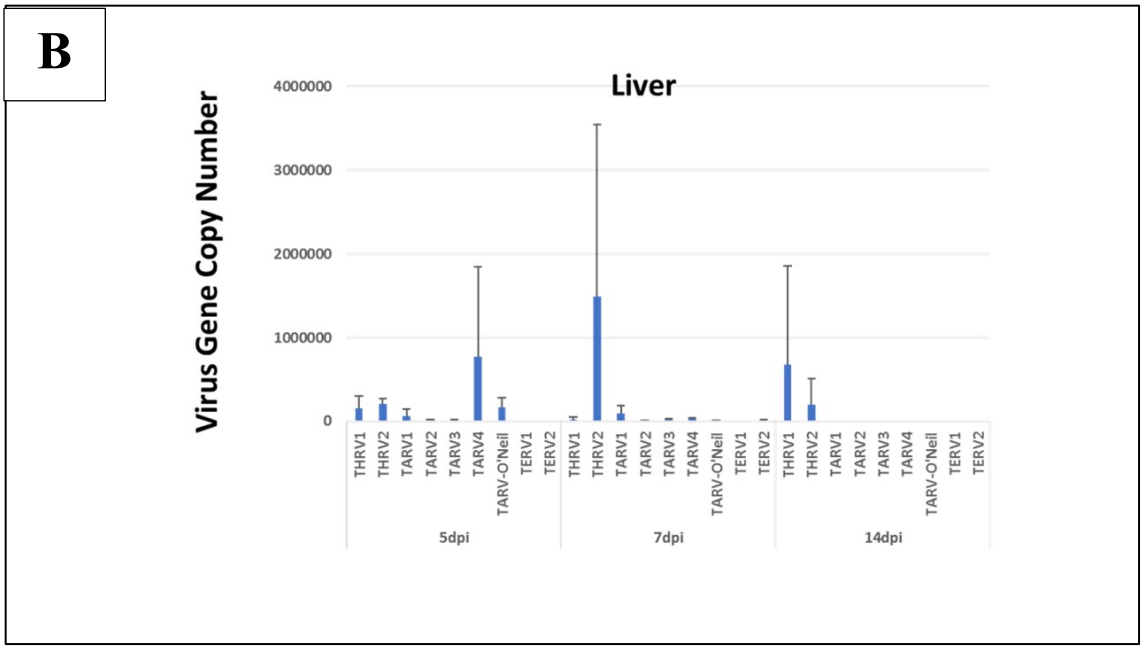
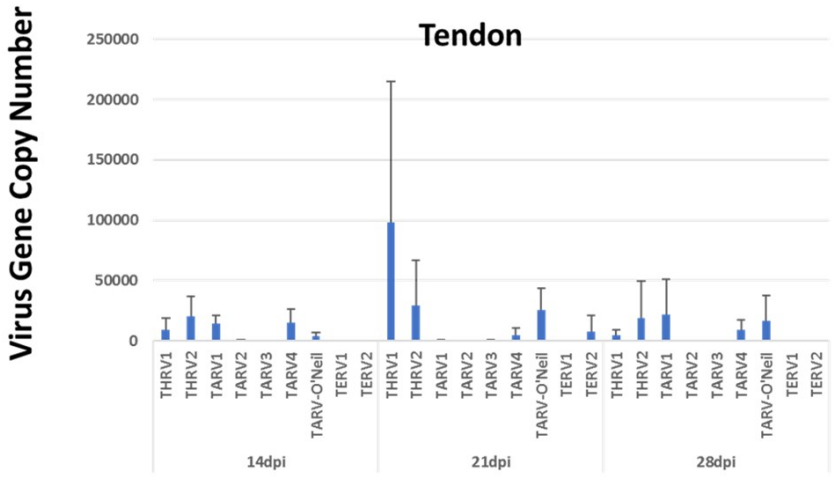


Figure 7.5: (A) Virus gene copy number in tendons at 14-, 21-, and 28-days post inoculation. (B) Histologic lesion scores in tendons at 7-, 14-, 21-, and 28-days post inoculation. Groups with different letters at one time point show statistically significant difference ($p<0.05$).

A



B

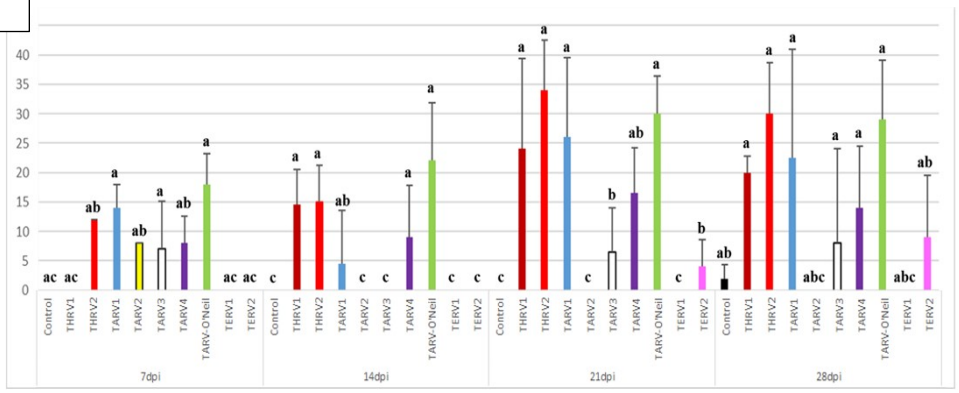
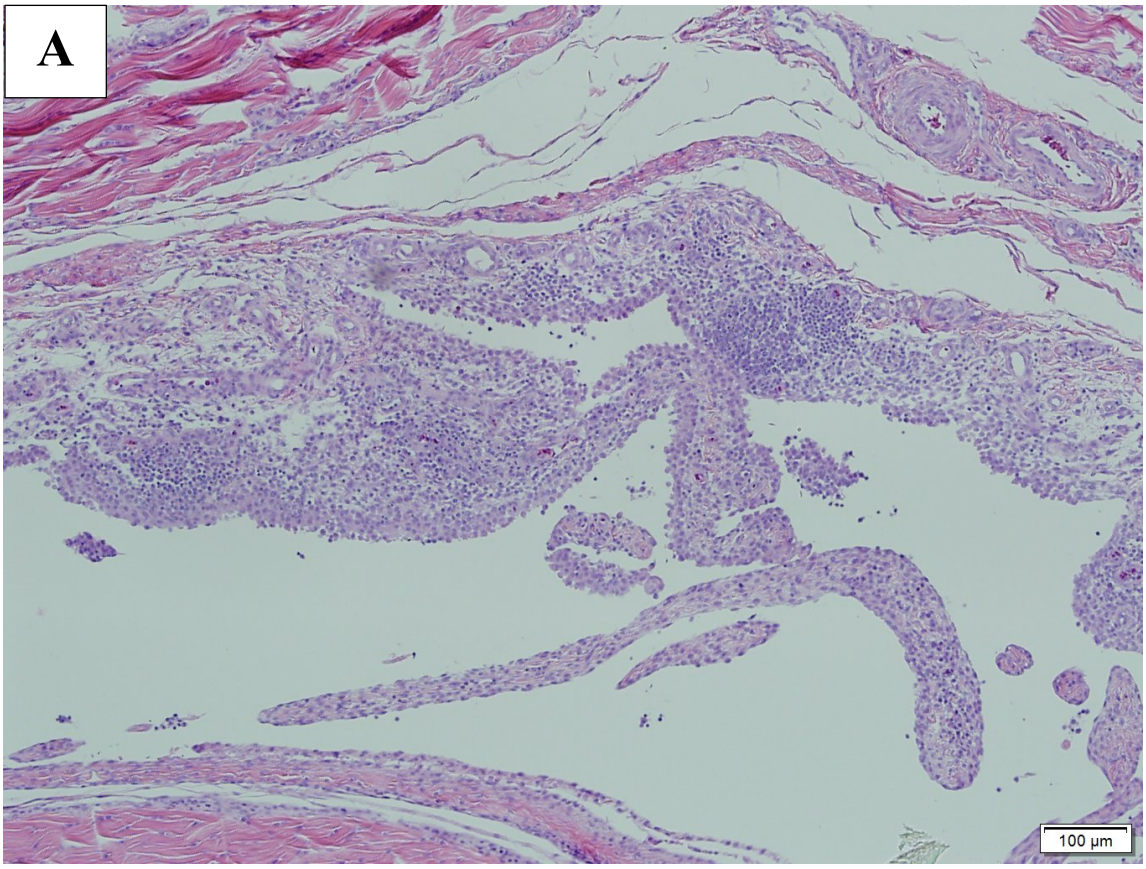
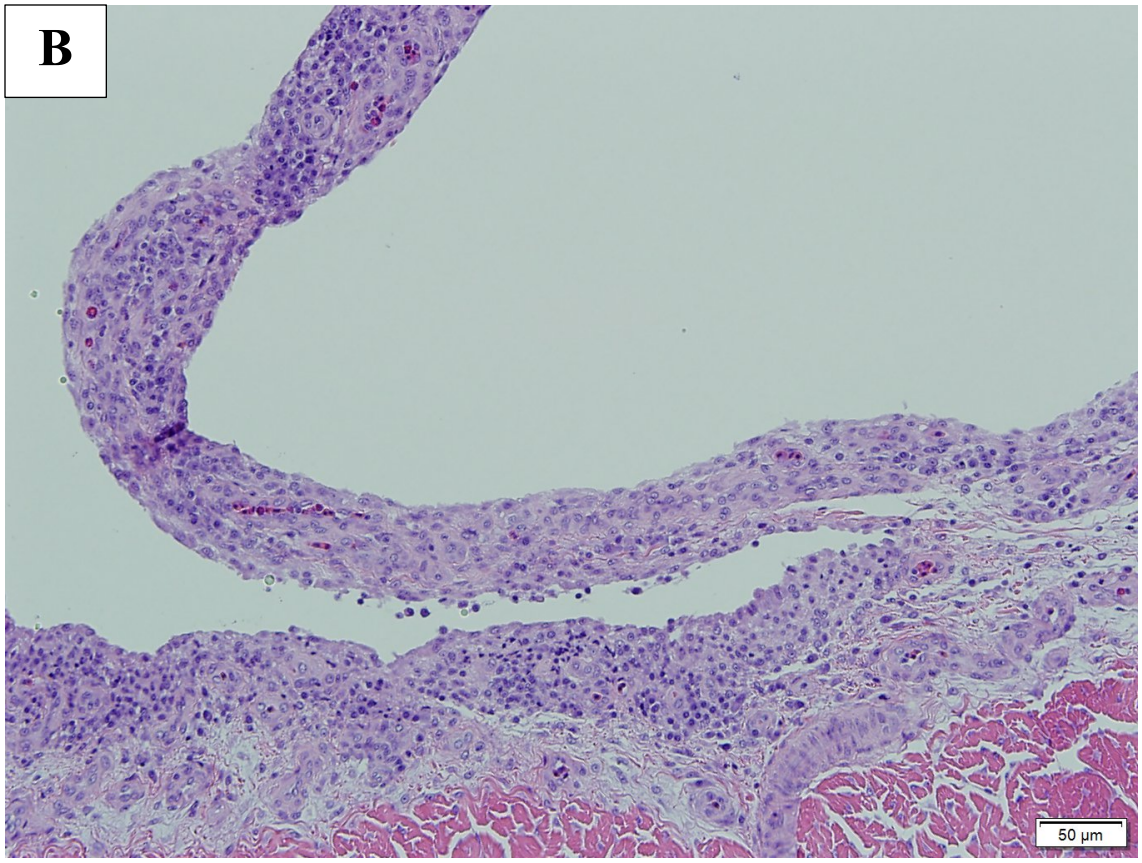
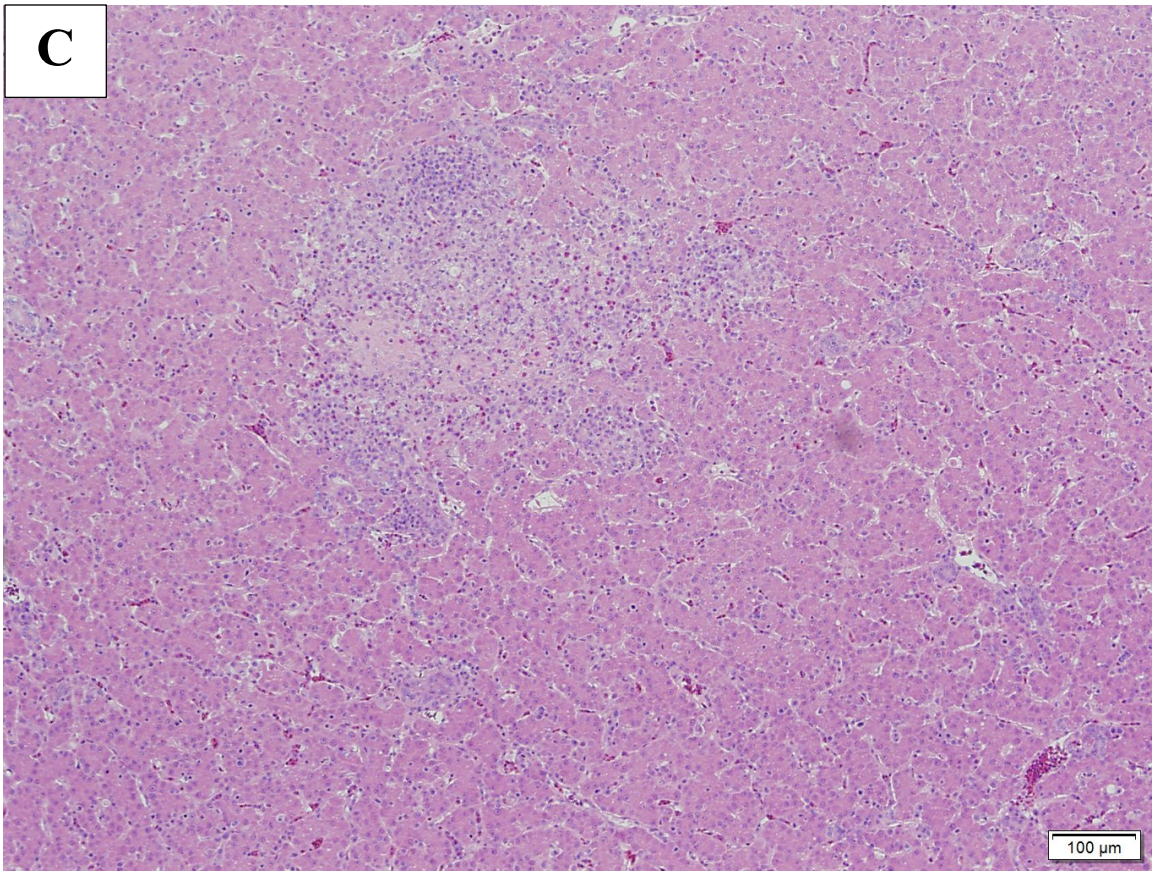
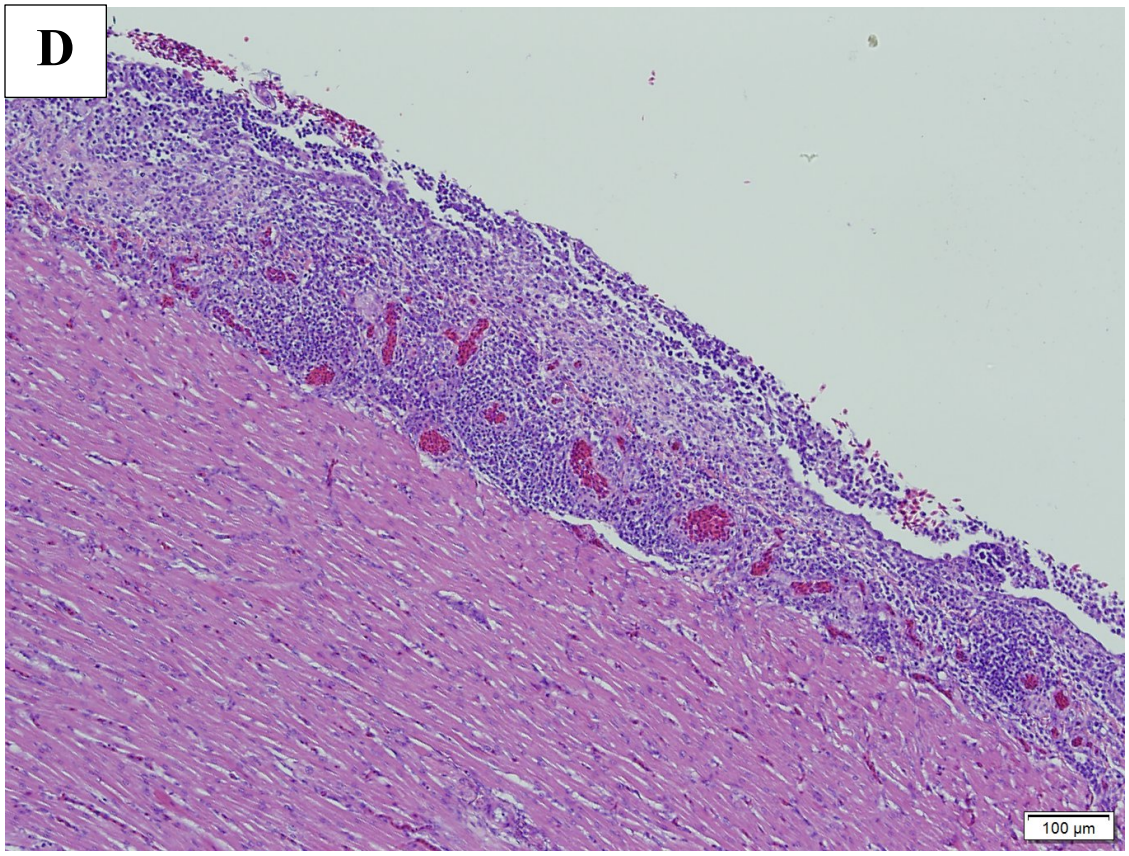


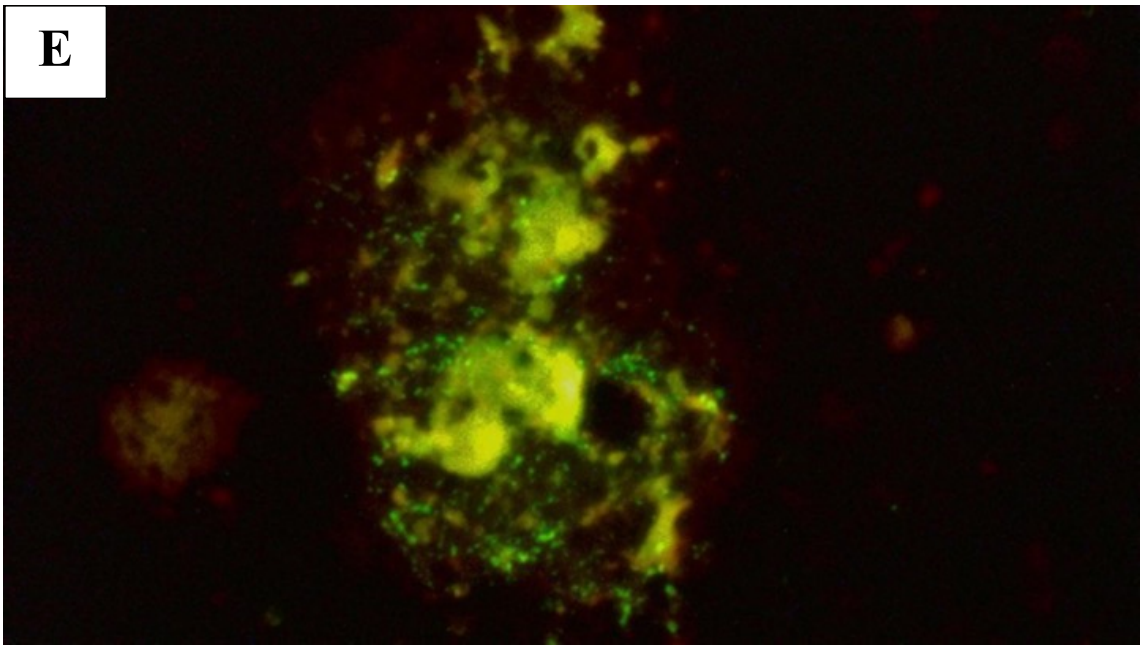
Figure 7.6: Histologic lesions in tendon, liver and heart. a) Villous nodular synovial hyperplasia: The villous include hyperplastic synoviocytes, lymphocytic infiltration and fibrosis; b) villous nodular hyperplasia with germinal center-like lymphoid aggregates; c) hepatocellular necrosis with mononuclear cell infiltration; d) lymphocytic epicarditis; e) Direct fluorescent antibody (FA) of THRV2 positive liver and spleen: QT-35 cells are displaying fluorescence indicating THRV2 replication.




B
50 µm


C
100 µm


D
100 µm


E

CHAPTER 8

AGE SUSCEPTIBILITY AND HORIZONTAL TRANSMISSION OF TURKEY ARTHRITIS REOVIRUS

The material in this chapter has been prepared for publication:
Rahul Kumar, Tamer A. Sharafeldin, Sagar M. Goyal, Sunil K. Mor, and Robert E. Porter. Age susceptibility and horizontal transmission of turkey arthritis reovirus. Microb. Pathog.

Introduction

Turkey arthritis reovirus (TARV) usually infects tom turkeys at the ages of 12-16 weeks of age resulting in lameness. The disease is characterized by uni- or bilateral lameness due to swelling of the hocks, periarticular fibrosis, tenosynovitis, occasional erosion of the articular cartilage on distal tibiotarsus, and rupture of the gastrocnemius or digital flexor tendon (Mor et al., 2013b; Porter, 2018). This leads to substantial economic losses in terms of increased culling, mortality, increased condemnation rates, poor feed efficiency and low rates of weight gain (Lu et al., 2015; Porter, 2018) in addition to creating well-being issues in turkey flocks (Lu et al., 2015; Mor et al., 2014; Tang et al., 2015). We previously reproduced the disease by oral inoculation of naïve birds at one week of age. The virus replicated in gastrocnemius tendons and produced tenosynovitis and varying degrees of lameness (Sharafeldin et al., 2014; Sharafeldin et al., 2015a, 2015b). Although a challenge model was developed in one-week-old turkey poults, we still do not know if there is an age-related resistance to TARV infection as has been described in chickens with chicken arthritis reovirus (CARV).

The turkey industry has adopted a strategy to vaccinate breeder turkeys with autogenous TARV vaccines in an effort to provide poults with passive immunity (Sellers, 2017). However, cases of TARV-associated lameness in turkeys continue to be reported, indicating that these vaccines are not entirely effective, especially against newer strains of the virus (Porter, 2018). It has been established that chicken reovirus is transmitted both vertically from infected eggs (Al-Muffarej et al., 1996) and horizontally by feces or aerosolized virus (Jones and Georgiou, 1984; Roessler and Rosenberger, 1989). It is possible that vertical and horizontal transmission of TARV in turkeys may occur by the

same phenomenon, but it is not established. This challenge study was designed to determine the age susceptibility (28 days or less) of turkey poults to oral infection with TARV. Another objective was to determine if infected turkeys could transmit TARV horizontally to age-matched sentinel turkeys.

Materials and Methods

Virus: Turkey reovirus (strain TARV O'Neil) was propagated and titrated in Japanese quail fibrosarcoma (QT-35) cells. The 50% tissue culture infective dose ($TCID_{50}$) was calculated by using the Reed and Muench (1938) method.

Experimental design: Day-old turkey poults (n=145) were procured from a non-vaccinated, reovirus-free flock. Meconium, intestine, and tendon samples from ten poults were collected and tested by real time RT-PCR (rRT-PCR) to ensure they were free from TARV infection. Serum samples from these birds were tested by an enzyme-linked immunosorbent assay (ELISA) to rule out the presence of anti-reovirus antibodies. The poults were randomly divided into seven groups, which were then placed in seven different air-filtered, isolators. Food and water were supplied *ad libitum*. Groups 1-5 of 15 poults each were inoculated orally with 0.2 ml of TARV O'Neil virus (~10^6 $TCID_{50}$/ml) at 2, 7, 14, 21 and 28 days of age (DOA), respectively (Table 8.1). Birds in group number 6 (sham inoculated control, n=34) were inoculated with virus-free DMEM and group 7 consisted of 26 birds to serve as a source of sentinel birds. Four birds from the sentinel group (group 7) were moved to each of the virus-inoculated group (groups 1-5) at 2-days post inoculation (dpi). The birds were examined daily for any overt clinical sign or mortality. Birds displaying signs of severe illness were euthanized according to

the IACUC and Research Animal Resources (RAR) guidelines. Five of the virus-inoculated birds plus two sentinels from each group were euthanized at 7 and 14 dpi. Three birds from each of the sham-inoculated group were euthanized at 2, 7, 14, 21 and 28 DOA, respectively, to establish a baseline for virus gene copy numbers and histologic lesion scores (negative controls) and to establish that experimental turkey age-groups were negative for TARV prior to challenge. At necropsy, gross lesions were noted followed by collection of ileocecum and hock joint with gastrocnemius and digital flexor tendons, which were subjected to real time RT-PCR (rRT-PCR). Ileocecum was chosen for PCR because our previous work indicated that reovirus replication is greatest at that site following oral challenge. Histopathology was performed on sections of gastrocnemius tendons and sheaths.

Sample processing: For rRT-PCR, one gram of individual intestinal samples was homogenized in Hanks' balanced salt solution (HBSS) for 2 min using a Stomacher (Model 80, Seward, Ltd., UK) to prepare a 10% suspension. Individual tendon samples were homogenized in phosphate buffered saline (PBS) contained in Geno/Grinder tubes for 2 cycles of 4 minute each using a Geno/Grinder (SPEX Sample Prep 2010 Geno/Grinder®, Thomas Scientific, Swedesboro, NJ). Tissue homogenates were centrifuged at 1800xg for 10 min at 4°C. The supernatant was decanted and frozen at -80 °C until further use.

Virus gene copy number: Virus gene copy numbers in the intestine and tendons were calculated using a universal avian reovirus rRT-PCR available at the Veterinary Diagnostic Laboratory, University of Minnesota (https://www.vdl.umn.edu/node/15341). The gene copy number was calculated using ten-fold dilutions of TARV-positive RNA

included with each 96-well plate for establishing a standard curve. The gene copy numbers of intestine and tendons at different times were calculated and subjected to appropriate statistical tests as described below.

Histopathology: Formalin-fixed soft tissues were trimmed, processed, and stained with hematoxylin and eosin (H&E). Formalin fixed hock joints were decalcified in ethylene diamine tetra-acetic acid prior to processing for histopathological examination. Lesions in the gastrocnemius tendons were scored using a previously described histologic lesion scoring system (Sharafeldin et al., 2014).

Statistics: To eliminate skewness, variability of data and small sample size, natural log of virus gene copy numbers in intestine and tendons were calculated. One-way ANOVA followed by a non-parametric Kruskal Wallis test and pairwise Wilcox test with continuity correction and "Benjamini & Hochberg (BH)" as p value adjustment method was used to test the significance of difference in virus gene copy numbers in tissues (intestine and tendon) at 7 and 14 dpi, respectively, and in histologic lesion scores in gastrocnemius tendons. Statistical significance was determined at p value < 0.05. Statistical analysis was done in R (R Core Team, 2017) and figures were produced using the package ggplot2 (Wickham, 2009).

Results

Clinical disease and gross lesions: During the entire period of the study, no clinical signs of lameness or mortality specific to reovirus were observed. At all ages and euthanasia time-points, no reovirus-specific gross lesions were observed.

Virus gene copy number in inoculated birds: The highest virus gene copy number in intestine was observed at 7 dpi in the group inoculated at 2 DOA. Groups inoculated at 2, 7 and 14 DOA did not show significant differences in the virus gene copy numbers (Figure 8.1A). Similarly, groups inoculated at 21 and 28 DOA did not have significant differences (Figure 8.1A). On the other hand, the groups inoculated at 21 and 28 DOA were positive for reovirus but had significantly lower virus gene copy numbers in intestine as compared to the groups inoculated at 2, 7 and 14 DOA (Figure. 8.1A). None of the groups inoculated at different DOA showed significant differences in virus gene copy numbers in tendons at 7 dpi (Figure. 8.1B. At 14 dpi, the virus gene copy numbers in the intestine were very low and did not show any significant difference among any of the groups inoculated at different DOA (Figure. 8.1C. At 14 dpi, the virus gene copy number in tendons of groups inoculated at 2 and 7 DOA were numerically higher than groups inoculated at 14, 21 and 28 DOA but the differences were not significant (Figure. 8.1D).

Virus gene copy numbers in sentinel birds: All sentinel turkeys were susceptible to TARV infection through horizontal transmission, likely via the fecal-oral route. At 7 dpi, the virus gene copy number in the intestines of sentinel turkeys was highest in the group inoculated at 7 DOA followed by 2 DOA and were lower in groups inoculated 14, 21 and 28 DOA (Figure. 8.2A). At 7dpi, in tendons (Figure 8.2B), the virus gene copy numbers increased from group inoculated at 2 DOA to 14 DOA and then decreased and then again increased in groups inoculated at 21 and 28 DOA. At 14 dpi in intestine (Figure 8.2C), the virus gene copy numbers gradually decreased in groups inoculated on 2 to 28 DOA. At 14 dpi in tendons of sentinel turkeys (Figure 8.2D), the virus gene copy numbers were

highest in the group inoculated at 2 DOA but did not follow any pattern shown by groups inoculated at other time points.

Histopathology of gastrocnemius tendons of inoculated birds: The histologic tendon lesions comprised of hypertrophy and hyperplasia of synoviocytes progressing to villous-nodular structures with infiltration of lymphoplasmacytic cells and fibroplasia at later stage. Histologic tendon lesions consistent with TARV infection were observed in all challenged and sentinel turkeys at all ages of challenge. At both 7dpi and 14dpi euthanasia time points, histologic lesions were observed in all age groups inoculated with the virus. At 7 dpi (Figure 8.3 A), the histologic lesion scores increased from group inoculated at 2 DOA to group inoculated at 7 DOA and decreased in group inoculated at 14 DOA but increased again in group inoculated at 21 DOA and then declined in group inoculated at 28 DOA. The histologic lesion score in birds inoculated at 7 DOA were significantly higher in comparison to groups inoculated at 14 DOA. The group inoculated at 14 DOA had a significantly lower histologic lesion score in comparison to the group inoculated at 21 DOA. The group inoculated at 21 DOA had significantly higher histologic lesion score in comparison to groups inoculated at 28 DOA (Fig. 8.3A). At 7 dpi, no specific pattern in histologic lesions scores of groups inoculated at different DOA was observed. At 14 dpi (Figure 8.3B), the groups inoculated with virus at different DOA showed decreasing histologic lesion scores with age of inoculation and were not significantly different from each other (Figure. 8.3B).

Histopathology of gastrocnemius tendons in sentinel birds: The sentinel birds were added 2 dpi to the groups inoculated with virus at different DOA. Histological lesions of tenosynovitis were seen in these birds. The lesions consisted of prominent lymphocytic

infiltration of the sub-synovium and mild hyperplasia of synoviocyte at 7 dpi along with mild fibroplasia and lymphoid nodule formation at 14 dpi. At 7dpi, the histologic lesions in tendon decreased in groups inoculated at 2, 7 and 14 DOA, respectively, but increased in group inoculated at 21 DOA and then decreased slightly in groups inoculated at 28 DOA (Fig 8.4A). At 14 dpi, the histologic lesions did not follow any pattern, but highest lesion scores were observed in sentinel birds added to the group inoculated at 2 DOA followed by 14, 28 and 21 DOA, respectively (Fig 8.4B). The histologic lesions in sentinel birds added to the groups inoculated at 7 DOA were very low in comparison to other groups (Fig 8.4B).

Discussion

While TARV has been resulting in economic losses due to increased culling of lame birds, there has no experimental studies to establish an understanding of transmission dynamics. The adopted vaccination programs can give maternal immunity for up to 2-3 weeks of age (Gharaibeh and Mahmoud. 2013; De Herdt et al., 2016). We did not know whether susceptibility of birds to infection changes as they age. To show age-related susceptibility in poults, we used the classic highly pathogenic TARV strain (TARV O'Neil) at a titer of (10^6 $TCID_{50}$/mL). Our established oral inoculation and histologic lesions scoring system in experimental model of TARV infection were applied in this study (Sharafeldin et al., 2014; 2015a).

The virus gene copy numbers in intestine at 7 dpi in groups inoculated at 2, 7 and 14 DOA was approximately double than that at 14 dpi in the corresponding groups. This decrease in virus replication at 14 dpi was probably due to the production of antiviral

cytokines after 1 week of virus inoculation (Sharafeldin et al., 2015b). On the other hand, the groups inoculated at 21 and 28 DOA had nearly a similar rate of virus replication and similar virus gene copy numbers in the intestine at both 7dpi and 14 dpi. This suggests that although the turkey poults in our study were susceptible to TARV infection at all ages (2, 7, 14, 21 and 28 days), the highest virus replication, based on gene copy numbers, occurred during the first two weeks of life. Change in the dynamics and/or efficacy of antiviral cytokines in older birds may be the reason behind the same rate of virus replication at 7 and 14dpi in groups inoculated at 21 and 28 DOA, which need to be studied in future. Apparently, the turkeys became less susceptible to TARV challenge after 14 DOA. Reovirus infection in chicken inoculated (orally or intra tracheal or by footpad route) at 2 weeks of age or older has also been shown to be less severe (Wood and Thorton, 1981; Jones and Georgiou, 1984; Roessler and Rosenberger, 1989). This age-related susceptibility in poults is probably due to their inability to prevent virus dissemination and tissue destruction (except tendons), which are followed by viremia. It is worth noting that although turkey poults showed the highest TARV replication at age 2 weeks or younger, all turkeys 28 days of age or less were susceptible to TARV infection and could transmit the infection horizontally. This age-related susceptibility pattern can vary with reovirus strain as the pathogenicity of arthrotropic reoviruses differs depending on intrinsic strain characters (Gouvea and Schnitzer, 1982). The differences in the pathogenic potential of reovirus strains were demonstrated under experimental conditions (Afaleq and Jones, 1989; Sharafeldin et al., 2014). Possibly, some arthrotropic strain that normally causes asymptomatic infection can be pathogenic under stressful managemental conditions or immunosuppression, but Ngunjiri et al., (2019) could not prove this

hypothesis in dexamethasone induced immunosuppression in turkey poults. Immunosuppression followed by managemental stress and coinfection with other reovirus strains or other enteric etiologies can trigger pathogenicity of an otherwise asymptomatic reoviruses (Barnes et al., 1982; Pantin-Jackwood et al., 2007b; Spackman et al., 2010; Mor et al., 2013a; Ongor et al., 2015; Johnson et al., 2018; Ngunjiri et al., 2019).

Leg tendons showed a similar rate of virus replication and virus gene copy numbers in all virus-inoculated age groups at 7 and 14 dpi. At 14 dpi, groups inoculated at 2 and 7 DOA had numerically higher virus gene copy numbers but not statistically significant than other groups. Presence of some extreme outlier created this insignificance. We expect that with a larger sample size the difference would be significant. This suggests that the tendons of turkey poults up to 7 DOA are most susceptible to TARV infection as compared to older age groups. Roessler and Rosenberger (1989) reported similar results in chickens where the presence of virus in tendons was also somewhat dependent on the age of chickens at inoculation, as indicated by lower incidence of infected tendons in chickens inoculated at 1 week of age or more as compared to those inoculated at 1-day of age.

Histologic lesion scores in leg tendons showed variability (low in age groups 2, 14, and 28 DOA to high in age groups 7 and 21 DOA) within and among different age groups at 7 dpi, the scores at 14 dpi were consistently high with no significant difference among different groups. The group inoculated at 28 DOA had numerical low lesions scores, which are not statistically significant due to some outliers. With a larger sample size, this group might have a significantly lower histologic lesions score. The variation in

histologic lesions scores within and among different age groups can be attributed to the slow progressing and subacute to chronic nature of TARV pathogenesis. Previous studies have reported that TARVs induce consistent moderate to high histologic lesion scores as early as 14 dpi in turkey poults inoculated at 7DOA. (Sharafeldin et al., 2014; 2015a; 2015b) and variation among different birds is expected before 14 dpi.

From virus gene copy numbers and tendon lesion scores, it seems that turkeys are still susceptible to the infection at an older age. The slow progressing nature (Sharafeldin et al., 2015a; 2016) of the reovirus in turkeys makes it more likely to be chronic unlike chicken arthritis reovirus (CARV) which is likely to be acute in chickens. Roessler and Rosenberger (1989) reported that CARV inoculation in chickens at 2 or more weeks of age resulted in mild infection and the microscopic lesions were absent, except in the persistently infected gastrocnemius tendons. The severity of microscopic tendon lesions in turkeys may not depend on the age of infection but on the chronicity and persistence of infection in the tendons. Upregulation of IL-6 and IFN-γ cytokine gene expression in experimentally infected turkeys correspond with lymphocytic infiltration in gastrocnemius tendon sheath, indicating the involvement of these cytokines in the slow progression in development of tenosynovitis (Sharafeldin et al., 2015b). Since reoviruses are known to persist in infected turkeys (Sharafeldin et al., 2015a), it is possible that infection occurs early in young poults and remains subclinical until the marketing age, when progressive tendon fibrosis and reduced tensile strength, complicated by the high body weight of tom turkeys, predisposes the birds to lameness and periodic gastrocnemius tendon rupture (Sharafeldin et al. 2016).

Experimental reovirus infection in young chickens demonstrated virus localization in hock joints for many weeks (Jones and Onunkwo, 1978; Marquardt et al, 1983). The results of the present study indicate that infection of older birds may lead to significantly shorter duration of virus persistence in hock joints leading to a gradual decrease in the histologic lesion scores with advanced age at infection. The other factors that probably contribute to a decrease in histologic lesion score with advancing age is the change in the number of fibroblasts, direction of fibers, and mineralization in the tendons of the hock joints as described by Abdalla (1979).

Introduction of sentinels to virus-inoculated groups at 2 dpi rules out the probability of transmission due to the virus inoculum itself, making this a more robust model for studying horizontal transmission of TARV. Sentinel birds added 2 dpi to groups inoculated with TARV O'Neil at different DOA showed viral replication and virus gene copies in intestine and tendons and histologic tendon lesions at 7 and 14 dpi similar to those in virus-inoculated birds. The virus gene copy number in sentinel birds added to group inoculated at 7 DOA was even higher (10^5) than in inoculated birds (10^3) at 7 dpi. The probable reason for this difference in the rate of virus replication may be the actual number of dpi. The sentinels were added to the inoculated groups after 2 days of inoculation and were euthanized at 7 dpi, which is actually 5 dpi for the sentinel birds. Previous studies have reported that virus replication in intestine reaches its peak between 4-7dpi and decreases thereafter (Sharafeldin et al., 2015b; Ngunjiri et al., 2019). In an experimental study with nine different TARV strains, peak virus replication was observed at 5dpi and decreased after 7 dpi in intestine of birds inoculated with different viral strains (unpublished). This decrease in virus gene copy number in inoculated birds may

be due to production of antiviral cytokines 7 dpi as reported by Sharafeldin et al. (2015b). In general, variability of virus copy number and histologic lesion scores of sentinel birds within and among different age groups was expected because it was dependent on the dose of infection transmitted to every single sentinel bird (uncontrolled) and due to the small number of sentinel birds in this experiment.

Virus gene copy numbers in sentinel birds further decreased to very low level at 14 dpi further indicating that the extent of reovirus replication is greatest during the first 14 days of life, but all birds are still susceptible to infection at 28 days of age. This histological pattern of lymphoplasmacytic tenosynovitis was consistent with both field cases and the experimental model of TARV infection in turkey poults (Sharafeldin et al., 2015a). Our results of histologic lesions in sentinels are supported by the observations of Sharafeldin et al. (2014) but are in contrast to Ngunjiri et al. (2019). Different virus strains were likely used in these studies, and our use of a highly pathogenic field TARV may be responsible for the discrepancy. Virus transmission to sentinels likely occurred through pecking of contaminated feces and litter by the sentinels (Jones and Georgiou, 1984; Macdonald et al., 1978; Mor et al., 2015) or through contaminated aerosols (Roessler and Rosenberger, 1989). The sentinel data were not subjected to statistical analysis due to the small sample size (n=4) of sentinels introduced to each group inoculated at different DOA and euthanized at 7 (n=2) and 14 dpi (n=2).

The resistance of older birds to reovirus infection may also be due to mature and functional humoral and T-cell mediated immune responses. Immunological unresponsiveness to reovirus infection in young birds is partly due to delayed antibody production and lack of helper T-cells (Seto, 1981). Maturation of macrophages may also

play a role in age susceptibility to reovirus infection because macrophages are the target cells for reovirus and macrophages found in older chickens are more effective in resolving the infection (Bülow and Klasen, 1983). The presence of virus replication in tendons in groups inoculated at older age indicates that reovirus can infect tendons in immunocompetent birds with mature immune system as evidenced by the inflammatory lesions in the hock joint. The joints and tendons act as sequestered sites, thus preventing virus elimination by the immune system (Jones and Georgiou, 1985).

The relative virulence and pathogenicity of a virus depends on its infectivity, ability to multiply within the host cells and infect other cells, spread to other tissues, and overcome host's defense mechanisms. The pathogenicity of CARV is influenced by several factors such as age (Wood and Thorton 1981; Jones and Georgiou 1984; Ruff and Rosenberger 1985; Roessler and Rosenberger, 1989), genetics (Jones and Kibenge 1984) and immunological responsiveness (Montgomery et al., 1986; Kibenge et al., 1987). The same factors may also be responsible for determining the pathogenicity TARV in turkeys.

The findings indicate that turkeys of ages 28 days or less are susceptible to infection with TARV following oral challenge, resulting in TARV replication in both intestine and gastrocnemius tendon, lymphoplasmacytic gastrocnemius tenosynovitis, and infected birds can transmit the infection and tenosynovitis to sentinel turkeys of equal age. Additional studies are indicated to determine the actual age at which turkeys show true resistance to TARV infection and subsequent horizontal transmission. These findings suggest that novel vaccination strategies may be necessary to control TARV in a production facility. Vaccination strategies may be designed to provide active immunity or maternally derived antibodies to turkey poults for longer duration because this study

showed that poults are susceptible to reovirus infection up to 28 days of age. The prolonged susceptibility and horizontal transmission of TARV in turkeys, when compared to the pathogenesis of similar disease in chickens, indicates that it may be necessary to vaccinate commercial meat-type turkey poults during grow out in order to reduce transmission to flock mates.

Table 8.1. Summary of experimental design of Age susceptibility study

Group	Group name (Age of Inoculation)	Virus Ch (O'Neil) at indicated age	Euthanasia (Age in days)
1	Challenge @ 2-day-age (n=15)	2nd day	9, 16
2	Challenge @ 7-day-age (n=15)	7th day	14, 21
3	Challenge @ 14-day-age (n=15)	14th day	21, 28
4	Challenge @ 21-day-age (n=15)	21st day	28, 35
5	Challenge @ 28-day-age (n=15)	28th day	35, 42
6	Negative Control (n=34)	MEM	9, 14, 16, 21, 28, 35, 42
7	Sentinels (n=26)		

Figure. 8.1: Virus gene copy numbers (Ln) in birds inoculated at different days of age **(A)** in intestine at 7 dpi. **(B)** in tendon at 7 dpi. **(C)** in intestine at 14 dpi. **(D)** in tendon at 14 dpi. Line plots having different alphabets have significant difference at $p<0.05$. Gene copy numbers in negative control turkeys (not shown) were 0.

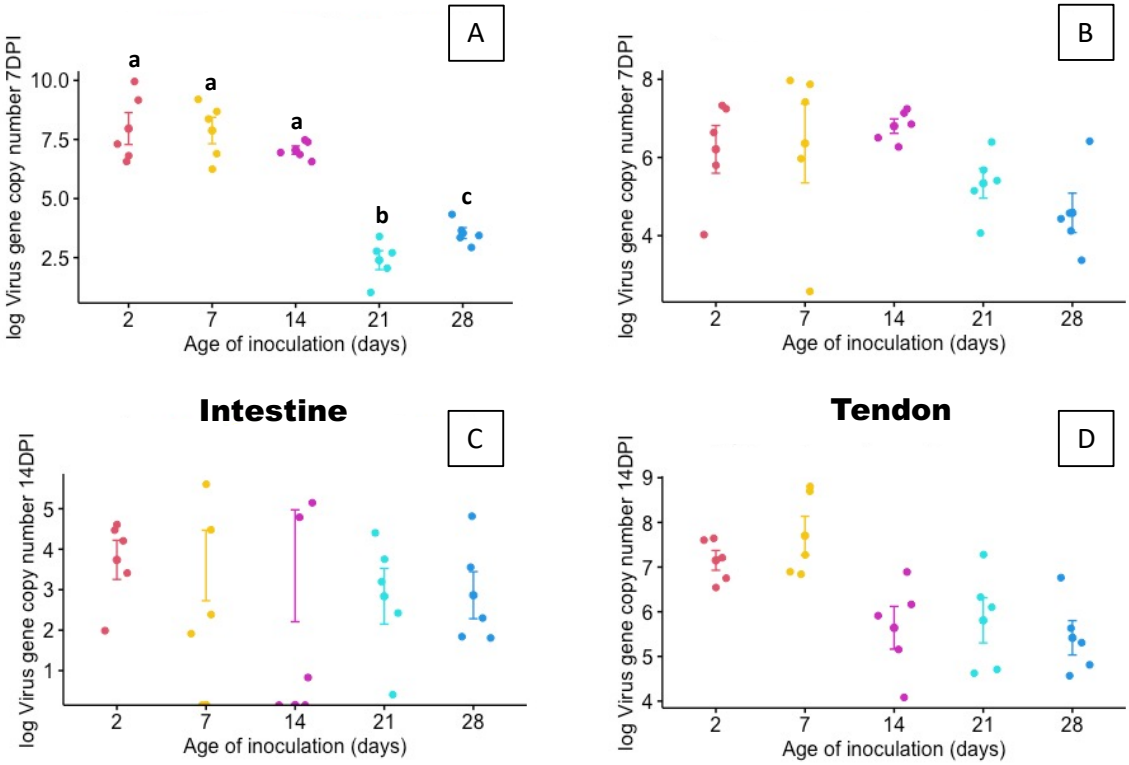


Figure. 8.2: Virus gene copy numbers (Ln) in sentinel birds at different days of age **(A)** in intestine at 7 dpi. **(B)** in tendon at 7 dpi. **(C)** in intestine at 14 dpi. **(D)** in tendon at 14 dpi. Statistical comparisons are not made due to small sample sizes (n=2).

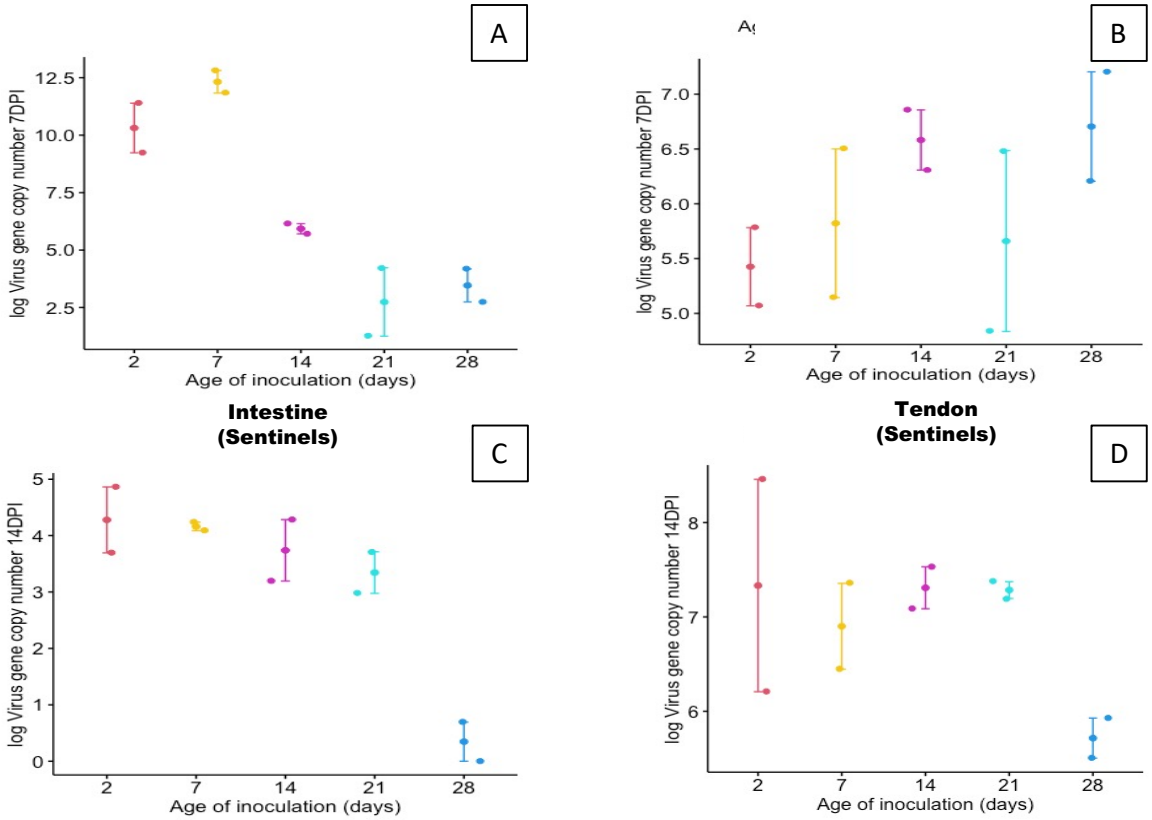


Figure. 8.3: **(A)** Tendon histologic lesion scores at 7 dpi. **(B)** Tendon histologic lesion scores at 14 dpi. Line plots having different alphabets have significant difference at $p<0.05$. Tendon lesions scores for negative control turkeys were negligible (not shown).

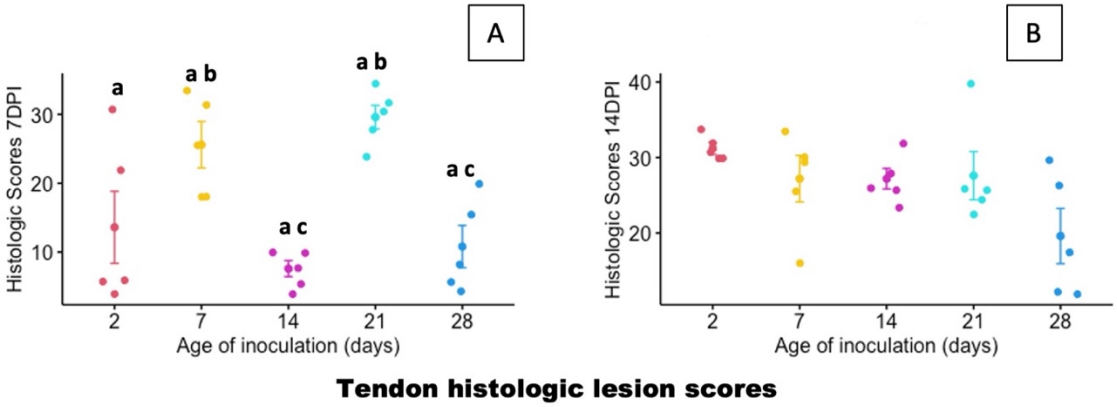
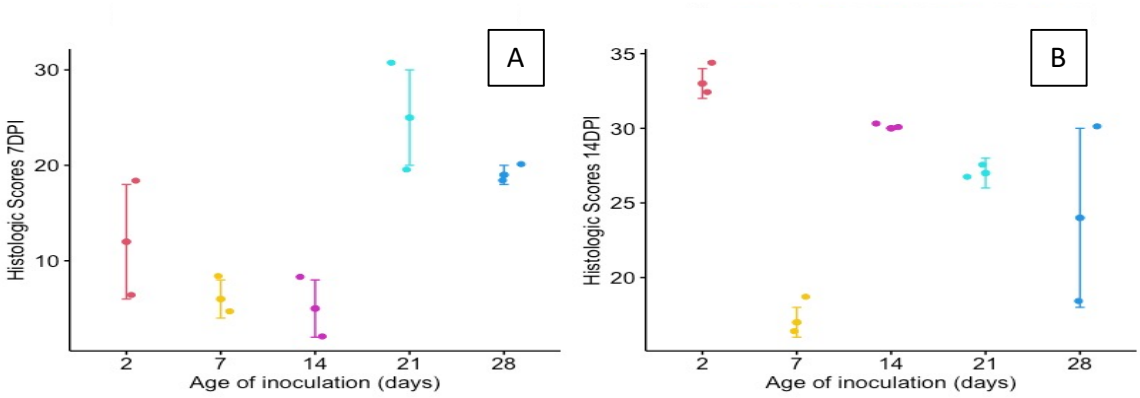


Figure. 8.4: Histologic lesion scores in tendons of sentinel birds at **(A)** 7 dpi. **(B)** 14 dpi. Statistical comparisons are not made due to small sample sizes (n=2).



CHAPTER 9

EFFICACY OF A LIVE VIRUS VECTORED VACCINE AGAINST TURKEY ARTHRITIS REOVIRUS HOMOLOGOUS AND HETEROLOGOUS VIRUS CHALLENGE

The material in this chapter has been prepared fro publication:
Rahul Kumar, Robert E. Porter, Sagar M. Goyal and Sunil K. Mor. 2021. Efficacy of a live virus vectored vaccine against turkey arthritis reovirus homologous and heterologous virus challenge. Vet. Microb.

Introduction

Turkey arthritis reovirus (TARV) causes lameness in turkeys, generally at 12-17 weeks of age. A reovirus was isolated from the gastrocnemius tendons of turkeys affected with arthritis/tenosynovitis in the1980s (Levisohn et al., 1980; Page et al., 1982), but this condition was not observed again for nearly 25 years when it was reported by Mor et al., (2013). Thereafter, several authors reported TARV-associated outbreaks of lameness in market age turkeys (Lu et al., 2015; Tang et al., 2015). These outbreaks result in substantial economic losses to turkey farmers in the form of increased culling, increased condemnation rates, poor feed efficiency and low rates of weight gain (Lu et al., 2015; Porter, 2018). The disease has been experimentally reproduced to confirm the involvement of reovirus (Sharafeldin et al., 2014, 2015) and the infection has consistently been associated with uni- or bilateral lameness due to swelling of the hocks, periarticular fibrosis, tenosynovitis, occasional erosion of the articular cartilage on distal tibiotarsus and rupture of the gastrocnemius or digital flexor tendon (Mor et al., 2013; Sharafeldin et al., 2014, 2015; Porter, 2018).

The TARV is a member of genus *Orthoreovirus* in the family *Reoviridae* containing double-stranded segmented RNA genome in double-shelled capsid. The 10 segments of viral genome are classified as L class (L1-L3), M class (M1-M3) and S class (S1-S4) (Spandidos and Graham, 1976) based on their electrophoretic mobility. Reoviral genome has 12 open reading frames (ORFs), which encode eight structural and four nonstructural proteins (Benavente and Martinez-Costas, 2007). Sigma C (SC) protein translated by the third ORF of S1 segment is an outer capsid cell attachment protein (Schnitzer et al., 1982; Martinez- Costas et al., 1997; Grande et al., 2002) and is the most

diverse among reovirus proteins. This protein is the main immunogenic surface protein containing type- and broad-specific neutralizing epitopes (Martinez- Costas et al., 1997). Sigma B (SB) protein of S3 segment is a major component of the viral outer capsid and contains a group-specific neutralizing epitope (Wickramasinghe et al., 1993). The SC protein alone or in combination with SB protein has been used in formulating subunit vaccines against avian reovirus infections (Wu et al., 2005; Lin et al., 2008; Bi et al., 2016; Goldenberg et al., 2016).

Since 2011, breeder turkeys in the U.S. have been vaccinated for TARV with autogenous killed virus, but commercial market turkeys have not been vaccinated. Setbacks to a successful vaccination program for TARVs is the development of multiple variant TARV strains and the absence of commercial vaccine. Recently, custom made autogenous vaccines are being used by the turkey industry to vaccinate breeder turkeys with prevalent pathogenic TARV strains. Breeders are being vaccinated to check the suspected vertical transmission and to provide maternally derived antibodies to the progeny for protection against the infection in the initial days of their life. However, these autogenous vaccines are poorly characterized and are showing variable efficacy, especially against the variant viruses (Porter, 2018). Antigenic and genetic variants different from the vaccine strains have been reported from progeny and unvaccinated breeder flocks in the U.S. (Mor et al., 2013, 2014a, 2014b, 2015; Sharafeldin et al., 2015; Tang et al., 2015; Sellers, 2017). Outbreaks of TARV-associated lameness continue to be reported, and affected turkeys are commonly submitted to diagnostic labs .

This study describes a live recombinant pichinde virus-vectored bivalent codon optimized turkey arthritis reovirus (rPICV-TARV) vaccine that was developed using the

Sigma C (SC) and Sigma B (SB) protein coding genes of TARV SKM121 strain (Kumar et al., 2021) based on the molecular characterization and whole genome sequencing of TARVs circulating in the US (similar to CARV WGS analysis in Chapter 3). This study was designed to assess the safety and efficacy of rPICV-TARV vaccine against homologous and heterologous virus challenge in the vaccinated poults. Another objective was to characterize the immunogenicity of the vaccine and the transmissibility between vaccinated and non-vaccinated pen mates.

Materials and Methods

Virus: Turkey reovirus strains TARV O'Neil (ON) and SKM121 (SKM) were propagated and titrated in Japanese quail fibrosarcoma (QT-35) cells. The 50% tissue culture infective dose ($TCID_{50}$) was calculated by using the Reed and Muench (1938) method. The bivalent codon optimized live recombinant pichinde virus vectored vaccine (PICV-TARV) was grown in BHK-21 cells and titrated in Vero cells by the plaque forming unit method (Lan et al., 2009).

Experimental design: Day-old turkey poults (n=180) were procured from a non-vaccinated and reovirus infection free flock. Ten poults were euthanized on the day of arrival. Serum samples were tested for reovirus antibodies by ELISA, and meconium, intestine and tendon samples were tested by real time RT-PCR (rRT-PCR) to ensure that the poults were not infected with reovirus prior to the study. The poults were randomly divided into eight groups namely (1) negative control (NC), (2) vaccine control (VC), (3) vaccinated and challenged with TARV SKM121 (V-SKM), (4) sentinels challenged with TARV SKM121 (Sen-SKM), (5) non-vaccinated and challenged with TARV SKM121

(SKM) (to serve as SKM challenge positive control), (6) vaccinated and challenged with TARV O'Neil (V-ON) strain, (7) sentinels challenged with TARV O'Neil (Sen-ON) and (8) non-vaccinated and challenged with TARV O'Neil (ON) (to serve as ON challenge positive control). The eight groups of birds were housed separately in air-filtered isolators. Food and water were supplied *ad libitum*.

The detailed experimental plan is shown in table 9.1. Briefly, Poults were vaccinated with a primary dose of rPICV-TARV vaccine (0.2 ml, $3x10^7$ PFU/ml) by oral route at 2 days of age (doa). In groups Sen-SKM and Sen-ON, 12 birds/group were wing banded and added as sentinels after 2 days of primary vaccination (4 doa). Poults were boosted intranasally (except the sentinels and NC) with 0.2 ml ($3x10^7$ PFU/ml) of PICV-TARV vaccine at 9 doa. On day 14, blood samples were collected from the non-vaccinated, vaccinated and sentinel birds for serology. Birds in all groups (except those in NC and VC) were challenged orally with 0.2ml ($3.2x10^7$ $TCID_{50}$/ml) of TARV SKM121 or TARV O'Neil at 15 doa. Groups NC and VC were sham inoculated with 0.2 ml of virus culture media (MEM). The birds were examined daily for any overt clinical signs or mortality. Birds displaying signs of severe illness were euthanized according to the IACUC and Research Animal Resources (RAR) guidelines. Five birds from each group were euthanized on 21, 28 and 35 doa. Body weight of the euthanized birds were noted before sample collection at 28 and 35 doa. At necropsy, gross lesions were noted followed by collection of intestines (ileocecum) and hock joint with gastrocnemius and digital flexor tendons for real time RT-PCR (rRT-PCR) and histopathology.

ELISA and serum neutralization test (SNT): Blood serum was tested for anti-TARV antibody using a commercial ELISA available at the Veterinary Diagnostic Laboratory,

University of Minnesota (UMVDL) (https://www.vdl.umn.edu/node/14381). The sera were also tested for serum neutralization antibodies against virus strains TARV SKM12 and TARV O'Neil to characterize the homologous and heterologous virus-neutralizing capability of antibodies produced by the vaccinated and sentinel birds. The SN test was performed by Aviserve, Inc., Delaware. Briefly, the heat inactivated serum samples were 4-fold serially diluted in a 96 well plate, and 25 µl of reovirus preparations (100 $TCID_{50}$) was added to all wells except negative control wells. Subsequently, the virus-sera mixture was incubated at 37 °C for 1 hour before adding onto freshly seeded primary hepatocellular carcinoma epithelial cell from a male leghorn chicken (LMH) (5×10^5 cell/well) with 10% fetal calf serum and incubated for 4-5 days. Virus infected and uninfected cells were used as positive and negative controls, respectively. Virus controls, cell controls, and serum controls were included on each plate. The plates were observed daily for the appearance of reovirus specific cytopathic effects (CPE) e.g., cell swelling, syncytia formation, detachment from monolayer. Medium was removed on appearance of CPE and the cells were stained with a 1% crystal violet prepared in 10% buffered formalin for 2–3 min, followed by washing with warm tap water. Plates were air dried and antibody titers were recorded as the reciprocal of the highest dilution of serum that inhibited virus induced CPE in at least 50% of the cell monolayer.

Processing of tissue samples: Individual intestinal (ileocecum) samples (1 gram) were homogenized in Hanks' balanced salt solution (HBSS) for 1-2 min using a Stomacher (Model 80, Seward, Ltd., UK) to prepare a 10% suspension. Individual tendon samples (1 gram) were homogenized in phosphate buffered saline (PBS) for 2 cycles of 4 minute each in Geno/Grinder tubes using Geno/Grinder (SPEX Sample Prep 2010

Geno/Grinder®, Thomas Scientific, Swedesboro, NJ). Tissue homogenates were centrifuged at 1800 xg for 10 min at 4°C. The supernatant was decanted and frozen at -80 °C until tested by rRT-PCR.

Nucleic acid extraction: Nucleic acids (RNA) were extracted from 50 µL of each intestinal and tendon sample homogenates. Nucleic acid extraction was conducted using a MagMAX™ Pathogen RNA/DNA Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) on a Kingfisher-Flex instrument (Thermo Fisher Scientific) following manufacturer's instructions. Nucleic acids were eluted in 90 µL of elution buffer.

Virus gene copy number: Virus gene copy numbers in intestine and tendon samples were estimated using a universal avian reovirus rRT-PCR available at the Veterinary Diagnostic Laboratory, University of Minnesota (https://www.vdl.umn.edu/node/15341). A standard curve was constructed using ten-fold serial dilutions of TARV-positive RNA included with each 96-well plate. The gene copy numbers were calculated in intestine and tendon samples collected at different time points and were subjected to appropriate statistical analysis.

Histopathology: Soft tissues were fixed in formalin, trimmed, processed, and stained with hematoxylin and eosin (H&E). Formalin-fixed hock joints were decalcified in EDTA prior to processing for histopathological examination. Lesions in the gastrocnemius tendons were scored using a previously described histologic lesion scoring system (Sharafeldin et al., 2014).

Statistical analysis: Pairwise comparisons of body weights of birds in different groups was done using t-test. To neutralize the skewness, variability and non-normal distribution

of data and small sample size, natural log of virus gene copy numbers in intestine and tendons was taken. A non-parametric Kruskal Wallis test followed by pairwise Wilcoxon rank sum test with continuity correction and "Benjamini & Hochberg (BH)" as p value adjustment method was used to test the significance of difference in serum neutralizing antibody titers, virus gene copy (in intestine and tendons), and histologic lesion scores in gastrocnemius tendons. Statistical significance was determined at p value < 0.05. Statistical analysis was done in R (R Core Team, 2017) and figures were produced using the package ggplot2 (Wickham, 2009).

Results

Clinical disease and gross lesions: During the period of this study, no reovirus-specific clinical signs, mortality, or gross lesions were observed in vaccinated and/or virus challenged groups. At all euthanasia time points, no reovirus-specific gross lesions or abnormalities were observed.

ELISA and serum neutralization antibody titers: Sera from the vaccinated birds showed zero ELISA antibody titer and hence negative results, whereas based on our experience, the sera from experimentally infected birds usually show an ELISA antibody titer ranging from 740 to 1190 (unpublished data). Negligible to very low serum neutralization antibody titers against TARV O'Neil or TARV SKM121 were observed in sera from non-vaccinated negative control birds. SN antibody titers against TARV O'Neil or TARV SKM121in vaccinated, and sentinel birds varied from 64 to 256 and were significantly higher than the negative control birds (Figure 9.1A and B).

Body weight: At 28 doa (Figure 9.1C), the mean body weight of all the groups viz. NC, VC, V-SKM, Sen-SKM, SKM, V-ON, Sen-ON and ON did not differ significantly whereas, the mean body weights of virus-challenge positive control groups (SKM and ON) were numerically lower than the respective vaccinated and sentinel groups (V-SKM, Sen-SKM and V-ON, Sen-ON, respectively). The mean body weight of ON group was lower than all other groups. At 35 doa (Figure 9.1D), the mean body weight of ON challenge positive control group was significantly lower than the mean body weight of NC, VC, V-SKM, Sen-SKM and V-ON but did not differ significantly Sen-ON and SKM positive control groups. The mean body weights of virus-challenge positive control groups (SKM and ON) were significantly lower than the NC and respective vaccinated groups (V-SKM and V-ON, respectively) whereas body weights did not differ significantly with the respective sentinel groups (Sen-SKM and Sen-ON, respectively). The mean body weight of SKM group is significantly lower than V-SKM and V-ON groups. The mean body weight of SKM group is significantly lower than the V-ON and Sen-ON groups and that of ON group is significantly lower than the V-SKM and Sen-SKM groups. The mean body weight of SKM group is numerically lower but did not differ significantly with the NC, VC, Sen-SKM and Sen-ON groups.

Virus gene copy number in intestine: At 21 doa (Figure 9.2A), virus gene copy numbers in NC and VC groups were significantly lower than the vaccinated-challenged (V-SKM and V-ON), sentinel-challenged (Sen-SKM and Sen-ON) and challenge-only (SKM and ON) groups. The highest virus gene copy numbers were observed in the ON group which are significantly higher than those of the NC, VC, V-SKM and SKM groups. The virus gene copy numbers in the ON group were numerically higher but did

not differ significantly with V-ON, Sen-ON and Sen-SKM groups. The virus gene copy numbers in SKM challenge-only group were numerically higher than the V-SKM and Sen-SKM groups but those differences were not statistically significant different. At 28 doa (Figure 9.2B), the virus gene copy numbers in all the groups did not differ significantly with each other. The virus gene copy numbers in the SKM and ON virus-challenge only groups were numerically higher than the respective vaccinated and sentinel challenge groups (V-SKM, Sen-SKM and V-ON, Sen-ON groups, respectively) but were not statistically significant. At 35 doa (Figure 9.2C), sentinel groups challenged with SKM and ON viruses (Sen-SKM and Sen-ON) had numerically higher virus gene copy numbers than the rest of the study groups, but those differences were not statistically significant. The virus challenge-only groups (SKM and ON) had numerically higher virus gene copy numbers than the vaccinated groups but were not statistically significant.

Virus gene copy number in tendons: At 21 doa (Figure 9.2D), the mean virus gene copy numbers in virus challenge-only groups (SKM and ON) were numerically higher than the respective vaccinated-challenge (V-SKM and V-ON) and sentinel-challenge (V-SKM and V-ON) groups but were not statistically significant. At 28 doa (Figure 9.2E), vaccinated, sentinel and virus challenge only groups of SKM and ON viruses had numerically similar virus gene copy numbers. At 35 doa (Figure 9.2F), virus gene copy number in Sen-SKM group is significantly lower than the ON challenge-only group. Additionally, the virus gene copy number in the virus challenge-only groups (SKM and ON) were numerically higher than their respective vaccinated (V-SKM and V-ON) and sentinel (Sen-SKM and Sen-ON) groups but were not statistically significant. Virus gene

copy numbers in NC and VC groups were significantly lower rest of the study groups at all time points (21, 28 and 35 doa)

Histopathologic lesion scores in gastrocnemius tendons: The histologic tendon lesions consisted of hypertrophy and hyperplasia of synoviocytes progressing to lymphoplasmacytic tenosynovitis and minimal fibroplasia at a later stage. At 21 doa (Figure 9.3A), the mean tendon inflammation scores did not differ significantly in all groups. At 28 doa (Figure 9.3B), the histologic lesion scores in the NC and VC groups were significantly lower than the vaccinated (V-SKM and V-ON) and sentinel (Sen-SKM and Sen-ON) groups but did not differ significantly from the virus challenge-only groups (SKM and ON). At 35 doa (Figure 9.3C), NC and VC groups had significantly lower histologic lesion scores than the rest of the vaccinated, sentinel and virus challenge-only groups. The mean tendon inflammation scores in the Sen-SKM groups were significantly lower than the SKM challenge-only groups in addition to V-ON and ON challenge only groups. The mean tendon inflammation score in the V-SKM group was significantly lower than V-ON and ON challenge-only groups.

Discussion

In chickens, vaccination for ARV is primarily done with live attenuated vaccine administered to young chicks followed by inactivated vaccine before egg laying (Giambrone et al., 1991). This regime was found to induce highest level of immune response in birds (Wood et al., 1986). The absence of a commercial vaccine against turkey arthritis reovirus poses the biggest hurdle in the vaccination program. The turkey industry has adopted a strategy of using polyvalent autogenous vaccines in breeders

(Sellers, 2017; Pitcovski and Goyal, 2021) prepared from the prevalent strains in their flocks. Custom made autogenous vaccines are not the long-term solution because there are inherent drawbacks with autogenous vaccines discussed in detail elsewhere (Sellers, 2017; Goldenberg et al., 2016). Emergence of variant reoviruses, especially variation in their cell attachment and outer capsid proteins is another hurdle in vaccination, because these variations cause inadequate protection provided by commercial vaccines in the vaccinated flock and their progeny. Our approach was to develop a live subunit vaccine to overcome these issues because subunit vaccines have potential advantages over the conventional and autogenous vaccines.

Live virus-vectored turkey arthritis reovirus vaccine provides an alternate to the use of autogenous vaccines that potentially promote the emergence of variant strains. The present study was designed to test the transmissibility and efficacy of rPICV-TARV vaccine against homologous and heterologous virus challenge. The rPICV-TARV vaccine expressing Sigma C (SC) and Sigma B (SB) antigenic proteins have been shown to elicit humoral immune response producing serum neutralizing antibodies in turkeys in our previous study (Kumar et al., 2021). Similarly, hemagglutinin and nucleoprotein genes of avian influenza virus (AIV) were carried by rPICV vector to stimulate humoral, and cell-mediated immune response providing protection against pathogenic AIV in mice (Dhanwani et al., 2016). Previous studies have reported various subunit vaccine expressing SC and SB proteins against chicken and duck reoviruses (Wu et al., 2005; Lin et al., 2008; Bi et al., 2016; Goldenberg et al., 2016). Subunit vaccines against infectious bursal disease (Pitcovski et al., 2003) and adenovirus infection (Fingerut et al., 2003; Pitcovski et al., 2005) have previously been proved to be efficacious.

Most vaccine programs to protect young poultry are devised to either vaccinate breeders and passively immunize the offspring with maternal antibodies or to directly vaccinate the young birds with a live vaccine (Jones, 2000). In our study two-days old turkey poults were primed with 0.2ml of rPICV-TARV vaccine ($3x10^7$ PFU/ml) by oral route followed by booster dose (0.2ml, $3x10^7$ PFU/ml) by intranasal route. Intranasal administration of booster dose was done to check the coarse spray administrability of our vaccine (Giambrone, 1991; Kumar et al., 2020). Poults were primed at two days and boosted at nine doa because birds are most susceptible to reovirus infection in their early life (Jones, 1984; Roessler and Rosenberger, 1989), and additional studies in our lab have shown that turkey are susceptible to infection with TARV at least up to four weeks of age (manuscript in preparation).

In our experience turkey poults experimentally infected with TARV produce moderate to high antibody titers to whole chicken reovirus (commercial ELISA). In our study a similar commercial reovirus ELISA showed negative results with no antibody titers in vaccinated poults. This is likely because the ELISA used in the study uses whole virus rather than subunits as antigen; hence, the whole virus did not optimally present adequate epitopes to detect antibodies to the SC and SB proteins produced by the subunit vaccine. The ELISA would have been more sensitive for our purposes if subunits were used as the determinant antigen rather than whole virus (Shieh et al., 2000; Liu et al., 2002; Lin et al., 2006). The ELISA using SC and/or SB proteins targets for type and group-specific neutralizing antibodies as the coated antigen were reported to be better in predicting the level of neutralization antibodies than ELISA using the whole virus (Yang et al., 2010; Lublin et al., 2011) and showed a good correlation between ELISA and SNT

(Yang et al., 2010). Therefore, we intend to use subunit antigens in ELISA in our future studies. Poults vaccinated by a combination of oral prime and intranasal boost by the bivalent codon optimized rPICV-TARV vaccine produced serum neutralizing (SN) antibodies to homologous (TARV SKM121) and heterologous (TARV O'Neil) viruses. Both vaccinated and sentinel poults produced SN antibody titers (64 to 256) that were greater than that of control poults, suggesting the vaccine can be transmitted from vaccinated to non-vaccinated pen mates and induce the same level of immune response (Fig 1A and B). Based on our experience, the SN antibody titer of TARV-infected poults ranges from 32 to 512 (unpublished data). The subunit vaccine can likely produce a higher level of immunization and increase the SN antibody titers if -if the period between the prime and booster dose is increased to three weeks. The rPICV based vaccine needs at least three weeks to form memory cells in the immunized host for producing better immune reaction (personal communication with Drs. Ly and Liang). Similarly, kumar et al., (2020) recommended a gap of 2-3 weeks between the prime and booster dose. The age at prime-boost (02 and 09 doa) and age at virus challenge (15 doa) was adopted in this study based on the previous research conducted on reovirus infection in chickens, with particular attention to the age susceptibility of chickens for reovirus infection. Chickens are most susceptible to reovirus infection during first 14 days of their life (Jones, 1984; Davis et al., 2013; Troxler et al., 2013; Niu et al., 2017; Perelman et al., 2019) and the birds become apparently resistant for reovirus infection in later age (Kerr and Olson, 1963; Jones and Georgiou, 1984; Roessler and Rosenberger, 1989). Current unpublished work in our lab indicates that turkey poults are susceptible to infection with

TARV for a much longer period, at least four weeks of age, which will allow us to modify the vaccine timing in our future studies.

By days 28 and 35 post vaccination, the vaccine control group (VC) birds showed no mortality and had mean body weights are like sham-inoculated negative control (NC) birds, suggesting that the vaccine is safe and without adverse effects. Additionally, mean body weight of virus challenge only groups (SKM and ON) were significantly lower than the respective vaccinated groups (V-SKM and V-ON, respectively) at 35 doa suggesting that the vaccine could check the replication of both the viruses in intestine thereby inhibiting the adverse effects of virus challenge translating into decreased body weight gain. The mean body weight of vaccinated groups (V-SKM and V-ON) was numerically higher than that of the respective sentinel groups (Sen-SKM and Sen-ON, respectively) but did not differ significantly. This decrease in the body weight gain of sentinel birds may be attributed to the higher population density in the sentinel groups.

At 21 and 28 doa, the virus gene copy numbers in intestine and tendon of vaccinated-challenged (V-SKM and V-ON) and sentinel-challenged (Sen-SKM and Sen-ON) groups were numerically lower than the corresponding virus-challenge only groups (SKM and ON, respectively) suggesting that the subunit vaccine can inhibit virus replication in intestine. These findings are likely attributable to the higher SN titers produced in the vaccinated and sentinel groups. Humoral immune response is the primary mechanism of providing protection against ARVs (Kibenge et al., 1987; Sellers, 2017) as antibodies produced against SC and SB proteins of ARVs inhibit virus attachment and causes lysis of virus and virus infected cells (van Loon et al., 2003). Additionally, the vaccine has provided similar protection against the homologous (TARV SKM121) and

heterologous (TARV O'Neil) virus challenge. Protection given by the humoral immune response to ARVs is dependent upon serotype and antigenic homogeneity, virulence of the virus, host age and levels of maternal derived antibodies (Rau et al., 1980; Takase et al., 1996). The vaccine was almost equally effective against heterologous virus challenge probably due to additional group-specific neutralizing antibodies induced by the SB protein of the vaccine. Precisely, SC protein elicits reovirus specific neutralizing antibodies (Shapouri et al., 1996; Goldenberg et al., 2011) and has been reported to elicit a strong mucosal immunity (Lin et al., 2012).

In all the vaccinated, sentinels and virus challenge only groups, the virus gene copy numbers in intestine and tendons were of the range of eight-fold log values at 21 doa, decreasing to two-fold log values at 28 doa in all the vaccinated, sentinels and virus challenge only groups. This decrease in the virus replication may be attributed to production of antiviral cytokines after 1 week of virus inoculation (Sharafeldin et al., 2015). At 35 doa, the virus gene copy numbers slightly increased in both the sentinel-challenged (Sen-SKM and Sen-ON) groups. The probable reason behind this increase in virus replication is decreased level of circulating antibodies after 20 days of vaccination. Additional studies to optimize the vaccine dose and vaccination timing will likely increase the resultant antibody titers and extent of immunization.

Histologic lesion scores in V-SKM and Sen-SKM groups were lower than the SKM challenge only group but V-ON, Sen-ON and ON groups did not follow this pattern at 28 and 35 doa. This is probably due to increased level of IL-6 and IFN-γ cytokine corresponded with lymphocytic infiltration in gastrocnemius tendon sheath, indicating the role of these cytokines in the development of tenosynovitis (Sharafeldin et al., 2015b).

The joints and tendons act as sequestered sites, thus preventing virus elimination by the immune system (Jones and Georgiou, 1985). The increase in the histologic lesion scores of vaccinated (V-SKM and V-ON) and sentinel (Sen-SKM and Sen-ON) groups is less than the increase in the scores of virus challenge only (SKM and ON) groups suggesting that the vaccine is possibly effective in controlling the tendon lesions.

The rPICV-TARV bivalent codon optimized vaccine has a potential to be used for vaccination against TARV infection. It can have promising economic benefits for the industry if breeder immunization is practiced preventing vertical transmission (Dobson et al., 1992). In birds, better immunity is achieved with a live vaccine before using inactivated vaccine in breeders (Wood et al., 1986). We plan to study the immunogenicity of this vaccine in breeder turkeys in future work. Additionally, it is worth investigating whether our vaccine would interfere with other turkey vaccines. The dose, route and number of shots will be further studied to develop immunization program for turkeys for field use. The additional advantages of our recombinant vaccine is that 1) the characterized gene segments (SC and SB) of new variants can be easily cloned into the rPICV virus which will immunize the birds against the variant strains; 2) other genes of outer capsid proteins e.g., M2 (MuB) can be cloned into the rPICV virus for enhancing the spectrum of our vaccine; 3) only gene segments translating to outer capsid proteins of variant TARVs are being used in the vaccine and not the whole viruses, eliminating the possibility of generation of variant TARVs. This study provides useful information that our vaccine could control and prevent the infection thereby it paves a foundation for a promising potent future vaccine. Lastly, the importance of strict biosecurity and best

management practices for maximizing vaccine efficacy in the prevention and control of reovirus infection in turkeys should not be ignored.

Table 9.1. Summary of experimental design for evaluating the efficacy of rPICV-TARV vaccine agasint a spectrum of virus challenge

Group Name	Group description (n)	Oral Vac (days)	Sentinel (days)	I/N vac (days)	Ch-SKM121 (days)	Ch-ON (days)	Sampling (age)
NC	Neg ctrl (12+5)	-	-	-	-	-	21-28-35
VC	V-ONLY (12+5)	2	-	9	-	-	21-28-35
V-SKM	V-Ch-SKM121 (12+5)	2	-	9	15	-	21-28-35
Sen-SKM	V+Sent-Ch-SKM121 (10+12)	2	4	9	15		21-28-35
SKM	Ch- SKM121 (12+5)	-	-	-	15	-	21-28-35
V-ON	V-Ch-ON (12+5)	2	-	9	-	15	21-28-35
Sen-ON	Vac+Sent-Ch-ON (10+12)	2	4	9	-	15	21-28-35
ON	Ch- ON (12+5)	-	-	-	-	15	21-28-35
	Sentinels (24)						

Figure. 9.1: A) SN Ab titer against SKM121; **B**) SN Ab titer against TARV O'Neil; **C**) BW at 28 doa; **D**) BW at 35 doa. Box plots having different alphabets have significant difference at $p<0.05$.

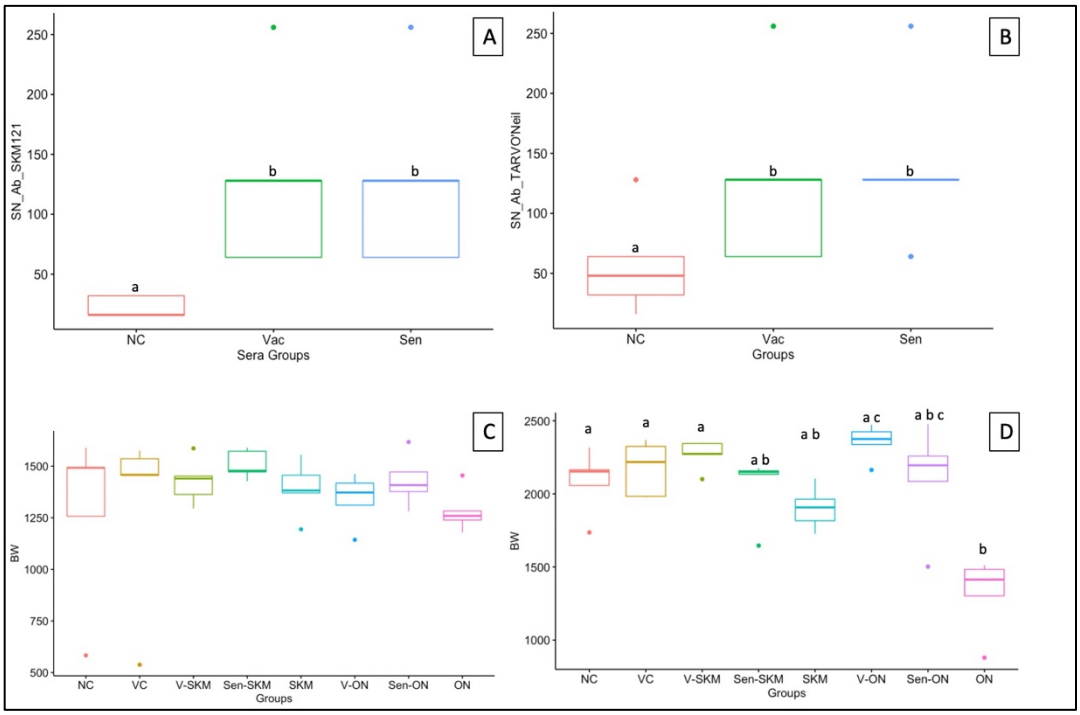


Figure. 9.2: Virus gene copy numbers **A**) Intestine at 18 doa; **B**) Intestine at 28 doa; **C**) Intestine at 35 doa; **D**) Tendons at 21 doa; **E**) Tendons at 28 doa; **F**) Tendons at 35 doa. Box plots having different alphabets have significant difference at $p<0.05$.

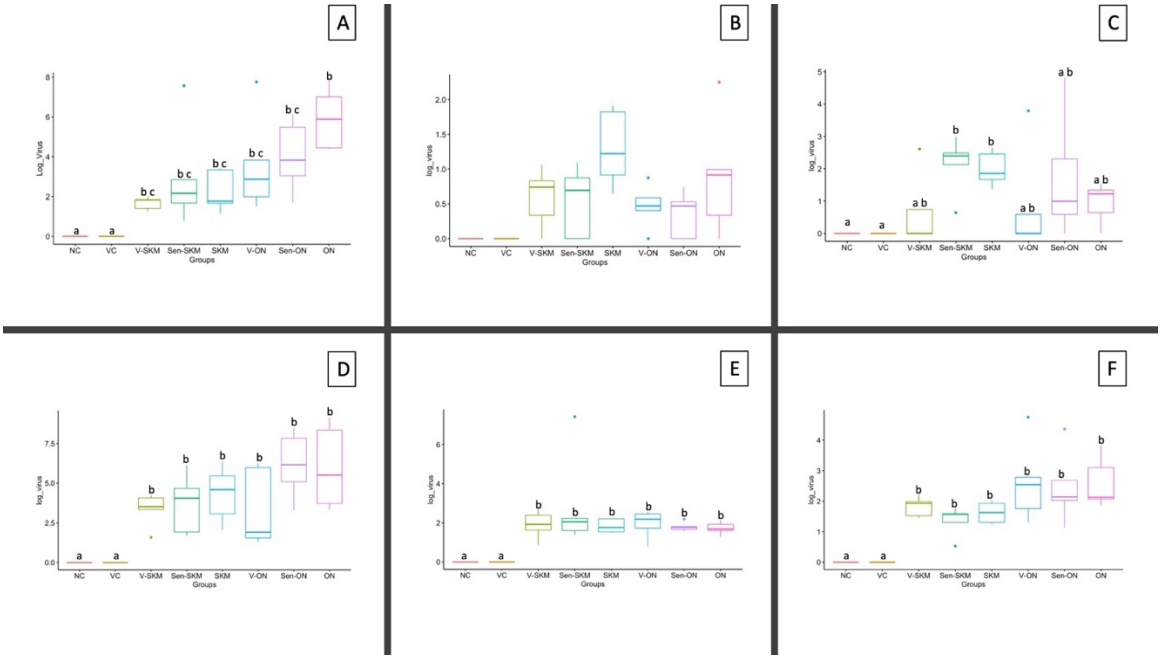
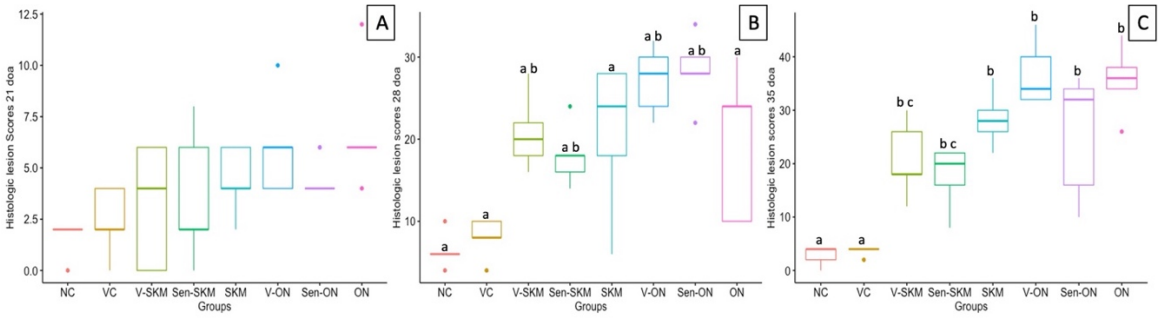


Figure. 9.3: Histologic lesion scores in tendons at **A**) 21 doa; **B**) 28 doa; **C**) 35 doa. Box plots having different alphabets have significant difference at $p<0.05$.



CHAPTER 10

GENERAL DISCUSSION, CONCLUSIONS, AND FUTURE DIRECTIONS

Avian reoviruses are associated with a variety of disease conditions in chickens and turkeys but viral arthritis/tenosynovitis is the most economically important because of poor carcass uniformity and condemnation of birds at the processing plants (Glass *et al.*, 1973; Dobson and Glisson, 1992; Perelman et al., 2019). The ARVs from chickens and turkeys are closely related but they are genetically distinct and are phylogenetically clustered in different groups (Sellers et al., 2004; Mor et al., 2013; Palamino-Tapia et al., 2018). Vaccination against chicken viral arthritis is the principal approach to control it in commercial poultry operations. An attenuated vaccine is administered to young birds. This is followed by an inactivated vaccine in breeders, so their chicks are protected by vertically transferred antibodies in egg yolk (Jones, 2000; Pitcovski and Goyal, 2020). For turkey arthritis reovirus, no commercial vaccine is available although some breeder companies use an autogenous killed vaccine.

Unfortunately, reovirus-associated arthritis/tenosynovitis in chickens and turkeys has re-emerged in the last decade, and outbreaks continue to be reported even from vaccinated flocks (Sellers, 2017). Genetic and antigenic variants appear to be responsible for the re-emergence, vaccination failure, and continuous outbreaks (Sellers, 2017; Egaña et al., 2019; Ayalew et al., 2020). In view of the emergence of variant strains, there is a need to develop new and effective vaccines to control CARV and TARV infections in commercial poultry.

The research in this thesis concerns the study of genetic variation and evolutionary dynamics of CARVs to select variant CARV strains for the development of a live virus-vectored vaccine. In chapters 3 and 4, whole genome sequencing of 35 field isolates of CARV from the US and 14 CARV isolates from Germany provided a

perspective on genetic diversity of reovirus in two different geographical locations. Based on the ML phylogenetic tree of σC gene sequences, the US isolates were clustered in all six genotype clusters (GC1 to GC6) as proposed by Egãna-Labrin et al. (2019) and Ayalew et al. (2020). The German isolates, on the other hand, were clustered in four GCs (GC1, GC2, GC4 and GC6). The study isolates formed subclusters within each GC and had high nt diversity. This indicates that autogenous vaccines may not be able to produce cross-protection against viral strains in the same cluster.

We found that the S1 (σC) genome segment showed noticeably higher divergence followed by M2 (μB) and L3 (λC) genes. The most expressed GC in the US isolates was GC1 followed by GC5 and GC2. In previous studies, the most expressed GC were GC1 and GC6 (Egãna-Labirin et al., 2019) and GC4 and GC5 (Palomino-Tapia et al, 2018). The GC3, GC4 and GC6 were the least expressed clusters in US isolates. The most predominant GC in German isolates was the recently evolved GC6 (46%), which is in contrast to earlier studies from Germany and France reporting higher prevalence of GC1 and GC4 (Kant et al., 2003; Troxler et al., 2013). These results are not surprising because previous studies have reported that the predominance of GCs varies with time and geographical location (Lu et al. 2015; Ayalew et al. 2017; Sellers, 2017; Palomino-Tapia et al. 2018). Egãna et al. (2019) showed a shift over time from GC1 to GC6 between 2015 to 2018. However, we did not observe a similar shift in either among German or the US isolates.

In contrast to clustering patterns based on the SC gene, the concatenated whole-genome sequences created a different phylogenetic clustering pattern, signifying a phylogenetically incongruent isolate topology. The assessment of the congruent

topologies of our isolates indicated frequent genetic reassortment among multiple co-circulating variants. The genetic variability among circulating CARVs is due to a combination of evolutionary mechanisms involving multiple cocirculating lineages and genetic reassortments. Regular molecular epidemiological studies using whole genome sequencing are necessary to determine the predominant GCs to be included in autogenous and/or vectored vaccines.

In chapter 5, we used a recombinant live Pichinde virus vector developed by using reverse genetics. Using this vector, we developed several monovalent and bivalent codon-optimized CARV vaccines. Two CARV strains were selected as vaccine candidates based on their clustering pattern of SC and SB gene sequences. The CARV-22 strain was selected from GC1 (vaccine cluster) to check the immunogenic efficiency of its SC and SB genes that had high genetic similarity with other vaccine strains. The other strain, CARV-196, was selected from GC5, which is an emerging and highly pathogenic genotypic cluster (Sellers, 2017; Ayalew et al., 2017; Egána et al., 2019). CARV-196 was used to determine the immunogenic potential of its SC and SB genes. A third strain, CARV-30, derived from GC6 (a recently evolved GC) served as a challenge virus to determine the spectrum of protection provided by our vaccines. Two gene segments, SC and SB, which encode variable outer capsid proteins, were selected as genes of interest because they induce type-specific and broad-specific neutralizing antibodies.

We used codon-optimized SC and SB gene sequences from CARV-22 and CARV-196 for inclusion in the Pichinde virus vector. The rPICV vector successfully carried and expressed SC and SB genes in all the monovalent and bivalent vaccines as confirmed by RT-PCR and DFA results. We then evaluated the safety, immunogenicity,

and efficacy of this vaccine against a spectrum of challenge viruses. Two formulations of the vaccine were used; V1 is a cocktail of two bivalent vaccines (CARV-22SC/SB and CARV-196SC/SB) and vaccine 2 (V2) is a cocktail of two monovalent vaccines (CARV-22SC and CARV-196SB). Chicks were primed at one day of age (doa) and boosted at 8 doa before being challenged with either CARV-22, CARV-196 or CARV-30 virus. Vaccinated birds showed high serum-neutralization antibody titers after booster vaccination. In some instances, we observed reduced virus replication and early clearing of virus from vaccinated birds.

Chapter 6 shows our study on the survival of the vaccine virus in water and litter to determine if the vaccine can be administered through drinking water and if horizontal transmission of vaccine to unvaccinated pen mates is possible. Using spiked samples of water and litter, we found that the vaccine virus was able to survive in autoclaved and non-autoclaved water and litter for approximately six hours at room temperature. Hence, it is possible to administer this vaccine to birds via drinking water as is true of other live vaccines. Since rPICV is a live virus-vectored vaccine, it can replicate in the gut of the birds and be shed in their feces. The six-hour survival time in litter should provide ample opportunity for the vaccine virus to be transferred to pen mates of vaccinated birds via the fecal-oral route.

In chapter 7, we studied the comparative pathogenesis of three types of turkey reoviruses, e.g., TERV, TARV, and THRV. All three types of viruses replicated in the intestine, confirming the enterotropic nature of reoviruses as has been reported previously (Sharafeldin *et al*., 2015a; Ngunjiri *et al*., 2019). None of the reoviruses caused histologic lesions in the intestine in contrast to previous studies (Sharafeldin *et al*., 2014).

Visceral organs showed peak virus replication within 7 dpi and declined thereafter, which may be due to the production of antiviral cytokines at 7 dpi (Sharafeldin *et al*., 2015b). In tendons, virus replication was observed for all three types of reoviruses, peaking at 21 dpi and declining after 28 dpi in contrast to previous studies (Sharafeldin *et al*., 2015b; Ngunjiri *et al*., 2019). All TARVs caused histologic lesions in tendons. Interestingly, THRV migrated to and replicated in tendons thus supporting anecdotal reports that both hepatitis and tenosynovitis may be caused by turkey reovirus in early and late phases of growth, respectively.

Chicks are most susceptible to reovirus infection in the first 7 days of their life after which they show less severe disease (Wood and Thorton, 1981; Jones and Georgiou, 1984; Roessler and Rosenberger, 1989). Information on age susceptibility of TARV, however, is not available. So, in chapter 8, we investigated the age susceptibility and transmission dynamics of TARV infection in turkey poults. Groups of turkey poults were inoculated orally with TARV at 7, 14, 21 and 28 days of age. Turkeys at 28 days of age or less were susceptible to TARV infection as evidenced by virus replication in intestine and tendons followed by lymphoplasmacytic tenosynovitis. It was further evident that turkeys infected at any age could transmit TARV horizontally to their age-matched controls (sentinels), which ultimately developed tenosynovitis. The results of this study can be extrapolated to adopt vaccination strategies to provide immunity to older birds by vaccinating meat-type turkeys during grow out to reduce transmission to flock mates.

Since no commercial vaccines is available against TARV, the turkey industry relies on the use of autogenous vaccines in breeder turkeys (Sellers, 2017; Pitcovski and Goyal, 2021). However, autogenous vaccines are not efficient in the face of variant

TARV strains. We have developed a bivalent codon optimized rPICV-TARV live vaccine, expressing SC and SB antigenic genes. We administered this vaccine to turkey poults orally at 2 doa followed by a booster dose at 9 doa. Sentinel birds were placed in contact with vaccine-inoculated birds at 4 doa. Groups of birds were then challenged with one of the two TARVs (SKM121 or TARV O'Neil) at 15 doa. The vaccine virus was able to transmit horizontally to unvaccinated age-matched pen mates, which developed same level of serum neutralizing antibodies as that of the vaccinated birds. The vaccine inhibited the replication of virus in the intestine and tendons leading to early clearance of virus. In addition, the tenosynovitis lesion scores in vaccinated birds were lower than in unvaccinated birds.

FUTURE DIRECTIONS

The results of whole genome sequencing of US and German isolates of CARV indicate variability in these viruses. It is important, therefore, to continue to characterize newer isolates of the virus over time and geographical areas to proactively figure out the divergence and change in pathogenicity of variant CARVs. This approach can possibly help predict future outbreaks and be of help in devising mitigation strategies in terms of strain selection for vaccine development. Additionally, the most immunogenic genes can be identified in real-time and be included in subunit vaccines quickly. In future studies, it may also be worthwhile to insert another immunogenic gene segment M2 (μB), in addition to SC and SB genes, from different strains into our rPICV-based CARV and TARV vaccines to determine their immunogenic potential and efficacy against a spectrum of challenge viruses.

The results of our vaccine studies should be considered preliminary in nature. In future, it is important to inoculate rPICV-CARV and rPICV-TARV vaccines to breeders to characterize passive immunization of their progeny as well as susceptibility of the progeny to subsequent viral challenge. The half-life of antibodies in both the breeders and their progeny should also be studied. Studies are also indicated to determine the appropriate dose, route, and number of doses to achieve the optimum level of immunity in vaccinated breeders and their progeny up to 28 days or perhaps older birds. Vaccination of poults at 2-3 weeks of age should also be explored for protection of poults susceptible to TARV infection at an older age.

REFERENCES

References

Abdalla, O.S.M.A.N., 1979. Ossification and mineralization in the tendons of the chicken (Gallus domesticus). *Journal of Anatomy*, *129*(Pt 2), p.351.

Al-Afaleq, A. I. and R. C. Jones. 1989. Pathogenicity of three turkey and three chicken reoviruses for poults and chicks with particular reference to arthritis/tenosynovitis. Avian Pathol 18:433–440.

Al-Afaleq, A. I., J. M. Bradbury, R. C. Jones, and A. M. Metwali. 1989. Mixed infection of turkeys with Mycoplasma synoviae and reovirus: field and experimental observations. Avian Pathol 18:441–453

Al-Afaleq, A.I., Jones, R.C., 1989. Pathogenicity of three turkey and three chicken reoviruses for poults and chicks with particular reference to arthritis/tenosynovitis. Avian Pathol.18, 433-440.

Al Afaleq, A.I. and Jones, R.C., 1991. A trypsin-sensitive avian reovirus: Isolation and experimental infection of poults and chicks. *Avian Pathology*, *20*(1), pp.5-16.

Al-Afaleq, A.I., Savage, C.E., Johnson, C.P. and Jones, R.C., 1997. Experimental inoculation of mice with trypsinresistant and trypsin-sensitive avian reoviruses. *Journal of comparative pathology*, *117*(3), pp.253-259.

Al Afaleq, A. and Jones, R.C., 1994. A comparison of single and repeated oral infection of chicks with two avian reoviruses. *Research in veterinary science*, *57*(1), pp.96-99.

Al-Muffarej, SI, Savage, CE and Jones, RC, 1996. Egg transmission of avian reoviruses in chickens: Comparison of a trypsin-sensitive and a trypsin-resistant strain. *Avian Pathology* , *25* (3), pp. 469-480.

Andrey, P., Dmitry, A., Dmitry, M., Alla, L. and Anton, K., 2020. Using SPAdes De Novo Assembler. *Current protocols in bioinformatics*, *70*(1), p.e102.

Attoui H, Fang Q, Jaafar FM, Cantaloube JF, Biagini P, de Micco P. and de Lamballerie X. 2002. Common evolutionary origin of aquareoviruses and orthoreoviruses revealed by genome characterization of Golden shiner reovirus, Grass carp reovirus, Striped bass reovirus and golden ide reovirus (genus Aquareovirus, family Reoviridae). *Journal of General Virology*, *83*(8), pp.1941-1951.

Attoui H, Jaafar FM, Belhouchet M, Biagini P, Cantaloube JF, de Micco P. and de Lamballerie X. 2005. Expansion of family Reoviridae to include nine-segmented dsRNA viruses: isolation and characterization of a new virus designated Aedes pseudoscutellaris reovirus assigned to a proposed genus (Dinovernavirus). *Virology*, *343*(2), pp.212-223.

Attoui H, Mertens PPC, Becnel J, Belaganahalli S, Bergoin M, Brussaard CP, Chappell JD, Ciarlet M, del Vas M, Dermody TS, Dormitzer PR, Duncan R. Fang Q, Graham R, Guglielmi KM, Harding RM, Hillman B, Makkay A, Marzachí C, Matthijnssens J, Milne RG, Mohd Jaafar F, Mori H, Noordeloos AA, Omura T, Patton JT, Rao S, Maan M, Stoltz D, Suzuki N, Upadhyaya NM, Wei C, Zhou H. 2012. Family Reoviridae. In: Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Edited by King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ. San Diego, CA: Academic Press pp 541-637.

Ayalew LE, Gupta A, Fricke J, Ahmed KA, Popowich S, Lockerbie B, Tikoo SK, Ojkic D. and Gomis S. 2017. Phenotypic, genotypic and antigenic characterization of emerging avian reoviruses isolated from clinical cases of arthritis in broilers in Saskatchewan, Canada. *Scientific reports*, *7*(1), pp.1-13.

Ayalew LE, Ahmed KA, Mekuria ZH, Lockerbie B, Popowich S, Tikoo SK, Ojkic D. and Gomis S. 2020. The dynamics of molecular evolution of emerging avian reoviruses through accumulation of point mutations and genetic re-assortment. *Virus evolution*, *6*(1), p.veaa025.

Back, A. and Nagaraja, K.V. 1996. Pathogenicity of chickens reovirus strains S1133 and 1733 for turkeys. Proc International Symposium on Adenovirus and Reovirus Infections in Poultry, Rauischholzhausen, Germany, 245—250.

Barnes, H.J., Wheeler, J. and Reed, D., 1982. Serologic evidence of infectious bursal disease virus infection in Iowa turkeys. *Avian Diseases*, pp.560-565.

Barta, V., Springer, W.T. and Millar, D.L., 1984. A comparison of avian and mammalian cell cultures for the propagation of avian reovirus WVU 2937. *Avian diseases*, pp.216-223.

Bányai, K., Palya, V., Benkő, M., Bene, J., Havasi, V., Melegh, B. and Szűcs, G., 2005. The goose reovirus genome segment encoding the minor outer capsid protein, σ1/σC, is bicistronic and shares structural similarities with its counterpart in muscovy duck reovirus. *Virus genes*, *31*(3), pp.285-291.

Bányai, K., Dandár, E., Dorsey, K.M., Mató, T. and Palya, V., 2011. The genomic constellation of a novel avian orthoreovirus strain associated with runting-stunting syndrome in broilers. *Virus genes*, *42*(1), pp.82-89.

Belhouchet, M., Mohd Jaafar, F., Firth, A.E., Grimes, J.M., Mertens, P.P. and Attoui, H., 2011. Detection of a fourth orbivirus non-structural protein. *PLoS one*, *6*(10), p.e25697.

Benavente, J. and Martínez-Costas, J., 2007. Avian reovirus: structure and biology. *Virus research*, *123*(2), pp.105-119.

Bains, B.S., MacKenzie. M., Spradbrow, P.B., 1974. Reovirus-Associated Mortality in Broiler Chickens. Avian Diseases; 18:472-476.

Bagust, T.A. and Westbury, H.A., 1975. Isolation of reoviruses associated with diseases of chickens in Victoria [Australia]. Letter to the Editor. *Australian veterinary journal*.

Barry, C. and Duncan, R., 2009. Multifaceted sequence-dependent and-independent roles for reovirus FAST protein cytoplasmic tails in fusion pore formation and syncytiogenesis. *Journal of virology*, *83*(23), pp.12185-12195.

Baron, M.D., Iqbal, M. and Nair, V., 2018. Recent advances in viral vectors in veterinary vaccinology. *Current opinion in virology*, *29*, pp.1-7.

Bi, Z., Zhu, Y., Chen, Z., Li, C., Wang, Y., Wang, G. and Liu, G., 2016. Induction of a robust immunity response against novel duck reovirus in ducklings using a subunit vaccine of sigma C protein. *Scientific reports*, *6*(1), pp.1-11.

Bodelón, G., Labrada, L., Martınez-Costas, J. and Benavente, J., 2001. The avian reovirus genome segment S1 is a functionally tricistronic gene that expresses one structural and two nonstructural proteins in infected cells. *Virology*, *290*(2), pp.181-191.

Bodelón, G., Labrada, L., Martínez-Costas, J. and Benavente, J., 2002. Modification of late membrane permeability in avian reovirus-infected cells: viroporin activity of the S1-encoded nonstructural p10 protein. *Journal of Biological Chemistry*, *277*(20), pp.17789-17796.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics.;30(15):2114-2120.

Boyle, D.B.; Coupar, B.E. Construction of recombinant fowlpox viruses as vectors for poultry vaccines. Virus Res. 1988, 10, 343–356.

Bublot, M.; Pritchard, N.; Swayne, D.E.; Selleck, P.; Karaca, K.; Suarez, D.L.; Audonnet, J.-C.; Mickle, T.R. Development and use of fowlpox vectored vaccines for avian influenza. Ann. N. Y. Acad. Sci. 2006, 1081, 193–201.

Bracewell, C.D. and Wyeth, P., 1981. Infectious stunting of chickens. *The Veterinary Record*, *109*(3), pp.64-64.

Brandariz-Nuñez, A., Menaya-Vargas, R., Benavente, J. and Martinez-Costas, J., 2010. Avian reovirus μNS protein forms homo-oligomeric inclusions in a microtubule-independent fashion, which involves specific regions of its C-terminal domain. *Journal of virology*, *84*(9), pp.4289-4301.

Bruhn, S., Bruckner, L. and Ottiger, H.P., 2005. Application of RT-PCR for the detection of avian reovirus contamination in avian viral vaccines. *Journal of virological methods*, *123*(2), pp.179-186.

Buchmeier, M.J., De La Torre, J.C., Peters, C.J., 2007. Arenaviridae: the viruses and their replication, p 1791–1827. In Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (ed), Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.

Bülow, V.V. and Klasen, A., 1983. Effects of avian viruses on cultured chicken bone-marrow-derived macrophages. *Avian Pathology*, *12*(2), pp.179-198.

Burns, C.C., Diop, O.M., Sutter, R.W. and Kew, O.M., 2014. Vaccine-derived polioviruses. *The Journal of infectious diseases*, *210*(suppl_1), pp.S283-S293.

Bull, J.J., Smithson, M.W. and Nuismer, S.L., 2018. Transmissible viral vaccines. *Trends in microbiology*, *26*(1), pp.6-15.

Burri, D.J., Palma, J.R.D., Kunz, S. and Pasquato, A., 2012. Envelope glycoprotein of arenaviruses. *Viruses*, *4*(10), pp.2162-2181.

Calvo, P.G., Fox, G.C., Parrado, X.L.H., Llamas-Saiz, A.L., Costas, C., Martínez-Costas, J., Benavente, J. and van Raaij, M.J., 2005. Structure of the carboxy-terminal receptor-binding domain of avian reovirus fibre sigmaC. *Journal of molecular biology*, *354*(1), pp.137-149.

Campbell, D., Barker, D.I.K. and Wobeser, G., 2004. Reovirus in crows–an emerging disease. *Wildlife Health Centre Newsletter. Can Cooperative Wildlife Centre*, *10*(1), p.8.

Campbell, D., Welch, K., Ojkic, D., Swinton, J.. 2008. Winter mortality in crows. Canadian Cooperative Wildlife Health Centre Newsletter;13(2): 11.

Caterina, K.M., Frasca Jr, S., Girshick, T. and Khan, M.I., 2004. Development of a multiplex PCR for detection of avian adenovirus, avian reovirus, infectious bursal disease virus, and chicken anemia virus. *Molecular and cellular probes*, *18*(5), pp.293-298.

Chao, L., 1997. Evolution of sex and the molecular clock in RNA viruses. *Gene*, *205*(1-2), pp.301-308.

Cavanagh, D. 2007. Coronavirus avian infectious bronchitis virus. Vet. Res. 38: 281–297.

Chen, Z., Zhu, Y., Li, C. and Liu, G., 2012. Outbreak-associated novel duck Reovirus, China, 2011. *Emerging infectious diseases*, *18*(7), p.1209.

Chen, P.N., Liu, H.J., Shien, J.H. and Lee, L.H., 2004. Antibody responses against avian reovirus nonstructural protein σNS in experimentally virus-infected chickens monitored by a monoclonal antibody capture enzyme-linked immunosorbent assay. *Research in veterinary science*, *76*(3), pp.219-225.

Chen, H., Yan, M., Tang, Y. and Diao, Y., 2019. Pathogenicity and genomic characterization of a novel avian orthoreovius variant isolated from a vaccinated broiler flock in China. *Avian Pathology*, *48*(4), pp.334-342.

Chénier, S., Boulianne, M. and Gagnon, C.A., 2014. Postvaccinal reovirus infection with high mortality in breeder chicks. *Avian diseases*, *58*(4), pp.659-665.

Cheng, R.H., 2008. Structural evolution of reoviridae revealed by oryzavirus in acquiring the second capsid shell. *Journal of virology*, 82:11344-53.

Chua, K.B., Voon, K., Crameri, G., Tan, H.S., Rosli, J., McEachern, J.A., Suluraju, S., Yu, M. and Wang, L.F., 2008. Identification and characterization of a new orthoreovirus from patients with acute respiratory infections. *PloS one*, *3*(11), p.e3803.

Culver, F., F. Dziva, D. Cavanagh, and M. P. Stevens. 2006. Poult enteritis and mortality syndrome in turkeys in Great Britain. Vet Rec 159: 209–210.

Chellappa, M.M., Dey, S., Gaikwad, S., Pathak, D.C. and Vakharia, V.N., 2017. Rescue of a recombinant Newcastle disease virus strain R2B expressing green fluorescent protein. *Virus genes*, (3), pp.410-417.

Choi, Y. and Chang, J., 2013. Viral vectors for vaccine applications. *Clinical and experimental vaccine research*, *2*(2), pp.97-105.

Chulu, J.L., Huang, W.R., Wang, L., Shih, W.L. and Liu, H.J., 2010. Avian reovirus nonstructural protein p17-induced G2/M cell cycle arrest and host cellular protein translation shutoff involve activation of p53-dependent pathways. *Journal of virology*, *84*(15), pp.7683-7694.

Chiu, H.C., Huang, W.R., Liao, T.L., Wu, H.Y., Munir, M., Shih, W.L. and Liu, H.J., 2016. Suppression of vimentin phosphorylation by the avian reovirus p17 through inhibition of CDK1 and Plk1 impacting the G2/M phase of the cell cycle. *PLoS One*, *11*(9), p.e0162356.

Chi, P.I., Huang, W.R., Lai, I.H., Cheng, C.Y. and Liu, H.J., 2019. Correction: The p17 nonstructural protein of avian reovirus triggers autophagy enhancing virus replication via activation of phosphatase and tensin deleted on chromosome 10 (PTEN) and AMP-activated protein kinase (AMPK), as well as dsRNA-dependent protein kinase (PKR)/eIF2 α signaling pathways. *Journal of Biological Chemistry*, *294*(31), pp.11676-11676.

Conzo, G., Magnino, S., Sironi, G., Lavazza, A., Vigo, P.G., Fioretti, A. and Kaleta, E.F., 2001. Reovirus infection in two species of Psittaciformes recently imported into Italy. *Avian Pathology*, *30*(1), pp.43-47.

Costas, C., Martínez-Costas, J., Bodelón, G. and Benavente, J., 2005. The second open reading frame of the avian reovirus S1 gene encodes a transcription-dependent and CRM1-independent nucleocytoplasmic shuttling protein. *Journal of virology*, *79*(4), pp.2141-2150.

Cowen, B.S. and Braune, M.O., 1988. The propagation of avian viruses in a continuous cell line (QT35) of Japanese quail origin. *Avian diseases*, pp.282-297.

Dalton, P.J. and Henry, R., 1967. Tenosynovitis in poultry. *Veterinary Record*, *80*(21), p.638.

Dandár, E., Bálint, Á., Kecskeméti, S., Szentpáli-Gavallér, K., Kisfali, P., Melegh, B., Farkas, S.L. and Bányai, K., 2013. Detection and characterization of a divergent avian reovirus strain from a broiler chicken with central nervous system disease. *Archives of virology*, *158*(12), pp.2583-2588.

Darteil, R., Bublot, M., Laplace, E., Bouquet, J.F., Audonnet, J.C. and Rivière, M., 1995. Herpesvirus of turkey recombinant viruses expressing infectious bursal disease virus (IBDV) VP2 immunogen induce protection against an IBDV virulent challenge in chickens. *Virology*, *211*(2), pp.481-490.

Davis, J.F., Kulkarni, A. and Fletcher, O., 2012. Myocarditis in 9-and 11-day-old broiler breeder chicks associated with a reovirus infection. *Avian diseases*, *56*(4), pp.786- 790.

Davis, J.F., Kulkarni, A. and Fletcher, O., 2013. Reovirus infections in young broiler chickens. *Avian diseases*, *57*(2), pp.321-325.

Day, J.M., Pantin-Jackwood, M.J., Spackman, E., 2007. Sequence and Phylogenetic analysis of the S1 genome segment of turkey-origin reoviruses. Virus Genes 35, 235-242.

Day, J.M., Spackman, E. and Pantin-Jackwood, M.J., 2008. Turkey origin reovirus-induced immune dysfunction in specific pathogen free and commercial turkey poults. *Avian diseases*, *52*(3), pp.387-391.

Day, J.M., 2009. The diversity of the orthoreoviruses: molecular taxonomy and phylogentic divides. *Infection, Genetics and Evolution*, *9*(4), pp.390-400.

De Carli, S., Wolf, J.M., Gräf, T., Lehmann, F.K., Fonseca, A.S., Canal, C.W., Lunge,

V.R. and Ikuta, N., 2020. Genotypic characterization and molecular evolution of avian reovirus in poultry flocks from Brazil. *Avian Pathology*, *49*(6), pp.611-620.

De Herdt, P., Broeckx, M., Van Driessche, F., Vermeiren, B., Van Den Abeele, G. and Van Gorp, S., 2016. Improved performance of broilers and broiler breeders associated with an amended vaccination program against reovirosis. *Avian diseases*, *60*(4), pp.841-845.

De la Torre, D., Astolfi-Ferreira, C.S., Chacón, R.D., Puga, B. and Piantino Ferreira, A.J., 2021. Emerging new avian reovirus variants from cases of enteric disorders and arthritis/tenosynovitis in Brazilian poultry flocks. *British Poultry Science*, *62*(3), pp.361-372.

Deshmukh, D.A. and Pomeroy, B.S., 1969a. Avian reoviruses. I. Isolation and serological characterization. *Avian diseases*, pp.239-243.

Deshmukh, D.A. and Pomeroy, B.S., 1969b. Avian Reoviruses. II. Physicochemical Characterization and Classification. Avian Diseases; 13:243-251.

Dey, S., Chellappa, M.M., Pathak, D.C., Gaikwad, S., Yadav, K., Ramakrishnan, S. and Vakharia, V.N., 2017. Newcastle disease virus vectored bivalent vaccine against virulent infectious bursal disease and newcastle disease of chickens. *Vaccines*, *5*(4), p.31.

Dobson, K.N. and Glisson, J.R., 1992. Economic impact of a documented case of reovirus infection in broiler breeders. *Avian diseases*, pp.788-791.

Domingo, E.J.J.H. and Holland, J.J., 1997. RNA virus mutations and fitness for survival. *Annual review of microbiology*, *51*(1), pp.151-178.

Donnelly, J.J., Friedman, A., Martinez, D., Montgomery, D.L., Shiver, J.W., Motzel, S.L., Ulmer, J.B. and Liu, M.A., 1995. Preclinical efficacy of a prototype DNA vaccine: enhanced protection against antigenic drift in influenza virus. *Nature medicine*, *1*(6), pp.583-587.

Dhanwani, R., Zhou, Y., Huang, Q., Verma, V., Dileepan, M., Ly, H. and Liang, Y., 2015. A novel live Pichinde virus-based vaccine vector induces enhanced humoral and cellular immunity after a booster dose. *Journal of virology*, *90*(5), pp.2551-2560.

Drastini Y, McKenna PK, Kibenge FSB, Lopez A. 994. Chymotrypsin and trypsin sensitivities of avian reoviruses. Canadian Journal of Veterinary Research; 58:75-78.

Duncan, R., and Sullivan K. 1998. Characterization of Two Avian Reoviruses That Exhibit Strain-Specific Quantitative Differences in Their Syncytium-Inducing and Pathogenic Capabilities. *Virology*. 250(2):263-272. doi:10.1006/VIRO.1998.9371.

Duncan, R., 1999. Extensive sequence divergence and phylogenetic relationships between the fusogenic and nonfusogenic orthoreoviruses: a species proposal. *Virology*, *260*(2), pp.316-328.

Dutta, S.K. and Pomeroy, B.S., 1967a. Isolation and characterization of an enterovirus from baby chicks having an enteric infection I. Isolation and pathogenicity. *Avian diseases*, *11*(1), pp.1-9.

Dutta, S.K. and Pomeroy, B.S., 1967b. Isolation and characterization of an enterovirus from baby chicks having an enteric infection II. Physical and chemical characteristics and ultrastructure. *Avian diseases*, *11*(1), pp.9-15.

Ellis, M.N., Eidson, C.S., Fletcher, O.J. and Kleven, S.H., 1983. Viral tissue tropisms and interferon production in white leghorn chickens infected with two avian reovirus strains. *Avian diseases*, pp.644-651.

Egaña-Labrin, S., Hauck, R., Figueroa, A., Stoute, S., Shivaprasad, H.L., Crispo, M., Corsiglia, C., Zhou, H., Kern, C., Crossley, B. and Gallardo, R.A., 2019. Genotypic characterization of emerging avian reovirus genetic variants in California. *Scientific reports*, *9*(1), pp.1-10.

Emonet, S.F., Garidou, L., McGavern, D.B. and Juan, C., 2009. Generation of recombinant lymphocytic choriomeningitis viruses with trisegmented genomes stably expressing two additional genes of interest. *Proceedings of the National Academy of Sciences*, *106*(9), pp.3473-3478.

Endo-Munoz, L.B., 1990. A western blot to detect antibody to avian reovirus. *Avian Pathology*, *19*(3), pp.477-487.

Fahey, J.E., 1955. Chronic respiratory disease of ducks. *Poultry Science*, *34*(2), pp.397-399.

Fahey, J.E., 1956. A virus in chronic respiratory disease of turkeys. *Nature*, *177*(4498), pp.90-91.

Farkas, S.L., Marton, S., Dandár, E., Kugler, R., Gál, B., Jakab, F., Bálint, Á., Kecskeméti, S. and Bányai, K., 2016. Lineage diversification, homo-and heterologous reassortment and recombination shape the evolution of chicken orthoreoviruses. *Scientific reports*, *6*(1), pp.1-9.

Farkas, S. L., R. Varga-Kugler, S. Marton, G. Lengyel, V. Palya, and K. Banyai. 2018. Genomic sequence and phylogenetic analyses of two novel orthoreovirus strains isolated from Pekin ducks in 2014 in Germany. Virus Research 257:57–62.

Franca, M., Crespo, R., Chin, R., Woolcock, P. and Shivaprasad, H.L., 2010. Retrospective study of myocarditis associated with reovirus in turkeys. *Avian diseases*, *54*(3), pp.1026-1031.

Fehling, S.K., Lennartz, F. and Strecker, T., 2012. Multifunctional nature of the arenavirus RING finger protein Z. *Viruses*, *4*(11), pp.2973-3011.

Fingerut, E., Gutter, B., Gallili, G., Michael, A. and Pitcovski, J., 2003. A subunit vaccine against the adenovirus egg-drop syndrome using part of its fiber protein. *Vaccine*, *21*(21-22), pp.2761-2766.

Flatz, L., Hegazy, A.N., Bergthaler, A., Verschoor, A., Claus, C., Fernandez, M., Gattinoni, L., Johnson, S., Kreppel, F., Kochanek, S. and Van Den Broek, M., 2010.

Development of replication-defective lymphocytic choriomeningitis virus vectors for the induction of potent CD8+ T cell immunity. *Nature medicine*, *16*(3), pp.339-345.

Gallardo, R. A. et al. Molecular epidemiology of reoviruses in California. 2017In: Proceedings of the 66th of the Western Poultry Disease Conference, Sacramento, California, 54–55.

Gershowitz, A. and Wooley, R.E., 1973. Characterization of two reoviruses isolated from turkeys with infectious enteritis. *Avian diseases*, pp.406-414.

Gharaibeh, S., Mahmoud, K. and Al-Natour, M., 2008. Field evaluation of maternal antibody transfer to a group of pathogens in meat-type chickens. *Poultry science*, *87*(8), pp.1550-1555.

Giambrone, J.J. and Solano, W., 1988. Serologic comparison of avian reovirus isolates using Virus Neutralization and an Enzyme-Linked Immunosorbent Assay. *Avian diseases*, pp.678-680.

Giambrone, J.J., Dormitorio, T. and Lockaby, S.B., 1992. Coarse-spray immunization of one-day-old broilers against enteric reovirus infections. *Avian diseases*, pp.364-368.

Goldenberg, D., M. Pasmanik-Chor, M. Pirak, N. Kass, A. Lublin, A. Yeheskel, D. Heller, and J. Pitcovski 2010. Genetic and antigenic characterization of sigma C protein from avian reovirus. Avian Pathol. 39: 189–199.

Goldenberg, D., Lublin, A., Rosenbluth, E., Heller, E.D. and Pitcovski, J., 2016. Optimized polypeptide for a subunit vaccine against avian reovirus. *Vaccine*, *34*(27), pp.3178-3183.

Gough, R.E., Alexander, D.J., Collins, M.S., Lister, S.A. and Cox, W.J., 1988. Routine virus isolation or detection in the diagnosis of diseases in birds. *Avian Pathology*, *17*(4), pp.893-907.

Gouvea, V., Schnitzer, T.J., 1982. Pathogenicity of avian reoviruses: examination of six isolates and a vaccine strain. Infect. Immun. 38, 731–738.

Gouvea, V.E.R.A. and Schnitzer, T.J., 1982. Pathogenicity of avian reoviruses: examination of six isolates and a vaccine strain. *Infection and immunity*, *38*(2), pp.731-738.

Glass, S.E., Naqi, S.A., Hall, C.F. and Kerr, K.M., 1973. Isolation and characterization of a virus associated with arthritis of chickens. *Avian diseases*, pp.415-424.

Gonzalez-Lopez, C., Martínez-Costas, J., Esteban, M. and Benavente, J., 2003. Evidence that avian reovirus σA protein is an inhibitor of the double-stranded RNA-dependent protein kinase. *Journal of general virology*, *84*(6), pp.1629-1639.

Goodwin, M.A., Davis, J.F., McNulty, M.S., Brown, J. and Player, E.C., 1993a. Enteritis (so-called runting stunting syndrome) in Georgia broiler chicks. *Avian Diseases*, pp.451-458.

Goodwin, M.A., Davis, J.F. and Player, E.C., 1993b. Reovirus-associated enteritis in Georgia broiler chicks. *Avian diseases*, pp.229-233.

Gaudry, D., Tetkoff, J. and Charles, J.M. 1972. A propos d'un nouveau virus isole chez le canard de Barbarie. *Bull. Soc. Sei Vet. et Med. Comparée*, 74: 137–143.

Ghetas, A.M., Thaxton, G.E., Breedlove, C., Van Santen, V.L. and Toro, H., 2015.
Effects of adaptation of infectious bronchitis virus Arkansas attenuated vaccine to embryonic kidney cells. *Avian diseases*, *59*(1), pp.106-113.
Grande, A., Rodriguez, E., Costas, C., Everitt, E. and Benavente, J., 2000. Oligomerization and Cell-Binding Properties of the Avian Reovirus Cell-Attachment Protein ςC. *Virology*, *274*(2), pp.367-377.
Grande, A., Costas, C. and Benavente, J., 2002. Subunit composition and conformational stability of the oligomeric form of the avian reovirus cell-attachment protein σC. *Journal of general virology*, *83*(1), pp.131-139.
Guardado-Calvo, P., Vazquez-Iglesias, L., Martinez-Costas, J., Llamas-Saiz, A.L., Schoehn, G., Fox, G.C., Hermo-Parrado, X.L., Benavente, J. and van Raaij, M.J., 2008. Crystal structure of the avian reovirus inner capsid protein σA. *Journal of virology*, *82*(22), pp.11208-11216.
Guy, J.S., Levy, M.G., Ley, D.H., Barnes, H.J. and Gerig, T.M., 1987. Experimental reproduction of enteritis in bobwhite quail (Colinus virginianus) with Cryptosporidium and reovirus. *Avian diseases*, pp.713-722.
Guy JS, Levy MG, Ley DH, Barnes HJ, Gerig TM. 1988. Interaction of Reovirus and Cryptosporidium baileyi in Experimentally Infected Chickens. Avian Diseases; 32:381-390.
Guy, J.S., Barnes, H.J., Smith, L.G. and Breslin, J., 1997. Antigenic characterization of a
turkey coronavirus identified in poult enteritis-and mortality syndrome-affected turkeys. *Avian Diseases*, pp.583-590.
Guy, J. S., L. G. Smith, J. J. Breslin, J. P. Vaillancourt, and H. J. Barnes. 2000. High mortality and growth depression experimentally produced in young turkeys by dual infection with enteropathogenic Escherichia coli and turkey coronavirus. Avian Dis 105–13.
Heffels-Redmann, U., Müller, H. and Kaleta, E.F., 1992. Structural and biological characteristics of reoviruses isolated from Muscovy ducks (Cairina moschata). *Avian Pathology*, *21*(3), pp.481-491.
Heggen-Peay, M. A., F. W. Qureshi, B. Edens, P. S. Sherry, P. H. Wakenell, C. L. O'Connell, and K. A. Schat 2002. Isolation of a reovirus from poult enteritis and mortality syndrome and its pathogenicity in turkey poults. Avian Dis 46:360–369.
Hemzani, E., M. Meroz, A. Weisz, G. Ayali, N. Kass, S. Mikhlin, E. Berman, and Y. Samberg. 1996. Isolation of an avian reovirus with unique antigenicity from a tenosynovitis outbreak. Proceedings International Symposium on

Adenovirus and Reovirus Infections in Poultry, Rauischholzhausen, Germany, 269–278.

Hieronymus, D.R.K., Kleven, S.H., Villegas, P., 1983a. Identification and serological differentiation of several reovirus strains isolated from chickens with suspected malabsorption syndrome. Avian Diseases; 27:246-254.

Hieronymus, D., Villegas, P., Kleven, S.. 1983b. Characteristics and Pathogenicity of Two Avian Reoviruses Isolated from Chickens with Leg Problems. Avian Diseases; 27:255-260.

Hlinak, A., Müller, T., Kramer, M., Mühle, R.U., Liebherr, H. and Ziedler, K., 1998. Serological survey of viral pathogens in bean and white-fronted geese from Germany. *Journal of wildlife diseases*, *34*(3), pp.479-486.

Hill, J.E., Rowland, G.N., Latimer, K.S. and Brown, J., 1989. Effects of cyclosporine A on reovirus-infected broilers. *Avian diseases*, pp.86-92.

Hollmén, T., Franson, J.C., Kilpi, M., Docherty, D.E., Hansen, W.R. and Hario, M., 2002. Isolation and characterization of a reovirus from common eiders (Somateria mollissima) from Finland. *Avian Diseases*, *46*(2), pp.478-484.

Hollmén, T., Docherty, D.E., 2007. Orthoreoviruses., in: Thomas, N.J., Hunter, D.B., Atkinson, C.T. (Eds.), Infectious Diseases of Wild Birds. Blackwell Pub., Ames, Iowa, pp. 177-181.

Howard, D.B. and Harvey, B.K., 2017. Assaying the stability and inactivation of AAV serotype 1 vectors. *Human gene therapy methods*, *28*(1), pp.39-48.

Hoang, D.T., Chernomor, O., von Haeseler, A., Minh, B.Q., Vinh, L.S., 2017. UFBoot2: Improving the ultrafast bootstrap approximation. *Molecular Biology Evolution*, in press. https://doi.org/10.1093/molbev/msx281

Hsu, C.J., Wang, C.Y., Lee, L.H., Shih, W.L., Chang, C.I., Cheng, H.L., Chulu, J.L., Ji, W.T. and Liu, H.J., 2006. Development and characterization of monoclonal antibodies against avian reovirus σC protein and their application in detection of avian reovirus isolates. *Avian Pathology*, *35*(4), pp.320-326.

Huang, W.R., Chiu, H.C., Liao, T.L., Chuang, K.P., Shih, W.L. and Liu, H.J., 2015. Avian reovirus protein p17 functions as a nucleoporin Tpr suppressor leading to activation of p53, p21 and PTEN and inactivation of PI3K/AKT/mTOR and ERK signaling pathways. *PLoS One*, *10*(8), p.e0133699.

Huang, Q., Shao, J., Lan, S., Zhou, Y., Xing, J., Dong, C., Liang, Y., Ly, H., 2015. In vitro and in vivo characterizations of the Pichinde viral NP exoribonuclease function. J Virol http://dx.doi.org/10.1128/JVI.00009-15.

Huhtamo, E., Uzcátegui, N.Y., Manni, T., Munsterhjelm, R., Brummer-Korvenkontio, M., Vaheri, A. and Vapalahti, O., 2007. Novel

orthoreovirus from diseased crow, Finland. *Emerging infectious diseases*, *13*(12), p.1967.

Huang, L.K., Liao, S.C., Chang, C.C. and Liu, H.J., 2006. Expression of avian reovirus σC protein in transgenic plants. *Journal of virological methods*, *134*(1-2), pp.217-222.

Ide, P.R. and Dewitt, W., 1979. Serological incidence of avian reovirus infection in broiler-breeders and progeny in Nova Scotia. *The Canadian Veterinary Journal*, *20*(12), p.348.

Ide, P.R., 1982. Avian reovirus antibody assay by indirect immunofluorescence using plastic microculture plates. *Canadian Journal of Comparative Medicine*, *46*(1), p.39.

Islam, M.R., Jones, R.C., 1988. An enzyme-linked immunosorbent assay for measuring antibody titre against avian reovirus using a single dilution of serum. *Avian Pathology*, *17*(2), pp.411-425.

Jaafar, F.M., Attoui, H., Mertens, P.P., de Micco, P. and de Lamballerie, X., 2005. Structural organization of an encephalitic human isolate of Banna virus (genus Seadornavirus, family Reoviridae). *Journal of general virology*, *86*(4), pp.1147-1157.

Ji, W.T., Lee, L.H., Lin, F.L., Wang, L. and Liu, H.J., 2009. AMP-activated protein kinase facilitates avian reovirus to induce mitogen-activated protein kinase (MAPK) p38 and MAPK kinase 3/6 signalling that is beneficial for virus replication. *Journal of general virology*, *90*(12), pp.3002-3009.

Jindal, N., Patnayak, D.P., Chander, Y., Ziegler, A.F., Goyal, S.M., 2010a. Detection and molecular characterization of enteric viruses from poult enteritis syndrome in turkeys. *Poultry Science*. 89, 217-226.

Jindal, N., Patnayak, D.P., Ziegler, A., Lago, A., Goyal, S.M., 2009. A retrospective study on poult enteritis syndrome in Minnesota. *Avian Disease*. 53, 268-275.

Jindal, N., Patnayak, D.P., Chander, Y., Ziegler, A.F., Goyal, S.M., 2010a. Detection and molecular characterization of enteric viruses from poult enteritis syndrome in turkeys. *Poultry Science*. 89, 217-226.

Jindal, N., Patnayak, D.P., Chander, Y., Ziegler, A.F., Goyal, S.M. 2010b. Detection and molecular characterization of enteric viruses in breeder turkeys. *Avian Pathology*. 39, 53-61.

Jindal, N., Mor, S.K., Patnayak, D.P., Ziegler, A.F., Goyal, S.M., 2014. Molecular characterization of S3 gene of turkey reovirus. *Avian Pathology*. 43, 224-230.

Jindal, N., Chander, Y., Patnayak, D.P., Mor, S.K., Ziegler, A.F., Goyal, S.M., 2012. A multiplex RT-PCR for the detection of astrovirus, rotavirus, and reovirus in turkeys. *Avian Disease*. 56, 592-596.

Johnson, D.C. and Van der Heide, L., 1971. Incidence of tenosynovitis in Maine broilers. *Avian diseases*, pp.829-834.

Johnson, D.C., 1972. Diagnosis, pathology, and etiology of tenosynovitis in broilers and broiler breeders. *Avian diseases*, pp.1067-1072.

Johnson, T.J., Youmans, B.P., Noll, S., Cardona, C., Evans, N.P., Karnezos, T.P., Ngunjiri, J.M., Abundo, M.C., Lee, C.W., 2018. A consistent and predictable commercial broiler chicken bacterial microbiota in antibiotic-free production displays strong correlations with performance. *Applied Environmental Microbiology*. 84.

Joklik, W.K., 1983. The reovirus particle. In *The Reoviridae* (pp. 9-78). Springer, Boston, MA.

Jones, R.C., Jordan, T.W. and Lioupis, S., 1975. Characteristics of reovirus isolated from ruptured gastrocnemius tendons of chickens. *The Veterinary Record*, *96*(7), pp.153-154.

Jones, R.C., 1976. Reoviruses from chickens with hydropericardium. *The Veterinary Record*, *99*(23), pp.458-458.

Jones, R.C. and Onunkwo, O., 1978. Studies on experimental tenosynovitis in light hybrid chickens. *Avian Pathology*, *7*(1), pp.171-181.

Jones, R.C. and Georgiou, K., 1984. Reovirus-induced tenosynovitis in chickens the influence of age at infection. *Avian pathology*, *13*(3), pp.441-457.

Jones, R.C. and Kibenge, F.S., 1984. Reovirus-induced tenosynovitis in chickens: The effect of breed. *Avian pathology*, *13*(3), pp.511-528.

Jones, R.C. and Georgiou, K., 1985. The temporal distribution of an arthrotropic reovirus in the leg of the chicken after oral infection. *Avian Pathology*, *14*(1), pp.75-85.

Jones, R.C., Islam, M.R. and Kelly, D.F., 1989. Early pathogenesis of experimental reovirus infection in chickens. *Avian Pathology*, *18*(2), pp.239-253.

Jones, R.C., Al-Afaleq, A., Savage, C.E. and Islam, M.R., 1994. Early pathogenesis in chicks of infection with a trypsin-sensitive avian reovirus. *Avian Pathology*, *23*(4), pp.683-692.

Jones, R.C. 2000. Avian reovirus infections. Rev Sci Tech. 19:614–25.

Jones, R.C., 2008. Reovirus Infections. In: Saif, Y.M., Barnes, H.J., Glisson, J.R., Fadly, A.M., McDougald, L.R., Swayne, D.E., (Eds.) Diseases of Poultry, 12th edn (pp. 309-328). Ames: Iowa State University PressJones, R.C., 2013. Reovirus infections. *Diseases of poultry*, pp.351-373.

Jones, R.C., 2000. Avian reovirus infections. *Revue Scientifique et Technique-Office International des Epizooties*, *19*(2), pp.614-619.

Jones, R.C., 2013. Reovirus infections. In: Swayne, D.E. (Ed), Diseases of Poultry. WileyBlackwell, Ames Iowa, pp. 351373.

Jung, K.M., Bae, E.H., Jung, Y.T. and Kim, J.W., 2014. Use of IgY antibody to recombinant avian reovirus σC protein in the virus diagnostics. *Acta virologica*, *58*(2), pp.108-113.

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K., Von Haeseler, A. and Jermiin, L.S., 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature methods*, *14*(6), pp.587-589.

Kant, A., Balk, F., Born, L., van Roozelaar, D., Heijmans, J., Gielkens, A. and ter Huurne, A., 2003. Classification of Dutch and German avian reoviruses by sequencing the sigma C protein. *Veterinary research*, *34*(2), pp.203-212.

Kapczynski, D., Sellers, H., Simmons, V. and Schultz-Cherry, S., 2002. Sequence analysis of the S3 gene from a turkey reovirus. *Virus Genes*, *25*(1), pp.95-100.Kaptein, L.C.M., Greijer, A.E., Valerio, D. and Van Beusechem, V.W., 1997. Optimized conditions for the production of recombinant amphotropic retroviral vector preparations. *Gene Therapy*, *4*(2), pp.172-176.

Karesh, W.B., Uhart, M.M., Frere, E., Gandini, P., Braselton, W.E., Puche, H. and Cook, R.A., 1999. Health evaluation of free-ranging rockhopper penguins (Eudyptes chrysocomes) in Argentina. *Journal of Zoo and Wildlife Medicine*, pp.25-31.

Kerr, K.M. and Olson, N.O., 1969. Pathology of chickens experimentally inoculated or contact-infected with an arthritis-producing virus. *Avian diseases*, pp.729-745.

Kibenge, F.S., Robertson, M.D. and Wilcox, G.E., 1982. Staphylococcus aureus isolated from poultry in Australia II. Epidemiology of strains associated with tenosynovitis. *Veterinary microbiology*, *7*(5), pp.485-491.

Kibenge, F.S., Wilcox, G.E. and Pass, D.A., 1983. Pathogenicity of four strains of staphylococci isolated from chickens with clinical tenosynovitis. *Avian Pathology*, *12*(2), pp.213-220.

Kibenge, F.S., Gwaze, G.E., Jones, R.C., Chapman, A.F. and Savage, C.E., 1985. Experimental reovirus infection in chickens: Observations on early viraemia and virus distribution in bone marrow, liver and enteric tissues. *Avian Pathology*, *14*(1), pp.87-98.

Kibenge, F.S. and Dhillon, A.S., 1987. A comparison of the pathogenicity of four avian reoviruses in chickens. *Avian diseases*, pp.39-42.

King AMQ, Adams MJ, Lefkowitz EJ, Carstens EB (2012) Virus taxonomy:

Classification and nomenclature of viruses: ninth report of the international committee on taxonomy of viruses. 30-31.

Koci, M. D. and S. Schulz-Cherry. 2002. Avian astroviruses. Avian Pathol 31: 213–227.

Kotani, H., Newton III, P.B., Zhang, S., Chiang, Y.L., Otto, E., Weaver, L., Blaese, R.M., Anderson, W.F. and McGarrity, G.J., 1994. Improved methods of retroviral vector transduction and production for gene therapy. *Human gene therapy*, *5*(1), pp.19-28.

Kouwenhoven, B., Davelaar, FG and Van Walsum, J., 1978a. Infectious proventriculitis causing runting in broilers. *Avian Pathology*, *7* (1), pp. 183-187.

Kouwenhoven, B., Vertommen, M. and Van Eck, J.H.H., 1978b. Runting and leg weakness in broilers; involvement of infectious factors. *Veterinary Science Communications*, *2*(1), pp.253-259.

Kouwenhoven, B., Vertommen, M. and Goren, E., 1988. Investigations into the role of reovirus in the malabsorption syndrome. *Avian Pathology*, *17*(4), pp.879-892.

Kuntz-Simon, G., Le Gall-Reculé, G., de Boisséson, C. and Jestin, V., 2002. Muscovy duck reovirus σC protein is atypically encoded by the smallest genome segment. *Journal of general virology*, *83*(5), pp.1189-1200.

Kumar, P., Sharafeldin, T.A., Kumar, R., Huang, Q., Liang, Y., Goyal, S.M., Porter, R.E., Ly, H. and Mor, S.K., 2021. Development of a Recombinant Pichinde Virus-Vectored Vaccine against Turkey Arthritis Reovirus and Its Immunological Response Characterization in Vaccinated Animals. *Pathogens*, *10*(2), p.197.

Lan, S., McLay Schelde, L., Wang, J., Kumar, N., Ly, H. and Liang, Y., 2009. Development of infectious clones for virulent and avirulent pichinde viruses: a model virus to study arenavirus-induced hemorrhagic fevers. *Journal of virology*, *83*(13), pp.6357-6362.

Langmead, B. and Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. *Nature methods*, *9*(4), pp.357-359.

Lawson, B., Dastjerdi, A., Shah, S., Everest, D., Núñez, A., Pocknell, A., Hicks, D.,

Horton, D.L., Cunningham, A.A. and Irvine, R.M., 2015. Mortality associated with avian reovirus infection in a free-living magpie (Pica pica) in Great Britain. *BMC veterinary research*, *11*(1), pp.1-7.

Lee, S.G., Kim, S., Robbins, P.D. and Kim, B.G., 1996. Optimization of environmental factors for the production and handling of recombinant retrovirus. *Applied microbiology and biotechnology*, *45*(4), pp.477-483.

Lee, L.H., Shien, J.H. and Shieh, H.K., 1998. Detection of avian reovirus RNA and comparison of a portion of genome segment S3 by polymerase chain reaction and restriction enzyme fragment length polymorphism. *Research in veterinary science*, *65*(1), pp.11-15.

Lee, K.J., Novella, I.S., Teng, M.N., Oldstone, M.B. and de la Torre, J.C., 2000. NP and L proteins of lymphocytic choriomeningitis virus (LCMV) are sufficient for efficient transcription and replication of LCMV genomic RNA analogs. *Journal of virology*, *74*(8), pp.3470-3477.

Levisohn, S., A. Gur-Lavi, and J. Weisman. 1980. Infectious synovitis in turkeys: isolation of a tenosynovitis virus-like agent. Avian Pathol 9:1–4.

Li, Y., Reddy, K., Reid, S.M., Cox, W.J., Brown, I.H., Britton, P., Nair, V. and Iqbal, M., 2011. Recombinant herpesvirus of turkeys as a vector-based vaccine against highly pathogenic H7N1 avian influenza and Marek's disease. *Vaccine*, *29*(46), pp.8257-8266.

Lin, Y.L., Shen, J.H. and Lee, L.H., 2006. A monoclonal antibody-based competitive enzyme-linked immunosorbent assay for detecting antibody production against avian reovirus protein σA. *Journal of virological methods*, *136*(1-2), pp.71-77.

Lin, Y.H., Lee, L.H., Shih, W.L., Hu, Y.C. and Liu, H.J., 2008. Baculovirus surface display of σC and σB proteins of avian reovirus and immunogenicity of the displayed proteins in a mouse model. *Vaccine*, *26*(50), pp.6361-6367.

Liu, Q., Zhang, G., Huang, Y., Ren, G., Chen, L., Gao, J., Zhang, D., Han, B., Su, W., Zhao, J. and Hu, X., 2011. Isolation and characterization of a reovirus causing spleen necrosis in Pekin ducklings. *Veterinary microbiology*, *148*(2-4), pp.200-206.

Liu, H.J. and Giambrone, J.J., 1997. In situ detection of reovirus in formalin-fixed, paraffin-embedded chicken tissues using a digoxigenin-labeled cDNA probe. *Avian diseases*, pp.447-451.

Liu, H.J., Chen, J.H., Liao, M.H., Lin, M.Y. and Chang, G.N., 1999a. Identification of the σ C-encoded gene of avian reovirus by nested PCR and restriction endonuclease analysis. *Journal of virological methods*, *81*(1-2), pp.83-90.

Liu, H.J., Liao, M.H., Chang, C.D., Chen, J.H., Lin, M.Y. and Tung, M.C., 1999b. Comparison of two molecular techniques for the detection of avian reoviruses in formalin-fixed, paraffin-embedded chicken tissues. *Journal of virological methods*, *80*(2), pp.197-201.

Liu, H.J., Kuo, L.C., Hu, Y.C., Liao, M.H. and Lien, Y.Y., 2002. Development of an ELISA for detection of antibodies to avian reovirus in chickens. *Journal of virological methods*, *102*(1-2), pp.129-138.

Liu, H.J., Lee, L.H., Hsu, H.W., Kuo, L.C. and Liao, M.H., 2003. Molecular evolution of avian reovirus: evidence for genetic diversity and reassortment of the S-class genome segments and multiple cocirculating lineages. *Virology*, *314*(1), pp.336-349.

Liu, H.J., Lin, P.Y., Lee, J.W., Hsu, H.Y. and Shih, W.L., 2005. Retardation of cell growth by avian reovirus p17 through the activation of p53 pathway. *Biochemical and biophysical research communications*, *336*(2), pp.709-715.

Liu, H.J., Lee, L.H., Hsu, H.W., Kuo, L.C. and Liao, M.H., 2013. Molecular evolution of avian reovirus: evidence for genetic diversity and reassortment of the S class genome segments and multiple cocirculating lineages. Virology. 314: 336-349.

Lin, Y.L., Shen, J.H. and Lee, L.H., 2006. A monoclonal antibody-based competitive enzyme-linked immunosorbent assay for detecting antibody production against avian reovirus protein σA. *Journal of virological methods*, *136*(1-2), pp.71-77.

Lin, K.H., Hsu, A.P., Shien, J.H., Chang, T.J., Liao, J.W., Chen, J.R., Lin, C.F. and Hsu, W.L., 2012. Avian reovirus sigma C enhances the mucosal and systemic immune responses elicited by antigen-conjugated lactic acid bacteria. *Vaccine*, *30*(33), pp.5019-5029.

Lozano, L.F., Bickford, A.A., Castro, A.E., Swartzman-Andert, J., Chin, R., Meteyer, C., Cooper, G., Reynolds, B. and Manalac, R.L., 1989. Association of Reoviridae particles in an enteric syndrome of poults observed in turkey flocks during 1988. *Journal of Veterinary Diagnostic Investigation*, *1*(3), pp.254-259.

Lu, H., Tang, Y., Dunn, P.A., Wallner-Pendleton, E.A., Lin, L. and Knoll, E.A., 2015. Isolation and molecular characterization of newly emerging avian reovirus variants and novel strains in Pennsylvania, USA, 2011–2014. *Scientific reports*, *5*(1), pp.1-11.

Lublin, A., Goldenberg, D., Rosenbluth, E., Heller, E.D. and Pitcovski, J., 2011. Wide-range protection against avian reovirus conferred by vaccination with representatives of four defined genotypes. *Vaccine*, *29*(47), pp.8683-8688.

Lüschow, D., Werner, O., Mettenleiter, T.C. and Fuchs, W., 2001. Protection of chickens from lethal avian influenza A virus infection by live-virus vaccination with infectious laryngotracheitis virus recombinants expressing the hemagglutinin (H5) gene. *Vaccine*, *19*(30), pp.4249-4259.

Malkinson, M.P.K.W., Perk, K. and Weisman, Y., 1981. Reovirus infection of young Muscovy ducks (Cairina moschata). *Avian Pathology*, *10*(4), pp.433-440.

Marquardt, J., Hermanns, W., Schulz, L.C. and Leibold, W., 1983. A persistent reovirus infection of chickens as a possible model of human rheumatoid arthritis (RA). *Zentralblatt für Veterinärmedizin Reihe B*, *30*(1-10), pp.274-282.

Martinez-Costas, J., Varela, R. and Benavente, J., 1995. Endogenous enzymatic activities of the avian reovirus S1133: identification of the viral capping enzyme. *Virology*, *206*(2), pp.1017-1026.

Martínez-Costas, J., Grande, A., Varela, R., García-Martínez, C. and Benavente, J., 1997. Protein architecture of avian reovirus S1133 and identification of the cell attachment protein. *Journal of virology*, *71*(1), pp.59-64.

Martínez-Costas, J., González-López, C., Vakharia, V.N. and Benavente, J., 2000. Possible involvement of the double-stranded RNA-binding Core protein ςA in the resistance of avian Reovirus to interferon. *Journal of virology*, *74*(3), pp.1124-1131.

Martínez-Sobrido, L., Zúñiga, E.I., Rosario, D., García-Sastre, A. and de la Torre, J.C., 2006. Inhibition of the type I interferon response by the nucleoprotein of the prototypic arenavirus lymphocytic choriomeningitis virus. *Journal of virology*, *80*(18), pp.9192-9199.

Mast, J., Van Den Berg, T., Letellier, C., Kerkhofs, P., Zeller, H. and Meulemans, G., 2006. Electron microscopic demonstration of a reovirus-like agent in carrion crows (Corvus corone) associated with clinical symptoms similar to West Nile Virus infection. *Vlaams Diergeneeskundig Tijdschrift*, *75*(3), pp.235-238.

MacKenzie, M.A. and Bains, B.S., 1976. Tenosynovitis in chickens. *Australian veterinary journal*, *52*(10), pp.468-470.

Macdonald, J.W., Randall, C.J., Dagless, M.D. and McMartin, D.A., 1978. Observations on viral tenosynovitis (viral arthritis) in Scotland. *Avian Pathology*, *7*(4), pp.471-482.

McAleer, W.J., Buynak, E.B., Maigetter, R.Z., Wampler, D.E., Miller, W.J. and Hilleman, M.R., 1992. Human hepatitis B vaccine from recombinant yeast. 1984. *Biotechnology (Reading, Mass.)*, *24*, pp.500-502.

McDonald, S.M. and Patton, J.T., 2009. Core-associated genome replication mechanisms of dsRNA viruses. In *Viral Genome Replication* (pp. 201-224). Springer, Boston, MA.

McDonald, S.M., Nelson, M.I., Turner, P.E. and Patton, J.T., 2016. Reassortment in segmented RNA viruses: mechanisms and outcomes. *Nature Reviews Microbiology*, *14*(7), pp.448-460.

McLay L, Liang Y, Ly H. 2014. Comparative analysis of disease pathogenesis and molecular mechanisms of New World and Old-World arenavirus infections. J Gen Virol 95:1–15.doi.org/10.1099/vir.0 .057000-0.

Meanger, J., Wickramasinghe, R., Enriquez, C.E., Robertson, M.D. and Wilcox, G.E., 1995. Type-specific antigenicity of avian reoviruses. *Avian Pathology*, *24*(1), pp.121-134.

Meanger, J., Wickramasinghe, R., Enriquez, C.E. and Wilcox, G.E., 1997. Immune response to avian reovirus in chickens and protection against experimental infection. *Australian veterinary journal*, *75*(6), pp.428-432.

Menendez, N.A., Calnek, B.W. and Cowen, B.S., 1975a. Experimental egg-transmission of avian reovirus. *Avian diseases*, pp.104-111.

Menendez, N.A., Calnek, B.W. and Cowen, B.S., 1975b. Localization of avian reovirus (FDO isolant) in tissues of mature chickens. *Avian diseases*, pp.112-117.

Joklik, W.K., 1983. The reovirus particle. In *The Reoviridae* (pp. 9-78). Springer,

Boston, M.A., Meteyer, C., Docherty, D., Ip, H., Ramsey, N., Saito, E. and Oaks, L., 2009, August. Reovirus-associated necrotizing enteritis in American crows. In *Program and abstracts of the 58th annual international conference, Wildlife Disease Association, Blaine, Washington* (pp. 2-7).

Meyer, B. and Ly, H., 2016. Inhibition of innate immune responses is key to pathogenesis by arenaviruses. *Journal of virology*, *90*(8), pp.3810-3818.

Martella, V., Bányai, K., Matthijnssens, J., Buonavoglia, C. and Ciarlet, M., 2010.

Zoonotic aspects of rotaviruses. Vet Microbiol140: 246–255.

McFerran, J.B., Connor, T.J., and McCracken, R.M., 1976. Isolation of adenoviruses and reoviruses from avian species other than domestic fowl. *Avian Diseases*, pp.519-524.

McNulty, M.S., Allan, G.M., Connor, T.J., McFerran, J.B. and McCracken, R.M., 1984. An entero-like virus associated with the runting syndrome in broiler chickens. *Avian Pathology*, *13*(3), pp.429-439.

Meanger, J., Wickramasinghe, R., Enriquez, C.E., Robertson, M.D. and Wilcox, G.E., 1995. Type-specific antigenicity of avian reoviruses. *Avian Pathology*, *24*(1), pp.121-134.

Meulemans, G. and Halen, P., 1982. Efficacy of some disinfectants against infectious bursal disease virus and avian reovirus. *The Veterinary record*, *111*(18), pp.412-413.

Montgomery, R.D., Villegas, P., Dawe, D.L. and Brown, J., 1985. Effect of avian reoviruses on lymphoid organ weights and antibody response in chickens. *Avian diseases*, pp.552-560.

Montgomery, R.D., Villegas, P., Dawe, D.L. and Brown, J., 1986a. A comparison between the effect of an avian reovirus and infectious bursal disease virus on selected aspects of the immune system of the chicken. *Avian diseases*, pp.298-308.

Montgomery, R.D., Villegas, P. and Kleven, S.H., 1986. Role of route of exposure, age, sex, and type of chicken on the pathogenicity of avian reovirus strain 81-176. *Avian diseases*, pp.460-467.

Mor, S.K., Abin, M., Costa, G., Durrani, A., Jindal, N., Goyal, S.M. and Patnayak, D.P., 2011. The role of type-2 turkey astrovirus in poult enteritis syndrome. *Poultry science*, *90*(12), pp.2747-2752.

Mor, S.K., Sharafeldin, T.A., Abin, M., Kromm, M., Porter, R.E., Goyal, S.M., Patnayak, D.P., 2013a. The occurrence of enteric viruses in light turkey syndrome. Avian Pathol. 42, 497-501.

Mor, S.K., Sharafeldin, T.A., Porter, R.E., Ziegler, A., Patnayak, D.P., Goyal, S.M., 2013b. Isolation and characterization of a turkey arthritis reovirus. Avian Dis. 57, 97-103.

Mor, S.K., Verma, H., Sharafeldin, T.A., Porter, R.E., Jindal, N., Ziegler, A. and Goyal, S.M., 2014a. Characterization of S class gene segments of a newly isolated turkey arthritis reovirus. *Virology*, *464*, pp.33-44.

Mor, S.K., Sharafeldin, T.A., Porter, R.E. and Goyal, S.M., 2014b. Molecular characterization of L class genome segments of a newly isolated turkey arthritis reovirus. *Infection, Genetics and Evolution*, *27*, pp.193-201.

Mor, S.K., Armién, A.G., Reed, L., Schott, R. and Goyal, S.M., 2014. Detection and molecular characterization of a reovirus in black-capped chickadees (Poecile atricapillus) from Minnesota, USA. *Journal of wildlife diseases*, *50*(4), pp.928-932.

Mor, S.K., Bekele, A.Z., Sharafeldin, T.A., Porter, R.E. and Goyal, S.M., 2015a. Efficacy of five commonly used disinfectants against turkey arthritis reovirus. *Avian diseases*, *59*(1), pp.71-73.

Mor, S.K., Verma, H., Sharafeldin, T.A., Porter, R.E., Ziegler, A.F., Noll, S.L. and Goyal, S.M., 2015b. Survival of turkey arthritis reovirus in poultry litter and drinking water. *Poultry science*, *94*(4), pp.639-642.

Mor, S.K., Marthaler, D., Verma, H., Sharafeldin, T.A., Jindal, N., Porter, R.E. and Goyal, S.M., 2015. Phylogenetic analysis, genomic diversity and classification of M class gene segments of turkey reoviruses. *Veterinary microbiology*, *176*(1-2), pp.70-82.

Mor, S.K., Verma, H., Sharafeldin, T.A., Porter, R.E., Ziegler, A.F., Noll, S.L. and Goyal, S.M., 2015. Survival of turkey arthritis reovirus in poultry litter and drinking water. *Poultry science*, *94*(4), pp.639-642

Meulemans, G., Dekegel, D., Charlier, G., Froyman, R., Van Tilburg, J. and Halen, P., 1983. Isolation of orthoreoviruses from psittacine birds. *Journal of comparative pathology*, *93*(1), pp.127-134.

Mustaffa-Babjee, A., Spradbrow, P.A. and Omar, A.R., 1973. Characterization of an avian reovirus isolated in Queensland. *Journal of comparative pathology*, *83*(3), pp.387-400.

Mandelli, G., Rampin, T. and Finazzi, M., 1978. Experimental reovirus hepatitis in newborn chicks. *Veterinary Pathology*, *15*(4), pp.531-543.

Murphy, F.A., Gibbs, E.P.J., Horzinek, M.C., Studdert, M.J., 1999. Reoviridae, in: Anonymous Veterinary Virology, 3rd ed. Academic Press, San Diego, CA, USA, pp. 391-404.

Montgomery, R.D., Villegas, P., Dawe, D.L. and Brown, J., 1986. A comparison between the effect of an avian reovirus and infectious bursal disease virus on selected aspects of the immune system of the chicken. *Avian diseases*, pp.298-308.

Nersessian, B.N., Goodwin, M.A., Kleven, S.H. and Pesti, D., 1985a. Studies on orthoreoviruses isolated from young turkeys. I. Isolation and characterization. *Avian diseases*, pp.755-767.

Nersessian, B.N., Goodwin, M.A., Page, R.K. and Kleven, S.H., 1985b. Studies on orthoreoviruses isolated from young turkeys. II. Virus distribution in organs and serological response of poults inoculated orally. *Avian diseases*, pp.963-969.

Nakaya, T., Cros, J., Park, M.S., Nakaya, Y., Zheng, H., Sagrera, A., Villar, E., García-Sastre, A. and Palese, P., 2001. Recombinant Newcastle disease virus as a vaccine vector. *Journal of virology*, *75*(23), pp.11868-11873.

Ngunjiri, J.M., Ghorbani, A., Jang, H., Waliullah, S., Elaish, M., Abundo, M.C., Mahesh, K.C., Taylor, K.J., Porter, R.E. and Lee, C.W., 2019. Specific-pathogen-free Turkey model for reoviral arthritis. *Veterinary microbiology*, *235*, pp.170-179.

Ni, Y. and Ramig, R.F., 1993. Characterization of avian reovirus-induced cell fusion: the role of viral structural proteins. *Virology*, *194*(2), pp.705-714.

Ni, Y. and Kemp, M.C., 1995. A comparative study of avian reovirus pathogenicity: virus spread and replication and induction of lesions. *Avian diseases*, pp.554-566.

Nibert, M.L., Schiff, L.A., 2001. Reoviruses and their replication. Fundamental virology, Philadelphia: Lippincott-Raven Press;4th Edition:793-842.

Nguyen, L.T., Schmidt Ha v, Haeseler A, Minh BQ. 2015. *IQ-TREE: a fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. Mol Biol Evol*, *32*(1), pp.268-274.

Niu, X., Zhang, B., Yu, X., Zhang, X., Dou, Y., Tang, Y. and Diao, Y., 2017. Preparation and evaluation of goose reovirus inactivated vaccine. *BMC veterinary research*, *13*(1), pp.1-9.

Nuismer, S.L. and Bull, J.J., 2020. Self-disseminating vaccines to suppress

zoonoses. *Nature Ecology & Evolution*, *4*(9), pp.1168-1173.
Ogasawara, Y., Ueda, H., Kikuchi, N. and Kirisawa, R., 2015. Isolation and genomic characterization of a novel orthoreovirus from a brown-eared bulbul (Hypsipetes amaurotis) in Japan. *Journal of General Virology*, *96*(7), pp.1777-1786.
O'Hara, D., Patrick, M., Cepica, D., Coombs, K.M. and Duncan, R., 2001. Avian reovirus major μ-class outer capsid protein influences efficiency of productive macrophage infection in a virus strain-specific manner. *Journal of virology*, *75*(11), pp.5027-5035.
Olson, N.O., 1959. Transmissible synovitis of poultry. *Laboratory investigation; a journal of technical methods and pathology*, *8*, pp.1384-1393.
Olson, N.O. and Solomon, D.P. 1968. A natural outbreak of synovitis caused by the viral arthritis agent. *Avian Diseases*, 12: 311–316.
Olson, N.O. and Khan, M.A., 1972. The effect of intranasal exposure of chickens to the Fahey-Crawley virus on the development of synovial lesions. *Avian diseases*, pp.1073-1078.
Olson, N.O. and Weiss, R., 1972. Similarity between arthritis virus and Fahey-Crawley virus. *Avian diseases*, pp.535-540.
Ongor, H., Bulut, H., Cetinkaya, B., Akan, M., Tonbak, S., Mor, S.K. and Goyal, S.M., 2015. Detection of astrovirus, coronavirus and haemorrhagic enteritis virus in Turkeys with poult enteritis mortality syndrome in Turkey. *The Journal of Poultry Science*, *52*(3), pp.232-237.
Ortiz-Riano, E., Cheng, B.Y.H., de la Torre, J.C. and Martínez-Sobrido, L., 2013. Arenavirus reverse genetics for vaccine development. *The Journal of general virology*, *94*(Pt 6), p.1175.
Page, R.K., Fletcher Jr, O.J. and Villegas, P., 1982a. Infectious tenosynovitis in young turkeys. *Avian diseases*, pp.924-927.
Page, R.K., Fletcher, O.J., Rowland, G.N., Gaudry, D. and Villegas, P., 1982b. Malabsorption syndrome in broiler chickens. *Avian Diseases*, pp.618-624.
Page, R.K., 1983. Pale bird syndrome: still much confusion [Chickens]. *Poultry Digest (USA)*.
Palomino-Tapia, V., Mitevski, D., Inglis, T., van der Meer, F. and Abdul-Careem, M.F., 2018. Molecular characterization of emerging avian reovirus variants isolated from viral arthritis cases in Western Canada 2012–2017 based on partial sigma (σ) C gene. *Virology*, *522*, pp.138-146.
Pantin-Jackwood, M.J., Spackman, E., Michael Day, J. and Rives, D., 2007. Periodic monitoring of commercial turkeys for enteric viruses indicates continuous presence of astrovirus and rotavirus on the farms. *Avian diseases*, *51*(3), pp.674-680.

Pantin-Jackwood, M.J., Day, J.M., Jackwood, M.W. and Spackman, E., 2008. Enteric viruses detected by molecular methods in commercial chicken and turkey flocks in the United States between 2005 and 2006. *Avian diseases*, *52*(2), pp.235-244.

Park, M.S., Steel, J., García-Sastre, A., Swayne, D. and Palese, P., 2006. Engineered viral vaccine constructs with dual specificity: avian influenza and Newcastle disease. *Proceedings of the National Academy of Sciences*, *103*(21), pp.8203-8208.

Pass, D.A., Robertson, M.D. and Wilcox, G.E., 1982. Runting syndrome in broiler chickens in Australia. *The Veterinary Record*, *110*(16), pp.386-387.

Palya, V., Glávits, R., Dobos-Kovács, M., Ivanics, É., Nagy, E., Bányai, K., Szücs, G., Dá, Á. and Benkö, M., 2003. Reovirus identified as cause of disease in young geese. *Avian Pathology*, *32*(2), pp.129-138.

Pennycott, T., 2004. Mortality in budgerigars in Scotland: pathological findings. *The Veterinary Record*, *154*(17), pp.538-539.

Pelliccia, M., Andreozzi, P., Paulose, J., D'Alicarnasso, M., Cagno, V., Donalisio, M., Civra, A., Broeckel, R.M., Haese, N., Silva, P.J. and Carney, R.P., 2016. Additives for vaccine storage to improve thermal stability of adenoviruses from hours to months. *Nature communications*, *7*(1), pp.1-7.

Perelman, B., Krispin, H., Solomon, A., Elrom, K. and andFarnoushi, Y., 2019. Use of Controlled Exposure as a Novel Method for Reovirus Arthritis/Tenosynovitis Prevention. A Preliminary Report. *Israel Journal of Veterinary Medicine*, *74*(4), pp.163-172.

Pinschewer, D.D., Perez, M. and de la Torre, J.C., 2003. Role of the virus nucleoprotein in the regulation of lymphocytic choriomeningitis virus transcription and RNA replication. *Journal of virology*, *77*(6), pp.3882-3887.

Pitcovski, J., Fingerut, E., Gallili, G., Eliahu, D., Finger, A. and Gutter, B., 2005. A subunit vaccine against hemorrhagic enteritis adenovirus. *Vaccine*, *23*(38), pp.4697-4702.

Popkin, D.L., Teijaro, J.R., Lee, A.M., Lewicki, H., Emonet, S., de la Torre, J.C. and Oldstone, M., 2011. Expanded potential for recombinant trisegmented lymphocytic choriomeningitis viruses: protein production, antibody production, and in vivo assessment of biological function of genes of interest. *Journal of virology*, *85*(15), pp.7928-7932.

Porter, R., 2018. Turkey reoviral arthritis update. 2018 MPF Convention Proceedings: Turkey Health Workshop (Midwest Poultry Federation).

Qi, X., Lan, S., Wang, W., Schelde, L.M., Dong, H., Wallat, G.D., Ly, H., Liang, Y. and Dong, C., 2010. Cap binding and immune evasion revealed by Lassa nucleoprotein structure. *Nature*, *468*(7325), pp.779-783.

Quinn, P.J., Markey, B.K., 2003. Reoviridae, in: Quinn, P.J., Markey, B.K. (Eds.), Concise Review of Veterinary Microbiology. Blackwell, Ames, Iowa, pp. 116-117.

Rau, W.E., Van der Heide, L., Kalbac, M. and Girshick, T., 1980. Onset of progeny immunity against viral arthritis/tenosynovitis after experimental vaccination of parent breeder chickens and cross-immunity against six reovirus isolates. *Avian diseases*, pp.648-657.

R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rhyan, J.C. and Spraker, T.R., 2010. Emergence of diseases from wildlife reservoirs. *Veterinary pathology*, *47*(1), pp.34-39.

Reece, R.L. and Frazier, J.A., 1990. Infectious stunting syndrome of chickens in Great Britain: field and experimental studies. *Avian Pathology*, *19*(4), pp.723-758.

Rigby, C.E., Pettit, J.R., Papp-Vid, G., Spencer, J.L. and Willis, N.G., 1981. The isolation of salmonellae, Newcastle disease virus and other infectious agents from quarantined imported birds in Canada. *Canadian Journal of Comparative Medicine*, *45*(4), p.366.

Ritter, G.D., Ley, D.H., Levy, M., Guy, J. and Barnes, H.J., 1986. Intestinal cryptosporidiosis and reovirus isolation from bobwhite quail (Colinus virginianus) with enteritis. *Avian diseases*, pp.603-608.

Robertson, M.D. and Wilcox, G.E., 1984. Serological characteristics of avian reoviruses of Australian origin. *Avian Pathology*, *13*(3), pp.585-594.

Robertson, M.A., 1986. Avian reovirus. *Vet. Bull.*, *56*, pp.155-174.

Roessler, D.E. and Rosenberger, J.K., 1989. In vitro and in vivo characterization of avian reoviruses. III. Host factors affecting virulence and persistence. *Avian diseases*, pp.555-565.

Rosenberger, J.K., and Olson, N.O., 1997. Viral arthritis. In Diseases of poultry, 10th Ed. (B.W. Calnek with H.J. Barnes, C.W. Beard, L.R. McDougald & Y.M. Saif, eds). Mosby-Wolfe, London, 711-20.

Rosenberger, J.K., Rosenberger, S.C., Markis, M., Rosenberger, J.P., 2013a. Pathogenicity and control of recent (2011-2012) reovirus isolates from broiler and turkey flocks presenting with viral arthritis and tenosynovitis. The 2013American Association of Avian Pathologist Annual Convention held in Chicago, Illinios, USA from July 20-23, 2013.

Rosenberger, S.C., Rosenberger, J.K., Markis M., Rosenberger, J.P., 2013b.

Characterization of recent (2011-2012) reovirus isolates from broiler and turkey flocks presenting with viral arthritis and tenosynovitis using in vitro antibody dependent virus neutralization assays. The 2013 American Association of Avian Pathologist Annual Convention held in Chicago, Illinios, USA from July 20-23, 2013.

Rosenberger, J.K., Stener, F.F., Botts, K., Lee, K.P., Margolin, A., 1989. In vitro and in vivo, characterization of avian reoviruses. I. Pathogenicity and antigenic relatedness of several avian reovirus isolates. Avian Dis. 33, 535-544.

Rosenberger JK, Sterner FJ, Botts S, Lee KP, Margolin A. In vitro and in vivo Characterization of Avian Reoviruses. I. Pathogenicity and Antigenic Relatedness of Several Avian Reovirus Isolates. Avian Diseases 1989; 33:535-544.

Rosenberger, J. K. and N. O. Olson. 1997. Viral arthritis. In B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald, and Y. M. Saif (eds.). Diseases of Poultry, 10th ed. Iowa State University Press; Ames, IA, 711–718.

Reed, L.J. and Muench, H., 1938. A simple method of estimating fifty per cent endpoints. *American journal of epidemiology*, *27*(3), pp.493-497.

Reuter, J.D., Fang, X., Ly, C.S., Suter, K.K. and Gibbs, D., 2012. Assessment of hazard risk associated with the intravenous use of viral vectors in rodents. *Comparative medicine*, *62*(5), pp.361-370.

Reynolds, D.L., Saif, Y.M. and Theil, K.W., 1987. A survey of enteric viruses of turkey poults. *Avian diseases*, pp.89-98.

Ruff, M.D. and Rosenberger, J.K., 1985. Concurrent infections with reoviruses and coccidia in broilers. *Avian Diseases*, pp.465-478.

Sahu, S.P. and Olson, N.O., 1975. Comparison of the characteristics of avian reoviruses isolated from the digestive and respiratory tract, with viruses isolated from the synovia. *American journal of veterinary research*, *36*(6), pp.847-850.

Sakaguchi, M., Nakamura, H., Sonoda, K., Okamura, H., Yokogawa, K., Matsuo, K. and Hira, K., 1998. Protection of chickens with or without maternal antibodies against both Marek's and Newcastle diseases by one-time vaccination with recombinant vaccine of Marek's disease virus type 1. *Vaccine*, *16*(5), pp.472-479.

Salsman, J., Top, D., Boutilier, J. and Duncan, R., 2005. Extensive syncytium formation mediated by the reovirus FAST proteins triggers apoptosis-induced membrane instability. *Journal of virology*, *79*(13), pp.8090-8100.

Salvato, M., Shimomaye, E. and Oldstone, M.B., 1989. The primary structure of the lymphocytic choriomeningitis virus L gene encodes a putative RNA polymerase. *Virology*, *169*(2), pp.377-384.

Sanchez-Cordon, P.J., Hervas, J., De Lara, F.C., Jahn, J., Salguero, F.J. and Gomez-Villamandos, J.C., 2002. Reovirus infection in psittacine birds (Psittacus erithacus): morphologic and immunohistochemical study. *Avian diseases*, *46*(2), pp.485-492.

Samberg, Y. and M. Meroj. 1996. Experiments with an autogenous reovirus oil emulsion vaccine. Proc. International Symposium on Adenovirus and Reovirus Infections in Poultry: Rauischholzhausen, Germany, 305–311.

Savage, C.E. and Jones, R.C., 2003. The survival of avian reoviruses on materials associated with the poultry house environment. *Avian Pathology*, *32*(4), pp.417-423.

Schnitzer, T.J., Ramos, T. and Gouvea, V., 1982. Avian reovirus polypeptides: analysis of intracellular virus-specified products, virions, top component, and cores. *Journal of virology*, *43*(3), pp.1006-1014.

Schnitzer, T.J., 1985. Protein coding assignment of the S genes of the avian reovirus S1133. *Virology*, *141*(1), pp.167-170.

Schwartz, L.D., Gentry, R.F., Rothenbacher, H. and Van der Heide, L., 1976. Infectious tenosynovitis in commercial white Leghorn chickens. *Avian diseases*, pp.769-773.

Sedghi, S., Feizi, A., Mokhtari, S. and Urmia, I., 2013. Clinical Evaluation the efficacy of Tri-Reo vaccine in broiler breeders by ELISA method. *Life Science Journal*, *10*(4s).

Sellers, H.S., Linnemann, E.G., Pereira, L. and Kapczynski, D.R., 2004. Phylogenetic analysis of the sigma 2 protein gene of turkey reoviruses. *Avian diseases*, *48*(3), pp.651-657.

Sellers, H.S., Linnemann, E.G., Gauthiersloan, V., Collett, T., 2013. Diagnostic report: Isolation and characterization of novel reoviruses from clinical cases of tenosynovitis. The 2013 American Association of Avian Pathologist Annual Convention held in Chicago, Illinios, USA from July 20-23, 2013.

Sellers, H.S., 2017. Current limitations in control of viral arthritis and tenosynovitis caused by avian reoviruses in commercial poultry. *Veterinary microbiology*, *206*, pp.152-156.

Senne, D.A., Pearson, J.E., Miller, L.D. and Gustafson, G.A., 1983. Virus isolations from pet birds submitted for importation into the United States. *Avian diseases*, pp.731-744.

Seto, F. Early development of the avian immune system. Poult. Sci. 60:1981-1995. 1981.Shapouri, M.R., Arella, M. and Silim, A., 1996. Evidence for the multimeric nature and cell binding ability of avian reovirus σ3 protein. *Journal of General Virology*, *77*(6), pp.1203-1210.

Sharafeldin, T.A., Mor, S.K., Bekele, A.Z., Verma, H., Goyal, S.M. and Porter, R.E., 2014. The role of avian reoviruses in turkey tenosynovitis/arthritis. *Avian Pathology*, *43*(4), pp.371-378.

Sharafeldin, T.A., Mor, S.K., Bekele, A.Z., Verma, H., Noll, S.L., Goyal, S.M., Porter, R.E., 2015a. Experimentally induced lameness in turkeys inoculated with a newly emergent turkey reovirus. Vet Res. 24, 46(1):11.

Sharafeldin, T.A., Mor, S.K., Sobhy, N.M., Xing, Z., Reed, K.M., Goyal, S.M., Porter, R.E., 2015b. A newly emergent turkey arthritis reovirus shows dominant enteric tropism and induces significantly elevated innate antiviral and T helper-1 cytokine responses. Plos One. 11, 10(12).

Sharafeldin, T.A., Mor, S.K., Goyal, S.M., Porter, R.E., 2016. Altered biomechanical properties of gastrocnemius tendons of turkeys infected with Turkey arthritis reovirus. Vet Med Int. 7829138. https://doi.org/10.1155/2016/7829138

Shivaprasad, H.L., Franca, M., Woolcock, P.R., Nordhausen, R., Day, J.M. and Pantin-Jackwood, M., 2009. Myocarditis associated with reovirus in turkey poults. *Avian diseases*, *53*(4), pp.523-532.

Spackman, E., Pantin-Jackwood, M., Michael Day, J. and Sellers, H., 2005. The pathogenesis of turkey origin reoviruses in turkeys and chickens. *Avian Pathology*, *34*(4), pp.291-296.

Spackman, E., Kapczynski, D. and Sellers, H., 2005. Multiplex real-time reverse transcription–polymerase chain reaction for the detection of three viruses associated with poult enteritis complex: turkey astrovirus, turkey coronavirus, and turkey reovirus. *Avian Diseases*, *49*(1), pp.86-91.

Spackman, E., Day, J.M. and Pantin-Jackwood, M.J., 2010. Astrovirus, reovirus, and rotavirus concomitant infection causes decreased weight gain in broad-breasted white poults. *Avian diseases*, *54*(1), pp.16-21.

Shen, P.C., Chiou, Y.F., Liu, H.J., Song, C.H., Su, Y.P. and Lee, L.H., 2007. Genetic variation of the λA and λC protein encoding genes of avian reoviruses. *Research in veterinary science*, *83*(3), pp.394-402.

Shien, J.H., Yin, H.S. and Lee, L.H., 2000. An enzyme-linked immunosorbent assay for the detection of antibody to avian reovirus by using protein σ B as the coating antigen. *Research in veterinary science*, *69*(2), pp.107-112.

Shih, W.L., Hsu, H.W., Liao, M.H., Lee, L.H. and Liu, H.J., 2004. Avian reovirus σC protein induces apoptosis in cultured cells. *Virology*, *321*(1), pp.65-74.

Shmulevitz, M., Yameen, Z., Dawe, S., Shou, J., O'Hara, D., Holmes, I. and Duncan, R., 2002. Sequential partially overlapping gene arrangement in the tricistronic S1 genome segments of avian reovirus and Nelson Bay reovirus: implications for translation initiation. *Journal of virology*, *76*(2), pp.609-618.

Smart, I.J., Barr, D.A., Reece, R.L., Forsyth, W.M. and Ewing, I., 1988. Experimental reproduction of the runting-stunting syndrome of broiler chickens. *Avian Pathology*, *17*(3), pp.617-627.

Sommerstein, R., Flatz, L., Remy, M.M., Malinge, P., Magistrelli, G., Fischer, N., Sahin, M., Bergthaler, A., Igonet, S., Ter Meulen, J. and Rigo, D., 2015. Arenavirus glycan shield promotes neutralizing antibody evasion and protracted infection. *PLoS pathogens*, *11*(11), p.e1005276.

Songserm, T., Zekarias, B., Van Roozelaar, D.J., Kok, R.S., Pol, J.M.A., Pijpers, A. and ter Huurne, A.A.H., 2002. Experimental reproduction of malabsorption syndrome with different combinations of reovirus, Escherichia coli, and treated homogenates obtained from broilers. *Avian diseases*, *46*(1), pp.87-94.

Songserm, T., Van Roozelaar, D., Kant, A., Pol, J., Pijpers, A. and ter Huurne, A., 2003. Enteropathogenicity of Dutch and German avian reoviruses in SPF white leghorn chickens and broilers. *Veterinary research*, *34*(3), pp.285-295.

Spandidos, D.A. and Graham, A.F., 1976. Physical and chemical characterization of an avian reovirus. *Journal of virology*, *19*(3), pp.968-976.

Spradbrow, P.B. and Bains, B.S., 1974. Reoviruses from chickens with hydropericardium. *Australian veterinary journal*, *50*(4), p.179.

Springer, W.T., Olson, N.O., Kerr, K.M. and Fabacher, C.J., 1983. Responses of specific-pathogen-free chicks to concomitant infections of reovirus (WVU-2937) and infectious bursal disease virus. *Avian diseases*, pp.911-917.

Spenser, E.L., 1991. Common infectious diseases of psittacine birds seen in practice. *Veterinary Clinics of North America: Small Animal Practice*, *21*(6), pp.1213-1230.

Savage, C.E. and Jones, R.C., 2003. The survival of avian reoviruses on materials associated with the poultry house environment. *Avian Pathology*, *32*(4), pp.417-423.

Steinhauer, D.A. and Holland, J.J., 1987. Rapid evolution of RNA viruses. *Annual Reviews in Microbiology*, *41*(1), pp.409-431.

Souza, S.O., De Carli, S., Lunge, V.R., Ikuta, N., Canal, C.W., Pavarini, S.P. and Driemeier, D., 2018. Pathological and molecular findings of avian reoviruses from clinical cases of tenosynovitis in poultry flocks from Brazil. *Poultry science*, *97*(10), pp.3550-3555.

Stott, J.L., 1999. Reoviridae, in: Hirsh, D.C., Zee, Y.C. (Eds.), Veterinary Microbiology. Blackwell Science, Malden, MA, pp. 430-438.

Su, Y.P., Su, B.S., Shien, J.H., Liu, H.J. and Lee, L.H., 2006. The sequence and

phylogenetic analysis of avian reovirus genome segments M1, M2, and M3 encoding the minor core protein μA, the major outer capsid protein μB, and the nonstructural protein μNS. *Journal of virological methods*, *133*(2), pp.146-157.

Subramanyam, P. and Pomeroy, B.S., 1960. Studies on the Fahey-Crawley virus. *Avian Diseases*, *4*(2), pp.165-175.

Stading, B., Ellison, J.A., Carson, W.C., Satheshkumar, P.S., Rocke, T.E. and Osorio, J.E., 2017. Protection of bats (Eptesicus fuscus) against rabies following topical or oronasal exposure to a recombinant raccoon poxvirus vaccine. *PLoS Neglected Tropical Diseases*, *11*(10), p.e0005958.

Swayne, D.E., Garcia, M., Beck, J.R., Kinney, N. and Suarez, D.L., 2000. Protection against diverse highly pathogenic H5 avian influenza viruses in chickens immunized with a recombinant fowlpox vaccine containing an H5 avian influenza hemagglutinin gene insert. *Vaccine*, *18*(11-12), pp.1088-1095.

Swayne, D. E., and J. R. Glisson. 2013. Diseases of poultry. Wiley-Blackwell, Ames, Iowa.

Takase K, Nishikawa H, Katsuki N, Yamada S. 1984a. Pathogenicity of avian reoviruses isolated from tendons and tendon sheaths of chickens with leg weakness. *The Japanese Journal of Veterinary Science,* 46:467-473.

Takase, K., Nishikawa, H., Nonaka, F. and Yamada, S., 1984b. Isolation of avian reoviruses from tendons and tendon sheaths of chickens with leg weakness and some characteristics of the isolates. *Journal of the Japan Veterinary Medical Association (Japan)*.

Takase, K., Fujikawa, H. and Yamada, S., 1996. Correlation between neutralizing antibody titre and protection from tenosynovitis in avian reovirus infections. *Avian Pathology*, *25*(4), pp.807-815.

Takehara, K., Kawai, C., Seki, A., Hashimoto, N. and Yoshimura, M., 1989. Identification and characterization of an avian reovirus isolated from black-tailed gull (Larus crassirostris). *The Kitasato archives of experimental medicine*, *62*(4), pp.187-198.

Tang, K.N., Fletcher, O.J. and Villegas, P., 1987. The effect on newborn chicks of oral inoculation of reovirus isolated from chickens with tenosynovitis. *Avian diseases*, pp.584-590.

Tang, K.N. and Fletcher, O.J., 1987. Application of the avidin-biotin-peroxidase complex (ABC) technique for detecting avian reovirus in chickens. *Avian diseases*, pp.591-596.

Tang, Y. and Lu, H., 2015a. Genomic characterization of a broiler reovirus field strain detected in Pennsylvania. *Infection, genetics and evolution*, *31*, pp.177-182.

Tang, Y. and Lu, H., 2015b. Genomic characterization of a novel avian arthritis orthoreovirus variant by next-generation sequencing. *Archives of virology*, *160*(10), pp.2629-2632.

Tang, Y., Lu, H., Sebastian, A., Yeh, Y.T., Praul, C.A., Albert, I.U. and Zheng, S.Y., 2015. Genomic characterization of a turkey reovirus field strain by Next-Generation Sequencing. *Infection, Genetics and Evolution*, *32*, pp.313-321.

Ter Veen, C., De Bruijn, N.D., Dijkman, R. and De Wit, J.J., 2017. Prevalence of histopathological intestinal lesions and enteric pathogens in Dutch commercial broilers with time. *Avian Pathology*, *46*(1), pp.95-105.

Tourís-Otero, F., Cortez-San Martín, M., Martínez-Costas, J. and Benavente, J., 2004. Avian reovirus morphogenesis occurs within viral factories and begins with the selective recruitment of sigmaNS and lambdaA to microNS inclusions. *Journal of molecular biology*, *341*(2), pp.361-374.

Thi Nguyen, M.A., Klaere, S. and von Haeseler, A., 2011. MISFITS: evaluating the goodness of fit between a phylogenetic model and an alignment. *Molecular biology and evolution*, *28*(1), pp.143-152.

Theophilos MB, Huang JA, Holmes IH. Avian reovirus sigma C protein contains a putative fusion sequence and induces fusion when expressed in mammalian cells. Virology 1995; 208:678–84, http://dx.doi.org/10.1006/viro.1995.1199.

Trapido H, Sanmartin C (1971) Pichinde virus, a new virus of the Tacaribe group from Colombia. Am J Trop Med Hyg 20:631–641

Trifinopoulos, J., Nguyen, L.T., von Haeseler, A. and Minh, B.Q., 2016. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic acids research*, *44*(W1), pp.W232-W235.

Troxler, S., Rigomier, P., Bilic, I., Liebhart, D., Prokofieva, I., Robineau, B. and Hess, M., 2013. Identification of a new reovirus causing substantial losses in broiler production in France, despite routine vaccination of breeders. *Veterinary Record*, *172*(21), pp.556-556.

Tsukamoto, K., Kojima, C., Komori, Y., Tanimura, N., Mase, M. and Yamaguchi, S., 1999. Protection of chickens against very virulent infectious bursal disease virus (IBDV) and Marek's disease virus (MDV) with a recombinant MDV expressing IBDV VP2. *Virology*, *257*(2), pp.352-362.

Valentino, K. and Poronsky, C.B., 2016. Human papillomavirus infection and vaccination. *Journal of pediatric nursing*, *31*(2), pp.e155-e166.

Valtierra, H.N., 2008. Stability of viral pathogens in the laboratory environment. *Applied Biosafety*, *13*(1), pp.21-26.

van den Brand, J.M., Manvell, R., Paul, G., Kik, M.J. and Dorrestein, G.M., 2007.

Reovirus infections associated with high mortality in psittaciformes in The Netherlands. *Avian Pathology*, *36*(4), pp.293-299.

Van der Heide, L., Geissler, J. and Bryant, E.S., 1974. Infectious tenosynovitis: serologic and histopathologic response after experimental infection with a Connecticut isolate. *Avian diseases*, pp.289-296.

Van der Heide, L. and Kalbac, M., 1975. Infectious tenosynovitis (viral arthritis): characterization of a Connecticut viral isolant as a reovirus and evidence of viral egg transmission by reovirus-infected broiler breeders. *Avian diseases*, pp.683-688.

Van der Heide, L., 1977. Viral arthritis/tenosynovitis: a review. *Avian pathology*, *6*(4), pp.271-284.

Van der Heide, L., Kalbac, M. and Brustolon, M., 1983. Development of an attenuated apathogenic reovirus vaccine against viral arthritis/tenosynovitis. *Avian diseases*, pp.698-706.

Van der Heide, L., 1996. Introduction on avian reovirus. *Proc. International Synposium on Adenovirus and Reovirus Infection in Poultry, 1996*, pp.138-142.

Vindevogel, H., Meulemans, G., Pastoret, P.P., Schwers, A. and Calberg-Bacq, C.M., 1982. Reovirus infection in the pigeon. In *Annales de Recherches Vétérinaires* (Vol. 13, No. 2, pp. 149-152).

Van Loon, A.A.W.M., Koopman, H.C., Kosman, W., Mumczur, J., Szeleszczuk, O., Karpinska, E., Kosowska, G. and Lütticken, D., 2001. Virology: Isolation of a new serotype of avian reovirus associated with malabsorption syndrome in chickens. *Veterinary quarterly*, *23*(3), pp.129-133.

Varela, R. and Benavente, J., 1994. Protein coding assignment of avian reovirus strain S1133. *Journal of virology*, *68*(10), pp.6775-6777.

Varela, R., Martínez-Costas, J., Mallo, M. and Benavente, J., 1996. Intracellular posttranslational modifications of S1133 avian reovirus proteins. *Journal of virology*, *70*(5), pp.2974-2981.

Vasserman, Y., Eliahoo, D., Hemsani, E., Kass, N., Ayali, G., Pokamunski, S. and Pitcovski, J., 2004. The influence of reovirus sigma C protein diversity on vaccination efficiency. *Avian diseases*, *48*(2), pp.271-278.

Veits, J., Lüschow, D., Kindermann, K., Werner, O., Teifke, J.P., Mettenleiter, T.C. and Fuchs, W., 2003. Deletion of the non-essential UL0 gene of infectious laryngotracheitis (ILT) virus leads to attenuation in chickens, and UL0 mutants expressing influenza virus haemagglutinin (H7) protect against ILT and fowl plague. *Journal of general virology*, *84*(12), pp.3343-3352.

Walsum, J.V., 1977. Contribution to the aetiology of synovitis in chickens, with special reference to non-infective factor. III. *Tijdschrift voor diergeneeskunde*, *102*(14), pp.793-800.

Wechuck, J.B., Ozuer, A., Goins, W.F., Wolfe, D., Oligino, T., Glorioso, J.C. and Ataai, M.M., 2002. Effect of temperature, medium composition, and cell passage on production of herpes-based viral vectors. *Biotechnology and bioengineering*, *79*(1), pp.112-119.

Wickham, H., 2009. *ggplot2: elegant graphics for data analysis*. Springer New York.

Wickramasinghe, R., Meanger, J., Enriquez, C.E. and Wilcox, G.E., 1993. Avian reovirus proteins associated with neutralization of virus infectivity. *Virology*, *194*(2), pp.688-696.

Wilcox, G.E., Robertson, M.D. and Lines, A.D., 1985. Adaptation and characteristics of replication of a strain of avian reovirus in Vero cells. *Avian Pathology*, *14*(3), pp.321-328.

Woolcock, P.R. and Shivaprasad, H.L., 2008. Electron microscopic identification of viruses associated with poult enteritis in turkeys grown in California 1993–2003. *Avian diseases*, *52*(2), pp.209-213.

Wood, G.W., Muskett, J.C. and Thornton, D.H., 1986. Observations on the ability of avian reovirus vaccination of hens to protect their progeny against the effects of challenge with homologous and heterologous strains. *Journal of comparative pathology*, *96*(2), pp.125-129.

Wood, G.W., Nicholas, R.A.J., Hebert, C.A. and Thornton, D.H., 1980. Serological comparisons of avian reoviruses. *Journal of comparative pathology*, *90*(1), pp.29-38.

Wood, G.W. and Thornton, D.H., 1981. Experimental infection of broiler chickens with an avian reovirus. *Journal of comparative pathology*, *91*(1), pp.69-76.

Wu, H., He, Z., Tang, J., Li, X., Cao, H., Wang, Y. and Zheng, S.J., 2016. A critical role of LAMP-1 in avian reovirus P10 degradation associated with inhibition of apoptosis and virus release. *Archives of virology*, *161*(4), pp.899-911.

Xu, W., Patrick, M.K., Hazelton, P.R. and Coombs, K.M., 2004. Avian reovirus temperature-sensitive mutant tsA12 has a lesion in major core protein σA and is defective in assembly. *Journal of virology*, *78*(20), pp.11142-11151.

Xu, W. and Coombs, K.M., 2009. Conserved structure/function of the orthoreovirus major core proteins. *Virus research*, *144*(1-2), pp.44-57.

Yin, H.S. and Lee, L.H., 1998. Identification and characterization of RNA-binding activities of avian reovirus non-structural protein sigmaNS. *Journal of general virology*, *79*(6), pp.1411-1413.

Xie, Z., Qin, C., Xie, L., Liu, J., Pang, Y., Deng, X., Xie, Z. and Khan, M.I., 2010. Recombinant protein-based ELISA for detection and differentiation of antibodies against avian reovirus in vaccinated and non-vaccinated chickens. *Journal of virological methods*, *165*(1), pp.108-111.

Xing, J., Ly, H. and Liang, Y., 2015. The Z proteins of pathogenic but not nonpathogenic arenaviruses inhibit RIG-I-like receptor-dependent interferon production. *Journal of virology*, *89*(5), pp.2944-2955.

Yang, Z.J., Wang, C.Y., Lee, L.H., Chuang, K.P., Lien, Y.Y., Yin, H.S., Tong, D.W., Xu, X.G. and Liu, H.J., 2010. Development of ELISA kits for antibodies against avian reovirus using the σC and σB proteins expressed in the methyltropic yeast Pichia pastoris. *Journal of virological methods*, *163*(2), pp.169-174.

Yin, H.S. and Lee, L.H., 1998. Development and characterization of a nucleic acid probe for avian reoviruses. *Avian Pathology*, *27*(4), pp.423-426.

Yin, H.S., Shien, J.H. and Lee, L.H., 2000. Synthesis in Escherichia coli of avian reovirus core protein ςA and its dsRNA-binding activity. *Virology*, *266*(1), pp.33-41.

Yin, H.S., Su, Y.P. and Lee, L.H., 2002. Evidence of nucleotidyl phosphatase activity associated with core protein σA of avian reovirus S1133. *Virology*, *293*(2), pp.379-385.

Zhang, X., Ji, Y., Zhang, L., Harrison, S.C., Marinescu, D.C., Nibert, M.L. and Baker, T.S., 2005. Features of reovirus outer capsid protein μ1 revealed by electron cryomicroscopy and image reconstruction of the virion at 7.0 Å resolution. *Structure*, *13*(10), pp.1545-1557.

Zhang, Y., Liu, M., Shuidong, O., Hu, Q.L., Guo, D.C., Chen, H.Y. and Han, Z., 2006. Detection and identification of avian, duck, and goose reoviruses by RT-PCR: goose and duck reoviruses are part of the same genogroup in the genus Orthoreovirus. *Archives of virology*, *151*(8), pp.1525-1538.

Zhang, X., Lei, X., Ma, L., Wu, J. and Bao, E., 2019. Genetic and pathogenic characteristics of newly emerging avian reovirus from infected chickens with clinical arthritis in China. *Poultry science*, *98*(11), pp.5321-5329.

Zhong, L., Gao, L., Liu, Y., Li, K., Wang, M., Qi, X., Gao, Y. and Wang, X., 2016. Genetic and pathogenic characterisation of 11 avian reovirus isolates from northern China suggests continued evolution of virulence. *Scientific reports*, *6*(1), pp.1-10.

Zhou, N.N., Senne, D.A., Landgraf, J.S., Swenson, S.L., Erickson, G., Rossow, K., Liu, L., Yoon, K.J., Krauss, S. and Webster, R.G., 1999. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. *Journal of virology*, *73*(10), pp.8851-8856.

Zhang, Y., Guo, D., Liu, M., Geng, H., Hu, Q., Liu, Y. and Liu, N., 2007.

Characterization of the σB-encoding genes of Muscovy duck reovirus: σC–σB-ELISA for antibodies against duck reovirus in ducks. *Veterinary microbiology*, *121*(3-4), pp.231-241.

Zhang, K.Y., Gao, Y.Z., Du, M.Z., Liu, S., Dong, C. and Guo, F.B., 2019. Vgas: a viral genome annotation system. Front Microbiol 10: 184.