PATHOGENICITY OF VARIANT FIELD ISOLATES OF AVIAN REOVIRUS AND MOLECULAR CHARACTERIZATION OF BRAZILIAN VARIANTS FROM COMMERCIAL BROILERS

by

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(Under the Direction of Holly S. Sellers)

ABSTRACT

Avian reoviruses are the causative agent of arthritis/tenosynovitis in broilers and turkeys. Since 2011, it has been observed an increased incidence of variant reoviruses inducing tenosynovitis in commercial poultry flocks in the U.S and worldwide, raising the question of cross species infection. Two turkey reoviruses isolates from clinical cases of tenosynovitis in commercial turkey breeders, 105057 and 105208, demonstrated to be pathogenic to commercial broilers, causing tenosynovitis with clinical signs of lameness (105057) and also, enteric disease (105208). Additionally, viral shedding was poor, demonstrating limited horizontal transmission. In commercial turkeys these isolates caused clinical disease and were efficiently shed. In addition, a chicken arthritis reovirus was inoculated in turkeys, but did not cause disease. Taken together, turkey arthritis reoviruses 105057 and 105208 represent a threat for the poultry industry. Additionally, it was determined for the first time the molecular characterization of arthritis reovirus variants from lame commercial broilers from Brazil.

INDEX WORDS: Avian reovirus, chicken reovirus, turkey reovirus, tenosynovitis, arthritis, myocarditis, broilers, turkeys, FTA® cards

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DEDICATION

I would like to dedicate this thesis to my parents that always encouraged me to study, and especially to my wife Angelica for all her love and support.

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CHAPTER 1

INTRODUCTION

Avian reoviruses (ARV) have a worldwide distribution among poultry flocks (4), but are also the causative agents of tenosynovitis in chickens and turkeys (5, 7). ARV causing tenosynovitis was first isolated in chickens in 1968 and soon after, commercial vaccines were developed, providing good protection to chickens (11), although sporadic outbreaks have been documented over the years. In turkeys, turkey arthritis reovirus (TARV) was first described in 1980, but only a few cases were reported after that, however, enteric disease associated to reovirus in turkeys has played a major role for many years (3).

In 2011, turkey arthritis reovirus reemerged, causing clinical signs of lameness and swelling of hock joints in commercial turkeys in Minnesota. Reoviruses were isolated and characterized as variants, that is, highly distinct genetically compared with commercial vaccines strains and closely related field strains (6). An increased incidence of tenosynovitis caused by reovirus was observed since 2011 in broilers as well, even in progeny from reovirus-vaccinated breeder flocks (9). Molecular characterization, again, revealed high divergence to vaccine related strains (3, 6). The emergence of reovirus variants inducing tenosynovitis in commercial poultry and the close proximity of sites of turkey and broiler production in the U.S brought the concern about interspecies pathogenicity of new variants.

The establishment of reovirus to be the causative agent of tenosynovitis is clear when virus is isolated from tendons of affected birds (9). Reovirus can be isolated in chicken liver cells, chickens embryo fibroblast and VERO cells, and characteristic cytopathic effect of syncytia formation is observed in monolayers of infected cells (2). Isolates are characterized using RT-PCR followed by sequencing (genotyping) of the sigma C. The sigma C protein is the target for molecular characterization of avian reoviruses and is the minor outer capsid protein, responsible for cell attachment, as well as, induction of type specific neutralizing antibodies (9). Pairwise comparison of the prediction of amino acid sequence is performed to examine the degree of sequence identity between field strains and reference strains from GenBank. Construction of phylogenetic trees and analysis for conservation of the σ C protein sequences reveals that avian reoviruses group in 5 distinct genotype clusters, according to amino acid identity (6). Therefore, to evaluate if amino acid similarities translate into some level of cross protection with current vaccine strains, cross neutralization test is performed using a panel of antisera from commercial vaccine strains and hyperimunne sera from field isolates, and titer obtained in this test determine serological relatedness between strains. Additionally, *in vivo* studies are a useful tool to establish pathogenicity of new strains of avian reovirus (9).

Various samples from clinical cases of tenosynovitis have been submitted to the Poultry Diagnostic and Research Center (PDRC) at The University of Georgia, since 2011. In the present work, two field isolates were investigated, TARVs 105057 and 105208, from commercial turkey breeders, in commercial broilers. Then, these two isolates, in addition to a chicken arthritis reovirus (CARV) isolate, 94826, were tested in

commercial turkeys. These studies aimed to determine the pathogenicity of these isolates in chickens and turkeys and whether they can reproduce disease as observed in the original clinical case from which they were isolated. TARV 105057 and 105208 were pathogenic to commercial broilers, demonstrated by the presence of arthritis and tenosynovitis, as well as enteric disease. In turkeys, both TARVs reproduced the disease in susceptible poults; however, the CARV was not pathogenic to turkeys.

Variants of avian reovirus have been reported worldwide. In Europe some variants of reovirus associated with tenosynovitis and malabsorption have been previously documented (10, 12). In Brazil, the first report of arthritis induced by reovirus in commercial broilers was in 1975 (1). However, recently, reovirus was detected from young immunosuppressed poults and molecular characterization indicated genetic similarity to some turkey reoviruses from the United States (8). In 2015, an increased incidence of condemnations caused by arthritis was observed in a slaughterhouse in Brazil. Five FTA cards containing impressions of hearts, synovial fluid and tendons from lame broiler flocks from the same region of the slaughterhouse were submitted to PDRC for RT-PCR and sequencing. New variants of avian reovirus were detected and genetically characterized. This was the first molecular characterization of avian reovirus variants in commercial broilers associated to high condemnation caused by arthritis in Brazil.

In the present work, it was established that two turkey arthritis reoviruses, recently isolated from commercial turkey breeders, cause arthritis and enteric disease in commercial broilers, however, a variant chicken reovirus tested was harmless to turkeys. Additionally, the present work provided, for the first time, molecular characterization of

Brazilian avian reovirus variants from commercial broilers with clinical signs of arthritis, suggesting that avian reovirus is still producing variants worldwide.

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CHAPTER 2

LITERATURE REVIEW

Introduction

Avian reoviruses are widespread in commercial poultry flocks and are involved in many disease conditions, including arthritis, tenosynovitis, enteric and respiratory diseases, myocarditis, hepatitis and the so-called stunting/malabsorption syndrome, however, is estimated that 85-90% of reovirus isolates are nonpathogenic (4, 31). The name reovirus was proposed in 1959, when the virus was isolated from respiratory and enteric tract, being called respiratory enteric orphan (REO) virus (77). Avian reovirus (ARV) was first isolated by Fahey and Crawley from birds with respiratory disease in 1954 (12), however, in 1968 viral arthritis/tenosynovitis in chickens (69) was first reported. Affected birds showed clinical signs of swelling and edema of the hock joint, wing joint and digital flexor tendons, with gastrocnemius tendon rupture, and was referred to as virus arthritis agent (69). In turkeys, reovirus causing tenosynovitis was first isolated in 1980 from a 15-week-old flock, where birds had clinical signs of lameness, swollen hock joints and stiffness in leg joints. Arthritis and tenosynovitis caused by reovirus have been extensively been reported in chickens since its first isolation until the present day, however, in turkeys, reovirus was predominantly associated with enteric disease for nearly 30 years. Since 2011, turkey arthritis reemerged in the United States of America causing significant economic impact (48, 59, 61, 81).

Economic losses from viral arthritis/tenosynovitis are due to poor growth and feed conversion, mainly through the inability of lame birds to reach feed and downgrading quality of carcasses at slaughter due to the unsightly appearance of affected hock joint (31).

Literature Review

Viral Classification

The family *Reoviridae* is the largest of the eight recognized double-stranded RNA (dsRNA) virus families and has a wide host range which includes insects, plants, fish, reptiles, birds, mammals, arachnids, fungi, arthropods and crustaceans (57). Avian reovirus (ARV) belongs to the *Orthoreovirus* genus, one of 11 genera in the *Reoviridae* family. The *Orthoreovirus* genus is divided into two subgroups, fusogenic and non fusogenic, based on the ability to cause syncytia formation in cell culture, and they have been isolated from a broad range of mammalian, avian, and reptilian hosts (31). Avian reovirus (ARV) differs from mammalian reovirus in its ability to induce fusion of host cells (106), but lacks hemagglutination activity (16).

Physiochemical properties

Avian Reoviruses are non-enveloped, have icosahedral symmetry and are 70 to 80 nm in diameter (16, 90). The genome is composed of 10 segments of double-stranded RNA, classified by size into 3 classes, large (L1, L2 and L3), medium (M1, M2, M3) and small (S1, S2, S3 and S4), based on electrophoretic mobility (4). The segments encode for at least 8 structural and four nonstructural proteins (6, 101). The proteins encoded by the three classes are designated lambda (λ) for the L-class, mu (μ) for the M-class and

sigma (σ) for the S class. There are 3 lambda structural proteins (λA, λB and λC), two mu (μA, μB), three sigma (σA, σB and σC) and μBN and μBC, that originate by post-translational cleavage of their precursor μB. There are two nonstructural proteins, μNS and σNS, encoded by the M3 and S4 genes, respectively. Additionally, nonstructural proteins p10 and p17 are encoded by the first two cistrons of the tricistronic avian reovirus S1 genome segment (4, 80, 84). Nine of the segments are monocistronic and encode a single protein, while segment S1 is tricistronic with partially overlapped reading frames encoding three proteins (4, 85).

Reoviruses are ether resistant (77) and some strains resistant to 2% formaldehyde. Partial inactivation with 2% phenol after 24 hours at room temperature has been reported, as well as complete inactivation with 100% ethyl alcohol (58). Avian reoviruses are stable between pH 3.0 to 9.0 and resistant to lipid solvents, however, they can be inactivated at 56°C in less than 1 hour (90). ARV can survive for 10 days in feathers, wood shavings, glass rubber and galvanic material (16, 31). Also, no hemagglutination activity has been observed with chicken, bovine, turkey or human erythrocytes (16). Avian reovirus sensitivity to trypsin is variable, not related to antigenic configuration or species of origin. In trypsin-sensitive reovirus strains, replication is poor in intestine following oral exposure, limiting dissemination to other tissues (91).

Viral Genome

L class

The L (large) class of avian reovirus is composed of three segments (L1, L2 and L3), and each one encode a distinct protein, designated by λ (A, B, C) (101).

Lambda A (λA)

The L1 gene, is 3958 nucleotides long, codes for the λA protein (a 1293 residue protein) that forms the core capsid shell that encloses both the virus genome segments and the viral RNA polymerase, and that is used as a scaffold for subsequent core assembly (109). Protein λA associates very rapidly with avian reovirus factories in infected cells, and it is diffusely distributed in the cytoplasm of transfected cells when expressed alone, but becomes associated with globular inclusions when co-expressed with μNS, suggesting that μNS mediates the recruitment of λA into viral factories (93).

Lambda B (λB)

The segment L2 encodes λB protein, which is a minor component of the viral core. ARV L2 is 3829 base pairs in length and is predicted to encode λB, an RNA dependent RNA polymerase protein of 1259 amino acids in length and required for viral propagation (4, 108).

Lambda C (λC)

Lambda C is a 142 Kda protein encoded by the L3 genome segment, a 3907 nucleotide long segment of the avian reovirus genome and forms core turret proteins of 1285 residues. It is known that the core turret mediates guanylyltransferase reaction in cap formation (27).

M Class

The M class is formed by 3 segments (M1, M2, M3), which encodes *μA, μB and μNS,* respectively (101).

Mu A (μA)

The M1 segment is formed by 2283 nucleotides and encodes protein μA, a 732 amino acid protein that forms the minor core (66). The properties and function of this protein are not well known in avian species, but its mammalian reovirus counterpart μ2 has been shown to interact with microtubules and with the nonstructural protein μNS, and these interactions are thought to be responsible for anchoring viral factories to microtubules, and therefore most mammalian reovirus strains form factories with a filamentous arrangement (71). The reovirus μ2 protein has been proposed to be a transcriptase cofactor, playing a role in viral RNA synthesis, but it remains the least understood, functionally and structurally, of the eight proteins found in virions (114).

Mu B (μB)

The M2 segment, a 2158 nucleotide long, is responsible for the primary translation product of the avian reovirus genome, μB, the major outer capsid protein (53). The μB protein is formed by 676 amino acids and is involved in virus entry and transcriptase activation, suggesting that a host-specific influence on ARV entry and/or uncoating may affect the likelihood of the virus establishing a productive infection in a macrophage cell (67). μB molecules cleave near the amino terminus to produce a myristoylated amino-terminal peptide, termed μBN, and a large carboxy-terminal protein, termed μBC, being both μB and its products structural products of the reovirion (4, 102).

Mu NS (μNS)

The M3 genome segment encodes the nonstructural protein μNS, which is 1996 nucleotides long and contains a long open reading frame that encodes a polypeptide of 635 amino acid residues starting at nucleotide 25 and ending at nucleotide 1929. uNS

amino acid sequence reveled the existence of two heptapeptide repeat motifs between positions 451-472 and 540-599, with primarily hydrophobic amino acids in the first and fourth positions, as characteristic of alpha helical regions of proteins to form coil coil super helices (94). It is suggested that a proportion of the μNS molecules present in infected cells are cleaved near the N-terminus to yield a 15 kDa-amino-terminal fragment, designated μNSN, and a 55 kDa-carboxy-terminal protein, designated μNSC (4). μNS interacts with σNS but not with μNSC. They co-localize in avian reovirus infected cells supporting association during viral infection (94). The expression of individual viral proteins in transfected cells has shown that avian reovirus μNS is the only viral protein capable of forming inclusions when expressed individually, suggesting that μNS is the minimal viral factor required for inclusion formation (94).

S class

The S class is formed by 4 segments (S1, S2, S3 and S4), which encodes structural proteins σA, σB and σC, as well as nonstructural proteins p10, p17 and σNS (101).

Sigma C (σC)

Segment S1 gene is 1644 nucleotide long and encodes protein σC in avian reoviruses, which is known to be responsible for viral-cell attachment (4, 101). Sigma C protein is formed by 326 amino acids residue and is an outer capsid protein of avian reovirus, encoded by the 3rd proximal cistron of the S1 genome segment and is a homotrimer in its native state; however, it has been shown that its cell attachment activity is exclusively associated with its oligomeric form (4, 19). Sigma C consists of two domains: the “head”, which is located at the C-terminal end of the protein; and the

“shaft”, located at the N-terminus. The carboxy-terminal domain is predicted to play a key role in receptor binding and responsible for primary host cell attachment. The carboxy-terminal globular domain has a beta-barrel-fold with the same overall topology as the mammalian reovirus fiber (sigma1). However, the monomers of the avian reovirus σC trimer show a more splayed-out arrangement than in the mammalian σ1 structure. Also, there are two triple beta-spiral repeats of the shaft or stalk domain. The presence of heptad repeats amino-terminal to the triple beta-spiral repeats suggests that the portion of the shaft domain contains a triple alpha-helical coiled-coil structure (20). σC is the only viral protein present in soluble extracts of infected cells capable of attachment to avian cell monolayers. Sigma C also elicits reovirus-specific neutralizing antibodies. Moreover, the attachment of σC to cultured cells can be completed by purified reovirions, and the attachment of avian reovirions to permissive cells can be blocked by pre-incubating the cells with protein σC. A C-terminal fragment of protein σC (residues 151–326) containing the receptor-binding globular domain has recently been crystallized. This fragment has the same topology as the head domain of the mammalian reovirus cell attachment protein, plus two repeats of a triple beta-spiral at residues 157–194. The structure of a receptor-binding fragment from Sigma C, encompassing amino acid residues 160–191, forms a triple β-spiral while residues 196–326 form a b-barrel head domain (20).

It is also suggested that σC induces apoptosis (84). Further studies demonstrated evidence of apoptosis in injured tissues following ARV infection and detection of the σC protein by RT-PCR suggests a correlation between virus replication and apoptosis in chicken tissues (41).

Protein p10

The S1 segment of avian reovirus genome is 1644 nucleotide long and is a functionally tricistronic gene that encodes the nonstructural protein p10 from open reading frame 1 (5). p10 is a multifunctional protein that plays key roles in virus-host interaction. The p10 protein is involved in cell arrest growth, and enhances membrane permeability, occurring during late infection times. Protein p10 has been shown to display fusogenic activity (5, 86). Therefore, membrane leakiness is observed following transient expression of p10 in several cell lines. In addition, the fusogenic extracellular NH2-terminal domain of p10 appears to be dispensable for permeabilizing activity, because its deletion entirely abolished the fusogenic activity of p10, without affecting its ability to associate with cell membranes and to enhance membrane permeability (6).

Protein p17

The S1 segment of avian reovirus genome also encodes the nonstructural protein p17 (5) in the second open reading frame (4). p17 accumulates in the nucleus of infected cells and mutational analysis identified a functional nuclear localization signal near its C-terminus. In addition, p17 is a nucleocytoplasmic shuttling protein with nucleocytoplasmic distribution coupled to transcriptional activity of the cell (8).

Sigma A (σA)

Protein σA, (416 amino acids) encoded by the S2 segment (1326 nucleotide length), is a component of the inner core shell and possesses sequence-independent dsRNA-binding activity (52, 53). The role of protein σA in binding dsRNA was tested *in vitro* by incubating the dsRNA with σA in high salt concentrations and resulted in high affinity binding. Avian reoviruses are highly resistant to the antiviral effect of interferon

and it is suggested that the double-stranded RNA (dsRNA) binding σA protein might play an important role in that resistance, therefore, protein σA is capable of reversing the interferon-induced antiviral state by down-regulating double-stranded RNA-dependent protein kinase activity (PKR) (11, 18). σA distribution colocalizes with μNS in the cytoplasmic viral factories and a minority but significant fraction co-localized with fibrillarin in the nucleus. σA can be detected in the nucleus of avian cells, suggesting that its nuclear distribution is controlled by species specific factors or alternative posttranslational modification (103).

Sigma B (σB)

The S3 (1101 nucleotide long) encoded protein, σB, is a major component of the reovirion outer capsid (102). σB protein is conserved among avian reoviruses, formed by 367 amino acids, σB is a highly variable protein and possesses group-specific neutralizing epitopes (24, 111).

Sigma NS (σNS)

The nonstructural protein σNS (367 amino acids) is encoded by the avian reovirus S4 genome segment, a 1192 nucleotide long gene, having nucleic acid binding activity, in particular, affinity of σNS for ssRNAs and no detectable binding activity to dsRNA and dsDNA (112, 113). The protein σNS participates in ARV morphogenesis, initiated by μNS forming reovirus factories and recruiting together with λA and σNS to induce core assembly and core coating (93). Current findings also showed that σNS is present in large ribonucleoprotein complexes in the cytoplasm of avian reovirus infected cells, indicating that it exists closely associated with ssRNAs (95).

Replication cycle

Avian reovirus uses the outer capsid protein σC to interact with the cell surface receptor. The cell receptor for avian reovirus is not known (4). For mammalian reoviruses, sequences in the N-terminus of the σ1(the analog of ARV σC) tail bind to a carbohydrate, which is known to bind to sialic acid in either α2,3 or α2,6 linkages and the C-terminus of the σ1 head binds to junctional adhesion molecule-1 (JAM-1). In contrast, sialic acid unlikely plays a role in avian reovirus attachment, however, avian reoviruses are able to attach and replicate, not only in avian cells, but also in most mammalian cells, suggesting that the avian reovirus receptor is a ubiquitous cell surface protein (2).

Reoviruses are non-enveloped and gain entry into the cell by receptor-mediated endocytosis, and may pass through changes in the viral particle, including proteolysis and/or conformational modifications of specific capsid proteins, required to acquire membrane-crossing ability (7, 51). Also, it has been proposed that ARV entry is caveolin mediated and dynamin 2 dependent (28). After endocytosis, avian reovirus requires transport to early endosomes before an acidic pH dependent step presumably leads to the release of viral cores into the cytoplasm, resulting in productive infection.

Virus uncoating occurs in intracellular vacuoles, largely dependent on endosome-mediated proteolytic processing of the major outer capsid protein μBC (4). Following uncoating, viral cores are released into the cytoplasm to initiate transcription of the viral genome (4).

Avian reovirus transcripts, which are identical to the positive strands of their encoding genes, possess a type-1 cap at their 5’ ends, lack a polyadenylated 3’ tail, and contain short untranslated regions at their 5’ and 3’ends. Transcription of reovirus

requires an ATP-dependent dsRNA helicase for duplex unwinding (54). The active site of the avian reovirus RNA polymerase is suggested to be contained within the minor core protein σb. Nine of the ten transcripts of avian reovirus are monocistronic and they use their 5' most proximal AUG for initiation of translation; the start codons of these transcripts are surrounded by a strong translation initiation context (38). The exception is S1 mRNA, which is tricistronic, and expresses three proteins from three partially overlapping reading frames, the first and the second cistrons express nonstructural protein p10 and p17, respectively, and the third cistron expresses the outer capsid protein σC (5).

The viral mRNAs are presumably synthesized within the inner core and acquire their cap structures when leaving the particle through the channels of the hollow turrets formed by pentamers of the avian reovirus capping enzyme, the lambda C protein (115). Furthermore, mRNAs play two different roles in infected cells, first they program viral protein synthesis at the ribosomes, and second they serve as templates for the synthesis of the genome minus strands. It was experimentally demonstrated *in vitro* that viral gene expression starts with the synthesis of all 10 viral mRNAs (54). Synthesis of avian reovirus polypeptides can be detected at early infection times and later, most of the polypeptides synthesized within the infected cells are from viral origin. The viral transcripts are produced in variable amounts in the intracellular levels of the viral proteins, with μBC, σB and σNS being the most abundant, while λB, μA and the three S1-encoded proteins the least abundant. The mechanism by which avian reovirus shuts down cellular protein synthesis is not known (4). Enzymes catalyze the synthesis of multiple ssRNAs that are copies of one strand of each segment of the dsRNA genome.

The mechanism of RNA transcription on the dsRNA templates is similar to that on dsDNA templates (87).

The synthesis of ssRNAs and dsRNAs forms during virion formation, and the maturation of complete virions occur along microtubules within cytoplasmic factories that are made up of ribosomes and function as "extranuclear" nuclei for viral biogenesis. As a result, the double-strand templates remain in this location, while single-strand transcripts are exported to the surrounding cytoplasm, where they encode viral proteins. These proteins return to the factories where they are assembled into progeny virions (87). Immunofluorescent microscopy of infected cells demonstrated that avian reovirus factories possess globular morphology and, unlike the factories observed in cells infected with most mammalian reoviruses, they are not microtubule-associated. Therefore, it is suggested that cores are assembled within the first 30 minutes after the synthesis of their polypeptide components, and that reovirion morphogenesis is completed over the next 30 minutes by the subsequent addition of outer capsid proteins (93).

Core assembly and core coating take place exclusively within viral factories of infected cells, in a temporally coordinated fashion. Moreover, it is suggested that viral morphogenesis starts with μNS forming reovirus factories and recruiting proteins σNS and λA. Viral proteins assemble, partially or totally, in the cytoplasm of infected cells (93). The assortment and packing mechanism of the 10 segments of viral mRNA is not well known, but it is suggested that RNA-binding activity of σNS and its early μNS-mediated association to viral inclusions, must play an important role in these processes (95). Viral release is not well understood for avian reovirus, but it is likely that

nonstructural protein p10 plays a key role in virus release and dissemination to neighboring cells, because of its permeabilizing and cell-fusion activities (6).

Genetic Diversity

Reassortment plays a major role in reovirus genetic diversity (45). Reassortment is based on the use of 2 parental genomes to produce a progeny segment of genome (reassortant) (30). The evaluation of mammalian reovirus in cells showed - by phylogenic tree analysis of σ2 - that reassortment of reovirus gene segments occurs in nature or in coevolution of serotype-specific gene sets. Reoviruses use a specific assortment mechanism with very high specificity and efficiency (30). They have a nonrandom nature for reassortment and may be explained by the two parent virions that infect the same cell, establishing separate zones of replication between which RNAs must be exchanged for reassortment to occur. If this exchange process is inefficient, then reassortants will be observed at a decreased frequency. Reovirus reassortants commonly contain one or more mutations relative to the parental genes from which they are derived and that some of these mutations may be essential for the viability and/or fitness of these reassortants (65). *In vivo* testing in mice demonstrated the role of host factors reovirus reassortment, since they were detected in more than one organ, in different mice and in different days (105). A study conducted with avian reoviruses evaluated genotypes, pathotypes, phylogenetic profiles, and the paired identity analysis revealed that genetic diversity was related more to the date and place of isolation than to host species and pathotypes. This result may be caused by the nonrandom reassortment and the parental role in the process. Additionally, it was demonstrated that S1 class segment produces the majority of genetic diversity through reassortment, especially in σC protein (45). The same author suggests that

reassortment of ARV is an important mechanism for evolution, involving co-circulating lineages, driving reassortment to generate new variants of the virus, different pathotypes and genotypes.

Incidence and distribution

Reovirus infections are prevalent in chickens, turkeys, ducks and many other avian species. Avian reoviruses are spread among commercial poultry flocks worldwide. Viral arthritis is primarily observed in meat-type chickens, but can also occur in layers and turkeys. However, isolations of nonpathogenic reoviruses in chickens and turkeys from the respiratory and digestive tracts are estimated to be greater than 80% (91).

Transmission

Avian reovirus can be transmitted vertically and horizontally (16, 116). The natural route of infection is mainly by fecal-oral (25, 78). Egg transmission of avian reovirus has been observed in several outbreaks in broilers originating from the same hatchery (16). It is known that egg transmission can occur in infected breeders, but the rate of transmission is around 2% and occurs between 17 to 19 days post infection (56). Depending on virus titer, experimental infection in eggs can kill embryos. Embryos inoculated with low titer can survive and virus can be isolated. Broiler breeders positive for avian reovirus can have a decrease of hatchability and increased early chicken mortality of approximately 5% for a period of 15 weeks (98). Avian reovirus inoculated in embryos can induce 100% of mortality between 72-96h and the alterations observed include dwarf embryos and subcutaneous hemorrhages (90). Experimentally, preferable routes for inoculation are oral, intranasal, intratracheal and footpad. These routes have been used in previous studies in chickens and turkeys (1, 25, 26, 81). The footpad route

of inoculation has been demonstrated to induce earlier and higher mortality rate than in orally challenged broilers and SPF chickens using tenosynovitis inducing strains of ARV, when inoculated at day of age (25, 63). In contrast, Sahu *et.al* (78, 79) inoculated chickens with different strains of ARV by the same routes, but in broilers at 2 weeks of age, and no mortality was observed. It was concluded so, that the pathogenicity of reoviruses depends not only on the type of strain but also the age of the bird. Mustaffa-Babjee *et al.* (63) also reported that mortality and lesions were more severe in young chicks after reovirus infection than in older birds. In turkeys, transmission has been suggested to occur by fecal-oral route, when sentinel poults were infected after contact with orally challenged poults. Experimentally, tenosynovitis is more severe in turkeys inoculated with turkey arthritis reovirus by footpad, followed by intratracheal and oral routes, respectively (81).

Pathogenesis and pathophysiology

Viral arthritis/tenosynovitis is predominantly a disease of broilers, but can also be seen in breeders (34) and is an important cause of leg weakness (31). Tenosynovitis has also been observed in layer pullets that have lameness (97). The greater susceptibility of meat type chickens may be related to the rapid growth and physical changes in tendons and legs. Also, a delay in immune response may be an explanation for this greater susceptibility, as broilers reovirus seroconversion is one week delayed when compared to light breeders (34). Additionally, the bursa of Fabricius of heavy breeds have a relatively slower growth rate and, prior to their regression, are relatively smaller than the bursa of light breeds, and this may have an impact in the immune response (17).

Arthritis and tenosynovitis associated with reovirus in turkeys was first described in 1980 in 15-week-old turkeys (40). In this report, gross lesions consisted of swollen intertarsal (hock) joints with histological lesions of hyperplastic synovium and inflammatory cell infiltrates in the subsynovium. In the 80's there were additional reported cases of reovirus-induced tenosynovitis in turkeys (70), however, for many years until 2011, turkey reovirus had been associated with primarily enteric diseases in poults (59). Interestingly, in 2011 novel turkey arthritis reoviruses (TARV) strains from tendons of turkeys with tenosynovitis/arthritis were isolated and characterized in Minnesota. Genetic characterization of S4 gene sequences showed a relatively high degree of homology (88.7 to 99.8%) between TARV and turkey enteric reovirus (TERV) and only 78% nucleotide identity between TARV and chicken arthritis reovirus (61). Therefore, poults challenged at 6 days of age with these turkey arthritis reovirus isolates, reproduced tenosynovitis showing lymphocytic tenosynovitis of the gastrocnemius and digital flexor tendon (81). Furthermore, it was reported an increased incidence of avian reovirus causing severe arthritis/tenosynovitis, pericarditis and depressed growth in commercial broilers and turkeys in Pennsylvania. The molecular analysis of Sigma C gene revealed that most of the isolates were genetically distinct from vaccines strains, belonging to different genotype clusters (48), consequently, traditional vaccination with commercial vaccines did not appear to confer any protection against field ARV infections (48, 49).

The main clinical sign of viral arthritis/tenosynovitis is swelling of one or both hock (tibiotarsal-tarsometatarsal) joints, causing acute lameness. This condition is rare in broilers less than four to five weeks of age and is commonly seen up to sixteen weeks of age, with a peak incidence at approximately seven weeks (31). In turkeys, flocks usually

are affected from 10 to 18 weeks of age (40, 61, 70). Pathogenicity is dependent of age, route, strain and type of bird (25, 34).

Broiler breeders can be affected during peak production; morbidity is variable but usually below 10% and mortality is low. The lesions seen are swelling and inflammation of the hock joint and in the most severe cases, rupture of the gastrocnemius tendon and erosion of the articular cartilage can occur. When both hock joints are severely affected, the bird is immobilized. Occasionally, one or more digital flexor tendons are ruptured. Rupture of the gastrocnemius is accompanied by hemorrhage, which in turn causes green discoloration of the skin at the joint (31). Development of tendon lesions may be detrimental to virus persistence in the tendon (34). Macroscopic lesions in chickens may include liver, spleen and/or bursal necrosis, hemorrhages and congestion in the bursa, spleen and kidneys; pericarditis, nephritis and tenosynovitis (25).

A reovirus field isolate from 22-day-old broiler chickens with leg weakness and swollen hocks, caused high mortality 21 days post infection in experimentally infected day old chickens, however, mortality decreased according to age when older birds were inoculated, providing direct evidence that disease caused by avian reovirus is age dependent. Furthermore, the presence of the virus does not always reproduce the disease; this may be due to differences of pathogenicity of the isolates or factors such as infection site, susceptibility or age of the birds (116). Induction of lesions by reovirus is due to the presence and dissemination of the virus to affected tissues, as observed with virulent strains inoculated via the footpad, resulting in high mortality, swollen feet and hock joints (64). The route of inoculation also influences the severity of lesions following infection (90). Milder disease is observed following oral and intranasal inoculation. This is likely

due to the affinity of the virus for respiratory and enteric epithelium, where it tends to remain localized. On the other hand, footpad and subcutaneous inoculation results in distribution of the virus to a wider range of cells and this appears to increase tissue invasion and a more generalized infection.

Viral entry by the natural oral route initially induces replication in the intestine and bursa, followed by viral spread via blood to other tissue or organs. When birds are inoculated orally, virus detection occurs first in the spleen and liver after 2 days post inoculation then in the heart, kidney, hock joint and then the bursa after 4 days post inoculation. The peak of virus replication occurs 4-6 days post inoculation in the liver and spleen (64). The liver is known to be a primary target organ for reovirus proliferation (50).

In viral arthritis, infected cells are located in the synovial stroma and peritendinous tissues. Virus can be detected in many tissues, including intestine, bursa, lung, kidney, spleen, liver, heart and hock joint. ARV that replicates in the intestine but cannot spread or replicate in other tissues may primarily cause malabsorption syndrome but if an infection can spread and replicate efficiently in other tissues, including the hock joint, it may manifest as viral arthritis or both viral arthritis and malabsorption syndrome (64).

Disease outcome following infection with avian reovirus is highly variable. Highly pathogenic ARV isolated from chicken tendons can kill 100% of day old chicks and can also kill embryos (64). ARV can cause immunosuppression due to the lesions in bone marrow, spleen and bursa. In enteric reovirus infection, virus persists for a longer period in ileum, cecal tonsils and rectum (50).

Virus shedding in broilers takes up to 3 weeks and for lighter breeders 2 weeks. There is a poor correlation of virus isolation with the presence of gross lesions, where virus may not be isolated from birds showing marked joint lesions, especially in broilers after 12 weeks of age (34).

Viremia can last up to 10 days post infection and virus can be recovered from whole blood after 5 days post inoculation (37). Although intestines show higher level of infection after oral inoculation, much of the virus is excreted via feces and relatively little appears in the liver, in contrast with footpad inoculation (37).

Histopathological lesions can be observed at 6-8 days post infection in orally infected birds with a very virulent tenosynovitis strain of ARV. In the heart, pericarditis, characterized by diffuse lymphocytic infiltration in the pericardium and in adjacent myocardium can be observed. In the liver, scattered foci of coagulative necrosis and highly vacuolated hepatocytes can be observed while in the spleen, necrosis is observed with associated deposition of eosinophilic material. In the bursa, ARV leads to hypertrophy of connective tissue, infiltration of heterophils and lymphocytes, and lymphoid depletion; in the hock joint, infiltration of primary heterophils, followed by hyperplasia of synovial membrane and stroma cells, as well as diffuse lymphocytic infiltrates (64). Chickens with clinical signs of tenosynovitis can show histopathological evidences of tendovaginitis, synovitis and chronic osteoarthritis, moreover, myocarditis and epicarditis characterized by infiltrations of mononuclear cells are observed (16).

Other observations in chickens showing arthritis/tenosynovitis may include heterophilic myocarditis, lymphoid cell hyperplasia in spleen, lungs, brain, cecal tonsils and proventriculus (25, 36). In turkeys, observations of reovirus induced tenosynovitis

include histological findings of lymphocytic tenosynovitis in the gastrocnemius and digital flexor tendon sheaths (81).

Despite the emergence of new turkey and chicken arthritis variants since 2011, turkey-origin ARV strains are antigenically distinct from chicken-origin ARV strains. The turkey-origin ARV strains are considered a separate virus subtype within the Orthoreovirus genus (9, 48, 61, 89). However, studies have been conducted to evaluate the pathogenesis of ARV variants and new strains in cross-species transmission. Sharafeldin *et.al*, 2014 challenged 6 days old poults with chicken arthritis reovirus, but there was no evidence of tenosynovitis, although virus was detected in tissues by RT-PCR (81). On the other hand, two different strains of TARV, when inoculated in chickens by footpad, induced gastrocnemius lymphocytic tenosynovitis. In contrast, no lesions were observed in chicks inoculated intratracheally and orally with the same strains of TARVs (82).

Local immunity

Evaluation of local immune response against reovirus in the gastrointestinal tract of mice showed that production of IgA is potentially primed by inflammatory mediators and innate immune factors due to the viral replication, moreover, aging reduces the potential of systemic and intestinal response against the antigen (13). Local immunity is affected by age, route of infection and trypsin sensitivity of virus (62). According to studies, day old chickens infected by oral or subcutaneous routes with trypsin resistant (strain R2) avian reovirus do not produce IgA in the intestine, however, between 7 days and 3 weeks of age, IgA is substantially detectable in the intestine. In contrast, sensitive

trypsin strain (strain TR1), when orally inoculated does not induce IgA until 3 weeks, however, IgG is detectable in the serum (62).

Cell mediated immunity

Cell mediated immune response may play an important role in avian reovirus infections. It has been demonstrated that broilers with maternal antibodies against reovirus and vaccinated subcutaneously with live attenuated vaccine at 1 day old and challenged at 3 weeks of age were protected against avian reovirus, even when a high challenge dose was used. It seemed that protection induced in broilers by the attenuated reovirus vaccine may not have been entirely humoral because in protected birds no antibodies against reovirus were detected by enzyme-linked immunosorbent assay at the time of challenge. Protection in these birds might, therefore, have been induced by cellular immunity (100). Further studies in SPF and broilers revealed that virus is controlled in the absence of actively produced antibodies, and is independent of B lymphocytes, suggesting that cellular immunity is sufficient for protection of broilers with maternal antibodies against reovirus infection following early age vaccination with live reovirus vaccine (98). By using monoclonal antibodies specific for B and T cells against reovirus arthritis virus, it was shown that in the acute phase of infection, CD8+ T cells production is low (72), but, in the subacute phase an increased number of CD8+, CD4+, IgM and plasma cells occur and in the chronic phase, CD4+ T cells are predominant (73). The same study suggested an important role of macrophages in the early stages of viral arthritis infection, through the production of soluble mediators, such as nitrous oxide, that serve to prevent cell lysis and promote viral clearance from the host.

Humoral Immunity

As previously described, avian reovirus affects young chickens at early ages, being susceptible since day of hatch and resistance increase with age. Reovirus-specific antibodies are able to provide protective immunity by different mechanisms. They may prohibit attachment of the virus to target cells, may facilitate lysis of viral particles or virus-infected cells following complement binding or may contribute to Fc-receptor-mediated phagocytosis and/or cytolysis (99). Maternal derived antibody (MDA) to offspring play an important role in early protection, however, it was demonstrated that MDA- negative chickens can mount detectable levels of reovirus specific antibodies, but may not be sufficient to control infection. It has been demonstrated that humoral response mature in chickens as they grow (62). Neutralizing antibodies can be detected 7—10 days following infection, and precipitating antibodies at approximately 2 weeks. The importance of antibody in establishing protection is not well understood, because birds may become persistently infected in the presence of high levels of circulating antibody (35). Relative protection afforded by antibody appears to be related to serotype homogeneity, virus virulence, host age, and antibody titer (74, 76, 92). With respect to avian reoviruses, young chicks have been suggested to have a less immunocompetent immune system compared to older birds (32) which may be related to the age-dependent changes in the B cell numbers in the chicken gastrointestinal tract, for example (3). It has been reported that the development of germinal centers in the gut which are vital in the immune system are antigen-driven. Young birds are less exposed to environmental antigen, and this makes their germinal centers less developed and consequently not as immunocompetent as older birds (23).

Control

The premises of biosecurity are important to try to keep flocks free or controlled from avian reovirus, once the virus is ubiquitous and can be transmitted horizontally and vertically, and its control might be difficult. Good management and adequate cleaning and disinfection of the poultry house with validated efficient products can be an important tool to prevent infection and dissemination of the pathogen (91).

Vaccination

Vaccination has been aimed to the transfer of maternal antibody to the progeny (98). In order to provide early protection to chickens, vaccination programs have been designed to deliver passive maternal antibody to the progeny by vaccinating breeders with live attenuated and inactivated vaccines, providing protection to chickens since day of age, when they are more susceptible (98). The neutralization titer in yolk is similar to the breeders during the laying period, proving that the transference of maternal antibody occurs, however, to provide protection, the antibody must be homologous to the challenge virus (55).

A vaccination program for breeders is generally comprised of several live attenuated vaccinations administered up to 12 weeks followed by several inactivated oil emulsion vaccines administered prior to the start of egg production. Commercial vaccines in the U.S are based on the S1133 strain or in combination with other strains, such as 1733, 2408 or 2177, to provide a wider spectrum of protection, not only for arthritis but also for malabsorption syndrome (47, 99, 107).

Many vaccines are based on the S1133 strain isolated in the United States of America. The use of monovalent or multivalent vaccines commercially available may not

provide efficient protection against heterologous serotypes (31, 55). Some companies choose to vaccinate broilers with live attenuated vaccines at day of age in the hatchery with S1133 or 2177, for example (100). Besides, *in ovo* vaccines have been experimentally developed for early protection, but to date they are not commercially available (22).

The use of recombinant vaccines has been studied to achieve effective protection for poultry. DNA recombinant vaccine construction using *Salmonella Typhimurium* as a vector and having σB and σC expressed together, showed to be a possible combination for oral recombinant vaccine against avian reovirus, determined by the production of serum IgG and small intestine IgA. This vaccine construction showed to be relatively protective against challenge, however, S1133 strain vaccine produces higher IgG and IgA titers, being more protective to challenge than combined σB – σC recombinant vaccine (104). A DNA recombinant vaccine construction using *Enterococcus faecium* as a vector to express avian reovirus σC protein was experimentally tested. Preliminary results in mice indicated induction of IgA systemically and in the spleen, mainly when administered intranasal. Further studies are needed to determine its efficacy in chickens and to challenge (42).

Diagnostics

Virus Isolation

In chickens with clinical signs of arthritis, virus isolation is indicated. The preferred samples to submit for best results include whole leg (including the sesamoid bone, digital flexor tendon, articular cartilage at the hock joint and gastrocnemius tendon) from affected birds (33). The timing of sample collection is important as experimental

data suggests that optimal sampling should be conducted between 5 to 8 days post infection. Field samples must be sent on ice and even though reovirus is resistant to temperature, samples must be stored at 4°C for short storage or -80°C for long storage. Virus isolation in embryonated SPF chicken eggs can be performed in 6-9 day-old embryos via yolk sac or in 9-11 day old embryos via inoculation in the chorioallantoic membrane. Mortality and embryo lesions may be seen after 7 days post inoculation, moreover, the chorioallantoic membrane can be harvested and group specific antigens can be detected by Agar Gel Precipitation test. Reovirus affected embryos can be hemorrhagic, with purplish discoloration, and surviving embryos are slightly dwarfed, with enlarged mottled liver, and splenomegaly (68).

Reovirus grows in a variety of primary cell lines, growing in suspension and monolayers efficiently, depending on pH, temperature (37°C) and strain (87). Isolation in cultured cells is better achieved in chicken liver cells than chicken kidney cells and chicken fibroblast, having the cytopathic effect of syncytia formation as a characteristic of avian reovirus in infected cells (21).

RT-PCR

The reverse transcription-polymerase chain reaction (PCR) is routinely used to detect reovirus RNA in samples. To establish a universal reverse transcriptase (RT)-PCR for detecting all ARVs, PCR primers chosen from conserved regions among ARV isolates are required (43). Avian reovirus has 10 segments of genome divided in size classes (2) and many of the viral proteins encoded by the segments have been used as targets for RT-PCR detection (39, 60, 114). The use of restriction enzyme analysis after RT-PCR has been utilized to characterize ARV isolates but this has largely been replaced

by RT-PCR, and sequencing is now available. Using sequencing, it is possible to determine, building a phylogenetic tree, distinct groups, genotypes and whether a new variant strain has been introduced into a flock or if a strain has spread from one flock to another (46). Genotyping based on amplification of the S1 gene segment encoding the Sigma C protein followed by sequencing is used for genetic characterization of field isolates (48).

Serology

Many serologic methods are used for avian reovirus diagnostics. Indirect fluorescent antibody (IFA) assay can be used to detect serum antibodies for avian reovirus, using Vero cultured cells, antiserum, a fluorescent conjugated serum and microscope (29). Agar gel precipitation (AGP) test is utilized for detection of reovirus specific group (75), however, IFA sensibility is higher than AGP (29). .

Enzyme linked immunosorbent assay (ELISA) for avian reovirus was first described in 1978 (88), the technique has a high level of sensitivity and reproducibility, and also allows automation. ELISA has become the method of choice for screening a large number of serum samples, being possible to use whole virus or a recombinant ARV protein as the antigen (83). The use of recombinant protein σB and σC, which induce neutralizing antibodies, is demonstrated to be more correlated to virus neutralization comparing to the conventional whole virus assay (44, 110) and can increase sensitivity and specificity (110).

Virus neutralization (VN)

VNs can be performed in different cell lines, commonly chicken embryo liver cells, chicken embryo fibroblasts and VERO cells, to name a few. The alpha and beta

protocols are used to perform virus neutralization. The beta virus neutralization was proposed by Giambrone (14), where in a 96 well containing confluent monolayer of VERO cells, a constant virus containing about 100 PFU was added, and serum sample diluted making serial two fold dilutions. On the other hand, the alpha method uses constant serum and diluted virus (10). Virus neutralization is an important tool for differentiating different serotypes of viruses. Serological evaluation of variants strains of avian reovirus results in a lack or no neutralization observed by VN using standard vaccine as antigen, suggesting that birds were challenged by a different serotype of ARV, and that flocks vaccinated with commercial vaccines may not be protected (96). Cross neutralization studies have been used for the identification of different serotypes. In these studies, a panel of isolates (antigens) and antisera are tested. Different serotypes are detected by the weak or lack of neutralization, demonstrating that this assay is serotype specific (15, 26).

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CHAPTER 3

PATHOGENICITY OF TWO TURKEY ARTHRITIS REOVIRUS FIELD ISOLATES IN COMMERCIAL BROILERS[A]

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Summary

The pathogenicity of two turkey reoviruses, 105057 and 105208, isolated from clinical cases of tenosynovitis in commercial turkey breeders from Wisconsin was evaluated in commercial broilers. Lame birds with swollen tendons and footpads were observed in some birds challenged by footpad with field isolate 105057. In addition, ruptured digital flexor tendons and microscopic evidence of tenosynovitis and myocarditis, characteristic of reovirus infection, were present in this group. Birds challenged orally with 105057 had milder tenosynovitis, but a higher prevalence of hydropericardium. While only mild tenosynovitis was observed in birds challenged with reovirus isolate 105208, diarrhea was observed from 14-28 days post challenge, contributing to a significant decrease in body weight, compared to the other experimental groups. Reovirus was detected by RT-PCR in tendons and hearts from groups challenged by footpad with both isolates, but little to no virus shedding was observed. Altogether, turkey reoviruses 105057 and 105208 were pathogenic in commercial broilers and capable of causing clinical disease.

Key words: avian reovirus, arthritis, tenosynovitis, turkey, broilers, myocarditis, lameness.

Abbreviations: ARV = avian reovirus; CAM = chorioallantoic membrane; d.p.i = days post inoculation; ELISA = enzyme-linked immunosorbent assay; FP = footpad; HVT-LT = Herpesvirus of turkeys-laryngotracheitis; OR = oral; PBS = phosphate-buffered saline; p.i = post inoculation; RT-PCR = reverse transcriptase polymerase chain reaction; SPF = specific pathogen free; TARV = turkey arthritis reovirus; TCID50 = tissue culture infectious dose; TK = turkey; VN = virus neutralization.

Introduction

Avian reoviruses are widespread in commercial poultry flocks and involved in many disease conditions, including arthritis, tenosynovitis, enteric and respiratory diseases, myocarditis, hepatitis and the so-called stunting/malabsorption syndrome, however, is estimated that 85-90% of reovirus isolates are nonpathogenic (1, 8).

Chicken and turkey reoviruses are members of the avian *Orthoreovirus* genus, in the *Reoviridae* family, but are genetically distinct (17, 19). The *Reoviridae* family is the largest of the eight recognized double-stranded RNA (dsRNA) virus families and has a wide host range which includes insects, plants, fish, reptiles, birds, mammals, arachnids, fungi, arthropods and crustaceans (17). The Reovirus genome encodes 10 segmented proteins, classified in 3 size classes, large (L1, L2 and L3), medium (M1, M2, M3) and small (S1, S2, S3 and S4), based on electrophoretic mobility. The segments encode for at least 8 structural and four nonstructural proteins (2, 33). The S1 segment encodes the Sigma C (σC), which is known to be the cell attachment protein and a major antigenic determinant for avian reovirus (ARV) (5, 26). The σC encoding region of the S1 gene is the target for diagnostic RT-PCR and sequencing of this product is the basis for genotypic characterization (14). The *Orthoreovirus* genus is divided into two subgroups, fusogenic and non fusogenic, based on the ability to cause syncytia formation in cell culture (8).

In the last few decades reovirus has been associated with a complex of economically important enteric diseases alongside other viruses in turkeys, including, poult enteritis complex (PEC), poult enteritis and mortality syndrome (PEMS), poult enteritis syndrome or PES and light turkey syndrome or LTS (7). Spackman et.al (31)

reported that turkey reovirus caused mild diarrhea and depression in SPF and commercial poults, as well as a decrease in body weight. In the same study, it was demonstrated that the turkey reovirus poorly replicates and did not cause disease in chickens. In 2009, myocarditis associated with reovirus in 17-day-old poults with anorexia, growth depression and increased mortality was documented (30). Recently, there have been numerous cases of reovirus-induced viral arthritis in turkeys (TARVs). Reoviruses were isolated from lame turkeys and caused lameness in experimentally infected birds, specifically as a result of rupture of the gastrocnemius tendon in turkeys over 8 weeks of age (28).

Since 2011, the incidence of reovirus-induced tenosynovitis in commercial chicken and turkey flocks has increased in the United States and elsewhere in the world, causing significant clinical disease and economic losses. Most of the ARV-infected birds suffer from severe arthritis, tenosynovitis, hydropericardium and decreased flock uniformity. High morbidity (up to 20% to 40%) has been observed in ARV-affected flocks, and flock mortality can be as high as 10% (15). Molecular characterization of σC revealed that most of these newly ARV isolates are genetically distinct from vaccine strains (S1133, 1733, and 2048) and conventional vaccination does not appear to be protective (15).

The increased incidence of variant reovirus inducing arthritis/tenosynovitis in commercial chicken and turkey flocks, and the close proximity of some poultry production sites in the U.S raises the question of cross species infection. . The aim of the present study is to evaluate the pathogenicity of two turkey reovirus field isolates from clinical cases of tenosynovitis in turkeys in commercial broilers.

Material and Methods

Cell lines

QM7 (quail muscle cells; ATCC reference CRL-1962™) and VERO (African green monkey kidney cells; ATCC reference CCL-81™) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% Penicillin + Streptomycin and Fungizone (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with 5% CO_2. QM7 cells were used for virus propagation and Vero cells were used for virus neutralization, virus isolation and titration of the field isolates.

Viruses

Two turkey reoviruses were isolated from the gastrocnemius and digital flexor tendons of lame birds from commercial flocks from Wisconsin, United States of America, from clinical cases of tenosynovitis were used in this study. Specifically, field isolates TK/WI/105057 Tendon/2014 (referred to as 105057 from here on out) and TK/WI/105208 Tendon/2014 (referred to as 105208 from here on out). For isolate 105057, primary isolation was made from tendons in the second passage of primary chicken embryo liver cells. Cytopathic effect consisting of syncytial cell formation characteristic of reovirus was observed in cell culture and reovirus confirmed by RT-PCR. For isolate 105208, primary isolation was made from tendons in turkey embryos inoculated via the chorioallantoic membrane (CAM) in the first passage. Lesions characteristic of reovirus were observed in the embryos and the CAMs were harvested and submitted for RT-PCR confirmation. Both isolates were propagated and titrated in QM7 cells (3). Titers for 105057 and 105208 viruses are shown in Table 3.1.

Birds

A hundred and twenty (120) day-of-hatch commercial broilers were divided into 6 groups (n=20) and placed in 3 isolation houses divided by treatment and route of inoculation (Table 3.2). Twenty five additional chicks from the same flock were bled for serology. Food and water was provided *ad libitum.* Birds were only vaccinated *in ovo* with HVT-LT in the hatchery.

Experimental Design

At day of age, all birds were weighed and distributed uniformly prior to inoculation. Chicks from each group were inoculated at day of age with either isolate 105057 at $10^{3.5}$ TCID50/bird, 105208 at $10^{3.1}$ TCID50/bird or Phosphate Buffered Saline (PBS) via left footpad (FP) or oral (OR) route. Chicks inoculated with the same virus but different route were placed in the same colony house but with no contact, separated by a PVC fence covered with a cardboard divider. All birds were observed daily for clinical signs, including lameness, swelling of the hock joint and/or swelling of the footpad. Body weight, footpad and hock joint measurements, as well as cloacal swabs, were collected individually at 7, 14, 21 and 28 days post inoculation (d.p.i). Two weeks post inoculation (p.i), ten birds per group were humanely euthanized for post mortem examination and tissue collection. Digital flexor tendons and hearts were collected for virus detection by RT-PCR. Birds exhibiting obvious signs of lameness with joint swelling prior to the end of the experiment were euthanized, necropsied and samples collected for reovirus detection. At four weeks p.i, the remaining birds were humanely euthanized. Hearts and hock joint swabs were collected for virus detection by RT-PCR. Digital flexor tendons,

heart and sections of duodenum and jejunum were also harvested and fixed in 10% neutral buffer formalin for histopathological examination.

Hock joint and footpad measurements

The left hock joint (tendon) and footpad measurements were taken using a digital caliper. Tendon and footpad to body weight ratios were calculated by the formula, (tendon(mm)/body weight(g))x100 and (footpad(mm)/body weight(g))x100. The ratios were used for the evaluation of tendon and footpad swelling, normalized to the body weight of the individual bird.

Swab

Cloacal swabs were collected in 1ml of triphosphate buffer (TPB) + 2% Penicillin + Streptomycin and Fungizone for virus isolation and titration. Samples were centrifuged at 1500 rpm for 3 minutes and stored at -80°C. At 4 weeks p.i, swabs from joint fluid/digital flexor tendons from each bird were collected in 2ml of TPB and stored at -80°C until use for RT-PCR.

Serology

Commercial ELISAs were used to test serum for reovirus, *Mycoplasma synoviae* and *Mycoplasma gallisepticum* antibodies (IDEXX, Westbrook, Maine, USA) according to the manufacturer's instructions. In addition to ELISA, virus neutralizations were performed, using the beta method, with 105057 and 105208 utilized as antigens (3). Briefly, the VN assays were performed in 96 well plates containing confluent monolayers of VERO cells. Plates were incubated at 37°C+5% CO_2 for 96 hours and then VN titers, represented by the reciprocal of the last dilution where no cytopathic effect was observed, were recorded.

Clinical signs

Birds were observed daily clinical signs for the duration of the study. Birds that showed recumbency, inability to move and to reach food and water were considered lame and removed from the study.

Gross lesions and histopathology

Half of each group of birds were humanely euthanized at 14 and 28 d.p.i, and examined for gross lesions in visceral organs, intestines and intertarsal (hock) joints. Digital flexor tendons, along with the heart and intestines were collected from birds euthanized at 28 d.p.i., fixed in 10% neutral buffered formalin, and stained with hematoxylin and eosin for examination of lesions by light microscopy. The tendons from the left leg were scored for inflammation as previously described (27).

Virus isolation and titration

Prior to virus isolation and titration, cloacal swabs were thawed and centrifuged for 10 minutes at 1500 rpm at 4°C. Samples were filtered using 0.2μM GDX filters (Whatman, Fischer Scientific, Norcross, GA). Viral shedding was evaluated from cloacal swabs collected weekly for each bird in VERO cells (21, 34). Cell cultures were examined daily for 5 days for presence of cytopathic effect characteristic of reovirus. Titration was performed in 96 well plates containing confluent monolayers and titers calculated using the method of Reed and Muench (24).

RNA extraction and RT-PCR of Sigma B and C

Total viral RNA was extracted from the second passage of each virus in QM7 cells for amplification of the Sigma B encoding region of the S3 gene and Sigma C

encoding region of the S1 gene using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations.

RNA was extracted from the digital flexor tendons and hearts collected at 14 d.p.i from pools containing 5 samples each. Swabs from hock joint fluid collected at 28 d.p.i were tested individually, while hearts were pooled in groups of five samples each. Samples were homogenized in virus transport media (VTM). Following centrifugation, RNA was extracted from 0.2ml of the supernatants using the MagMAX™ Pathogen RNA/DNA Kit on the MagMAX™ Express-96 Deep Well Magnetic Particle Processor sample extractor (Thermo Fischer scientific, Waltham, MA, USA). RNA was stored at -80°C until further use.

Reverse transcription-polymerase chain reaction

A cDNA corresponding to the Sigma B encoding region of the S3 gene was amplified from the virus stocks using previously published primers (11). Likewise, the Sigma C encoding region of the S1 was generated by reverse transcription-polymerase chain reaction (RT-PCR) using Superscipt III RNase H- RT and Platinum Taq DNA polymerase with previously published S1P1 and S1P4 primers (11, 12). The 1 kb (S1) and 1.1kb (S3) amplified products were separated on a 1.0% agarose gel, stained with ethidium bromide, and visualized with an ultraviolet transilluminator. The cDNA fragment was excised, purified with the QIAEX II gel extraction kit (Qiagen, Inc. Germantown, MD), eluted in RNase free water, and stored at -80° C until sequenced.

Direct nucleotide sequencing of amplified products

Gel-purified PCR products were sequenced with S1P1 and S1P4 primers (S1), and S3F and S3R (S3) using Sanger sequencing chemistry on a 96-capillary Applied Biosystems 3730xl DNA analyzer at Retrogen, Inc. (San Diego, CA).

Sequence analysis

Nucleotide sequences were analyzed and consensus sequences were assembled from sequence files using the DNASTAR Lasergene 12 software (DNASTAR, Inc. Madison, WI, USA) suite. Nucleotide sequences were *in silico* translated into corresponding amino acid sequences. Amino acid sequences generated were aligned using Clustal Omega multiple sequence alignment algorithm (http://www.ebi.ac.uk/Tools/msa/clustalo/) and phylogenetic analyses were conducted using MEGA version 6.0 (32). Sigma C sequences for the 105057 and 105208 reoviruses were submitted to Genbank and assigned accession numbers KU886560 and KU886561 respectively. Previously published sequences used in this analysis and their corresponding accession numbers are as follows: 1733 (KF741712.1); S1133 (AY536919.1); 12-1167 (HE985301.1); 11-12523 (HE985296.1); 11-12525 (HE985298.1); TX99 (DQ996602.1); GA 441569 (DQ872798.1); AL 99159 (KJ879682.1); GA 97837 (KJ879660.1); 916 (AF297214.1); MS 97992 (KJ879667.1); 918 (AF297215.1); GA 40973 (DQ872797.1); GA 97350 (KJ879644.1); AVS-B (FR694197.1); 1017-1 (AF297216.1); NC 96816 (KJ803997.1); GA 12296 (JX983600.1); GEI 1097M (AF354219.1); 94826 (KJ803967.1); RAM1 (L38502.1); SOMERVILLE 4 (L07069.1); TARV MN3 (KF872233.1); TARV O'NEIL (KF872231.1); 601G(AF297217.1).

Sigma B sequences for the reoviruses analyzed in this study were submitted to GenBank and assigned the following accession numbers: 94826 (KU936100); 96139 (KU936101); 105057 (KU936098); 105208 (KU936099); Previously published sequences used in this analysis and their corresponding accession numbers are as follows: 138 (AF059721.1); AVS-B (FR694199.1); 916 (AY008383.1); 601G (AY008384.1); S1133 (KF741764.1); 919 (AF208034.1); 750505 (AF208035.1); TX99 (AY444910.1); TARV-MN1 (KF872265.1); D15/99 (AY114138.1); ZJ2000M (KF306090.1); CA (KJ569582.1).

Statistical analysis

One way ANOVA test was used to statistically analyze body weights, footpad/body weight ratios and tendon/body weight ratios at different time points. The Kruskal Wallis test was used for comparison of histologic inflammation scores at 28 d.p.i, (GraphPad Software, Inc., La Rolla, CA USA).

Results

Serology

Sera collected from twenty five chicks at day-of-hatch were positive for reovirus antibodies, with a GMT of 745. Chicks were negative for virus neutralizing antibodies to viruses 105057 and 105208 as determined by VN (data not shown). Sera collected from birds at 20 days of age were negative for *Mycoplasma gallisepticum and Mycoplasma synoviae* (data not shown).

Clinical signs

Clinical signs were observed in the 105057 FP group starting at day 5 through day 9 p.i and included 3/20 lame chicks, 5/20 birds with swollen left hock joints and 18/20 birds with swollen left footpads. In 105057 OR group, 1/10 bird at 21 d.p.i was lame and this condition progressed up to 28 d.p.i. No clinical signs of lameness or swollen footpads or hock joints were observed in the 105208 FP and OR groups. However, from 14 to 28 d.p.i, generalized diarrhea was observed in both groups (Table 3.3).

Body weight, footpad and tendon measurements

At 7 and 14 d.p.i, mean body weights from both footpad and orally challenged groups were not significantly different ($P>0.05$). At 21 and 28 d.p.i, a significant decrease ($P<0.0001$) in mean body weights was observed between the 105208 OR and 105208 FP groups when compared to the control and 105057 groups (Figure 3.1).

At 7 d.p.i, 105057 FP and 105208 FP groups had significantly ($P\leq0.0034$) swollen tendons compared to the controls. In addition, 105057 FP tendons were significantly more swollen than 105208 FP, as evidenced by increased tendon/body weight ratios (Figure 3.2A). At the same timepoint, 105057 FP group had significantly ($P\leq0.0003$) more swollen footpads when compared to control and 105208 FP group (Figure 3.3A). In orally inoculated groups, tendons from both challenged groups were significantly swollen compared to the control group (Figure 3.2B). In group 105057 OR, footpads were significantly ($P<0.0320$) more swollen compared to control but not to 105208 group (Figure 3.3B). At 14 d.p.i, group 105057 FP had significantly swollen tendons compared to 105208 FP, but none of these groups differed from control groups (Figure 3.2A). In

addition, 105057 FP group had significantly swollen footpads compared to control, but not when compared to 105208 FP (Figure 3.3A). At 21 d.p.i, group 105208 FP showed significantly swollen tendons and footpads compared to control and 105057 FP (Figure 3.2A and 3.3A). Also, group 105208 OR had significantly swollen tendons and footpads compared to the control and 105057 OR groups (Figure 3.2B and 3.3B). At 28 d.p.i, group 105208 FP showed significantly swollen tendons compared to control and 105057 FP (Figure 3.2A).

Gross lesions

At 14 d.p.i, no gross lesions were observed in control and 105057 groups, however, thin intestines with watery feces were observed in both 105208 groups (data not shown). At 28 d.p.i, gross lesions consisting of hydropericardium and swollen hock joints were observed in virus inoculated groups with variable severity. In orally challenged groups, hydropericardium was more prevalent compared to the footpad challenged groups. On the other hand, the presence of swollen hock joints were similar between groups, however, greater severity was observed in footpad challenged groups, including gastrocnemius tendon rupture (Table 3.4).

Virus detection

Hearts and tendons pooled at 14 d.p.i were positive for reovirus by RT-PCR in footpad challenged groups. At 28 d.p.i, pooled hearts from footpad challenged groups were positive. Moreover, 9/10, 1/10 and 4/10 tendon swabs from 105057 FP, 105057 OR and 105208 FP were positive, respectively (Table 3.7). Tendon and heart samples collected from a bird that died from 105208 OR was negative for reovirus (data not shown).

Virus Isolation and titration

Virus isolation from cloacal swabs collected weekly was performed in Vero cells. Virus was isolated in only 2/20 birds in group 105208 FP at 7 and 14 days. One positive sample for virus isolation at 7 d.p.i had a titer of 10^1 TCID50/0.1ml (Table 3.8).

Histopathology of internal organs

Challenged groups 105057 and 105208, regardless of route of inoculation, had lymphocytic myocarditis (Table 3.5) with or without lymphoid nodules at 28 d.p.i. Myocarditis was mostly observed in groups inoculated by the FP route (Figure 3.7). Mild lymphoplasmacytic inflammation in the duodenum and jejunum was observed in all groups with mild to moderate villus blunting (Table 3.6).

Tendon scores

Footpad challenged groups had higher tendon inflammation scores, but 105057 FP was the only group that had significantly ($P \leq 0.0182$) higher scores compared to the other groups at 28 d.p.i, except 105208 FP (Figure 3.4). Histological findings in the tendons of group 105057 FP included mild lymphoplasmacytic tenosynovitis with peritendonal fibrosis (Figure 3.8).

S1 sequence analysis

Phylogenetic analysis based on the Sigma C protein sequences of avian reovirus revealed that TARV 105057 and 105208 are grouped in Cluster 2 (Figure 3.5). TARV 105057 shared 98% amino acid similarity to TARV MN3. Isolate 105208 shared 99.7% similar to TARV O'NEIL. Both TARVs evaluated in this study shared approximately 54% amino acid similarity to vaccine strains (Table 3.9).

S3 sequence analysis

Phylogenetic analysis based on Sigma B protein sequence of TARVs 105057 and 105208 demonstrated that our isolates grouped together with turkey origin strains (Figure 3.6).

Discussion

Avian reovirus is ubiquitous among commercial poultry (8) but is also the causative agent of arthritis and tenosynovitis in broilers and turkeys (13, 22). Few reports were published in the 80's describing turkey reovirus as a cause of tenosynovitis in turkeys (13, 23) and after 30 years, new cases of turkey reovirus causing tenosynovitis have increasingly been reported in the U.S (15, 19).

In the present study, pathogenicity of two turkey reoviruses (105057 and 105208) field isolates from clinical cases of tenosynovitis in turkey breeders were evaluated in day old commercial broilers for 28 days. Chicks were inoculated via the oral route because this is the natural route of infection (6) or, by footpad which is a classical experimental challenge route to evaluate tenosynovitis caused by reovirus (9).

It was determined that TARV 105057 and 105208 are pathogenic to susceptible commercial broilers. The isolates affected the musculoskeletal system at an early age, more evident in TARV 105057, causing clinical signs of lameness from 5 to 9 d.p.i, and gross lesions at 28 d.p.i due to tenosynovitis. An enteric component was observed in birds inoculated with TARV 105208 resulting in diarrhea; however, tenosynovitis was also observed at 28 d.p.i suggesting a predilection for multiple systems in commercial

broilers. The enteric disease was an unexpected outcome, because this isolate was obtained from a clinical case of tenosynovitis, although, it is well known that certain strains of turkey reovirus are associated with enteric disease (31), less is understood about the ability of one virus to contribute to multiple disease presentations.

More severe lesions of tenosynovitis were observed in footpad challenged groups, as evidenced by more inflamed tendons determined histologically. It is well known that inoculation of some reovirus strains by footpad induce tenosynovitis earlier with more severe tendon lesions compared to the oral route (20). Because a significant number of birds orally challenged showed gross lesions of swelling hock joint and tenosynovitis, it is possible that these birds could have developed clinical signs of lameness if the study had lasted longer, since heavier birds are more prone to develop tendon rupture. This is supported by findings that established that presence of histologic evidence of tenosynovitis might not compromise the ability to walk until a particular weight threshold is reached (8, 27). In this present study, TARV 105208 and 105057 OR groups may not have reached a sufficient body weight capable to induce clinical signs of lameness. Furthermore, this result indicates that commercial broilers can become ill through oral inoculation, which is one of the natural routes of infection in field transmission. It is clear that early horizontal transmission of these TARVs can result in disease in broilers, being a potential threat for commercial flocks.

Despite the presence of clinical signs and viral detection by RT-PCR in groups challenged with both viruses, turkey reoviruses were not efficiently shed by broilers in this study (10% 105208 FP). Similar results were obtained by Spackman and coworkers when inoculating SPF chickens with turkey reovirus isolates from clinical cases of

enteritis, however, the isolates were not pathogenic to chickens (31). A previous study revealed that virus shedding in broilers takes up to 3 weeks and for lighter breeders 2 weeks, when challenged with chicken reovirus (10). In this present study, broilers were infected by turkey reoviruses, however, low levels of virus were shed, suggesting limited horizontal transmission under experimental conditions. Moreover, avian reovirus can also be transmitted vertically through the egg, and thus contribute to the spread to several flocks via the same breeder flock (16). However, this study did not evaluate vertical transmission of the turkey reoviruses described.

An increase in isolations of new variants of ARV from field cases of tenosynovitis in chickens and turkeys has recently been reported (15, 19), however, biological characterization of few isolates has been reported especially between species (27). In addition, little is known about the ability of these viruses to contribute to both musculoskeletal and enteric pathotypes of disease in poultry. The isolates evaluated in this study belong to genotype cluster 2 and it is known that this cluster contains reoviruses with varying pathotypes (Figure 3.5). In a previous study, two different TARVs (MN3 and O'NEIL) from cluster 2 were evaluated in one-week-old specific pathogen free chickens, but with the exception of microscopic lesions, no clinical disease was reported (29). Differences in results between studies are likely due to differences in reovirus pathotypes, as well as, age and type of bird. In the present study, commercial broilers were used, because in some locations they are in close proximity to commercial turkey flocks and there is the concern of the risk of transmission of turkey arthritis reovirus to broilers or vice versa, which might cause economic impact to the poultry industry. TARVs in our study caused disease in broilers when inoculated at day of age,

when birds are most susceptible (18), as evidenced by early occurrence of clinical signs. In spite of recent studies have attempted to elucidate pathogenicity of new variants of ARV, isolates tested were from cluster 2, including the ones in the present study, thus, more studies are necessary to determine if other variants from different clusters may be pathogenic to broilers or even induce more than one disease presentation.

The present study was conducted for a period of 28 days, with adequate air circulation, temperature and water *ad libitum*, providing a controlled environment for the birds for their best development. Commercial broiler flocks birds can stay until 42-45 days of age in the poultry house, resulting in higher body weight and more stress to the tendon as chickens grow (10). The environment inside a commercial poultry house can be challenging because of feed competition, high density, poor air quality and presence of immunosuppression agents. We provided clean wood shavings and the isolation houses used in this study were disinfected prior to chicks housing. It is known that reovirus can persist in wood shavings and equipment for many days when they are not clean and disinfected properly (8). Field conditions could exacerbate the outcomes of reovirus infection, or any infection for that matter, causing significant economic losses.

The present study contributes to the understanding of the pathogenicity of TARVs 105057 and 105208 in commercial broilers. Both isolates caused disease, 105057 caused clinical signs consistent with viral arthritis, while 105208 caused enteric disease and tenosynovitis. Nevertheless, limited viral shedding was demonstrated. The origin of the variant reoviruses in both turkey and chicken populations is unclear, but this study clearly demonstrates that chickens are susceptible to infection with at least some turkey reovirus isolates.

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Table 3.1. Titers for turkey reovirus isolates determined in QM7 cells after 2 passages.

Isolate	Titer
105057	$10^{5.4}$ TCID50/ml
105208	$10^{4.4}$TCID50/ml

Table 3.2. Experimental design by group, number of chickens, treatment and route of inoculation[A].

Groups	Chickens	Treatment	Route of inoculation
1	20	Control[B]	Footpad (FP)
2	20	Control[B]	Oral (OR)
3	20	105057	Footpad (FP)
4	20	105057	Oral (OR)
5	20	105208	Footpad (FP)
6	20	105208	Oral (OR)

[A] A hundred and twenty (120) day-of-hatch commercial broilers were divided into 6 groups (n=20) and placed in 3 isolation houses divided by treatment and route of inoculation

[B] Negative controls were mock challenge via foot pad or oral route with sterile PBS

Table 3.3. Prevalence of clinical signs observed from 1 to 28 d.p.i in broilers inoculated with PBS (control), turkey arthritis reoviruses 105057 and 105208 by footpad (FP) or oral (OR) route of inoculation.

Groups	Lame	Swollen hock joint	Swollen footpad	Mortality	Diarrhea
Control FP	0/20[C]	0/20	0/20	0/20	-
Control OR[A]	1/20	0/20	0/20	1/20	-
105057 FP	3/20	5/20	18/20	0/20	-
105057 OR	1/20	0/20	0/20	0/20	-
105208 FP	0/20	0/20	0/20	0/20	+
105208 OR[B]	0/20	0/20	0/20	1/20	+

[A] One bird from control oral was eliminated in the first week

[B] One bird in group 105208 orally challenge died at 20 d.p.i

[C] Number of affected birds/total

Table 3.4. Gross lesions and clinical signs observed at 28 d.p.i at necropsy in broilers inoculated with PBS (control), turkey arthritis reoviruses 105057 and 105208, by footpad (FP) or oral (OR) route of inoculation.

	Gross Lesions				
	Clinical signs		Swollen Hock Joint		
Groups	Lame	Hydropericardium	Mild	Moderate	Severe
Control FP	0/10[A]	0/10	0/10	0/10	0/10
Control OR	0/9	0/9	0/9	0/9	0/9
105057 FP	0/10	1/10	4/10	0/10	1/10
105057 OR	1/10	6/10	3/10	3/10	0/10
105208 FP	0/10	2/10	2/10	3/10	2/10
105208 OR	0/9	4/9	2/9	4/9	0/9

[A] Number of affected birds/total

Table 3.5. Prevalence of myocarditis at 28 d.p.i in broilers inoculated with PBS (control), turkey arthritis reoviruses 105057 and 105208, by footpad (FP) or oral (OR) route of inoculation.

	Myocarditis	
Groups	Mild	Moderate
Control FP	0/10[A]	0/10
Control OR	3/9	0/9
105057 FP	7/10	1/10
105057 OR	4/10	0/10
105208 FP	6/10	0/10
105208 OR	3/9	0/9

[A] Number of affected birds/total

Table 3.6. Histopathological evaluation of inflammation and villus blunting in duodenum and jejunum at 28 d.p.i from broilers inoculated with PBS (control), turkey arthritis reoviruses 105057 and105208 by footpad (FP) or oral (OR) route of inoculation.

	Duodenum		Jejunum		
	Inflammation	Villus Blunting	Inflammation	Villus Blunting	
Groups	Mild	Mild	Mild	Mild	Moderate
Control FP	3/3[A]	0/3	3/3	0/3	0/3
105057 FP	4/4	2/4	4/4	3/4	1/4
105057 OR	4/5	4/5	4/4	4/4	0/4
105208 FP	5/5	5/5	5/5	3/5	0/5
105208 OR	6/6	6/6	7/7	7/7	0/7

[A] Number of affected birds/total

Table 3.7. Detection of reovirus by RT-PCR in S1 gene in hearts and tendons at 14 d.p.i, and hearts and tendon swabs at 28 d.p.i. from broilers inoculated with PBS (control), turkey arthritis reoviruses 105057, or 105208 by footpad (FP) or oral (OR) routes of inoculation.

	14 d.p.i		28 d.p.i	
Groups	Heart[A]	Tendon[A]	Heart[A]	Tendon Swab[B]
Control FP	-	-	-	0/10[C]
Control OR	-	-	-	0/9
105057 FP	+	+	+	9/10
105057 OR	-	-	-	1/10
105208 FP	+	+	+	4/10
105208 OR	-	-	-	0/9

[A] Samples were pooled

[B] Individual samples

[C] Number of positive detection/total

Table 3.8. Virus Isolation of reovirus in Vero cells from cloacal swabs collected at 7, 14, 21 and 28 d.p.i. from broilers inoculated with PBS (control), turkey arthritis reoviruses 105057, 105208 by footpad (FP) or oral (OR) routes of inoculation.

Groups/ d.p.i	7	14	21	28
Control FP	0/20	0/20	0/10	0/10
Control OR	0/20	0/19	0/9	0/9
105057 FP	0/20	0/20	0/10	0/10
105057 OR	0/20	0/20	0/10	0/10
105208 FP	2/20[AB]	2/20[B]	0/10	0/10
105208 OR	0/20	0/20	0/9	0/9

[A] 1 sample from 105208 FP at 7 days had titer of 10^1 TCID50/0.1ml

[B] Number of positive isolations/total of samples

Table 3.9. Comparison of nucleotide and deduced amino acid sequences of the Sigma C protein of TARVs 105057, 105208 and selected reovirus reference strains.

% Amino acid similarity							
	1	2	3	4	5	6	7
1.S1133	100	96.5	53.5	54.2	54.5	54.2	54.2
2. 1733		100	55.2	55.8	55.8	55.8	55.8
3. 105057			100	97.7	97.7	98.1	97.4
4. 105208				100	99.4	99.7	99.0
5. TARV MN3					100	99.7	99.0
6. TARV O'NEIL						**100**	**99.4**
7.TKY 2342/2003							**100**

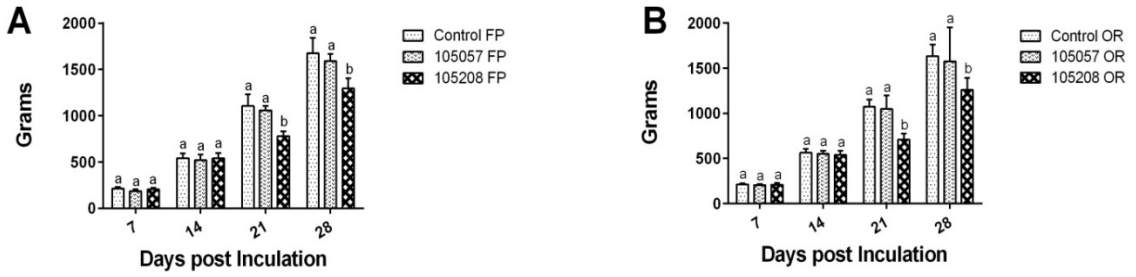

Figure 3.1. Mean body weight of broilers at 7, 14, 21 and 28 d.p.i for unchallenged controls, turkey arthritis reoviruses 105057 and 105208 by footpad (FP) (1A) or oral (OR) (1B) routes of inoculation. Significant differences between groups are indicated by use of different lowercase letters ($P<0.0001$, one way ANOVA).

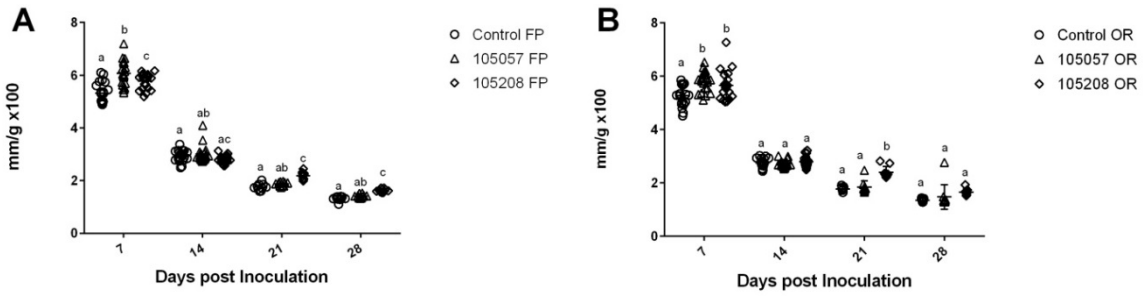

Figure 3.2. Mean Tendon/Body weight ratios (tendon (mm) / body weight (g) x 100) of broilers at 7, 14, 21 and 28 d.p.i for controls, turkey arthritis reoviruses 105057 and 105208 by footpad (FP) (2A) or oral (OR) (2B) route of inoculation. Different lowercase letters indicate significant differences between groups (FP groups at 7 d.p.i $P \leq 0.0034$; at 14 d.p.i $P \leq 0.0170$; at 21 d.p.i $P \leq 0.001$; at 28 d.p.i $P \leq 0.0147$; OR groups at 7 d.p.i $P \leq 0.0234$; at 21 d.p.i $P \leq 0.0001$ one way ANOVA).

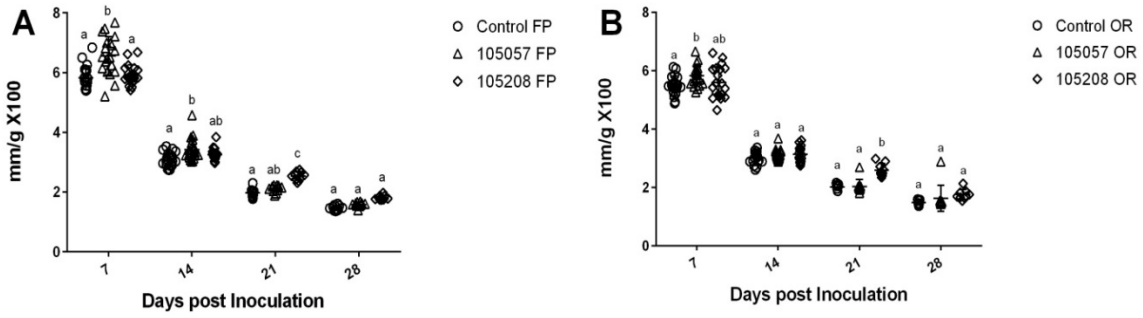

Figure 3.3. Mean Footpad/Body weight ratio (footpad (mm) / body weight (g)x100) of broilers at 7, 14, 21 and 28 d.p.i for controls, turkey arthritis reoviruses 105057 and 105208 by footpad (FP) (2A) or oral (OR) (2B) route of inoculation. Different lowercase letters indicate significant differences between groups (FP groups at 7 d.p.i $P \leq 0.0003$; at 14 d.p.i $P \leq 0.0320$; at 21 d.p.i $P \leq 0.0001$; OR groups at 7 d.p.i $P \leq 0.0320$; at 21 d.p.i $P \leq 0.0001$ one way ANOVA).

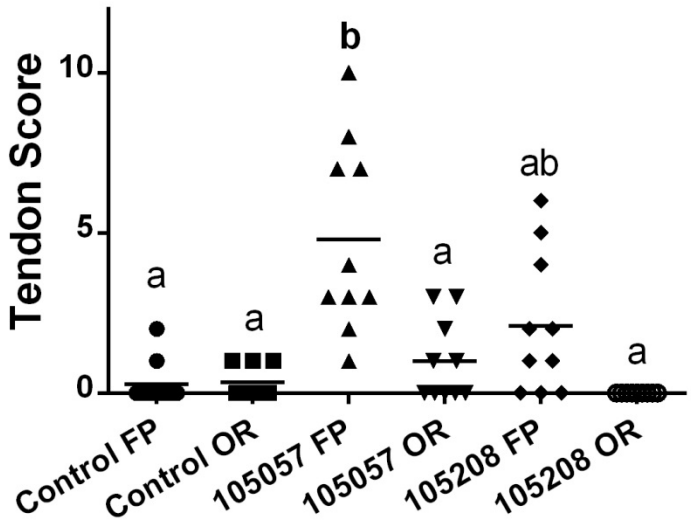

Figure 3.4. Histological inflammation tendon scores from digital flexor from left leg of broilers at 28 d.p.i for controls, turkey arthritis reoviruses 105057 and 105208, by footpad (FP) or oral (OR) inoculation. Different lowercase letters indicate significant differences between groups ($P \leq 0.0182$, Kruskal Wallis Anova).

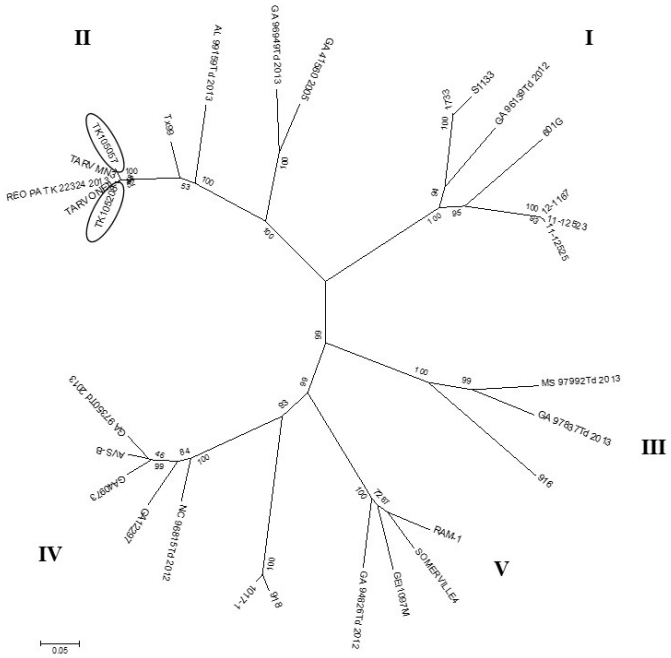

Figure 3.5. Phylogenetic tree based on Sigma C protein of avian reoviruses. Multiple alignments of the amino acid sequence of Sigma C were performed in Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and an unrooted phylogram was generated using the Neighbor-Joining method (25) in MEGA6 (32). The optimal tree with the sum of branch length = 3.29910677 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (4). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (35) and are in the units of the number of amino acid substitutions per site. The five previously described genotypic clusters are identified by roman numerals (11). Commercial vaccines strains (S1133 and

1733, GenBank KF741712.1, AY536919.1) belong to cluster I. TARVs 105057 and 105208 belong to cluster II and are circled.

Figure 3.6. Phylogenetic tree based on Sigma B protein of avian reoviruses. Multiple alignments of the amino acid sequence of Sigma B were performed in Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and an unrooted phylogram was generated using the Neighbor-Joining method(25) in MEGA6 (32). The optimal tree with the sum of branch length = 3.22048594 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (4). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (35) and are in the units of the number of amino acid substitutions per site. Reoviruses are clustered by specie. Previously identified reovirus strains are represented in Phylogenetic tree: 138

(AF059721.1); AVS-B (FR694199.1); 916 (AY008383.1); 601G (AY008384.1); S1133 (KF741764.1); 919 (AF208034.1); 750505 (AF208035.1); TX99 (AY444910.1); TARV-MN1 (KF872265.1); D15/99 (AY114138.1); ZJ2000M (KF306090.1); CA (KJ569582.1). TARVs 105057 and 105208 are circled in the turkey origin group of avian reoviruses. Chickens reoviruses are grouped separately.

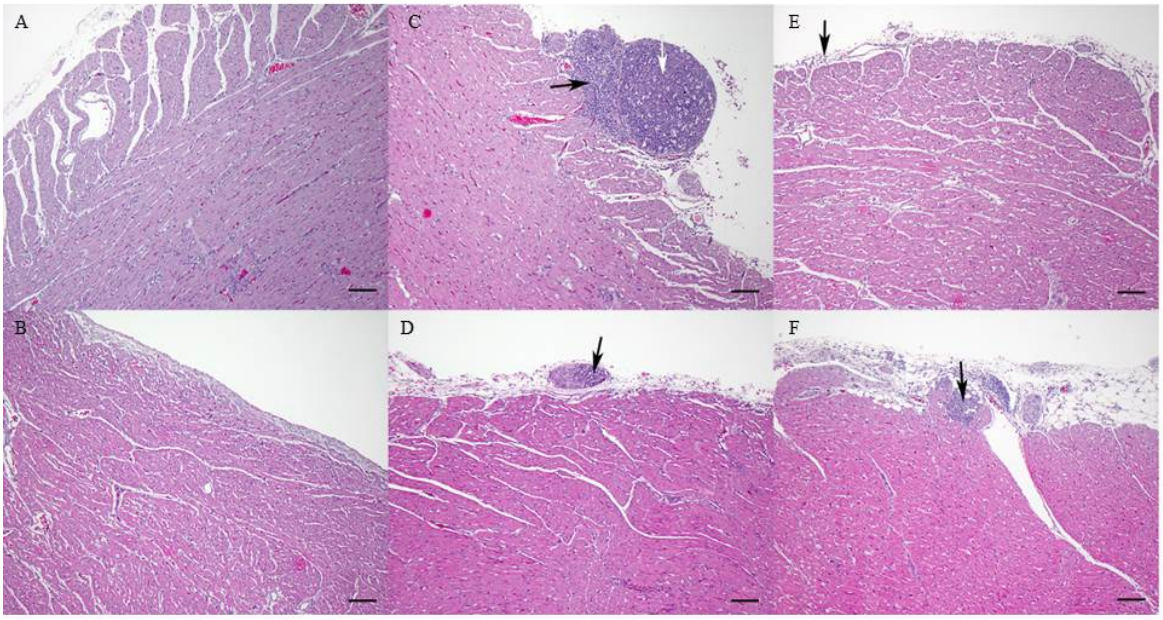

Figure 3.7. Photomicrographs of hematoxylin and eosin stained hearts from broilers from different challenge groups and routes of inoculation at 28 d.p.i. Black arrows indicate lymphocytes and the white arrow a lymphoid nodule in the following sections: (7A) Control FP; (7B) Control OR; (7C) 105057 FP; (7D) 105057 OR; (7E) 105208 FP; (7F) 105208 OR. Bars = 100µm.

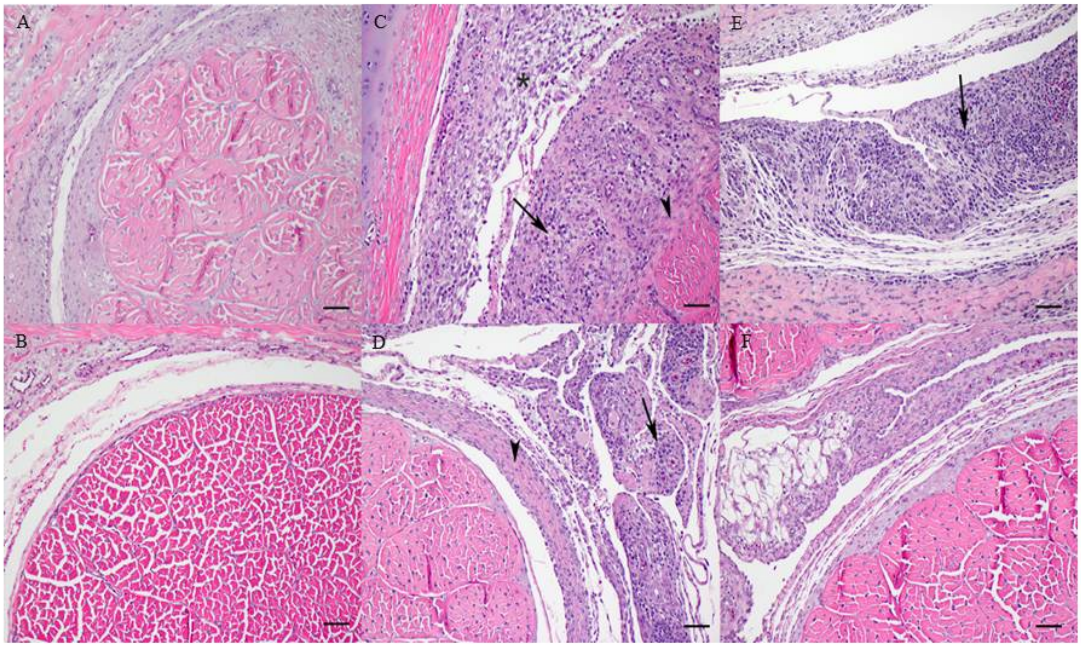

Figure 3.8. Photomicrograph of hematoxylin and eosin stained left leg digital flexor tendons from broilers from groups challenged with different viruses and routes of inoculation at 28 d.p.i. Black arrows indicate inflammatory infiltrate composed of lymphocytes and plasma cells in tendon sheaths and synovial membrane; arrowheads indicate fibrosis; and asterisk indicates synovial cell hyperplasia in the following sections: (7A) Control FP; (7B) Control OR; (7C) 105057 FP, (7D) 105057 OR, (7E) 105208 FP and (F) 105208 OR. Bars = 50µm.

CHAPTER 4

PATHOGENICITY OF TURKEY AND CHICKEN ARTHRITIS REOVIRUSES IN COMMERCIAL TURKEYS[A]

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Summary

The incidence of arthritis and tenosynovitis has increased in the last few years with the emergence of new variants of reovirus in the U.S and worldwide. In this study, two field isolates from clinical cases of tenosynovitis in commercial turkey breeders (TARV 10505 and 105208) and one field isolate from commercial broilers (CARV 94826) were tested to evaluate its pathogenicity in turkeys. Footpad and oral routes of inoculation were used for the challenge. Turkey arthritis reovirus (TARV) isolates, 105057 and 105208 caused clinical disease in turkeys, evidenced by clinical signs of lameness and presence of lymphocytic tenosynovitis and myocarditis. In contrast, chicken arthritis reovirus (CARV) isolate 94826 did not induce tenosynovitis in turkeys, but only mild myocarditis. Using RT-PCR, reovirus was detected in tendons of TARV-challenged groups, but not in turkeys challenged with the CARV isolate. In addition, viral shedding was observed to varying degrees in TARV-challenged groups but not in the CARV-challenged group suggesting poor fecal-oral transmission of CARV in turkeys.

Key words: avian reovirus, chicken reovirus, turkey reovirus, tenosynovitis, arthritis, myocarditis

Abbreviations: ARV = avian reovirus; CAM = chorioallantoic membrane; CARV = chicken arthritis reovirus; d.p.i = days post inoculation; ELISA = enzyme-linked immunosorbent assay; FP = footpad; HVT-LT = Herpesvirus of turkeys-laryngotracheitis; LTS = light turkey syndrome; OR = oral; PB = phosphate buffered saline; p.i = post inoculation; PEC = poult enteric complex; PES = poult enteric syndrome; RT-PCR = reverse transcriptase-polymerase chain reaction; TARV = turkey arthritis reovirus; TCID50 = tissue culture infectious dose 50; TK = turkey; VN = virus neutralization.

Introduction

For over 30 years the role of reoviral disease in turkeys has been associated with enteric disease/syndromes, among them light turkey syndrome (LTS), poult enteric complex (PEC) and poult enteric syndrome (PES) (3, 12, 21), characterized by combinations of diarrhea, dehydration, weight loss, anorexia, growth depression and high mortality (9). Turkey reovirus was first reported as a causative agent of arthritis and tenosynovitis in turkeys in 1980 (26). Furthermore, in 2009 Shivaprasad *et.al* (32) first documented the occurrence of myocarditis caused by reovirus in young poults wherein lymphoid depletion was also observed in the bursa of Fabricius. In 2011, an increased incidence of reovirus-induced turkey arthritis was observed and subsequently isolated from the tendons of lame commercial turkeys with tenosynovitis and gastrocnemius tendon rupture (22).

In chickens, reovirus has also been associated with cloacal pasting and mortality (5), ulcerative enteritis (15), enteric disease (5), respiratory disease (6), hepatitis (19), runting and stunting syndrome (8), and more definitively as the causative agent of viral arthritis/tenosynovitis (24).

Avian reoviruses (ARV) are members of the *Orthoreovirus* genus, one of the 12 genera of the *Reoviridae* family (2, 20) and are ubiquitous in commercial poultry (13). ARV has been detected in different species of birds, including chickens (25), turkeys (33), ducks (38), pigeons (36) and many others, however, more attention has been given to turkey and chicken reoviruses due to the economic impact in commercial flocks (11,

13). Although turkey and chicken reoviruses belong to the same genus, they are genetically distinct (22).

Newly emerging avian reoviruses causing arthritis and tenosynovitis in chickens and turkeys have been isolated from commercial poultry flocks since 2011 (18). The molecular analysis of Sigma C, the least conserved segment of the reovirus genome (17), revealed that a majority of recently identified variants are genetically distinct from vaccine strains and this may imply a reduction in protection induced by immunization with current commercial vaccines (18, 22). The aim of the present study was to evaluate the pathogenicity of two TARV and one CARV field isolates, from clinical cases of tenosynovitis, in commercial turkeys.

Material and Methods

Cell lines

QM7 (quail muscle cells, ATCC reference CRL-1962™) and VERO (African green monkey kidney cells, ATCC reference CCL-81™) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 2% Penicillin + Streptomycin and Fungizone (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a 5% CO_2 incubator. QM7 cells were utilized for virus propagation and Vero cells were utilized for virus neutralization, virus isolation and titration.

Viruses

Two turkey reoviruses were isolated from the tendons of lame commercial turkey breeders from Wisconsin, United States of America, and identified as TK/WI/105057

Tendon/2014 (referred to as 105057 from here out) and TK/WI/105208 Tendon/2014 (referred to as 105208 from here out). Both isolations were obtained from clinical case submissions to the Poultry Diagnostic and Research Center (University of Georgia), with tenosynovitis as the primary reason for submission. For isolate 105057, primary isolation was made from tendons in the second passage of primary chicken embryo liver cells. Cytopathic effect characteristic of reovirus was observed and confirmed by RT-PCR. For isolate 105208, primary isolation was made from tendons in the first passage in SPF turkey embryos inoculated via the chorioallantoic membrane inoculation (CAM). Lesions characteristic of reovirus were observed in the embryos. CAMs were submitted for RT-PCR confirmation and genotyping. Both isolates were propagated, passed twice and titrated in QM7 cells (4). Additionally, a chicken arthritis reovirus was used in this study, CK/GA/94826 Tendon/2012 (referred to as 94826 from here out), from a clinical case of tenosynovitis in commercial broilers from Georgia, USA. Primary isolation was made from the tendons submitted in the second passage of primary chicken embryo liver cells. Cytopathic effect characteristic of reovirus was observed and confirmed by RT-PCR. Isolate 94826 was propagated in primary chicken embryo liver cells. Titers for 105057, 105208 and 94826 viruses, as determined by Reed and Muench (28), are shown in Table 4.1.

Turkeys

One hundred, day-of-hatch commercial turkeys were divided into 8 groups (4 groups n=13 and 4 groups n=12) and placed in 4 isolation houses divided by treatment and route of inoculation (Table 4.2). Food and water was provided *ad libitum.*

Experimental Design

At 4 days of age, all birds were weighed prior to inoculation. Poults from each group were inoculated with either isolate 105057 at $10^{3.5}$ TCID50/bird, 105208 at $10^{3.1}$ TCID50/bird, 94826 at $10^{3.5}$ TCID50/bird or Phosphate Buffered Saline (PBS) via the left footpad (FP) or by oral (OR) instillation. Poults inoculated with the same virus but by a different route were placed in the same colony house, in different pens separated by a PVC fence covered with a cardboard divider (Table 4.2). All birds were observed daily for clinical signs, including lameness, swelling of the hock joint and/or swelling of the footpad. Body weight, footpad and hock joint measurements, as well as cloacal swabs, were collected individually at 6, 13, 20, 27 and 32 days post inoculation (d.p.i). At 32 d.p.i all birds were humanely euthanized for post mortem examination and tissue collection. Hearts and swabs from the hock joint and synovial fluid were collected for virus detection by RT-PCR. Digital flexor tendons, heart and intestinal sections of jejunum were collected and fixed in 10% neutral buffer formalin for histopathological examination. Birds that showed obvious signs of lameness with joint swelling before the end of the experiment were euthanized, necropsied and samples collected for reovirus detection.

Hock joint and footpad measurements

Left hock joint (tendon) and footpad measurements were taken using a digital caliper. A tendon and footpad to body weight ratio was determined by the formula, (tendon(mm)/body weight(g))x100 and footpad/body weight ratio by (footpad(mm)/body

weight(g))x100. The ratios were used for the evaluation of tendon and footpad swelling, normalized by the body weight.

Swab

Cloacal swabs were collected for virus isolation and titration in 1ml of tryptose phosphate buffer (TPB) + 2% Penicillin + Streptomycin and Fungizone. Samples were centrifuged at 1500 rpm for 3 minutes and stored at -80°C. At 32 d.p.i, swabs from joint fluid and digital flexor tendons from each bird were collected in 2ml of TPB and stored at -80°C for RT-PCR.

Serology

Commercial ELISA was used to test sera for reovirus, *Mycoplasma synoviae* and *Mycoplasma gallisepticum* antibodies (IDEXX, Westbrook, Maine, USA) according to the manufacturer's instructions. In addition to ELISA, the virus neutralization (VN) test, using the beta method, was performed with 105057, 105208 and 94826 antigens (4). Briefly, the VN assay was performed in 96 well plates containing confluent pre-formed monolayers of VERO cells. Plates were incubated at 37°C+5% CO_2 for 96 hours and then VN titers, as represented by the reciprocal of the last dilution where no cytopathic effect was observed, were recorded.

Clinical signs

Birds were observed daily for clinical signs throughout the study. Birds that showed recumbency, inability to move, being unable to reach food and water were considered lame and removed from the study. Removed birds were necropsied and digital flexor tendon and hearts were collected for reovirus detection by RT-PCR.

Gross lesions and histopathology

All birds were humanely euthanized at 32 d.p.i, and examined for gross lesions in the visceral organs, intestines and intertarsal (hock) joints. Gastrocnemius and digital flexor tendons, along with the heart and intestines were collected and fixed in 10% neutral buffered formalin, stained with hematoxylin and eosin for examination of lesions by light microscopy. The digital flexor tendons from the left leg were scored for inflammation as previously described (30).

Virus Isolation and titration

Prior to virus isolation and titration, cloacal swabs were thawed and centrifuged for 10 minutes at 1500 rpm at 4°C. Samples were filtered using 0.2μM GDX filters (Whatman, Fischer Scientific, Norcross, GA). Viral shedding was evaluated from cloacal swabs collected at 6, 13, 20, 27 and 32 d.p.i from each bird in VERO cells (23, 37). Cell cultures were examined daily for 5 days for presence of cytopathic effect characteristic of reovirus. Titration was performed in VERO cells in 96 well plates containing confluent pre-formed monolayers and titers calculated using the method of Reed and Muench (28).

RNA extraction and RT-PCR

Tendon swabs from joint fluid and hearts collected at 32 d.p.i were tested for reovirus RNA detection individually and by pools of 5 samples, respectively. Virus transport media (VTM) was used to homogenize tissue or tissue pools. Following centrifugation, RNA was extracted from 0.2ml supernatants from the tendon swabs using the MagMAX™ Pathogen RNA/DNA Kit on MagMAX™ Express-96 Deep Well Magnetic Particle Processor sample extractor (Thermo Fischer scientific, Waltham,

MA, USA). The RNeasy® kit (Qiagen, Hilden, Germany) was used for RNA extraction from the heart samples. RNA was stored at -80°C until use. RT-PCR using S1 primers was performed on all RNA as previously described (14).

Statistical analysis

One way ANOVA test was used for the evaluation of body weight, footpad/body weight ratio and tendon/body weight ratio and Kruskal Wallis test for comparison of histologic inflammation tendon scores (GraphPad Software, Inc., La Rolla, CA USA).

Results

Serology

Sera collected from ten poults prior to inoculation were negative for reovirus antibodies by ELISA. Birds were negative for virus neutralizing antibodies to 105057, 105208 and 94826 antigen as determined by VN (data not shown). Sera collected from birds at 20 days of age were negative for *Mycoplasma gallisepticum and Mycoplasma synoviae* by ELISA (data not shown).

Clinical signs and mortality

One (1/12) poult in group 94826 (Oral) was lame at 8 d.p.i, but this condition lasted only 2 days and this bird appeared to recover. Similarly, two (2/13) poults in group 105057 FP were lame, one at 7 and another at 14 d.p.i and both recovered two days later. 105057 OR group had one (1/12) lame poult at 7 d.p.i and this bird was removed from the study. Two (2/13) poults in 105208 FP group showed signs of lameness at 4 d.p.i, one died and the other was removed from the study at 5 d.p.i. One poult died at day of

housing in this same group. No gross lesions were observed in birds that died during the study (Table 4.3).

At 20 d.p.i a breach in the barrier separating birds inoculated with the same virus but by different routes of inoculation was identified and corrected in all the isolation houses. It was not possible to determine with certainty the original groupings of the birds and therefore the data collected from timepoint 20 d.p.i forward could reflect a mixed population of birds.

Body weight, tendon and footpad measurements

There was no significant difference ($P>0.05$) in body weights when challenge groups were compared to control groups, however, a significant decrease was observed at 27 ($P\leq0.0097$) and 32 d.p.i ($P\leq0.0118$) in the orally challenged group 105057 compared to 105208 OR and 94826 OR (Figure 4.1).

Significantly swollen footpads ($P\leq0.0303$) and tendons ($P\leq0.0492$) were observed at 6 d.p.i in turkey reovirus footpad inoculated groups when compared to 94826 and control groups (Figures 4.2 and 4.3) as evidenced by higher footpad/tendon to body weight ratios.

Gross lesions at necropsy

At 32 d.p.i, hydropericardium was observed in four (4/13) turkeys in group 105057 FP. No other gross lesions were observed in any of the other groups irrespective of treatment (Table 4.4).

Histopathology

Lymphocytic epicarditis and myocarditis were observed in all challenged groups except in 94826 FP at 32 d.p.i. The lesions were mostly mild and more prevalent in 105057 FP group (Table 4.5 and Figure 4.5).

Most of the microscopic changes in the intestines of group 94826 FP were heterophilic inflammation in Meckel's diverticulum, whereas, 94826 OR had focal septic granuloma in Meckel's and focal mild lymphocytic inflammation. Lymphocytic inflammation in the jejunum was observed in all groups challenged with TARVs, more prevalent in turkeys challenged with virus 105057 (Table 4.6).

Group 105057 FP was the most affected group in this study, with mild to severe lymphocytic and proliferative tenosynovitis. Groups inoculated with 105208 had mild proliferative tenosynovitis. Mild proliferative tenosynovitis was also observed in groups inoculated with virus 94826, however, these were very mild changes that were not significant when compared to controls. Microscopic findings included mild hyperplasia of the synovial cells lining the tendons sheaths and mild to severe lymphocytic infiltrates and fibroplasia in the tendon sheaths. The orally challenged group 105057 had mild lymphocytic tenosynovitis.

Tendons score for inflammation

Inflammation scores obtained from microscopic evaluation of the tendons were statistically analyzed using the Kruskal-Wallis test. Groups were compared to each other regardless of route of inoculation. Statistically significant higher inflammation score was detected for group 105057 FP ($P \leq 0.015$) when compared to negative controls and

challenged groups, except 105057 OR (Figure 4.4). The inflammation scores for group 105057 OR were significantly higher ($P \leq 0.005$) compared to oral groups, except 105208. No significant differences were observed in groups inoculated with isolate 94826, compared to the control and TARV challenged groups (Figure 4.4).

Virus shedding

Virus shedding was detected only in groups challenged with turkey reoviruses at 6 and 13 d.p.i, except in group 105208 FP wherein no virus was isolated at 6 d.p.i. The highest prevalence of viral shedding was observed in group 105057 OR, with 1/3 of the birds shedding virus, albeit at low levels, $\leq 10^1$ TCID50/0.1ml, and the highest titer was 10^3 TCID50/0.1ml, obtained from 105057 FP group at 6 d.p.i. At 13 d.p.i, viral shedding was detected in two birds (2/12) from 105208 OR and in one for each of 105057 and 105208 FP groups. Levels of shedding were low in both TARV groups at 13 d.p.i (< or = to 10^1 TCID50/0.1ml) (Table 4.7).

Virus detection

Reovirus was detected by RT-PCR in tendon swabs at 32 d.p.i only in groups inoculated with turkey reovirus (Table 4.8). All hearts and tendon swabs from chicken reovirus inoculated groups were negative for reovirus by RT-PCR. Tendons collected from group 105208 FP mortality and eliminated birds were positive.

Discussion

Tenosynovitis was first reported in turkeys by Levisohn and coworkers when a virus agent was isolated from joint fluid from 15 week old turkeys exhibiting clinical

signs of lameness with stiff hock joints (16). Two years later, Page *et.al* described two cases of tenosynovitis caused by reovirus affecting 5-8 week old young poults that were experiencing clinical signs of lameness, swollen hock joints with increased mortality. Moreover, virus was isolated and inoculated by footpad in susceptible poults and the disease was successfully reproduced. Recently, numerous variants of avian reovirus have been reported from clinical cases of viral arthritis and tenosynovitis in commercial poultry flocks in the U.S (18, 22) and elsewhere around the world.

In the present study, commercial poults were challenged with two TARVs and one CARV, isolated from clinical cases of tenosynovitis in commercial turkey breeders and broilers, respectively, to evaluate the pathogenicity of the viruses in turkeys. Two routes of inoculation were used, oral and footpad. These routes have been tested in previous studies of reovirus infection in turkeys, being oral the natural route, while footpad is experimentally used for tenosynovitis induction (1, 27).

Field isolates 105057 and 105208 were inoculated in susceptible poults and both viruses were pathogenic, causing clinical signs of lameness from 4 to 14 d.p.i. Reovirus infection was evidenced by lymphocytic tenosynovitis and myocarditis, in addition, reovirus was detected in tendons by RT-PCR. As these TARVs were originally isolated from 19 and 13 weeks old commercial turkey breeders, respectively, this result indicates that tenosynovitis may impact not only breeders, but also meat type turkeys if induced early in the life of a turkey poult, which could potentially result in economic losses.

An important result of this study was the observation of clinical signs not only in footpad challenged groups, but also in orally challenged groups, as observed in 105057

OR, moreover, inflammation in tendons between these groups were similar. Footpad inoculation has been a preferred route to evaluate tenosynovitis induced by reovirus in chickens and turkeys, due to the faster induction of clinical signs (26, 30), however, our results indicated that TARV 105057 was similarly pathogenic through both routes of inoculation, suggesting that natural route of fecal-oral transmission can induce tenosynovitis similarly to the experimental footpad route for this virus. On the other hand, clinical signs were observed in 105208 FP but not in orally challenged group, despite the similarity of the tendon inflammation between them. Previous studies have shown that lameness and swollen hock joints are usually observed in turkeys older than 10 weeks in field conditions (18, 30). The presence of clinical signs of lameness has been suggested to be related to weight-bearing forces on the tendons of older birds which may result in tendon rupture. In turkeys, this correlation was observed to occur at 8 weeks, peaking at 16 weeks post challenge (31). Because poults in this study were evaluated for only 32 days, it is possible that clinical signs would have been more significant if the poults were allowed to age and have their body weight increased (30, 31).

Myocarditis was mostly mild and observed in all challenged groups, except 94826 FP. The occurrence of myocarditis in young turkeys was first documented by Shivaprasad *et.al* (32) and has been reported in studies involving transmission of new TARVs in poults, being an indicative of reovirus infection (30). In broilers, reovirus causing myocarditis has been extensionally documented (13). Besides myocarditis, hydropericardium was observed in some turkeys in our study (30% in 105057 FP), suggesting association with reovirus infection. In previous work, pericarditis was

observed in a case report of tenosynovitis in turkeys in 1982 (26) and more recently was documented in Pennsylvania, related to new emergent turkey reovirus variants from commercial flocks older than 8 weeks (18). Differences in results may be indicative of differences in strains and age when turkeys were evaluated.

One important result of this study was that TARVs challenged groups were able to shed the virus through the cloaca. This information indicates that transmission may occur, as the virus showed to efficiently replicate in turkeys and also was excreted; probably, horizontal transmission could have occurred through the fecal-oral route, as previously reported by other studies (30). In addition, reovirus can be vertically transmitted, but this was not evaluated during this study. These results indicate that TARVs evaluated in the present work can be spread in commercial turkey flocks.

To prevent and control vertical and horizontal transmission of reovirus, vaccination of chicken breeders has historically shown to be efficient (34), however, only against homologous serotypes (10). Because the variants tested in this study are genotypically distinct from commercially available vaccines, the use of an autogenous vaccine containing field isolates 105057 and 105208 could provide the best opportunity to prevent disease and transmission (35). Moreover, conventional screening of vaccinated flocks with autogenous vaccine is inefficient using commercially ELISA kits, due to heterologous serotype used to coat the plate, and a secondary chicken antibody, resulting in low binding affinity (7). On the other hand, virus neutralization using homologous antigen can be used to detect different serotypes, however it is not automated and it is time consuming, being not practical for screening.

Chicken arthritis reovirus 94826 did not cause disease in turkeys during the study. Originally, this virus was isolated from a field case of tenosynovitis in commercial broilers and experimentally induced tenosynovitis in broilers in studies conducted at PDRC (30). In the present study, CARV 94826 was shown to poorly replicate in turkeys, as indicated by RT-PCR and virus isolation; however myocarditis was suggestive of reovirus infection. It seems that turkeys are more resistant to chicken arthritis reovirus and the potential for transmission is low. Similar results were obtained by Afaleq and Jones (1). In contrast, Sharafeldin *et.al* detected reovirus by RT-PCR in turkey tendons inoculated with CARV at 1 week of age, although no evidence of tenosynovitis was reported in that study. It is reasonable to conclude that CARV 94826 can infect poults, however, this isolate is not capable to induce tenosynovitis or being transmitted efficiently among turkeys due to poor viral shedding.

Neither the turkey nor the chicken arthritis reovirus significantly affected body weights when compared to control groups until 32 d.p.i, however in the orally challenged group 105057, the mean body weight decreased over time compared to other challenged groups. It is suggestive that body weights in this group would have significantly decreased compared to control if the study had lasted longer. Experimentally, poults challenged with pathogenic TARV had decreased body weights from 12 to 18 weeks p.i., due to occurrence of more clinical signs of lameness as birds aged (31). The difference in mean body weights between challenged groups in our study may have been caused by lesions in tendons of more severely affected birds with tenosynovitis as observed in

group 105057 OR, which likely had more difficult to walk and reach the feeders and consequently had reduced feed consumption.

Altogether, we established that both turkey arthritis reoviruses 105057 and 105208 were pathogenic to turkeys, causing clinical disease consistent with what was observed clinically in commercial turkey breeders from the original case. Furthermore, virus was shed in both groups indicating that horizontal transmission may occur. On the other hand, CARV 94826 caused infection, as evidenced by microscopic lesion of myocarditis, however this virus did not produce neither tenosynovitis nor virus shedding, suggesting limited pathogenicity and transmission of CARV in turkeys. Future studies may be needed to evaluate the long term effect of these isolates in older turkeys and if they can be efficiently transmitted from infected to naïve birds.

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Table 4.1. Titers for chicken and turkey reovirus isolates determined in CEF[1] and QM7[2] cells, respectively.

Isolate	Titer
94826[1]	$10^{5.8}$ TCID50/ml
105057[2]	$10^{5.4}$ TCID50/ml
105208[2]	$10^{4.4}$ TCID50/ml

Table 4.2. Experimental design by group, number of chickens, treatment and route of inoculation[A].

Groups	# Poults	Treatment	Route of Inoculation
1	13	Control [B]	Footpad (FP)
2	12	Control [B]	Oral (OR)
3	13	94826	Footpad
4	12	94826	Oral
5	13	105057	Footpad
6	12	105057	Oral
7	13	105208	Footpad
8	12	105208	Oral

[A] One hundred (100) day-of-hatch commercial turkeys were divided into 8 groups and placed in 4 isolation houses divided by treatment and route of inoculation.

[B] Negative controls were mock challenged via foot pad or oral route with sterile PBS.

Table 4.3. Prevalence of clinical signs, elimination and mortalities from 1 to 32 d.p.i in turkeys inoculated with PBS (control), turkey arthritis reoviruses 105057 and 105208, by footpad (FP) or oral (OR) routes of inoculation.

Groups	Lame	Eliminated	Mortality
Control FP	0/13[A]	0/13	0/13
Control OR	0/12	0/12	0/12
94826 FP	0/13	0/13	0/13
94826 OR	1/12	0/12	0/12
105057 FP	2/13	0/13	0/13
105057 OR	2/12	1/12[B]	0/12
105208 FP	2/13	1/13[B]	2/13[B]
105208 OR	0/12	0/12	0/12

[A] Number of birds affected/total

[B] Euthanized lame birds and mortalities after challenge were evaluated for reovirus detection by RT-PCR in tendon and hearts. Tendons from two turkeys from group 105208 FP were positive.

Table 4.4. Gross lesions at necropsy at 32 d.p.i. in turkeys inoculated with PBS (control) TARVs 105057 and 105208 and CARV 94826 by footpad (FP) or oral (OR) routes of inoculation.

Groups	Hydropericardium
Control FP	0/14[A]
Control OR	0/11
94826 FP	0/12
94826 OR	0/13
105057 FP	4/13
105057 OR	0/11
105208 FP	0/12
105208 OR	0/10

[A] Number of birds affected/total

Table 4.5. Prevalence of myocarditis at 32 d.p.i in turkeys inoculated with PBS (control), TARVs 105057, 105208 and CARV 94826, by footpad (FP) or oral (OR) routes of inoculation.

	Myocarditis	
Groups	**Mild**	**Moderate**
Control FP	0/14[A]	0/14
Control OR	0/11	0/11
94826 FP	0/12	0/12
94826 OR	2/13	0/13
105057 FP	6/13	1/13
105057 OR	3/11	0/11
105208 FP	2/12	0/12
105208 OR	1/10	0/10

[A] Number of birds affected/total

Table 4.6. Histopathological evaluation of inflammation and villus blunting in the Jejunum of turkeys at 32 d.p.i with PBS (control),TARVs 105057, 105208 and CARV 94826 by footpad or oral route of inoculation.

	Jejunum		
	Inflammation		Villus blunting
Groups	Mild	Moderate	Mild
Control FP	3/7[A]	0/7	2/7
94826 FP	1/8	0/8	2/8
94826 OR	3/10	0/10	3/10
105057 FP	6/10	1/10	5/10
105057 OR	6/11	0/11	6/11
105208 FP	1/12	0/12	1/12
105208 OR	4/10	1/10	4/10

[A] Number of birds affected/total

Table 4.7. Virus Isolation of reovirus in Vero cells from cloacal swabs collected at 6, 13, 20[D], 27[D] and 32[D] d.p.i. from turkeys inoculated with PBS (control), turkey arthritis reoviruses 105057, 105208 and CARV 94826, by footpad (FP) or oral (OR) routes of inoculation.

Groups /d.p.i	6	13
Control FP	0/13[E]	0/13
Control OR	0/12	0/12
94826 FP	0/13	0/13
94826 OR	0/12	0/12
105057 FP	2/13[A]	1/13[C]
105057 OR	4/12[A]	1/11[B]
105208 FP	0/11	1/10 [C]
105208 OR	1/12	2/12 [C]

[A] At 6 days of age 1 sample from 105057 FP had titer of 10^3 TCID50/0.1ml and 1 sample from 105057 OR had titer of 10^1 TCID50/0.1ml. Other samples had titer $<10^1$ TCID50/0.1ml

[B] At 13 days of age 1 sample from 105057 OR had titer of 10^1 TCID50/0.1ml.

[C] At 13 days of age 1 sample from 105057 FP, sample from 105208 FP and 2 samples from105208 Oral had titers $<10^1$ TCID50/0.1ml.

[D] Timepoints where no virus was isolated.

[E] Number of positive isolations/total

Table 4.8. Detection of reovirus by RT-PCR in S1 gene in and hearts and tendon swabs at 32 d.p.i. from turkeys inoculated with PBS (control), TARV 105057, 105208 and CARV 94286, by footpad (FP) or oral (OR) route of inoculation.

Groups	Heart[A]	Tendon Swabs[B]
Control FP	0/14[C]	0/14
Control OR	0/11	0/11
94826 FP	0/12	0/12
94826 OR	0/13	0/13
105057 FP	0/13	10/13
105057 OR	0/11	10/11
105208 FP	0/10	2/10
105208 OR	0/12	2/12

[A] Samples were pooled

[B] Individual samples

[C] Number of positive samples/total

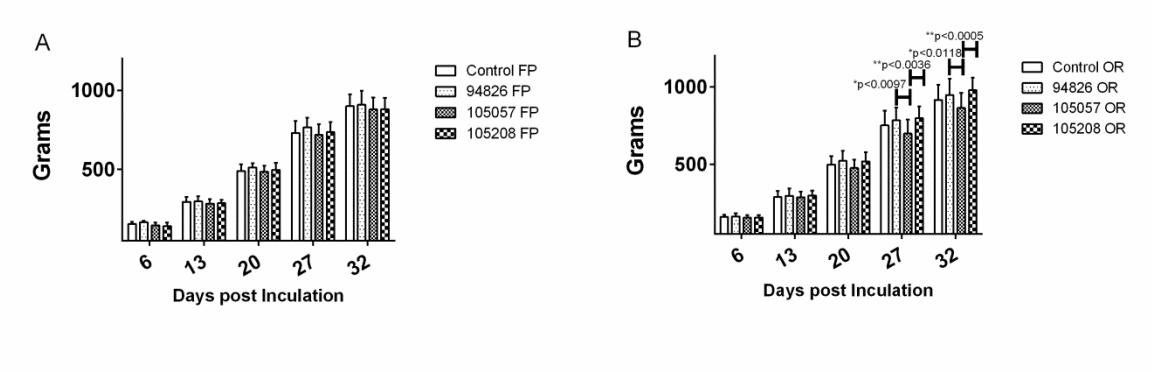

Figure 4.1. Mean body weight of turkeys at 6, 13, 20, 27 and 32 d.p.i for unchallenged controls, turkey arthritis reoviruses 105057 and 105208 and CARV 94826 by footpad (FP) (4.1.A) route and (4.1.B) oral (OR) route of inoculation. Significant difference between groups 94826 and 105208 compared to 105057 FP at 27 d.p.i (*P<0.0097 and **P< 0.0036), respectively. Significant difference between groups 94826 and 105208 compared to105057 OR at 32 d.p.i (*P<0.0118 and** P<0.0005, respectively, one way ANOVA test)

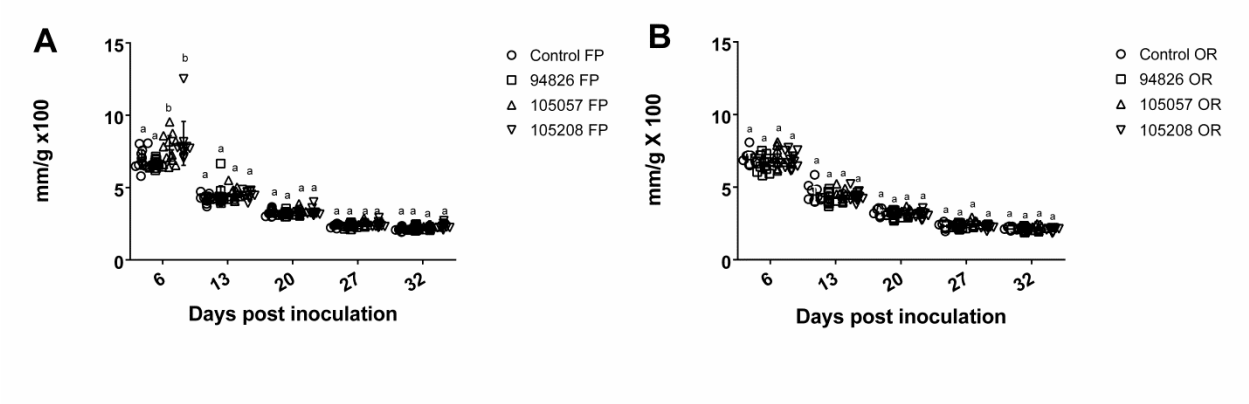

Figure 4.2. Mean Footpad/Body weight ratio of turkeys (tendon (mm) / body weight (g)x100) at 6, 13, 20, 27 and 32 d.p.i for unchallenged controls, turkey arthritis reoviruses 105057 and 105208 and CARV 94826 by footpad (FP) (4.2.A) and (4.2.B) oral (OR) route of inoculation. Different lowercase letter indicate significant differences between groups (P≤0.0303, one way ANOVA test).

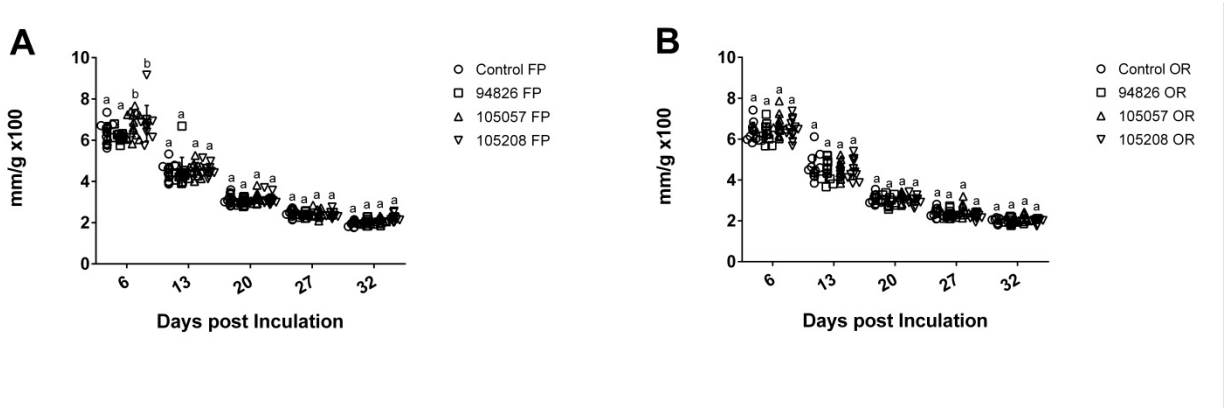

Figure 4.3. Mean Tendon/Body weight ratio of turkeys (tendon (mm) / body weight (g)x100) at 7, 14, 21, 28 d.p.i for unchallenged controls, turkey arthritis reoviruses 105057 and 105208 and CARV 94826: (4.3.A) footpad (FP) route and (4.3.B) oral (OR) route of inoculation. Different lowercase letter indicate significant differences between groups ($P<0.0492$, one way ANOVA).

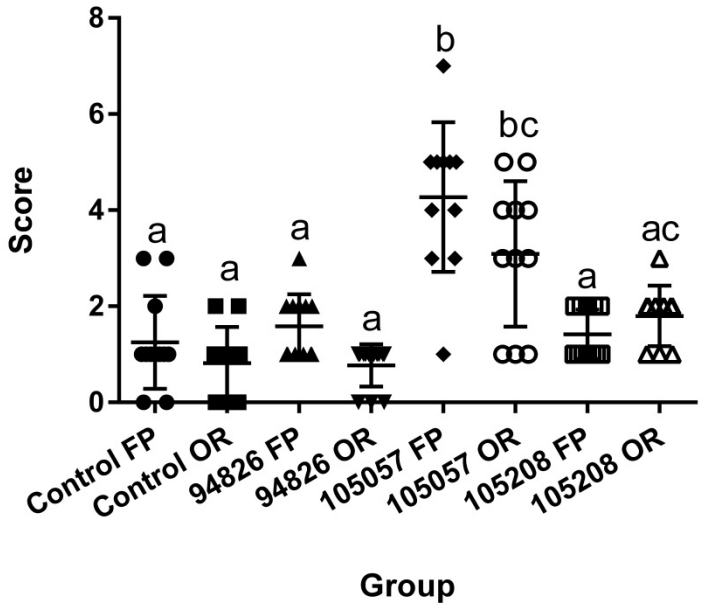

Figure 4.4. Histological inflammation tendon scores from digital flexor from left leg of turkeys at 28 d.p.i for unchallenged controls, turkey arthritis reoviruses 105057 and 105208 and CARV 94826 by footpad (FP) or oral (OR) inoculation. Different lowercase letters indicate significant differences between groups (105057 FP $P<0.015$ and 105057 OR $P<0.005$ Kruskal Wallis).

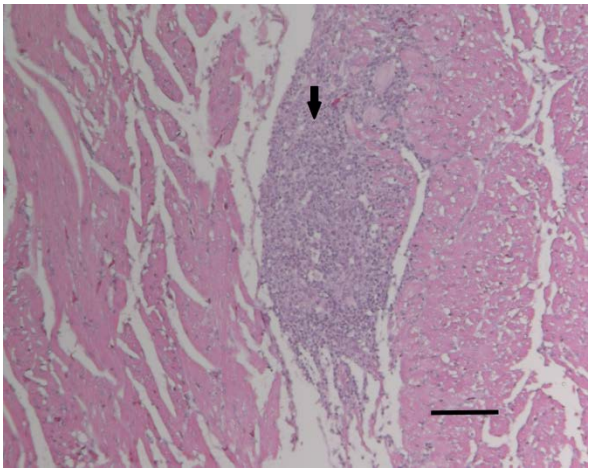

Figure 4.5. Photomicrographs of hematoxylin and eosin stained heart from challenge group 105057 FP turkey at 32 d.p.i. Black arrows indicate lymphocytic myocarditis. Bars = 100µm

CHAPTER 5

DETECTION AND MOLECULAR CHARACTERZATION OF AVIAN REOVIRUSES FROM COMMERCIAL BROILERS IN BRAZIL[A]

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To be submitted to Avian Diseases

Summary

An increased incidence of condemnations caused by greenish hock joints in commercial broilers was observed in a slaughterhouse in June of 2015 in Santa Catarina, Brazil. Five FTA® cards with tissue impressions were submitted to the Poultry Diagnostic and Research Center for reovirus RT-PCR and genotyping. The samples submitted were positive for reovirus by RT-PCR of the Sigma C encoding region of the S1 gene and the product was sequenced. New variants of avian reovirus were identified based on the *in silico* translated sequence of the Sigma C protein. Numerous variant reoviruses from clinical cases of viral arthritis and tenosynovitis have recently been reported in the U.S., France, Israel, Canada, to name a few. To date, this is the first report and molecular characterization of similar reovirus variants from clinically diseased commercial broilers from Brazil.

Key words: avian reovirus, broilers, condemnations, genotyping, green legs, Sigma C, slaughterhouse, variants.

Abbreviations: ARV = avian reovirus; RT - PCR = reverse transcription-polymerase chain reaction; = SDS = Sodium Dodecyl Sulfate

Introduction

Avian reovirus (ARV) belongs to the genus *Orthoreovirus*, subfamily *Spinareovirinae* in the *Reoviridae* family. Reoviruses have a segmented, double-stranded, RNA (dsRNA) genome comprised of three large (L1-L3), three medium (M1-M3), and four small (S1-S4) genomic segments (12).

Reovirus is the causative agent of viral arthritis and tenosynovitis in broilers, causing economic losses due to poor growth and feed conversion, mainly through inability of lame birds to reach feed and downgrading quality of carcasses at slaughter due to the unsightly appearance of affected hock joint (8). Viral arthritis/tenosynovitis is predominantly a disease of broilers, but can also be seen in breeders. Meat type chickens greater susceptibility may be related to the rapid growth and physical changes in tendons and legs. The main lesion of viral arthritis and tenosynovitis is a swelling of one or both hock (tibiotarsal-tarsometatarsal) joints, causing acute lameness. Tenosynovitis can lead to rupture of the Gastrocnemius tendon in chickens accompanied by hemorrhage, which in turn causes green discoloration of the skin at the joint (8).

In the present work, FTA® cards containing tissue impressions from hearts, synovial fluid, gastrocnemius and digital flexor tendons from lame commercial broilers in Brazil were submitted to the Poultry Diagnostic and Research Center (University of Georgia) for reovirus detection by RT-PCR followed by sequencing analysis for genotype identification.

History

In June of 2015, a high incidence of condemnations caused by greenish hock joints was observed in a slaughterhouse in the state of Santa Catarina, Brazil, resulting in

significant financial loss. Previously, condemnations associated with leg issues (viral arthritis or tenosynovitis) were insignificant (<0.01%), however, in June a 5% increase over the average was reported, with condemnations for some flocks peaking at 25%. In general, flocks were composed of female commercial broilers, weighing between 1,350g to 1,450g and housed in a negative pressure darkhouse. Clinical signs included greenish and swollen hock joints between 17 to 32 days of age. None of the breeder flocks and respective broiler progeny flocks evaluated in this study were vaccinated for reovirus.

Materials and methods

FTA® card samples for Reovirus RT-PCR and genotyping

Five Whatman FTA® cards were submitted to the Poultry Diagnostic and Research Center (University of Georgia, College of Veterinary Medicine, Athens, GA) for Reovirus RT-PCR and genotyping and were identified by accession numbers Brazil_111049_2015 (KU596803), Brazil_111057_2015 (KU896804), Brazil_111065_2015 (KU896805), Brazil_111089_2015 (KU896806), and Brazil_111109_2015 (KU896807). The FTA® cards contained pooled organ impressions consisting of heart, synovial fluid, gastrocnemius and digital flexor tendons, from commercial broilers.

RNA extraction

Total viral RNA was extracted from FTA® cards using the High Pure Viral RNA kit (Roche Diagnostics, Indianapolis, IN) with some modifications. Four to eight, 3mm disks were excised from each card sample using an FTA® punch and mat, and incubated in 0.5% SDS with 1mg/ml proteinase K at 37°C for 1 hour. The working solution

(prepared according to manufacturer's recommendations) was added to each sample, FTA® disks pelleted by centrifugation, supernatant transferred to the High Pure columns and RNA extracted per manufacturer's recommendations. Purified RNA was stored at -80°C until use.

Reverse transcription-polymerase chain reaction

A cDNA corresponding to the Sigma C encoding region of the S1 gene was generated by reverse transcription-polymerase chain reaction (RT-PCR) using Superscipt III RNase H- Reverse transcriptase (RT) and Platinum Taq DNA polymerase with previously published S1P1 and S1P4 primers (10). The 1 kb amplicons were separated on a 1.0% agarose gel, stained with ethidium bromide, and visualized with an ultraviolet transilluminator. The cDNA fragment was excised, purified with the QIAEX II gel extraction kit (Qiagen, Inc. Germantown, MD), eluted in RNase free water, and stored at -80° C until sequenced.

Direct nucleotide sequencing of amplified products

Gel-purified PCR products were sequenced with S1P1 and S1P4 primers using Sanger sequencing chemistry on a 96-capillary Applied Biosystems 3730xl DNA analyzer at Retrogen, Inc. (San Diego, CA).

Sequence analysis

Nucleotide sequences were analyzed and consensus sequences assembled from raw sequence files using the DNASTAR Lasergene version 12 software (DNASTAR, Inc. Madison, WI, USA) suite. Nucleotide sequences were *in silico* translated into corresponding amino acid sequences. Amino acid sequences generated were aligned using Clustal Omega multiple sequence alignment algorithm

(http://www.ebi.ac.uk/Tools/msa/clustalo/) and phylogenetic analyses were conducted using MEGA version 6.0 (18). Sequences generated in this study were submitted to GenBank and assigned the following accession numbers shown in parentheses: 111049 identified as Brazil_111049_2015 (KU596803), 111057 identified as Brazil_111057_2015 (KU896804), 111065 identified as Brazil_111065_2015 (KU896805), 111089 identified as Brazil_111089_2015 (KU896806), and 111109 identified as Brazil_111109_2015 (KU896807). The following reference sequences and corresponding GenBank accession numbers were also used in the multiple alignment and phylogenetic analyses for comparison: 1733 (KF741712.1); S11133 (AY536919.1); 12-1167 (HE985301.1); 11-12523 (HE985296.1); 11-12525 (HE985298.1); Tx99 (DQ996602.1); GA 41560 (DQ872798.1); AL 99159 (KJ879682.1); GA 97837 (KJ879660.1); 916 (AF297214.1); MS 97992 (KJ879667.1); 918 (AF297215.1); GA 40973 (DQ872797.1); GA 97350 (KJ879644.1); AVS-B (FR694197.1); 1017-1 (AF297216.1); NC 96816 (KJ803997.1); GA 12296 (JX983600.1); GEI 1097M (AF354219.1); 94826 (KJ803967.1); RAM1 (L38502.1); SOMERVILLE 4 (L07069.1); TARV MN3 (KF872234.1); TARV O'NEIL (KF872231.1); 601G(AF297217.1).

Results and discussion

In the present study, pooled tissues from FTA® card impressions were positive for avian reovirus by RT-PCR. The nucleotide and deduced amino acid sequences of the Sigma C protein of 111049, 111057, 111065, 111089 and 111109 were compared with previously published avian reoviruses (Table 5.1). Variants 111049 and 111065 shared a 95% and 96% similarity with the previously reported chicken isolates 11-12523 and 11-

12525 (19), respectively, and a 98.7% similarity with each other. In addition, 111049 and 111065 were 74% similar to commercial vaccine strains S1133 and 1733. Variants 111057 and Brazil 111109 share an 83% and 82 % amino acid similarity to chicken isolate GEI 97M, respectively and a 90% similarity with each other. Variant 111089 was 96% similar, at the amino acid level, to GA 94826 (Table 1). Amino acid sequences obtained for 111057, 111109 and 111089 were less than 50% similar to commercial vaccine strains (Table 5.1).

Phylogenetic analysis of Sigma C sequences revealed that variants 111049 and 111065 belonged to genotype cluster 1. This cluster contains variants 11-12523 and 11-12525, isolated in France from clinical cases of tenosynovitis in broilers. Commercial vaccine strains S1133, 1733 and others however, are located in a distinct subgroup separate from the variants strains in cluster 1. Variants 111057, 111089, 111109 are grouped in genotype cluster 5 (Figure 5.1), along with a prevalent group of variant viruses (identified in the U.S. as Group 1 VA variants), isolated between 2011-present, from clinical cases of tenosynovitis in commercial broilers. Tenosynovitis was reproduced experimentally in commercial broiler studies performed with several field isolates from this subgroup in cluster 5 (15). Broilers used in these experiments were progeny from reovirus vaccinated breeders and the results suggested that commercial vaccines do not provide sufficient protection against these variants.

The increased incidence of reovirus-induced arthritis/tenosynovitis in commercial broilers has been reported in the last couple of years worldwide (13, 19, 21). Genetic characterization of reovirus isolates, from 2011 until 2013 in the U.S. and Canada from clinical cases of tenosynovitis in commercial broilers, are distinct from commercial

vaccines strains. While two prevalent groups of variants belonging to two distinct genotypic clusters have been identified and characterized, isolation/detection of additional variants from clinical cases of tenosynovitis continues to date (17). In France, reovirus variants inducing tenosynovitis in vaccinated flocks were reported to be the cause of substantial economic losses due to lameness, stunting and non-uniform bodyweights (19). Similarly in Israel, isolations and molecular characterization of highly divergent variants, based on the Sigma C sequence, have been reported, also suggesting poor protection in vaccinated flocks (6).

In general, breeder flocks and respective broiler progeny flocks, from the Brazilian company analyzed in this study, were not vaccinated, therefore, broilers sampled were susceptible to reovirus infection. Vertical transmission has been described in the literature and occurs at a low rate, however, following horizontal transmission, reovirus can efficiently spread among flocks, once infected chickens shed virus through the feces (1, 14). It is reasonable to propose both vertical and horizontal transmission is occurring in the region of the flocks sampled in this study, due to early occurrence of clinical signs, even though no breeder samples were evaluated in this study.

Although reovirus vaccines have historically provided very good protection against clinical disease (20), it has been well documented that complete protection is best provided when homologous serotypes are utilized in the live attenuated and inactivated vaccines (7, 21). Vaccination of broiler breeders is essential in providing progeny early protection against infection via maternally derived antibodies (4). Because the variants characterized in this study are genotypically distinct from commercially available vaccines (Table 5.1), it is unlikely that optimal protection against clinical disease would

be provided if only current commercial vaccines were utilized in either breeders or broilers. In this case, inclusion of an autogenous vaccine containing a field isolate/s from affected birds alongside a commercial reovirus vaccine program in breeders may provide the best opportunity to control disease and subsequently reduce condemnations at the processing plant. By implementing a reovirus vaccination program, several benefits would result including reduction in the potential for vertical transmission from breeders to broilers, as well as, transfer of maternal antibodies to the progeny for protection against early infection, thus reducing horizontal transmission (6). Since it is not possible to isolate viruses from the FTA® card samples, virus isolation attempts, from affected birds, would need to occur in a local laboratory if an autogenous vaccine was to be pursued.

Clinical signs of swelling and greenish hock joints reported in this study have already been described elsewhere in clinical cases of tenosynovitis caused by reovirus in commercial broilers (8). Reovirus infection in flocks with clinical signs seems to occur early in life and rapid growth can cause stress in the tendon, leading to tendon rupture and hemorrhage (9, 11). In this study, the percentage of condemnations increased substantially (from 0.01% to 5%) resulting in significant economic loss. In addition to high condemnations, downgrading of carcasses, increased feed conversion and issues in slaughtering process can also cause financial losses due to ARV (3, 19).

To our knowledge, this is the first report of detection and molecular characterization of avian reovirus variants in commercial broilers in Brazil, associated with high condemnations caused by arthritis. Avian reovirus was first isolated in Brazil in 1975 in broilers and layers (2) and recently, turkey reovirus was isolated from

immunosuppressed young poults. Sequencing based on Sigma B (S3 gene) revealed high similarity of this Brazilian isolate with turkey reoviruses strains circulating in the United States (15). Unfortunately, no Sigma C sequences were reported thus, it was not possible to compare the variants detected in this study with the turkey reoviruses from Brazil. In summary, chicken reovirus variants were detected in samples from commercial broilers in the state of Santa Catarina, Brazil, one of the main chicken producer states in Brazil and ultimately were responsible for economic losses due to high condemnations.

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Table 5.1. Nucleotide and amino acid identity of Sigma C protein encoding region of the S1 gene among Brazilian variants and other avian reoviruses.

		% Amino acid identity										
		1	2	3	4	5	6	7	8	9	10	11
Nucleotide	1. S1133	**100**	96.5	47.7	46.8	47.1	46.1	46.8	73.5	73.9	73.9	73.5
Identity (%)	2.1733	99.1	**100**	48.4	48.4	48.7	47.7	47.7	76.5	76.8	76.8	76.5
	3. GA 94826	53.4	54.5	**100**	82.9	81.3	79.7	96.8	50.6	50.3	50.3	50.3
	4. GEI 1097	52.7	52.8	80.7	**100**	83.5	81.6	81.6	51.3	51.0	51.0	51.0
	5. 111109	54.7	54.9	78.1	78.7	**100**	90.3	80.6	49.7	49.4	50.6	50.6
	6. 111057	53.4	53.6	76.0	78.1	92.2	**100**	79.0	47.7	47.4	47.4	47.4
	7. 111089	52.6	52.7	97.9	80.3	77.7	75.9	**100**	49.4	49.0	49.0	49.0
	8. 111065	75.5	75.9	55.0	54.9	52.9	52.6	54.6	**100**	98.7	95.8	95.8
	9. 111049	76.3	76.7	55.2	55.2	53.2	53.2	54.8	98.4	**100**	95.5	95.2
	10. 11-12523	76.5	76.9	55.3	54.9	52.0	51.5	54.9	95.9	95.7	**100**	99.7
	11. 11.12525	76.4	76.8	55.3	80.7	52.0	51.5	54.9	95.7	95.5	99.8	**100**

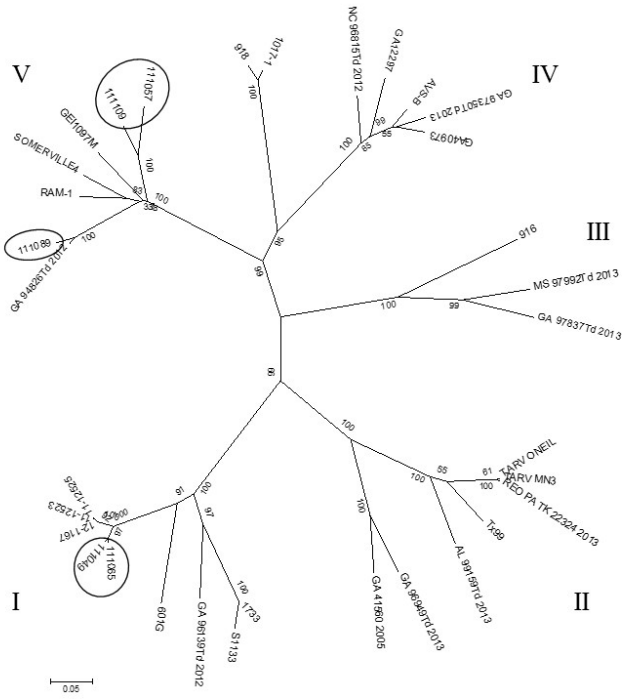

Figure 5.1. Multiple alignments of the amino acid sequence of Sigma C were performed in Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and an unrooted phylogram was generated using the Neighbor-Joining method (16) in MEGA6 (18). The optimal tree with the sum of branch length = 3.51620929 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (5). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (22) and are in the units of the number of amino acid substitutions per site. The five previously described genotypic clusters are identified by roman numerals (10). Commercial vaccines strains (S1133 and 1733, GenBank KF741712.1, AY536919.1) belong to cluster I. Brazilian variants 111049 and 111065 belong to a distinct subgroup in cluster I and are

circled. Variants 111057, 111089 and 111109 belong to the distant cluster V (identified in the U.S. as Group 1 VA variants) and are circled.

CHAPTER 6

DISCUSSION

Avian reovirus was for the first time reported to be the causative agent of tenosynovitis in chickens in 1968 and in turkeys in 1980 (5, 9). In chickens, since this first report, reovirus has been observed for decades in sporadic cases of tenosynovitis, causing clinical signs of swollen hock joint and lameness (4). In turkeys, after the first report, few cases were reported in the 80's and then turkey reovirus was associated to enteric diseases, including light turkey syndrome (LTS), poult enteric complex (PEC) and poult enteric syndrome (PES) (3, 10). Enteritis caused by reovirus occurs in young turkey poults of 1–7 weeks of age and the incidence is higher until 3 weeks of age and decreases as birds get older. Affected turkeys have ruffled feathers, diarrhea, depression, reduced weight gain and increased mortality. In most cases the intestinal contents are frothy and watery. Currently, there is no commercial turkey reovirus vaccine available for use in turkeys (3).

For more than 20 years there were no reports of lameness associated with turkey reovirus, until 2011 when a turkey reovirus causing tenosynovitis was isolated from gastrocnemius tendons and tibiotarsal joint fluid from >12week old commercial turkeys (8). Time related, it was observed an increased incidence of tenosynovitis in commercial broilers (6). The emergence of cases of tenosynovitis caused by reovirus in poultry has been reported not only in the U.S but also worldwide (12, 16). Genetic characterization revealed that these isolates are genetically distinct from commercial vaccines strains and

traditional vaccination programs are not effective to protect poultry flocks. The close proximity of some poultry production sites in the U.S raises the question of cross species infection.

We have tested 3 field isolates of avian reoviruses from clinical cases of tenosynovitis in chickens and turkeys. Two of them were isolated from turkey breeders and were named TARVs 105057 and 105208, and one from chickens, named CARV 94826, according to their submission case number at PDRC. Genetic characterization of sigma C gene reveled that these isolates are genetically distinct from commercial vaccine strains based on amino acid similarity, isolates 105057 and 105208 belonging to the distant cluster 2, and isolate 94826 belonging to cluster 5 (also called group 1 variant).

The pathogenicity of turkey reovirus isolates 105057 and 105208 was evaluated in commercial broilers and turkeys by challenging birds by oral and footpad route of inoculation. In addition, turkeys were challenged with chicken arthritis reovirus isolate 94826.

Results from the studies provided evidence that TARVs 105057 and 105208 were pathogenic to commercial broilers and turkeys. The isolates affected musculoskeletal system at an early age, causing clinical disease, evidenced by clinical signs of lameness, lymphocytic tenosynovitis and myocarditis, reproducing disease similar to the original clinical case in turkey breeders in which these viruses were isolated. Interestingly, in broilers, TARV 105208 caused enteric disease resulting in diarrhea; however, tenosynovitis was also observed, suggesting a predilection for multiple systems. In contrast, lymphocytic tenosynovitis was induced in the gastrocnemius of chicks inoculated only via the footpad route at 2 and 3 week post inoculation (P.I) but with no

evidence of clinical evidence of lameness was reported by Sharafeldin et.al (14) testing distinct variant strains of TARVS in specific pathogen free chickens. Turkeys challenged with a strain of variant CARV did not produced tenosynovitis after 4 weeks P.I. In agreement with previous publications, in the present study, footpad route induced more severe inflammation in tendons then oral route (13).

One important result in this study was that CARV 94826 did not cause clinical disease in commercial turkeys, evidenced by the lack of clinical signs, gross and histological lesions of tenosynovitis in digital flexor tendons, though, myocarditis was indicative of reovirus infection. It seems that turkeys are more resistant to chicken reoviruses (1, 13, 15).

Although, TARVS caused disease in broilers, the viral shedding was poor, indicating low replication and excretion of reovirus, suggesting that TARV might not be transmitted effectively in chickens (15). Similar results were obtained in turkeys challenged with CARV. It's conclusive that experimental cross infection can occur with different species origin reoviruses tested in this study, but transmission is limited. Another way of infection is through vertical transmission, but this was not evaluated in this present work. These results were obtained in a clean and controlled experimental condition, however, disease may be exacerbated by field conditions. In contrast, TARVs were efficiently shed by turkeys, suggesting high risk of transmission of these variants among turkey flocks.

Historically, vaccination has provided protection in chickens against reovirus infection, however the best protection is achieved when vaccines are homologous with field isolates (17). Vaccination of broiler breeders is essential for early protection of

progenies against infection via maternally derived antibodies, ensuring protection early in life when chickens are more susceptible; moreover, breeders get protected and avoid vertical transmission as well (18, 19). In turkeys, vaccination against reovirus causing arthritis is less known, because historically it hasn't been a major problem in the industry. With the emergence of numerous variants causing tenosynovitis in commercial poultry flocks, traditional vaccination is unlikely to provide protection to breeders and progenies. In this case, autogenous vaccines containing field isolates may be necessary to provide protection to breeders and also to transfer maternal antibodies to the progeny (7). It is reasonable to conclude that a combination of traditional vaccines combined with TARV 105057 and/or 105208 may be necessary to protect commercial poultry flocks against the imminent risk of infection with these TARVs.

New variants of reoviruses causing arthritis and tenosynovitis have been reported in the U.S and worldwide (12, 16). The economic losses due to arthritis are associated with increased mortality, non-uniform flocks and downgrading carcass in the slaughterhouse and many more (16).

In Brazil, arthritis caused by reovirus in broilers was first described in the 70's (2), but not much is known about genetic characteristics of reoviruses circulating in Brazilian poultry flocks. Recently, some isolates of turkey reoviruses causing immunosuppression in turkeys were characterized as variants, showing high amino acid similarity based on Sigma B protein to variants from the U.S (11).

In June of 2015, an increase in condemnations caused by arthritis was reported in a poultry company in Brazil. Greenish hock joints and lameness from 17 to 32 days of

age were the most evident clinical signs in affected flocks, moreover neither the breeders nor the broilers are vaccinated in this company.

Five FTA cards with heart, synovial fluid, gastrocnemius and digital flexor tendons impressions were submitted to PDRC for RT-PCR and sequencing from diseased broiler flocks. Through the present work, it was established for the first time the molecular characterization of five variants of reovirus from lame commercial broilers from Brazil.

As previously discussed, vaccination has played a major role in prevention and control of reovirus in commercial poultry flock and it is well known that complete protection is only provided when birds are vaccinated with homologous serotypes. Genotyping revealed that these five Brazilian variants are highly distinct to commercial vaccine strains in the amino acid level based on the analysis of Sigma C protein. Therefore, it is highly recommended that a vaccine program must include homologous variant serotypes that are circulating in these flocks to provide adequate protection. Furthermore, because clinical signs have been observed at early ages, it is evident that vertical transmission is occurring and also horizontal transmission has been contributing for dissemination of reovirus among these susceptible flocks.

Altogether, it was established that TARV variants 105057 and 105208 isolated from lame commercial turkey breeders are capable to experimentally reproduce the disease in commercial turkeys and are able to shed efficiently the virus, indicating a high risk of dissemination of this variants among turkey flocks. More interesting, this TARVs were able to cause disease in commercial broilers, not only tenosynovitis but also enteric disease, giving evidence that these new variants can be pathogenic to broilers; however

horizontal transmission was suggestive to be limited, but still a risk of vertical transmission. On the other hand CARV 94826 did not showed evidences to be a risk for turkeys, not inducing disease and having limited potential to spread among this species.

In addition, the establishment of five variants of chicken reovirus causing arthritis in commercial broiler in Brazil was a remarkable finding, giving evidence that variants are still emerging worldwide, and this represents a threat to the profitability of the poultry industry.

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