Turkey arthritis reovirus: pathogenesis and immune response

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Abstract

In 2011, turkey reoviruses were isolated from tendons and synovial fluids of >15-week-old lame turkeys displaying swollen joints and occasionally ruptured leg tendons in Midwest, USA. These reoviruses were tentatively called turkey arthritis reoviruses (TARV) to differentiate them from reoviruses isolated from intestinal contents and feces of turkeys namely turkey enteric reoviruses (TERV). TARV were found to be genetically distinct from chicken arthritis reoviruses (CARV). Five experiments were conducted to test the pathogenicity of TARV in turkeys and in chickens and to compare it with that of TERV and CARV. Additionally, this work investigated the virus pathogenesis and cytokine immune responses. TARV showed unique capability to induce significantly higher tenosynovitis scores in turkeys as compared with TERV and CARV which induced minimal scores. Clinical lameness was first displayed at 8 weeks of age in TARV-inoculated turkeys at 1 week of age. Lameness in infected group reached approximately 50% at 16 weeks of age. TARV did not induce any lesions in chickens via intratracheal or oral route. TARV inoculation via footpad route induced tenosynovitis in chickens at 2 and 3 weeks PI with no clinical lameness. In pathogenesis study, TARV displayed the greatest replication in intestines and bursa of Fabricius than in leg tendons of turkeys. Viral infection mediated effective antiviral cytokines immune response that limited virus replication in the intestines. Furthermore, viral infection mediated a significantly elevated T helper-1(Th1) cytokine response in intestines and tendons and minimal Th2 and Th17 cytokine response during the early stage (2 weeks) of infection. This work established an experimental model to study TARV which provides early end points that are indicative of disease pathogenicity. Additionally this work developed a new grading system for histologic tenosynovitis which can be used in a wide variety of experimental models. For lameness evaluation in turkeys, this work developed a grading system for gait scores. In summary, this work showed unique pathogenicity of the newly isolated TARV and added significant knowledge to TARV pathogenesis and immune response using the newly established reproducible experimental model and the newly developed grading systems for evaluation of tenosynovitis and clinical lameness.

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Chapter 1: Introduction, objectives and literature review

Introduction

Avian reovirus was first isolated from the respiratory tract of chickens displaying chronic respiratory disease (Fahey and Crawley, 1954). Fahey-Crawley strain of reovirus induced respiratory disease, liver necrosis, and tenosynovitis in experimentally inoculated chickens (Petek et al., 1967). The first clinical case of viral tenosynovitis/arthritis in *Mycoplasma synoviae*-negative chickens in USA was reported in 1968 (Olson and Solmon, 1968). Since then, several studies have described reovirus as the causative agent of tenosynovitis/arthritis in chickens (Rosenberger, 2003; Jones, 2000; van der Heidi, 1977).

Experimental inoculation of avian reovirus in specific-pathogen-free (SPF) chickens through different inoculation routes resulted in viremia with systemic distribution of the virus to respiratory, enteric, and reproductive organs in addition to hock joints and tendons (Menendez et al., 1975). The incubation period of avian reovirus varies based on age and breed of the infected bird and with the strain of virus (Robertson and Wilcox, 1986; Sterner et al., 1989).

Mucosal IgA induced by vaccination or maternally derived IgG can protect 'in contact' birds from acquiring infection from reovirus-challenged birds (Juerissen et al., 2000). Additionally, avian reoviruses that had higher multiplication rates induced significantly higher production of IL-6, IL-10 and INF-γ compared with reoviruses exhibiting a lower rate of multiplication (Shen et al., 2014).

In turkeys, association of turkey reoviruses with poult enteritis has been the subject of several reports (Lojkic et al., 2010; Mor et al., 2013a; Clavert, 2012; Jindal et al., 2009;

Jindal et al., 2010; Woolcock and Shivaprasad, 2007). Reovirus has also been isolated from joints and ruptured tendons of turkeys displaying tenosynovitis/arthritis (Levisohn et al., 1980; Page et al., 1982). However, experimental infection with turkey reoviruses failed to reproduce tenosynovitis/arthritis in turkeys (Al-Afaleq & Jones, 1989).

Recently, we isolated and characterized novel strains of turkey reovirus from tendons of turkeys with tenosynovitis/arthritis and tentatively named them as turkey arthritis reoviruses (TARV). Genetic characterization of S4 gene sequences showed that these TARV strains were distinct from chicken arthritis reoviruses (CARV) (Mor et al., 2013b).

The present study was undertaken to characterize the pathogenicity of TARVs as compared with that of turkey enteric reoviruses (TERV) and CARV; determine the long term pathogenicity of TARV; measure biomechanical changes in the gastrocnemius tendons of TARV-infected turkeys; describe the pathogenesis and immune response associated with TARV infection; and test the pathogenicity of TARV in chickens. The thesis consists of seven chapters starting with chapter one on review of literature on the pathogenicity, pathogenesis and immune response of mammalian and avian reoviruses. Chapters 2-6 address five different objectives of the study and chapter 7 consists of general discussion and conclusions.

Review of Literature

1. Introduction to reoviruses:

Reoviridae is a virus family that includes all reoviruses. These viruses were found to affect the respiratory and gastrointestinal tracts of the host "human" and originally were not associated with any known diseases. For that reason the agents were referred to as **r**espiratory **e**nteric **o**rphan (reo) viruses. These viruses can infect vertebrates, invertebrates and plants (Juklik, 1981). Reovirus was discovered in the early 1950s when scientists observed many cytopathogenic agents in samples of human alimentary tract and these agents were not poliomyelitis virus as had been anticipated (Robins et al., 1951; Ramos-Alvaris and Sabin, 1954).

2. Classification of reoviruses:

The family *Reoviridae* contains non-enveloped segmented double-stranded RNA (Juklik, 1981). The Reoviridae family has two subfamilies; *Sedoreovirinae* and *Spinareovirinae* (Carstens, 2010). *Sedoreovirinae* subfamily contains 6 genera; *Cardoreovirus* infects crabs, *Mimoreovirus* is environmental marine water virus (King et al., 2012), *Orbivirus* is arthropod-borne virus (Belhouchet et al., 2011), *Phytoreovirus* affects plants (Lu et al., 1988), *Rotavirus* causes diarrhea in humans and animals species (Martella et al., 2010) and *Seadornaviruses* are arthropod borne viruses infecting humans and animals (Mohd Jaafar et al., 2005). *Spinareovirinae* subfamily contains 11 genera; *Aquareovirus* infects aquatic species (Attoui et al., 2002), *Coltivirus* causes Colorado tick fever in human (Attoui et al., 1998), *Cypovirus* infects insects (Yang et al., 2012), host of *Dinovernavirus* is unknown (Attoui et al,

2005), *Figivirus* infects plants (Isogai et al., 1998), *Idnoreovirus* infects insects (King et al., 2012), *Mycoreovirus* infects fungi (Suzuki et al., 2004), *Orthoreovirus* infects mammals and birds. (Duncan, 1999), *Oryzavirus* (rice ragged stunt virus) infects rice (Miyazaki et al., 2008), *Piscinereovirus* infects fish (Kibenge et al, 2013) and *Crabreovirus* infects mud crab (Huang et al., 2012)) (Table1.1). Avian reoviruses are classified under genus *Orthoreovirus* that contains avian reoviruses and mammalian reoviruses.

3. Mammalian reoviruses:

Structure: The structure of mammalian reoviruses has been established by electron microscopy. Reovirus is non-enveloped and is encapsulated with two capsules (inner and outer capsule with diameters 50 and 75 nm, respectively). Both capsules have icosahedral symmetry (Joklik, 1981). Reovirus has a double-stranded RNA genome that is segmented (10-12 segments) (Martens 2004). These segments are divided into 3 classes based on their size; L class refers to large size segment of 3800 bp (three L segments; L1, L2 & L3), M class refers to medium size segments of 2200 bp (three M segments; M1, M2 & M3) and S class that refers to small size segments of 1100-1400 bp (4 segments; S1, S2, S3 & S4) (Joklik, 1985). These gene segments encode different proteins that form the reovirus particle. The inner capsid (core) is formed by λ1, λ2, λ3, μ2 and σ2 proteins that are encoded by L1, L2, L3, M2 and S2 segments, respectively, while the outer capsid is formed by μ1, σ1 and σ3 proteins that are encoded by M1, S1 and S3 segments, respectively (Stehle and Dermody, 2004). Viral membrane attachment is done by σ1 protein while μ1 works

on membrane penetration with help of σ3 that provides cap protection for μ1 (Leimann et al., 2002).

Table 1.1: Reovirus classification

Family	Subfamily	Genus	Host
Reoviridae	*Sedoreovirinae*	*Cardoreovirus*	Crabs
		Mimoreovirus	Marine water environment
		Orbivirus	Vertebrates (Arthropod borne)
		Phytoreovirus	Plants
		Rotavirus	Vertebrates
		Seadornaviruses	Vertebrates (Arthropod borne)
	Spinareovirinae	*Aquareovirus*	Aquatic species
		Coltivirus	Humans (Colorado tick fever)
		Cypovirus	Insects
		Dinovernavirus	Unknown
		Figivirus	Plants
		Idnoreovirus	Insects
		Mycoreovirus	Fungi
		Orthoreovirus	Vertebrates
		Oryzavirus	Plants (rice ragged stunt virus)
		Piscinereovirus	Fishes
		Crabreovirus	Mud crabs

3.1. Replication cycle:

Virus attachment: The outer capsid protein σ1 is responsible for virus particle attachment to the cell surface (Forrest and Dermody, 2003). The σ1 protein consists of a tail domain that penetrates the virion wall and a globular head domain which projects outside the virus (Fraser et al., 1990; Furlong et al., 1988). Some strains of

murine neurotropic reoviruses have a σ1 protein that contains receptor binding domains in both tail and head structures. The tail domain binds α-linked sialic acid (Chappell et al., 1997) while head domain binds junction adhesion molecule 1 (JAM1) (Barton et al., 2001) and is now called JAM-A. Domains of the σ1 protein of these reoviruses may be dissociated with intestinal proteases and this may explain the attenuated virulence of some reovirus strains when inoculated orally (Bodkins and Fields, 1989; Keroack and Fields, 1986). Another strainofneurotropic reoviruses has a σ1 protein tail domain that binds to cellular surface carbohydrates (Chappell et al., 2000). Some researchers propose binding of σ1 protein head domain to surface cellular proteinaceous receptors (Duncan et al., 1991; Turner et al., 1992).

Virus entry: Cellular surface receptor JAM-A, which ensures high affinity attachment of reovirus to the cell, was shown to play a minor role in reovirus internalization when mutant cells lacking JAM-A showed a high degree of reovirus infection. The outer capsid protein λ2 which is the structural base of σ1 protein (Furlong et al., 1988; Dryden et al., 1993) contains integrin-binding domains referring to the role of integrins in reovirus entry (Breun et al., 2001). This was confirmed when β1 integrin subunit-specific monoclonal antibodies inhibited reovirus infection (Maginnis et al., 2006). Some research has referred to the role of clathrin-dependent endocytosis in reovirus cell entry (Sturzenbecker et al., 1987; Birsa et al., 1979; Borsa et al., 1981). Reovirus and clathrin were shown to co-localize during virus entery (Ehrlich et al., 2004).

After reovirus is internalized in a host cell it is coated by a vesicle and then transported in microtubules (Georgi et al., 1990) to endosomes where it accumulates with other virus particles (Mainou and Dermody, 2012). Inside the vesicles, reovirus undergoes a disassembly process that starts with formation of infectious subvirion particle (ISVP), which lacks outer capsid protein σ3, has conformational changes in σ1 attachment protein and has cleaved outer capsid protein μ1 that was exposed after removal of σ3 (Danthi et al., 2013). The μ1 protein is cleaved into μ1N and μ1C, and then the μ1C cleaves into δ and ϕ fragments; these cleavage steps play an unknown role in virus penetration. The mutant form of μ1 that is unable to cleave to δ and ϕ fragments was able to induce virus penetration (Chandran and Nibert, 1998).

Transformation of ISVP to ISVP like particle (ISVP*) is associated with μ1 protein conformational changes (cleavage) that may lead to penetration of endosomal membranes and release of active core particles for transcription (Chandran et al., 2002, 2003; Leimann et al., 2002).

Viral replication and assembly: After endosomal proteolysis of the reovirus particle, the transcriptionally active core that contains the virus transcription machinery is released into the cytoplasm to synthesize full-length single-stranded RNA (ssRNA) for each viral segment (Banerjee and Shatkin, 1970; Chang and Zweerink, 1971). The positive-sense ssRNA may be translated to form viral protein or may act as a template for complimentary RNA strands to form double-stranded RNA (dsRNA) (Li et al., 1980; Schonberg et al., 1971; Shatkin and Kozak, 1983). Formation of dsRNA is the first step in assortment of gene segments of the progeny

virion (Antczak and Joklik, 1992). The virus replication cycle ends with the synthesis of outer capsid proteins around the dsRNA-containing core to assemble a new virion (Zweering et al., 1976).

Replication and assembly takes place within viral inclusions in cytoplasm of infected cells that contain dsRNA, viral proteins, and complete and incomplete assembled virions (Fields et al., 1971). Viral nonstructural proteins, μNS and σNS, as well as minor core protein μ2 are important for genesis and maturation of viral inclusions within the cytoplasm of the infected cell (Arnold et al., 2008; Kobayashi et al., 2006). The μNS protein is coded by M3 gene segment; it associates with viral mRNA, core protein μ2 and nonstructural protein σNS (Broering et al., 2002; Becker et al., 2003; Miller et al., 2003). This association enables μNS protein to recruit λ1, λ2, λ3 and σ2 core proteins (Broering et al., 2004) and also prevents the assembly of outer capsid and prolongs the transcription (Broering et al., 2000) to maximizes RNA and protein production. Thus, μNS plays a crucial role in viral inclusion formation and secures a suitable environment for RNA replication and virus assembly (Kobayashi et al., 2009).

Viral release: The assembled virion may release from infected cells without inducing cell death (Lai et al., 2013) or it may induce apoptosis and cell death (Forest and Dermody, 2003) prior to release.

3.2. Pathogenesis:

Apoptosis and cellular signaling pathways: Many mammalian reoviruses induce cellular signaling transduction pathways which may lead to apoptosis (Danthi et al., 2013). Reovirus activates nuclear factor NF-κB signaling pathway in host cells within 2-4 hours post infection (PI) and this potentiates apoptosis (Connolly et al., 2000). In addition, reovirus may induce the mitogen-activated protein kinases (MAPKs) signaling pathway in infected cells within 10-12 hours PI and may persist until 20-30 hour PI (Clark et al., 2001). MAPKs activate apoptotic signaling in reovirus infected cells (Clark et al., 2004). Inhibition of p38 MAPK reduces reovirus growth even in cells that have an activated RAS pathway, which is a reovirus replication activator (Norman et al., 2004; Duncan et al., 1978; Strong et al., 1998). Reovirus-infected cell culture showed activation of transcription factor IRF-3 within 2-4 hours PI. IRF-3 is an inducing factor of apoptosis and Interferon-β (IFN-β) production in reovirus-infected cells (Norman et al., 2002).

Reovirus-induced apoptosis requires an extrinsic apoptotic pathway and intrinsic (mitochondrial damage) initiation (Danith et al., 2013). The extrinsic pathway is initiated by a TNF-related apoptosis-inducing ligand (TRAIL) and its corresponding death receptors (DR4 and DR5), which are up-regulated within 4 hours after infection (Clark et al., 2000). Death receptors associated with TRAIL initiate apoptosis through caspase 8 activation (Blatt and Glick, 2001). TRAIL showed a peak after NF-κB activation (Connolly et al., 2000; Clark et al., 2000). The extrinsic apoptotic TRAIL pathway and its death receptors have been shown to be regulated by type I IFN

(Chawla-Sarker et al., 2003; Shigeno et al., 2003). Reovirus-induced mitochondrial injury constitutes activation of the intrinsic apoptotic pathway via the release of cytochrome c that activates caspase 9 and caspase 3 (Li et al., 1997; Verhagen et al., 2000; Du et al., 2000; Juza et al., 2001; Li et al., 1998).

In vivo apoptosis induced by reovirus has been reported in neurons after intracranial or oral inoculation (Oberhaus et al., 1997; O'Donnell et al., 2005). In addition, the innate antiviral immune response plays a role in regulation of reovirus-induced myocardial injury. Non-myocarditis reovirus strains were found to induce high levels of type I IFN compared with myocarditis reoviruses (Sherry et al., 1998). NF-κB is required for reovirus-induced apoptosis in CNS, while also protecting the heart from damage induced by virus through activation of type I IFN (O'Donnell et al., 2005).

Viremia: Reovirus viremia varies among strains. A reovirus strain was detected in blood of 1-day-old mice at 4 days after subcutaneous inoculation, and virus titer was the highest in buffy coat followed by whole blood and then plasma (Jenson et al., 1966). Reovirus viremia results from infected tissue cells or tissue macrophages releasing individual virions, membrane-bound particles that contain virions, or cell-free viral aggregates into blood vessels (Jenson et al., 1966). Junction adhesion molecule-A (JAM-A) is expressed on epithelial cells and plays an important role of disseminating reovirus into the circulation by helping passage through endothelial cells. In enteric mucosa the role of JAM-A was dispensable (Antar et al., 2009). Reovirus may prefer to use enteric mucosal M cells for trancytosis to reach lymphoid

aggregates and then access the circulation (Wolf et al., 1981); however, trancytosis is not restricted to M cells and other enteric mucosal cells can be used (Coyne et al., 2009). JAM-A facilitates transmigration of reovirus-infected leukocytes, transcytosis through endothelium, or reovirus may disassemble JAM-JAM adhesions between endothelial cells and use JAM-A for trancytosis (Coyne et al., 2009). The σ1 protein in reovirus showed an important key role in virus spread from intestinal lymphatics to blood stream (Boehmi et al., 2009).

3.3. Immune response:

Innate immune response: The innate immune response mediated by reovirus infection is regulated through a network of cytokines that are produced by different local tissue cells. The innate immune response varies depending on virus tropism, virus replication and route of inoculation (Bodkin and Fields, 1989; Weiner et al., 1977, 1980, 1988). Neurotropic reovirus mediates activation of microglia and astrocytes (gliosis) that leads to production of inflammatory cytokines and secondary CNS damage (Farina et al., 2007; Farnk-Cannon et al., 2009). Type I interferon (IFNα/β) is released in response to reovirus infection followed by activation of interferon-stimulated gene ISG that activates antiviral protein, pattern recognition receptors (PRR), inflammatory cytokines, and the chemokines tumor necrosis factor-alpha (TNF-α) and (C-X-C motif) ligand 10 (CXCL10) (Hu et al., 2008; Kawai and Akira, 2008; Lehnardt, 2010; Romieu-Mourez et al., 2009; Kraft et al., 2009; Park and Bowers, 2010). The inflammatory cytokines may be produced extensively from

glial cells and astrocytes by pathways other than type I interferon (Schittone et al., 2012).

Reoviruses that cause myocarditis can increase some cytokines and decrease others. This variation sometimes mediates antiviral activity and has a protective effect against myocarditis (Miyamoto et al., 2009). Interleukin-6 (IL-6) can increase in response to reovirus infection (Miyamoto et al., 2009). IL-6 is known to be a pro-inflammatory cytokine, but IL-6 production can also decrease the viral titer and reduce the extent of heart injury (Kanda et al., 1996).

Dendritic cells have an active role in the immune response to reovirus; they recognize the virus by PRR-like toll-like receptors (PRR/TLR) (Schulz et al., 2005). When the PRR/TLR recognizes reovirus, it produces inflammatory cytokines, including type I IFNs, IL-12 and TNF-α (Diebold et al., 2004). Dendritic cells can promote non antigen-restricted killing by natural killer cells (Erington et al., 2008). This particular property of dendritic cells has resulted in the use of reovirus as a tumor treatment factor (Hirasawa et al., 2002; Alain et al., 2002).

Cell mediated adaptive immune response: When reovirus is inoculated through the oral route, it either contacts intraepithelial lymphocytes (IEL) (London et al., 1987) or passes through M-cells and reaches the gut-associated lymphoid tissue to stimulate a T-cell response (London et al., 1990). The type of T-helper (Th) response depends mostly on the route of reovirus administration. Mucosal viral infection tends to mediate more robust Th1 response than parenteral injection (Pacheco et al., 2000). It has been reported that the Th1 response, including production of IL-12 and INF-γ,

is involved in cell-mediated immunity, while the Th2 response, including production of IL-4 and IL-5, is involved in inducing humoral immunity (Finkelman et al., 1990; Mosmann et al., 1986). Following oral infection of reovirus in mice, Th1 was the predominant cell-mediated response (Fan et al., 1998). Populations of dendritic cells played a role in determining the type of T-helper response; lymphoid dendritic cells induced Th1 and myeloid dendritic cells induced Th2 production (Maldonado-Lopez, 1999; Pulendran et al., 1999). Reovirus induced production of $CD4^+$/$CD8^+$ T cells in gut-associated lymphoid tissue (GALT) and respiratory-associated lymphoid tissue (RALT) in mice (Periwal and Cebra, 1999).

Intraepithelial lymphocytes (IEL) are $CD3^+$ cells (T cells) with the majority expressing the $CD8^+$ phenotype. There are two populations of IEL based on expression of T cell receptors (TCR); $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ (Hayday et al., 2001; Cheroutre, 2004, 2005). Experimental inoculation of reovirus in mice activated IELs with $TCR\alpha\beta^+Thy\text{-}1^+CD8^+$ phenotype and these cells were cytotoxic similar to those in lamina propria and Peyer's patches. These IELs used perforin, Fas-FasL and TRAIL pathways for their cytotoxic effect (Bharhani et al., 2005). $TCR\alpha\beta^+Thy\text{-}1^+CD8^+$, $TCR\alpha\beta^+Thy\text{-}1^+CD4^+$ and $TCR\alpha\beta^+Thy\text{-}1^+CD4^+CD8^+$ IEL populations in intestines increased significantly 7-10 days after oral inoculation with reovirus (Bharhani et al., 2007).

Humoral immune response: From intestinal mucosa, reovirus reaches the dendritic cells in the GALT to activate naïve B cells into specific B-cell colonies that secrete IgA into the lumen (Cebra et al., 1989; Organ and Rubin, 1998). The same

happens when reovirus reaches respiratory mucosa to gain access to RALT (Morin et al., 1994; Periwal and Cebra, 1999). In addition, the primed naïve B-cells produce IgG that, along with mucosal IgA and cytotoxic T-cells, clears or neutralizes reovirus on the mucosal surface (Barkon et al., 1996; Tyler et al., 1993; Virgin and Tyler, 1991). However, IgA is the most important component in mucosal protection against reovirus and without it mucosal protection is not ensured even with high titer of serum IgG (Silvey et al., 2001).

When intestinal mucosal cells are exposed to reovirus, they express increased numbers of immunoglobulin receptors that facilitate the transcytosis of IgA (Pal et al., 2005). IgA prevents the contact of a pathogen with mucosal cells through attaching to the pathogen in the mucus layer to facilitate clearance (Cone, 1999; Lamm, 1997; Russell et al., 1999). In addition, IgA can interfere with surface proteins on a pathogen to prevent attachment to receptors on mucosal epithelium (Svanborg-Eden and Svennerholm, 1978) or IgA can intercept the pathogen in the vesicular compartment of epithelial cells (Bosmel et al., 1998; Burns et al., 1996; Mazanec et al., 1992). Furthermore, the pathogen can be expelled back to the lumen by action of IgA (Kaetzel et al., 1991; Mestecky et al., 1999). Monoclonal IgA that was specific for "σ1 reovirus protein" protected Peyer's patches from reovirus infection while "σ1 reovirus protein"-specific monoclonal IgG could not demonstrate the crucial role of mucosal IgA in reovirus clearance (Hutchings et al., 2004).

The cell-mediated immune response has been shown to affect the quality and intensity of the humoral immune response. Reovirus-specific IgA and IgG exerted

significantly higher effects in β-microglobin (MHC-I domain)$^{-/-}$ mutant mice compared with controls. This shows that MHC-I restricted $CD8^{+}$ cells can modulate the humoral immune response during reovirus infection (Major and Cuff, 1997). In addition, it has been shown that production of Th1 cytokines enhances immunoglobulin class switching to IgG2A (Finkelman et al., 1990; Mosmann et al., 1986). However, this change between Th1 and Th2 response does not affect IgA production (Mathers and Cuff, 2004).

4. Avian reoviruses:

4.1. Classification and discovery: Avian reoviruses are classified under genus *Orthoreovirus* of family *Reoviridae* (Martens et al., 2004). They are viruses that induce clinical diseases in poultry (tenosynovitis/arthritis and enteritis) leading to economic losses to the poultry industry (Rosenberger et al., 1989). The first documented avian reovirus (Fahey-Crawley strain) was isolated from the respiratory tract of chickens with chronic respiratory disease; however, at that time it was only recognized as a virus (Fahey and Crawley, 1954). The Fahey-Crawley strain was confirmed to be an avian reovirus when it induced respiratory disease, liver necrosis, and tenosynovitis in inoculated chickens (Petek et al., 1967). The first clinical case of viral tenosynovitis/arthritis in Mycoplasma synoviae-negative chickens in USA was reported in 1968 (Olson and Solmon, 1968) and the etiological agent was referred to as viral arthritis agent (VAA) (Olson and Kerr, 1966). Reovirus was then isolated from tendons of 28-day-old broiler chickens with tenosynovitis/arthritis and ruptured tendons (Jones et al., 1975).

4.2. Structure and biology: Avian reovirus is 70 - 80 nm in diameter and is a non-enveloped, icosahedral-shaped virion with a double-shelled arrangement of capsid proteins (Jones, 2000). The virus contains 10 dsRNA gene segments that are divided into 3 groups according to their sizes; large segments (L1, L2, L3) that encode proteins λA, λB and λC, medium segments (M1, M2, M3) that encode proteins μA, μB and μNS) and small segments (S1, S2, S3, S4) that encode proteins σc-P10-P17, σA, σB and σNS (Benavente and Martinez-Costaz, 2007). Outer capsid proteins (σc, μB, σB) play an important role in virus attachment (σc) to cell surface receptors and penetration (μB) of the host cell (Duncan et al., 1996; Grande et al., 2000; Shapouri et al., 1996). After attachment the virus is internalized into intracellular vesicles and the acidic proteolytic environment results in uncoating of the virus with cleavage of μB protein and penetration into the cytosol (Duncan et al., 1996). Inner core proteins (λA, λB, λC, μA, σA) and non-structural proteins (μNS, P10, P17, σNS) then modulate the replication cycle of the virus. The viral mRNAs are synthesized within the inner core and acquire their cap structures at the time of leaving the virion through the channels of hollow "turrets" formed by λC protein (Zhang et al., 2005).

The positive-strand mRNAs play two important roles in protein synthesis at the ribosome and serving as a template for negative-strand RNA of the newly formed viral genome (Nibert and Schiff, 2001). The inner core proteins λA and λB that are enclosing the RNA segments and RNA polymerase and they are suggested to be recruited by μNS protein in to the viral factories (Touris-Otero et al., 2004a). The μNS protein is required for intracytoplasmic factory formation and for virus assembly and morphogenesis (Touris-Otero et al., 2004b). The inner core σA protein binds

independently to dsRNA and this enables it to prevent the activation of the dsRNA-dependent protein kinase PKR, thus preventing interferon action against the virus genome (Gonz´alez-L´opez et al., 2003; Martinez-Costas et al., 2000; Yin et al., 2000). The σNS protein binds to ssmRNA and the minimum RNA size for binding is between 10 and 20 nucleotides (Touris-Oteroet al., 2005). The biological role of P17 and σB in virus replication is unclear (Benavente and Martinez-Costaz, 2007). Although all viral mRNA transcripts are produced in equal concentrations, the amount of the different viral proteins that are produced can vary, suggesting that avian reovirus protein production is regulated at the translation level (Benavente and Martinez-Costaz, 2007).

Avian reovirus can trigger apoptosis in infected cells by different mechanisms either before or after viral gene expression (Shih et al., 2004). The σC protein expression is involved in the induction of apoptosis (Lin et al., 2011). Additionally, avian reovirus is characterized by formation of cell-cell fusion (syncytia formation), which is mostly mediated by P10 protein (Liu et al., 2008).

Table 1.2: Structure and biology of mammalian and avian orthoreoviruses

	Mammalian reoviruses	Avian reoviruses
Proteins encoded by RNA segments:		
L1	λ3	λA
L2	λ2	λB
L3	λ1	λC
M1	μ2	μA
M2	μ1/μ1C	μB
M3	μNS	μNS
S1	σ1	σC, P10 and P17
S2	σ2	σA
S3	σNS	σB
S4	σ3	σNS
Virus attachment	By σ1 with JAM-A receptors	By σC and cell receptors is unknown
Uncoating	σ3 is removed σ1 undergoes conformational changes	Outer capsid proteins are removed and μB is exposed
Penetration	μ1 cleavage then penetration	μB is cleaved then penetration
Assembly	μNS, σNS and μ2 form reovirus inclusions and μNS recruits core proteins (λ1, λ2, λ3 and σ2)	μNS protein recruits inner core proteins into viral factories
Syncytia	Non fusogenic except baboon reovirus	Fusogenic mediated by P10 protein

4.3. Epidemics: Though avian reoviruses have been isolated from different species of birds, reoviruses associated with tenosynovitis/arthritis are usually found in chickens and turkeys (Rosenberger, 2003). Avian reoviruses have been isolated from clinically ill ducks, geese, pigeons and psittacine birds, but the only host-pathogen relationship was described in geese and ducks (Palya et al., 2003; Robertson and Wilcox, 1986). Avian reovirus survived in poultry house materials like wood, feathers and egg shells (10 days) and drinking water (10 weeks) (Savage and Jones, 2011). Certainly the ability of a virus to survive in a poultry house plays important role in the persistence of virus infection. Most avian reoviruses isolated in the environment are non-pathogenic (Jones et al., 2000).

Virus transmission is affected by many factors. Young chickens are more susceptible than old birds to infection by reovirus and are more likely to show clinical signs. Conversely, older birds can become infected, but are less susceptible to clinical disease (Kerr and Olson 1964; Jones and Georgiou, 1984; Rosenberger, 1983). Avian reoviruses can be transmitted via various routes. Vertical transmission through the egg was reported by many authors (Al-Mufarrej et al., 1996; Menendez et al., 1975; van der Heide and Kalbac, 1975) while the fecal-oral route of horizontal transmission is common (Jones and Onunkwo, 1978). Furthermore, fecal contamination of broken skin was proposed as a route by which avian reovirus can access leg tendons and joints (Al-Afaleq and Jones, 1990). Breed susceptibility to infection with avian reovirus can vary in chickens. Though heavy meat-type chickens are mostly reported to be infected with reovirus induced arthritis, light egg layers infection and clinical disease has been occasionally reported (Schwartz et al., 1976). In addition, broilers

were more susceptible than white leghorn chickens to reovirus arthritis (Jones and Kibenge, 1984)

4.4. Pathogenesis and tissue spread: Experimental infection of avian reovirus in specific-pathogen-free (SPF) chickens through oral, intranasal, or intratracheal route resulted in viremia with distribution of reovirus to respiratory, enteric, reproductive organs, hock joint and tendons (Menendez et al., 1975). Virus was found in erythrocytes and lymphocytes in peripheral blood at 30 hours PI, followed by distribution of virus to internal organs within 3-5 days PI (Kibenge et al., 1985). Intestines and bursa of Fabricius are the primary sites of virus multiplication and within 2-4 days these organs serve as a portal of entry for viral spread to other internal organs (Jones et al., 1989). Hock joint was also reported to be an important site for chicken reovirus multiplication (Jones and Kibenge, 1984; Sahu and Olson, 1975; Walker et al., 1972). Chicken reovirus can target liver where it multiplies extensively to cause hepatitis and mortality within 10 days PI (Jones and Guneratne, 1984). Avian reovirus tissue tropism has a high degree of correlation with the S1 segment, which encodes the σC protein (Meanger et al., 1999).

The incubation period of avian reovirus varies based on age and breed of the infected bird along with the strain of the virus (Robertson and Wilcox, 1986; Sterner et al., 1989). In a study on reovirus challenge in chickens, the incubation period varied from 1 day for footpad route of inoculation to 11 days for intravenous and intramuscular inoculation, and up to 9-13 days in case of intratracheal or contact infection (Neighbor et al., 1994). Viral shedding was shown at two weeks PI via the oral route (Kibenge and Dhillon, 1987). In another study, viral shedding peaked at 1-2

weeks PI but decreased after 3 weeks PI (Islam et al., 1988; Al-Afaleg and Jones, 1994). Chicken arthritis reovirus can persist in legs of inoculated chickens for 285 days (Olson & Kerr, 1967).

4.5. Immune response: Mucosal IgA provided from a previous infection (vaccination) or maternally derived IgG can protect the contacts' mucosal epithelium, the respiratory or digestive tract from viral challenged birds (Juerissen et al., 2000). Maternally derived antibody can protect 1-day-old chicks from clinical disease associated with reovirus infection (Grindstaff, 2008; van der Heide et al., 1976). When a virus infects a bird, it induces an innate immune response that is mediated through natural killer cells (NK), dendritic cells (DC), macrophages and heterophils. Measurement of cytokine production has been used to detect or characterize the action of these inflammatory cells. Interleukins IL-1, IL-6 and tumor necrosis factor (TNF-alpha) have been used as indicators of macrophage activity (Juerissen et al., 2000). The degree of the innate immune response, cytokine production and clinical disease is directly proportional to the extent of virus multiplication. In one study, avian reoviruses that had higher multiplication rates induced significantly higher production of IL-6, IL-10 and INF-γ compared with reoviruses exhibiting a lower rate of multiplication (Shen et al., 2014). Avian reovirus can activate some cellular signaling pathways like PI3-kinase, NF-κB and Stat-3 that lead to inflammation mediated by IL-6 (Lin et al, 2010).

The innate immune response initiates lymphocyte proliferation to form either T-cell clones or B-cell clones (Juerissen et al., 2000). The amount of avian reovirus-specific IgA produced during infection can be affected by the route of administration

and age of the host. Chickens at 1-3 weeks of age produced higher titers of mucosal IgA than 1-day-old chicks and the oral route of inoculation induced greater amounts of mucosal IgA than the subcutaneous route (Mukiibi-Muka and Jones, 1999). In the same study, anti-reovirus IgG production occurred only in serum and not locally in enteric mucosa, and the IgG production was not affected by the route of inoculation or age. The avian reovirus σC protein was shown to increase the titer of systemic and mucosal IgA when it was introduced with lactic acid bacteria compared with control bacteria lacking σC protein (Lin et al., 2012). This shows that some fractions of avian reovirus are immunogenic and can be used to enhance the immune response to avian reovirus infection.

$CD8^+$ T cells are superior to $CD4^+$ T cells in clearing avian reovirus (Songserm et al., 2003). Previous work has shown that subacute avian reovirus infection in chickens (8-14 days PI) is characterized by infiltration of both $CD4^+$ and $CD8^+$ T cells as well as IgM^+ B cells, while acute infection (2-6 days PI) mobilizes $CD8^+$ T cells and chronic infection (more than 14 days PI) is characterized by infiltration of $CD4^+$ T cells with few IgM^+ B cells. This pattern of lymphocyte response that was detected in joints by immunohistochemistry was similar to lymphocytic activation in human rheumatoid arthritis (Pertile et al., 1996a). In fact, others indicate that avian reovirus infection may be a good model for human rheumatoid arthritis (Sahu and Olson, 1975; Walker et al., 1975). Antinuclear antibodies or anti-collagen antibodies have been detected in the serum of avian reovirus-infected chickens, indicating that avian reovirus might be associated with an autoimmune response in the host (Islam et al., 1990; Pradhan et al., 1987). Avian reovirus also has the ability to suppress

lymphocyte proliferation (Neelima et al., 2003; Pertile et al., 1996b), which might explain the observations of clinical immunosuppression associated with avian reovirus infection.

4.6. Clinical disease and pathology: There are field reports of broiler chickens, either 3-4 weeks or 6-7 weeks of age, experiencing reovirus infection with lameness, swollen joints and stunted growth in 6-8% of birds. In another report, an infected broiler flock displayed 5% swollen joints and tendons, markedly decreased body weight, 5% mortality and nearly 90% of the flock was seroconverting for reovirus antibodies (Rosenberger, 2003; Glass et al., 1973; Johnson and van der Heide, 1971). Lameness and tendon rupture in >12 weeks old male chickens have been reported with avian reovirus infection (Johnson and van der Heide, 1971; Jones et al., 1975). Swollen joints and tendon rupture will disturb the gait in infected birds. Avian reovirus was isolated from birds with runting-stunting and malabsorption syndrome, but the same clinical disease could not be reproduced in experimentally infected chickens (Page et al., 1982; Davis et al., 2013). Other work showed that avian reoviruses 2408 and 1733 caused stunting and feathering abnormalities when inoculated into 1 and 7-day-old chicks (Rosenberger et al., 1989). Finally, avian reovirus was reported to induce nervous signs in SPF chickens (Van de Zande and Kuhn, 2007).

Intertarsal joint and tendon swelling is often the primary gross lesion in chicken reoviral arthritis. Petechie can develop on the synovial membranes and small erosions on the joint articular surface. Joints of reovirus-infected birds can contain increased volume of fluid that is straw-colored or blood-tinted, but not turbid. In chronic cases, fibrous adhesion of gastrocnemius and digital flexor tendons to their sheaths can

impair smooth movement of the tendons (McNulty, 1993; Rosenberger and Olson, 1997; van der Heidi, 1977). It was demonstrated that the gastrocnemius tendon of 10 and 18-week-old chickens infected with reovirus had significantly lower tensile strength and higher resistance to stretch compared to control birds (Mohamed et al., 1995). The susceptibility of leg tendons of older and heavier birds to rupture has been documented in numerous reports and studies (Hill et al., 1989b; McNulty, 1993; Rosenberger and Olson, 1997; van der Heide, 1977; Jones, 2000; Rosenberger, 2003). Experimental inoculation of 1-day-old, SPF chickens with chicken reovirus via the oral and footpad routes produced necrosis and congestion of liver, spleen, kidney and bursa of Fabricius with tenosynovitis, myocarditis and pericarditis (Hieronymus et al., 1983).

Histologically, at the acute stage (1-2 weeks PI) of infection after footpad inoculation, the gastrocnemius tendon sheath was expanded by edema, synoviocyte hypertrophy and hyperplasia, and infiltrates of lymphocytes and macrophages in the subsynovium. Later, in the chronic stage, fibroplasia was prominent and fibrous connective tissue accumulated in the subsynovium along with villous-like processes extending from the synovium. Periostitis and increased osteoclast production can also be observed at chronic stages (Kerr and Olson, 1964; Rosenberger et al., 2003). In chickens that were orally inoculated with reovirus, by 7-8 weeks PI, there was fibrosis in gastrocnemius tendon and tendon sheath resulting in adhesion and immobilized tendon (van der Heide et al., 1974). Reovirus infection has been associated with erosions in the hock joint articular cartilage accompanied with subchondral bony exostoses that might immobilize the joint (Kerr and Olson, 1969).

This histologic picture of reovirus tenosynovitis could resemble that of Staphylococcus aureus or Mycoplasma synoviae infection (Jones, 2000), but lymphocytic inflammation is generally characteristic for avian reovirus while Mycoplasma and Staphylococcus infection show purulent inflammation (Hill et al., 1989a). Microscopic lesions of reovirus infection can be observed in visceral organs as well. Myocarditis and pericarditis have been described in chickens infected with reovirus. These lesions are characterized by heterophilic and lymphocytic infiltration the in myocardium and lymphocytic aggregates within the epicardium; these lesions in chickens were considered to be almost diagnostic for reovirus arthritis infection (Kerr an Olson, 1969; Tang et al., 1987; Olson and Solomon, 1968). Additionally, another study demonstrated hepatic necrosis within 3 weeks PI in chickens infected with reovirus at 1 day of age (Gouvea and Schintzer, 1982).

Ultrastructural changes associated with reovirus infection in chickens have been described. Gastrocnemius tendon and sheath of broilers that were orally infected with reovirus at 1 day of age had fibroblastic lesions, consisting of cytoplasmic vacuolization, loss of ribosomes from the endoplasmic reticulum, and mitochondrial disruption from 1-5 weeks PI (Hill et al., 1989b).

4.7. Vaccination: Avian reovirus is hardy in the environment, is transmitted both vertically and horizontally, and testing of cloacal swabs is not a reliable sample to confirm virus absence. Vaccination has become an effective way to prevent chicken viral arthritis (Jones, 2000). The production focus is to protect the chicks from reovirus infection since young birds are more susceptible to infection than adults (Jones and Georgiou, 1984; Roessler and Rosenberger, 1989). Subcutaneous

vaccination at one day of age with attenuated S1133 avian reovirus strain was protective against reovirus infection (van der Heide et al., 1983). Administration of a coarse spray of S1133 at one day of age. The vaccine did not produce serum neutralizing antibodies, but birds were protected from reovirus infection and S1133 did not interfere with the immune response of other vaccine viruses when administered in combination. Application of S1113 in a coarse spray or drinking water produced a high titer of antireovirus antibodies (Giambrone and Hathcock, 1991). SPF chicks treated with cyclophosphamide to deplete B cells and then vaccinated with a subcutaneous injection of live attenuated avian reovirus at 7 days of age were shown to be protected from reovirus challenge at two weeks post vaccination. This finding suggesed that cell-mediated immunity played an independent role in protection (van Loon et al., 2003).

In ovo vaccination at the 18^{th} day of incubation was 70% protective against reovirus infection and the post-hatch mortality was significantly decreased as compared to the non-vaccinated control group (Guo et al., 2003). In ovo vaccination induces significantly higher $CD4^+$ and $CD8^+$ T cells compared with non-vaccinated control birds while B-cell numbers were the same in both groups. In ovo attenuated live virus vaccination should be administered to eggs containing maternally derived antibodies otherwise it might induce immunosuppression (Guo et al., 2004).

In general, the administration of live attenuated vaccines in young chicks has not been effective in inducing protection against avian reovirus infection and this was attributed to underdevelopment of the chick immune system (Mukiibi-Muka, 1997). In addition, S1133 avian reovirus vaccine can interfere with Marek's disease vaccine

if these are administered simultaneously (Rosenberger, 1983; Schnitzer et al., 1983). The most effective and recommended live vaccine regimen is to administer vaccine to chicks that have passively absorped anti-reovirus antibodies derived from vaccinated breeders with a high serum antibody titer (van der Heide, 1996; Wood et al., 1996).

Vaccination of breeders by live and/or killed vaccine provides passive immunity to chicks (van der Heide et al., 1976). Killed S1133 was used in broiler breeders and it produced short-lived high serum titers (Rau et al., 1980; van der Heide, 1977), while live attenuated S1133 (47^{th} passage) administered subcutaneously or orally to 10 to15-week-old broiler breeders protected progeny against challenge with the same virulent virus (Rau et al., 1980; Van der Heide, 1977; Edison et al., 1979; Van der Heide and Page, 1980). This same attenuated S1133 did not protect against other serotypes of reovirus (Rau et al., 1980). A tetravalent live attenuated avian reovirus vaccine gave protection against the challenge with all the four genotypes of virulent avian reoviruses (typed based on sigma C protein) (Lublin et al., 2011). It is recommended to prime breeders with live attenuated vaccine at early stage of life then give killed vaccine at 6 weeks of age and prior to lay. This will ensure having high titer of serum protective antibodies that will protect progeny (Giambrone, 1985).

Subunit reovirus vaccines have been recently developed and are showing promise. A baculovirus recombinant expressing sigma B and sigma C provided significantly higher antibody titer against these proteins compared with controls that received baculovirus that did not express sigma proteins or received PBS (Lin et al., 2008). In addition, sigma C protein can be expressed on the surface of transgenic plants, thus

opening the way to administer virus protein in a subunit vaccine through feed composed of transgenic plants (Huang et al., 2006).

5. Turkey reoviruses:

5.1. Clinical disease: Turkey reoviruses have been associated with enteritis in turkey poults (Lojkic et al., 2010; Mor et al., 2013a; Clavert, 2012; Heggen-Peay et al., 2002; Jindal et al., 2009; Jindal et al., 2010; Woolcock and Shivaprasad, 2007; Nersessian et al., 1985). The reovirus-associated enteric diseases vary in severity and are characterized by different names or syndromes. Some clinical diseases were associated with decreased body weight and diarrhea (Clavert, 2012; Mor et al., 2013; Jindal et al, 2009, 2010) while others (like poult enteritis and mortality syndrome) are severe diseases (Heggen-Peay et al., 2002).

Reovirus, rotavirus and astrovirus were reported to be the main viruses associated with turkey and poult enteritis (Jindal et al., 2010). A combination of these three viruses was inoculated orally into turkey poults and produced decreased body weight and diarrhea as shown in field cases (Mor et al., 2013; Spackmann et al., 2010) while inoculation of individual viruses (including reovirus) failed to reproduce clinical disease (Spackman et al., 2010). Experimental inoculation of other turkey reovirus strains into poults resulted in decreased body weight and diarrhea (Nersessian et al., 1986; Spackman et al., 2005). Association of turkey reoviruses with myocarditis in turkeys has also been reported (Shivaprasad et al., 2009; Farnca et al., 2010). Reovirus was isolated from ruptured tendons of turkeys with tenosynovitis/arthritis but Koch's postulates were not fulfilled (Levisohn et al., 1980; Page et al., 1982).

Page et al. (1982) were able to isolate reovirus from affected tendon and then reproduce the clinical disease by inoculation of the footpad of turkey pouls, but the report did not indicate that reovirus was successfully reisolated from experimentally infected poults (Page et al., 1982). In other reports reovirus was found to be associated with immunosuppression in turkeys (Pantin-Jackwood et al., 2007; Day et al., 2008; Rosa et al., 2013).

5.2. Pathology: In previous studies, turkeys infected with enteric reovirus developed diarrhea with pasty (fecal covered) vent feathers and vent skin that was necrotic and hyperemic. Necropsy revealed small intestines and ceca that were distended with watery contents and gas (Nersessian et al., 1986; Spackman et al., 2005). Microscopically, intestines showed mild crypt enterocyte hyperplasia with lymphocytic infiltration in the submucosa at 1-2 weeks PI (Pantin-Jackwood et al., 2007; Day et al., 2008). Reovirus was isolated from a heart of 17-day-old turkey poult that showed increased fluid in the pericardial sac with dilated ventricles and pale epicardium (Shivaprasad et al., 2009). Microscopically, there was multifocal lymphocytic infiltration in epicardium and myocardium with macrophages and plasma cells infiltration within necrotic myocardium (Pantin-Jackwood et al., 2007; Shivaprasad et al., 2009). Immunosuppressed turkeys infected with reovirus showed atrophy of the bursa of Fabricius with microscopic evidence of lymphoid depletion in both bursa and spleen at 1-2 weeks PI (Spackman et al., 2005; Pantin-Jackwood et al., 2007; Day et al., 2008).

The gross lesions observed in turkey reovirus induced tenosynovitis/arthritis consisted of swollen intertarsal (hock) joints with histological lesions of hyperplastic

synovium and inflammatory cell infiltrate in the subsynovium (Levisohn et al., 1980). Page et al., 1982 reported on two cases of lameness and reluctance of movement in 5- and 8-week-old turkeys. Reovirus was isolated from these cases and tenosynovitis was reproduced inoculating the virus into footpads of 1-day-old turkey poults (Page et al., 1982). However, another report showed that reovirus strains isolated from turkeys with tenosynovitis/arthritis did not induce tenosynovitis/arthritis when inoculated into footpads of 1-day-old poults (Al-Afaleq & Jones, 1989).

5.3. Molecular characterization: There is little work done on molecular characterization of turkey reoviruses. Sequencing of different segments of turkey reoviruses along with correlating pathogenicity with virus strain will enable us to understand the role of different reoviral proteins in virus pathogenesis and virulence. This information will aid in development of effective vaccines to control the clinical diseases induced by turkey reoviruses. In addition, sequence data of different turkey reovirus strains will increase our knowledge about sources of newly emerging viruses and possible mutations and reassortments occurring among different avian reoviruses.

Turkey reovirus (NC98) S3 gene segment that encodes σB protein was sequenced and found to have 64% identity with chicken reoviruses and 53% identity with miscovy duck reovirus (Kapczynski et al., 2002; Seller et al., 2004). A turkey reovirus strain isolated in Brazil showed 98-100% identity with turkey reovirus strains in USA based on S3 gene segment sequence (Rosa et al., 2014). Another study sequenced S3 gene segemt of turkey reoviruses that was detected in poult with enteritis. They found that turkey reoviruses had 88.9 to 100% identity among themselves, 59.5 to 63.5% and 69.2 to 72.6% with geese-ducks reoviruses group and

chicken reoviruses groups respectively (Jindal et al., 2014). Recently, turkey reoviruses isolated from tendons of lame turkeys with ruptured tendons showed high degree of homology (88.7 to 99.8%) among turkey reoviruses based on S4 gene sequence and only 78% nucleotide identity between turkey reoviruses and chicken reoviruses (Mor et al., 2013b). Next generation sequencing seems to be promising in detection of reovirus in enteric population and sequencing whole genome of viruses. Reovirus was detected in turkey gut among much viral population using metagenomic next generation approach (Day et al., 2010).

5.4. Other causes of lameness in turkeys: Since lameness is a common problem in turkeys and reovirus is just one factor associated with it, it is important to focus on other possible causes of lameness in turkeys for differential diagnosis. Musculoskeletal disorders that are induced by rapid rate of growth in turkeys can lead to lameness (Julian, 2005). Spondylolisthesis (kinky-back) is ventral dislocation of the anterior end of the 4^{th} thoracic vertebrae that resulting in compression and malacia of the spinal cord, resulting in posterior paralysis and lameness. The lesion can be observed in longitudinal sections of the vertebral column (Crespo and Shivaprasad, 2003). Tibial dyschondroplasia (TD) is a genetic disorder in which cartilage at bone epiphysis fails to ossify leading to weak legs and lame birds (Farquharson and Jefferies, 2000). The proximal epiphysis of tibia contains a cartilage mass (Julian et al., 1998). Valgus–varus deformity is a deviation of the hock joint either laterally or medially. It is associated with rapid growth rate and may show gastrocnemius tendon slippage (Riddell, 1992). Vitamin B complex and mineral deficiency (Ferguson et al.,

1978; Riddell, 1992, 1996) can result in perosis or chondrodystrophy whose clinical signs are similar to valgus–varus deformity.

Tibial rotation is another disorder that occurs “mostly unilateral” in turkeys and results from improper egg storage and hatchery temperature (Thorp, 1992; Julian and Gazdzinski, 1999; Crespo and Shivaprasad, 2003). Gastrocnemius tendon rupture may occur due to non -infectious causes like rapid growth rate and hight body weight (Julian, 1994, 1998b). Shaky leg lameness occurs mostly in male turkeys 8-18 weeks of age in which turkeys are reluctant to stand and walk. There is no specific known non- infectious cause for shaky legs but improving litter quality, particularly maintaining dry litter , and other factors that lead to footpad ulcers may decrease the incidence of shaky legs (Buyse et al., 1996). Epiphyseal separation occurs mostly in turkeys with osteochondrosis of the femoral head and this may result in spontaneous epiphyseal separation of the head of the femur (Julian, 1985). Fractures with lameness may occur spontaneously due to calcium deficiency leading to lameness (Julian, 2005). Deep pectoral myopathy (DPM) in turkeys resulted from increased muscle activity with increased production of lactic acid, which causes damage to the muscle cells with swelling and edema (Julian, 2005). Rickets can be a cause of lameness in turkeys and results from calcium and vitamin D deficiency in feed (Metz et al., 1995)

Mycoplasma synoviae induces arthritis/tenosynovitis in turkeys, and the clinical signs can resemble tenosynovirus/arthritis of viral etiology. PCR and culture are the best ways to confirm presence or absence of Mycoplasma (Landman and Feberwee, 2012). Culture to detect bacterial causes of lameness in turkeys like Staphylococcus aureus and E.coli (Narin, 1973) is the best way to confirm presence and absence of

these bacteria. Turbidity of synovial fluid and microscopical picture of heterophil and blue bacterial colonies can be another indicator of bacteria. Turkeys that are raised in cages are known to have numerically high incidence of leg problems and mortality (Chen et al., 1991).

5.5. Gait scoring systems for lameness evaluation: Accurate detection of lameness requires a gait scoring system that includes all variations of gait abnormalities. A 4-point gait scoring system has been described in turkeys (Ferket et al., 2009). However, this system was unable to address all the abnormalities in turkey gait. Previously designed 6-point (0-5) gait scoring system (Kestin et al., 1992) and its modification (Garner et al., 2002) in chickens are not suitable for turkeys. These chicken gait scoring systems include some behavioural actions that are different in turkeys. For example: fast running of chickens when approached as a sign of healthy chicken and healthy legs does not apply for turkeys which do not run like chickens whenapproached.

Chapter 2: The role of avian reoviruses in turkey tenosynovitis/arthritis

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Summary

Turkey arthritis reovirus (TARV) has been isolated from the gastrocnemius tendons and tibiotarsal joint fluid of lame, >12-week-old male turkeys in the Midwest. Two experiments were conducted to compare the pathogenicity in turkeys of three TARVs (TARV-MN2, TARV-MN4 and TARV-O'Neil) one turkey enteric reovirus (TERV), and one chicken arthritis reovirus (CARV). Two hundred ul of virus was inoculated by oral, intratracheal, or footpad route into 6-day-old poults placed in isolator units. Poults were necropsied at 1 and 4 weeks post infection (PI) in Experiment 1 and at 2 and 4 weeks PI in Experiment 2. Reovirus was detected by RT-PCR and virus isolation in tendons of TARV-inoculated poults at 1, 2 and 4 weeks PI. In general, TARVs produced lymphocytic tenosynovitis of the gastrocnemius and digital flexor tendon sheaths without inflammation of the tendons proper. In Experiment 1, poults inoculated with TARV-MN2 and TARV-O'Neil had significantly higher gastrocnemius tendon inflammation scores, as determined by histology, than those inoculated with TERV-MN1 or CARV-MN1. In Experiment 2, poults inoculated with TARV-MN2 and TARV-O'Neil had significantly higher gastrocnemius tendon inflammation scores than those inoculated with TARV-MN4 and virus-free medium (negative control group). Koch's postulate was fulfilled when TARV-MN2 and TARV-O'Neil were re-isolated from tendons of poults that had originally been challenged with either of these viruses. Results of these experiments indicate that TARVs have a unique ability to induce gastrocnemius tenosynovitis in turkeys and that administration of TARV-O'Neil through oral or intratracheal route is a reproducible model to study pathogenesis of TARV infection.

Introduction

Viral tenosynovitis/arthritis was first reported in 1968 in *Mycoplasma synoviae*-negative chickens in the USA (Olson & Solomon, 1968). In this report a virus, referred to as virus arthritis agent, was associated with swelling and edema of hock joint, wing joint and digital flexor tendons. Lameness associated with a reovirus in turkeys was first described in 1980 in 15-week-old turkeys with tenosynovitis/arthritis, in which reovirus was isolated from tendons of affected turkeys (Levisohn et al., 1980). Gross lesions consisted of swollen intertarsal (hock) joints with histological lesions of hyperplastic synovium and inflammatory cell infiltrate in the subsynovium. Page et al. (1982) reported two cases of lameness and reluctance of movement in 5- and 8-week-old turkeys. Viruses isolated from these cases produced tenosynovitis when inoculated into footpads of 1-day-old turkey poults. However, another report showed that reovirus strains isolated from turkeys and chickens with tenosynovitis/arthritis did not induce tenosynovitis/arthritis when inoculated into footpads of 1-day-old poults (Afaleq & Jones, 1989). There are no recent (< 20 years) reports of lameness associated with turkey reovirus although turkey enteric reovirus (TERV) has been identified as a cause of diarrhea, poult enteritis, and light turkey syndrome in turkeys (Jindal et al., 2010; Mor et al., 2013a).

In 2011, Minnesota Veterinary Diagnostic Laboratory isolated and characterized novel turkey arthritis reovirus (TARV) strains from tendons of turkeys with tenosynovitis/arthritis. Genetic characterization of S4 gene sequences showed relatively high degree of homology (88.7 to 99.8%) between TARV and TERV and only 78% nucleotide identity between TARV and CARV (chicken arthritis reovirus; Mor et al.,

2013b). In this current study, we describe and compare the pathogenicity in turkey poults of three TARV strains and one strain each of CARV and TERV.

Materials and methods:

Birds: In Experiment 1**,** three hundred and sixty 1-day-old male turkey poults were purchased from a Minnesota turkey hatchery. Twenty poults were placed in each of 18 filtered air isolators (20 birds per isolator); 15 isolators housed virus-inoculated and sentinel birds (see Experimental Design for details) and the three remaining isolators housed mock-inoculated control birds. In Experiment 2**,** one-day-old poults (n=151) from another Midwest turkey hatchery were divided into 10 different filtered air isolators (nine isolators contained 15 birds each and the tenth had 16 birds). The birds in both experiments were continuously supplied with food and water *ad libitum*. In each experiment, serum samples from five birds were obtained at one day of age and tested for reovirus antibodies. In addition, fecal samples collected from 1- and 6-day-old poults prior to inoculation were tested for reovirus by reverse transcription-polymerase chain reaction (RT-PCR; Mor et al., 2014).

Viruses: Three strains of TARV (TARV-MN2, TARV-MN4 and TARV O'Neil) were isolated from gastrocnemius/digital flexor tendons of turkeys with lameness and swollen hocks (periarticular fibrosis and joint effusion) with or without ruptured tendons. TERV-MN1 and CARV-MN1 were previously isolated from feces of turkeys with enteritis and from gastrocnemius tendons of chickens with classical gross and histological lesions of chicken viral arthritis, respectively. All viruses were grown and titrated on QT-35 cells and showed the following titers: TARV-MN2, TARV-MN4, TARV-O'Neil and CARV-

MN1 = $10^{5.5}$ $TCID_{50}$/ml; TERV-MN1= $10^{3.17}$ $TCID_{50}$/ml. The viruses were used at passage 2 to 7; TARV-MN2 and TARV-MN4 were at 5 passages, TARV-O'Neil at 3 passages, CARV-MN1 at 2 passages, and TERV-MN1 at 7 passages.

Experimental design: In Experiment 1, groups of 6-day-old male poults were inoculated in blind fashion with 0.2 ml of a virus via oral, intra-tracheal (IT) or footpad (FP). Each of the 15 isolators housed 20 birds apiece; 15 poults that were inoculated with one virus by a single route and an additional five poults that served as non-inoculated sentinels. The birds in the remaining three isolators were inoculated with 0.2 ml of virus-free medium by one of the three routes (as negative controls). All birds were observed daily for lameness and swollen red joints by two individuals who were blinded to the virus and route of inoculation used. At 1 week PI, five poults from each isolator (3 inoculated and 2 sentinels) were euthanized. Four separate pools each of right and left leg gastrocnemius and digital flexor tendons (RLT and LLT), liver/spleen/heart (LSH), and intestinal contents (INT) were collected for virus detection by RT-PCR and virus isolation. Samples from inoculated and sentinel poults were pooled separately. Swabs from joint fluid and tendons were tested for aerobic bacteria by culture and for *Mycoplasma gallisepticum* and *M. synoviae* by culture and PCR. Hock joint, liver, spleen, intestines, bursa of Fabricius and heart were collected and fixed in 10% neutral-buffered formalin for histologic examination. At 4 weeks PI, the remaining poults were euthanized and samples were collected. Poults that showed obvious signs of lameness with joint swelling before the end of the experiment were euthanized and samples collected as described in the experimental design.

In Experiment 2, all 15 poults in each of the nine isolators were inoculated with a virus without any sentinels. Only three viruses were used for inoculation; TARV-MN2, TARV-MN4 and TARV-O'Neil. Sixteen control poults in the tenth isolator were inoculated with virus-free medium via oral (five poults), IT (five poults) or FP route (six poults). At 2 weeks PI, five poults from each isolator were euthanized and samples were collected as in Experiment 1. At 4 weeks PI, the remaining poults were euthanized and samples were collected.

Sample pooling: Pooling of similar organs for virus detection by RT-PCR and virus isolation was done only in Experiment 1; samples collected in Experiment 2 were tested individually. At one week PI, four separate pools of the right and left gastrocnemius and digital flexor leg tendons, liver/spleen/heart, and small intestine were collected separately from each poult and stored (-80^{0}C) for later analysis. Immediately prior to analysis the matching tissues from poults in each inoculation group were pooled to produce four separate tissue pools each for the inoculated and sentinels birds from each isolator. This resulted in 8 pools per isolator for a total of 144 pools at 1 week PI and the same number at 4 weeks PI.

Detection of virus and viral RNA: Hanks' balanced salt solution (HBSS) with 2% donor horse serum was used to homogenize tissue or tissue pools. Following centrifugation, the supernatants were decanted and then frozen at -80^{0}C for subsequent analysis by RT-PCR and virus isolation (Mor et al., 2013b, 2014). In Experiment 1, RNA extraction from pooled samples was done using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). Extracted RNA was tested for reovirus S4 gene using Onestep

RT-PCR Kit (Qiagen, Valencia, CA). The amplified products were separated on a 1.5% agarose gel. Appearance of 1100 bp band indicated positive sample. In Experiment 2, samples were homogenized in HBSS individually with no pooling. RNA was extracted and tested by RT-PCR. In both experiments, RT-PCR positive samples were subjected to virus isolation through two blind passages in QT35 cells (Mor et al., 2013b).

Serology: A commercial enzyme-linked immunosorbent assay (ELISA) was used to test for reovirus antibodies (IDEXX, Westbrook, ME) in serum samples of 5 birds / experiment (total 10 birds) before inoculation. The test was done according to manufacturer's instructions.

Clinical Signs: Birds were observed twice daily by two different blinded individuals. Birds that showed recumbency, inability to move, disturbed gait, splayed legs and/or swollen red tibiotarsal and foot joints, were recorded as lame birds. Individual birds were manipulated and examined using airtight rubber sleeves and gloves that were built into each isolator. A bird was euthanized if lameness reached a point of complete inability to move and to reach feed and water.

Gross lesions and histopathology: Birds were euthanized by exposure to CO_2 gas and examined for gross lesions in visceral organs, intestine and intertarsal (hock) joint. The intertarsal joints (comprising of gastrocnemius tendon, digital flexor tendon, distal tibiotarsus and proximal tarsometatarsus) along with heart, duodenum, jejunum, iliocecal junction with cecal tonsils, liver, spleen, and bursa of Fabricius were fixed in 10% neutral buffered formalin. Intact intertarsal joint, consisting of distal tibiotarsus and proximal tarsometatarsus, was decalcified in EDTA solution for 10 days after fixation. Samples

were then embedded in paraffin, sectioned at 3-5µm, and stained with hematoxylin and eosin.

Histologic inflammation scoring system: The gastrocnemius tendon sheath along the hock joint, the distal tibiotarsal physis and proximal tarsometatarsal physis served as the three microscopic fields (TT, JS and TM; Figure 1) for histological scoring based on a predictable inflammation pattern. Each region was scored as a 100X field according to three criteria; synovium, subsynovium and additional histological features. Synovium was scored as 0= normal single squamous epithelium layer, 1= single layer of hypertrophied synoviocytes, 2= 2-4 layers of hyperplastic synoviocytes, and 3= >4 layers of hyperplastic synoviocytes (Figure 2). Subsynovium was scored as 0= <10 lymphocytes, 1= 10-50 lymphocytes, 2= 50-100 lymphocytes, and 3 = too numerous to count (Figure 3). Additional histological features scored in the subsynovium were the presence of lymphoid follicles = 1 point, fibroplasia = 1 point, and dilated subsynovial blood vessels = 1 point (Figure 4). The sum of scores for synovium and subsynovium at TT, JS and TM regions were added to arrive at a score for one leg. The histologic inflammation score for each poult was the sum of scores for both right and left legs.

Statistics: Non parametric statistical analysis "Mann Whitney U test" was used to identify significant differences between the median histologic inflammation scores of different treatment groups and subgroups at $P<0.05$ (NCSS 9 Statistical Software, NCSS LLC., Kaysville, UT)

Results

Mortality, lameness and swollen joints: In Experiment 1, mortality was nearly the same in all infected groups. Poults developed lameness (5-6 birds/60) in all infected groups within 4 weeks PI. In addition, some poults showed swollen and red intertarsal joints (1-2 birds/60) at 3 weeks PI in all infected groups except the CARV group (Table 1). In Experiment 2, there were two dead birds; one each in TARV-O'Neil and TARV-MN4 groups. In Experiment 2, lameness was seen in TARV-O'Neil group inoculated with different routes (1 bird/route).

Gross pathology: Other than swollen intertarsal joints in Experiment 1, no remarkable gross lesions wereobserved in internal organs in Experiment 1 or 2 (Figure 5).

Virus detection: In Experiment 1, 18 fecal samples examined before inoculation at 1 and 6-days of age were positive for reovirus by RT-PCR. When sequenced for S4 gene the PCR products matched with TARV-MN4 and TERV lineages. In TARV-MN2 and TARV-O'Neil groups, reovirus was identified by RT-PCR in tendons of orally inoculated poults at 1 week PI. In TARV-O'Neil and TERV-MN1 groups, reovirus was identified by RT-PCR in tendons of intratracheally inoculated poults at 1 week PI. Reovirus was only identified by RT-PCR in internal organs at 1 week PI in poults inoculated by the IT route with TERV-MN1. In the TARV-MN4 group, reovirus was identified in intestine of orally inoculated and footpad-inoculated poults at 1 and 4 weeks PI, respectively. In the TARV-O'Neil group, reovirus was detected by RT-PCR in tendons of sentinel birds at 1 and 4 weeks PI. TARV-MN2 sentinel poults had reovirus -positive tendons at 1 week PI (Table 2).

In Experiment 2, ten fecal samples obtained prior to inoculation were negative for reovirus by RT-PCR. At 2 weeks PI, reovirus was identified by RT-PCR in tendons of the TARV-O'Neil (all routes), TARV-MN4 (all routes) and TARV-MN-2 (oral route) groups. At 4 weeks PI, viruses were identified by RT-PCR in tendons of TARV-O'Neil (oral and IT routes) and TARV-MN4 (oral and FP routes) groups, but not in TARV-MN2 (Table 3).

When these RT-PCR-positive samples (10.4% and 9.1% in Experiment 1 and Experiment 2, respectively) were subjected to virus isolation, reovirus was successfully isolated from the tendons of TARV-MN2 and TARV-O'Neil groups. Of the 30 samples positive by RT-PCR, 22 were positive by virus isolation. In Experiment 2, 36 of 51 RT-PCR positive samples yielded virus by virus isolation. Two blind passages in QT-35 cells were performed to determine if a sample was virus positive or negative. Ten viral isolates from poults inoculated with TARV-MN2 and TARV-O'Neil were sequenced at the S4 gene and all of them gave match with the virus that was inoculated.

Serology (ELISA): In Experiment 1, sera collected from five poults prior to inoculation were positive for reovirus antibodies but sera of five poults tested in Experiment 2 were negative.

Histopathology of internal organs: Different treatment groups showed mild to moderate, diffuse lymphocytic myocarditis with or without pericarditis in both experiments at 1, 2 and 4 weeks PI. Myocarditis was observed most often in TARV-O'Neil and TARV-MN4 groups in both experiments. Mild to moderate enteritis and typhlitis, characterized by increased heterophils in the lamina propria of villi, were

observed in the small intestines and ceca of the TERV-MN1 group and all TARV groups at 1 week PI in Experiment 1. In Experiment 2, enteritis was more commonly observed in birds inoculated with TARV-O'Neil than those with TARV-MN2 or TARV-MN4 at 2 weeks PI.

Histopathology of gastrocnemius tendon: In Experiment 1, only the TARV-O'Neil groups (all routes) had significantly higher inflammation scores than the other groups at 1 week PI, and there was no significant difference between inflammation scores of the other treatment groups. In Experiment 2, inflammation scores for TARV-MN2 and TARV-O'Neil groups were significantly higher than those of the negative control and TARV-MN4 groups at 2 weeks PI. Additionally, inflammation scores for TARV-O'Neil group were significantly higher than those of TARV-MN2 group ($P<0.05$).

Birds that showed swollen and red hock joints at 3 weeks PI had varied histologic alterations in gastrocnemius tendon. Poults inoculated with TARV-O'Neil had the highest histologic inflammation scores appearing as lymphocytic tenosynovitis. Negative control birds showed moderate heterophilic tenosynovitis and exudate accumulation in the intertarsal joint space. Bacterial cocci were observed in the joint space and corresponding bacterial cultures were positive for staphylococci. Samples from other birds were negative for aerobic bacteria and mycoplasmas.

In Experiment 1, at four weeks PI, TARV-MN2, TARV-MN4 and TARV-O'Neil produced significantly higher histologic inflammation scores than those produced by TERV-MN1, CARV-MN1 and virus-free culture medium (all routes together; Figure 6a). In Experiment 2, TARV-MN2 and TARV-O'Neil groups had significantly higher

histologic inflammation scores than TARV-MN4 group and group receiving virus-free culture medium (all routes together; Figure 6b). In both experiments, poults inoculated through oral and intratracheal routes showed significantly higher histologic inflammation scores in TARV-MN2 and TARV-O'Neil groups as compared to those in other groups. TARV-O'Neil inoculated by footpad route produced significantly higher inflammation scores compared to the intratracheal or oral route of inoculation ($P<0.05$) in experiments 1 and 2 (Figure 7). In Experiment 1, there were no significant differences in histologic inflammation scores among the three routes of inoculation. In Experiment 2, the TARV-MN4 footpad route group had significantly higher scores ($P<0.05$) than the oral and intratracheal groups. The TARV-O'Neil footpad and intratracheal groups had significantly higher scores than the oral route group ($P<0.05$).

Discussion

In this study we challenged turkey poults with a variety of reoviruses associated with enteritis or arthritis in turkeys to determine if the TARVs have a particular ability to induce tenosynovitis. Three routes of inoculation (oral, intratracheal and footpad) were used. These routes of inoculation have been used in previous studies on reovirus infection in chickens (Islam et al., 1988; Jones et al., 1995) and turkeys (Al-Afaleq & Jones, 1989; Pantin-Jackwood et al., 2007). Although one-day-old poults in Experiment 1 were positive for reovirus before inoculation, we continued with the experiment because several years of diagnostic PCR testing of feces have indicated that Minnesota poults are usually positive for reovirus and our intent was to evaluate the effect of TARVs on turkeys based in Minnesota. Additionally, we suspected that the virus detected by RT-

PCR prior to Experiment 1 was enteric reovirus or a non-pathogenic reovirus as are the majority of reoviruses in turkeys (Jones, 2000). In Experiment 1, poults in all groups experienced variable mortality and lameness from 1-3 weeks PI while poults in Experiment 2 largely showed no clinical signs with just two deaths and only 3 cases of lameness (in TARV-O'Neil group at 4 weeks PI). We believe that the source of the birds, cage flooring and several isolator management issues in Experiment 1 were the reason for this variation. Turkeys that are raised in cages are known to have numerically high incidence of leg problems and mortality (Chen et al., 1991). We were able to correct problems with isolator humidity, temperature and lighting prior to Experiment 2.

In Experiment 1, reoviruses were detected at 1 week PI in tendons of all treatment groups by RT-PCR except in TARV-MN4-inoculated groups. At the same time TARV-MN2, TARV-MN4 and TARV-O'Neil-inoculated poults had significantly higher gastrocnemius tendon inflammation scores than TERV-MN1, CARV-MN1 and control group at 4 weeks PI. TERV-MN1 and CARV-MN1 did not induce tenosynovitis although reovirus was identified in tendons of TERV-MN1 and CARV-MN1 groups by RT-PCR. Sequencing of the S4 gene segment of positive samples detected in birds before inoculation indicated that this virus matched with TARV-MN4 lineage. Our previous work with reovirus S4 gene sequencing has shown that TARVs are grouped into two lineages; one includes TARV-MN2 and TARV-O'Neil and the other includes TARV-MN4 along with a variety of turkey enteric reoviruses (Mor et al., 2013b). GenBank (National Center for Biotechnology Information) lacks complete S4 gene sequences for other S and M segments and thus we were not able to confirm whether this pre-study contaminant in

Experiment 1 was indeed TARV-MN4, another enteric reovirus or a non-pathogenic reovirus in the same lineage.

In Experiment 1, reovirus was identified in the tendons of sentinel poults in TARV-MN2 (1 week PI) and TARV-O'Neil (1 and 4 weeks PI) groups. Two sentinel poults that were PCR-positive for reovirus at 1 week PI had no histologic evidence of tenosynovitis. Virus transmission to sentinels likely occurred through the fecal-oral route or through broken skin as has been previously proposed with studies on transmission of chicken reovirus (Jones & Onunkwo, 1978; Afaleq & Jones, 1990). Sentinel poults were 1-week-old when they were in contact with infected birds and were likely susceptible to infection at that age. Chickens are more susceptible to infection by arthritis reovirus at 1 day to 2 weeks of age (Jones & Georgiou, 1984).

We have established that turkey poults inoculated with TARV can show histologic evidence of tenosynovitis at 2-3 weeks PI without demonstrating overt lameness. In the field, most cases of lameness associated with TARV are seen after 13 weeks of age. We believe that the tenosynovitis does not compromise the ability to walk until a particular weight threshold is reached in the growing turkey, as has been suggested with viral arthritis of chickens, (Jones, 2000). There is an extensive history of research on chicken reoviral arthritis that lends itself to comparison with turkey reoviral arthritis.
Although S4 gene sequencing of TARV and CARV shows separate lineages with only moderate homology (Mor et al., 2013b), the association of TARVs with field cases of gastrocnemius tendon rupture and the histologic appearance of lymphocytic tenosynovitis with lymphoid nodule formation induced in turkeys by TARV closely resembles previous

clinical and histological descriptions of CARV in chickens (Islam et al., 1988; Olson & Weiss, 1972). Additionally, it has been established that reoviruses associated with tenosynovitis in chickens can be transmitted by both horizontal and transovarian routes and hence breeder vaccination has been used as a control method in chickens (van der Heide, 2000). Chicken arthritis reovirus can persist in inoculated legs of chickens for 285 days (Olson & Kerr, 1967) with varying susceptibility to disease depending on chicken breed/conformation (Jones and Onunkwo, 1978) and age, with greatest susceptibility in chicks less than two weeks of age (Jones & Georgiou, 1984). We have established the causality of tenosynovitis and TARV infection in turkeys, fulfilling Koch's postulate, showing the horizontal transmission to sentinels and have established a reproducible turkey model to study infection with TARV. Our future studies will investigate the probable routes of transmission, age susceptibility and long term pathogenesis of TARV infection.

Acknowledgement: The authors thank Dr. Jack Rosenberger for providing TARV-O'Neil for the study.

Figure2.1: Gastrocnemius tendon histologic scoring sites (1a) intertarsal (hock) joint. TT, TM and JS correspond to levels of tibiotarsal physis, tarsometatarsal physis and joint space, respectively. (1b) general appearance of a 100x microscopic field, containing tendon and subsynovium, that was scored. Hematoxylin and eosin stain, bar = 500µm.

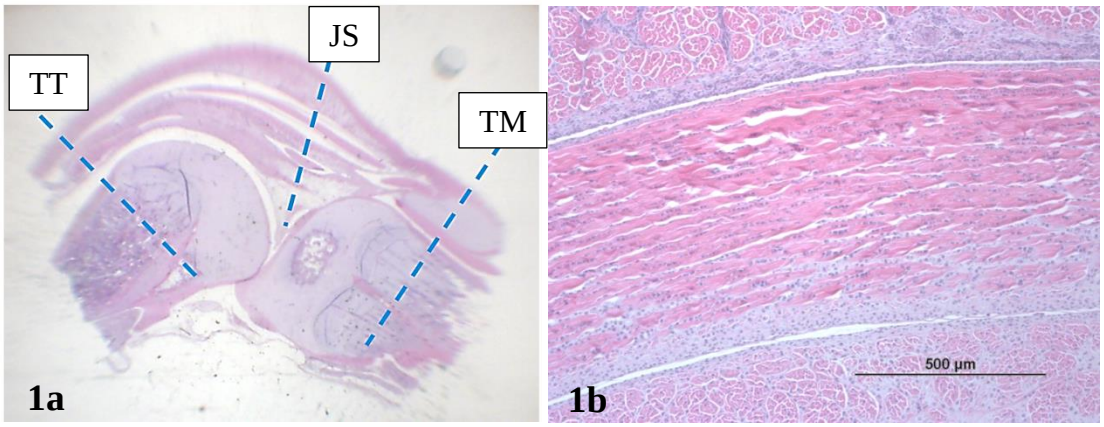

Figure 2.2.Gastrocnemius tendon histologic scoring scale for synovium; (2a) normal single squamous epithelium layer = 0, bar = 100µm, (2b) single layer of hypertrophied synovial epithelium = 1, bar = 100µm, (2c) 2-4 layers of hyperplastic synoviocytes = 2, bar = 100µm and (2d)>4 layers of hyperplastic synoviocytes = 3, bar = 100µm. Hematoxylin and eosin stain.

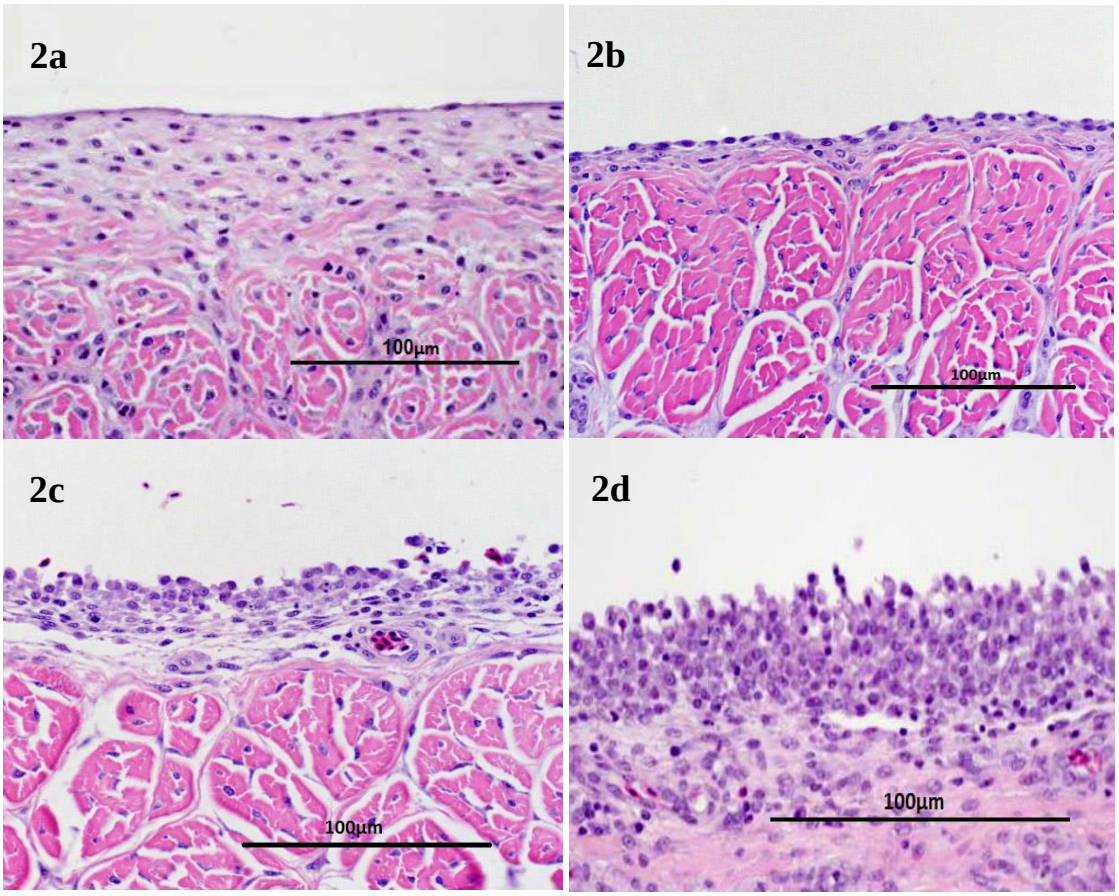

Figure 2.3. Gastrocnemius tendon histologic scoring scale for subsynovium for gastrocnemius tendon; (3a) < 10 lymphocytes = 0, bar = 200µm (3b) 10-50 lymphocytes = 1, bar = 200µm (3c) 50-100 lymphocytes = 2, bar = 200µm, and (3d) = lymphocytes too numerous to count (TNTC) = 3, bar = 200µm. Hematoxylin and eosin stain.

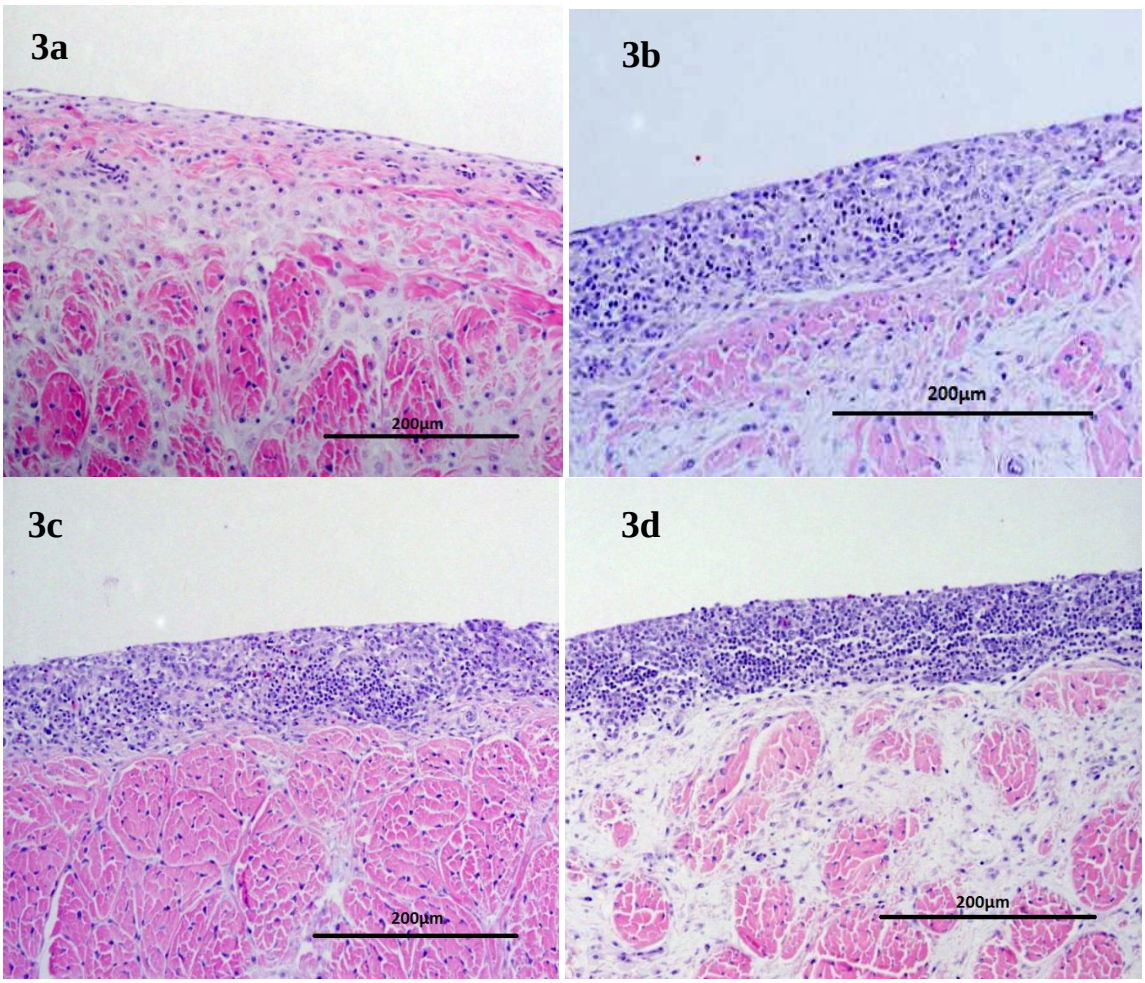

Figure 2.4. Additional histologic features in gastrocnemius tendon subsynovium that were scored; (4a) presence of lymphoid follicles (white arrow) = 1; congested blood vessels (black arrow) = 1, bar = 500µm. (4b) fibrosis (white arrows) = 1, bar = 200µm. Hematoxylin and eosin stain.

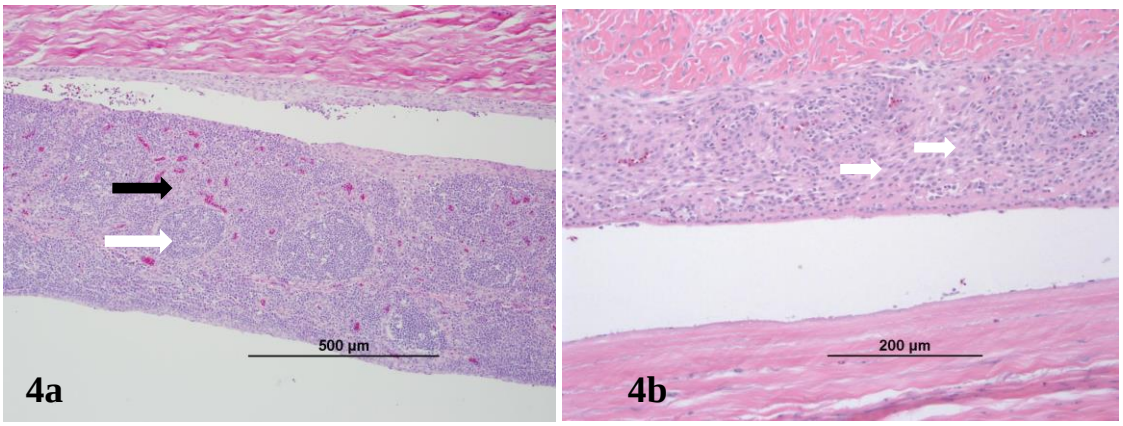

Figure 2.5: Turkeys with swollen hock joints at 3 weeks PI (Experiment 1); (5a) TARV-MN4 administered by oral route, (5b) TARV-O'Neil administered by intratracheal route.

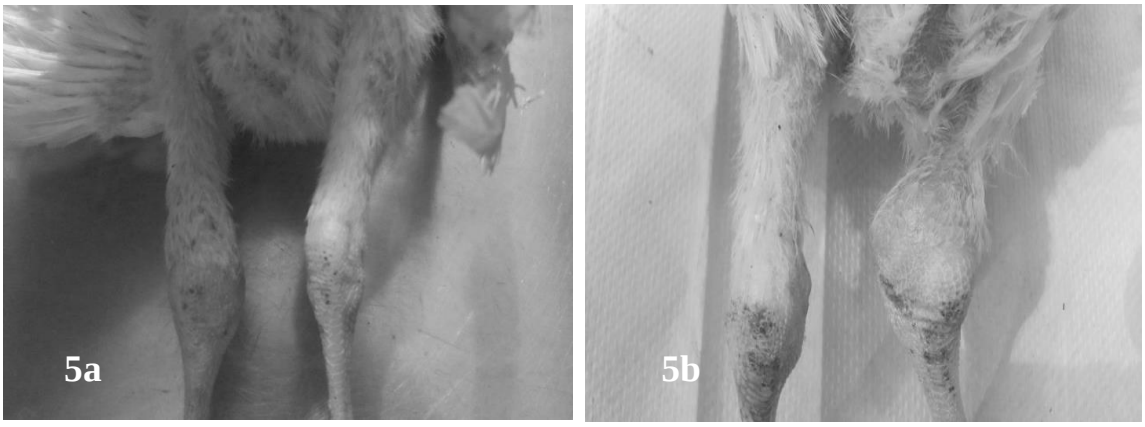

Figure 2.6: Gastrocnemius tendon histologic inflammation scores at 4 weeks PI in different group regardless of route of inoculation. (6a) Experiment 1, (6b) Experiment 2. Different uppercase letters indicate significant differences between groups ($P < 0.05$, Mann-Whitney U test).

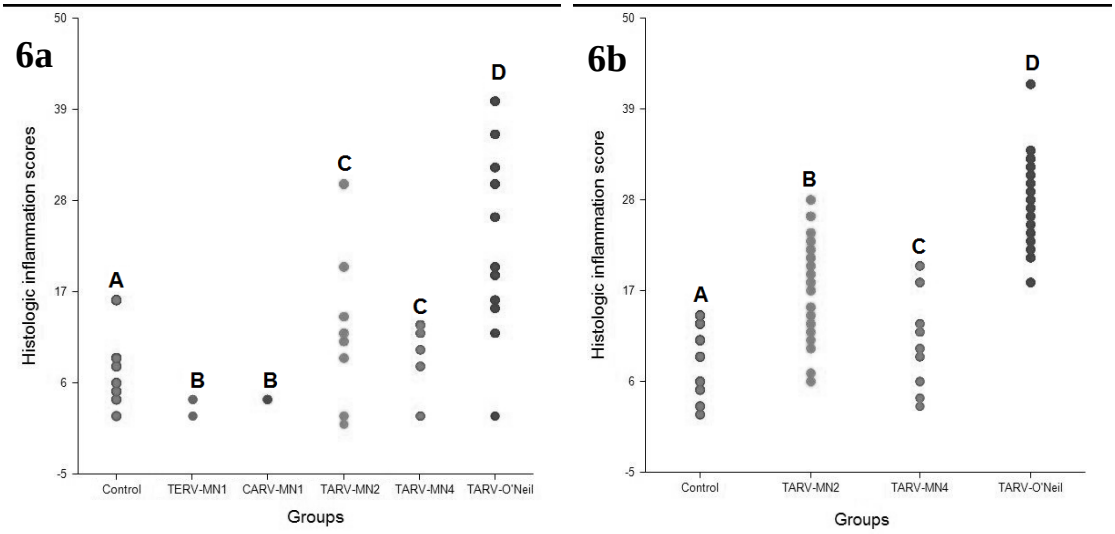

Figure 2.7: Gastrocnemius tendon histologic inflammation scores for all routes of inoculation at 4 weeks PI. (7a) oral, (7b) intratracheal and (7c) footpad inoculation in Experiment 1; (7d) oral, (7e) intratracheal, and (7f) footpad inoculation in Experiment 2.

Different uppercase letters indicate significant differences between groups ($P < 0.05$, Mann-Whitney U test).

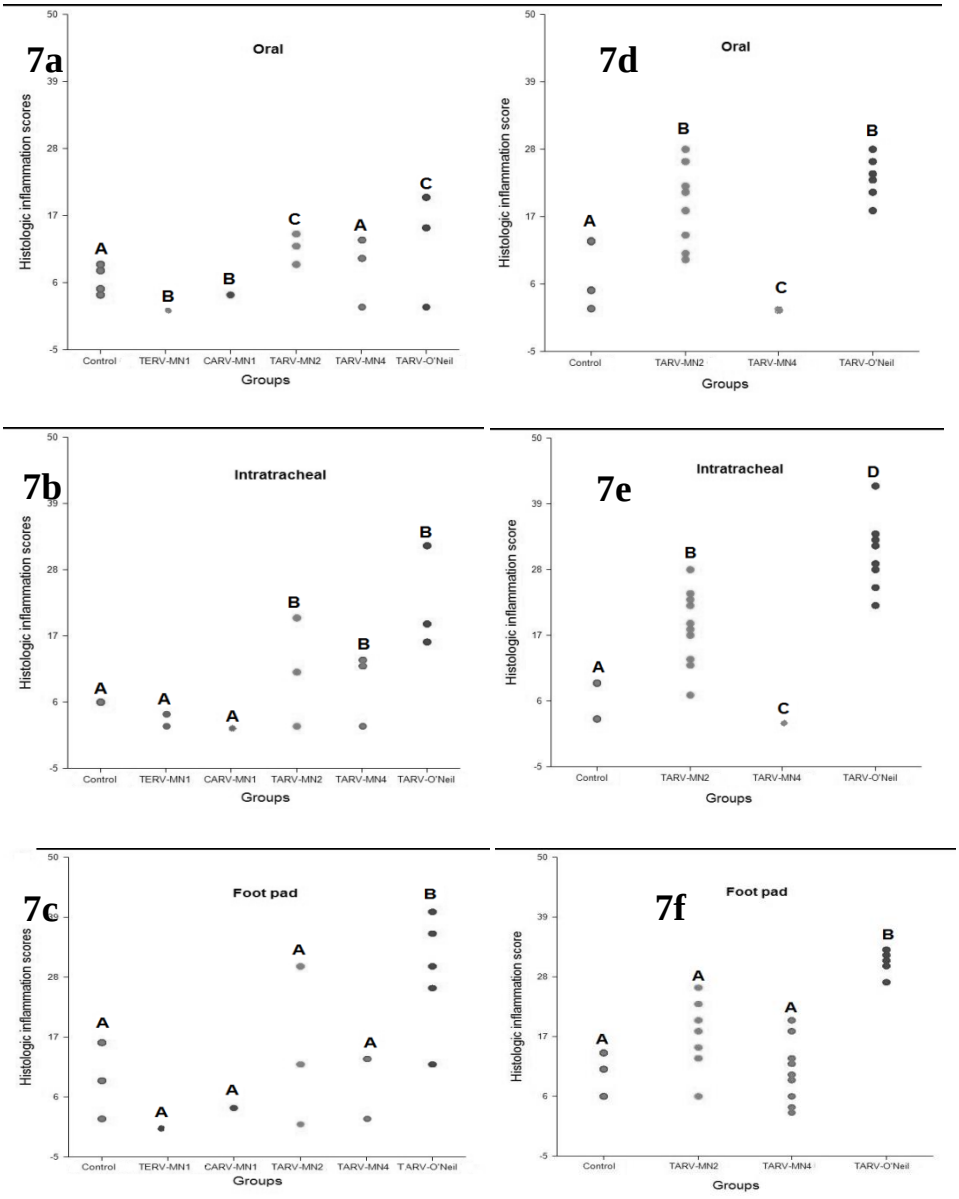

Table 2.1. Presence of lameness and swollen joints in turkey poults (Experiment 1).

Virus/inoculation Route		No. of poults showing lameness (per 20 birds)	Time of earliest observation of lameness (Weeks PI)	Swollen joint (3 wks PI)
TARV-MN2	Oral[a]	2	1	0
	IT	2	1,3	1
	FP	1	1	0
TARV-MN4	Oral	2	3	2
	IT	2	4	0
	FP	2	4	0
TARV-O'Neil	Oral	3	3,4	1
	IT	1	3	1
	FP	2	4	0
TERV-MN1	Oral	2	1,3	1[b]
	IT	1	1	0
	FP	3	3	1[b]
CARV-MN1	Oral	1	1	0
	IT	2	1	0
	FP	3	1,4	0
Control	Oral	1	4	0
	IT	3	1,3,4	1
	FP	0	-	1

[a]Inoculation routes were oral, intratracheal (IT) and footpad (FP)

[b]Sentinel turkey poults

Table 2.2. Detection of reovirus by RT-PCR in tissues of turkey poults (Experiment 1).[a]

Virus	Inoculated poults						Sentinel poults					
	1 week PI			4 weeks PI			1 week PI			4 weeks PI		
	FP[b]	IT	Oral	FP	IT	Oral	FP	IT	Oral	FP	IT	Oral
TARV/MN2	-	-	RLT[c] LLT	-	-	-	-	-	RLT	-	-	-
TARV/MN4	-	-	INT	INT	-	-	-	-	-	-	-	-
TARV/O'Neil	-	LLT	RLT	-	-	-	RLT LLT	-	INT	-	LLT	-
TERV/MN1	-	LSH RLT	-	-	-	INT	-	-	-	-	-	-
CARV/MN1	-	-	RLT	-	-	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-	-	-	-

[a]Tissues positive by RT-PCR are underlined and are labelled as to source; - = negative by RT-PCR.

[b]Inoculation routes were footpad (FP), intratracheal (IT) and oral

[c]The tissues tested were right leg tendon (RRT),left leg tendon (LLT), intestine (INT), and a pool of liver, spleen, and heart (LSH)

Table 2.3. Detection of reovirus by RT-PCR in tissues of turkey poults (Experiment 2)

Virus/inoculation routes		2 weeks PI				4 weeks PI			
		RLT[b]	LLT	LSH	INT	RLT	LLT	LSH	INT
TARV/MN2	Oral[a]	1/5[c]	0/5	0/5	0/5	0/10	0/10	0/10	0/10
	IT	0/5	0/5	0/5	0/5	0/10	0/10	0/10	0/10
	FP	0/5	0/5	0/5	1/5	0/10	0/10	0/10	0/10
TARV/MN4	Oral	1/5	2/5	2/5	2/5	1/10	4/10	0/10	0/10
	IT	1/5	1/5	0/5	0/5	0/9	0/9	0/9	0/9
	FP	1/5	2/5	1/5	2/5	2/10	3/10	1/10	0/10
TARV/O'Neil	Oral	2/5	2/5	0/5	0/5	2/10	1/10	1/10	0/10
	IT	0/5	1/5	0/5	0/5	4/9	3/9	3/9	1/9
	FP	1/7	2/7	0/7	1/7	0/7	0/7	0/7	0/7
Control	Oral	0/2	0/2	0/2	0/2	0/3	0/3	0/3	0/3
	IT	0/2	0/2	0/2	0/2	0/3	0/3	0/3	0/3
	FP	0/2	0/2	0/2	0/2	0/4	0/4	0/4	0/4

[a]Inoculation routes were oral, intratracheal (IT) and footpad (FP).

[b]The tissues tested were right leg tendon (RRT), left leg tendon (LLT), intestine (INT), and a pool of liver, spleen, and heart.

[c]Positive results are underlined. Ratios represent number positive /number of poults inoculated per group.

Chapter 3: Experimentally induced lameness in turkeys inoculated with a newly emergent turkey reovirus

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Summary

Newly emergent turkey arthritis reoviruses (TARVs) have been isolated from cases of lameness in male turkeys over 10 weeks of age. In a previous study, experimental inoculation of TARV in one-week-old turkey poults produced lymphocytic tenosynovitis at four weeks post inoculation but without causing clinical lameness. This study was undertaken to determine if TARV infection at an early age can lead to clinical lameness in birds as they age. One-week-old male turkeys were inoculated orally with a TARV (strain TARV-O'Neil) and monitored for the development of gait defects until 16 weeks of age. At 4, 8, 12 and 16 weeks of age, a subset of birds was euthanized followed by the collection of gastrocnemius tendon, digital flexor tendon, and intestines for virus detection by rRT-PCR and for histologic inflammation scoring. Clinical lameness was first displayed in TARV-infected turkeys at 8 weeks of age and ruptured gastrocnemius tendons were also seen at 12-16 weeks of age. The virus was detected in gastrocnemius tendon of 4- 8- and 12-week-old turkeys but not in 16-week-old turkeys. Histologic inflammation scores of tendons at each of the four time points were significantly higher in the virus-inoculated group than in the control group ($P<0.01$). Lesions began as lymphocytic tenosynovitis with mild synoviocyte hyperplasia at four weeks of age and progressed to fibrosis as the birds aged. These results demonstrate the potential of TARV to infect young turkeys experimentally and to produce subclinical tenosynovitis that becomes clinically demonstrable as the turkeys age.

Introduction

Initially, turkey arthritis reoviruses (TARVs) were detected in tendons of lame, 15-week-old turkeys with swollen intertarsal (hock) joints showing histological lesions of synovial hyperplasia and inflammatory cellular infiltrates in the subsynovium (Levisohn et al., 1980; Page et al., 1982). In an effort to reproduce the disease, three different turkey reoviruses were inoculated into the footpad of 1-day-old poults but no clinical disease or histologic lesions were observed (Al-afaleq and Jones 1989). Subsequently, there were no documented reports on reovirus-induced lameness in turkeys until 2013, when we isolated TARVs from lesions of tenosynovitis/arthritis in turkeys (Mor et al., 2013b). The samples used for TARVs isolation were gastrocnemius and digital flexor tendons of 12-to15-week-old lame turkeys.

Partial sequence analysis of the avian reovirus S4 gene showed 88.7 to 99.8% nucleotide identity between a panel of TARVs and turkey enteric reoviruses (TERVs); however, the nucleotide identity of TARVs with chicken arthritis reoviruses (CARVs) was only 78% (Mor et al., 2013b). Experimental inoculation of TARVs through oral, intratracheal, and footpad routes in 1-week-old turkey poults produced histologic lesions of marked lymphocytic tenosynovitis with synovial hyperplasia at 2 and 4 weeks post inoculation (PI). These lesions were absent in poults inoculated with TERV or CARV (Sharafeldin et al., 2014). Although TARV-inoculated birds showed high histologic scores of tenosynovitis and reovirus could be re-isolated from the gastrocnemius and digital flexor tendons, clinical lameness was not observed for up to 4-weeks PI (5 weeks of age) when the study was terminated (Sharafeldin et al., 2014).

The aims of the present work are; to determine whether infection in young poults progresses to clinical lameness as they age from 1 to 16 weeks, identify the age at which lameness occurs, and determine if there is a correlation between histological tenosynovitis and clinical lameness. In addition, this work provides a detailed description of lesion progression induced by TARV in turkeys and addresses the potential of the experimental model to study TARV pathogenesis and immune response in turkeys in the future studies.

Materials and methods

Birds: One hundred and sixty 1-day-old male turkey poults purchased from a Midwest poultry hatchery were divided into two groups (80/group) and placed in two isolation units. Meconium samples from another ten birds were collected at 1-day of age and examined for reovirus by real time reverse transcription- polymerase chain reaction (rRT-PCR) (Mor et al., 2014). Serum samples from an additional 10 birds were tested for anti-reovirus antibodies using a commercial enzyme-linked immunosorbent assay (ELISA; IDEXX, Westbrook, ME). Birds were supplied feed and water *ad libitum*. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of University of Minnesota.

Virus: TARV-O'Neil strain of TARV isolated from leg tendons of lame turkeys in Minnesota was kindly supplied by Dr. Jack Rosenberger, AviServe LLC, Newark, Delaware. The virus was grown and titrated on Japanese quail fibrosarcoma cell line (QT-35) cells and had a titer of $10^{5.5}TCID_{50}$/ml.

Experimental Design: The poults were divided into two groups and housed separately in isolation rooms. One group of poults was orally inoculated at 1 week of age with 0.2ml of

TARV-O'Neil virus. The negative control group was inoculated with virus-free MEM. At 4, 8, 12 and 16 weeks of age, all birds were weighed and their gaits were scored using a newly developed, turkey-specific gait scoring system (Table 1). After weighing and gait scoring at each of the four time points, 10 birds from each group were euthanized by exposure to carbon dioxide gas followed by the collection of tissue samples as described below. Intertarsal (hock) joint, including distal tibiotarsus and proximal tarsometatarsus, was excised and fixed in 10% neutral-buffered formalin for histopathology. Right and left leg tendons (distal part of gastrocnemius and digital flexor tendons) and 2cm intestinal segments (along with their digesta) were excised and stored at -20^0C for rRT-PCR. At the end of the experiment, the remaining birds (16 weeks of age) were euthanized after recording their body weights and gait scores. At each time point, Dacron swabs were inserted into the sheaths of excised gastrocnemius tendons from the ten turkeys and placed as a pool into 3ml of brain-heart infusion broth for PCR tergeting *Mycoplasma gallisepticum* and *M. synoviae*. Additionally, the gastrocnemius tendon sheaths of five birds from each treatment group were swabbed and cultured for aerobic bacteria and Mycoplasma.

Gait scoring system: A six-point (0-5) scoring system was designed to score the gait of turkeys and to quantify lameness (Table 1). Each bird was observed and scored separately and any recumbent bird was gently prodded to determine if they could stand or walk independently. Turkeys with gait scores of 3 or more were categorized as clinically lame.

Histopathology and histologic inflammation scoring: Bones comprising the intertarsal joint with adjacent gastrocnemius tendon were preserved in 10% neutral-buffered formalin. After decalcification in EDTA solution, the tissues were trimmed, processed,

embedded in paraffin, sectioned at 3-4µm, placed on glass slides, stained with hematoxylin and eosin (H&E), examined under an Olympus BX40 microscope and photomicrographs were taken by Olympus DP71 digital microscope camera. Tenosynovitis was scored histologically using a previously described histologic inflammation scoring system (Sharafeldin et al., 2014).

Virus detection: Samples were homogenized in Hanks' balanced salt solution (HBSS) containing 2% donor horse serum. The homogenates were then centrifuged and the supernatant stored at -80^{0}C until subsequent RNA extraction and rRT-PCR. RNA extraction was done by QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). Extracted RNA was tested by rRT-PCR for reovirus S4 gene (Mor et al., 2014) using one step RT-PCR Kit (Qiagen, Valencia, CA). This rRT-PCR showed higher sensitivity than virus isolation and detected as few as 10 viral gene copies (data not shown).

Statistical Analysis: The correlation coefficient between tenosynovitis (as determined histologically) and clinical lameness was calculated at 4, 8, 12 and 16 weeks of age. Detection of significant differences between the means of histologic inflammation scores and gait scores of different groups was done using the non-parametric statistical analysis "Mann Whitney U test" (NCSS 8 Statistical Software, NCSS LLC, Keysville, UT).The difference between averages of body weights was tested by two sample t-test.

Results

Gait Scoring and lameness: At the age of four-weeks, none of the turkeys in either the inoculated or the control group showed any clinical signs of lameness and their average gait score was 0. However, the gait scores in the inoculated group were significantly higher than those of control group at the ages of 8, 12 and 16 weeks ($P<0.05$) (Fig.1).

Clinical lameness (gait score >3) was observed at 8 weeks of age and progressively increased as the inoculated birds aged. Clinical lameness was observed in 26%, 30%, and 48% of inoculated birds at 8, 12, and 16 weeks of age, respectively. There was a minimal increase in gait scores in turkeys of control group from 4 to 12 weeks of age but 5 of 39 birds (13%) showed lameness at 16 weeks (Fig. 2). Virus-inoculated turkeys showed swollen hock joints with breast blisters and breast buttons with a single bird showing ruptured gastrocnemius tendon at 16 weeks (Fig. 3).

Histologic inflammation scores: Mean histologic inflammation scores of gastrocnemius tendon sheaths were significantly higher ($P<0.01$) in the inoculated group as compared to the control group at all ages (4, 8, 12 and 16-weeks) (Fig. 4). The lesions consisted of prominent lymphocytic infiltration of subsynovium and mild synoviocyte hyperplasia at 4 and 8 weeks, along with mild fibroplasia and lymphoid nodule formation at 8 weeks, progressing to decreased lymphocytic infiltration and increasing fibrosis at 12 weeks, and finally a prominent subsynovial fibrosis at 16 weeks (Fig. 5).

Correlation between tenosynovitis and gait score: The gait score and histologic inflammation score for each bird were used to calculate correlation coefficient, which was low (0.1) at 4 weeks and progressively increased until a strong positive correlation (0.9) was observed between tenosynovitis and high gait score (lameness) at 16 weeks (Fig. 6).

Body weight: The average body weight of inoculated birds was significantly lower ($P<0.05$) than that of control birds at 12 and 16 weeks (Fig. 7).

Virus detection: Fecal and serum samples taken from 1-day-old poults were negative for reovirus and antireovirus antibodies, respectively. In addition, samples from 1, 4, 8, 12

and 16-week-old turkeys were negative for aerobic bacteria by culture and negative for *Mycoplasma synoviae* and *M. gallisepticum* by culture and PCR. Reovirus was detected by rRT-PCR in 4 of 10 tendon and 3 of 10 intestine samples of 4-week-old turkeys in the virus-inoculated group. In 8-week-old birds, 5 of 10 tendons and 4 of 10 intestines were positive while at 12 weeks, only 2 of 10 tendon and 2 of 10 intestinal samples were positive for reovirus by rRT-PCR. All tissues collected from negative control turkeys at all ages were negative for reovirus by rRT-PCR.

Discussion

Previous work in our laboratory showed that poults orally inoculated with TARV at 1-week of age developed lymphocytic tenosynovitis by 4 weeks PI but did not show clinical lameness (Sharafeldin et al., 2014). Our present study sought to determine if tenosynovitis induced by TARV would progress to clinical lameness as the birds aged, to characterize the lameness through gait scoring, and to determine the relationship between tenosynovitis and gait scores. The study also sought to describe progression of histologic lesions from ages 4 to 16 weeks. The study aimed in general to test the potential of the experimental model for future experimental studies.

Lymphocytic infiltration of the gastrocnemius tendon sheath was prominent in 4-week-old turkeys and progressively decreased by the time the turkeys were 16 weeks of age, while subsynovial fibroplasia was initially observed in 8-week-old turkeys and was most prominent in 16-week-old turkeys. These results resemble those shown in a long term study on chicken reoviral arthritis (Hill et al., 1989b), in which oral inoculation of 1-day-old chicks with chicken reovirus led to the development of gastrocnemius lymphocytic

tenosynovitis and fibroplasia by 10 weeks of age and predominant fibrosis by 20 weeks of age.

The fibrosis of tendon and tendon sheath might lead to a decrease in the tensile strength of tendons as the birds age and become heavier, especially in turkeys that have high growth rate. This may explain why clinical lameness and tendon rupture were seen in older age birds (at 8-week-old), but not at younger ages. In addition, tendon adhesion with heavy weight might have led to tendon rupture (Jones, 2000). Similar results were shown in chickens infected with chicken arthritis reovirus; at 10 weeks PI there was fibrosis and adhesion of the gastrocnemius tendon, which along with the stress of heavier body weight resulted in tendon failure and rupture (Hill et al., 1989a).

Lameness evaluation in this work required a gait scoring system that includes all variations of gait abnormalities. For that reason, a previous 4-point gait scoring system in turkeys (Ferket et al., 2009) did not suit our requirements. The previously designed 6-point (0-5) gait scoring system designed for chickens (Kestin et al., 1992) and its modifications (Garner et al., 2002) were also not suitable because of behavioral variation between chickens and turkeys. Our newly designed 6-point (0-5) gait scoring system is specific for turkeys in which scores of 3 and above are considered as clinically lame. There was a clear distinction between score 2 and score 3 to avoid confusion and to have a clear cut point that distinguishes between lame and non-lame birds. Though the average gait scores of the infected group were not more than 3 but it was significantly higher than average gait scores in the control group.

Clinical lameness was first observed in 8-week-old turkeys with 25.8% of inoculated turkeys being lame. The percentage of lame birds in the inoculated group progressively increased at 12 weeks (30.4%) and 16 weeks (48.5%) while negative control turkeys, for the most part, remained clinically normal. The correlation coefficient between tenosynovitis score and degree of lameness was low (0.2) in 4-week-old turkeys; these birds had high tendon inflammation scores (tenosynovitis) but did not show clinical lameness. These findings were in agreement with our previous work that showed no clinical lameness in 5-week-old turkeys that had been orally inoculated with TARV at 1 week of age, despite the presence of high gastrocnemius tendon inflammation scores. In the present study, the correlation between tenosynovitis and gait score increased dramatically from 8 to 16-week-old turkeys with a peak of 0.9 at 16 weeks. This high correlation coefficient was attributed to increased clinical lameness in tenosynovitis birds and this was associated with increased body weight (Fig. 6). Studies on reovirus infection in chickens have demonstrated a strong association of lameness with fibrosis of the gastrocnemius tendon and tendon sheath, tendon adhesion and eventual rupture as the chickens increased in body weight (Hill et al., 1989a). In addition, it has been shown that the greater weight gain in meat producing chickens might affect the physical consistency of tendons during reovirus infection leading to clinical lameness (Kibenge and Wilcox, 1983).

TARV-infected turkeys had significantly lower average body weights than control groups at 12 and 16 weeks of age. Because reduced body weights were observed when gait scores started to increase at 8 weeks, we suspect that the discomfort associated with lameness made it more difficult for the affected turkeys to compete for food with other

birds and likely reduced the visits to the feeder. Additionally, because TARV induced mild enteritis in turkeys (Sharafeldin et al., 2014) the reduced body weight could also be the result of impaired digestion.

In a previous study, two-day-old turkeys inoculated with enteric reoviruses showed significantly lower body weight than controls at 2 to 9 days PI (Spackman et al., 2005). This might be due to the age of inoculation since younger birds have been shown to be most susceptible to reovirus infection. In our study, we inoculated turkeys at 1 week of age. The effect of age of inoculation on TARV infection has not yet been studied in turkeys but this age susceptibility has been demonstrated in chickens challenged with reovirus (Jones and Georgiou, 1984). TARV not only affects the carcass quality (e.g., hock swelling, breast blisters) of turkeys but, under experimental conditions also results in a significantly lower body weight at market age.

In this study, the virus was detected by rRT-PCR in 20%-50% of tendon and intestinal samples in infected turkeys at 4, 8 and 12 weeks of age with the peak of virus detection in 8-week-old turkeys. In field cases, we originally diagnosed tenosynovitis and successfully isolated reovirus from tendons of 16-week-old turkeys, but under experimental conditions we were not able to detect reovirus in tendons of 16-week-old turkeys by using rRT-PCR which showed higher sensitivity in TARV detection than virus isolation (Mor et al., 2014). Reovirus has been detected in field cases of chickens with tenosynovitis/arthritis at 11 weeks of age and younger (Jones et al., 1982). It appears that experimental conditions are different from field conditions, perhaps in terms of overall viral load and viral shedding and cycling in field turkeys. In addition, although we do not

know when field turkeys are naturally infected with TARV, our work indicates that surveillance of turkeys for TARV should occur weeks before any anticipated lameness occurs (mostly observed in field cases >12-week-old) in order to increase detection of virus, particularly if field poults are naturally infected vertically through the egg or at a very young age, as has been demonstrated in chickens (van der Heide, 1977).

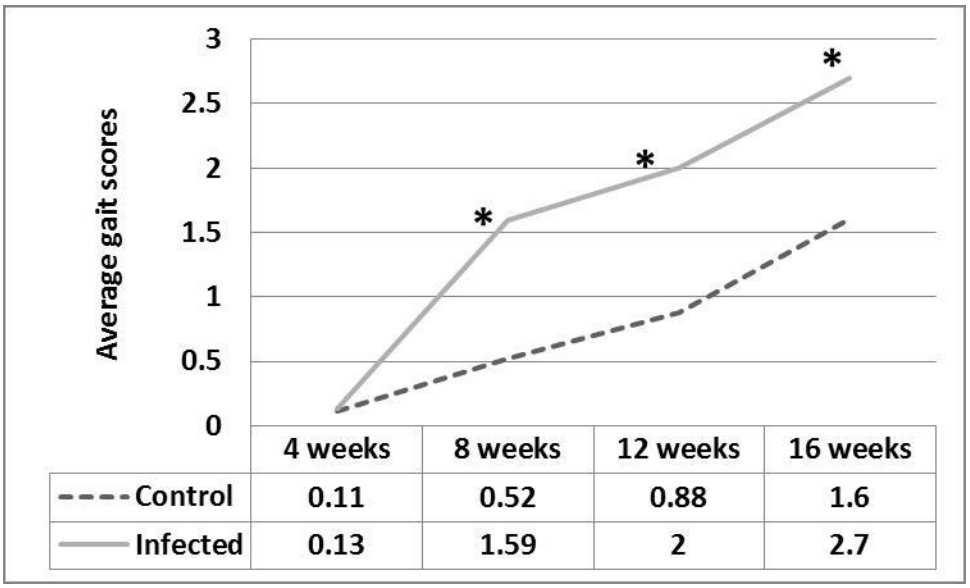
In conclusion, infection of poults with TARV causes lymphocytic gastrocnemius tenosynovitis that over time progresses to subsynovial fibroplasia and fibrosis. These changes along with increased body weight lead to clinical lameness and occasional tendon rupture as the birds age. In addition, TARV is most readily detected in infected turkeys prior to or as soon as the lameness is observed. We developed a gait-scoring system that should be useful for veterinarians or production personnel to characterize early alterations in gait, thus identify earlier onset of TARV infection and increase the opportunity for detection of TARV in infected turkeys. These findings augment the similarity of the experimental model (inoculation of TARV-O'Neil orally at 1-week-old) with the field condition and prove the potentiality of using this model in future immunopathogenesis studies.

Acknowledgements

The authors thank Dr Jack Rosenberger for providing TARV-O'Neil for the study. This work was supported by the University of Minnesota Rapid Agricultural Research Fund.

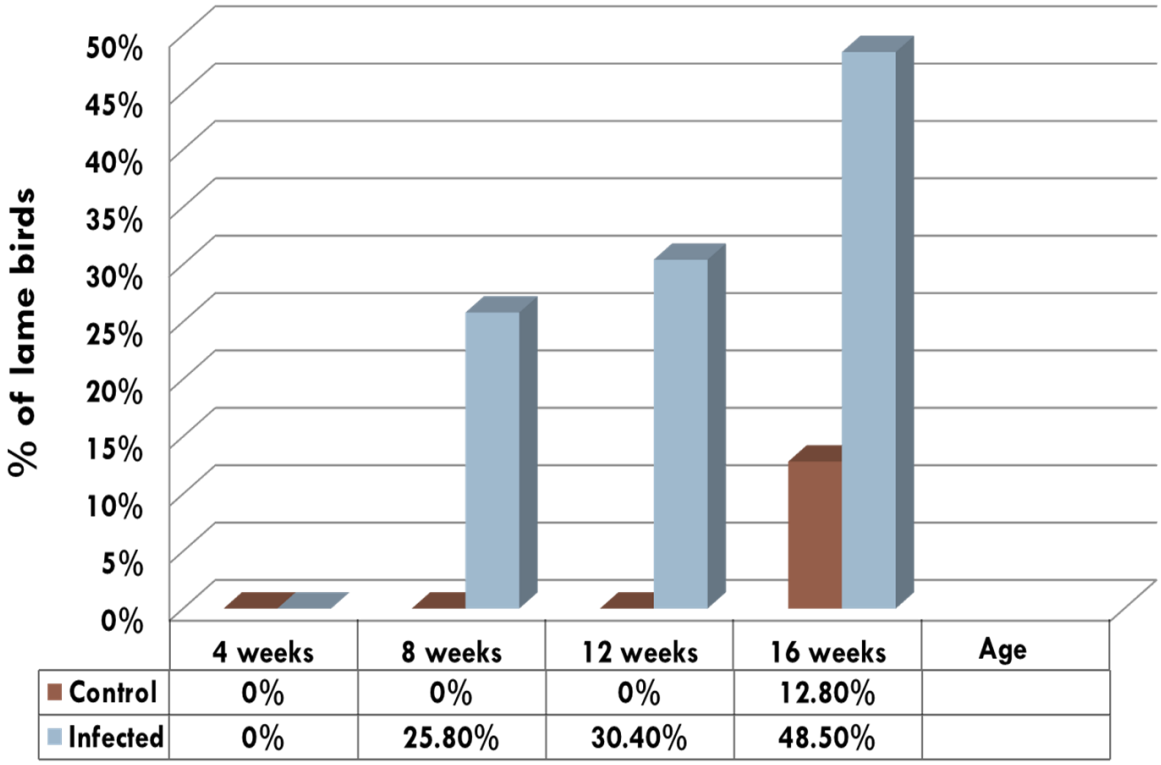
Figure 3.1. Average gait scoring system at different time points. The averages of gait scores of infected birds are significantly higher than non infected controls at 8, 12 and 16 weeks of age

* = Significant difference at P<0.05



	4 weeks	8 weeks	12 weeks	16 weeks
Control	0.11	0.52	0.88	1.6
Infected	0.13	1.59	2	2.7

Figure 3.2. Percentage of lame birds in infected and non-infected control. Lameness is predominant in infected birds at 8, 12 and 16 weeks of age.



	4 weeks	8 weeks	12 weeks	16 weeks	Age
Control	0%	0%	0%	12.80%	
Infected	0%	25.80%	30.40%	48.50%	

Figure 3.3. A) Hemorrhage at the site of ruptured tendon (white arrow) in 16-week-old turkey. Scale bar, 1 cm. B) Shredded tendon fragment (black arrow) surrounded by erythrocytes and exudate. H&E stain (40X magnification).

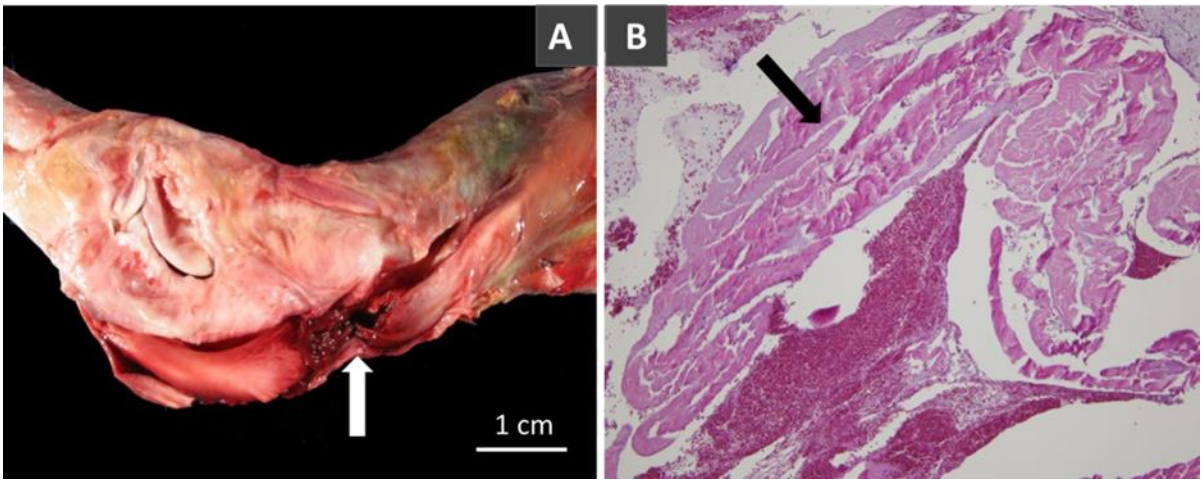


Figure 3.4. Averages of histologic inflammation scores in gastrocnemius tendon sheath. Infected birds had significantly higher averages of histologic inflammation scores at 4, 8, 12 and 16 weeks of age.

* Significant difference at P<0.01

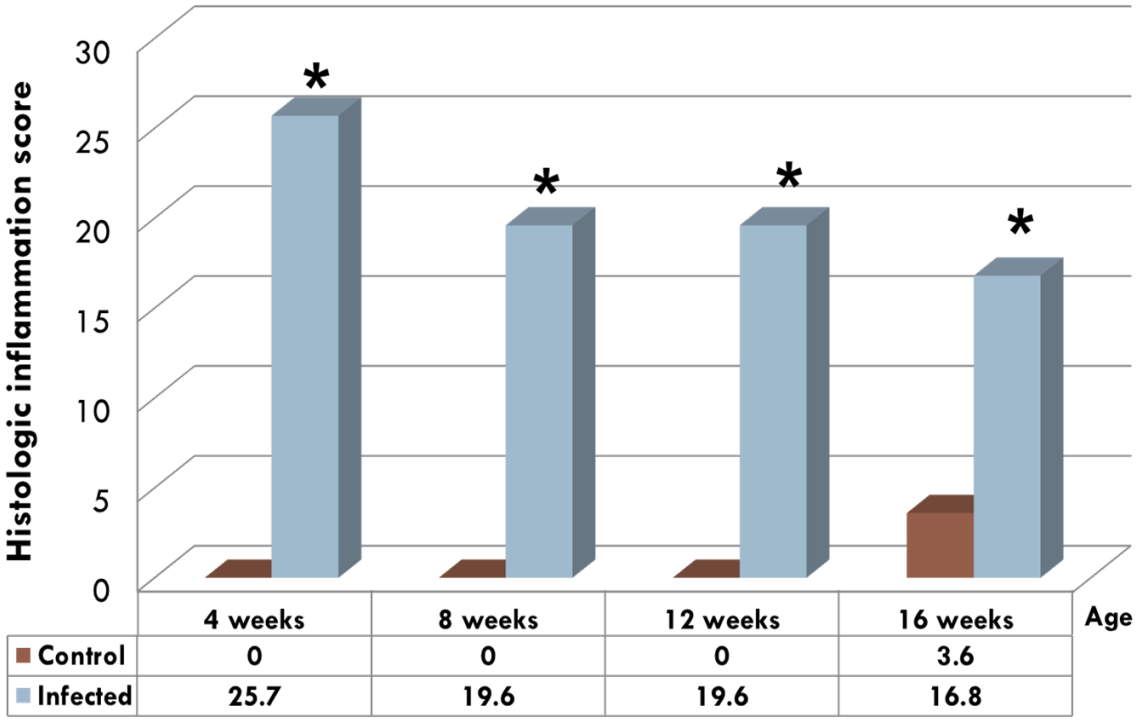


Figure 3.5. Lesion progression in infected birds from 4 to16 weeks of age: A) Prominant lymphocytic infiltration (White up-down arrow) (4-week-old); B) Lymphocytic nodules (Black arrow) present and fibroplasia (White up-down arrow) starts (8-week-old); C) Fibrosis increases (White up-down arrow) and lymphocytic infiltration (Black arrow) decreases (12-week-old); D) Fibrosis (White up-down arrow) is prominent (16-week-old). H&E stain (40X magnification).

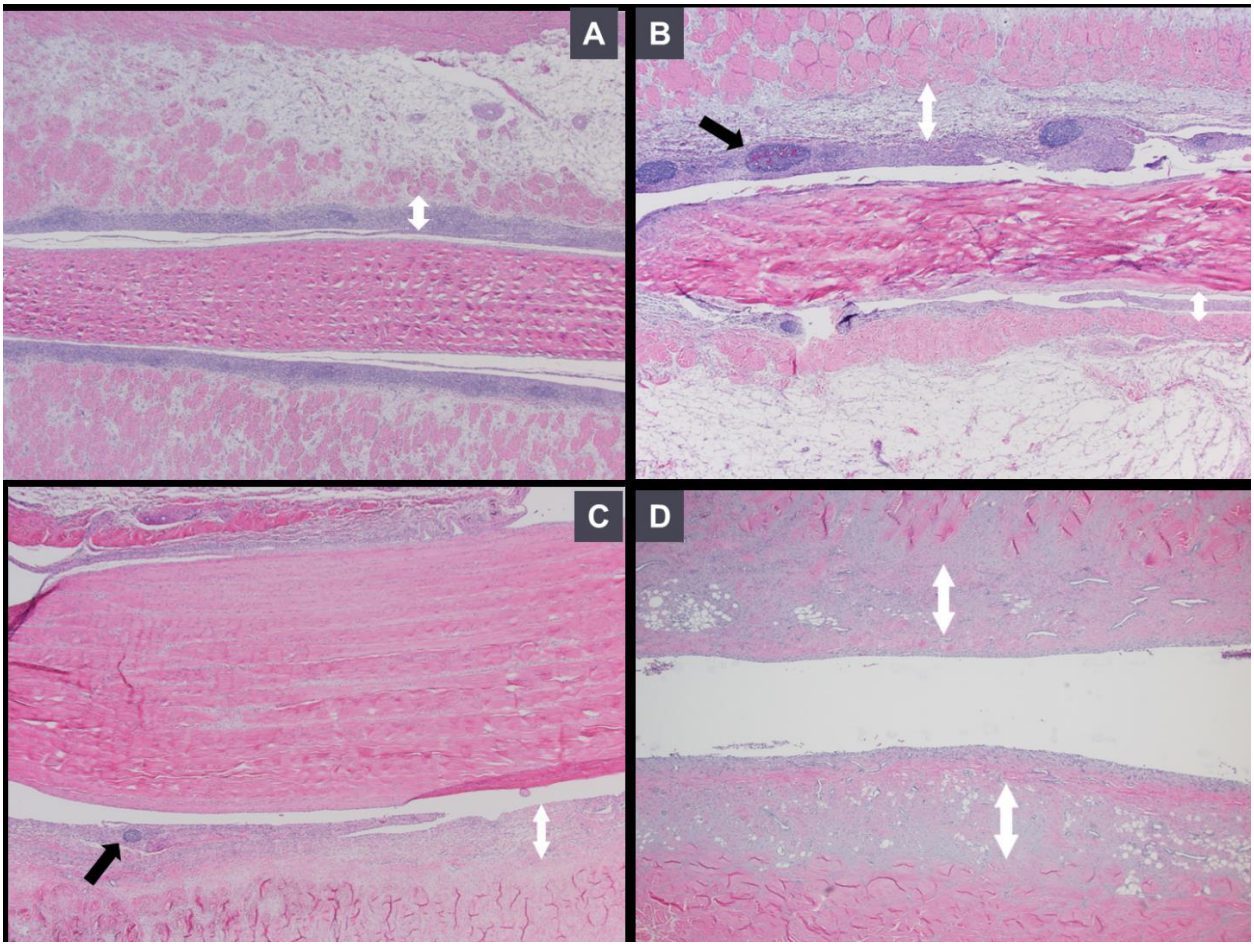


Figure 3.6. Correlation coefficient between gastrocnemius tendon histologic inflammation score and gait score. The correlation is low at 4 weeks of age as there were no bird showed clinical lameness though they had high histologic inflammation scores the correlation gradualy inceases to reach 0.9 at 16 weeks of age.

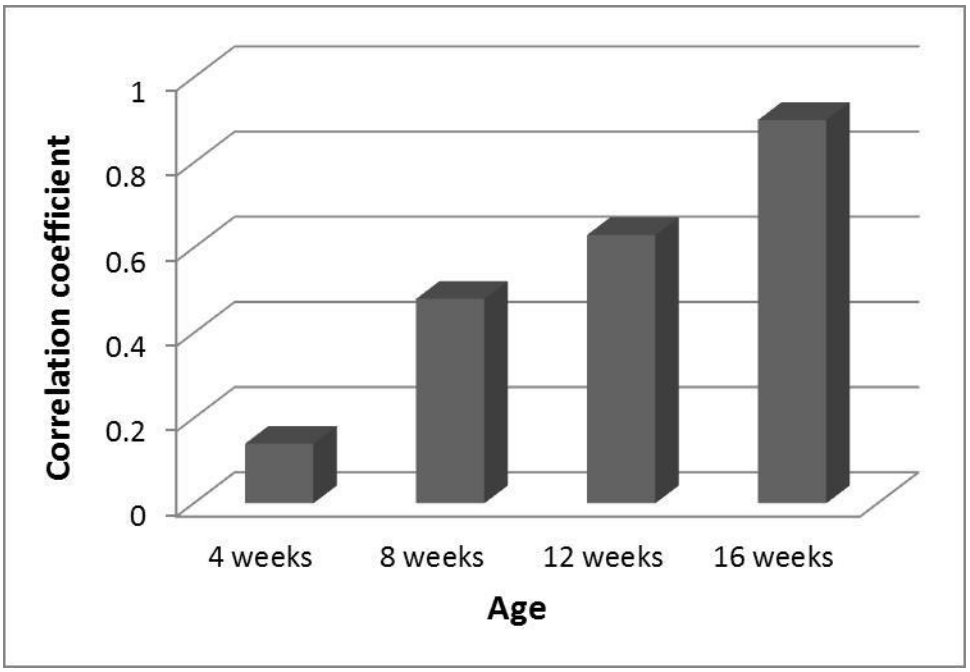


Figure 3.7. Average body weights at different time points. Only at 12 and 16 weeks of age, infected birds had a significantly lower body weight compared with non infected control.

*Significant difference at P<0.05

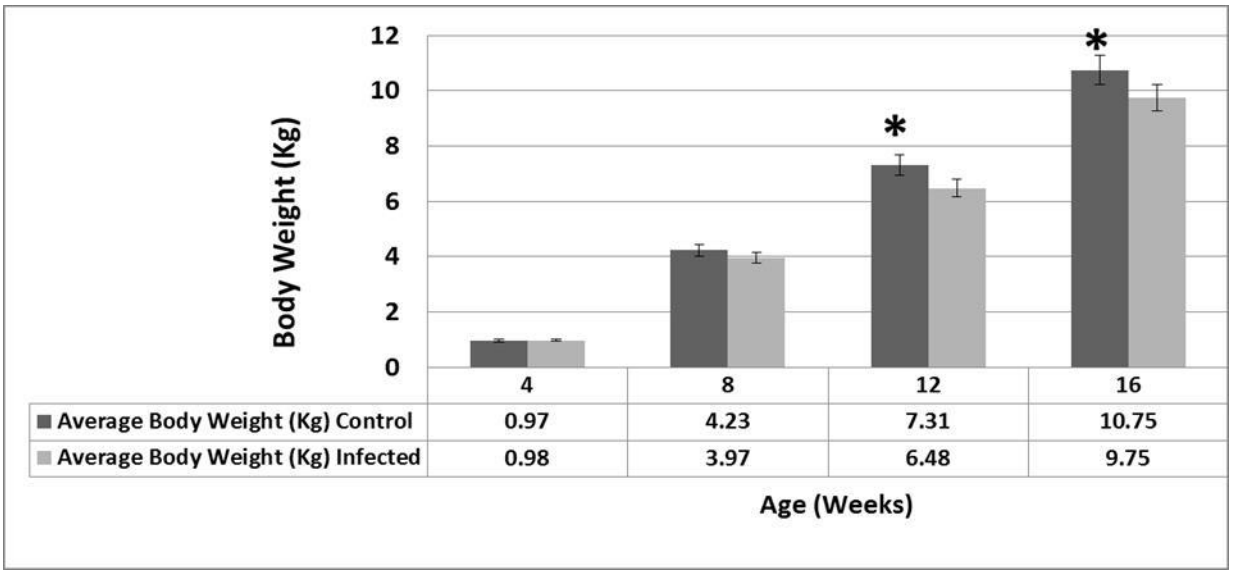


Table 3.1. Newly developed six-point (0-5) gait scoring system for turkeys:

Score	Description
0[N]	▪ Flex and extend legs smoothly and easily ▪ No reluctance in movement ▪ Walk actively with upright breast ▪ Legs are parallel ▪ Stand with no shaking in legs or labored breathing
1[N]	▪ Flex and extend legs smoothly and easily ▪ Little reluctance in movement ▪ Walk actively with upright breast ▪ Legs are parallel ▪ Legs shake during standing ▪ No anatomical changes in legs (swelling, valgus or varus)
2[N]	▪ Flex and extend legs smoothly and easily ▪ Little reluctance in movement ▪ Walk actively with upright breast ▪ Legs are parallel ▪ Legs shake during standing ▪ Mild anatomical changes in legs (swelling, valgus or varus)
3[L]	▪ Staggered movement and dropped keelbone ▪ Unilateral significant hock joint swelling, varus or valgus ▪ Marked bilateral defect, but can walk and stand for more than 30 seconds
4[L]	▪ Staggered movement and dropped keelboneMarked bilateral hock joint swelling, varus or valgus ▪ Cannot walk and stand for more than 30 seconds
5[L]	▪ Completely recumbent ▪ Stand and walk for seconds if it is pushed to walk ▪ Bird then prefers to walk on hocks "creeping" ▪ Bird may not be able to stand and creeps when initiated to move

[N]: non-lame **[L]:** lame

Chapter 4: Biomechanical properties of gastrocnemius tendons of TARV-infected turkeys

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Summary

Turkey arthritis/tenosynovitis reoviruses (TARV) can induce lameness and tenosynovitis in commercial turkeys. In some cases, the tenosynovitis is associated with gastrocnemius tendon rupture, suggesting that the reovirus infection alters tendon biomechanical properties. One-week-old turkey poults were orally inoculated with O'Neil strain of TARV. Groups of birds were removed, euthanized, and necropsied at 4, 8, 12 and 16 weeks of age. Lameness was first observed at 8 weeks of age and then was also present at 12 and 16 weeks. The left intertarsal joint with adjacent gastrocnemius tendon was excised and placed in formalin for histological examination. The right gastrocnemius tendon proximal to the intertarsal joint was dissected from adjacent tissue and remained attached to the tarsometatarsus and foot. The right foot and tarsometatarsus were embedded in polyurethane and the gastrocnemius tendons were trimmed to 2mm width. Tensile strength and modulus of elasticity were analyzed with a MTS 858 Material Testing System, which stressed each tendon to the point of rupture. There were no differences in tensile strength and modulus of elasticity between gastrocnemius tendons of infected and control turkeys at 4, 8 and 12 weeks post infection, but gastrocnemius tendons of TARV-infected 16-week-old turkeys displayed significantly lower ($P<0.05$) tensile strength and modulus of elasticity compared to tendons of non-infected control turkeys. Histology of gastrocnemius tendon and sheath revealed lymphocytic tenosynovitis beginning at 4 weeks of age, continuing through 8 and 12 weeks, and progressing to subsynovial and peritendon fibrosis at 12 to16 weeks of age. We propose that peritendon fibrosis is one of the key features contributing to decreased tensile strength and decreased elasticity of gastrocnemius tendon in TARV-infected turkeys.

Introduction

Turkey reoviruses have often been associated with enteric diseases in turkeys (Lojkic et al., 2010; Mor et al., 2013a; Clavert, 2012; Heggen-Peay et al., 2002; Jindal et al., 2009; Jindal et al., 2010; Woolcock and Shivaprasad, 2007; Nersessian et al., 1985). In the early 1980's reoviruses were isolated from tendons and joints of lame commercial turkeys (Levisohn et al., 1980; Page et al., 1982). However, attempts to reproduce the disease in turkeys were unsuccessful (Al-Afaleq & Jones, 1989). Recently, turkey reoviruses were isolated from gastrocnemius and digital flexor tendons and synovial fluid of lame 15-week-old lame turkeys, some of which had ruptured gastrocnemius or digital flexor as well as consistent histological evidence of lymphocytic tenosynovitis. These turkey arthritis/tenosynovitis reoviruses, defined as TARV, are genetically distinct from chicken reoviruses (Mor et al., 2013 b). Experimental inoculation of 1-week-old turkey poults with TARV via oral, intratracheal and footpad routes produced histological lesions of gastrocnemius lymphocytic tenosynovitis at 4 weeks post inoculation (PI) while turkey enteric reovirus (TERV) and chicken arthritis reovirus (CARV) did not (Sharafeldin et al., 2014). Additionally, a reproducible model of turkey reoviral tenosynovitis and lameness was established by oral inoculation of 1-week-old turkey poults with TARV (O'Neil strain). With this model, the turkeys first displayed clinical signs of lameness at 8 weeks of age, which increased in severity by the ages of 12 and 16 weeks (Sharafeldin et al., 2014b). The aim of the present work was to compare the biomechanical properties of gastrocnemius tendons of TARV- infected and noninfected turkeys at 4, 8, 12 and 16 weeks of age. Additionally, the histological alterations in the gastrocnemius tendons of these turkeys were also described in order to determine if any differences in

biomechanical properties could be associated with morphological changes in the tendons. Establishing a correlation between changes in tendon biomechanical properties with histologic and clinical findings in TARV- infected turkeys is an important objective to explain the unique pathogenicity of TARV compared with turkey enteric reoviruses.

Material and methods

Turkeys: One hundred and eighty, one-day-old commercial male turkey poults were supplied with food and water ad libitum. Feces and serum of 10 birds were tested for reovirus by real-time reverse transcription-polymerase chain reaction (rRT-PCR) (Mor et al., 2014) and for reovirus antibodies, respectively, at the time of their arrival to isolation.

Virus: The TARV-O'Neil strain isolated from gastrocnemius/digital flexor tendons of lame turkeys was used. The virus was grown and titrated on QT-35 cell line and had a titer of $10^{5.5}$ $TCID_{50}$/ml.

Experimental design: The poults were divided into two groups and housed separately in two isolation rooms. One group of poults was orally inoculated at 1 week of age with 0.2ml ($10^{5.5}$ $TCID_{50}$) of TARV-O'Neil. The non-infected control group was inoculated with virus-free MEM. At 4, 8, 12 and 16 weeks of age, all birds were weighed and six birds from each group were euthanized by exposure to carbon dioxide gas followed by immediate collection of tissue samples. Intertarsal (hock) joint (including distal tibiotarsus and proximal tarsometatarsus), of the right leg was excised and fixed in 10% neutral-buffered formalin for histopathology and the remaining of this leg tendons (distal part of gastrocnemius and digital flexor tendons) were excised and stored at -20^0C for rRT-PCR. The left leg was collected, skin removed, wrapped in gauze moistened with

phosphate-buffered saline and kept frozen (-20^{0}C) until used for biomechanical testing. At the end of the experiment, the remaining birds (16 weeks of age) were euthanized after recording their body weights. At each time point, Dacron swabs were inserted into the sheaths of excised gastrocnemius tendons from the ten turkeys and placed as a pool into 3ml of brain-heart infusion broth for PCR targeting Mycoplasma gallisepticum and M. synoviae. Additionally, the gastrocnemius tendon sheaths of 10 birds from each treatment group were swabbed and cultured for aerobic bacteria and Mycoplasma.

Histopathology: The intertarsal joint with gastrocnemius tendon was preserved in 10% neutral-buffered formalin. Tissues were then decalcificied in EDTA solution, trimmed, processed and stained with hematoxylin and eosin (H&E). The stainedglass slides were examined under an Olympus BX40 microscope and photomicrographs were taken with an Olympus DP71 digital microscope camera. Special staining was done by trichrome for detection of collagen which is indicative of fibrosis.

Virus detection: Samples were homogenized in Hanks' balanced salt solution (HBSS) with 2% donor horse serum. The homogenates were then centrifuged and the supernatant RNA was extracted by QIAamp Viral RNA Mini Kit for rRT-PCR (Mor et al., 2014) by QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA).

Biomechanics: A 36mm segment of the tendon halfway between the attachment to the tarsometatarsus and the insertion to the gastrocnemius muscle complex was trimmed to 2 mm wide (Fig. 1), using a custom made dual blade punch, allowing for the focal point of stress in each tendon (midsection) to have uniform length and width. The thickness of each tendon at the trimmed midsection was determined by placing the trimmed segment

of each tendon between the pressure plates of a highly sensitive table top electromagnetic loading device (Endura TEC, ELF 3200 series, EnduraTEC Systems Corporation, Minnetonka, MN).

The gastrocnemius tendon tensile strength and modulus of elasticity were measured with the MTS 858 Electromagnetic Material Testing System (MTS, MN, USA). The foot and tibiosarsus with attached muscle were embedded in polyurethane potting compound (Fastcast, Goldenwest MFG, Inc., Oak Ridge, CA). The potting cylinder was then anchored at the base of the loading frame of the single pole electromagnetic device and the gastrocnemius tendon with portion of gastrocnemius muscle was attached to the upper hydraulic arm with a fixed alligator tooth screw clamp as shown (Fig 2). Force was exerted on each tendon to the point of stretch and complete rupture at the scalloped mid-section. Six tendons were analyzed for each experimental group, and measurements on tendons that did not rupture at the predetermined midsection were omitted in final calculations.

Statistical Analysis: Significant differences ($P<0.05$) between tensile strength and elastici modulus of infected and non-infected control groups at 4, 8, 12 and 16 weeks of age were determined by using the non-parametric statistical analysis "Mann Whitney U test" (NCSS 8 Statistical Software, NCSS LLC, Keysville, UT).

Results

Body weight and tendon rupture: The average body weight of inoculated birds was significantly lower ($p<0.05$) than that of control birds at 12 and 16 weeks (Sharafeldin et

al., under review). Additionally, there was a ruptured gastrocnemius tendons of 1 bird at 16 weeks of age in infected group.

Histopathology: At 4 and 8 weeks of age, prominent lymphocytic infiltration with mild synoviocyte hyperplasia was observed in gastrocnemius tendon sheath while mild fibrosis was observed only at 8 weeks of age. Lymphocytic infiltration decreased at 12 weeks with increased subsynovial fibrosis, which also involved the peritendon, and was predominant in 16-week-old turkeys. Masson trichrome stain, to detect collagen and elastin, revealed a progressive increasein deposition of collagen in the subsynovium and in some instances on the peritendon surface of the gastrocnemius tendon and tendon sheath of 12 and 16-week-old infected turkeys (Fig. 4).

Virus and bacteria detection: Fecal and serum samples taken from 1-day-old poults at the beginning of the trial were negative for reovirus and antireovirus antibodies by rRT-PCR and ELISA, respectively. No aerobic bacteria, *Mycoplasma synoviae* or *M. gallisepticum* was detected in tendon swabs taken from 1, 4, 8, 12 and 16-week-old turkeys by culture and PCR. TARV was detected by rRT-PCR or virus isolation in 4 of 10 tendons at 4 weeks, 5 of 10 tendons at 8 weeks, and 2 of 10 tendons at 12 weeks. No virus was detected in tendons of 16-week-old turkeys. Tendons collected from negative control turkeys at all ages were negative for reovirus by rRT-PCR.

Biomechanics: Biomechanical measurement of 5 gastrocnemius tendons were omitted from the study because the tendons did not rupture at the predetermined midsection while measurement of 32 tendons were deemed accepted. In all instances, the aberrant ruptures occurred at the end or clamped region of the tendon. The mean tensile strength and modulus of elasticity of gastrocnemius tendons of infected and non-infected groups had

no significant differences at 4, 8 and 12 weeks of age; however, by 16 weeks of age the gastrocnemius tendons of infected turkeys had a significantly lower ($P<0.05$) mean tensile strength and modulus of elasticity compared to tendons of non-infected turkeys (Table1).

Discussion

The aim of the present work was to observe the effect of TARV infection on gastrocnemius tendon tensile strength and modulus elasticity in turkeys. Clinical cases of tendon rupture associated with reovirus infection are generally observed in male turkeys older than 12 weeks (David Mills, personal communication). In our original challenge study with TARV, inoculation of 1-week-old turkey poults induced tenosynovitis, but lameness was not observed by 4 weeks PI (Sharafeldin et al., 2014). This observation directed us to study the long term pathogenicity of turkey reovirus. TARV (O'Neil strain) inoculation in 1-week-old turkey poults via the oral route is a reproducible model to study the pathogenicity of TARV (Sharafeldin et al., 2014). This might serve as a useful model for human tenosynovitis/ arthritis and rheumatoid arthritis. Avian reovirus infection in chickens was suggested to be a good model for human rheumatoid arthritis (Sahu and Olson, 1975; Walker et al., 1972). Antinuclear antibodies or anti-collagen antibodies have been detected in the serum of avian reovirus-infected chickens, indicating that avian reovirus might be associated with an autoimmune response in the host (Islam et al., 1990; Pradhan et al., 1987).

Subsynovial fibrosis first developed in 8-week-old infected turkeys and became the predominant change by 16 weeks of age. Mean tendon tensile strength and modulus of

elasticity (resistance to stretch) was significantly lower ($p<0.05$) in 16-week-old infected turkeys compared to non-infected turkeys. Mean tendon tensile strength in control turkeys was significantly lower at 8-week-old compared with controls at other time points (Table 1). We do not have explanation for that significant decrease at 8 weeks although these values were not significantly different from those of infected 8-week-old turkeys. Perhaps the reduced tensile strength of tendon in 8-week-old control turkeys is spuriously low because only 4 tendons from that group were analyzed as a result of aberrant rupture in two tendons, which were omitted from the study. The means of tendon tensile strength were the same in infected turkeys at all-time points ($P<0.05$). Mean tendon elasticity in control turkeys were the same at 4, 8 and 12 weeks while elasticity was significantly increased in non-infected 16-week-old turkeys ($P<0.05$) (Table1).

Fiberous (collagen deposition) of gastrocnemius tendon may be responsible for decreasing the tensile strength and modulus of elasticity in infected turkeys. Previous studies on collagen composition and arrangement in turkey tendon demonstrated progressive changes in procollagen content and organization as the turkey grows (Knott et al., 1997). In our study, induction of substantial inflammation and edema in the gastrocnemius subsynovium and tendon, as well as reovirus replication in these tissues, might have altered physiological collagen deposition, ultimately altering tendon tensile strength and elasticity.

A study on gastrocnemius tendon biomechanics in reovirus-infected chickens showed that tendons of chickens infected at one day of age had significantly lower mean tensile strength compared with non-infected chickens at 6, 10 and 18 weeks ($P\leq 0.05$). This work also reported a gradual increase of the mean tendon tensile strength of control birds

which was significantly higher only at 18 weeks compared to other time points. Additionally, the mean modulus of elasticity in controls was significantly lower than that of infected birds only at 18 weeks (Mohamed et al., 1995).

Changes in the biomechanical properties of tendon in the turkeys in our study may be a result of decreased movement of infected birds. A previous work showed that immobilized chickens have 10-30% decreased tendon strength and 70% decreased tendon elasticity (Foutz et al., 2007). These findings were attributed to the decreased organization and diameter of collagen fibers.

Finally, the progressive deposition of fibrous connective tissue on and around the tendon of reovirus-infected turkey as they age combined with the progressive increase in body weight are likely predisposing factors in inducing lameness and in some instances tendon rupture. In chickens, it was proposed that fibrosis and adhesions with increased age in heavy weight birds are responsible for gastrocnemius tendon and digital flexor tendon rupture (Jones, 2000).

In conclusion, TARV-infected turkeys developed gastrocnemius lymphocytic tenosynovitis by 4 weeks of age, progressing to fibrosis by 16 weeks of age (Sharafeldin et al., 2014b). Clinical lameness was observed at 8 weeks and was observed in 50% of infected birds at 16 weeks. This lameness was accompanied with tendon rupture in a small percentage of 16-week-old turkeys. The progressive lameness and occasional tendon rupture represent altered tendon function (reduced tensile strength and reduced modulus of elasticity) during the course of infection in a turkey with rapid growth rate.

Figure 4.1. The midsection of the gastrocnemius tendon was cut as shown to create a predetermined region for measuring tendon failure.

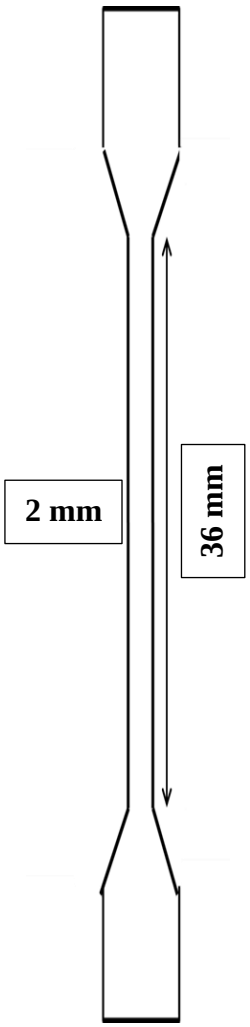


Figure 4.2. The leg of a 4-week old turkey is fixed in the material testing system apparatus. Metatarsus and foot are embedded and fixed at the base of the loading frame and gastrocnemius muscle with portion of tendon is clamped to the upper moveable hydraulic arm. The predetermined point of tendon failure is in the midregion of the exposed tendon (Arrow).

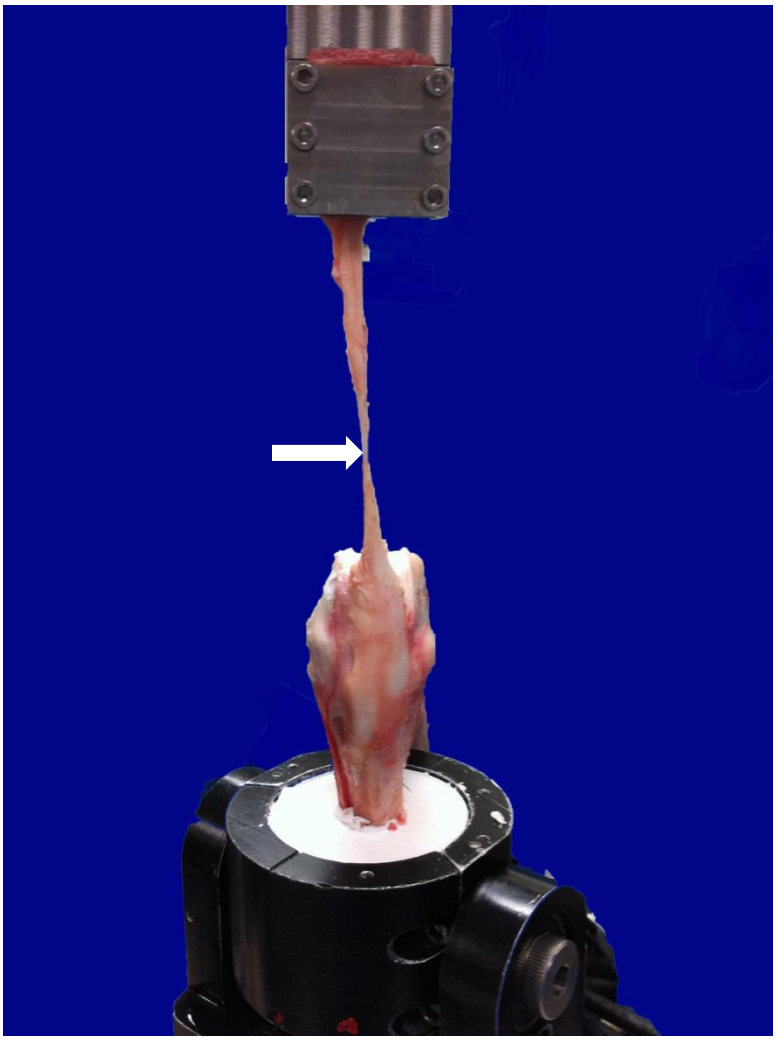


Figure 4.3. **A)** Stress/strain curve in 16-week-old turkeys. **B)** Stress/strain curves of of infected versus non-infected control turkeys (16 weeks of age). **A)** The shape of the normal curve and elevation reflected the ability of tendon to tolerate stress (tendile strength) until certain point at which tendon cannot tolerate stress (point of failure) then the curve decline after rupture. **B)** The curve of median 16-week-old infected bird (green) is shallower than the curve of the median of 16-week-old non infected control (blue). This reflects significantly lower tensile strength and elasticity at $P<0.05$.

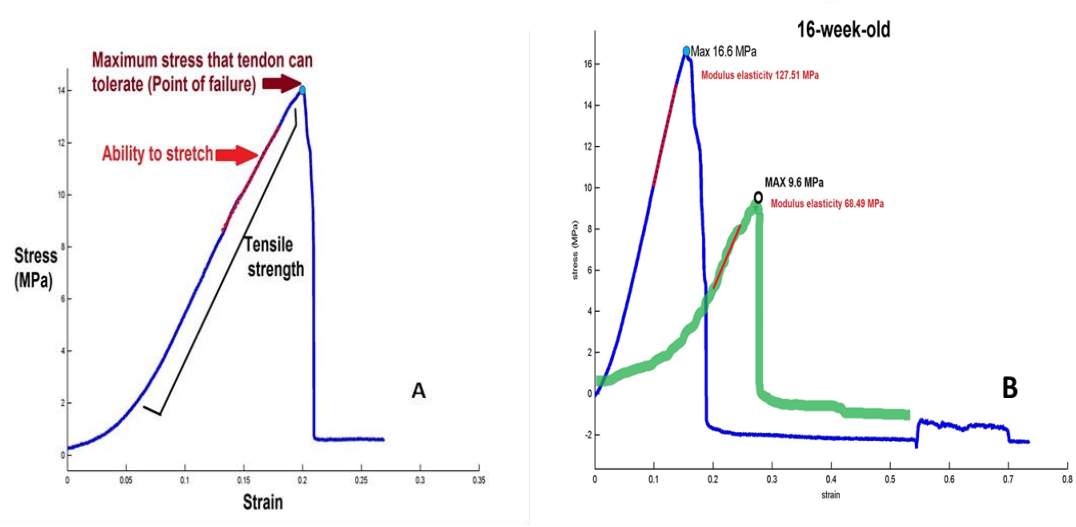
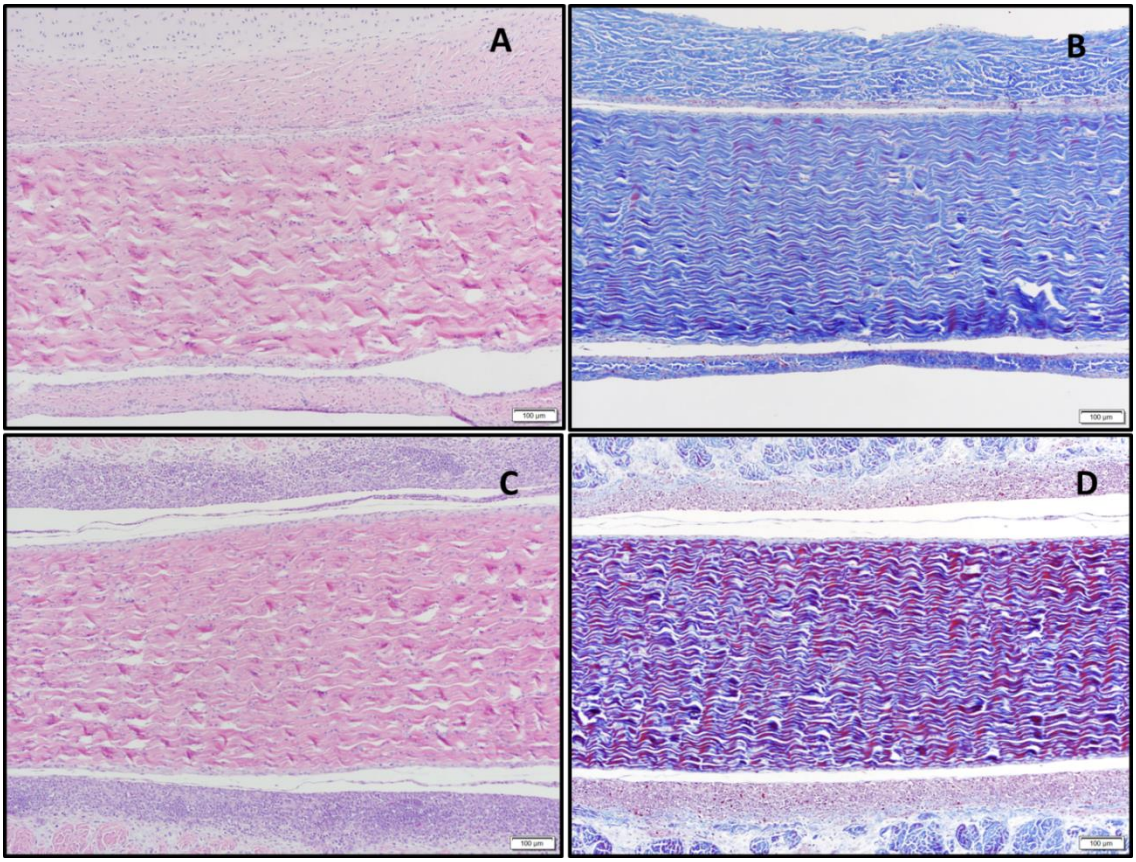
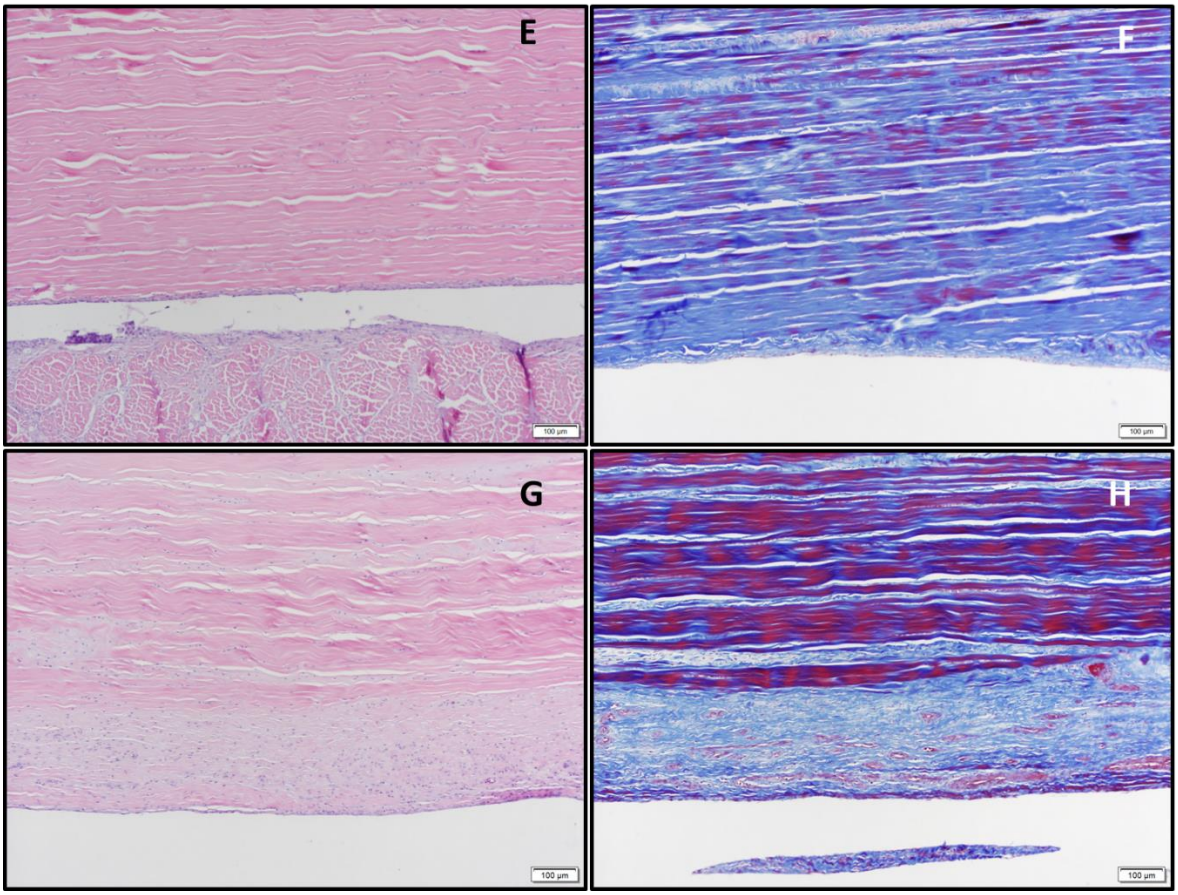


Figure 4.4. Development of histologic lesions from 4 to 16 week-old with special Mason Trichrome stainig.. A) 4 week control H&E; B) 4 weeks control MT; C) 4 weeks infected H&E; D) 4 weeks infected MT; E) 16 weeks control H&E; F) 16 weeks control MT; G) 16 weeks infected H&E; H) 16 weeks infected MT. Inflammatory cells (lymphocytes) in subsynovium in C and D. Collagen (indicative for fibroplasia) deposition in the tendon (blue fibrils) in G and H.




E
F
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100 µm
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100 µm

Table 4.1: Tendon tensile strength and elasticity of gastrocnemius tendon at different time points after infection.

Age (weeks)	Group	Number of tendons	Tensile strength (MPa) Mean +S.D.	Modulus elasticity (MPa) Mean ± S.D.
4	Control	6	15.4 ± 4.86	75.86 + 29.37
	Infected	6	12.6 ± 11.01	67.10 + 41.51
8	Control	4[+]	9.03 [a] ± 2.12	41.45 + 16.54
	Infected	6	12.78 ± 6.70	51.86 + 22.60
12	Control	5	11.76 ± 4.55	57.34 + 2.12
	Infected	6	9.03 ± 5.56	50.76 + 23.08
16	Control	5	19.36 ± 6.59	150.40 + 59.34
	Infected	5	9.84* ± 3.75	74.32* + 22.15

* Significantly different from control mean at the same age at $P<0.05$ Mann Whitney U test.

MPa (megapascal)= 145.038 pounds/square inch

[a] Significantly different from the means of controls of other ages at $P<0.05$ Mann Whitney U test.

[+] n = 6 per group. Tendon measurements were omitted if the tendon did not rupture at the predetermined midsection.

S.D. = standard deviation

Chapter 5: Immunopathogenesis of a newly emergent turkey arthritis reovirus in turkeys

Tamer A. Sharafeldin, Sunil K. Mor, Nader M. Sobhy, Zheng Xing, Kent Reed, Sagar M. Goyal and Robert E. Porter

Summary

Newly emergent turkey arthritis reoviruses (TARV) were isolated from tendons of lame 15-week-old tom turkeys that displayed ruptured leg tendons. Experimentally, TARVs induced remarkable tenosynovitis in gastrocnemius tendon. The current study characterized the location and extent of virus replication as well as the cytokine response induced by TARV during the first two weeks of infection. One-week-old male turkeys were inoculated orally with TARV (O'Neil strain). Virus gene copy numbers were determined in intestines, internal organs and tendons at 12 hours and 1, 2, 3, 4, 7 and 14 days PI (dpi). Cytokine profiles were measured in intestines, spleen and leg tendons at 0, 4, 7 and 14 dpi. The peaks of the gene copy numbers were reached in jejunum, cecum and bursa of Fabricius at 4 dpi. Virus gene copy numbers increased dramatically in leg tendons at 7 and 14 dpi while minimal average virus copy numbers were detected in internal organs and blood during the same period. Virus was detected in cloacal swabs at 1-2 dpi and peaked at 14 dpi. A remarkable elevation of IFN-α and IFN-β was observed in intestines at 7 dpi as well as a prominent T helper-1 response (Th1) (IFN-γ) at 7 and 14 dpi. IFN-γ and IL-6 were elevated in gastrocnemius tendons at 14 dpi. This shows the enterotropism of the virus and the early shedding in feces. Additionally, the virus induced an elevation of antiviral cytokines that remarkably limited the viral replication in intestines at 7 dpi. The virus replication mediated a dominant Th1 response in intestines and leg tendons which execluded the possibility of immunoglobulin or autoimmune mediated reactions that would be mediated by Th 2 and Th17 responces.

Introduction

Turkey reoviruses have often been associated with a variety of turkey enteric diseases (Lojkic et al., 2010; Mor et al., 2013a; Clavert, 2012; Heggn-Peay et al., 2002; Jindal et al., 2009; Jindal et al., 2010; Woolcock and Shivaprasad, 2007; Nersessian et al., 1985). Turkey enteric reoviruses (TERV) were initially found to replicate in intestines and bursa of Fabricius of turkeys by 2-5 days post inoculation (dpi) via oral route inoculation (Pantin-Jackwood et al., 2007). TERV was shown to induce bursal atrophy and lymphocytic depletion in spleen and bursa of Fabricius of experimentally infected 3-day-old specific pathogen free and commercial turkey poults but not in 3-week-old poults (Day et al., 2008; Spackman et al., 2005).

Reoviruses were first isolated from ruptured tendons of turkeys with tenosynovitis/arthritis in 1980 (Levisohn et al., 1980; Page et al., 1982) suggesting a possible link between reovirus infection and lameness. However, early attempts to fulfill Koch's postulates were not successful because reovirus strains isolated from turkeys with tenosynovitis/arthritis did not induce tenosynovitis/arthritis when inoculated into footpads of 1-day-old poults (Al-Afaleq & Jones, 1989). Recently, we isolated turkey reovirus from gastrocnemius and digital flexor tendons of 15 to 18-week-old lame turkeys in the Midwestern USA and showed them to be genetically distinct from chicken reoviruses (Mor et al., 2013b). These newly identified viruses, which are tentatively called turkey arthritis reoviruses (TARV), showed a unique ability, unlike TERV and chicken arthritis reovirus (CARV), to induce tenosynovitis by 4 weeks post challenge in 1-week-old turkey poults inoculated via oral, intratracheal and footpad routes. Additionally, Koch's

postulates for TARVs were fulfilled (Sharafeldin et al., 2014a). Clinical lameness of increasing severity was observed at 7, 11 and 15 weeks PI in turkey poults that were orally inoculated at 1-week of age (Sharafeldin et al., 2014b).

The aim of the present work was to characterize the pathogenesis and the resulting cytokine profile of TARV infection in turkey poults to understand reovirus- host interaction and the role of immune response in viral pathogenesis

Materials and methods

Poults: Eighty, one-day-old male turkey poults were purshased from a commercial turkey hatchery. Five birds were bled upon their arrival and serum was tested for reovirus antibodies by using avian reovirus (REO) ELISA kits (IDEXX, Westbrook, ME). In addition, fecal samples were collected from ten 1-day-old poults and tested for reovirus by real-time reverse transcription-polymerase chain reaction (rRT-PCR) (Mor et al., 2014).

Virus: The TARV-O'Neil strain of TARV isolated from gastrocnemius/digital flexor tendons of lame turkeys was kindly supplied by Dr. Jack Rosenberger, AviServe LLC, Newark, Delware was used. The virus was grown and titrated on QT-35 cells and showed a titer of $10^{5.5}$ $TCID_{50}$/ml.

Experimental design: Poults were divided into two groups of 40 each and were placed in air filtered isolators and were supplied with food and water *ad-libitum*. One group was inoculated at 7 days of age orally with 0.2 ml TARV-O'Neil and the second group was inoculated with 0.2 ml of virus-free MEM. Five birds from each group were euthanized at

0, 1/2, 1, 2, 3, 4, 7 and 14 days post inoculation (dpi) and samples were collected as given below.

Histopathology: Samples from intestines, bursa of Fabricius, heart, liver, spleen, kidney, and intertarsal joint with gastrocnemius tendon were collected and fixed in 10% neutral-buffered formalin. Tissues were then trimmed, processed, embedded in paraffin, sectioned at 5 microns, and stained with hematoxylin and eosin (H&E) for histologic examination.

Viral gene copy number: Duodenum, jejunum, cecum, bursa of Fabricius, cloacal swab, heart, liver, spleen, kidney, and gastrocnemius and digital flexor tendons were collected and 100 mg of each sample were homogenized in Hanks' balanced salt solution (HBSS) containing 2% donor horse serum. The homogenate was then centrifuged at 1500 xg for 20 min and supernatant was subjected to RNA extraction using QIAamp Viral RNA mini kit (Qiagen, Valencia, CA). RNA from 200µl sample of whole blood (with anticoagulant) was extracted using TRIZOL RNA extraction protocol (Life Technologies, Carlsbad, CA). Copy number of the S4 gene was then determined by a previously developed quantitative real-time RT-PCR method specific for turkey reovirus S4 gene (Mor et al., 2014) using OneStep RT-PCR kit (Qiagen, Valencia, CA).

Cytokine profile: Samples of intestines, spleen, and tendons were tested for the presence of mRNA of eleven cytokines at 0, 4, 7 and 14 dpi. Selected cytokines included proinflammatory cytokines interleukin 6 (IL-6), tumor necrosis factor- σ (TNF-α); antiviral cytokines interferon-α (IFN-α), IFN-β; IL-2; T helper1 (Th1) (IFN-γ, IL-12); T helper 2 (Th2) (IL-4, IL-5); T helper 17 (Th17) IL-17; and housekeeping calibrator gene

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Segments of duodenum, jejunum, cecum, spleen, and leg tendons (gastrocnemius and digital flexor) were collected, immersed in RNA Later and kept frozen at -20 C until use. Tissue (100mg) was homogenized with RLT lysis buffer in tubes with ceramic beads and was left to settle for 10-15 minutes. The supernatant was subjected to total RNA extraction using RNeasy mini kit (Qiagen, Valencia, CA). RNA was subjected to reverse transcription using Primscript RT master mix (TAKARA BIO INC., Otsu, Shiga, Japan). Resulting DNA product was analyzed by PCR using SYBR® Premix Ex Taq™ II (TAKARA BIO INC.). For reactions, the turkey cytokine mRNA sequences were indetified in the NCBI GenBank and primers were designed to amplify part of these sequences (amplicons) (Table 1). The PCR reaction included the following stages; holding stage (50°C for 60 sec and 95°C for 30 sec), PCR cycling stage (95°C for 5 sec and 60°C for 30 sec) up to 40 cycles and melting curve stage (95°C for 15 sec, 60°C for 60 sec and 95°C for 15 sec). Expression was determined using $2^{-\Delta\Delta C}{}_T$ method. The $\Delta\Delta C_T = (\Delta C_T$ target cytokine gene - ΔC_T Calibrator (GAPDH)) $_{\text{Time}}$ x - (ΔC_T target cytokine gene - ΔC_T Calibrator (GAPDH)) $_{\text{Time 0}}$ (Livak and Schmittgen, 2001).

Statistical analysis: The detection of significant differences in the average fold change between infected group and non-infected groups was done using the non-parametric statistical analysis “Mann Whitney U test” (NCSS 8 Statistical Software, NCSS LLC, Keysville, UT) with all time points compared with values measured at time zero.

Results

Histopathology: No significant lesions were observed in sections of internal organs, intestines or intertarsal joint and tendons until 14 dpi, when gastrocnemius tendons showed tenosynovitis characterized by mild to moderate, diffuse subsynovial infiltration of lymphocytes (Figure 1).

Virus gene copy number (Figure 2): Detectable S4 gene copy numbers were shown at 1-2 dpi in different intestinal segments, bursa of Fabricius, and cloacal swab samples. The average gene copy number was 10 copies/100mg in jejunum, bursa of Fabricius, and cloacal swabs and 200 copies/100mg in duodenum and cecum. Average gene copy number was the highest in jejunum, cecum and bursa of Fabricius (3000-6000 copies/100mg) at 4 dpi while in duodenum and cloacal swab, the average gene copy numbers were 100 copies/100mg. At 7 dpi, a remarkable decline in the average gene copy number was observed in all intestinal segments followed by slight elevation at 14 dpi, especially in duodenum and cloacal swabs (300-500 copies/100mg).

In liver, kidney, spleen and heart, average gene copy number was <100 copies/100mg at all-time points. The average gene copy number approached 100 copies/100mg at 3 dpi in spleen and liver and at 7 dpi in liver. Tendons had low average copy number early but showed a dramatic increase at 7 (nearly 200 copies/100mg) and 14 dpi (nearly 500 copies/100mg). In blood, the average copy number remained under 100 copies/200 µl at all-time points and peaked at 4 dpi.

Cytokine profiling: **Proinflammatory and anti-inflammatory cytokines:** Fold changes of IL-6 CT values compared with zero time were significantly higher in infected group

than non-infected control in duodenum and jejunum at 4 and 7 dpi. IL-6 was significantly elevated in tendons of infected birds compared to controls at 14 dpi. Average fold changes of TNF-α Ct values were significantly higher in infected group compared with non-infected control only in jejunum at 7 dpi. The IL-10 transcript showed significant higher fold change in infected groups compared with non-infected control in duodenum and jejunum at 4 dpi and in jejunum and spleen at 7 dpi.

Antiviral cytokines: IFN-α and IFN-β showed significant higher fold change in infected groups compared with non-infected control in only jejunum and cecum at 7 dpi.

T helper 1, 2 and 17: IL-12 showed significant higher fold change in infected group compared with non-infected control in jejunum and cecum at 7 dpi while IFN-γ had a significant elevation compared with non-infected control in jejunum at 7 and 14 dpi and in tendons at 14 dpi. Th2 (IL-4 and IL-5) and Th17 (IL-17) cytokines did not show statistically significant differences in fold change with zero time control between infected and non-infected control groups. IL-2 in duodenum and jejunum of infected control had a significantly higher fold changes than non-infected control at 4 and 7 dpi.

Discussion

The present work aimed to study the tissue distribution and tropism of a newly emergent turkey reovirus associated with tenosynovitis/arthritis. Understanding the tropism of the newly emergent virus will help to characterize the pathogenesis of this virus in tendons. Furthermore, following the cytokine profile induced by the infection will enhance our knowledge about the mechanism of viral pathogenicity.

The rRT-PCR quantitative technique used in this report could detect as low as 10 gene copies of the virus (Mor et al., 2014), which demonstrated intestines and bursa of Fabricius as the main sites of viral replication based on the S4 gene copy numbers detected, peaking at 4 dpi. These findings agree with previous studies, which showed that intestines and bursa of Fabricius were the initial sites of replication of several turkey enteric reoviruses in turkeys within 2-5 dpi (Pantin-Jackwood et al., 2007) and chicken reovirus in chickens within 2-4 dpi (Jones et al., 1989). The virus gene copy number was low in internal organs and blood compared with intestines and bursa of Fabricius while gene copies increased dramatically in gastrocnemius tendon at 7 dpi, peaking at 14 dpi. Although the viral RNA was detected in blood at 2 dpi and peaked at 4 dpi, this low level of viremia was not associated with general systemic clinical illness at these time points. Chicken reoviruses have been shown to initially replicate in intestines, reach the blood at 2-3 dpi, and subsequently the internal organs within 3-5 dpi (Kibenge et al., 1985). A chicken reovirus targeted liver of experimentally infected chickens causing hepatitis and mortality within 10 dpi (Jones and Guneratne, 1984). The weak viremia in our study explains why there was no early systemic disease induced by TARV-O'Neil and why the virus did not reach internal organs in numbers as high as in intestines and bursa of Fabricius.

No studies have been previously conducted to determine reovirus pathogenesis in turkey tendons. Chicken reoviruses inoculated into chickens targeted the hock joint, which was reported to be an important site for virus replication (Jones and Kibenge, 1984; Sahu and Olson, 1975; Walker et al., 1972). In this report, we showed that TARV replicated and reached the peak of gene copy numbers at 14 dpi, and only at that time lymphocytic

tenosynovitis was observed in the gastrocnemius tendon sheath of infected birds. There were no lesions in tendons at earlier time points when the virus gene copy number was low. These data indicate that inflammation of the tendon sheath was associated with the presence of a detectable virus titer.

The gene copy numbers in the cloacal swab are indicative of the early shedding of the virus starting at 1-2 dpi and peaked at 14 dpi. This may explain how young birds could be rapidly infected in the field. In one study, the shedding of chicken reovirus was reported at two weeks PI via oral route (Kibenge and Dhillon, 1987) and in another study chicken reovirus shedding peaked at 1-2 weeks PI, but decreased after 3 weeks PI (Islam et al., 1988; Al-Afaleq and Jones, 1994). TARV-O'Neil in this study had a faster shedding in turkeys than other studied chicken reoviruses in chickens. This impression of faster shedding is due to early detection by the highly sensitive technique (rRT-PCR) (Mor et al., 2014) that was not available at the time of the mentioned chicken literatures.

The virus gene copy numbers peaked at 4 dpi in intestines and bursa of Fabricius and remarkably declined at 7 dpi; this can be correlated with the antiviral effect of IFN-α and IFN-β, which were significantly elevated at 7 dpi in jejunum and cecum of infected birds. This shows that interferons played an important antiviral role in limiting replication of TARV in intestines.

We analyzed cytokine profile in multiple tissues of turkeys infected with the reovirus in order to understand the immunopathogenicity. Significant elevation of IL-2 in intestines of infected group at 4 and 7 dpi confirmed the proliferation of lymphocytes in response to viral infection. This proliferation unlikely caused infiltration of lymphocytes in the

intestinal lamina propria but it was within the gut associated lymphoid tissues (GALT) like Peyer's patches, cecal tonsils, bursa of Fabricius or other intestinal lymphoid aggregates. Pro-inflammatory cytokines IL-6 and TNF- α were significantly elevated in duodenum and jejunum of infected birds at 4 and 7 dpi and in jejunum at 7 dpi, respectively. Though this was a statisticaly significant elevation, it seemed not to be effective because there were no leukocytes infiltration, dilated blood vessels or exudation (that are usually associated with elevated proinflammatory cytokines) in intestines at those time points. The same is true of IL-10 which showed statistically significant elevation in intestines of infected birds at 4 and 7 dpi but it was not effective enough for down regulation of Th1 cytokines in infected birds.

Th1 cytokines IFN-γ and IL-12 were significantly higher in intestines of infected birds compared with non-infected control while Th-2 (IL-4 and IL-5) and Th-17 (IL-17) did not show any significant elevation in infected birds. This dominant Th1 response in intestines at 7 and 14 dpi over Th2 response excluded the possibility of immunoglobulins playing a role in immune response during early course of infection and the possibility of an autoimmune reaction that would be mediated by IL-17 (Komatsu and Takayanagi, 2014). The elevated IL-10 might be indicative of the activity of regulatory T (T reg) over the minimal Th17 response and this was confirmed by the absence of destructive bone lesions in the previous long term pathogenicity trials in turkeys (Sharafeldin et al., 2014b). Destructive bone lesions would be mediated by Th17 (Komatsu and Takayanagi, 2015).

Comparing the cytokine response with viral replication and histologic alteration in leg tendons helps determine the events occuring in leg tendons during the course of viral infection. IL-6 and IFN-γ showed a significantly higher fold increase than that of non-infected controls only at 14 dpi. This increase corresponds to subsynovial lymphocytic infiltration in gastrocnemius tendon sheath, which was first observed at 14 dpi. It is likely that this lymphocytic infiltration was associated with viral replication that reached the peak at 14 dpi. Reovirus replication was shown to induce certain cytokine proliferation when chicken reoviruses of high multiplication rate induced significantly higher production of IL-6, IL-10 and INF-γ compared to reoviruses of low multiplication rate (Shen et al., 2014). In our study, virus replication was accompanied by inflammation in leg tendons but not in intestines although virus replication was higher in intestines. Virus replication might be associated with inflammatory cells that comprise the GALT and were seen infiltrating the tendon sheath. Additionally, little is known about the cellular release of avian reoviruses and whether or not this phnomenon is associated with cell lysis or not. Mammalian reoviruses may release from infected cells without inducing cell death (Lai et al., 2013) or may induce apoptosis and cell death (Forest and Dermody, 2003) prior to release. We have not observed any syncytia formation by histologic examination though avian reovirus is characterized by formation of cell-cell fusion (syncytia formation), which is mostly mediated by P10 protein (Liu et al., 2008).

Future studies using electron microscopy, specific immunohistochemistry and transcriptome analysis will be very helpful in determining the virus replication cycle details including adhesion, assembly and release as well as the cells that the virus replicate in and use for local and systemic spread.

In conclusion, the turkey arthritis reovirus when administered orally is initially enterotropic followed by viremia and replication in tendons. The enterotropic virus is shed in feces early during the course of infection. Additionally, the virus evokes an effective antiviral cytokine response that limits viral replication and a dominant Th1 response. Immunoglobulin as an autoimmune/immune complex reaction, which would be mediated by Th2 and Th17 respectively, appears to play a little or no role early in infection. Neither Th2 nor Th17 is elevated in infected birds during the first 2 weeks of infection.

Figure 5.1: Histologic inflammation in gastrocnemius tendon at 14 dpi

A) Infected 14 dpi. The magnified square shows the subsynovial lymphocytic infiltration. B) Non infected control at 14 dpi.

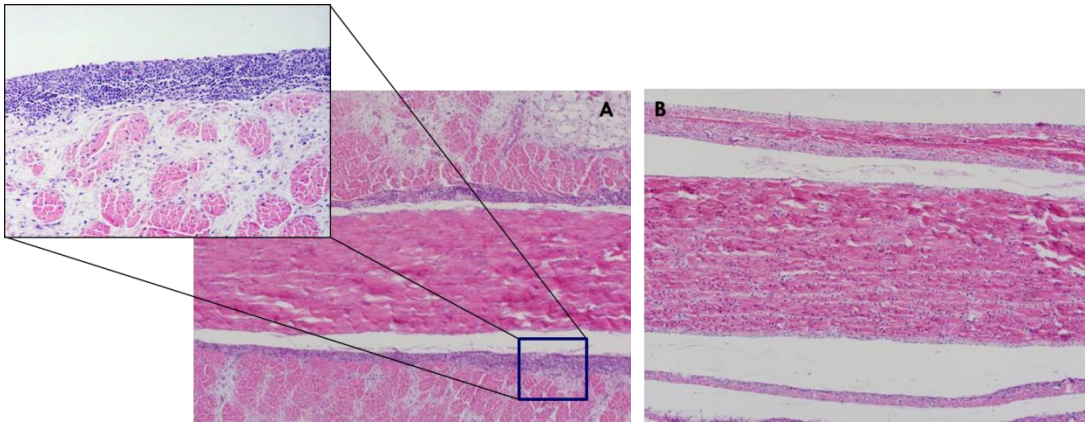
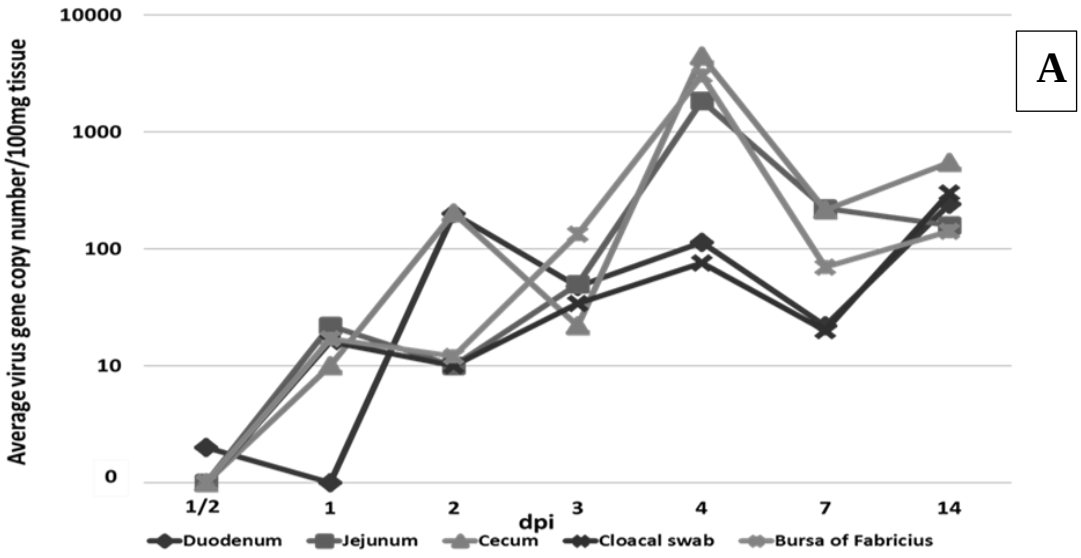
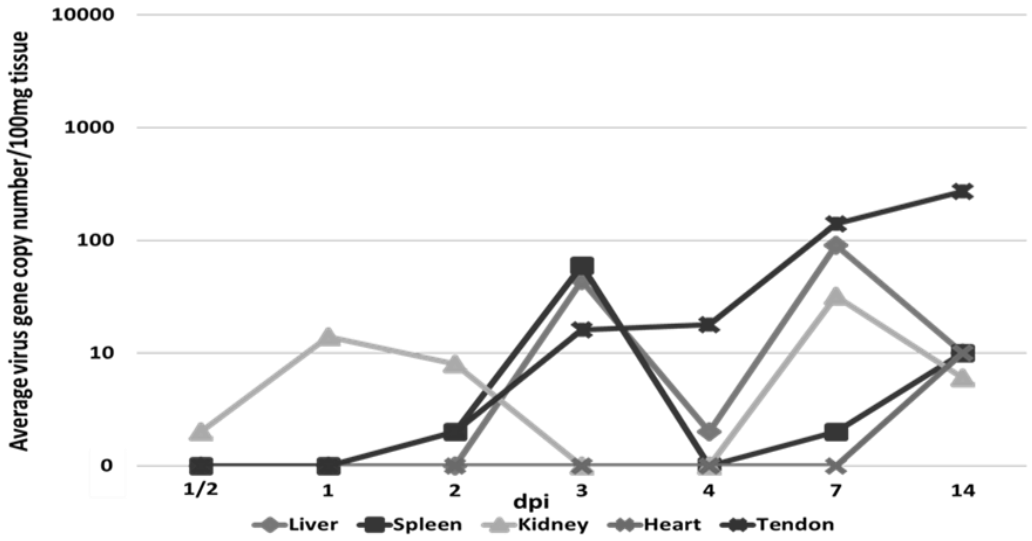


Figure 5.2. The virus gene copy number: A) Intestines and bursa of Fabricius. Virus gene copy number peaks in jejunum, cecum and bursa of Fabricius at 4 dpi and a remarkable decline at 7 dpi. Gene copy number in cloacal swab as indicative for virus shedding shows detectable titer starting from 1-3 dpi and peak at 14 dpi; B) Internal organs have minimal gene copy numbers(<100 copies/100mg) and a remarkable elevation in tendons at 7 and 14 dpi; C) Blood has minimal gene copy number (<100copies/200µl) that peaks at 4 dpi.



B


Average virus gene copy number/100mg tissue
10000
1000
100
10
0
1/2
1
2
3
4
7
14
dpi
Liver
Spleen
Kidney
Heart
Tendon

C

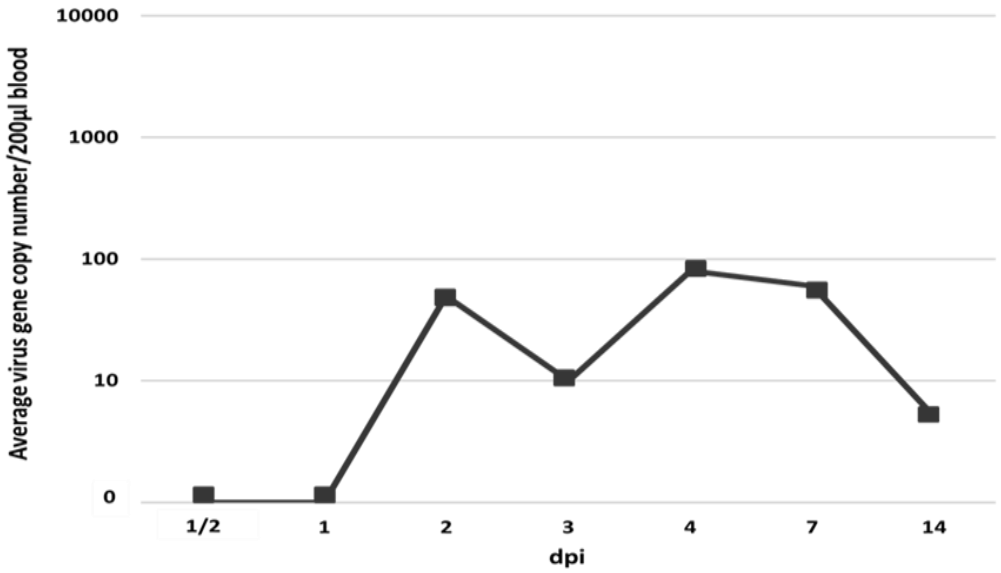
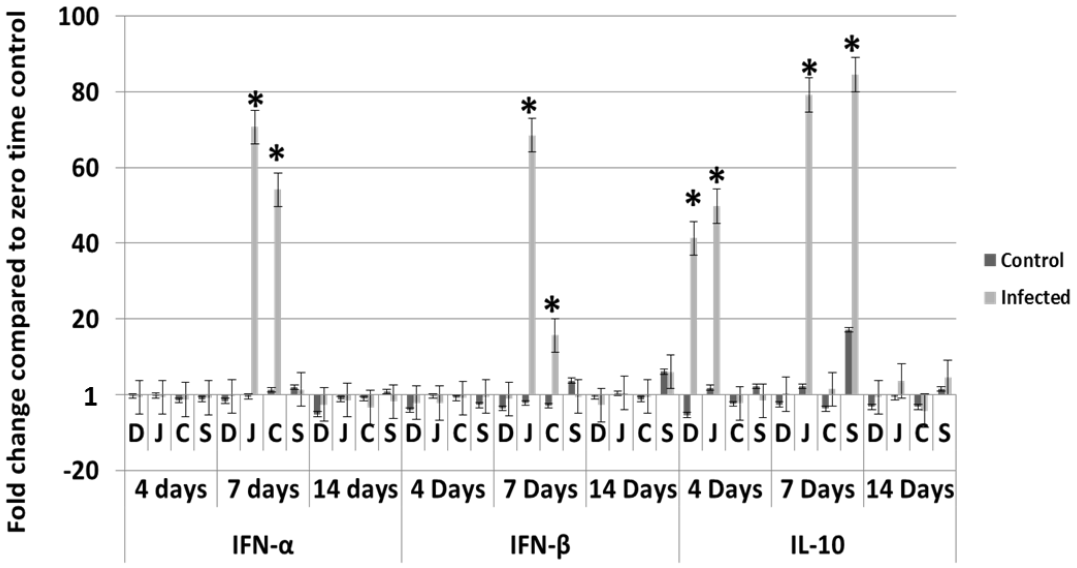

Average virus gene copy number/200µl blood
10000
1000
100
10
0
1/2
1
2
3
4
7
14
dpi

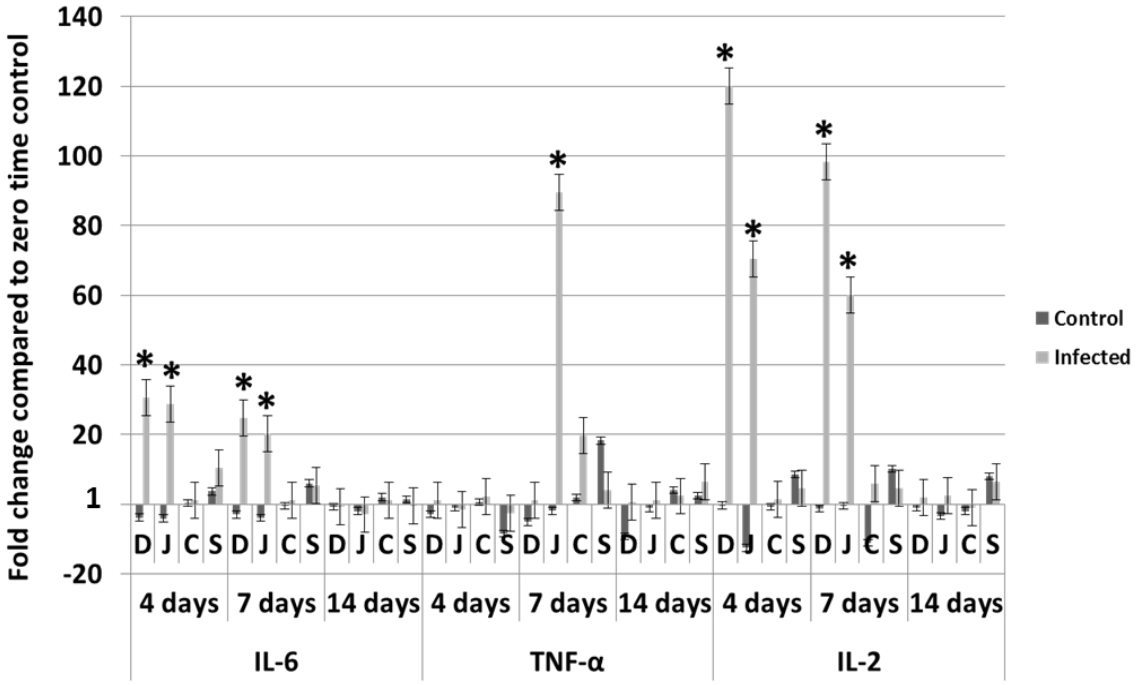
Figure 5.3. Fold change in antiviral cytokines (IFN-α and IFN-β) and antiinflammatory IL-10. There is statistical significant elevation of IFN-α and IFN-β in jejunum and cecum of infected birds at 7 dpi. IL-10 is significatnly elevated in duodenum and jejunum of infected birds at 4 dpi and in jejunum and spleen of infected birds at 7 dpi.



D: duodenum, **J**: jujenum, **C**: cecum, **S** spleen, **Days** refers to days post inoculation (dpi).

* Significant difference between infected group and non infected group within the same organ at $p<0.05$ Mann Whitney U test.

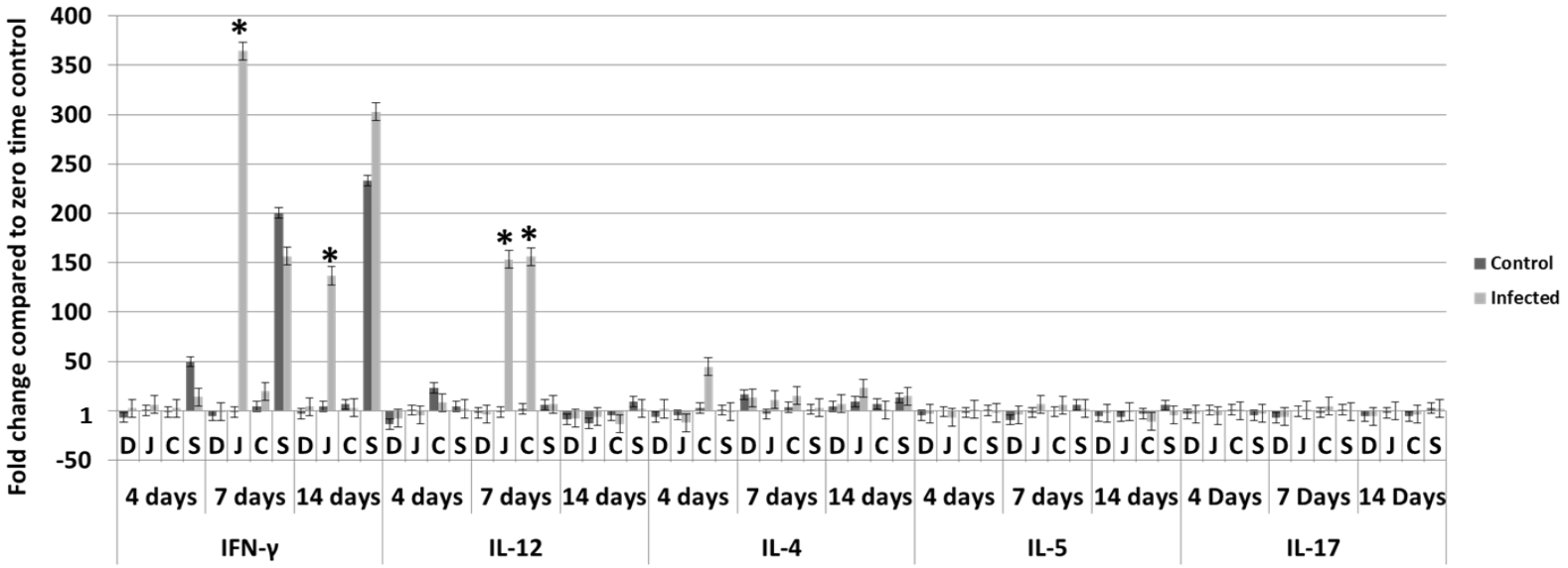
Figure 5.4. Fold change in proinflammatory cytokines (IL-6 and TNFα) and IL-2. IL-6 and IL-2 are significantly elevated in dudenum and jejunum of infected birds at 4 and 7 dpi. TNF-α has significantly higher fold increase in infected birds compared with non infecte control in jejunum at 7 dpi.



D: duodenum, **J**: jujenum, **C**: cecum, **S** spleen, **Days** refers to days post inoculation (dpi).

* Significant difference between infected group and non infected group within the same organ at $p<0.05$ Mann Whitney U test.

Figure 5.5. The cytokines changes in Th1, Th2 and Th17 cytokines. Fold changes in infected and non infected control birds compared with zerotime shows significant elevation of Th1 cytokine IFN-γ in the jejunum of infected birds at 7 and 14 dpi.The other Th1 cytokine IL-12 shows significant elevation in jejunum and cecum of infected birds at 7 dpi. Th2 (IL-4 and IL-5) and Th17(IL-17) are not showing any significant difference between infected and non infected control birds at $p<0.05$



D: duodenum, **J**: jujenum, **C**: cecum, **S** spleen, **Days** refers to days post inoculation (dpi). * Significant difference between infected group and non infected group within the same organ at $p<0.05$ Mann Whitney U test.

Table 1: List of cytokine genes and their primers used:

Cytokine	Accession number	Primers	Product size
GAPDH	GQ184819.1	F 5'CTGGCAAAGTCCAAGTGGTG3' R 5'TCCCATTCTCAGCCTTGACA3'	123 bp
IL-2	AJ007463.1	F 5'TGGAGCATCGCTATCACCAG3' R 5'TTGCTGACTGCACTCCTTGA3'	136 bp
IL-4	XM_003210493.1	F 5'TTCCTGTGGCAAGATGAACG3' R 5'CTGCAGGTTCTTGTGGCAGT3'	124 bp
IL-5	XM_003210491.1	F 5'TGACGAAAGCTGCATCAAAA3' R 5'CTCTTGCCAGGTTTGCTGTG3'	134 bp
IL-6	XM_003207130.1	F 5'GCTTCGACGAGGAGAAATGC3' R 5'AGCACAGCGATTCGACATTC3'	120 bp
IL-10	AM493432	F 5'TGGGCCTGAAGATGACAATG3' R 5'CTCCCCCATGGCTTTGTAGA3'	131 bp
IL-12	AJ564203.1	F 5'TCCAAAGACTGGGCCAAAAG3' R 5'CTCCAGCAGCAGAAGGCTCT3'	121 bp
IL-17	XM_003204633.1	F 5'CCATTGCTGTTGGTGTTGCT3' R 5'GGCATCCAGCATCTCCTTTC3'	115 bp
IFN-α	U28140.1	F 5'GCCTCCTCAACCAGATCCAG3' R 5'TGATGGTGAGGTGAGGGTTG3'	108 bp
IFN-β	XM_003213368.1	F 5'CCGTTCTGGAAAGCAAGGAC3' R 5'GTGTGCGTGGTCAATCCAGT3'	119 bp
IFN-γ	AJ000725	F 5'ACCTGGCCAAGCTTCAGATG3' R 5'TGGCTCCTTTTCCTTTTGGA3'	115 bp
TNF-α	XM_003210543.1	F 5'TGACTTGGCTGTCGTGTGGT3' R 5'GGCATTGCAATTTGGACAGA3'	119 bp

Chapter 6: Pathogenicity of turkey arthritis reoviruses in chickens

Tamer A. Sharafeldin, Sunil K. Mor, Harsha Verma , Aschalew Z. Bekele, Liliya Ismagilova, Sagar M. Goyal and Robert E. Porter

Summary

Turkey arthritis reoviruses (TARVs) were isolated recently from gastrocnemius and digital flexor tendons of lame turkeys with swollen joints and tenosynovitis. These TARVs were genetically different from chicken arthritis reoviruses (CARVs) and produced gastrocnemius tenosynovitis when inoculated into turkey poults. The purpose of this study was to determine the pathogenicity of TARVs in chickens. One-week-old, SPF chicks were inoculated with either a TARV (TARV-MN2 or TARV-O'Neil) or CARV via oral, intratracheal or footpad routes. At two and three weeks post inoculation (PI), a subset of chicks from each group was euthanized followed by collection of tissues for real-time RT-PCR (rRT-PCR), virus isolation and histopathology. Chickens inoculated with CARV via intratracheal and footpad routes developed gastrocnemius lymphocytic tenosynovitis at 2 and 3 weeks PI. Both TARV-MN2 and TARV-O'Neil induced gastrocnemius lymphocytic tenosynovitis in chicks inoculated via the footpad route only at 2 and 3 weeks PI. Though there was no evidence of clinical lameness, the virus was present in leg tendons, internal organs and intestines of all TARV-inoculated chicks via all routes as indicated by rRT-PCR and virus isolation. These results indicate that TARVs do not produce gastrocnemius tenosynovitis in chicks by 3 weeks PI when administered via the most probable natural route (e.g., oral and intratracheal). Further studies are needed to determine the long term effects these viruses might play in inducing lameness in chickens.

Introduction

The first clinical case of viral tenosynovitis/arthritis in *Mycoplasma synoviae*-negative chickens in the USA was reported by Olson and Solomon in 1968. Affected chickens had swelling and edema of intertarsal joints, wing joints and digital flexor tendons. Inoculation of chickens with chicken arthritis reovirus (CARV) via footpad demonstrated inflammation in tendon sheath of chickens as early as 1 week post inoculation (PI) (Kerr and Olson, 1969). Both naturally and experimentally infected birds showed heart lesions. Reovirus subsequently was isolated from tendons of 28-day-old broiler chickens displaying tenosynovitis/arthritis and ruptured tendons (Jones et al., 1975). Experimental inoculation of 1-day-old, specific-pathogen-free (SPF) chickens with chicken reovirus via the oral and footpad routes produced necrosis and congestion of liver, spleen, kidney and bursa of Fabricius in addition to causing tenosynovitis, myocarditis and pericarditis (Hieronymus wt al., 1983).

Chicken reovirus replicates primarily in enterocytes and bursa of Fabricius at 12 hours PI (Jones et al., 1989) and then reaches tissues and organs within 48 hours PI. Reovirus viremia occurs within 30 hours PI and the highest virus titer in cloacal swabs is seen from 1-5 days PI (Kibenge et al., 1985). Reovirus is transmitted mainly by the fecal oral route (Jones et al., 1978) but can also be transmitted from hens to chicks via eggs (Al-Mufarrej et al., 1996; Menendez et al., 1975; van der Heide, 1976). Infection through broken skin of legs may lead to virus localization in hock joints of chickens (Al-Afaleq et al., 1990)

Reoviruses were first identified in turkeys with tenosynovitis/arthritis (Levisohn et al., 1980, Page et al., 1982). After these reports in the 1980s, reovirus-induced lameness in

turkeys was unreported until we isolated turkey arthritis reoviruses (TARV) from gastrocnemius and digital flexor tendons of several lame turkeys in the Midwest (Mor et al., 2013b). These TARVs were found to be genetically different from chicken reoviruses (Mor et al., 2013b). In an experimental study, three different TARVs produced lymphocytic tenosynovitis within 4 weeks post inoculation (PI) via oral, intratracheal and footpad routes when inoculated at 1 week of age (Sharafeldin et al., 2014) but did not induce clinical lameness in infected turkeys. Clinical lameness appeared at 7 weeks PI and the percentage of lame turkeys increased at 11 and 15 weeks PI (Sharafeldin et al., 2014b).

With significant turkey and broiler production in the U.S., there are concerns about the risk that TARVs may pose to chickens. For example, three reoviruses isolated from turkeys with tenosynovitis could produce erosive arthritis and tenosynovitis at 3 weeks PI when inoculated into the footpad of 1-day old chicks (Al-Afaleq et al., 1989). However, inoculation of turkey enteric reovirus (TERV) did not produce any clinical illness in SPF chickens (Nersessian et al., 1986, Spackman et al., 2005). The aim of the present study was to determine the pathogenicity of two newly isolated TARVs in chickens and compare it with that of a chicken arthritis reovirus (CARV).

Materials and Methods

Viruses: The isolation and characterization of TARVs and their pathogenicity in turkeys have been described (Mor et al., 2013b, Sharafeldin et al., 2014). In this study, two different strains of TARV (TARV-MN2 and TARV-O'Neil) were used. For comparison, a pathogenic CARV (strain 2048) (Rosenberger et al., 1989) kindly supplied by Dr. Jack

Rosenberger, AviServe LLC, Newark, Delaware was used. All viruses were grown and titrated on QT-35 cells and had an average titer of $10^{5.5}TCID_{50}$/ml.

Birds: One hundred and twenty 1-day-old SPF white leghorn chicks (Charles River Laboratories, Wilmington, MA) were divided into 12 groups (10 birds/ group) and placed in 12 different filtered air isolators. Birds were supplied with food and water *ad libitum*.

Experimental design: Each isolator contained ten chicks that were inoculated with the same virus and by the same route. Six-day-old chicks were inoculated in blind fashion with 0.1 ml of a virus via oral, intratracheal or footpad route. Control chicks were inoculated with virus-free Minimum Essential Medium (MEM). Two individuals, who were blinded to the type of virus and route of inoculation for each isolator, observed the chicks daily for clinical signs (lameness and swollen red joints) and mortality until the termination of the experiment. At 2 and 3 weeks PI, 5 birds from each isolator were removed and euthanized. The birds were necropsied, gross lesions were recorded, and tissues [right leg gastrocnemius and digital flexor tendons (RLT); left leg gastrocnemius and digital flexor tendons (LLT); a pool of liver, spleen, heart, and bursa of Fabricius (LSHB); and intestinal contents (INT)] were collected from each bird for rRT-PCR, histopathology, and virus isolation. Procedures of bird housing, inoculation and euthanasia were approved by Institutional Animal Care and Use Committee (IACUC), University of Minnesota.

Virus detection: Self designed, TARV-specific rRT-PCR was performed (Mor et al., 2014) followed by virus isolation on positive tendon samples. Forward primer 5'-ATCATGGCT GGGTTTGTGCC–3' and reverse primer 5'-

AGAACGAATTTGTARGCGACCA-3' were designed to amplify 99 bp fragments from S4 gene. A TaqMan probe, 5'- FAM-TGAG MGTGATGACTTTACYCC –TAMRA-3' was similarly selected. The Real time RT-PCR reactions were carried using One-step RT -PCR Kit (Qiagen, Valencia, CA). Each reaction mixture (25 µl) had 23 µl of reagent mix (5µl, 5X reaction buffer, 1.0 µl enzyme mix, 0.2 µl RNase inhibitor, 300nM of each primer and 200 nM of TaqMan probe) and 2 µl of RNA. The PCR cycling conditions started with 50ºC for 30 min, 95ºC for 15 min and then 45 cycles of a two-step cycle (denaturation at 95ºC for 15s; annealing and extension at 56ºC for 45s).

Histopathology: Tissue samples (intestines, intertarsal joint, heart, liver, spleen, and bursa of Fabricius) were fixed in 10% neutral buffered formalin. Bone samples were decalcified in EDTA solution for 1 week after fixation. Tissues were then trimmed, dehydrated, embedded in paraffin, sectioned at 3-5 µm and stained with hematoxylin and eosin. A previously described histologic inflammation scoring system was used to evaluate histopathological lesions (Sharafeldin et al., 2014). Briefly, three 100x fields at the level of the tibiotarsal physis, tarsometatarsal physis, and intertarsal joint space along the gastrocnemius tendon were scored for inflammation in synovial epithelium and subsynovium. Synovium was scored as either 0= Normal, single synoviocyte layer, 1= Single layer of hypertrophied synoviocytes, 2= 2-4 layers of hyperplastic synoviocytes or 3= more than 4 layers of hyperplastic synoviocytes. Subsynovium scores were 0= < 10 lymphocytes, 1= 10-50 lymphocytes, 2= 50-100 lymphocytes or 3 = >100 (too numerous to count). Other lesions scored were lymphoid follicles= 1 point, fibroplasia= 1 point and dilated subsynovial blood vessels= 1 point. Scores of synovium, subsynovium and other lesions along the three levels of gastrocnemius tendon were added together as a total

score for one leg. Scores of right and left legs were added to arrive at the final score for each chicken (Fig. 1).

Statistics: Mann Whitney U test was used to test the significant difference between the group histologic inflammations scores (NCSS 9 Statistical Software, NCSS LLC., Kaysville, UT).

Results

Clinical signs and mortality: Chicks in all groups, except the CARV-footpad group, were active with no evidence of lameness. Chicks that were inoculated with CARV through the footpad route were often recumbent and developed swollen and reddened right shanks, hocks and feet by 4 days PI. Two birds inoculated with CARV died 4 days PI; one was inoculated by the intratracheal route and the other was inoculated by the footpad route. No gross lesions were observed in the two birds that died during the study. Aside from the swollen right hock, shank and tarsus occurring in all chicks in the CARV-footpad group there were no gross lesions observed in other treatment and control groups.

Virus detection: TARV-MN2 was detected by rRT-PCR in tendons of chicks inoculated by oral and footpad routes at 2 weeks PI and in tendons of chicks inoculated by all routes at 3 weeks PI. Chicks that were inoculated with TARV-O'Neil through oral, intratracheal and footpad routes were positive by rRT-PCR at 2 and 3 weeks PI (Table 1). None of the CARV-inoculated bird was positive by TARV-specific rRT-PCR. Virus isolation from tendon samples followed by S4 gene sequencing confirmed that the isolated virus was the same as the inoculated virus.

Histopathology: Internal organs showed no histologic lesions except lymphocytic epicarditis and myocarditis in CARV group at 2 and 3 weeks PI in all routes. Heterophilic enteritis in duodenum, jejunum, and cecum was seen at 2 weeks PI in the CARV group inoculated by oral and IT routes. The CARV- inoculated group had significantly higher histologic gastrocnemius tendon inflammation scores (all routes together) than those inoculated with TARV-MN2 and TARV-O'Neil at 2 and 3 weeks PI ($p<0.05$). The birds inoculated with TARV-MN2 and TARV-O'Neil had higher histologic inflammation scores (all routes together) than the negative control but the difference was not significant ($p<0.05$). At 2 and 3 weeks PI, no histologic lesions were observed in gastrocnemius tendon and intertarsal joints of chicks inoculated with TARV-MN2 or TARV-O'Neil by the oral and IT routes. However, chicks inoculated in the footpad with TARV-O'Neil developed gastrocnemius lymphocytic tenosynovitis, graded as high inflammation scores by 2 and 3 weeks PI. At 2 weeks PI the TARV-O'Neil-footpad group had histologic inflammation scores that were significantly lower than the CARV-footpad group (positive control) ($P<0.05$) (Fig. 3A). At 3 weeks PI, TARV-O'Neil-footpad group had a histologic inflammation score that was significantly higher than the negative control but was similar to that seen in the CARV-footpad group (positive control) ($p<0.05$) (Fig. 3B). By 3 weeks PI chicks inoculated with CARV by both footpad and IT routes showed gastrocnemius lymphocytic tenosynovitis with high inflammation scores while those inoculated with the oral route showed minimal inflammation scores (Fig. 3B).

Discussion

This study evaluated the pathogenic effects of two TARVs in chickens for up to three weeks PI. In previous pathogenicity studies chickens were inoculated at 1 day of age. We

used chickens at 1 week of age to try to mimick the field situation in which maternally derived antibodies can protect chicks from reovirus infection in the first 7 days of age (Jones, 2000). We believe that the results from inoculation at 7 days of age will be more reliable under field conditions than those obtained byinoculation of chickens at 1 day of age since the age of infection can affect the susceptibility of chickens (Jones and Georgiou, 1984). We did inoculation via oral and intratrachial routes (possible natural routes of infection). Additionally, we inoculated via footpad route which was suggested to be a possible route for reovirus infection and localization in leg tendons and joints via broken skin (Al-Afaleq and Jones, 1990). TARV-MN2 and TARV-O'Neil produced no clinical signs, no gross lesions and no microscopic lesions in internal organs of chickens, although the respective viruses were detected in all tissues at 3 weeks PI. Only TARV-MN2 and TARV-O'Neil footpad route of inoculation produced histologic gastrocnemius lymphocytic tenosynovitis but without any clinical lameness. In a previous study, footpad route inoculation of 1-day-old chickens with reoviruses isolated from turkeys with tenosynovitis/arthritis produced erosive arthritis, gross joint lesions and mortalities (Al-Afaleq and Jones, 1989).

The results of rRT-PCR and virus isolation indicated that TARVs can infect and multiply in chickens but do not produce clinical disease for up to 3 weeks PI (4 weeks of age). These two viruses (TARV-MN2 and TARV-O'Neil) did produce histologic lymphocytic tenosynovitis in one-week-old turkeys within 4 weeks PI (Sharafeldin et al., 2014a). TARV-MN2 and TARV-O'Neil inoculated by oral and IT routes did not produce histological lesions of tenosynovitis by 3 weeks PI, although the viruses were detected in tendons, intestines and internal organs of inoculated chickens. The TARV-O'Neil footpad

group showed a mild lymphocytic tenosynovitis at 2 weeks PI and the inflammation score was significantly lower than that of the CARV footpad group. However, at 3 weeks PI, histologic inflammation scores of TARV-O'Neil and CARV footpad groups were similar ($P<0.05$) indicating the progression of inflammation from 2 weeks to 3 weeks PI. In turkeys, TARV-MN2 and TARV-O'Neil induced tenosynovitis in one-week-old turkeys within 4 weeks PI via oral, intratracheal and footpad routes (Sharafeldin et al., 2014a).

The long term clinical effects of TARV-induced tenosynovitis in chicks are not known. Perhaps after 3 weeks PI, TARV-O'Neil footpad inoculation could have induced gross lesions and a clinical disease in chickens or a delayed immune response could have elicited more severe tenosynovitis. In turkeys, TARV-O'Neil inoculation via oral route at 1 week of age produced clinical lameness at 8 weeks of age (Sharafeldin et al., 2014b). Histologic tenosynovitis associated with TARV- O'Neil infection was shown to be an early endpoint (indicator) for the clinical disease in turkeys (Sharafeldin et al., 2014b). In chickens, oral and intratracheal TARV-O'Neil inoculation did not show the early endpoint (histologic tenosynovitis) at 3 weeks PI while was shown only in footpad route which we think it is unlikely a possible route of infection in the field situation especially in the young age chickens. Assuming that intratracheal and oral routes are the natural routes of infection, TARV-MN2 and TARV-O'Neil are not likely a possible cause of lameness in chickens.

In conclusion, TARV-MN2 and TARV-O'Neil, two reoviruses that induce tenosynovitis and lameness in turkeys, can infect chickens via multiple routes and multiply in internal organs and tendons. Chicks inoculated at one week of age, had detectable rRT-PCR and

virus isolation in tendon samples at 2 and 3 weeks PI. Only chickens inoculated with TARV-O'Neil by the footpad route showed gastrocnemius tenosynovitis as severe as CARV footpad inoculated chickens at 3 weeks PI. The possible natural routes (oral and intratracheal are not producing a diseases while experimental footpad route is. This raises the conclusion that TARV is not a possible cause of lamenss in chickens assuming that oral and intratracheal routes are the possible natural routes.

Figure 6.1. Calculation of histologic inflammation score for each bird

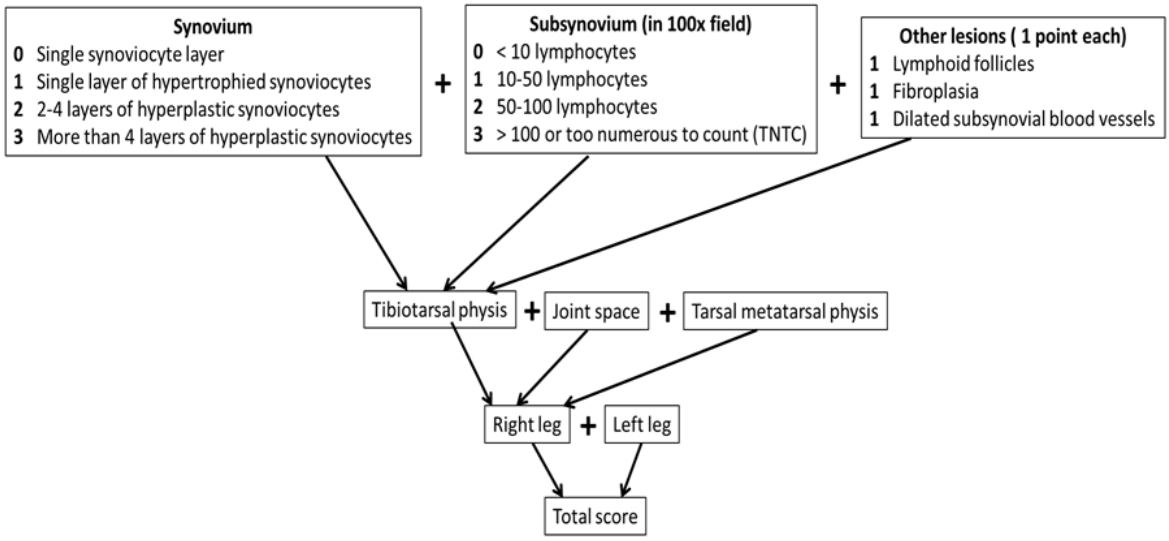


Figure 6.2. TARV-O'Neil gastrocnemius tendinitis (T) and tenosynovitis (TS) in chickens (2 weeks PI, footpad route)

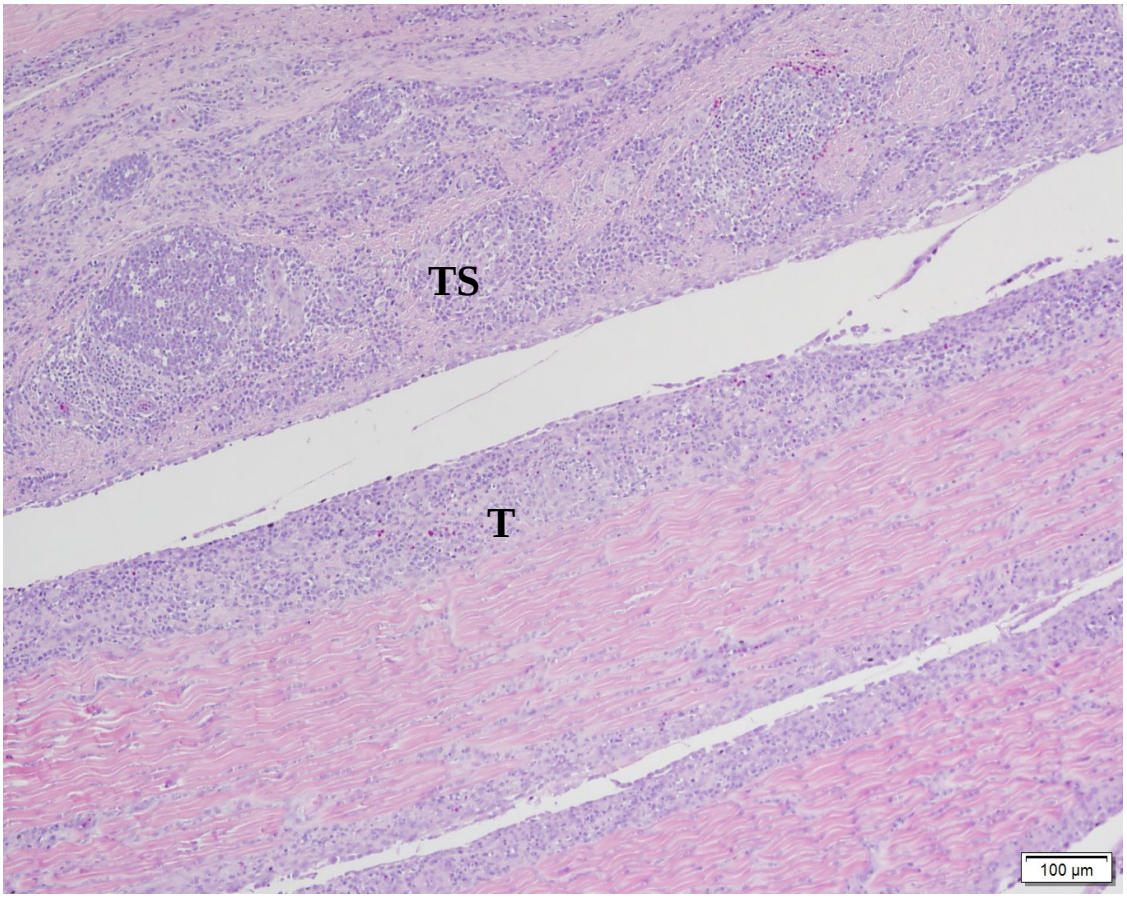
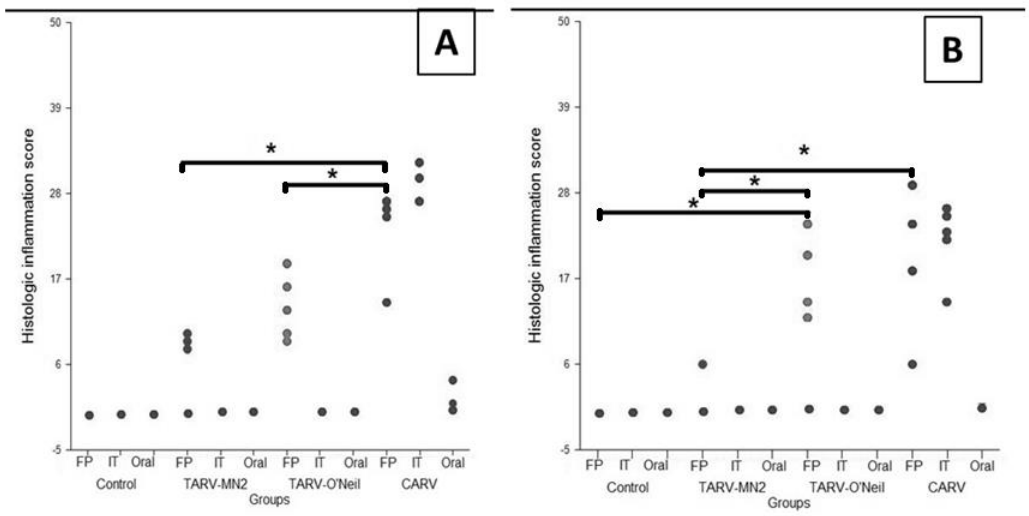


Figure 6.3. Histologic inflammation scores of gastrocnemius tendon and sheath: A) At 2 weeks PI, groups that were inoculated withTARV-MN2 and TARV-O'Neil via footpad route showed detectable tenosynovitis scores which were significantly lower than score of positive control group inoculated with CARV via footpad route. B) At 3 weeks PI, the tenosynovitis score of TARV-O'Neil footpad route group shows a progressive tenosynovitis score that is not significantly different from CARV footpad group scores. TARV-MN2 and TARV-O'Neil groups inoculated via oral and intratracheal groups do not display any score of tenosynovitis at 2 and 3 weeks PI.



Mann Whitney U test: Histologic inflammation scores in different treatment groups with different routes (Each group represents 5 birds). *P< 0.05.

Table 6.1. Virus detection by turkey reovirus specific rRT-PCR

		2 weeks PI				**3 weeks PI**			
		RLT	LLT	LSHB	INT	RLT	LLT	LSHB	INT
TARV-MN2	Oral	0/5	**2/5**	**1/5**	**2/5**	**5/5**	**4/5**	**2/5**	**1/5**
	IT	0/5	0/5	0/5	0/5	**5/5**	**4/5**	**5/5**	**1/5**
	FP	**4/5**	**4/5**	**4/5**	**1/5**	**2/5**	**2/5**	**3/5**	**2/5**
TARV-O'Neil	Oral	**5/5**	**5/5**	**3/5**	**5/5**	**4/5**	**3/5**	**4/5**	**5/5**
	IT	**4/5**	**5/5**	**4/5**	**5/5**	**5/5**	**5/5**	**5/5**	**5/5**
	FP	**4/5**	**2/5**	**1/5**	**3/5**	**3/5**	**4/5**	**5/5**	**5/5**
CARV	Oral	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	IT	0/4	0/4	0/4	0/4	0/5	0/5	0/5	0/5
	FP	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

RLT: right leg tendon, LLT: left leg tendon, LSHB: Liver, spleen, heart, bursa of Fabricius, INT: Intestine, FP: footpad, IT: intratracheal.

Positive results are bold and underlined

Chapter 7: General Discussion and Conclusions

Avian reoviruses have been known to cause enteric disease in chickens and turkeys and tenosynovitis/arthritis in chickens. In turkeys, avian reovirus isolation was reported from tendons and joints of turkeys with tenosynovitis/arthritis in 1980 and 1982, but the disease could not be reproduced experimentally. Thirty years later in 2011, we isolated avian reoviruses from tendons and joints of 16-week-old lame turkeys in the Midwest. This problem caused significant economic losses and showed varing degrees of morbidity and mortality. The aim of this thesis was to study the pathogenicity, pathogenesis and immune response elicited by these newly emergent turkey reoviruses (TARVs) that are genetically distinct from chicken reoviruses (Mor et al., 2013a).

For the study in chapter 2, we experimentally inoculated 1-week-old commercial turkey poults with 3 turkey arthritis reoviruses (TARV-MN2, TARV-MN4 and TARV-O'Neil) via oral, intratracheal and footpad routes and followed the inoculated poults for 4 weeks post inoculation (PI). The aim was to compare the pathogenicity of these 3 TARV strains with a turkey enteric reovirus (TERV-MN1) and a chicken arthritis reovirus (CARV-MN1). In two experiments, TARV-MN2 and TARV-O'Neil showed consistent capability to induce high histologic inflammation scores in gastrocnemius tendon sheath within 4 weeks PI but no clinical lameness was observed. TERV-MN1 and CARV-MN1 showed minimal histologic inflammation scores that were significantly lower than those produced by TARV-MN2 and TARV-O'Neil. We were able to reproduce the disease and fulfill Koch's postulates using TARV-MN2 and TARV-O'Neil. TARV- O'Neil appeared to be the most pathogenic dtrains because it produced the highest tendon inflammation scores, and therefore was the isolate chosen for our experimental challenge model. The most pathogenic strain (TARV-O'Neil) based on the results of lymphocytic tenosynovitis

inoculation via footpad, intratracheal and oral routes did not show significantly different ($P<0.05$) histologic tenosynovitis scores. For that reason, we established TARV-O'Neil inoculation via oral route to be the best reproducible experimental model to study TARV pathogenicity. Oral route inoculation is a possible natural route of infection and it is the easiest route for experimental inoculation.

In chapter 3, we inoculated 1-week-old turkey poults with TARV-O'Neil via the oral route and followed them for up to 16 weeks of age. In this chapter, we also developed a new gait scoring system in turkeys to evaluate lameness. The 6 point grading system (0-5) included all the variations that were observed in lame turkeys, considered the behavioral variation between chickens and turkeys and had a distinct point to differentiate lame versus non-lame turkeys. Brids in infected groups developed histologic tenosynovitis/arthritis at the age of 4 weeks (3 weeks PI), which was consistent with the results obtained in the previous study (see chapter 2). Additionally, infected turkeys developed clinical lameness (25%) at 8 weeks of age (7 weeks PI) and the percentage of lame birds increased at 12 and 16 weeks of age (30% and and 48% respectively). Control birds did not show any lameness at 4, 8 and 12 weeks of age while at 16 weeks of age, 5 birds out of 39 (12.8%) had lameness which we attributed to non-infectious causes. Histologically, the lesions in gastrocnemius tendon sheath started with lymphocytic tenosynovitis at 4 and 8 weeks of age, then fibroplasia began to develop by 12 weeks with a decline of lymphocytic infiltration. At the age of 16 weeks, fibroplasia was the most prominent change which might have resulted in tendon rupture observed in a few infected birds.

In chapter 4, we characterized the biomechanical change in properties (tensile strength and elasticity modulus) of TARV-O'Neil-infected turkeys from the experiment described in chapter 3. We compared tensile strengths and elasticity modulus of gastrocnemius tendons of infected and non-infected control turkeys at 4, 8, 12 and 16 weeks. There were no significant differences except in 16-week-old infected turkeys that had gastrocnemius tendons with significantly lower mean tensile strength and elasticity modulus compared with those of gastrocnemius tendons of control turkeys. This confirms that the prominent fibrosis occuring in tendons of 16-week-old infected turkeys, played a key role in decreasing the tensile strength and modulus elasticity, which could promote the tendon ruptures.

In chapter 5, 1-week-old turkey poults were inoculated with TARV-O'Neil via oral route and samples (intestines, internal organs, leg tendons and blood) were collected at 0, ½, 1, 2, 3, 4, 7, and14 days PI (dpi). This experiment aimed to describe virus pathogenesis and the immune response modulated by TARV-O'Neil infection in turkeys. Virus replication was higher in intestines and bursa of Fabricius than in gastrocnemius tendon and the virus was shed in feces as early as 3 days PI (dpi). The virus modulated an effective antiviral cytokine immune response (IFN-α and IFN-β) at 7dpi that induced a sharp decline in virus replication in intestines. Additionally, the virus induced T-helper 1 cytokine (IFN-γ) response evident at 7 and 14 dpi in intestines and at 14 dpi in tendons. Th 2 and Th17 cytokines did not show any significant elevation in infected birds compared with non-infected control; this execludes the possibility of immunoglobulin or autoimmune mediated reaction in the early course of infection.

In chapter 6, in response to concerns of poultry producers regarding cross-species pathogenicity of TARVs, we studied the pathogenicity of TARV strains in chickens. We inoculated 1-week-old chickens with TARV-MN2 and TARV-O'Neil and a pathogenic CARV as a positive control via oral, intratracheal and footpad routes. TARV-O'Neil via footpad route induced a high histologic inflammation score in gastrocnemius tendon sheath at 2 weeks PI, which increased at 3 weeks PI to be the same as the score of CARV footpad inoculated chickens. This experiment raised the concern about the demonstrated ability of TARVs to infect chickens even if they do not induce a clinical disease.

In summary, this thesis introduced new information about a newly emergent virus. The pathogen-disease relationship was established in turkeys and in chickens and the experimental model was established successfully to mimic the field problem with acceptable early end points (histologic inflammation scores). Furthermore, tissue spread and shedding and correlation with modulated immune response was described (host-pathogen interaction). Finally, two new scoring systems were developed; one for histologic inflammation in gastrocnemius tendons as an early end point for the experimental model and the other for lameness evaluation. The latter should be helpful to the veterinarians to assess lame turkeys in the field.

Limitations of the study

The limitations of this study are as follows:

(i) Obtaining SPF turkeys was very difficult and there was no guarantee of persistence of the SPF state during shipping. We used commercial turkeys from a reovirus unvaccinated breeder flocks and the poults' feces and serum were tested for reovirus and antibodies, respectively on arrival.

(ii) The optimum time and route of inoculation were not known as these are new previously untested viruses. Birds were inoculated at 1-week-old (not too young or too old) and multiple routes with multiple virus groups were used to establish the optimum conditions to the experimental bird model.

(iii) There are no established turkey immunoglobulin-specific ELISA for turkey reovirus. The available chicken reovirus ELISA kits helped to screen serum positive and negative turkey poults upon arrival. However, we could not make further use of this kit as the specificity and sensitivity to turkey immunoglobulin were not established.

(iv) There were no specific immunohistochemistry reagents for turkey reoviruses or for turkey cytokines. Although the antigen could not be visualized, we could estimate the virus gene copy number by a newly developed turkey reovirus specific rRT-PCR (Mor et al., 2014) and SYBERGreen PCR helped to identify cytokine profile mediated by virus infection.

(v) Experimentally, Using histologic inflammation scoring helped as an early successful end point in detecting viral effects as early as 2 weeks PI. The virus induced clinical lameness at 7 weeks PI. Experiments would have taken long time to conduct.

Future directions

Although there is significant new information in this work, there is much to do in the future. Studying the age susceptibility in different TARV strains is nessecary to understand the mechanism behind the unique pathogenicity of TARV in turkeys. Furthermore, developing turkey reovirus specific immunohistochemistry will help describe the cellular pathogenesis and cells targeted by the virus during different stages of infection. Additionally, this virus multiplies mostly in intestines and this requires

understanding the interaction among different components of gut environment which necessarily play a crucial rule in viral pathogenesis.

Developing an effective vaccination and protection policy, is a top priority to minimize the economic loss resulting from this virus infection in turkeys. Based on the pathogenicity results and molecular analysis and sequencing, choice of a proper vaccine strain and estimating its efficacy in protection against experimental infection and immune response modulated by this vaccine will result in establishing effective protection.

Testing the pathogenicity of more TERV strains in turkeys with state of immunosuppression will confirm if TARV strains develop a clinical disease (tenosynovitis and lameness) under special conditions or it had a unique capability to induce the disease.

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