Characterization, diagnosis and environmental survival of turkey arthritis reoviruses

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Abstract

During the Spring and Summer of 2011, the University of Minnesota Veterinary Diagnostic Laboratory (MVDL) received 14 submissions of 12- to 18-week-old tom turkeys that were recumbent with wing tip bruises ("wing walkers") and unilateral or bilateral swelling of the hock (tibiotarsal) joints. Gastrocnemius or digital flexor tendons were occasionally ruptured. A total of five turkey arthritis reoviruses (TARV-MN1 through TARV-MN5) were isolated in QT-35 cells and in embryonated chicken eggs from specific-pathogen-free chickens. The identity of the isolates was confirmed by electron microscopy, reverse transcription-polymerase chain reaction (RT-PCR) and gene sequence analysis. Additionally, blast analysis on the basis of 880bp nucleotide sequence of S4 gene confirmed all isolates as reovirus.

Phylogenetic analysis divided the five isolates into two subgroups: subgroup I containing TARV-MN1, TARV-MN2, TARV-MN3 and TARV-MN5 and subgroup II containing TARV-MN4. Isolates in subgroup I had a similarity of 97% to 100% with each other while subgroup II (TARV-MN4) had a similarity of only 89.2% with subgroup I viruses. This isolate showed 90% to 93% similarity with U.S. strains of turkey enteric reoviruses (TERVs) while the four isolates in subgroup I had 89% to 97.6% similarity with TERVs. These results indicate divergence within TARVs as well as between TARVs and TERVs, which needs to be confirmed by complete genome sequence analysis. Experimental studies to determine the role of TARV, TERV, and classical chicken reovirus (CRVs) in turkey arthritis have recently been completed. It has been found that TARVs are able to produce tenosynovitis in turkey poults but not TERV or CRV.

We isolated additional TARV strains from 2012 to 2104 and also received two isolates from a laboratory in Delaware. We characterized the S class gene segments of 12 TARVs and compared it with that of a TERV. Phylogenetic analysis of S2, S3 and S4 genome segments revealed grouping of all TARVs into two lineages while, on the basis of S1 genome segment, only one lineage was found. All TARVs had 95% to 100% nucleotide identity based on sigma C protein sequences (S1 segment) but varied from 90%-100%, 88.9%-100% and 88.7%-100% on the basis of S2, S3, and S4 genome segments, respectively. Point mutations as well as possible re-assortments were observed in TARVs throughout the S class.

We then did complete M class gene analysis of these 12 TARVs and included three more strains isolated in 2013 and 2014. Eight TERVs were isolated from fecal samples of turkeys and used for comparison. The aims of this study were to characterize turkey reovirus (TRV) based on complete M class genome segments, to determine genetic diversity within TARVs in comparison to TERVs and CRVs, and to find molecular markers that might be used to differentiate TARVs from TERVs. In this study, nt cut off values of 84%, 83% and 85% for the M1, M2 and M3 gene segments was proposed, generating 5, 7, and 3 genotypes, respectively. Phylogenetic analysis revealed point mutations and reassortments among TARVs, TERVs and CRVs.

On the basis of our results, we proposed M class genotype constellations (GCs) for avian reoviruses (including TARV, TERV, CRV, duck reovirus, and goose reovirus). The TARVs and TERVs were divided into three GCs of which GC2 was unique for TARVs and TERVs only. The maximum number of GCs (n=7) was formed by CRVs of which GC1 and GC3 were shared with TARVs and TERVs indicating reassortment

among TARVs, TERVs and CRVs. The newly proposed GC approach should help in the identification of virus strains that can be used for developing universal vaccine(s) against avian reoviruses (ARVs).

Strains of TARVs (n=7) and TERVs (n=3) were further studied for L class genome segment sequences. All three L class gene segments of TARVs and TERVs and their encoded proteins λA, λB, and λC were similar in size to those of CRV reference strain S1133. The conserved motifs such as C2H2 zinc-binding motif and conserved polymerase region were present in λA and λB, respectively. In λC protein, a conserved motif for ATP/GTP-binding site and S-adenosyl-L-methionine (SAM)-binding pocket for methyl transferase were observed in TARVs and TERVs with only one substitution as compared to CRV.

We proposed a new GC system for classification of avian reoviruses based on nt identity cut-off values for each L class. Based on this new genotype classification, all ARVs were divided into six, seven and eight genotypes in L1, L2 and L3 genes, respectively. Interestingly TARVs and TERVs grouped with three CRVs (two arthritic strains from Taiwan and one enteritic strain from Japan) in genotype L1-I and formed a different genotypes (L2-I, L3-I) from CRVs in L2 and L3 genes. The maximum nucleotide divergence was observed in genotypes of L1 and L2 genes. However, lower divergence at the amino acid level indicated that changes were mostly of synonymous type. Compared to L1 and L2 genes, the nonsynonymous changes were more in L3 gene. Point mutations and possible reassortments among TARVs, TERVs and CRVs were also observed.

One of the aims of this study was to develop a rapid diagnostic test for turkey reoviruses (both TARVs and TERVs). We developed a TaqMan real time RT-PCR (rRT-PCR) assay for this purpose using a primer-probe set designed from the conserved region of the S4 segment of the turkey reovirus genome. The newly developed rRT-PCR was specific for the detection of turkey reoviruses. The detection limit of this assay was 10 genome copies per reaction. For TARV-MN4 strain of turkey arthritis reovirus, one 50% tissue culture infectious dose ($TCID_{50}$) was equivalent to 11.6 ± 0.2 genome copies. The highest coefficient of variation for intra-experimental and inter-experimental variability was 0.08 and 0.06, respectively, indicating the reproducibility of the assay. This new test should be useful for the rapid detection of both TARVs and TERVs.

Another objective of our study was to compare the survival of TARVs in the environment (poultry litter and drinking water) with that of TERVs and CRV. Virus survival was studied in both autoclaved and non-autoclaved poultry litter and drinking water at room temperature (~25^0C). Three TARV isolates (TARV-O'Neil, TARV-MN2, TARV-MN4), one TERV (TERV-MN1) and one CARV isolate were used in this study. The viruses were propagated and titrated on QT-35 cells. In autoclaved de-chlorinated tap water all five viruses were able to survive for 9 to 13 weeks. In non-autoclaved water, all five viruses survived for $\geq$10 days. In autoclaved litter, the viruses survived for 6 to 8 weeks. In non-autoclaved litter, the survival was for 7 to 9 days only.

Another study was done to determine the efficacy of commonly used disinfectants in the turkey industry. We tested the antiviral efficacy of five disinfectants (Virocid, Keno X5, Synergize, One Stroke, and Tek Trol) against TARVs. For comparison, TERV and CARV were also included. At their recommended concentrations, all five

disinfectants inactivated more than 4 $\log_{10}$ $TCID_{50}$ of all viruses within 10 min indicating that commonly used disinfectants can be an effective tool in the control of these viruses.

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Chapter 1: Introduction, literature review and objectives

1. Introduction

Avian reoviruses (ARVs) belong to the genus orthoreovirus in the family *Reoviridae*. They are ubiquitous in domestic poultry with 80% of them being non-pathogenic (Jones, 2008). However, ARVs have also been implicated in different disease conditions such as enteritis, hepatitis, neurological disorder, myocarditis, respiratory distress and viral arthritis/tenosynovitis in chickens (Jones, 2008). Clinical disease associated with ARV is mostly dependent on age of the affected host, host immune status, virus pathotype, and route of exposure (oral, intratracheal, footpad, or subcutaneous). Economic losses are due to poor weight gain, uneven growth; poor feed conversion, increased morbidity and mortality, and reduced marketability of commercial chickens and turkeys (Jones, 2008).

In 1957, ARVs were isolated from naturally occurring cases of synovitis in chickens. Viral arthritis caused by chicken arthritis reovirus (CARV) has been well defined and its pathogenesis well established (Al Afaleq and Jones, 1989; van der Heide and Kalbac, 1975). Several different serotypes (at least 11 serotypes) and pathotypes of CARV have been described (Olson et al., 1957; Jones, 2008, Wood et al., 1980). Recently, variants of CARV have been isolated from lameness and tenosynovitis cases in 2.5 to 8-week-old commercial broiler chickens in Europe and North America (Rosenberger et al., 2013; Sellers et al., 2013; Troxler et al., 2013). Commonly used commercial vaccines are not effective against these CARV variants (Sellers et al., 2013; Troxler et al., 2013). A reovirus was isolated from the brain of a wild crow showing neurological signs. This virus was named the Tvarminne avian virus (TVAV). This virus appears to be a distinct reovirus species within the Orthoreovirus genus (Dandar et al., 2014). Recently, we described a reovirus in a chickadee (*Poecile atricapillus*) affected

with enteritis. The sequencing of S4 gene segment revealed close relationship of this virus to turkey reoviruses (Mor et al., 2014c).

For many years, turkey enteric reoviruses (TERVs) have been isolated from apparently healthy poults as well as from turkeys with poult enteritis complex (PEC),which include poult enteritis syndrome (PES) and light turkey syndrome (LTS) (Pantin-Jackwood et al., 2008; Jindal et al., 2010; Mor et al., 2013a). During late 2009, an unusual problem of lameness and swollen hock joints in commercial turkeys was reported in the upper Midwest of the U.S.A., which continues to this day. The disease caused substantial economic losses to turkey producers. We isolated reovirus from tendons and joint fluids of lame turkeys submitted to the Minnesota Veterinary Diagnostic Laboratory (MVDL) and tentatively named it as TARV (turkey arthritis reovirus), to differentiate it from TERV and CARV. Koch's postulates were fulfilled by experimental inoculation of these newly isolated TARVs in turkey poults (Sharafeldin et al., 2014a; 2014b). The problem of reovirus-associated lameness in turkeys appears to be re-emergence of a condition that was reported over 20 years ago (Levisohn et al., 1980; Page et al., 1982) although TERVs have been detected in enteritis-affected and apparently healthy turkey poults for a long time. We hypothesize that these newly isolated TARVs are genetically different from commonly detected TERVs. The main objectives of this study were to characterize these newly isolated TARVs, develop a molecular diagnostic test for rapid diagnosis and determine survival of TARVs in the environment.

This literature review focuses on reovirus infections in chickens, ducks, geese and turkeys with the main emphasis placed on turkey reoviruses (both TARVs and TERVs) including the characteristics of the viruses, modes of transmission and routes of exposure,

traditional methods of prevention and control, and laboratory methods for detecting infection.

2. Review of literature

2.1. Family *Reoviridae*

The family *Reoviridae* is a large and diverse family of non-enveloped, icosahedral viruses whose protein capsid is arranged in one, two or three concentric capsid layers, with an overall diameter of 60-90 nm. The name "*Reo*" is derived from respiratory enteric orphan virus since initial isolates were known to infect the respiratory and gastrointestinal tracts of humans without causing disease (King et al., 2012; Sabin, 1959). The family *Reoviridae* contains 15 genera divided into two subfamilies. (Attoui et al., 2011; Schiff et al., 2007).

The subfamily *Spinareovirinae* contains viruses that have relatively large spikes or turrets situated at the 12 icosahedral vertices of either the virus or core particle. The subfamily *Sedoreovirinae* includes viruses that do not have turret, giving them an almost spherical or "smooth" appearance. The transcriptionally active core particle of the spiked viruses (subfamily *Spinareovirinae*) appears to contain only a single complete capsid layer (which has been interpreted as having T = 1 or T = 2 symmetry) to which the projecting spikes or turrets are attached. In most cases, the core in the complete virion is surrounded by an incomplete protein layer (with T = 13 symmetry) that forms the outer capsid, which is penetrated by the projections on the core surface (Fig 1.1). These virus particles are, therefore, usually regarded as double-shelled (King et al., 2012).

In contrast, virions of the non-spiked viruses (subfamily *Sedoreovirinae*) have an inner protein layer, which may be relatively fragile, having structural similarities to the innermost shell of the spiked viruses (interpreted as having T = 2 symmetry). However, in transcriptionally active core particles, the subcore is surrounded and reinforced by a complete core-surface layer, which has T = 13 symmetry. These double-layered cores have no surface spikes and (in intact virions) are surrounded by another outer capsid shell, giving rise to three-layered virus particles that are equivalent to the two-layered particles of subfamily *Spinareovirinae*. Within the ‘‘turreted’’ group, the genus *Orthoreovirus* includes avian and mammalian reoviruses.

2.2. Genus *Orthoreovirus*

The Orthoreovirus genus can be divided into two groups: fusogenic and non-fusogenic. Fusogenic viruses have the ability to cause fusion of infected cells resulting in the formation of multinucleated, giant cells (syncytia) (Benavente, and Martinez-Costas, 2007). Fusogenic reoviruses infect mammals, birds and reptiles and form a genetically distinct clade from non-fusogenic mammalian reoviruses (MRVs; Day et al., 2009; Duncan and Sullivan, 1998). Most MRVs are non-fusogenic in nature but some are fusogenic e.g., Nelson Bay reovirus (NBV; most recently renamed as *Pteropine orthoreoviruse* or PRV) and bat, and baboon reoviruses. The orthoreoviruses are divided into five groups (I-V) (King et al., 2012); group I includes prototypical MRV strains including Ndelle virus; group II contains avian reoviruses (ARVs); group III includes bat orthoreoviruses formerly known as the Nelson Bay virus (NBV); groups IV and V include baboon (BRV) and reptilian orthoreoviruses (RRV), respectively.

2.3. Mammalian versus avian orthoreovirus

Although ARVs have similar structural and molecular composition as their mammalian counterparts, they do differ from MRVs in many aspects, e.g., ARVs lack hemagglutination property (Glass et al., 1973) and are fusogenic in nature (Duncan and Sullivan, 1998; Kawamura et al., 1965). In addition, ARVs only infect avian species and are associated with distinct pathological conditions, such as runting and stunting syndrome and tenosynovitis in chickens (Robertson and Wilcox, 1986). MRVs are propagated on established murine and human cell lines while most ARV strains grow only on primary cultures of avian cells (Jones, 2008), although some strains have been adapted to grow on mammalian cell lines (Robertson and Wilcox, 1986; Dandar et al., 2014).

2.4. History of avian reoviruses

2.4.1 Chicken reovirus

Chicken reovirus (CRV) was first isolated from chickens with chronic respiratory disease and was named as the Fahey-Corawley (FC) agent (Fahey and Crawley, 1954). Later, the FC agent was characterized and placed in the genus *Orthoreovirus* (Petek et al., 1967). Other diseases caused by CRVs in chickens include viral arthritis/tenosynovitis, respiratory disease, immunosuppression, inclusion body hepatitis, hydropericardium and hepatitis in young chicks, and the runting/malabsorption syndrome (Jones, 2008). Olson and Solomon (1968) reported the first clinical case of viral tenosynovitis/arthritis in U.S. chickens; these birds were shown to be negative for *Mycoplasma synoviae* infection, which is also a common cause of arthritis and tenosynovitis in poultry. The affected birds

had swelling and edema of hock and wing joints and digital flexor tendons. CRVs can also be isolated from apparently healthy chickens.

After arthritis, an enteric disease called runting-stunting syndrome (RSS), also known as "malabsorption syndrome" (MAS), is the second most common reovirus-associated illness in broilers. RSS was first reported in the 1940s, became well recognized in commercial broiler production in the 1970s, and has since been reported around the world (Rebel et al. 2006). Originally, RSS was thought to be caused by a reovirus but attempts to reproduce the syndrome with reovirus have not always been successful. Later, however, certain strains of reovirus (1733, 2408, CO_8) were shown to experimentally reproduce RSS and were used to develop vaccines. While vaccination of broilers for RSS is effective about 50% of the time, a consistent vaccination program for breeders often provides long term benefits (van der Heide, 2000) because immunity from breeder hens is passed to chickswhich helps to protect them from the disease. Recently, Dandar et al. (2011) reported a novel variant strain of reovirus (AVS-B) associated with RSS.

2.4.2 Turkey reovirus

Enteritis in turkey poults is well recognized as a major cause of morbidity and mortality. On the basis of clinical presentation these conditions have been named as poult enteritis and mortality syndrome (PEMS), poult enteritis complex (PEC), poult enteritis syndrome (PES), light turkey syndrome (LTS) and simply poult enteritis (PE) (Barnes et al., 2000; Jindal et al., 2009, 2010a; Mor et al., 2011, 2013a; Pantin-Jackwood et al., 2007; 2008; Woolcock et al., 2008). These are multifactorial disease syndromes and reovirus is

known to be associated with them. However, turkey reovirus can also be detected in apparently healthy turkey poults. In a study of 33 turkey flocks, Pantin-Jackwood et al. (2008) found reoviruses in 46% of the flocks. Jindal et al. (2010a) tested intestinal contents of 43 PES-affected flocks during 2007-2008 and reported the presence of reovirus in 40% flocks. The TERVs have been characterized genetically based on their S1, S3 and S4 genome segments and have been shown to form a distinct group in the *Reoviridae* family (Day et al., 2007, Jindal et al, 2010, Pantin-Jackwood et al., 2008). In addition, reovirus has also been reported to be associated with pericarditis and perihepatitis in turkeys (Shivaprasad et al., 2009).

Reovirus was also identified and isolated from cases of tenosynovitis/ arthritis in turkeys (Levisohn et al., 1980). Page et al. (198) reproduced tenosynovitis/arthritis when they inoculated reovirus isolated from tenosynovitis turkeys in the footpad of 1-day-old turkeys. Afaleq et al. (1989) also found that three turkey reoviruses that were isolated from tenosynovitis turkeys could produce tenosynovitis/arthritis when inoculated in the footpad of 1-day-old chickens. After these reports in the 1980s and early 1990s, no cases of turkey viral arthritis were reported in the published literature until we isolated TARV from arthritis cases in 12 to 18-week-old tom turkeys. The newly isolated viruses were partially characterized on the basis of S4 genome segment (Mor et al., 2013b).

2.4.3 Duck and goose reovirus

Duck reoviruses (DRVs) have been isolated from different species of ducks, including mallards, healthy Pekin ducks, and diseased ornamental ducks. All DRVs share a common group antigen with CRVs. Reovirus strains from Pekin ducks are able to cause

microscopic lesions of tenosynovitis in specific-pathogen-free chicks. Heffels-Redmann et al. (1992) reported that the two duck strains they examined were antigenically distinct from standard chicken strains. Classical Muscovy duck reovirus (MDRV) is the cause of the disease, which was first described in South Africa in the 1950s (Kaschula, 1950) and then in France (Gaudry et al., 1972). It typically affects young ducklings 2-4 weeks of age, causing diarrhea and difficulty in movement, high morbidity, and mortality of 10% or higher in the ducklings. Malkinson et al. (1981) isolated a reovirus from affected ducks in which they found necrotic foci in the liver, spleen and kidneys. In 2002, a new infectious disease emerged in Muscovy ducks and geese in China, which was named as hemorrhagic necrotic hepatitis. The novel types of reoviruses isolated from these cases were named as the novel duck reovirus (NDRV) (Chen et al., 2012; Liu et al., 2011; Yun et al., 2014).

Goose reovirus (GRV) is a causative agent of arthritis in geese. The disease was characterized by splenitis with miliary necrotic foci during the acute phase, and epicarditis, arthritis and tenosynovitis during the subacute/chronic phase (Palya et al., 2003). Recently, the complete genome sequences of DRVs and GRVs have been reported (Dandar et al., 2014; Wang et al., 2013; Yun et al., 2014; Zhang et al., 2007).

2.4.4 Reovirus in wild birds

The ARVs have also been isolated from cases of enteritis in wild avian species including pigeons, grey parrots and quails (McFerran et al. 1976; Meulemans et al. 1983; Ritter et al. 1986; Gough et al., 1988). Jones and Guneratne (1984) isolated a reovirus from the feces of a zoo wedge-tailed eagle *(Aquila andax)*. This virus caused microscopic lesions

of tenosynovitis in SPF chicks. A virus associated with mortalities in American woodcock *(Scolopax minor)* was identified as a reovirus by Doherty et al. (1994). An outbreak of disease in pheasants in Turkey attributed to reovirus infection was described by Mutlu et al. (1998). Curtis et al. (1992) reported cases of reovirus-associated tenosynovitis in 6-7-week-old pheasants.

Antibodies to ARVs have been detected in ostriches *(Struthio camelus)* in Zimbabwe, rockhopper penguins (*Eudyptes chrysocomes*) in Argentina and in bean geese (*Anser fabalis*) and white fronted geese (*Anser albifrons*) in Germany. All these reoviruses share a common group antigen with CRVs, but their importance as pathogens in the host species has not been determined (Jones, 2008). In 2002, a reovirus was isolated in Finland from a wild crow showing central nervous system signs. It was later fully characterized based on complete genome sequencing as a new virus named the Tvarminne avian virus (TVAV) (Dander et al. 2014; Huhtamo et al. 2007).

2.5. Orthoreovirus genome

The viral genome is segmented and ten segments are divided into three classes namely large (L), medium (M), and small (S), depending on their migration pattern on polyacrylamide gel electrophoresis (Benavente and Martınez-Costas, 2007; Varela and Benavente, 1994). The L and M genes are further subdivided into three segments each (L1, L2, L3 and M1, M2, M3) while the S gene has four segments (S1, S2, S3, S4; Benavente, and Martinez-Costas, 2007) (Fig. 1.1).

The reovirus genome has 12 open reading frames (ORFs), which encode for eight structural and four non-structural proteins. The structural proteins are an important part of

progeny virions while non-structural proteins are not a part of the mature virion but are only expressed in infected cells (Martinez-Costas et al., 1997). The proteins encoded by L, M and S genes are lambda (λ), mu (μ) and sigma (σ), respectively. Three structural proteins λA, λB and λC are encoded by L gene segments L1, L2, and L3, respectively. M1 and M2 segments encode two structural proteins (μA and μB) while M3 segment encodes a non-structural protein (μNS). The three σ proteins σC, σA, σB are encoded by the S1, S2, S3 segments, respectively, while the S4 segment encodes for non-structural protein σNS (Varela and Benavente, 1994; Varela et al., 1996). The S1 segment encodes for two additional non-structural proteins; p10 and p17. The S1 is tricistronic containing three ORFs for p10, p17 and σ C proteins (Bodelon et al., 2001; Shmulevitz et al., 2002) while S2, S3 and S4 have single ORFs that encode for σA, σB and σNS, respectively (Benavente, and Martinez-Costas, 2007).

2.6. Viral proteins

2.6.1. Protein λA

The largest gene segment (3958bp) of ARV is L1 which encodes protein λA of 1293 amino acids with a molecular weight of 142.3kDa (Table 1.1). This protein forms the inner core shell that encloses the viral genome segments and the viral RNA polymeraseand is used as a scaffold for subsequent core assembly (Fig 1.1). Protein λA, which associates very rapidly with avian reovirus factories in infected cells, is diffusely distributed in the cytoplasm of transfected cells when expressed alone but becomes associated with globular inclusions when co-expressed with μNS, suggesting that μNS

mediates the recruitment of λA into viral factories (Benavente, and Martinez-Costas, 2007, Touris-Otero et al., 2004).

2.6.2. Protein λB

The L2 gene segment is 3829 bp long and encodes protein λB which contains 1259 amino acids with 139.8kDa molecular weight (Table 1.1, Fig 1.1). This segment encodes the viral RNA-dependent RNA polymerase (RdRp), an essential enzyme for RNA virus replication (Xu et al., 2008).

2.6.3. Protein λC

The L3 gene (3907bp) encodes λC protein of 1285 amino acids with a molecular weight of ~141.9kDa (Table 1.1). This protein extends from the inner core to the outer capsid of the virion (Martinez-Costas et al., 1997; Zhang et al., 2005) (Fig 1.1). Pentamers of protein λC form turrets projecting from the five-fold axes of cores (Zhang et al., 2005). Affinity radiolabeling of the structural polypeptides contained in avian reovirus particles has revealed that protein λC is the viral capping enzyme. Thus, λC is the only structural protein that binds GMP through a phosphoamide linkage when viral particles are incubated with GTP, and the GMP moiety of the complex can be transferred to GDP and GTP acceptors, yielding the cap structure (Martinez-Costas et al., 1995).

2.6.4. Protein μA

The M1gene segment (2283bp) encodes protein μA (732 aa) which is a minor component of the inner capsid (Martinez-Costas et al., 1997) (Table 1.1, Fig 1.1). The deduced

amino acid sequence of this protein has been recently reported, but no studies on the properties and function of this protein have yet been described. Its MRV counterpart μ2 has been shown to interact with microtubules and with the nonstructural protein μNS. These interactions are thought to be responsible for anchoring viral factories to microtubules and, therefore, most MRV strains form factories with a filamentous arrangement (Parker et al., 2002).

2.6.5. Protein μB

The M2 (2158bp; Table 1.1) segment encodes μB protein of 676 amino acids with 82kDa molecular weight (Varela and Benavente, 1994). This is one of the major outer capsid proteins and plays an important role in virus penetration (Fig 1.1). This protein contains a myristoylation consensus sequence at its amino terminus. The μB protein confirmed to be is N-myristoylated by metabolic radiolabeling of ARV-infected cells with tritiated myristic acid (Varela et al., 1996). A large proportion of the μB molecules synthesized in infected cells undergo cleavage near the amino terminus to produce a myristoylated amino-terminal peptide (termed μBN) and a large carboxy-terminal protein (termed μBC).

Both μB and its cleavage products are structural components of the reovirion outer capsid (Martinez-Costas et al., 1997). Amino-terminal sequencing of μBC located the μB cleavage site between residues Asn-42 and Pro-43. Being a major structural reovirion component, μBC appears to play an important role in the internalization of ARV into the host cell. Thus, intralysosomal ARV uncoating is accompanied by two sequential μBC cleavages near its C-terminus that generate the δ and δ' polypeptides.

Indirect evidence suggests that these cleavages are necessary to promote interactions with lysosomal membranes and conformational changes, which are necessary to facilitate the release of transcriptionally active core particles to the cytoplasm (Duncan, 1996).

2.6.6. Protein μNS

The M3 gene segment (1996bp) expresses a 70 kDa nonstructural protein termed μNS (Varela and Benavente, 1994; Touris-Otero et al., 2004b) (Table 1.1). This 635-amino acid protein contains two predicted coiled coil segments located between positions 451-472 and 540-599. The segments are separated by an intervening sequence, suggesting that this protein may form homo- or hetero-oligomers (Touris-Otero et al., 2004b). Benavente et al. (2007) reported that a proportion of the μNS molecule present in infected cells is cleaved near the N-terminus to yield a 15 kDa-amino-terminal fragment (designated μNSN) and a 55 kDa-carboxy-terminal protein (designated μNSC). It is believed that μNS protein plays important roles in the early stages of virus morphogenesis (Touris-Otero et al., 2004b). Examination of transfected cells co-expressing μNS and several other viral proteins has revealed that μNS mediates the selective recruitment to inclusions of σNS and σA, but not other viral proteins tested (Touris-Otero et al., 2004a). The expression of individual viral proteins in transfected cells has shown that avian reovirus μNS is the only viral protein capable of forming inclusions when expressed individually, suggesting that μNS is the minimal viral factor required.

2.6.7. Protein p10

The first gene segment of S class is S1 (length 1643bp), which encodes three ORFs. The first ORF encodes a 10.3 kDa nonstructural protein named as p10 (Table 1.1). This is a type-1 transmembrane protein with a central transmembrane domain that separates ecto- and endodomains of approximately equal size (Shmulevitz and Duncan, 2000). The p10 protein is a member of the fusion-associated small transmembrane (FAST) protein family and its expression in transfected cells induces extensive cell-cell fusion, suggesting that p10 plays a key role in the fusogenic property of ARVs (Bodelon et al., 2001; Shmulevitz and Duncan, 2000). In addition, p10 protein contains a di-cysteine motif between the transmembrane and the endodomain; this motif is palmitoylated and this acylation is required for the fusion activity of p10 (Shmulevitz et al., 2003).

2.6.8. Protein p17

The second ORF of the S1 gene encodes a protein named p17 which has 146 amino acids and 16.9kDa molecular weight (Table 1.1). This protein has no sequence homology with any other viral or cellular proteins. A study revealed that p17 accumulates in the nucleus of both infected and transfected cells, and mutational analysis identified a functional nuclear localization signal near its C-terminus (Costas et al., 2005). This study further showed that p17 is a nucleocytoplasmic shuttling protein and that the nucleocytoplasmic distribution of p17 is coupled to the transcriptional activity of the cell.

2.6.9. Protein σC

The 3'-proximal cistron of the S1 gene encodes 326 aa protein named σC which has 34.9kDa molecular weight (Table 1.1). This protein forms the minor outer capsid but plays an important role in viral cell attachment (Fig. 1.1). The σC is the only viral protein present in soluble extracts of infected cells that is able to attach to avian cell monolayers and elicits reovirus-specific neutralizing antibodies (Martinez-Costas et al., 1997; Shapouri et al., 1996). It has been recently reported that σC causes apoptosis when expressed in transfected cells (Shih et al., 2004). However, the contribution of σC to ARV-induced apoptosis is not clear since viral gene expression is not required for ARV to induce apoptosis in cultured cells (Labrada et al., 2002). Protein σC is a homotrimer in its native state and its cell attachment activity is exclusively associated with its oligomeric form (Grande et al., 2002). A C-terminal fragment of protein σC (residues 151-326) contains the receptor-binding globular domain (Guardado Calvo et al., 2005) and has the same topology as the head domain of the MRV cell attachment protein plus two repeats of a triple beta-spiral (residues 157-194).

2.6.10. Protein σA

The S2 gene segment is 1324bp long and encodes protein σA (416 amino acids), which is a component of the inner core shell (Martinez-Costas et al., 1997) (Table 1.1, Fig 1.1) and possesses sequence-independent dsRNA-binding activity (Martinez-Costas et al., 2000; Yin et al., 2000). This protein binds dsRNA in vitro with very high affinity; the nucleic acid is not released from the σA-dsRNA complex upon incubation with high salt concentrations (Touris-Otero et al., 2005). Protein σA possesses a nonspecific nucleotidyl

phosphatase activity that is able to hydrolyze all four types of nucleoside triphosphates to their corresponding nucleoside di- and monophosphates and free phosphate (Yin et al., 2002). It has been reported that σA displays anti-interferon activity by preventing the activation of the dsRNA-dependent protein kinase PKR, and that this activity is probably linked to its capacity to bind and sequester dsRNA (Martinez-Costas et al., 2000; Gonzalez-Lopez et al., 2003). Thus, it appears that σA plays a key role in the resistance of ARV to the antiviral action of interferon.

2.6.11. Protein σB

The 1202bp long S3 gene segment encodes for a 367 amino acid protein σB, which is a major component of the reovirion outer capsid (Martinez-Costas et al., 1995; Varela et al., 1996) (Table 1.1, Fig 1.1). The σB protein associates spontaneously and very rapidly with σB and σBC in the cytosol of infected cells to form a ternary hetero-oligomeric complex that contains stoichiometrically equal amounts of the three viral proteins, and the three proteins incorporate into core particles as a pre-formed complex (Touris-Otero et al., 2004a, 2004b). In contrast with its MRV counterpart σ3, σB does not bind dsRNA in solution suggesting that it does not possess anti-interferon activity (Touris-Otero et al., 2005).

2.6.12. Protein σNS

The smallest segment of S class is S4 gene segment (1192bp long), which encodes for nonstructural protein σNS of 367 amino acids (Schnitzer, 1985; Varela and Benavente, 1994) (Table 1.1). This protein has been shown to bind ssRNA in vitro in a sequence-

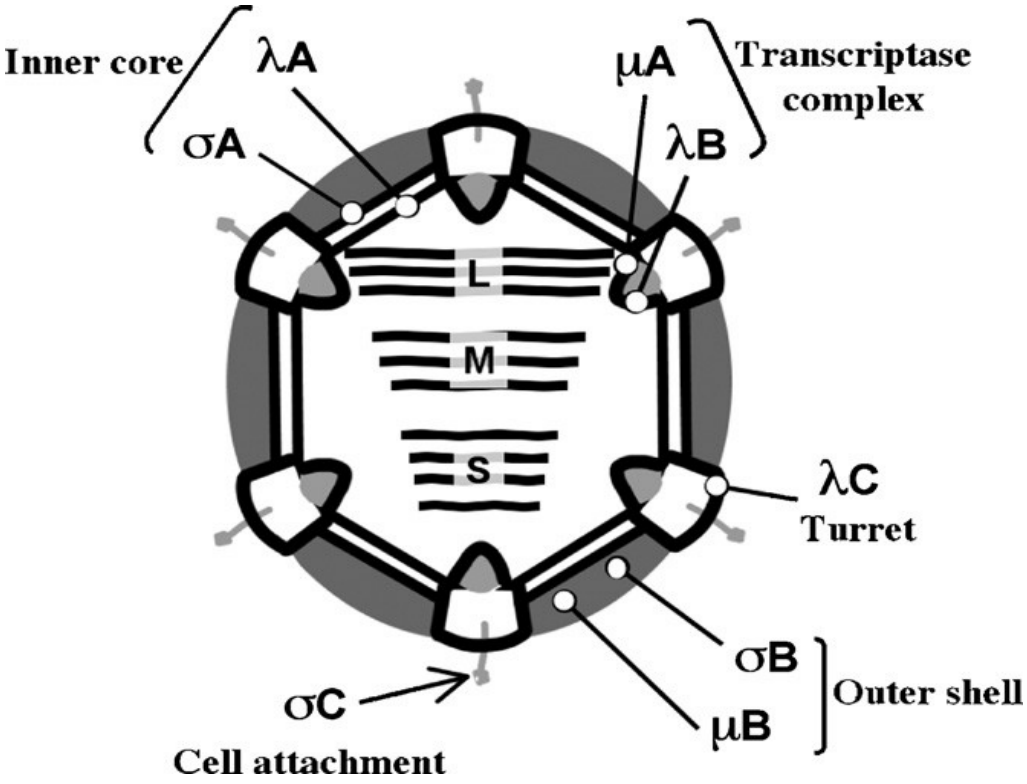
independent manner (Touris-Otero et al., 2005; Yin and Lee, 1998). It is present in large ribonucleoprotein complexes in the cytoplasm of infected cells, indicating that it exists in intimate association with ssRNAs in vivo (Touris-Otero et al., 2005). Mutagenic analysis has revealed that the five conserved basic residues that are important for RNA binding are dispersed throughout the entire σNS sequence, suggesting that this protein binds RNA through conformational domains (Touris-Otero et al., 2005). As a nonstructural RNA-binding protein that accumulates in viral factories of ARV-infected cells, σNS probably plays a key role in RNA packaging and replication (Benavente and Martinez-Costas, 2007).

Table 1.1. Proteins encoded by various genome segments of reoviruses

Segment	Nucleotide (bp)	ORF	Amino acids	Protein	Distribution	Function
L1	3958	21-3899	1293	λA	Inner core	Core shell scaffold
L2	3829	14-3790	1259	λB	Inner core	Putative transcriptase (RNA dependent RNA polymerase)
L3	3907	13-3867	1285	λC	Turret	Capping enzyme
M1	2283	13-2208	732	μA	Inner core	Putative transcriptase co-factor
M2	2158	30-2050	676	μB	Outer capsid	Penetration
M3	1996	25-1929	635	μNS	Nonstructural	Formation of viral factories Protein recruitment
S1	1643	25-318	98	p10	Nonstructural	Fusogenic/ permeabilising
		293-730	146	p17	Nonstructural	Unknown
		630-1607	326	σC	Outer capsid	Cell attachment
S2	1324	16-1263	416	σA	Inner core	dsRNA binding, anti-interferon activity
S3	1202	31-1131	367	σB	Outer capsid	Unknown
S4	1192	24-1124	367	σNS	Nonstructural	ssRNA binding

Figure 1.1. Protein profile of avian reovirus

Figure 1.1



(Adapted from Benavente and Martinez-Costas, Virus Research, 123:105-119)

2.7. Reassortments

Reassortment is common among segmented dsRNA viruses such as influenza virus, rotavirus and reovirus. During mixed infections, the segmented nature of reovirus genome allows genomes from different strains to undergo assortment, resulting in generation of progeny virions (reassortants) that contain a mixed set of gene segments from the two parental strains (Ramig and Ward, 1991). It is possible to identify parental origin of each gene segment in any reassortant when viewed in polyacrylamide gels. Suppose two strains that infect the same cell undergo a completely random assortment, the resulting progeny would have 2^n (where n is the number of gene segments) possible gene combinations of genome segments from the two parents. Thus, with 10 gene segments of reoviruses, there will be 2^{10} (1024) possible gene combinations, of which two are parental and the rest are reassortants. In practicality, only 3-25% progeny reassortants are produced from co-infection (Fields, 1971).

Precise understanding of these nonrandom segregation phenomena is lacking. However, two possible explanations are: (i) the failure of efficient reassortments results from homologous RNA segments that need to be exchanged while replicating in separate areas within an infected cell (Joklik and Roner, 1995) and (ii) viral RNAs and/or proteins from different parents may not interact effectively, so that potential reassortants would not survive (Roner et al., 1990). In addition, some genome segments or protein products from two different strains need accommodating mutations so that they can be productively paired (Nibert et al., 1996). That mixed infection/coinfection of different ARVs strains may lead to emergence of new strains has been proven in an experimental study by Ni and Kemp (1992). They co-infected chicken embryo fibroblasts with ARV

strain 883 and one of the three CRV strains (176, S1133, or 81-5). They reported that the selection of genome segments in coinfection was virus strain specific.

2.8. Diagnosis of reovirus

Virus isolation in embryonated chicken eggs, continuous cell lines or primary cells is most commonly used for the diagnosis of ARVs (Levisohn et al., 1980; al Afaleq et al., 1989). More sensitive molecular diagnostic techniques e.g., reverse transcription-polymerase chain reaction (RT-PCR), nested PCR, and multiplex PCR (Jindal et al., 2012; Pantin-Jackwood et al., 2008, Zhang et al., 2006) have also been developed and used for the detection of ARVs. Real time RT-PCR using the TaqMan® technology has recently been developed for the specific and sensitive detection of CRVs (Guo et al., 2011).

Serological methods such as virus neutralization, agar-gel precipitin assay, and enzyme-linked immunosorbent assay (ELISA) are often used for the detection of anti-ARV antibodies (Slaght et al., 1978; Adair et al., 1987). Unfortunately, most of the commercially available ELISAs cannot distinguish between antibodies from vaccinated and infected birds and they cannot differentiate antibodies produced by CRVs and TRVs. An ELISA has recently been developed to distinguish vaccine and wild type of ARV antibodies. Xie et al. (2010) used non-structural proteins as antigens to detect specific antibodies for ARV in serum samples from infected birds. Although these researchers were able to differentiate between infected and vaccinated sera, the sensitivity and specificity of the method were limited (61.1 to 88.9% of serum samples from infected

chickens tested positive, and 0 to 6.7% of serum samples from the vaccinated chickens tested positive).

Sigma C induces neutralizing antibodies and is the most variable protein among strains and isolates of ARVs. By using sigma C, Goldenberg et al. (2011) developed a differentiating ELISA, which enabled distinguishing between vaccine and field strains of the virus, identifying the infection source, and exclusively determining the level of protective antibodies. Whereas the whole virus detected antibodies against all strains, differentiating ELISA enabled differentiating between infected and vaccinated animals (DIVA) and in most cases, identifying the sigma C genotype.

2.9. Reovirus survival

Studies on the survival of pathogens in the environment are important in formulating preventive and control measures. Several reports indicate that CRVs are stable between pH 3.0 and pH 9.0 but are inactivated at 56°C in less than one hour (Jones, 2000). Some strains of CRV were found to be sensitive to trypsin (al-Afaleq and Jones, 1991). A few reports indicate that CRVs are relatively resistant to certain disinfectants. For example, they can survive in the presence of 2% formaldehyde at 4°C but their infectivity is affected by 2% phenol and partially by 100% ethyl alcohol (Meulemanns and Halen, 1982). The CARVs can survive in water for at least ten weeks with little reduction in virus titer (Savage and Jones, 2003). Litter and drinking water may play an important role in the transmission of reoviruses, but studies on survival of these viruses in poultry litter and drinking water are lacking.

2.10. Prevention and control

ARVs are ubiquitous in nature and hence maintaining an ARV-free flock is difficult. Strict biosecurity, good management practices, and vaccination are key methods to control ARV infection. Biosecurity and good management practices include minimizing entry into the barn, practicing all-in-all-out management, effective cleaning and disinfecting of the barn, and good record keeping. Wise use of effective disinfectants is also important. Several vaccines based on arthritic (1133) and enteric (1733, 2408) CRVs are available worldwide. Autogenous killed vaccines are also being used in flocks where commercial vaccines are not effective.

Because of the importance of this new disease condition (turkey lameness), I have planned my thesis work on comparative molecular characterization of TARV, TERV, and CARV. This will be helpful in differentiation of TARVs from TERVs and CARVs and may shed some light on the possibility of reassortments among these viruses. This work should be helpful in finding possible sources of TARV infection. In addition, I plan to develop an RT-PCR that can differentiate TARVs from TERVs and may be useful to quantitate virus load in field samples. I also plan to compare the survival of these viruses in litter and water and to test the efficacy of commonly used disinfectants against them. This information should be helpful in designing appropriate preventive and control measures.

Chapter 2: Isolation and characterization of a turkey arthritis reovirus

This work has been published as:

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1. Introduction

Reoviruses belong to genus Orthoreovirus in the family *Reoviridae* and contain a double-stranded RNA genome with 10 segments. The virus is non-enveloped with icosahedral symmetry and a particle size of 70-80nm (Spandidos and Graham, 1976). Based on migration pattern on polyacrylamide gel electrophoresis the 10 segments of the virus are grouped into large (L1, L2, L3), medium (M1, M2, M3), and small (S1, S2, S3, S4) (Benavente and Martinez-Costas, 2007; Varela and Benavente, 1994). The segmented genome encodes for eight structural and four non-structural proteins. The eight structural proteins are encoded as follows: 3 λ proteins (λ A, λ B and λ C) by L segments, two μ proteins (μ A and μ B) by M segments, and three σ proteins (σC, σA, σB) by the S segments (Varela and Benavente, 1994; Varela et al., 1996). Among non-structural proteins M3 and S4 segments encode two major non-structural proteins μNS and σNS, respectively, while p10 and p17 are two additional non-structural proteins encoded by the S1 segment (Bodelon et al., 2001; Shmulevitz et al., 2002).

Avian reoviruses differ from those of mammals; the former lack hemagglutination activity but can induce cell-cell fusion resulting in the production of syncytia in cell cultures (Glass et al., 1973). Both pathogenic and non-pathogenic strains of avian reovirus exist in nature with 85-90% of them being non-pathogenic (Jones, 2000). In addition to chicken and turkeys, reoviruses have been isolated from other avian species e.g., ducks and pigeons with diarrhea (Kaschula, 1950; Mcferran et al., 1976), grey parrots and quail with enteritis (Meulemans et al., 1983; Ritter et al., 1986), and American crows exhibiting nervous signs (Hustamo et al., 2007).

In chickens, avian reoviruses have been associated with various disease conditions such as malabsorption, "runting-stunting" syndrome, ulcerative enteritis, inclusion body hepatitis, cloacal pasting, and respiratory infections (Bagust and Westbury, 1975; Bains et al., 1974; Dutta and Pomeroy, 1969; Goodwin et al., 1993). In addition, they also cause lameness, arthritis and brittle bone disease/femoral head necrosis in chickens (van der Heide and Kalbac, 1975; Vertommen et al., 1980). For the purposes of clarity, we will consider turkey reovirus different than avian (chicken) reovirus.

Turkey enteric reoviruses have been isolated from cases of enteric diseases in turkeys such as the light turkey syndrome (LTS), poult enteritis complex (PEC), poult enteritis syndrome (PES), poult enteritis mortality syndrome (PEMS) (Jindal et al., 2010a, 2010b, Pantin-Jackwood et al., 2008), myocarditis (Shivaprasad et al., 2009), and lameness (arthritis/ tenosynovitis; Levisohn et al., 1980; Page et al., 1982). Recently, the Minnesota Veterinary Diagnostic Laboratory (MVDL) at the University of Minnesota saw an increase in the number of submissions of 15- to 18-week-old tom turkeys that were recumbent (lame) with wing tip bruises ("wing walkers") and uni- or bilateral swelling of hock (tibiotarsal) joints. Here we describe the isolation and partial molecular characterization (on the basis of the S4 gene) of turkey arthritis reoviruses from cases of lameness and tenosynovitis. The S4 gene is conserved and detects most diverse lineages of avian reoviruses (Pantin-Jackwood et al., 2008) as well as it codes for antigenic and pathogenic protein of the virus (Guo et al., 2011).

2. Materials and Methods

2.1. Source of samples

During the Spring and Summer of 2011, the MVDL at the University of Minnesota received 14 cases (one case refers to one flock) of 15- to 18-week-old tom turkeys from Minnesota with flock histories of lameness and arthritis. The lameness started as early as 10 weeks of age and affected at least 3-5% of the flock.

2.2. Gross lesions and histopathology

Dead or humanely euthanized lame tom turkeys were submitted to MVDL. In some cases, only the legs of the affected turkeys were submitted either fresh or frozen. The gastrocnemius and digital flexor tendons were removed and immersed in formalin. The formalized samples were placed in cassettes, embedded in paraffin, sectioned at 5 microns and stained with hematoxylin and eosin for histopathology. Tendons and/or joint fluids from these cases were examined for the presence of bacteria and viruses.

2.3. Bacterial isolation

The synovial swabs were tested for aerobic bacteria as well as for mycoplasma. For aerobic bacteria, the swabs were inoculated on sheep blood agar and MacConkey agar followed by incubation at 37^0C for 48 hours. For mycoplasma culture, the swabs were inoculated in Frey's broth and incubated at 37^0C for 2 weeks. The broth culture was tested by real time PCR for mycoplasma.

2.4. Virus Isolation

Tendon pools were homogenized as a 10% suspension in Hanks' balanced salt solution. Following centrifugation at 1,200 xg for 30 min, the supernatants were collected and inoculated into 11-day-old specific-pathogen-free embryonated chicken eggs via the allantoic route (200 µL per egg) and in 6-day-old specific-pathogen-free eggs via the yolk sac route. After four days of incubation at 37^0C, the allantoic fluids and embryo homogenates were processed for the next passage. A total of 3-5 blind passages were performed for each sample. In addition, the supernatants were inoculated onto QT-35 cells to determine if the virus can be grown in cell cultures. Up to five passages were given in these cells. The identity of the isolated virus was confirmed by negative contrast electron microscopy (Goyal et al., 1987) and reverse transcription-polymerase chain reaction (RT-PCR) (Jindal et al., 2010b).

2.5. Molecular characterization

RNA was extracted from homogenized tendon pools, infected allantoic fluids, embryo homogenates, and infected cell culture fluids by using a viral RNA mini kit (Qiagen, Valencia, CA). In brief, 140 µL of the sample was mixed with 560µL of AVL buffer and 5.6 µL of carrier RNA, allowed to react for 10 min, followed by the addition of 500µL of 100% ethanol. This mixture was transferred to RNA spin columns followed by two washings with RW1and RW2 buffer. The final elution was done in 50µL elution buffer. As a positive control, RNA was extracted from turkey enteric reovirus (SEP 108, kindly provided by Dr. J. M. Day, Southeast Poultry Research Laboratory, Athens, GA). Published primers of turkey reovirus S4 gene (σNS) were used for RT-PCR (Pantin-

Jackwood et al., 2008). The amplified products were run on 1.5% agarose gel and the appearance of a specific band at 1100bp confirmed the presence of the virus.

2.6. Sequencing

The amplified PCR products were purified using Qiagen PCR purification kit and then submitted for sequencing to the Biomedical Genomic Center (BMGC), University of Minnesota. The sequencing was done in both directions using the same primers as used in RT-PCR reactions. Forward and reverse sequences were aligned together using Sequencher software (www.msi.umn.edu) followed by BLAST analysis (www.ncbi.nlm.nih.gov). The nucleotide sequences thus obtained were aligned by the Clustal W method using MEGA 6.0 software (Tamura et al., 2013). The evolutionary distances were computed using the Maximum Composite Likelihood Model. A phylogenetic tree of aligned sequences was constructed by the neighbor-joining method using 1000 bootstrap replicate values.

2.7. GenBank Accession Numbers

Partial nucleotide sequences of S4 gene of reovirus were submitted to GenBank with the following accession numbers: JQ954690- JQ954694 for TARV-MN1 to TARV-MN5 and JQ954695 for ChARV-MN1 (a local isolate of chicken reovirus). The sequences from GenBank used for comparison are shown in Table 2.1.

3. Results

There was unilateral or bilateral swelling of hock joints in all affected turkeys. In many instances, the birds had periarticular and subcutaneous hemorrhage of the hock joint resulting from partial to complete tearing of the gastrocnemius or digital flexor tendon(s) (Fig. 2.1 and 2.2). There was increased serosanguinous fluid in the sheath of the origin of gastrocnemius tendon at the hock joint (Fig. 2.1 and 2.2). In some cases, there was subcutaneous hemorrhage under the skin of the hock joint. In mild cases, the joints and tendon sheaths contained increased amount of clear, straw-colored, or brown serosanguinous fluid. Histopathology of gastrocnemius and digital flexor tendons revealed lymphoplasmacytic infiltrates in the synovial sheath (Fig. 2.3). In some instances, likely chronic stages of lameness, the tendon sheaths exhibited synovial villonodular hyperplasia with mild lymphoplasmacytic inflammation (Fig. 2.4).

All samples were found negative for mycoplasma and for aerobic and anaerobic bacteria. No virus was isolated by the CAM route of embryonated chicken egg (ECE) inoculation. However, five of the 14 sample pools yielded reovirus in ECE when inoculated by the yolk sac route. Allantoic fluids and embryo homogenates were positive for reovirus when tested by RT-PCR. Negative contrast electron microscopy revealed the presence of non-enveloped spherical virions of ~70-80 nm size (Fig. 2.5). All five samples also yielded reovirus when inoculated in QT-35 cells. In both ECE and QT-35 cells, no virus was isolated at first passage; some were isolated at second passage and the others at passage 4 or 5. The isolated viruses were named as turkey arthritis reovirus (TARV-MN1 through TARV-MN5) to differentiate them from turkey enteric reoviruses.

Four of the isolates (TARV-MN1 to TARV-MN3 and TARV-MN 5) were from one hatchery source while TARV-MN4 was from a different hatchery.

Phylogenetic analysis was done on the basis of nucleotide sequence of the S4 gene. Blast analysis of sequences confirmed all viruses as reovirus. Phylogenetic analysis on the basis of 880bp nucleotides classified the five turkey isolates into two subgroups: the first subgroup contained TARV-MN1, -MN2, -MN3 and -MN5 while the second subgroup contained TARV-MN4. A local isolate of chicken reovirus (ChARV-MN1) isolated from chickens showing lameness was used for comparison. The chicken isolate grouped differently from the five TARVs showing only 78% similarity with the TARVs (Fig. 2.6).

The TARV-MN1, MN2, MN3 and MN5 had 97% to 100% similarity with each other. These four viruses showed similarity of 88.7% to 99.8% with enteric reoviruses detected in Minnesota turkey flocks with enteritis. Similarity with enteric reoviruses from healthy Minnesota flocks was 96.6% to 99.4%. Similarity with enteric reoviruses from other U.S. states was 89% to 97.6%. Isolate TARV-MN4 was different from these four TARVs with a similarity of only 89.2%. This isolate was 90% similar to enteric reoviruses from healthy flocks in Minnesota and 89.5% to 94.4% similar to those from enteritis-affected flocks in Minnesota. The similarity with U.S. isolates of enteric reoviruses was 90% to 93%. A local isolate of chicken reovirus (ChARV-MN1) from cases of lameness in chickens shared a similarity of only 78% with TARVs while it was 90.3% to 92.7% similar to chicken reoviruses associated with enteritis and runting stunting syndrome (RSS) in chickens.

4. Discussion

An outbreak of lameness/arthritis occurred in 15- to 18-week-old tom turkeys in Minnesota during the Spring and Summer of 2011. Flock histories suggested that lameness occurred as early as ten weeks of age and affected at least 3-5% of the flock with >1% mortality or culling per week. In severely affected flocks, up to 25% of birds were affected (Dr. David Mills, personal communication). Turkey arthritis reovirus (TARV) was isolated from tendons and/or joint fluids of birds from five of 14 flocks. No other pathogen was isolated.

This appears to be the first study on isolation of TARVs in Minnesota. In fact, there have been only three reports of reovirus isolation from the joints of turkeys from more than 20 years ago. In one instance, reovirus was isolated from the hock joints of lame turkeys, but virus did not localize in hock joint or cause lesions when inoculated into turkey poults (al Afaleq and Jones, 1989; 1991). In the second report, a reovirus-like agent was isolated from the hock joint of lame turkeys, but the infection was complicated by the presence of *Mycoplasma synoviae* (Levisohn et al., 1980), another common cause of tenosynovitis. Finally, a virus serologically similar to reovirus of chickens was isolated from 5- to 8-week-old turkeys with swollen hock joints (Page et al., 1982). The clinical and gross lesions observed in this study are very similar to those reported in the previous three studies.

Avian reoviruses have long been recognized as a cause of lameness (arthritis and tenosynovitis) in meat-type chickens, but turkeys are considered generally resistant to chicken-origin reoviruses (al Afaleq and Jones, 1989; 1990; 1991). Transmission of reoviruses in chickens has been shown to occur via feces, contaminated egg shells, and

by vertical transmission from infected hens to progeny (van der Heide and Kalbac, 1975). Lameness results from swelling of the tendon sheath of the shanks and gastrocnemius tendon immediately proximal to the hock. The affected birds are recumbent and are unable to extend the hocks. Reovirus-associated arthritis has also been reported in Hungarian geese (Palya et al., 2003). On hisopathologic analysis, infiltration of different mononuclear inflammatory cells in synovial layers of the tendon sheaths was observed. The mild lymphoplasmacytic synovitis and multifocal villonodular synovial hyperplasia of synovial membrane observed in our study are consistent with reovirus-associated arthritis in chicken and geese.

To the best of our knowledge, there are no previous reports on phylogenetic analysis of turkey arthritis reoviruses although enteric reoviruses from turkeys have been well characterized. Phylogenetic analysis of reovirus isolates in this study was done on the basis of the S4 gene. Pantin-Jackwood et al. (2008) reported that, as a group, turkey enteric reoviruses had 90% to 100% homology and did not demonstrate any geographical predilection. Similarly, Jindal et al. (2010a) reported that reoviruses associated with poult enteritis syndrome in Minnesota shared 91.6% to 99.3% homology with previously published enteric reoviruses. Both of these studies reported that turkey reoviruses clustered into one group and assortment occurred at the species level (chicken or turkey). In our study also the assortment occurred primarily by the species of origin (chicken and turkey) and all turkey isolates clustered into one group. The ChARV-MN1 shared 78% homology with turkey isolates of this study, which is in agreement with previous studies reporting 73.6% to 83.1% similarity between enteric reoviruses of chickens and turkeys (Jindal et al., 2010b, Pantin-Jackwood et al., 2008). Because there is no sequence of

turkey arthritis reovirus as well as less number of enteric reovirus S4 gene sequences in GenBank, this study did not reveals a specific pattern of assortment between enteric and arthritis reoviruses as well as assortment based on geographical locations. A better picture will come when more arthritis reoviruse sequences from different geographical locations will be available in GenBnak.

Based on phylogenetic divergence, the TARV isolates formed two subgroups. Subgroup I contain TARV isolates (TARV-MN1, 2, 3 and 5) with 88.7% to 99.8% homology with viruses associated with enteritis in Minnesota turkey flocks. Subgroup II contains a single isolate, the TARV-MN4, which had 10.8% divergence of from subgroup I. The hatchery that yielded subgroup II viruses was different than that from which subgroup I viruses were isolated.

Clark et al. (1990) who divided chicken reoviruses into three pathotypes on the basis of correlation between genotypes and specific disease: pathotype I caused transient digestive system disorder (TDSD), pathotype II caused viral arthritis syndrome (VAS), while pathotype III caused both TDSD and VAS. It is not yet known if a similar situation occurs in turkey enteric and turkey arthritis reoviruses.

The source of the TARV-MN viruses is not clear at this time. It is possible that infected poults have undiagnosed lameness at a young age which later recrudesces when the birds are much heavier and older. Dhillon et al. (1986) isolated reovirus from cases of arthritis/tenosynovitis in commercial layers farms in Western Washington. The source of infection was traced to a breeder flock that supplied progeny chicks to all of the affected farms. Enteric reoviruses have been detected in cases of poult enteritis syndrome in Minnesota turkeys for up to 9 weeks of age but not after that.

Phylogenetically, TARV 1, 2, 3 and 5 are closely related to enteric reoviruses which indicates the possibility that enteric reovirus may localize in hock joints in older age. We plan to do complete genome sequence analysis to figure out differences between arthritis-associated and enteritis-associated reoviruses in turkeys with the ultimate goal of finding specific molecular markers for both. Experimental studies are planned to determine if turkey reovirus is the primary cause of arthritis and lameness. If these turkey reoviruses demonstrate behavior similar to that of chicken reovirus, there is a strong possibility that the infection goes well beyond market-age turkeys and that infected breeder flocks might be propagating this virus and spreading it vertically to young birds, thus complicating efforts to detect and control reovirus infection in turkeys. In summary, this appears to be first report of isolation of reovirus from outbreak of lameness in a long time. Experimental studies are in progress to learn about the source of infection, mode of transmission, and methods of control for this pathogen.

Figure 2.1. Sixteen-week-old, reovirus-positive tom turkey with periarticular swelling and bruising of hock joint.

Figure 2.1

Figure 2.2. Sixteen-week-old, reovirus-positive tom turkey with rupture of gastrocnemius tendon at level of hock.

Figure 2.2

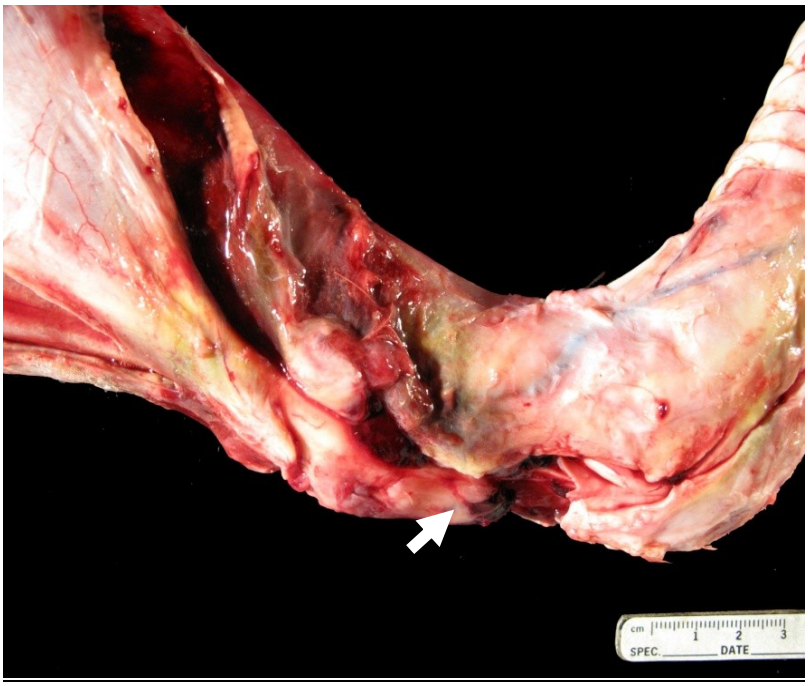


Figure 2.3. H&E staining histopath picture showing mild lymphoplasmacytic infiltrates.

Figure 2.3

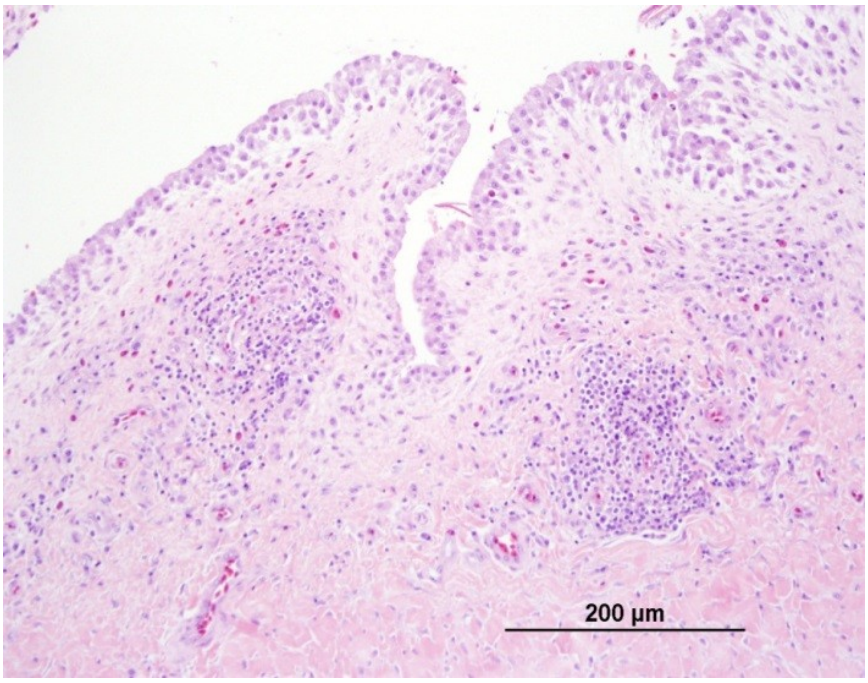


Figure 2.4. Tendon sheaths exhibiting synovial villonodular hyperplasia with mild lymphoplasmacytic inflammation.

Figure 2.4

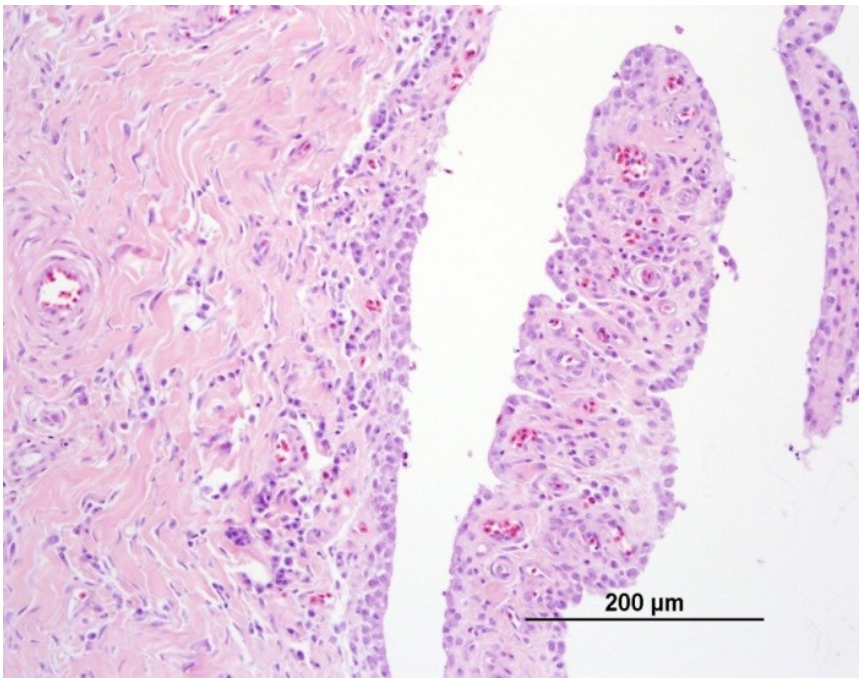


Figure 2.5. Electron Microphotograph, negative contrast of a non-enveloped spherical ~ 70- 80 nm dsRNA virion.

Figure 2.5

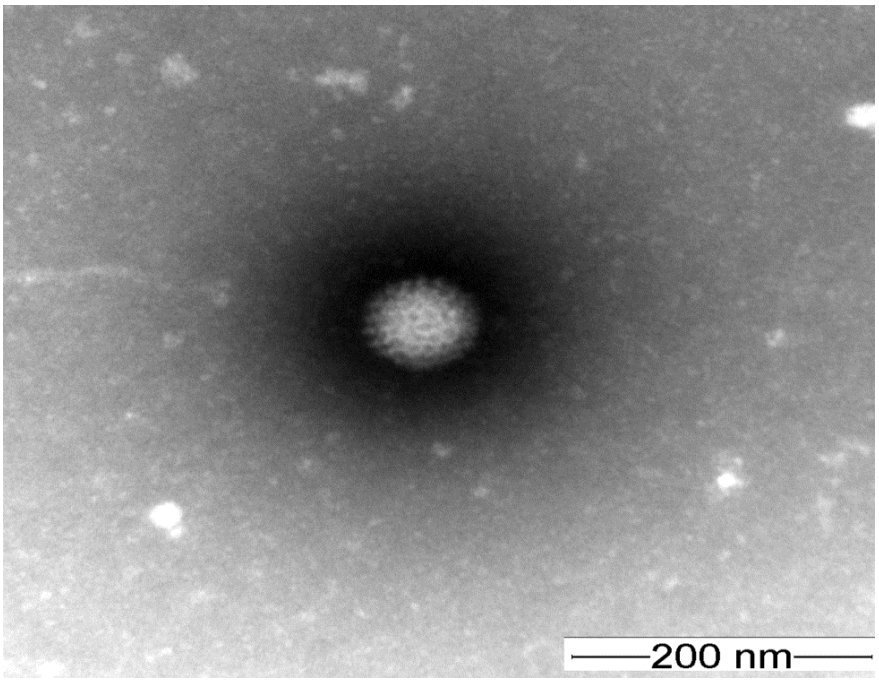
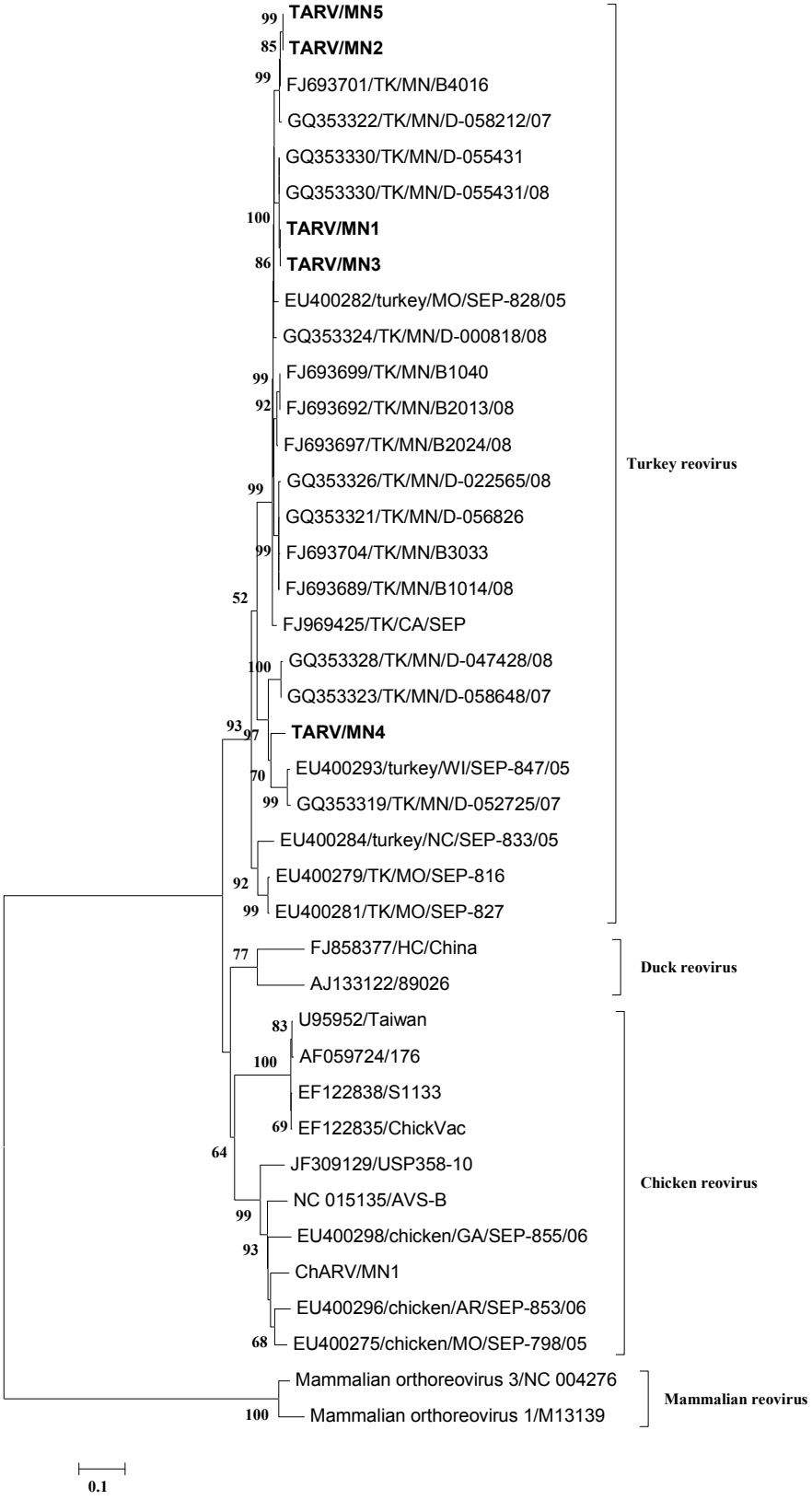


Figure 2.6. Phylogenetic analysis on the basis of 880bp nucleotides of S4 gene of reovirus by using MEGA 6.0. Neighbor-joining tree was constructed using the Kimura 2-parameter model with 1,000 bootstrap replicates and a 70% cut-off.

Figure 2.6


TARV/MN5
TARV/MN2
FJ693701/TK/MN/B4016
GQ353322/TK/MN/D-058212/07
GQ353330/TK/MN/D-055431
GQ353330/TK/MN/D-055431/08
TARV/MN1
TARV/MN3
EU400282/turkey/MO/SEP-828/05
GQ353324/TK/MN/D-000818/08
FJ693699/TK/MN/B1040
FJ693692/TK/MN/B2013/08
FJ693697/TK/MN/B2024/08
GQ353326/TK/MN/D-022565/08
GQ353321/TK/MN/D-056826
FJ693704/TK/MN/B3033
FJ693689/TK/MN/B1014/08
FJ969425/TK/CA/SEP
GQ353328/TK/MN/D-047428/08
GQ353323/TK/MN/D-058648/07
TARV/MN4
EU400293/turkey/WI/SEP-847/05
GQ353319/TK/MN/D-052725/07
EU400284/turkey/NC/SEP-833/05
EU400279/TK/MO/SEP-816
EU400281/TK/MO/SEP-827
Turkey reovirus
FJ858377/HC/China
AJ133122/89026
Duck reovirus
U95952/Taiwan
AF059724/176
EF122838/S1133
EF122835/ChickVac
JF309129/USP358-10
NC 015135/AVS-B
EU400298/chicken/GA/SEP-855/06
ChARV/MN1
EU400296/chicken/AR/SEP-853/06
EU400275/chicken/MO/SEP-798/05
Chicken reovirus
Mammalian orthoreovirus 3/NC 004276
Mammalian orthoreovirus 1/M13139
Mammalian reovirus
0.1

Chapter 3: Characterization of S class gene segments of a newly isolated turkey arthritis reovirus

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1. Introduction

Avian reoviruses (ARVs) belong to the family *Reoviridae* under genus *Orthoreovirus*. The ARVs are non-enveloped, double-stranded RNA viruses with icosahedral symmetry and a particle size of 70-80 nm (Varela and Benavente, 1994). As opposed to mammalian reoviruses, the ARVs are fusogenic with the ability to cause fusion of infected cells resulting in multinucleated syncytia formation (Benavente and Martinez-Costas, 2007). The viral genome consists of 10 segments that are divided into three classes namely large (L), medium (M), and small (S), depending on their migration pattern on polyacrylamide gel electrophoresis (Benavente and Martinez-Costas, 2007; Varela and Benavente, 1994). The L and M genes are further subdivided into three segments each (L1, L2, L3 and M1, M2, M3, respectively) while the S gene has four segments (S1, S2, S3, S4; Spandidos and Graham, 1976).

The ARV genome has 12 open reading frames (ORFs), which encode for eight structural and four non-structural proteins. The structural proteins are an important part of progeny virions while non-structural proteins are expressed only in infected cells (Martinez-Costas et al., 1997). The proteins encoded by L, M and S genes are lambda (λ), mu (μ) and sigma (σ), respectively. Three structural proteins λA, λB and λC are encoded by L gene segments L1, L2, and L3, respectively. M1 and M2 segments encode two structural proteins (μA and μB) while M3 segment encodes a non-structural protein (μNS). The three σ proteins σC, σA, σB are encoded by the S1, S2, S3 segments, respectively, while the S4 segment encodes for non-structural protein σNS (Varela and Benavente, 1994; Varela et al., 1996). The S1 segment encodes for two additional non-structural proteins; p10 and p17 (Bodelon et al., 2001; Shmulevitz et al., 2002). The σC

protein encoded by S1 gene possesses both type and broad specific epitopes while σB protein, which is a major outer capsid protein encoded by S3 gene, has group-specific neutralizing epitope (Wickramasinghe et al., 1993). The σA protein is responsible for double-stranded RNA (dsRNA) binding and resistance to interferon. The non-structural protein σNS (encoded by S4 gene) is responsible for single-stranded RNA (ssRNA) binding (Martinez-Costas et al., 2000; Yin and Lee, 1998).

ARVs are ubiquitous in domestic poultry with 85-90% of them being non-pathogenic (Jones, 2000). Of the diseases caused by ARVs, viral arthritis/tenosynovitis in chickens is the most common but respiratory disease, immunosuppression and enteric disease can also occur (Rosenberger, 2003). Turkey reoviruses (also called turkey enteric reoviruses or TERVs) have been detected in the gastrointestinal tracts of both healthy and enteritic turkeys. The TERVs form a distinct group within the *Reoviridae* family based on the genetic analysis of S1, S3 and/or S4 genome segments (Day et al., 2007; Jindal et al, 2010a; Pantin-Jackwood et al., 2008).

Recently, we isolated reoviruses from cases of arthritis and lameness in tom turkeys and partially characterized them on the basis of their S4 genome segment (Mor et al., 2013). To differentiate these viruses from TERVs, we have named them as turkey arthritis reoviruses (TARVs). In the early 1980s, reoviruses were detected in arthritic turkeys (Levisohn et al., 1980; Page et al., 1982) without any further reports on the occurrence of these viruses. Recently, we conducted an experimental study in turkey poults using TARVs and TERV isolates; only TARVs were able to produce tenosynovitis in turkey poults when inoculated orally at two weeks of age (Sharafeldin et al., 2014). In the field, TERVs have been detected in both enteritis- affected and apparently healthy

turkey poults for several years (Jindal et al., 2010a; 2010b; Pantin-Jackwood et al., 2008) but the problem of lameness in turkeys was not observed until recently in late 2009. Based on this information, we hypothesized that TARVs are genetically different from TERVs. In addition, new variants of chicken reovirus (CRV) causing lameness and arthritis in commercial broilers at the age of 2.5 to 8 weeks have been reported in Europe and North America since 2011 (Rosenberger et al., 2013a; Sellers et al., 2013; Troxler et al., 2013). Hence we undertook this study to characterize TARV isolates based on their S class genome segments and to compare them with CRVs and TERVs.

2. Materials and Methods

2.1. Virus isolates

A total of 12 isolates of TARVs were used in this study; ten were isolated in our laboratory from lame turkeys at the age of 5-18 weeks and two isolates (TARV-O'Neil and TARV-Crestview) were obtained from Dr. Jack Rosenberger of AviServe, who also isolated them from cases of turkey lameness. Of the 10 isolates from our laboratory, five (TARV-MN1, TARV-MN2, TARV-MN3, TARV-MN4, and TARV-MN5) have been partially characterized based on their S4 gene (Mor et al., 2013). Five newer isolates included in this study are: TARV-MN6, TARV-MN7, TARV-MN8, TARV-MN9, and TARV-MN10. One TERV isolate from our lab (TERV-MN1) was also used. All 13 viruses were isolated and propagated in QT-35 cells as described previously (Mor et al., 2013).

2.2. RNA extraction and RT-PCR

Cell culture supernatants from infected QT-35 cells were used for RNA extraction using a QIAamp viral RNA mini kit (Qiagen, Valencia, CA). Primers were designed mainly from 5' and 3' UTR regions by aligning with available sequences for CRVs and TERVs (Table S3.1). All RT-PCR reactions were carried out using Qiagen One step RT-PCR kit (Qiagen, Valencia, CA). The reactions (50µl volume) were run in an Eppendorf thermocycler for 30 min at 50^{o}C and 15 min at 95^{o}C (RT step) followed by 35 PCR cycles with denaturation at 94^{o}C for 1 min, annealing at respective temperatures for each primer for 1 min, and elongation at 72^{o}C for 1 min. Final elongation was done at 72^{o}C for 10 min. PCR products were visualized on 1.2% agarose gel in Tris-acetate-EDTA buffer by electrophoresis and the appearance of a specific band of expected size confirmed the amplification.

2.3. Sequencing

The amplified PCR products were purified using Qiagen PCR purification kit and then sequenced at the University of Minnesota Genomic Center (UMGC). The sequencing was done in both directions using the same primers as used in RT-PCR reactions. Forward and reverse sequences were aligned together using Sequencher 5.1software (www.genecodes.com) followed by BLAST analysis (www.ncbi.nlm.nih.gov). The nucleotide sequences thus obtained were aligned by the Clustal W method using MEGA 6.05 software (Tamura et al., 2013). The TARV and TERV sequences of this study were compared with ARV sequences of different segments available in GenBank. The evolutionary distances were computed using the *p*-distance method and phylogenetic

trees of aligned sequences were constructed using Maximum Likelihood method with 2000 bootstrap replicate values. The best Maximum Likelihood model for analysis of DNA and protein sequences of each segment was selected on the basis of the lowest BIC score (Bayesian Information Criterion) in MEGA 6.0 (Tamura et al., 2013). We used GTR (General Time Reversible) +G (Gamma distribution with 5 rate categories) +I (Evolutionary Invariable sites) and Hasegawa-Kishino-Yano (HKY) +G models for analysis of S1 and S4, respectively, and Kimura 2-Parameter (K2) +G model for analysis of S2 and S3 sequences. The amino acid histogram was constructed using Geneious Pro (Drummond et al., 2011). Chicken arthritis reovirus strain S1133 was used as a reference strain throughout this study. The term Lineage is used for differentiation of genetic linkages of S class genes and is defined as a cluster of genetically related viruses with less than 10% nucleotide divergence. However, direct epidemiological linkage was considered if there was less than 2% nucleotide divergence between strains (Liu et al., 2003). In this study, TARVs and TERVs within a lineage were further divided into groups based on more than 2% nt divergence for better understanding of relationship within TARVs and between TARVs and TERVs.

2.4. GenBank accession numbers

All 12 TARV sequences were submitted to GenBank with accession numbers KF87231-KF87242 for S1 gene sequences while S2, S3 and S4 genes sequences had accession numbers KF87243-KF87254, KF87255-KF87266, KF87267-KF87278, respectively. TERV-MN1 sequences were also submitted with accession numbers KJ700478, KJ700479, KJ700480 and KJ700481 for S1, S2, S3 and S4 gene, respectively.

3. Results

The sequences of different S class genome segments of 12 TARVs and one TERV were analyzed and compared with those of TERVs and with reoviruses of chicken, duck and goose (CRV, DRV, and GRV, respectively).

3.1. Analysis of S1 gene segment

Based on p10 (100aa, 300nt; ORF1) sequences of S1 gene, all TARVs from this study grouped together in lineage I with 97% to 100% nucleotide (nt) and 97%-100% amino acid (aa) identity with each other (Table 3.1). The TERVs, however, formed three distinct lineages. Within lineage I, some TERVs (TERV-MN1, NC/98, NC/SEP-R108 and NC/SEP-R61) had 93.0%-97.0% and 92.0%-97.0% nt and aa identity, respectively, with TARVs. Lineage II TERVs (TX 98, TX99 and PEMS/85) which had 87.5% to 90.5% nt and 87.0% to 90.5% aa identity with TARVs while lineage III TERVs (NC/SEP-R044) had only 61.8% to 64.0% and 54.0% to 56.4% nt and aa identity with TARVs. The CRV, DRV, and GRV had only 70.0% to 72.5% (74.0-76% aa), 48.8% to 50.0% (32.6-33.7% aa) and 34.0% to 35.0% (32.6-33.7% aa) nt identity with TARVs, respectively (Table 3.1).

The aa 1-22 at NH_2- terminal region of p10 had four amino acid substitutions (L2S, M4P, P5S, P6S) in TARVs and TERVs as compared to reference strain of CRV. Insertions and substitutions of amino acids were also observed in TARVs and TERVs when compared to CRV reference strain. The predicted transmembrane motif in TARVs and TERVs was 44-YL(A/V)(A/V)GGGVLLLLIVVVAVIY-63 as compared to 44-YLAAGGGFLLIVIIFALLY-62in CRV. Five TARVs (TARV-MN2, TARV-MN4,

TARV-MN5, TARV-MN6 and TARV-MN7) had V instead of A at the third position (46) of this motif and the remaining seven TARVs had A at this position. In addition TARV-MN6 had V at aa position 47 instead of A in the remaining 11 TARVs. There was an insertion of V at aa position 51 of this motif in all TARVs, TERV-MN1, and two of seven previously published TERVs (NC/98 and NC/SEP-R61). The five remaining TERVs had insertion of I instead of V.

Sequence analysis of ORF p17 (151aa, 453nt; ORF2) also gave almost the same tree topology and per cent identities as p10 (Table 3.1). However, the nuclear localization signal (NLS) motif in p17 of TARVs had different amino acid composition (119-VTAKRSRG(I/V)D-128) as compared to CRV reference strain (119-IAAKRGRQLD-128).

Based on ORF3 (σC; 326aa and 981nt) of S1 gene, the TARVs and TERV-MN1 grouped in lineage I. Previously published TERVs were again divided into three lineages but with more sequence divergences within and between lineages (Table 3.1). Lineage I contained all TARVs with 93.3% to 100% nt and 93%-100% aa identity. TARVs and TERVs in lineage I were further divided into five groups based on criteria of more than 2% nt divergence: group 1 included nine TARVs, group 2 had TERV-MN1, group 3 had TARV-MN4, group 4 had TARV-MN5 and TARV-MN6, and group 5 included three previously published TERVs (NC/SEP-R61, NC/98, NC/SEP/R108) (Fig. 3.6A). TERV-MN1 (group 2) had a minimum of 2% aa divergence from all TARVs with three aa substitutions (N69D, Q122R, N236S). One aa substitution (D41G) was found only in TARV-MN4.

We observed some aa substitutions only in certain isolates from a particular state such as TARV-MN6 isolate from North Carolina had seven amino acid substitutions (K66E, S71T, G94S, K132Q, S141N, D215E and A245T) while South Dakota isolate (TARV-MN7) had four aa substitutions (C27G, V38A, G52S, A245V). The Iowa isolate (TARV-MN10) had nine aa substitutions (S26N, L40I, D41K, T43N, L46W, L47V, S50Y, A60D, L65I), which were found only in the starting 1-70 aa region of σC. The previously published TERVs in lineage I (NC/98, NC/SEP-R108 and NC/SEP-R61) had 92.0%-95.5% nt identity (93.0%-95.7% aa) with TARVs. Three previously published TERVs (TX 98, TX99 and PEMS/85; Fig. 3.1) of lineage II had 82.0%-87.0% nt and 83.0%-87.0% aa identity with TARVs. Lineage III included one previously reported TERV (NC/SEP-R44), which had 40.2% to 44.6% nt and 32.3% to 33.2% aa identity with lineage I and II sequences. The CRV, DRV, and GRV sequences had 55.6% to 56.4%, 42.8% to 43.6% and 42.0% to 42.5% nt identity, respectively (Table 3.1).

The σC protein contained three conserved motifs, 221-AHCHGRRTDYMMS-233, 273-ASFPVDVSF-281 and 319-LTVRTGIDT-327 in all TARVs, all TERVs (except NC/SEP-R44), and CRVs. Most of the variations occurred from position 40 to 130 aa (Fig. 3.2). At position 16 to 155 of the reference CRV strain, we found heptapeptide repeat pattern in all TARVs with a polar amino acids at positions a and d of the heptad.

3.2. Analysis of S2 gene segment

Based on 1180 nt of S2 gene segment, TARVs were divided into two lineages (Fig. 3.3, Fig. 3.6B). Lineage I included all TARVs (except TARV-MN9 and TARV-MN10) and

TERV-MN1with 98.4% to 100% nt and 99.2% to 100% aa identity. Lineage II had TARV-MN9, TARV-MN10 and a previously published TERV isolate Muntrilj 06 (FJ606766) and had 90.1%-91.3% nt and 96.0% to 97.0% aa identity with lineage I sequences. The CRV, DRV, and GRV had 86.8% to 91.4%, 72.6% to 75.0% and 72.8% to 74.0% nt identity, respectively, with all TARVs (Table 3.1). Four aa substitutions (I45L, V52A, A106T, I225V) were observed in TARV-MN9 and TARV-MN10, which makes these isolates divergent from lineage I TARVs. The sequence of epitope II motif in σA protein was different (QWVVAGLVSAT/A) in all TARVs as compared to that in the reference strain of CRV (340-QWVMAGLVSAA-350). All TARVs had V at the fourth position (343) instead of M while four TARVs (TARV-Crestview, TARV-O'Neil, TARV-MN1 and TARV-MN3) had T instead of A at the last position (350) of this motif.

3.3. Analysis of S3 gene segment

The S3 gene sequence analysis on the basis of 1000 nt divided all TARVs, TERV-MN1 and previously published TERVs into two lineages (Fig. 3.4, Fig. 3.6C). Lineage I included 11 TARVs (except TARV-MN4) along with previously published TERVs from healthy and enteritis-affected poults from Minnesota and other states in the US. Within lineage I, all 11 TARVs, TERV-MN1 and previously published TERVs had 92.0% to 100% nt identity with each other. Within lineage I, TARVs and TERVs were further clustered into six different groups (Groups 1 to 6). Group 1 included seven TARVs, TERV-MN1, 12 previously published TERVs from apparently healthy turkey poults (TK/MN/B-AB16/2008, TK/MN/B-AB18/2008, TK/MN/B-AB19/2008, TK/MN/B-AB20/2008, TK/MN/B-AB21/2008, TK/MN/B-AB22/2008, TK/MN/B-AB23/2008,

TK/MN/B-AB24/2008, TK/MN/B-AB25/2008, TK/MN/B-AB26/2008, TK/MN/B-AB27/2008, TK/MN/B-AB29/2008), and three TERVs from enteritis-affected poults (TK/MN/D-AB14/2011, TK/MN/D-AB15/2011, TK/MN/D-AB06/2008) in Minnesota. Group 2 included TARV-MN5, TARV-MN6 and one previously published TERV (NC/SEP-R44). Groups 3, 5 and 6 included previously published TERVs while group 4 included two TARVs of this study (TARV-MN9 and TARV-MN10; Fig. 3.6C). Lineage II included TARV-MN4 and previously published TERV strains (TK/MN/D-AB01/2007, TK/MN/D-AB04/2008, TK/MN/D-AB08/2008, TK/MN/D-AB10/2009, TK/MN/D-AB11/2010, TK/MN/D-AB12/2010, and TK/MN/D-AB13/2010) from enteritis-affected turkey poults in Minnesota. Lineage II sequences had 88.9% to 90.0% nt and 95.0% to 95.3% aa identity with lineage I sequences. The CRV, DRV, and GRV had 68.2% to 72.2%, 59.8% to 60.5% and 59.7% to 60.3% nt identity, respectively, with TARVs (Table 3.1).

On comparing σB protein sequences we found nine C and H residues from aa position 38 to 76. Random amino acid substitutions were observed throughout the σB protein in all TARVs and TERVs but three amino acid substitutions (I100V, R138S, and Q146R) were observed only in lineage II sequences. A conserved CHCC zinc binding motif was also present from amino acid positions 51-75 similar to that in CRVs except three substitutions (T61A, L62P, and A64S) in all TARVs and TERVs. At position 69, we found substitution of Y to H in three TARVs (TARV-Crestview, TARV-O'Neil, TARV-MN1) and Y to C in two TARVs (TARV-MN5, TARV-MN6), respectively. At C-terminus of σB, a basic aa motif KKVSHYR (from 287-293 aa position in reference

strain) was observed in all TARVs and TERVs. Within this motif, all TARVs and TERVs had V at position 289 while all CRVs used for comparison had A at this position.

3.4. Analysis of S4 gene segment

Phylogenetic analysis based on 950 nt of S4 gene divided TARVs, TERV-MN1 and previously published TERVs into three lineages (Fig. 3.5, Fig. 3.6D). Lineage I included all TARVs (except TARV-MN4),14 TERVs (TK/MN/B1016/08, TK/MN/B1038/08, TK/MN/B1040/08, TK/MN/B2013/08, TK/MN/B2014/08, TK/MN/B2015/08, TK/MN/B2016/08, TK/MN/B2021/08, TK/MN/B2022/08, TK/MN/B2024/08, TK/MN/B2028/08, TK/MN/B3030/08, TK/MN/B3033/08, TK/MN/B4016/08) from healthy poults and five TERVs (TK/MN/D-049007/07, TK/MN/D-000818/08, TK/MN/D-022565/08, TK/MN/D-048814/08, TK/MN/D-055431/08) from enteritic turkeys with 96.8% to 100% nt and 97.7% to 100% aa identity with each other. In lineage I, all sequences were divided into six groups: group 1 included 10 TARVs (except TARV-MN4, TARV-MN5) and two TERVs (TK/MN/D-048814/08, TK/MN/D-055431/08) from enteritis-affected poults in Minnesota while TARV-MN5 clustered with a TERV (TK/MN/B4016/08) in group 3. TERV-MN1 clustered into group 2 along with 10 previously published TERVs from apparently healthy poults in Minnesota. Groups 4, 5 and 6 included TERVs from Minnesota and other states in the US (Fig. 3.6D). Lineage II had TARV-MN4 and two previously reported TERVs from Wisconsin (WI/SEP-847/05) and Minnesota (TK/MN/D-052725/07). Lineage II sequences had 88.7% to 90.2% nt and 97.3% to 97.5% aa identity with those of lineage I and III. Lineage III contained TERVs from apparently healthy poults from North Carolina and Missouri; they

had 89% to 92.5% nt identity with lineage I and II sequences. The CRV, DRV, and GRV had 77.4% to 79.8%, 75.0% to 76.6% and 75.7% to 76.3% nt identity with TARVs, respectively (Table 3.1). A conserved motif MLDMVDGRP (aa180-188 of CRV S1133), which is considered as epitope B on σNS, was present in all TARVs and TERVs. TERVs of lineage II had one substitution (M to I) at position 183.

3.5. Possible reassortment

Random point mutations were observed across the S class gene segments in TARVs. Phylogenetic trees of all four S class gene segments were analyzed to test the possibility of reassortment. TARVs separated into lineages I and II in S2, S3 and S4 gene segments' phylogeny and grouped together in lineage I in σC phylogeny. However, within lineage I, TARVs and TERVs were further divided into different groups such as five groups in σC and six each in S3 and S4 gene segments (Fig. 3.6, Table 3.2). Interestingly, TERV isolate (NC/SEP-R44), which formed lineage III in σC, was grouped together with TARV-MN5 and TARV-MN6 in group 2 of lineage I in S3 gene segment. TARV-Crestview, TARV-O'Neil, TARV-MN1 were in the same group and lineage in all four gene segments. Three TARVs (TARV-MN5 and TARV-MN6) clustered in the same group in S1, S2 and S3 phylogeny but were divided into two different groups in S4 gene segment phylogeny. TARV-MN4 grouped with lineage I TARVs in σC and S2 phylogeny but formed lineage II with TERVs in S3 and S4 gene segments. TERVs in lineages I and II in S3 gene formed different groups in lineages I, II and III in sigma C (Fig. 3.6, Table 3.2).

3.6. Comparison of TARVs based on hatchery source

All TARVs and TERV were isolated from commercial turkeys and not from breeder flock. However, when we traced back hatchery source of these TARV-infected birds, it was found that the source only two hatcheries (Hatchery A and B). The maximum number of isolates (n=10) were from hatchery A but had been isolated over a three year period (2011-2013). Six of 10 TARVs were isolated in 2011, one in 2012 and three in 2013. All 10 isolates related to hatchery A were isolated from four different states with maximum number from Minnesota. Two isolates were from hatchery B and the birds for these two isolates belonged to two different states and two different age groups. The maximum aa substitutions among TARVs from the same hatchery as well as TARVs from different hatcheries was observed in σC followed by σB protein. TERV-MN1 was related to hatchery B and was isolated from four-week-old turkey poults suffering from enteritis. Both TARVs and one TERV of hatchery B had some unique aa substitutions indicating importance of point mutation between different pathotypes of the same hatchery source.

3.7. Genotype versus pathotype

Geographical and temporal analysis of TARVs with TERVs was performed; aa substitutions were observed among isolates from different states and time of isolation. We also found aa substitutions among TARVs isolated from different age groups of affected birds. There was no distinction of genetic lineages based on pathotypes (enteritis or arthritis) since different pathotypes of TERVs and TARVs grouped together in different lineages of S class gene segments.

4. Discussion

The problem of lameness and arthritis caused by TARVs appears to be re-emerging in U.S. turkeys (Mor et al., 2013); the disease has been seen in the upper Midwest since late 2009 after its initial reports in the 1980s and 1990s (al Afaleq et al., 1989; 1991; Levisohn et al., 1980). At about the same time, new variants of CRV causing lameness in commercial broilers were reported in Europe and North America. Some of these variants are genetically different from previously reported CRVs making the commonly used commercial reovirus vaccines ineffective (Rosenberger et al., 2013a; Sellers et al., 2013; Troxler et al., 2013). This study was undertaken to characterize the S class gene segments of TARVs and to compare them with those of CRVs and TERVs.

Based on amino acid and nucleotide sequence alignment of the S1, S2, S3 and S4 gene segments, all TARVs grouped differently from CRVs, DRVs and GRVs although TARVs were related to CRVs followed by DRVs and GRVs based on S2 gene phylogeny. The criteria developed by the International Committee on Taxonomy of Viruses (ICTV) for species demarcation include nucleotide identity >75% within species versus <60% between species (King et al., 2012). The amino acid identity for species demarcation for conserved core proteins includes >85% identity within species and <65% between species. For divergent outer capsid proteins it should be >55% identity within a species and <35% between species. Based on these criteria, all TARVs and TERVs grouped together under genus *Orthoreovirus* in the family *Orthoreoviridae* but formed a different group from other avian reoviruses (CRV, DRV, and GRV).

The maximum nt divergence in S3 and S4 gene segments of TARVs followed by S1 gene (σC) and maximum aa divergence in σC protein followed by S3 and S4 gene

segments (Table 3.1) indicated higher non-synonymous changes than synonymous changes (Table 3.1). In chickens, Liu et al. (2003) reported maximum aa divergence of 53% in σC protein of CRVs. Sellers et al. (2013) reported two groups (groups 1 and 2) of new variants of CRVs based on σC protein with <50% aa similarity between the two groups. Troxler et al. (2013) characterized new variants of CRV causing lameness and tenosynovitis in free range and commercial broilers in France and reported three groups of new variants of CRVs based on σC protein. The possible reasons for lower divergence in TARVs could be: (i) eight of the 12 TARVs (six were from Minnesota, one from North Carolina and one from Wisconsin) were isolated during the onset of disease problem in 2011, (ii) All TARVs originated from two different hatcheries; 10 of these were isolated from different commercial turkey flocks from 2011 to 2013 in which the poults originated from a single hatchery (hatchery A) making it possible that we isolated similar types of strains at that time point. However, the 2012 and 2013 isolates were from different breeder flocks of hatchery A and (iii) the virus is a newly emerging pathogen and has not gone through selective pressure. We found maximum aa substitutions in σC followed by σB protein between TARVs from same or different hatcheries. This indicated that isolates from the same hatchery source were closely related but some divergence occurred over time, age and location of affected flocks.

We found random aa substitutions throughout the S class but did not find any specific sequence motif that could differentiate TARVs from TERVs. Jindal et al. (2014) found a total of 35 aa substitutions in σB protein of which 22 and four were observed in TERVs from enteritis-affected and apparently healthy poults in Minnesota, respectively, and the remaining were observed in both types of flocks. Further studies are necessary to

determine if these aa substitutions have any role in tissue tropism and pathogenicity of the virus. We did find conserved motifs (specifically in σC protein, Fig. 3.2) in all S class segments across different avian reovirus species indicating the possibility of developing a universal vaccine.

Reassortment and point mutations are common among segmented dsRNA viruses. All TARVs and TERVs were mainly divided into lineages and groups within lineages in different S gene segments (Fig. 3.6, Table 3.2) indicating the possibility of reassortment among TARVs as well as between TARVs and TERVs. Liu et al. (2003) reported reassortment among CRVs and concluded that each lineage of S class genome segment consisted of a mixture of different pathotypes of CRVs (enteritis vs arthritis). They further surmised that co-evolution of different pathotypes of CRVs may have occurred. Banyai et al. (2011) suggested that multiple reassortments and strong divergence were likely reasons for genetic heterogeneity in AVS-B strain of CRV.

Clustering pattern of turkey reoviruses in this study suggests that co-evolution of different pathotypes of turkey reoviruses may have occurred. The occurrence of TERVs in apparently healthy turkey flocks is well known (Jindal et al., 2010b; Pantin-Jackwood et al., 2008). If such flocks are infected with a TARV at some point, it may lead to exchange of genetic material thereby increasing the chances of reassortment. This is particularly true in breeder or layer flocks where birds are kept for a longer period of time and the viruses have enough time to undergo mutation and reassorment. Co-infection with different viral strains is one method by which new strains emerge. In an experimental study, Ni and Kemp (1992) co-infected chicken embryo fibroblasts with

ARV strain 883 and one of the three ARV strains (176, S-1133, and 81-5) and found that the selection of genome segments in co-infection was virus strain specific.

In a comparative pathogenicity study, we found that TARV-O'Neil was the most pathogenic followed by TARV-MN2 and TARV-MN4 (Sharafeldin et al., 2014a). However, we were not able to differentiate these different pathotypes on the basis of their genetic lineages. Our findings are consistent with those of Kant et al. (2003) who also did not find any correlation between genotypes, serotypes and pathotypes of different ARV strains. However, the divergence and clustering pattern of different S-class segments revealed that there may be involvement of multiple genes in pathology and serology as has been opined by Guo et al. (2012).

Compared to 11 serotypes of CRVs, TARVs have only a single serotype so far (Rosenberger et al., 2013b). Based on S class genome segments we also found fewer genetic variations in TARVs as compared to those in CRVs further reinforcing the notion that TARVs are probably newly emerging or re-emerging reoviruses. Surveillance studies on types of reoviruses circulating in breeder and commercial poults in different geographical areas are indicated to determine the source of these newly re-emerging pathogens.

To the best of our knowledge this is the first report on characterization of TARVs and TERV based on complete S class genome segments. The results indicate the presence of point mutations at nucleotide and amino acid levels as well as possible reassortments but not as high as in CRVs. We found more divergence within TARVs in the S1 and S3 genome segments and hence these two segments deserve more scrutiny for characterization of new reovirus isolates. Future studies should be conducted on survival

of infection and viral persistence as well as correlation of mutations with pathogenicity. Viral pathogenesis studies using different passages of the same viral isolates should be conducted to determine association of mutations in viral proteins with virulence. Recent reports on new variants of CRVs are alarming and indicate a strong need for continuing surveillance, epidemiological, and genetic studies on these viruses.

Table 3.1. Per cent identity of TARVs with other reoviruses based on complete S class gene sequences.

Viruses		Per cent identity of TARVs					
		S1			S2	S3	S4
		p10	p17	σ C			
TARVs	nt*	97.0-100 (n=12)	95.0-100 (n=12)	93.3-100 (n=12)	90.1-100 (n=12)	88.9-100 (n=12)	88.7-100 (n=12)
	aa**	97.0-100	96.0-100	93.0-100	96.0-100	95.0-100	97.3-100
TERVs	nt	61.8-97.0 (n=8)	47.0-97.0 (n=7)	40.2-95.5 (n=7)	40.6-100 (n=2)	88.0-100 (n=33)	88.7-100 (n=31)
	aa	54.0-97.0	31.3-99.0	32.3-97.3	35.0-97.0	95.0-100	97.0-100
CRVs	nt	70.0-72.5 (n=31)	62.8-64.4 (n=8)	55.6-56.4 (n=8)	86.8-91.4 (n=9)	68.2-72.2 (n=20)	77.4-79.8 (n=31)
	aa	74.0-76.0	60.0-62.6	50.0-52.0	95.2-97.6	76.0-79.8	91.0-92.0
DRVs	nt	48.8-50.0 (n=5)	36.5-37.4 (n=2)	42.8-43.6 (n=1)	72.6-75.0 (n=1)	59.8-60.5 (n=3)	75.0-76.6 (n=2)
	aa	32.6-33.7	41.6-42.7	28.2	90.8-91.7	59.5-60.5	90.5-91.2
GRV	nt	34.0-35.0 (n=2)	38.3-40.5 (n=1)	42.0-42.5 (n=1)	72.8-74.0 (n=1)	59.7-60.3 (n=1)	75.7-76.3 (n=2)
	aa	32.6-33.7	41.6-42.7	28.2	90.2-91.7	58.4-59.7	91.2-92.0

*nt= nucleotide; **aa= amino acid
TARV= Turkey arthritis reovirus; TERV= Turkey enteric reovirus; CRV= Chicken reovirus; DRV= Duck reovirus; GRV= Goose reovirus
n= number of sequences used for calculating per cent identity

Table 3.2. Lineages of TARVs and TERVs indicating possible re-assortments.

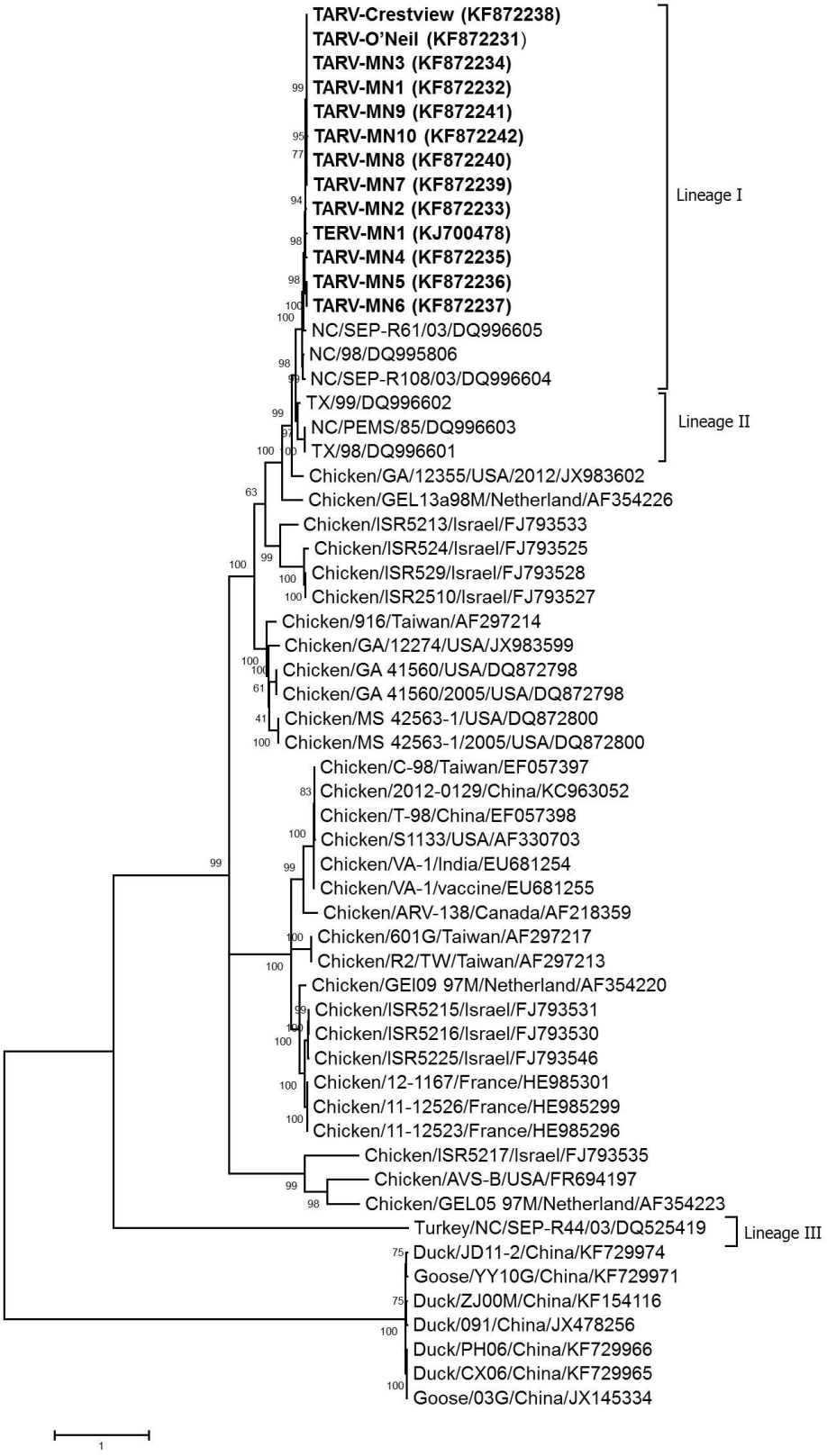
Isolate/strain name	Isolation source	State/ Country	Year of isolation	Lineages in S class genome segments			
				S1*	**S2**	**S3***	**S4***
TARV-Crestview	Tendon	Minnesota	2011	I(1)	I	I(1)	I(1)
TARV-O'Neil	Tendon	Minnesota	2011	I(1)	I	I(1)	I(1)
TARV-MN1	Tendon	Minnesota	2011	I(1)	I	I(1)	I(1)
TARV-MN2	Tendon	Minnesota	2011	I(1)	I	I(1)	I(1)
TARV-MN3	Tendon	Minnesota	2011	I(1)	I	I(1)	I(1)
TARV-MN4	Tendon	Minnesota	2011	I(3)	I	II	II
TARV-MN5	Tendon	Wisconsin	2011	I(4)	I	I(2)	I(3)
TARV-MN6	Tendon	North Carolina	2011	I(4)	I	I(2)	I(1)
TARV-MN7	Tendon	South Dakota	2012	I(1)	I	I(1)	I(1)
TARV-MN8	Tendon	Minnesota	2013	I(1)	I	I(1)	I(1)
TARV-MN9	Tendon	Minnesota	2013	I(1)	II	I(4)	I(1)
TARV-MN10	Tendon	Iowa	2013	I(1)	II	I(4)	I(1)
TERVs							
TERV-MN1	Feces	Minnesota	2011	I(2)	I	I(1)	I(2)
CA-SEP-N605	Feces	California	2008	-	-	-	I
MN-B-AB14	Feces	Minnesota	2008	-	-	I(1)	-
MN-B-AB15	Feces	Minnesota	2008	-	-	I(1)	-
MN-B-AB16	Feces	Minnesota	2008	-	-	I(1)	-
MN-B-AB18	Feces	Minnesota	2008	-	-	I(1)	-
MN-B-AB19	Feces	Minnesota	2008	-	-	I(1)	-
MN-B-AB20	Feces	Minnesota	2008	-	-	I(1)	-
MN-B-AB21	Feces	Minnesota	2008	-	-	I(1)	-
MN-B-AB22	Feces	Minnesota	2008	-	-	I(1)	-
MN-B-AB23	Feces	Minnesota	2008	-	-	I(1)	-
MN-B-AB24	Feces	Minnesota	2008	-	-	I(1)	-
MN-B-AB25	Feces	Minnesota	2008	-	-	I(1)	-
MN-B-AB26	Feces	Minnesota	2008	-	-	I(1)	-
MN-B-AB27	Feces	Minnesota	2008	-	-	I(1)	-
MN-B-AB28	Feces	Minnesota	2008	-	-	I(3)	-
MN-B-AB29	Feces	Minnesota	2008	-	-	I(1)	-
MN-D-AB01	Feces	Minnesota	2007	-	-	II	-
MN-D-AB02	Feces	Minnesota	2007	-	-	I(3)	-
MN-D-AB03	Feces	Minnesota	2007	-	-	I(3)	-
MN-D-AB04	Feces	Minnesota	2008	-	-	II	-
MN-D-AB05	Feces	Minnesota	2008	-	-	I(3)	-
MN-D-AB06	Feces	Minnesota	2008	-	-	I(1)	-
MN-D-AB08	Feces	Minnesota	2008	-	-	II	-
MN-D-AB10	Feces	Minnesota	2009	-	-	II	-
MN-D-AB11	Feces	Minnesota	2010	-	-	II	-
MN-D-AB12	Feces	Minnesota	2010	-	-	II	-
MN-D-AB13	Feces	Minnesota	2010	-	-	II	-
MN-D-AB15	Feces	Minnesota	2011	-	-	I(1)	-
MN-B4016	Feces	Minnesota	2008	-	-	-	I(3)
MN-B1016	Feces	Minnesota	2008	-	-	-	I(5)
MN-B2024	Feces	Minnesota	2008	-	-	-	I(2)

MN-B2028	Feces	Minnesota	2008	-	-	-	I(5)
MN-B2013	Feces	Minnesota	2008	-	-	-	I(2)
MN-B2022	Feces	Minnesota	2008	-	-	-	I(2)
MN-B2014	Feces	Minnesota	2008	-	-	-	I(2)
MN-D052725	Feces	Minnesota	2007	-	-	-	II
MN-D022565	Feces	Minnesota	2008	-	-	-	I(5)
MN-D000818	Feces	Minnesota	2000	-	-	-	I(4)
MN-D049007	Feces	Minnesota	2007	-	-	-	I(4)
MN-D055431	Feces	Minnesota	2008	-	-	-	I(1)
MO-SEP-816	Feces	Missouri	2005	-	-	-	III
MO-SEP-819	Feces	Missouri	2005	-	-	-	III
MO-SEP-828	Feces	Missouri	2005	-	-	-	I(5)
NC-SEP-R61-03	Feces	North Carolina	2003	I(5)	-	-	-
NC-98	Feces	North Carolina	1998	I(5)	-	I(5)	-
NC-SEP-R108-03	Feces	North Carolina	2003	I(5)	-	-	-
NC-PEMS-85	Feces	North Carolina	1985	II	-	I(5)	-
NC-SEP-R44-03	Feces	North Carolina	2003	III	-	I(2)	-
NC-SEP-832	Feces	North Carolina	2005	-	-	-	III
NC-SEP-833	Feces	North Carolina	2005	-	-	-	III
NC-SEP-835	Feces	North Carolina	2005	-	-	-	III
TX-98	Feces	Texas	1998	II	-	I(5)	-
TX-99	Feces	Texas	1999	II	-	I(6)	-
WI-SEP-847	Feces	Wisconsin	2005	-	-	-	II
ATCC-TEV-VR-818	Feces	North Carolina	1972	-	-	I(6)	-
BF	bursa of Fabricius	Brazil	-	-	-	III	-
Muntrilj06	Feces	Croatia	2007	-	II	-	-

(-) indicates that sequence of the particular segment is not available for comparison.
*Possible groups of lineage I TARVs and TERVs are shown in bracket.
TARV= Turkey arthritis reovirus; TERV= Turkey enteric reovirus

Figure 3.1. Phylogenetic analysis on the basis of nucleotide sequences (981bp) of S1 genome segment (σC protein). Tree was constructed using Maximum Likelihood method with General Time Reversible model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, +G parameter=2.4686). The rate variation model allowed for sites to be invariable (+I). The strain names (with accession numbers) in bold are of this study while the strain name (with accession number) unbold are previously published orthoreoviruses used for comparison.

Figure 3.1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TARV-O'Neil (KF872231)
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TARV-MN7 (KF872239)
TARV-MN2 (KF872233)
TERV-MN1 (KJ700478)
TARV-MN4 (KF872235)
TARV-MN5 (KF872236)
TARV-MN6 (KF872237)
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NC/98/DQ995806
NC/SEP-R108/03/DQ996604
TX/99/DQ996602
NC/PEMS/85/DQ996603
TX/98/DQ996601
Lineage I
Lineage II
Chicken/GA/12355/USA/2012/JX983602
Chicken/GEL13a98M/Netherland/AF354226
Chicken/ISR5213/Israel/FJ793533
Chicken/ISR524/Israel/FJ793525
Chicken/ISR529/Israel/FJ793528
Chicken/ISR2510/Israel/FJ793527
Chicken/916/Taiwan/AF297214
Chicken/GA/12274/USA/JX983599
Chicken/GA 41560/USA/DQ872798
Chicken/GA 41560/2005/USA/DQ872798
Chicken/MS 42563-1/USA/DQ872800
Chicken/MS 42563-1/2005/USA/DQ872800
Chicken/C-98/Taiwan/EF057397
Chicken/2012-0129/China/KC963052
Chicken/T-98/China/EF057398
Chicken/S1133/USA/AF330703
Chicken/VA-1/India/EU681254
Chicken/VA-1/vaccine/EU681255
Chicken/ARV-138/Canada/AF218359
Chicken/601G/Taiwan/AF297217
Chicken/R2/TW/Taiwan/AF297213
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Chicken/ISR5216/Israel/FJ793530
Chicken/ISR5225/Israel/FJ793546
Chicken/12-1167/France/HE985301
Chicken/11-12526/France/HE985299
Chicken/11-12523/France/HE985296
Chicken/ISR5217/Israel/FJ793535
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Chicken/GEL05 97M/Netherland/AF354223
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Lineage III
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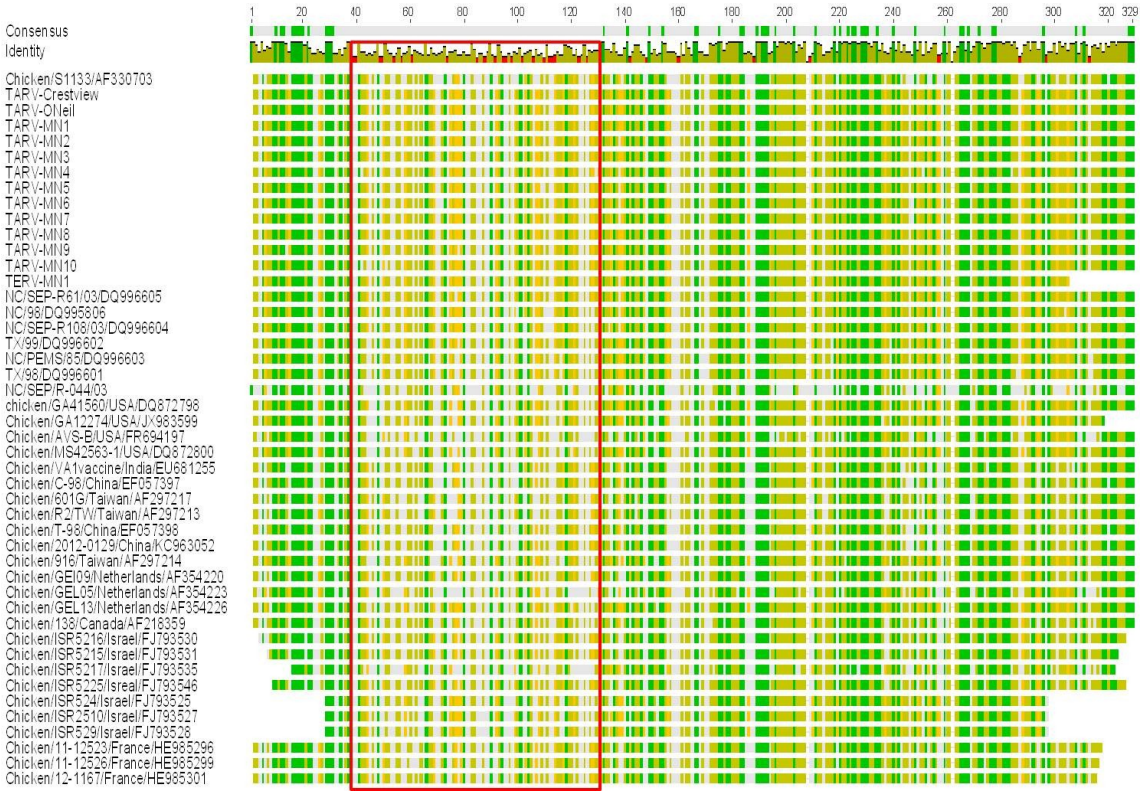
Goose/YY10G/China/KF729971
Duck/ZJ00M/China/KF154116
Duck/091/China/JX478256
Duck/PH06/China/KF729966
Duck/CX06/China/KF729965
Goose/03G/China/JX145334
1

Figure 3.2. Image of amino acid sequence comparison of σC protein of turkey arthritis virus with previously published orthoreoviruses of chicken and turkey origin. Each color represents a specific amino acid while amino acids in gray match the consensus amino acid. In the consensus histogram, the dark green regions (peaks) represent conserved residues while the red regions (valleys) represent divergent amino acids.

Figure 3.2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Consensus
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TARV-Crestview
TARV-ONeil
TARV-MN1
TARV-MN2
TARV-MN3
TARV-MN4
TARV-MN5
TARV-MN6
TARV-MN7
TARV-MN8
TARV-MN9
TARV-MN10
TERV-MN1
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NC/98/DQ995806
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TX/98/DQ996601
NC/SEP/R-044/03
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Chicken/GA12274/USA/JX983599
Chicken/AVS-B/USA/FR694197
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Chicken/C-98/China/EF057397
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Chicken/R2/TW/Taiwan/AF297213
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Chicken/2012-0129/China/KC963052
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Chicken/GEI09/Netherlands/AF354220
Chicken/GEL05/Netherlands/AF354223
Chicken/GEL13/Netherlands/AF354226
Chicken/138/Canada/AF218359
Chicken/ISR5216/Israel/FJ793530
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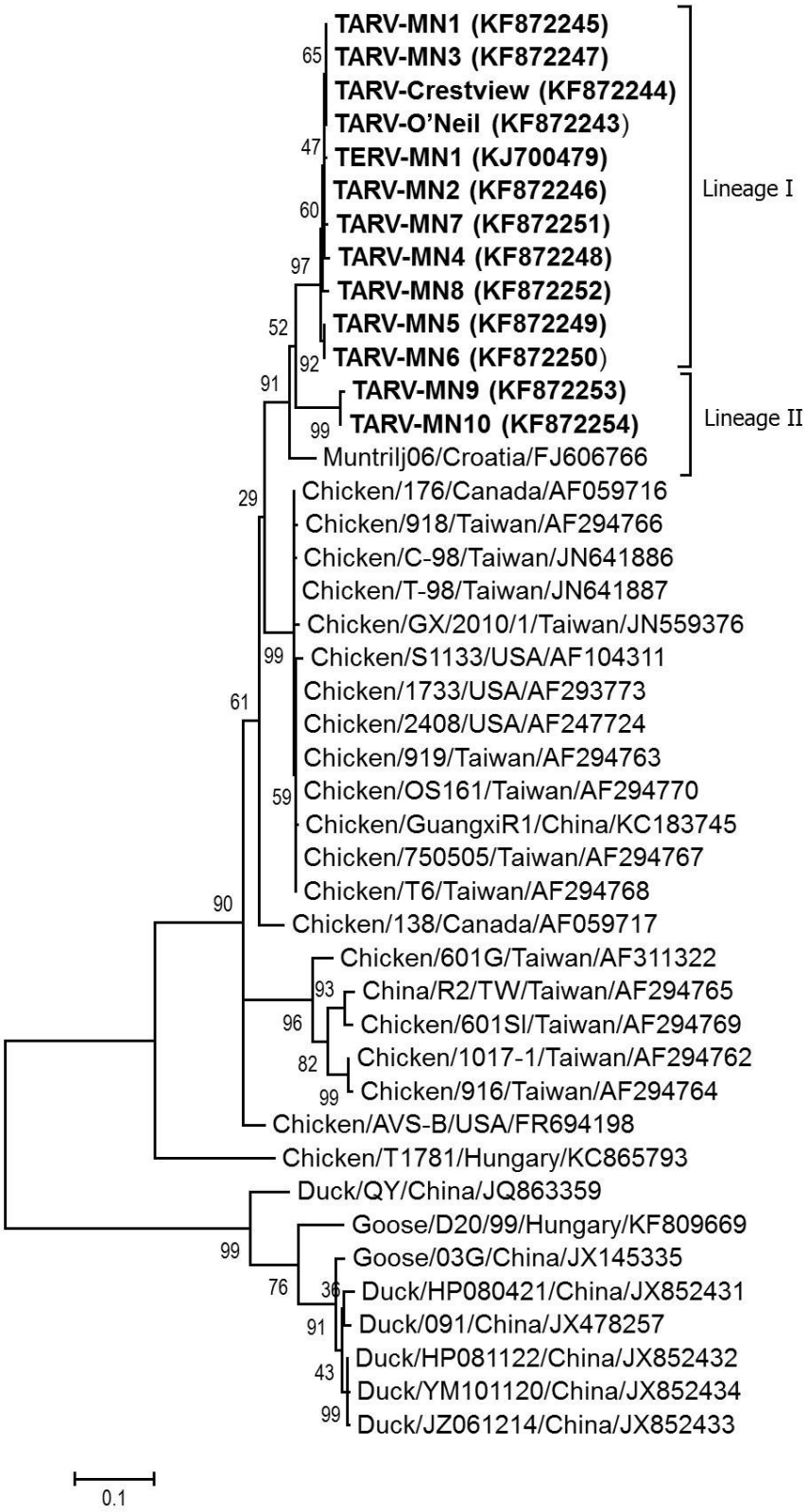
Chicken/11-12526/France/HE985299
Chicken/12-1167/France/HE985301

Figure 3.3. Phylogenetic analysis on the basis of nucleotide sequences (1180bp) of S2 genome segment. Phylogenetic tree was constructed using Maximum Likelihood method based on Kimura 2-parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, +G parameter=0.5720). The strain names (with accession numbers) in bold are of this study while the strain name (with accession number) unbold are previously published orthoreoviruses used for comparison.

Figure 3.3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TARV-MN3 (KF872247)
TARV-Crestview (KF872244)
TARV-O'Neil (KF872243)
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TARV-MN2 (KF872246)
TARV-MN7 (KF872251)
TARV-MN4 (KF872248)
TARV-MN8 (KF872252)
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Lineage I
TARV-MN9 (KF872253)
TARV-MN10 (KF872254)
Muntrilj06/Croatia/FJ606766
Lineage II
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Chicken/918/Taiwan/AF294766
Chicken/C-98/Taiwan/JN641886
Chicken/T-98/Taiwan/JN641887
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Chicken/S1133/USA/AF104311
Chicken/1733/USA/AF293773
Chicken/2408/USA/AF247724
Chicken/919/Taiwan/AF294763
Chicken/OS161/Taiwan/AF294770
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Goose/D20/99/Hungary/KF809669
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97
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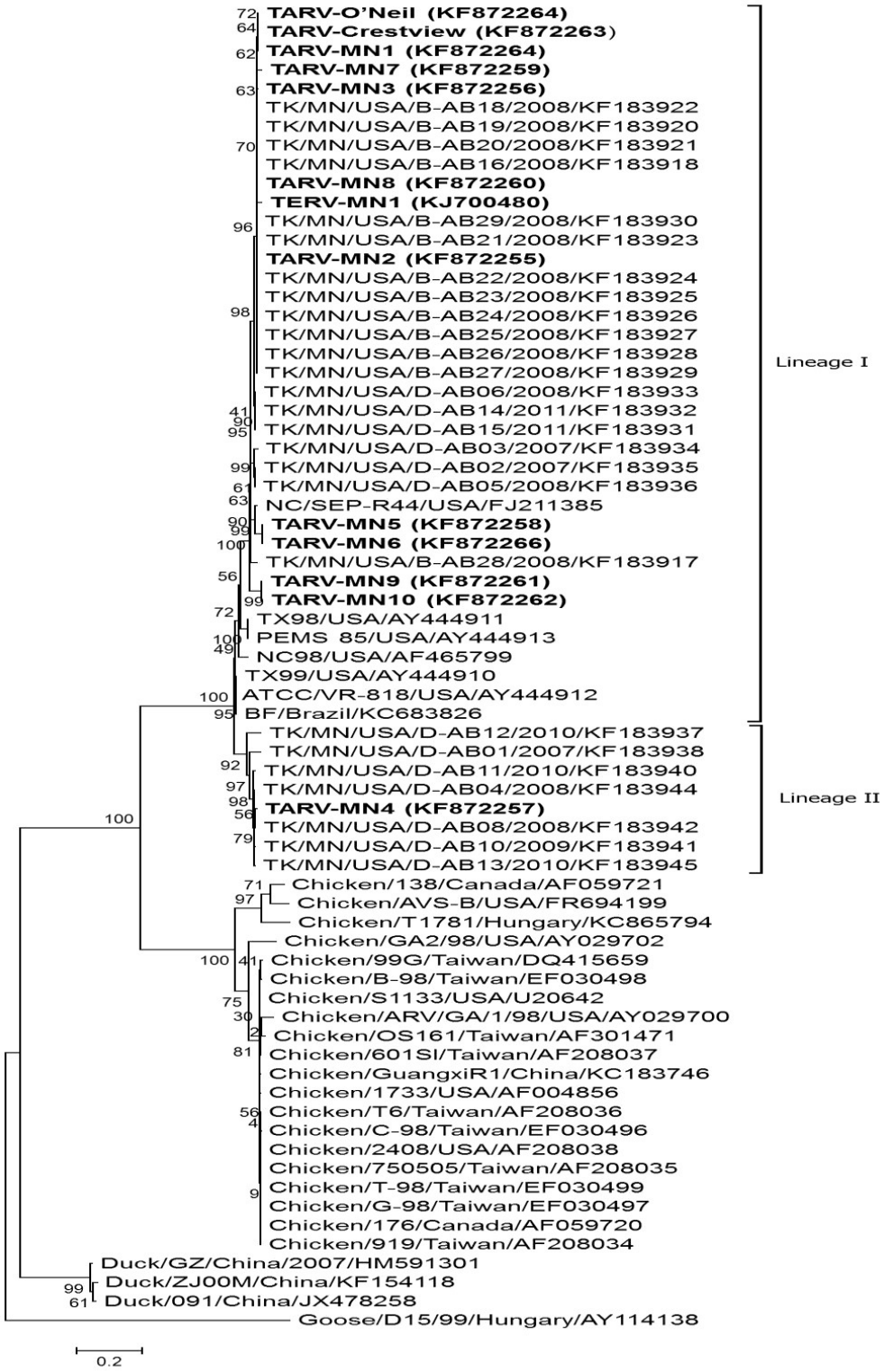
92
91
99
29
99
61
59
90
93
96
82
99
99
76
36
91
43
99
0.1

Figure 3.4. Phylogenetic analysis on the basis of nucleotide sequences (1000bp) of S3 genome segment. Phylogenetic tree was constructed using Maximum Likelihood method based on Kimura 2-parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, +G parameter=0.9665). The strain names (with accession numbers) in bold are of this study while the strain name (with accession number) unbold are previously published orthoreoviruses used for comparison.

Figure 3.4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TARV-MN7 (KF872259)
TARV-MN3 (KF872256)
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TK/MN/USA/B-AB20/2008/KF183921
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TERV-MN1 (KJ700480)
TK/MN/USA/B-AB29/2008/KF183930
TK/MN/USA/B-AB21/2008/KF183923
TARV-MN2 (KF872255)
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TK/MN/USA/B-AB23/2008/KF183925
TK/MN/USA/B-AB24/2008/KF183926
TK/MN/USA/B-AB25/2008/KF183927
TK/MN/USA/B-AB26/2008/KF183928
TK/MN/USA/B-AB27/2008/KF183929
TK/MN/USA/D-AB06/2008/KF183933
TK/MN/USA/D-AB14/2011/KF183932
TK/MN/USA/D-AB15/2011/KF183931
TK/MN/USA/D-AB03/2007/KF183934
TK/MN/USA/D-AB02/2007/KF183935
TK/MN/USA/D-AB05/2008/KF183936
NC/SEP-R44/USA/FJ211385
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TARV-MN6 (KF872266)
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TARV-MN10 (KF872262)
TX98/USA/AY444911
PEMS 85/USA/AY444913
NC98/USA/AF465799
TX99/USA/AY444910
ATCC/VR-818/USA/AY444912
BF/Brazil/KC683826
TK/MN/USA/D-AB12/2010/KF183937
TK/MN/USA/D-AB01/2007/KF183938
TK/MN/USA/D-AB11/2010/KF183940
TK/MN/USA/D-AB04/2008/KF183944
TARV-MN4 (KF872257)
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TK/MN/USA/D-AB10/2009/KF183941
TK/MN/USA/D-AB13/2010/KF183945
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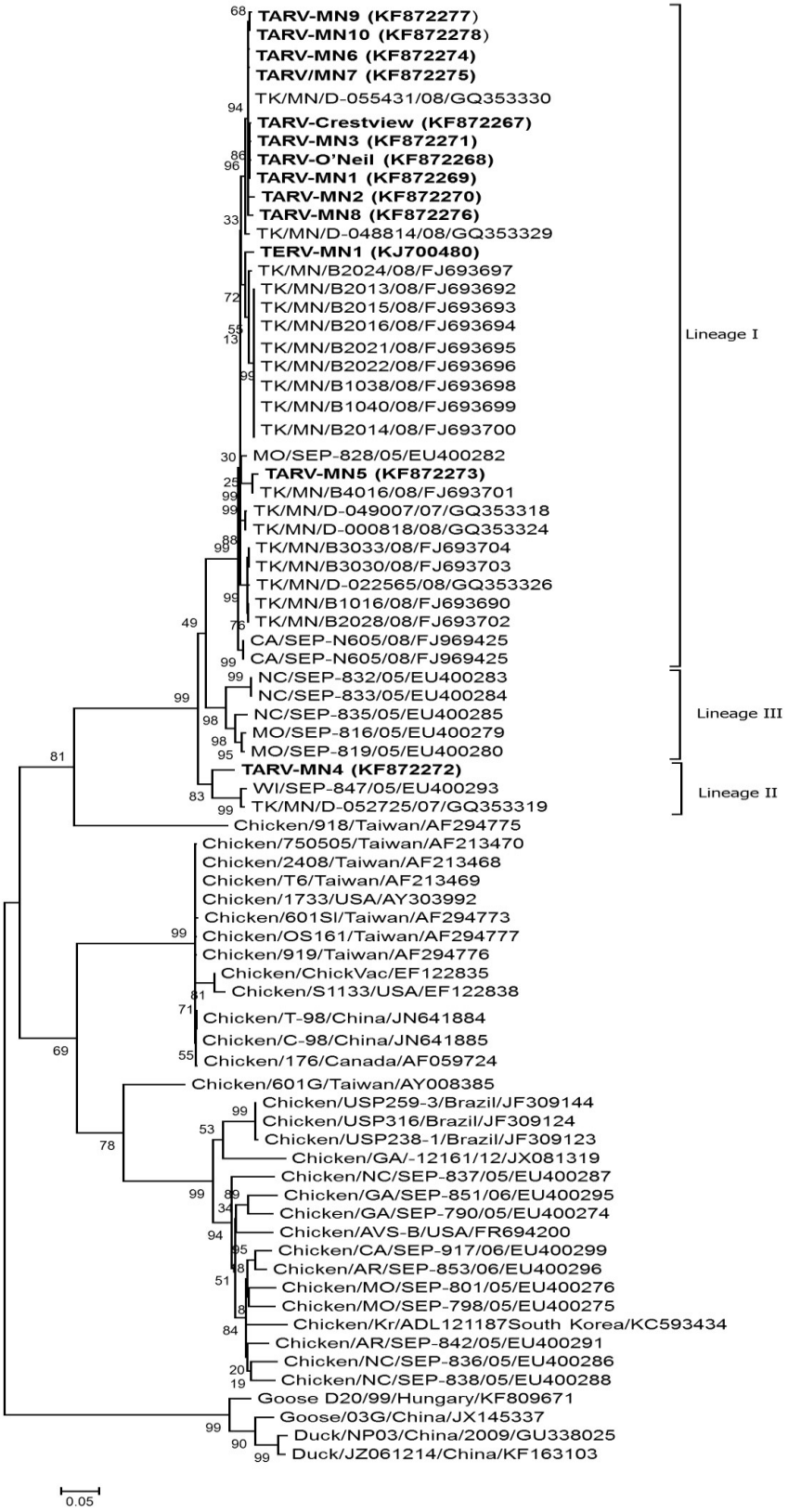
Chicken/AVS-B/USA/FR694199
Chicken/T1781/Hungary/KC865794
Chicken/GA2/98/USA/AY029702
Chicken/99G/Taiwan/DQ415659
Chicken/B-98/Taiwan/EF030498
Chicken/S1133/USA/U20642
Chicken/ARV/GA/1/98/USA/AY029700
Chicken/OS161/Taiwan/AF301471
Chicken/601SI/Taiwan/AF208037
Chicken/GuangxiR1/China/KC183746
Chicken/1733/USA/AF004856
Chicken/T6/Taiwan/AF208036
Chicken/C-98/Taiwan/EF030496
Chicken/2408/USA/AF208038
Chicken/750505/Taiwan/AF208035
Chicken/T-98/Taiwan/EF030499
Chicken/G-98/Taiwan/EF030497
Chicken/176/Canada/AF059720
Chicken/919/Taiwan/AF208034
Duck/GZ/China/2007/HM591301
Duck/ZJ00M/China/KF154118
Duck/091/China/JX478258
Goose/D15/99/Hungary/AY114138
0.2
Lineage I
Lineage II

Figure 3.5. Phylogenetic analysis on the basis of nucleotide sequences (950bp) of S4 genome segment. Phylogenetic tree was constructed by using Maximum Likelihood method based on Hasegawa-Kishino-Yano (HKY) model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, +G parameter=0.2606). The strain names (with accession numbers) in bold are of this study while the strain name (with accession number) unbold are previously published orthoreoviruses used for comparison.

Figure 3.5


TARV-MN9 (KF872277)
TARV-MN10 (KF872278)
TARV-MN6 (KF872274)
TARV/MN7 (KF872275)
TK/MN/D-055431/08/GQ353330
TARV-Crestview (KF872267)
TARV-MN3 (KF872271)
TARV-O'Neil (KF872268)
TARV-MN1 (KF872269)
TARV-MN2 (KF872270)
TARV-MN8 (KF872276)
TK/MN/D-048814/08/GQ353329
TERV-MN1 (KJ700480)
TK/MN/B2024/08/FJ693697
TK/MN/B2013/08/FJ693692
TK/MN/B2015/08/FJ693693
TK/MN/B2016/08/FJ693694
TK/MN/B2021/08/FJ693695
TK/MN/B2022/08/FJ693696
TK/MN/B1038/08/FJ693698
TK/MN/B1040/08/FJ693699
TK/MN/B2014/08/FJ693700
MO/SEP-828/05/EU400282
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TK/MN/D-000818/08/GQ353324
TK/MN/B3033/08/FJ693704
TK/MN/B3030/08/FJ693703
TK/MN/D-022565/08/GQ353326
TK/MN/B1016/08/FJ693690
TK/MN/B2028/08/FJ693702
CA/SEP-N605/08/FJ969425
CA/SEP-N605/08/FJ969425
NC/SEP-832/05/EU400283
NC/SEP-833/05/EU400284
NC/SEP-835/05/EU400285
MO/SEP-816/05/EU400279
MO/SEP-819/05/EU400280
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Chicken/750505/Taiwan/AF213470
Chicken/2408/Taiwan/AF213468
Chicken/T6/Taiwan/AF213469
Chicken/1733/USA/AY303992
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Chicken/OS161/Taiwan/AF294777
Chicken/919/Taiwan/AF294776
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Chicken/S1133/USA/EF122838
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Chicken/C-98/China/JN641885
Chicken/176/Canada/AF059724
Chicken/601G/Taiwan/AY008385
Chicken/USP259-3/Brazil/JF309144
Chicken/USP316/Brazil/JF309124
Chicken/USP238-1/Brazil/JF309123
Chicken/GA/-12161/12/JX081319
Chicken/NC/SEP-837/05/EU400287
Chicken/GA/SEP-851/06/EU400295
Chicken/GA/SEP-790/05/EU400274
Chicken/AVS-B/USA/FR694200
Chicken/CA/SEP-917/06/EU400299
Chicken/AR/SEP-853/06/EU400296
Chicken/MO/SEP-801/05/EU400276
Chicken/MO/SEP-798/05/EU400275
Chicken/Kr/ADL121187South Korea/KC593434
Chicken/AR/SEP-842/05/EU400291
Chicken/NC/SEP-836/05/EU400286
Chicken/NC/SEP-838/05/EU400288
Goose D20/99/Hungary/KF809671
Goose/03G/China/JX145337
Duck/NP03/China/2009/GU338025
Duck/JZ061214/China/KF163103
Lineage I
Lineage III
Lineage II
0.05

Figure 3.6. Possible reassortment within TARVs and in between TARVs and TERVs represented with phylogenetic trees based on nucleotide sequences of the S class gene segments [S1(σC;A), S2 (B), S3 (C), and S4 (D)]. The strain names (with accession numbers) in bold are of this study while the strain name (with accession number) unbold are previously published orthoreoviruses used for comparison.

Figure 3.6- A and B

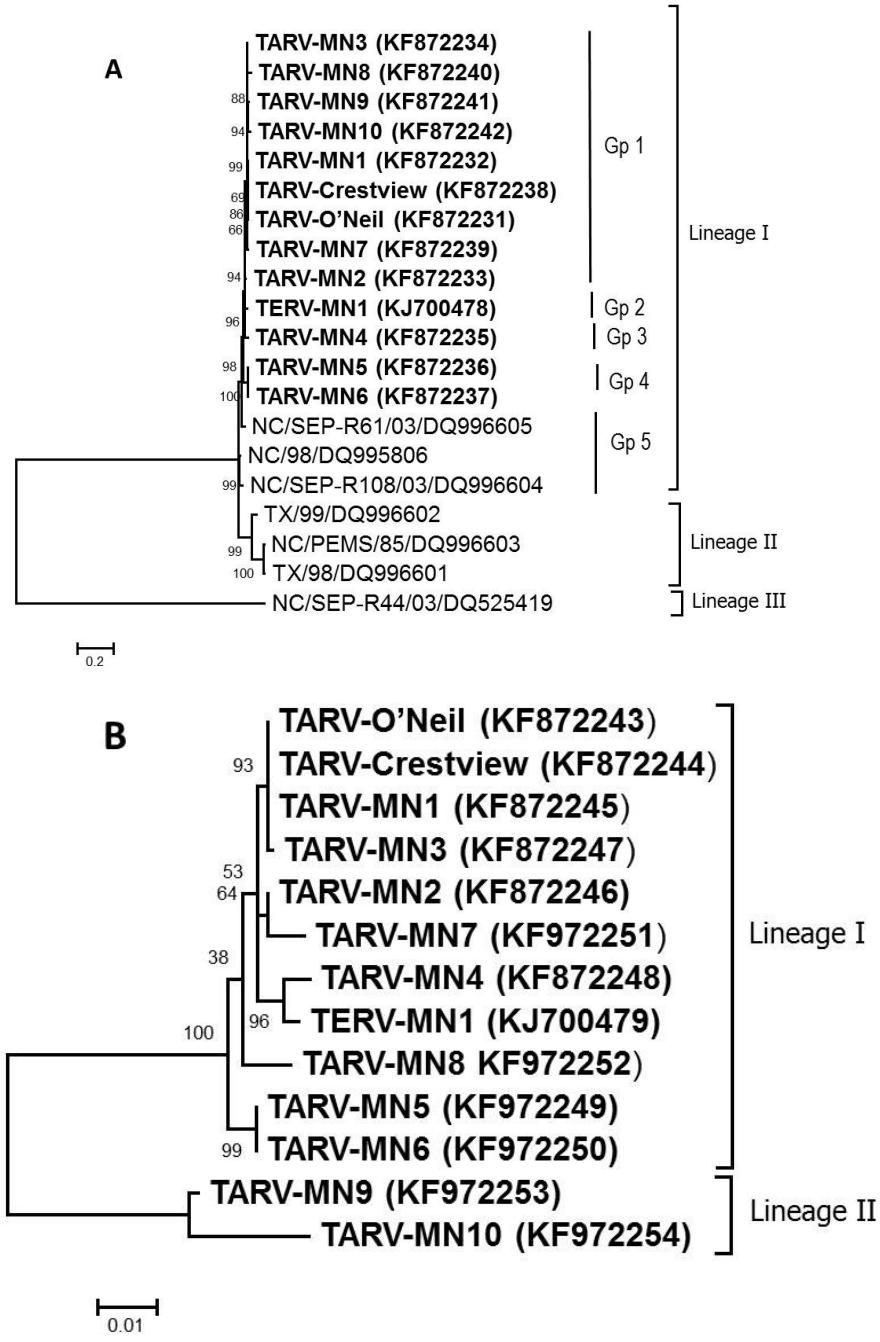
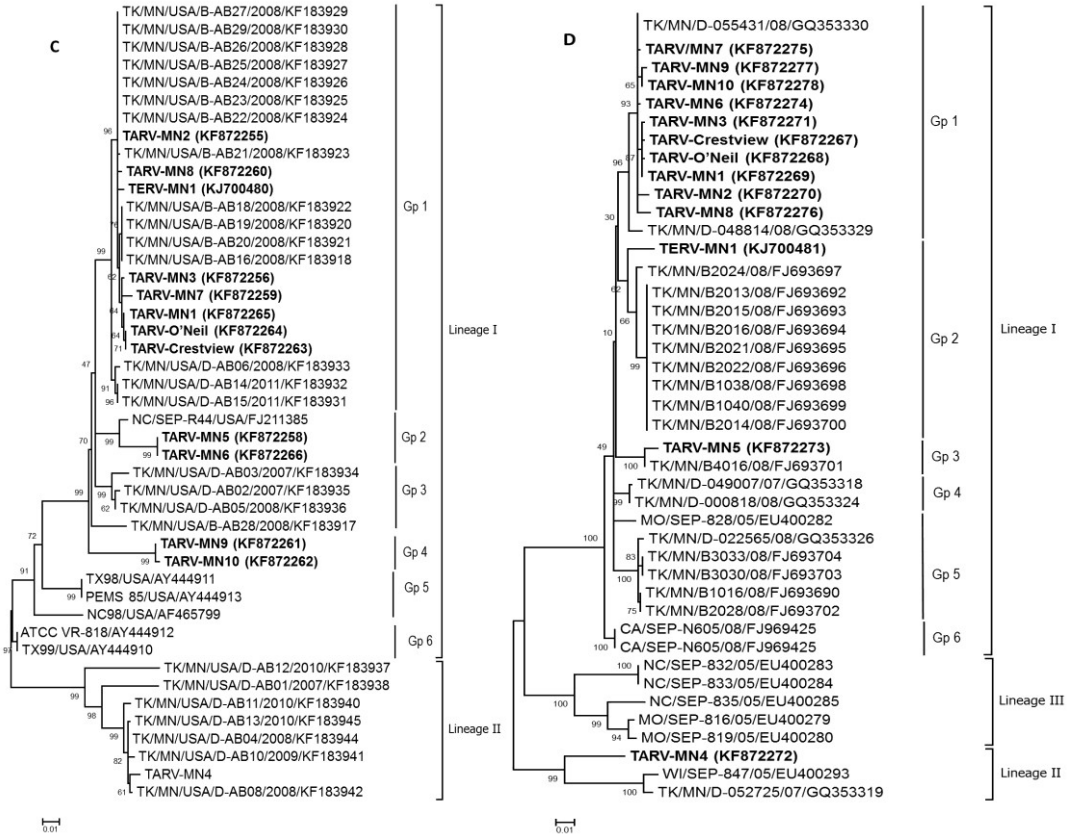


Figure 3.6- C and D



Chapter 4: Phylogenetic analysis, genomic diversity and classification of M class gene segments of avian reoviruses

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1. Introduction

Avian reoviruses (ARVs) are non-enveloped viruses with icosahedral symmetry and belong to genus Orthoreovirus in the family *Reoviridae* (Varela and Benavente, 1994). The ten genome segments of double stranded RNA are divided into three classes namely large (L), medium (M), and small (S), based on their migration pattern in polyacrylamide gel electrophoresis (Benavente and Martinez-Costas, 2007; Varela and Benavente, 1994). The L and M genes are further subdivided into three segments each (L1, L2, L3 and M1, M2, M3, respectively) while the S gene has four segments (S1, S2, S3, S4; Spandidos and Graham, 1976). The reovirus genome has 12 open reading frames (ORFs), which encode for eight structural and four non-structural proteins. The structural proteins are an important part of the progeny virions while non-structural proteins are expressed only in infected cells (Martinez-Costas et al., 1997).

The L, M and S gene segments encode lambda (λ), mu (μ) and sigma (σ) proteins, respectively. The M1 and M2 segments encode two structural proteins (μA and μB, respectively) while M3 gene segment encodes a non-structural protein (μNS), which has an important role in the early stages of virus morphogenesis (Benavente and Martinez-Costas, 2007; Touris-Otero et al., 2004). The μA protein is a minor component of the inner capsid and is believed to serve as a putative transcriptase co-factor. The μB protein is present in the outer capsid and is important in virus penetration into host cell after it is cleaved into μBN (small myristolylated amino-terminal peptide) and μBC (large carboxy-terminal fragment; Benavente and Martinez-Costas, 2007).

The genus *Orthoreovirus* includes two types of reoviruses e.g., fusogenic and nonfusogenic; the former induces cell-to-cell fusion resulting in the formation of syncytia

in infected cells. Most mammalian reoviruses (MRV) are non-fusogenic in nature except the Nelson Bay reovirus (NBV; recently re-named as *Pteropine orthoreovirus* or PRV) and bat and baboon reoviruses. The fusogenic reoviruses are divided into five groups (I-V) (King et al., 2012); group I includes prototypical MRV strains including Ndelle virus; group II contains ARVs; group III includes PRV; groups IV and V include baboons and reptilian orthoreoviruses, respectively.

The ARVs are ubiquitous in domestic poultry with 80% of them being non-pathogenic and are frequently found in clinically normal birds (Jones, 2008). However, ARVs have also been isolated from different disease conditions in poultry including enteritis, hepatitis, neurological disorder, myocarditis, respiratory distress and viral arthritis/tenosynovitis (Jones, 2008). It is believed that ARV-associated clinical disease is mostly dependent on the age and immune status of the host and on virus pathotype. Economic losses associated with ARVs in commercial poultry are due to poor weight gain, uneven growth, poor feed conversion, increased morbidity and mortality, and reduced marketability (Jones, 2008).

The ARV in chickens is commonly referred to as chicken reovirus (CRV) and its pathogenesis has been well defined (Al Afaleq and Jones, 1989; van der Heide and Kalbac, 1975). Several different serotypes (at least 11 serotypes) and pathotypes of CRV exist (Olson et al., 1957; Jones, 2008, Wood et al., 1980), which have been implicated mainly in tenosynovitis and runting-stutnting syndrome (RSS). Recently, variants of CRV have been isolated from cases of lameness and tenosynovitis in 2.5 to 8 week old commercial broiler chickens in Europe and North America (Rosenberger et al., 2013;

Sellers et al., 2013; Troxler et al., 2013). Most of the commercially available vaccines are not effective against these variants (Sellers et al., 2013; Troxler et al., 2013).

The ARVs isolated from turkeys are known as turkey reoviruses (TRVs) and they have been isolated not only from apparently healthy poults but also from cases of poult enteritis complex (PEC), poult enteritis syndrome (PES), and light turkey syndrome (LTS) (Pantin-Jackwood et al., 2008; Jindal et al., 2010; Mor et al., 2013a). In 1980s and early 1990s, TRV were isolated from arthritic joints of turkeys (Al Afaleq et al., 1991; Levisohn et al., 1980; Page et al., 1982) after which there were no reports of arthritis-associated reovirus in turkeys. Recently, we isolated reoviruses from tendons of 12- to 18-week-old market age tom turkeys from the upper Midwest area of the U.S. and experimentally reproduced tenosynovitis by oral inoculation of turkey poults (Mor et al., 2013b; Mor et al., 2014; Sharafeldin et al., 2014a; 2014b). We have tentatively named these viruses as TARV (turkey arthritis reovirus) as opposed to TERV (turkey enteric reovirus) isolated from healthy and enteritic poults.

The reoviruses isolated from ducks and geese are called duck reovirus (DRV) and goose reovirus (GRV), respectively. In addition, ARVs have been isolated from wild birds. For example, Tvarminne avian virus (TVAV) was isolated from the brain of a wild crow with neurological signs (Dander et al., 2014). We isolated an ARV from a chickadee affected with enteritis. Sequencing of S4 gene segment of this virus (Chickadee/2011/USA/MN/KJ475124) revealed close relationship to TRVs (Mor et al., 2014c).

The M class gene segments of CRV, DRV, and GRV have been described but this information is not available for either TERV or TARV (Banyai et al., 2011; Noad et al.,

2006; Su et al., 2006; Wang et al., 2013, Zhang et al., 2007) except that we have characterized the S class genome segments of TARVs (Mor et al., 2014). In the present study, we further characterize the TRVs on the basis of M class genome segments and compare them with those available ARV strains from GenBank. Based on our results, we propose a new M class genotyping classification system, which allows investigation of M class reassortment events.

2. Materials and Methods

2.1. Sample source

Dead or humanely euthanized lame turkeys (2-to-19-weeks of age) or fresh or frozen turkey legs from lame turkeys were submitted to the University of Minnesota Veterinary Diagnostic Laboratory (UMVDL). Tendons and/or joint fluids from these cases were subjected to reovirus isolation in QT-35 cells as previously described (Mor et al., 2013b). For histopathology, gastrocnemius and digital flexor tendons were removed and immersed in 10% buffered formalin. Fecal samples from cases of PES and LTS were also processed for virus isolation as previously described (Jindal et al., 2010a; Mor et al., 2013a).

2.2. Virus isolates

A total of 15 TARV isolates were included in this study. Thirteen TARV strains were isolated at UMVDL (TARV-MN1 to TARV-MN13) while 2 TARV strains (TARV-O'Neil and TARV-Crestview) were obtained from Dr. Jack Rosenberger, AviServe, Newark, Delaware. In addition, eight TERV strains (TERV-MN1 to TERV-MN8) of

turkey enteritis were isolated and used for comparison. All viruses were isolated and propagated in QT-35 cell line as described previously (Mor et al., 2013b; Mor et al, 2014).

2.3. RNA extraction and RT-PCR

The RNA was extracted from cell culture supernatants using a QIAamp viral RNA mini kit (Qiagen, Valencia, CA). Three primer sets were designed for each M class genome segment starting from 5' UTR to 3' UTR region by aligning all available ARV sequences in GenBank (Table S1). All RT-PCR reactions utilized Qiagen one step RT-PCR kit (Qiagen, Valencia, CA) with 50µl reaction volume. The reactions were run in an Eppendorf thermocycler with the following thermal cycling conditions: 30 min at 50^{o}C and 15 min at 95^{o}C (RT step) followed by 35 PCR cycles with denaturation at 94^{o}C for 1 min, annealing at respective temperature for each primer for 1 min, and elongation at 72^{o}C for 1 min with a final elongation of 72^{o}C for 10 min. PCR products were visualized in 1.2% agarose gel in Tris-acetate-EDTA buffer by electrophoresis and the appearance of a specific band of expected product size confirmed amplification.

2.4. Sequencing

The amplified PCR products were purified using Qiagen PCR purification kit according to manufacturer's guidelines and then submitted to the University of Minnesota Genomic Center (UMGC) for sequencing. The sequencing was performed using the product primers as used in RT-PCR reactions. Forward and reverse trace files were aligned

together using Sequencher 5.1 software (www.genecodes.com) followed by BLAST analysis (www.ncbi.nlm.nih.gov).

2.5. Illumina sequencing

The M2 gene of seven TARVs (TARV-MN2, TARV-MN4, TARV-MN9, TARV-MN10, TARV-MN11, TARV-MN12 and TARV-MN13) and four TERVs (TERV-MN1, TERV-MN2, TERV-MN5 and TERV-MN6) could not be amplified with either self-designed or published ARV primers. Hence, these isolates were sequenced using Illumina MiSeq Next Generation Sequencing. Large amount of high quality total RNA was extracted using Trizol LS reagent (Invitrogen, NY, USA) followed by RNA purification using QIAamp viral RNA mini kit (Qiagen, Valencia, CA). Extracted RNA was submitted to UMGC for library preparation and 250 cycles paired end reads on Illumina MiSeq.

2.6. Sequence analysis and phylogeny

The nucleotide sequences generated in this study (n=23) and those available in GenBank (n=33) were aligned using ClustalW method in MEGA 6.06 software (Tamura et al. 2013). The best substitution model for analysis of DNA and protein sequences of each segment was selected on the basis of the lowest BIC score (Bayesian Information Criterion) in MEGA 6.05. The nucleotide substitution model GTR (General Time Reversible) +G (Gamma distribution with 5 rate categories) was used to generate the M2 and M3 phylogenetic trees while the Hasegawa-Kishino-Yano (HKY)+ G models)+I (Evolutionary Invariable sites) was used to generate the M1 gene segment phylogenetic

tree. The Maximum Likelihood phylogenetic trees were statistically validated by using 1000 bootstrap replicates (Felsenstein J. 1985). Pairwise identity charts of nucleotides and amino acids were constructed with the percentage on the x axis and the frequency of each identity on the y axis (Ball, 2005).

2.7. GenBank accession numbers

The TARV (n=15) and TERV (n=8) nucleotide sequences were submitted to GenBank with accession numbers KJ874304 - KJ874326, KJ874281 - KJ874303, KJ874258 - KJ874280 for M1, M2 and M3 genes, respectively.

3. Results

3.1. Gross lesions and histopathology

A total of 15 TARV strains were isolated from turkeys showing signs of lameness, swollen hock joints, and gross pathological lesions such as periarticular fibrosis and increased yellow synovial fluid in gastrocnemius tendon sheath. In some cases, there was rupture of gastrocnemius tendon and/or digital flexor tendon (Fig. 4.1). From such cases TARV-O'Neil and TARV-MN12 were isolated. Histopathology of tendons revealed marked fibrosis of subsynovium along with minimal infiltration of lymphocytes (Fig. 4.2). When inoculated orally in 1-week-old turkey poults, TARVs produced tenosynovitis but TERV and CRV did not (Sharafeldin et al., 2014a).

3.2. M class gene sequence comparison, phylogenetic analysis, and classification of M class genes and encoded proteins

3.2.1. M1 gene and μA protein

The M1 gene segments of TRVs (both TARV and TERV) consisted of 2283 nucleotides (nt) and included 5' and 3' untranslated regions (UTR) of 12 and 72 nt, respectively. There were no insertions or deletions and the single ORF, ranging from nt 13 to 2211, encoded for protein μA of 732 amino acid (aa). However, seven unique aa substitutions were observed (G106V, A131T, Q296R, A395V, N602S, K675R, G730N) in TRVs that could differentiate them from CRVs. A basic aa motif 101-RSRLKVFQK/RRP-111 with one aa substitution (G106V) in TRVs further differentiated them from CRVs (RS/PRLKGFQKRP). The TRV protein μA had a sequence motif of LALDPPF at position 458-464 similar to N-6 adenine-specific DNA methylase (Timinskas et al., 1995; Su et al., 2006). No aa motif substitutions were observed that could differentiate the two TRV types (TARVs and TERVs).

Using the 15 TARV and eight TERV sequences generated in this study and the ARV M1 gene segment sequences available in GenBank, a pairwise identity chart and phylogenetic tree were constructed on nt and aa levels. The ARV sequences (both TRVs and CRVs) were different from those of mammalian reovirus (MRV) and TVAV with nt identity varying from 34.4% to 57.0% and 53.4% to 55.8%, respectively. Based on the nt phylogenetic tree and pairwise chart, an 84% cut off value was used, which resulted in the generation of five genotypes (M1-I to M1-V) of ARV (Fig. 4.3).

Genotype M1-I included all 23 TRV sequences in addition to 18 CRV sequences reported from Asia and North America. Genotype M1-II was composed of a single CRV

sequence (Chicken/Hungary/2012/T1781/KC865789) while genotype M1-III included three DRV sequences. Genotype IV had a single GRV sequence (D20) while genotype V contained one GRV (Goose/China/2003/03G) and seven DRV sequences (Fig. 4.4). The MI-III genotype was the most conserved with an nt identity of 99.3%-99.7%. The MI-I genotype contained the lowest nt identity of 84.8-100% although its aa identity was relatively high (95.1-100%) (Table 4.1). The TRV sequences were more closely related to CRV sequences than those of DRV and GRV as indicated by the phylogenetic tree (Fig. 4.4).

3.2.2. M2 gene and μB protein

The TRV M2 gene segment was 2158 bp long with 29 and 98 nt 5' and 3' UTR, respectively. The M2 gene segment encodes a single ORF (30 to 2060 nt), which translates the μB protein (676 aa in size). The TRV and CRV aa sequences shared residues (NP) at position 42 and 43, which is predicted cleavage site of the μB protein into smaller μBN and larger μBC protein. The large carboxy-terminal protein μBC was more variable among ARV sequences as compared to the μBN. The residue R at position 582 was also conserved in all ARV sequences.

Using the 23 TRV sequences generated in this study and the ARV M2 gene segments from GenBank, pairwise identity chart and phylogenetic tree were constructed. An 83% cut off value generated seven M2 genotypes (M2-I to M2-VII) of ARVs; the TRVs and CRVs were divided into three and four genotypes, respectively, with less than 75% nt identity between the genotypes. Genotype M2-I included nine TARVs, five TERVs and 12 CRVs (Fig. 4.5). Genotype M2-II had five TARVs and two TERVs and

no CRV. Genotype M2-III included one TARV, one TERV and four CRVs from Asia and USA. Genotype IV and V contained one and two CRV strains, respectively (Fig. 4.6). Genotype M2-VI included six DRVs and one GRV while M2-VII had three DRVs and one GRV.

Broad range of nt identities (86.5%-100%, 83.2%-99.7%, 83.1%-100%) were observed in genotypes M2-I, M2-III and M2-V but aa identities (94.7%-100%, 93.6%-99.6%, 94.2%-100%, respectively) were higher (Table 4.1). Four, nine and ten unique aa substitutions were observed in genotypes M2-I, M2-II and M2-III of TRVs, which was not seen in CRV, DRV or GRV sequences. No sequence motif was observed that could differentiate TARVs from TERVs. However, random aa substitutions were observed in TRV (both TARV and TERV) sequences. Based on M2 genome, the ARV sequences were different from MRV and TVAV sequences with nt identity varying from 46.7% to 64.9% and 64.1% to 67.6%, respectively.

3.2.3. M3 gene and μNS protein

The M3 gene segment of TRV was the smallest in M class (1996 nt). The 5' and 3' UTR were 24 and 64 nt long and the predicted ORF from nt position 25 to 1932. The single ORF encodes for 635 aa non-structural protein μNS. The two α-helical –coiled –coil structures are at position 451-472 and 540-599. On the basis of pairwise identity chart and phylogenetic tree, a cutoff value of 85% was used, which generated four ARV genotypes (M3-I to M3-IV) (Fig. 4.7). The TRV (TARV and TERV) sequences clustered together with the CRV sequences in M3-I genotype. However, 11 aa substitutions were unique to TARV and TERV sequences which were different from those of CRV, DRV,

and GRV. No substitutions or aa motifs were observed that could differentiate TERV and TARV strains.

Based on M3 gene and μNS protein sequence analysis, ARV sequences were different from MRV and TVAV sequences with only 32.1% -48.7% and 48.9%-51.1% nt identity, respectively (Fig. 4.8). Genotype M3-I contained all TARVs (n=15), all TERVs (n=8) and a large number of CRVs (n=14) from Asia and North America with 86.3%-100% nt and 92.1%-100% aa identities with each other (Table 4.1). Genotype M3-II included one Hungarian (T1781) and four Taiwanese (R2, 916SI, 918, 1017-1) strains of CRV with 89.2%-99.2% nt and 95.6%-99.2% aa identities. Genotype M3-III included all DRVs and one GRV (03G) with 87.3%-99.6% nt and 94.0%-99.6% aa identities (Table 4.1). Genotype M3-IV included one GRV (D20) from Hungary (Fig. 4.8).

3.2.4. M class genotype constellation and reassortment.

Using the proposed genotypes for the three M gene segments, M class genotype constellations (GC) were constructed, which yielded 12 different GCs (Table 4.2-4.3). The TRV strains consisted of 3 GCs (GC1-GC3) while the CRV strains consisted of 8 GCs (GC1 and GC-3 to GC9). The DRV and GRV strains consisted of 3 GCs (GC9-GC11) and 2 GCs (GC11 and GC12), respectively.

Only GC2 was unique to TRV strains while GC1 and GC3 were shared between TRV and CRV strains (Table 4.2). The GC1 consisted of 9 TARVs, 5 TERVs, and 11 CRVs. The TRVs were isolated from the US in 2011-2013 while the 11 CRV strains were reported from US, Canada, China, and Taiwan during 1970-2006. The GC3 was formed by TARV (n=1) and TERV (n=1) isolated from the US in 2011and three CRVs

reported from the US and Taiwan from 1986-2006. The Japanese chicken enteric strain (OS161) and Hungarian chicken neurotropic strain (T1781) formed the unique GC3 and GC8, respectively. The Taiwanese chicken origin enteric strains 916SI and 918 formed unique GC6 and GC7, respectively. When GCs of arthritic strains of ARVs were compared, TARVs formed three GCs (GC1, GC2, GC3) and arthritic strains of CRV also formed three GCs (GC1, GC3, GC5).

4. Discussion

Reovirus-associated turkey arthritis re-emerged in late 2009 and the problem still continues today as evidenced by regular receipt of lameness and arthritis cases by the UMVDL. It is important to understand the ecology of these viruses to try to mitigate their devastating effects on the poultry industry in general and the turkey industry in particular. In a previous study, we reported on the molecular characterization of these isolates based on complete S gene segments (Mor et al., 2014). In this study, we have characterized the M gene of TARVs and TERVs for the first time.

The presence of conserved residues and motifs such as N-6 adenine-specific DNA methylase and single stretch of basic aa in μA protein, cleavage aa residue (NP) in μB protein and two α-helical –coiled –coil structures in μNS protein are consistent with homologous μA, μB, and μNS proteins of CRVs, respectively (Noad et al., 2006; Su et al., 2006). The residue R at position 582 was also conserved in all ARV sequences which is predicted as a trypsin cleavage site for dissociation of μBC to generate intermediate subviral particle (ISVP)- associated peptides δ and ϕ during cell entry and uncoating (Noad et al., 2006).

However, genetic variations were observed that may help differentiate TRVs from CRVs. A total of 7 variations in μA, 4 to 10 in μB and 11 in μNS of TRVs were observed that differentiate them from CRVs, DRVs and GRVs. Currently, the TARV-Crestview strain is used as an inactivated, autogenous vaccine in the field (Dave Mills, personal communication). When this vaccine strain was compared with other TARVs and TERVs we found two and one unique aa residues that could be used as a marker to differentiate it from wild strains of TARVs (Mor, unpublished observations).

Phylogenetic analysis of M1 and M3 genes placed TARVs and TERVs into a single genotype while M2 gene divided them into three genotypes, which indicates possible reassortment among TARVs and TERVs and with CRVs. All TARVs and TERVs formed three GCs (GC1-GC3) indicating possible reassortment between the two types of turkey reoviruses (TARV and TERV). Based on our proposed GCs, the ARVs were divided into three GCs of which GC2 was unique to TRVs only.

The maximum number of GCs (n=7) was formed by CRVs of which GC1 and GC3 were shared with TARVs and TERVs indicating potential reassortments between TRVs and CRVs. An interesting finding was that the maximum number of TARVs (n=9), TERVs (n=5) and all North American CRV strains (n=5) (except strain AVS-B) formed GC1 indicating that GC1 is more prevalent in chicken and turkey populations in North America. The DRVs and GRVs did not share any GC with TARVs, TERVs and CRVs, indicating no reassortment between these viruses. The nt percent identity was higher than aa percent identity within the TRV sequences indicating that synonymous substitutions were higher than nonsynonymous changes. These findings are in agreement with

previous studies on M class gene analysis of CRVs, DRVs and GRVs (Noad et al., 2006, Su et al., 2006, Wang et al., 2013; Zhang et al., 2007).

In this study, nt cut off values of 84%, 83% and 85% were proposed for the M1, M2 and M3 gene segments, which generated 5, 7, and 3 genotypes, respectively. While previous CRV and DRV studies defined a cluster of genetically similar sequences with less than or equal to 10% divergence, our method uses nt percent identities and the phylogenetic tree to construct a cut-off value for each gene segment. This classification method has been used extensively for other viruses including astroviruses, Sapporo like viruses, noroviruses, hantaviruses, papillomaviruses, and rotaviruses (de Villiers et al., 2004; Maes et al., 2009; Marthaler et al., 2012; Schuffenecker et al., 2001; Zheng et al., 2006).

Banyai et al. (2014) described complete genome of a reptilian reovirus (RRV) isolate (47/02) from Bush viper and found broader range of similarities with available sequences of python-origin RRV strains, suggesting that RRVs from bush viper and python could be antigenically different based on sequence differences of sigma C protein. These authors further stressed on the importance of intra-species classification due to genetic diversity among RRVs.

The TARVs and TERVs shared GC1 and GC3 with some CRVs, which further indicates reassortment between chicken and turkey reoviruses. This has been experimentally proven that TARVs and TERVs are able to replicate in chickens and CRVs are able to replicate in turkeys (Rosenberger 2013a; Sharafeldin et al., 2014a; 2014b). Interestingly, the M2-III genotype contained TARV-MN4, TERV-MN5, an enteric CRV strain (AVS-B) isolated in 2006, and an arthritis strain (916S1) isolated in

1992 from Taiwan further indicating reassortment between different pathotypes of chicken and turkey reoviruses.

When we compared these genotype M2-III sequences based on GCs, the strain 916S1 formed a unique GC6 different from other M2-III sequences, which formed GC3. The TARV-MN4 was isolated from lame turkey poults at five weeks of age but TERV-MN5 was isolated from turkey poults suffering from enteritis at four weeks of age from different commercial flocks. However, these flocks belonged to the same hatchery, suggesting vertical transmission of these two types of strains. While the TARVs, TERVs, and DRVs did not group together in any of the M class gene segments, some of the same aa substitutions were observed that were not observed in CRVs.

In phylogenetic tree, DRV and GRV strains seemed to be have evolved together with CRVs, TARVs and TERVs compared to MRV strains, which suggests the possibility of co-evolution of CRVs, TARVs and TERVs together with DRVs and GRVs. Co-infection of different ARV strains may lead to emergence of new ARV strains, which has been proven in an experimental study by Ni and Kemp (1992). Chicken embryo fibroblasts were co-infected with ARV strain 883 and with 1 of 3 CRV strains (176, S1133, or 81-5), which indicated gene segment selection was virus strain specific. In future studies, it will be interesting to see outcome of co-infection of CRVs, TARVs and TERVs in different combinations to understand the reassortment among them.

It is interesting to note that TERVs have been isolated from apparently healthy and enteritis-affected turkeys for years (Jindal et al., 2010a, b; Pantin-Jackwood et al., 2008) but, until recently, there have been no reports on reovirus-associated lameness and arthritis in turkeys after it was first reported in the 1980s (al Afaleq et al., 1989; Levisohn

et al., 1980). After a hiatus of >20 years, the problem of turkey arthritis re-emerged in the upper Midwest area initially and then was reported from several US states in both commercial and breeder flocks.

Recently, new variants of CRVs associated with viral arthritis in broiler chickens have also been reported from Europe and North America (Rosenberger et al., 2013a; Sellers et al., 2013; Troxler et al., 2013). Based on virus neutralization test, it has been proved that new variants of CRVs and TARVs are antigenically different and do not cross neutralize (Rosenberger et al., 2013b). In addition, commercially available CRV vaccines have been found to be ineffective against these new variants. The occurrence of these two events simultaneously in two different host species (chickens and turkeys) raises the question on the source of these arthritis-associated reoviruses. Unfortunately, sequences from the CRV new variants are not yet available for comparison in GenBank but there could be three possibilities for the sudden appearance of these viruses: Firstly, sequencing data indicate that CRV strains are closely related to TARV and TERV strains and share one genomic constellation (GC1), which suggests possible common source of TARV and new CRFV variants. However, turkey and chicken hatcheries are two different sorts of units and do not share a common connection. However, possible virus transmission between these two types of units can occur via aerosols, wild birds, or by mechanical means (e.g., through fomites, personnel, or farm equipment).

Secondly, TARVs could be variants of TERVs present in apparently healthy breeder flocks. In a comparative study of CRVs, the enteric strain CO_8 was reported to cause tenosynovitis in 1-day-old specific-pathogen-free (SPF) chicks by the footpad inoculation but not by oral or subcutaneous inoculation (Tang et al., 1987). Similarly, the

neurotropic CRV strain T1781 isolated from the brain of 18-day-old broiler chickens produced tenosynovitis after inoculation by the footpad route in 1-day-old SPF chicks (Dandar et al., 2013). Some of the new variants of CRVs are able to cause both enteritis and tenosynovitis in chickens (Rosenberger et al., 2013a). So, there could be possibility that TERVs could be source of TARVs.

Thirdly, breeder companies would have changed their breeding characteristics, which inadvertently made new breeder flocks more susceptible to arthritic reoviruses. Jones and Kibenge (1984) studied effect of chicken breeds on infection with an arthrotropic CRV strain R2 by oral and footpad inoculation. The 1-day-old chicks of three different breeds: i) SPF light-hybrid, ii) commercial white leghorn egg-layer and iii) commercial Ross-1 broiler were inoculated and observed for 12 weeks of age. All three breed developed swelling of hock joint at 3-4 weeks of age, but tenosynovitis lesions were only observed in commercial Ross-1 broilers. Recently, Troxler et al. (2013) analyzed sigma C protein sequences of 21 CRVs from 17 broiler farms and five hatcheries in France. They succeeded in predicting a common origin of the virus as well as its horizontal and vertical transmission on the basis of distribution to the involved hatcheries and broiler breeder flocks. In future, screening of breeder and commercial turkey flocks for TRV strains and broilers for CRVs and their complete genome sequencing may help to differentiate these viruses and may help to predict the source of infection. In addition, wild birds commonly found around hatcheries should be screened for ARVs.

The three TARV isolates used in the pathogenicity study were TARV-O'Neil, TARV-MN2 and TARV-MN4. These isolates formed GC1, GC2, and GC3, respectively,

and produced tenosynovitis. However, TERV-MN1 was also grouped in GC2 although it did not produce tenosynovitis. An interesting finding was that TARV, TERV and CRV sequences of genotypes M1-I and M3-I formed different GCs. The presence of different GCs indicated that reassortments can occur within and between TERV, TARV, and CRV.

In conclusion, this appears to be the first report on molecular characterization and reassortment events of TARV and TERV strains based on complete M class genome segments. We have proposed a nt cut-off values to define ARV genotypes for the three M class gene segments, which should be useful in identifying new ARV genotypes in the future. In addition, CGs were identified for each avian host species. Future ARV studies should be conducted on complete genome sequencing to understand the reassortment events between co-infection of TRV and CRV strains as well as with DRV and GRV strains to understand the molecular evolution of ARV. Recent reports on new variants of CRV and TARV strains are alarming and indicate a strong need for continued surveillance, epidemiological and genetic studies to develop adequate vaccine.

Table 4.1. Nucleotide and amino acid identities of different avian reovirus genotypes. Upper part represents per cent nucleotide identity and lower highlighted part represents amino acids identities.

M1 genome segment

Genotypes	M1-I	M1-II	M1-III	M1-IV	M1-V
M1-I	84.8-100 95.1-100	79.4-81.3	72.7-73.9	72.8-73.9	73.1-74.5
M1-II	91.8-94.5	NA NA	73.4-73.6	73.5	73.4-74.1
M1-III	84.4-86.3	84.7-85.0	99.3-99.7 99.2-99.7	82.3-82.5	80.8-81.8
M1-IV	84.6-85.8	84.3	91.5-91.8	NA NA	79.7-80.5
M1-V	85.3-87.4	84.7-85.7	93.4-94.8	90.6-91.5	95.3-99.4 96.9-99.5

M2 genome segment

Genotypes	M2-I	M2-II	M2-III	M2-IV	M2-V	M2-VI	M2-VII
M2-I	86.5-100 94.7-100	74.0-75.4	74.0-75.9	73.5-75.3	73.0-74.5	75.2-77.8	68.2-69.4
M2-II	88.8-89.8	94.9-99.9 98.8-100	73.3-75.1	74.3-74.6	74.8-76.3	74.8-76.1	67.6-68.2
M2-III	86.8-89.8	88.2-89.8	83.2-99.7 93.6-99.6	74.7-76.8	72.7-74.6	75.4-77.3	66.2-67.4
M2-IV	86.1-87.4	87.0-87.3	88.9-90.8	NA NA	72.7-72.9	74.9-75.9	66.5-67.3
M2-V	84.8-86.1	88.8-90.1	86.9-88.2	85.4-86.0	83.1-100 94.2-100	74.2-75.5	67.3-69.1
M2-VI	88.6-90.5	90.2-91.6	88.3-91.4	87.1-88.0	88.1-89.3	87.1-99.2 95.4-99.6	67.2-67.9
M2-VII	74.9-76.8	77.2-78.4	75.0-77.1	74.7-75.4	76.5-77.0	75.3-76.9	92.1-99.5 96.8-99.6

M3 genome segment

	M3-I	M3-II	M3-III	M3-IV
M3-I	86.3-100 92.1-100	78.7-81.2	66.6-72.5	70.9-72.8
M3-II	88.0-92.9	89.2-99.2 95.6-99.2	66.9-72.1	71.2-71.9
M3-III	71.5-81.6	72.5-81.9	87.3-99.6 94.0-99.6	79.2-80.3
M3-IV	79.8-80.9	80.0-81.3	90.9-92.0	NA NA

Table 4.2. Genotypes of ARVs indicating reassortment

Virus strain	Origin	Source	Pathotype (*)	Year of isolation	Genotype Constellations (GC)
Genotype I-I-I					
Turkey					
TARVs					
TARV-Crestview	USA/MN	Tendon	VA	2011	1
TARV-O'Neil	USA/MN	Tendon	VA	2011	1
TARV-MN1	USA/MN	Tendon	VA	2011	1
TARV-MN3	USA/MN	Tendon	VA	2011	1
TARV-MN5	USA/WI	Tendon	VA	2011	1
TARV-MN6	USA/NC	Tendon	VA	2011	1
TARV-MN7	USA/SD	Tendon	VA	2012	1
TARV-MN8	USA/MN	Tendon	VA	2013	1
TARV-MN12	USA/MI	Tendon	VA	2013	1
TERVs					
TERV-MN3	USA/MN	Feces	PES	2011	1
TERV-MN4	USA/MN	Feces	PES	2011	1
TERV-MN6	USA/MN	Feces	LTS	2011	1
TERV-MN7	USA/MN	Feces	Normal	2012	1
TERV-MN8	USA/MN	Feces	Normal	2012	1
Chicken-CRV					
S1133	USA	Tendon	VA	1971	1
1733	USA	Feces	MAL	1983	1
2408	USA	Feces	MAL	1983	1
601SI	Taiwan	Tendon	VA	1992	1
T6	Taiwan	Lung	RES	1970	1
919	Taiwan		Normal	1992	1
R2	Taiwan	Tendon	VA	1992	1
C98	China	Tendon	VA	2006	1
T98	China	Tendon	VA	2006	1
GuangxiR1	China			2000	1
ARV138	Canada	Tendon	VA		1
ARV176	USA	Tendon	VA		1
Genotype I-II-I					
Turkey					
TARVs					
TARV-MN2	USA/MN	Tendon	VA	2011	2
TARV-MN9	USA/MN	Tendon	VA	2013	2
TARV-MN10	USA/IA	Tendon	VA	2013	2
TARV-MN11	USA/MN	Tendon	VA	2013	2
TARV-MN13	USA/MN	Tendon	VA	2014	2
TERVs					2
TERV-MN1	USA/MN	Feces	PES	2011	2
TERV-MN2	USA/MN	Feces	PES	2010	2
Genotype I-III-I					
Turkey					
TARV					
TARV-MN4	USA/MN	Tendon	VA	2011	3
TERV					

TERV-MN5	USA/MN	Feces	PES	2011	3
Chicken-CRVs					
750505	Taiwan	Tendon	VA	1986	3
601G	Taiwan	Tendon	VA	1992	3
AVS-B	USA	intestine	RSS	2006	3
Genotype I-IV-I Chicken- CRV					
OS161	Japan	Feces	MAL	1970	4
Genotype I-I-II Chicken-CRV					
R2	Taiwan	Tendon	VA	1992	5
1017-1	Taiwan	Feces	MAL	1992	5
Genotype I-III-II Chicken-CRV					
916SI	Taiwan	Feces	MAL	1992	6
Genotype I-V-II Chicken-CRV					
918	Taiwan	Feces	MAL	1992	7
Genotype II-V-II Chicken-CRV					
T1781	Hungary	Brain	CNS	2012	8
Genotype III-VI-III Duck-DRV					
MW9710	China	Liver	MDA		9
Genotype III-VII-III Duck-DRV					
ZJ2000M	China	Liver		2011	10
S14	China	Liver	MDA	1998	10
Genotype V-VI-III Duck-DRV					
ZZ	China	Liver			11
NP03	China	Liver	HNH	2003	11
J18	China	Liver	HNH	2008	11
091	China	Liver	LNS	2009	11
TH11	China	Liver	LNS	2011	11
ZJOOM	China	Liver		2000	11
89330	France	Liver	MDA	1989	11
Goose- GRV					
03G	China	Liver	HNH	2003	11
Genotype IV-VII-VI Goose-GRV					
D20	Hungary	Liver	GOA	1999	12

*VA= viral arthritis; MAS= malabsorption syndrome, RSS= runting-stunting syndrome, PES= poult enteritis syndrome, LTS= light turkey syndrome, RES= respiratory disease, CNS= central nervous system disease, HNH= hemorrhagic-necrotic hepatitis, LNS= large necrotic foci in spleen, MDA= ARV-Md-associated lesions, including necrotic foci in liver and spleen, GOA= ARV-Go-associated lesions, including necrotic foci in liver and spleen

Table 4.3. Genotype constellation of ARV in different avian species.

Species and number of GC	Genotype	Genotype constellation	Genotype constellation counts
Turkey (TRVs) (n=3)	I-I-I	1	14
	I-II-I	2	7
	I-III-I	3	2
Chicken (CRVs) (n=7)	I-I-I	1	11
	I-III-I	3	3
	I-IV-I	4	1
	I-I-II	5	2
	I-III-II	6	1
	I-V-II	7	1
	II-V-II	8	1
Duck (DRVs) (n=3)	III-VI-III	9	1
	III-VII-III	10	2
	V-VI-III	11	8
Goose (GRVs) (n=2)	V-VI-III	11	1
	IV-VII-VI	12	1

Figure 4.1. Gross findings showing: A) swollen tibiotarsal joints, (B) rupture of gastrocnemius and (C) digital flexor tendons.

Figure 4.1

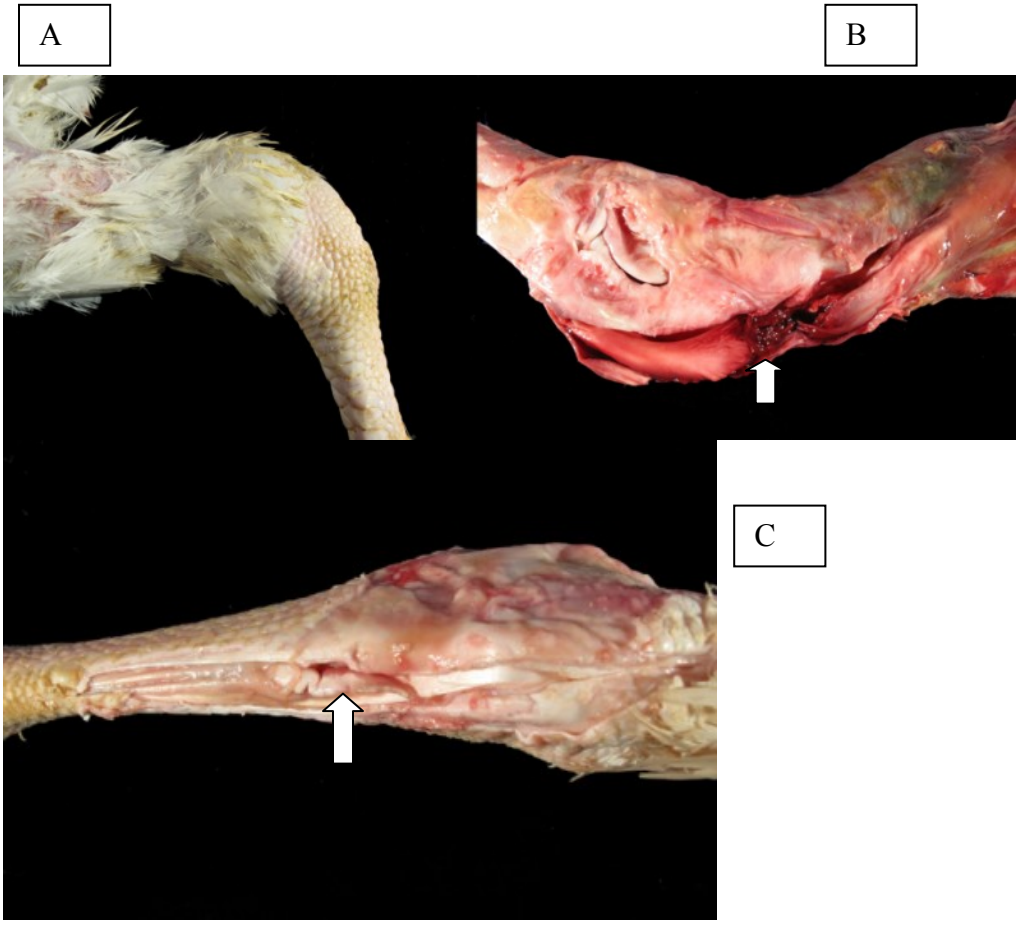


Figure 4.2. Micrograph of affected tendon showing fibrosis.

Figure 4.2

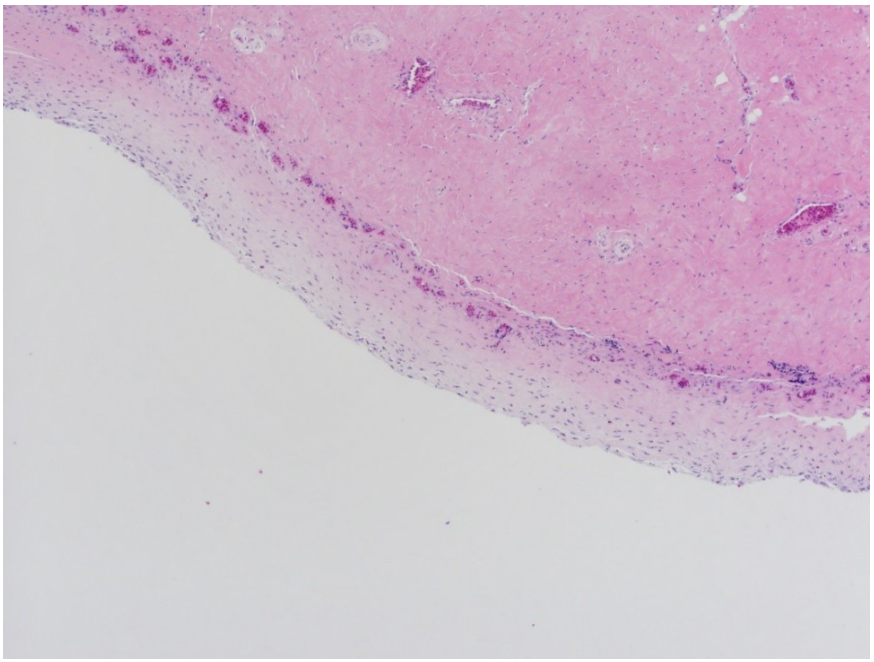


Figure 4.3. Pairwise identity frequency graph using complete ORF nucleotide sequences of M1 gene of 15 TARVs, eight TERVs and available ARV sequences in GenBank. The suitable nucleotide cut-off value (84%) is depicted by vertical solid line.

Figure 4.3

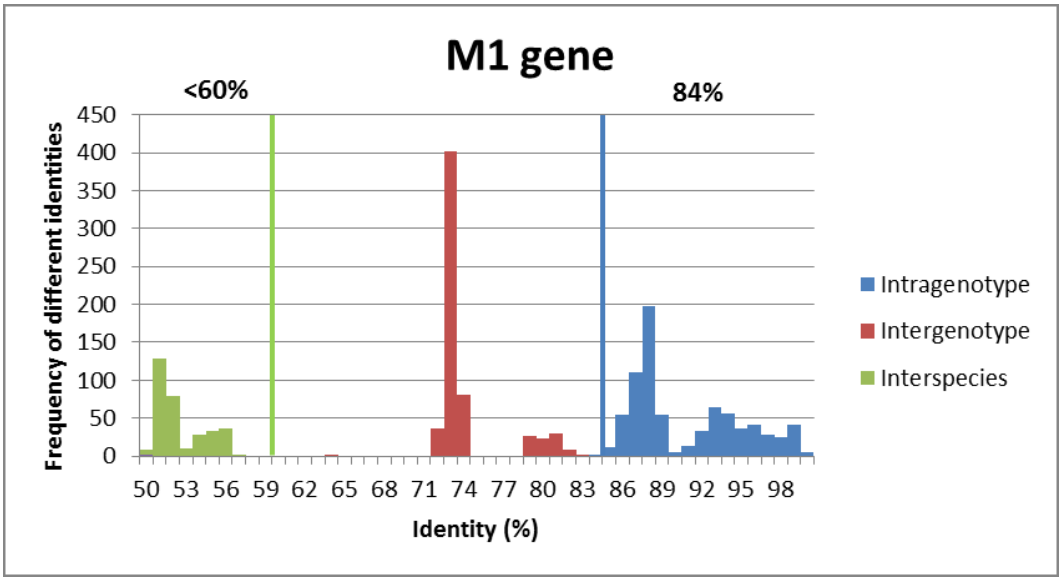
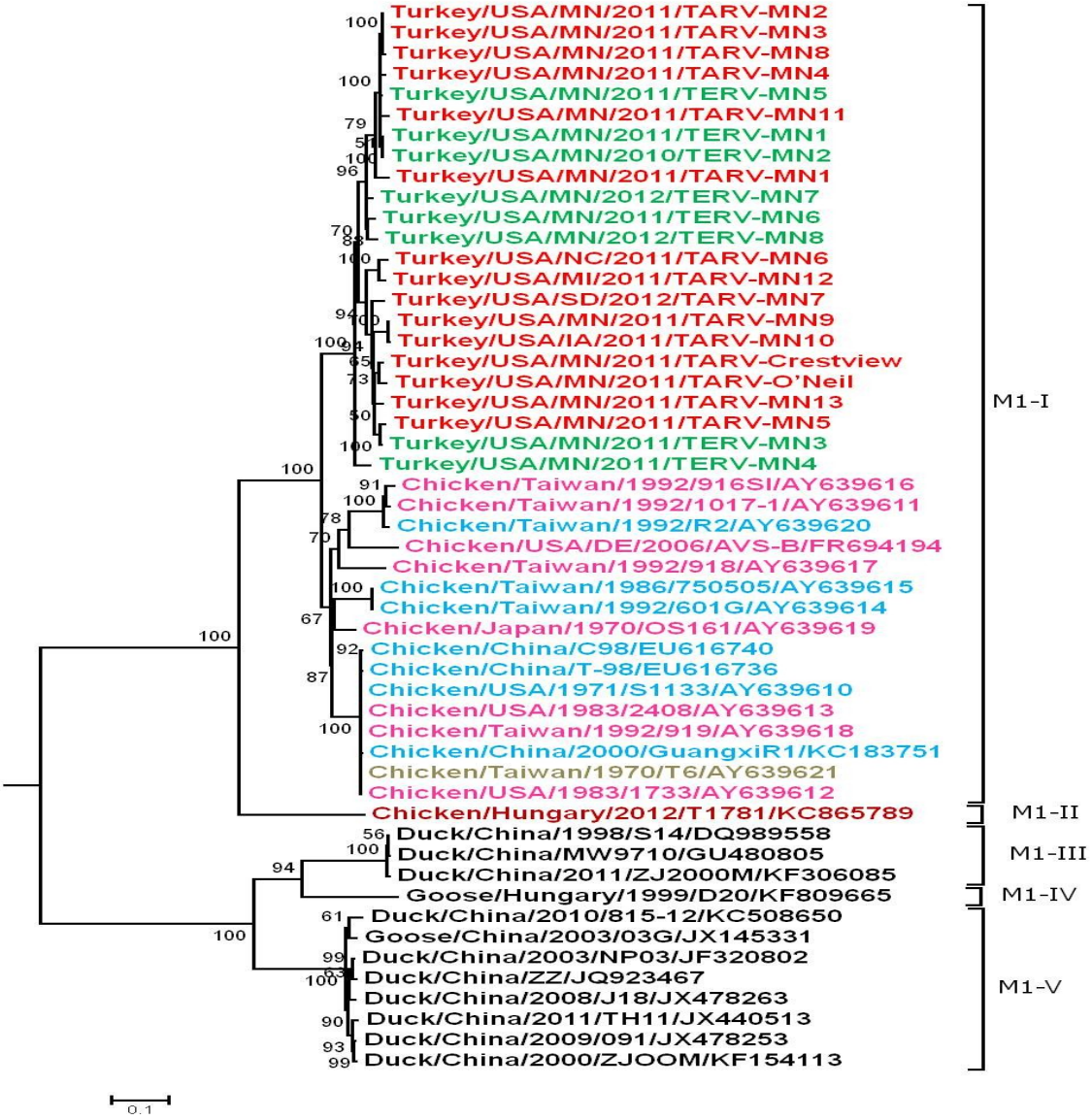

M1 gene
<60%
84%
Frequency of different identities
450
400
350
300
250
200
150
100
50
0
50 53 56 59 62 65 68 71 74 77 80 83 86 89 92 95 98
Identity (%)
Intragenotype
Intergenotype
Interspecies

Figure 4.4. Phylogenetic tree constructed based on complete ORF nucleotide sequences of M1 gene of ARV sequences. Tree was constructed in MEGA 6.06 using HKY+G+I model with Maximum Likelihood method and 100 bootstrap replicates.

Figure 4.4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Turkey/USA/MN/2011/TARV-MN3
Turkey/USA/MN/2011/TARV-MN8
Turkey/USA/MN/2011/TARV-MN4
Turkey/USA/MN/2011/TERV-MN5
Turkey/USA/MN/2011/TARV-MN11
Turkey/USA/MN/2011/TERV-MN1
Turkey/USA/MN/2010/TERV-MN2
Turkey/USA/MN/2011/TARV-MN1
Turkey/USA/MN/2012/TERV-MN7
Turkey/USA/MN/2011/TERV-MN6
Turkey/USA/MN/2012/TERV-MN8
Turkey/USA/NC/2011/TARV-MN6
Turkey/USA/MI/2011/TARV-MN12
Turkey/USA/SD/2012/TARV-MN7
Turkey/USA/MN/2011/TARV-MN9
Turkey/USA/IA/2011/TARV-MN10
Turkey/USA/MN/2011/TARV-Crestview
Turkey/USA/MN/2011/TARV-O'Neil
Turkey/USA/MN/2011/TARV-MN13
Turkey/USA/MN/2011/TARV-MN5
Turkey/USA/MN/2011/TERV-MN3
Turkey/USA/MN/2011/TERV-MN4
Chicken/Taiwan/1992/916SI/AY639616
Chicken/Taiwan/1992/1017-1/AY639611
Chicken/Taiwan/1992/R2/AY639620
Chicken/USA/DE/2006/AVS-B/FR694194
Chicken/Taiwan/1992/918/AY639617
Chicken/Taiwan/1986/750505/AY639615
Chicken/Taiwan/1992/601G/AY639614
Chicken/Japan/1970/OS161/AY639619
Chicken/China/C98/EU616740
Chicken/China/T-98/EU616736
Chicken/USA/1971/S1133/AY639610
Chicken/USA/1983/2408/AY639613
Chicken/Taiwan/1992/919/AY639618
Chicken/China/2000/GuangxiR1/KC183751
Chicken/Taiwan/1970/T6/AY639621
Chicken/USA/1983/1733/AY639612
Chicken/Hungary/2012/T1781/KC865789
Duck/China/1998/S14/DQ989558
Duck/China/MW9710/GU480805
Duck/China/2011/ZJ2000M/KF306085
Goose/Hungary/1999/D20/KF809665
Duck/China/2010/815-12/KC508650
Goose/China/2003/03G/JX145331
Duck/China/2003/NP03/JF320802
Duck/China/ZZ/JQ923467
Duck/China/2008/J18/JX478263
Duck/China/2011/TH11/JX440513
Duck/China/2009/091/JX478253
Duck/China/2000/ZJOOM/KF154113
M1-I
M1-II
M1-III
M1-IV
M1-V
0.1

Figure 4.5. Pairwise identity frequency graph using complete ORF nucleotide sequences of M2 gene of 15 TARVs, eight TERVs and available ARV sequences in GenBank. The suitable nucleotide cut-off value (83%) is depicted by vertical solid line.

Figure 4.5

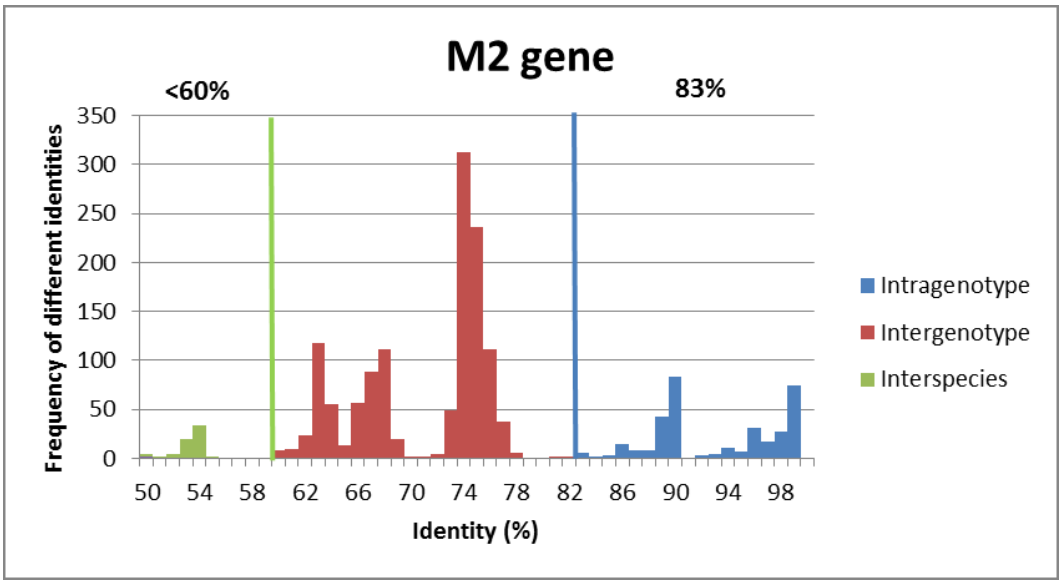
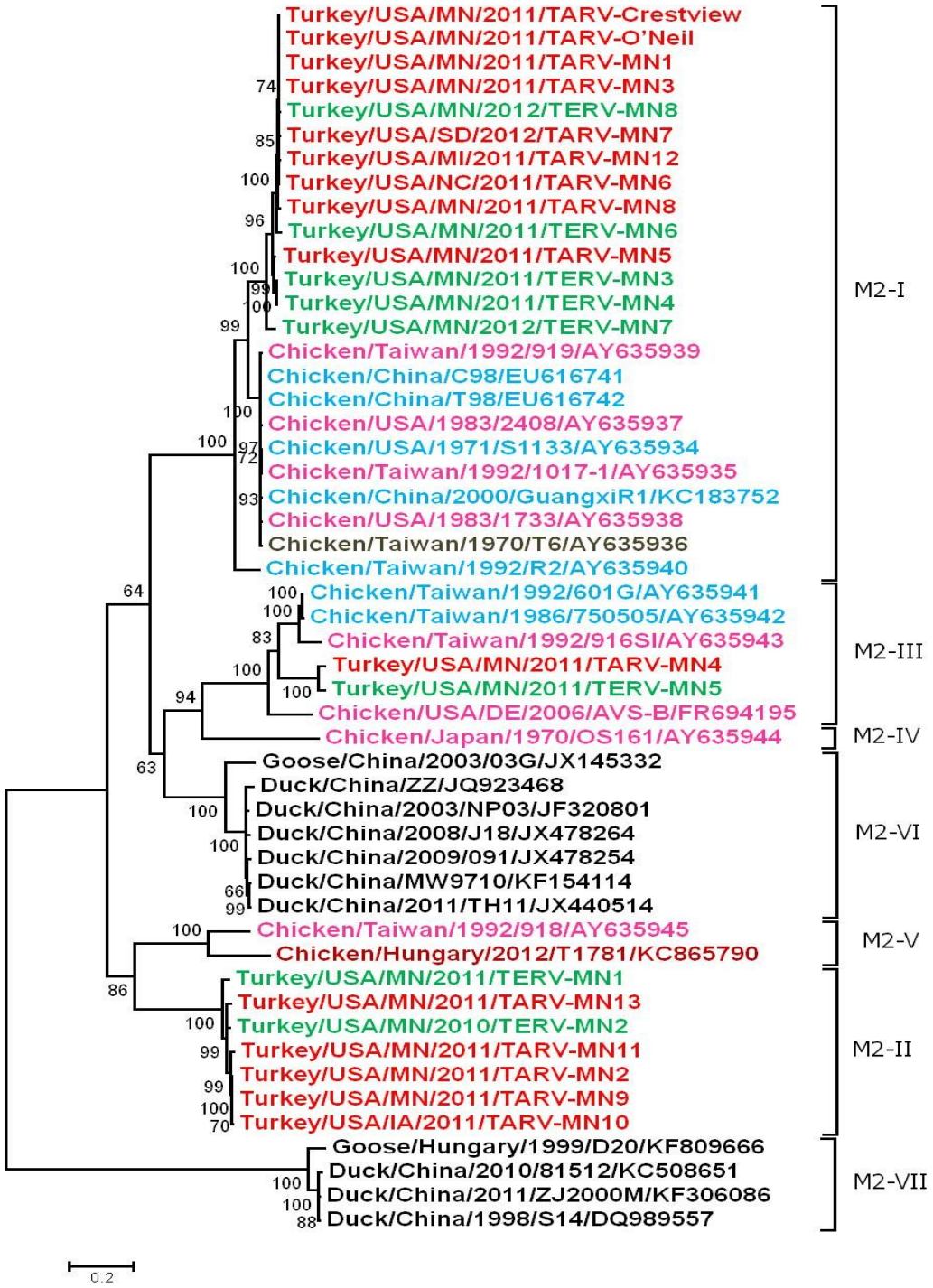

M2 gene
<60%
83%
Frequency of different identities
350
300
250
200
150
100
50
0
50 54 58 62 66 70 74 78 82 86 90 94 98
Identity (%)
Intragenotype
Intergenotype
Interspecies

Figure 4.6. Phylogenetic tree constructed based on complete ORF nucleotide sequences of M2 gene of ARV sequences. Tree was constructed in MEGA 6.06 using GTR+G model with Maximum Likelihood method and 100 bootstrap replicates.

Figure 4.6


Turkey/USA/MN/2011/TARV-Crestview
Turkey/USA/MN/2011/TARV-O'Neil
Turkey/USA/MN/2011/TARV-MN1
Turkey/USA/MN/2011/TARV-MN3
Turkey/USA/MN/2012/TERV-MN8
Turkey/USA/SD/2012/TARV-MN7
Turkey/USA/MI/2011/TARV-MN12
Turkey/USA/NC/2011/TARV-MN6
Turkey/USA/MN/2011/TARV-MN8
Turkey/USA/MN/2011/TERV-MN6
Turkey/USA/MN/2011/TARV-MN5
Turkey/USA/MN/2011/TERV-MN3
Turkey/USA/MN/2011/TERV-MN4
Turkey/USA/MN/2012/TERV-MN7
Chicken/Taiwan/1992/919/AY635939
Chicken/China/C98/EU616741
Chicken/China/T98/EU616742
Chicken/USA/1983/2408/AY635937
Chicken/USA/1971/S1133/AY635934
Chicken/Taiwan/1992/1017-1/AY635935
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Chicken/USA/1983/1733/AY635938
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Chicken/Taiwan/1992/601G/AY635941
Chicken/Taiwan/1986/750505/AY635942
Chicken/Taiwan/1992/916SI/AY635943
Turkey/USA/MN/2011/TARV-MN4
Turkey/USA/MN/2011/TERV-MN5
Chicken/USA/DE/2006/AVS-B/FR694195
Chicken/Japan/1970/OS161/AY635944
Goose/China/2003/03G/JX145332
Duck/China/ZZ/JQ923468
Duck/China/2003/NP03/JF320801
Duck/China/2008/J18/JX478264
Duck/China/2009/091/JX478254
Duck/China/MW9710/KF154114
Duck/China/2011/TH11/JX440514
Chicken/Taiwan/1992/918/AY635945
Chicken/Hungary/2012/T1781/KC865790
Turkey/USA/MN/2011/TERV-MN1
Turkey/USA/MN/2011/TARV-MN13
Turkey/USA/MN/2010/TERV-MN2
Turkey/USA/MN/2011/TARV-MN11
Turkey/USA/MN/2011/TARV-MN2
Turkey/USA/MN/2011/TARV-MN9
Turkey/USA/IA/2011/TARV-MN10
Goose/Hungary/1999/D20/KF809666
Duck/China/2010/81512/KC508651
Duck/China/2011/ZJ2000M/KF306086
Duck/China/1998/S14/DQ989557
M2-I
M2-III
M2-IV
M2-VI
M2-V
M2-II
M2-VII
0.2

Figure 4.7. Pairwise identity frequency graph using complete ORF nucleotide sequences of M3 gene of 15 TARVs, eight TERVs and available ARV sequences in GenBank. The suitable nucleotide cut-off value (85%) is depicted by vertical solid line.

Figure 4.7

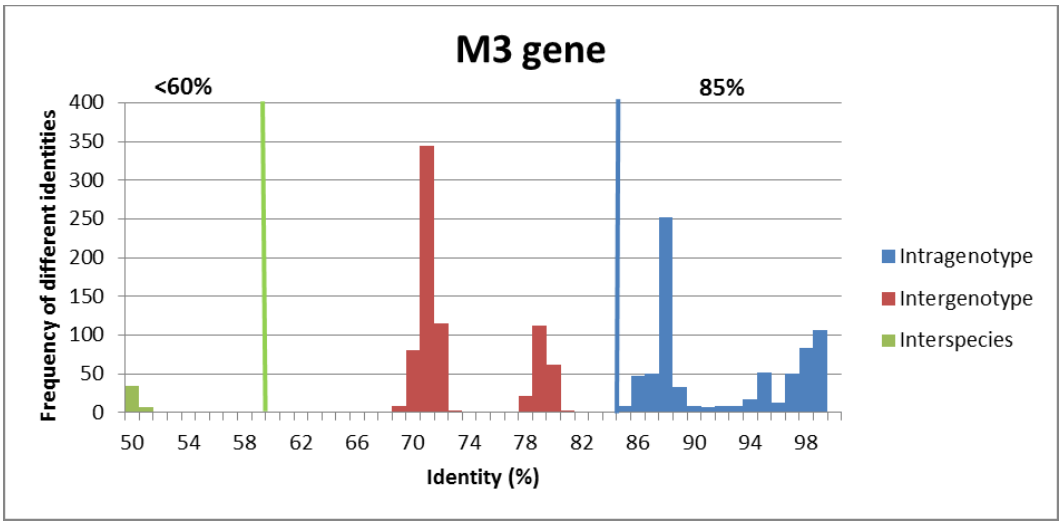
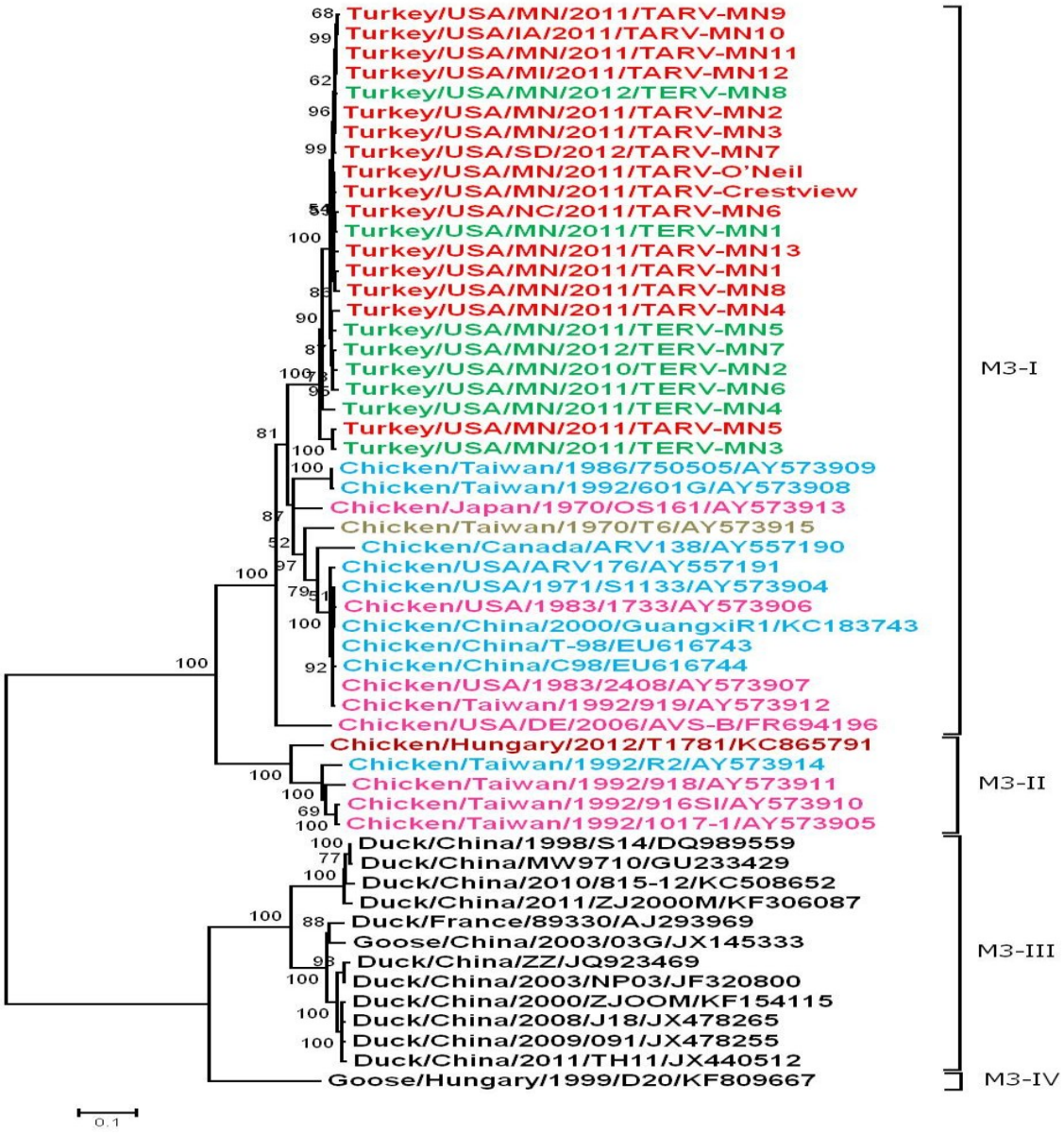

M3 gene
<60%
85%
Frequency of different identities
400
350
300
250
200
150
100
50
0
50
54
58
62
66
70
74
78
82
86
90
94
98
Identity (%)
Intragenotype
Intergenotype
Interspecies

Figure 4.8. Phylogenetic tree constructed based on complete ORF nucleotide sequences of M3 gene of ARV sequences. Tree was constructed in MEGA 6.06 using GTR+G model with Maximum Likelihood method and 100 bootstrap replicates.

Figure 4.8


Turkey/USA/MN/2011/TARV-MN9
Turkey/USA/IA/2011/TARV-MN10
Turkey/USA/MN/2011/TARV-MN11
Turkey/USA/MI/2011/TARV-MN12
Turkey/USA/MN/2012/TERV-MN8
Turkey/USA/MN/2011/TARV-MN2
Turkey/USA/MN/2011/TARV-MN3
Turkey/USA/SD/2012/TARV-MN7
Turkey/USA/MN/2011/TARV-O'Neil
Turkey/USA/MN/2011/TARV-Crestview
Turkey/USA/NC/2011/TARV-MN6
Turkey/USA/MN/2011/TERV-MN1
Turkey/USA/MN/2011/TARV-MN13
Turkey/USA/MN/2011/TARV-MN1
Turkey/USA/MN/2011/TARV-MN8
Turkey/USA/MN/2011/TARV-MN4
Turkey/USA/MN/2011/TERV-MN5
Turkey/USA/MN/2012/TERV-MN7
Turkey/USA/MN/2010/TERV-MN2
Turkey/USA/MN/2011/TERV-MN6
Turkey/USA/MN/2011/TERV-MN4
Turkey/USA/MN/2011/TARV-MN5
Turkey/USA/MN/2011/TERV-MN3
Chicken/Taiwan/1986/750505/AY573909
Chicken/Taiwan/1992/601G/AY573908
Chicken/Japan/1970/OS161/AY573913
Chicken/Taiwan/1970/T6/AY573915
Chicken/Canada/ARV138/AY557190
Chicken/USA/ARV176/AY557191
Chicken/USA/1971/S1133/AY573904
Chicken/USA/1983/1733/AY573906
Chicken/China/2000/GuangxiR1/KC183743
Chicken/China/T-98/EU616743
Chicken/China/C98/EU616744
Chicken/USA/1983/2408/AY573907
Chicken/Taiwan/1992/919/AY573912
Chicken/USA/DE/2006/AVS-B/FR694196
Chicken/Hungary/2012/T1781/KC865791
Chicken/Taiwan/1992/R2/AY573914
Chicken/Taiwan/1992/918/AY573911
Chicken/Taiwan/1992/916SI/AY573910
Chicken/Taiwan/1992/1017-1/AY573905
Duck/China/1998/S14/DQ989559
Duck/China/MW9710/GU233429
Duck/China/2010/815-12/KC508652
Duck/China/2011/ZJ2000M/KF306087
Duck/France/89330/AJ293969
Goose/China/2003/03G/JX145333
Duck/China/ZZ/JQ923469
Duck/China/2003/NP03/JF320800
Duck/China/2000/ZJOOM/KF154115
Duck/China/2008/J18/JX478265
Duck/China/2009/091/JX478255
Duck/China/2011/TH11/JX440512
Goose/Hungary/1999/D20/KF809667
M3-I
M3-II
M3-III
M3-IV
0.1

Chapter 5: Molecular Characterization of L class genome segments of a newly isolated turkey arthritis reovirus

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1. Introduction

Reoviridae is a large and diverse family of non-enveloped, icosahedral viruses whose protein capsid is arranged in one, two or three concentric capsid layers with an overall diameter of 60-90 nm. The virus family contains 15 genera divided into two groups, turreted or nonturreted, based on the presence of a "turret" protein situated at the 12 icosahedral vertices of the virus (Attoui et al., 2011; Schiff et al., 2007). The genus Orthoreovirus can be divided into two groups: fusogenic and non-fusogenic. The former have the ability to cause fusion of infected cells resulting in the formation of multinucleated, giant cells (syncytia) (Benavente and Martinez-Costas, 2007). Fusogenic reoviruses infect mammals, birds and reptiles and form a genetically distinct clade from non-fusogenic mammalian reoviruses (Day et al., 2009; Duncan, 1999). The recent taxonomic classification divides fusogenic reoviruses into five groups (I-V) (Attoui et al., 2011): group I includes mammalian orthoreoviruses (MRVs) including the Ndelle virus; group II has avian orthoreoviruses (ARVs); group III includes Nelson Bay virus (NBV) and related orthoreoviruses of bats; and groups IV and V include baboon (BRV) and reptilian orthoreoviruses (RRV), respectively.

The viral genome of ARVs is segmented and the ten segments are divided into three classes namely large (L), medium (M), and small (S), depending on their migration pattern on polyacrylamide gel electrophoresis (Benavente and Martınez-Costas, 2007; Varela and Benavente, 1994). The L and M genes are further subdivided into three segments each (L1, L2, L3 and M1, M2, M3) while the S gene has four segments (S1, S2, S3, S4; Benavente and Martinez-Costas, 2007). The proteins encoded by L, M and S

genes are lambda (λ), mu (μ) and sigma (σ), respectively. Three structural proteins λA, λB and λC are encoded by L gene segments L1, L2, and L3, respectively.

The predicted function and genetic variation of these L class gene segments and their encoded proteins have been reported for chicken reovirus (CRV), duck reovirus (DRV) and goose reovirus (GRV) (Dandar et al., 2014a; Shen et al., 2007; Wang et al, 2013; Xu et al., 2008; 2009; Yun et al., 2014). The L1 gene-encoded λA protein is thought to form the inner core shell that encloses both the virus genome segments and the viral RNA polymerase, which is used as a scaffold for subsequent core assembly. The L2-encoded λB protein is presumed to encode for the viral RNA-dependent RNA polymerase (RdRp), an essential enzyme for RNA virus replication (Xu et al., 2008). This protein extends from the inner core to the outer capsid of the avian reovirion (Martinez-Costas et al., 1997; Zhang et al., 2005). Pentamers of protein λC form the turrets projecting from the five-fold axes of viral cores (Zhang et al., 2005). Affinity radiolabeling of the structural polypeptides has revealed that protein λC is the viral capping enzyme. Thus, λC is the only structural protein that binds GMP through a phosphoamide linkage when virus particles are incubated with GTP, and the GMP moiety of the complex can be transferred to GDP and GTP acceptors, yielding the cap structure (Martinez-Costas et al., 1995).

Unfortunately, information on the major core proteins of L class is lacking for reoviruses of turkeys. In turkeys, two types of reoviruses have been reported which are tentatively named as turkey enteric reoviruses (TERVs) and turkey arthritis reoviruses (TARVs). The TERVs are the most common and have been detected in apparently healthy poults as well as in cases of turkey viral enteritis including poult enteritis

complex (PEC), poult enteritis syndrome (PES) and light turkey syndrome (LTS) (Jindal et al., 2010a; 2010b; Mor et al., 2013a; Pantin-Jackwood et al., 2008). Until the early 1990s; there were only three published reports on viral arthritis in turkeys. However, more than 20 years later the problem of reovirus-associated lameness/arthritis has been reported in 12-18-week-old market age tom turkeys in the upper Midwest area of the U.S. (Mor et al., 2013b; 2014a; 2014b; Sharafeldin et al., 2014a). The aim of this study was to characterize the major core proteins of L class genome segments of these newly isolated TARVs and to compare them with those of TERV, CRV, DRV and GRV.

2. Materials and Methods

2.1. Virus isolates

Seven strains of TARVs isolated from cases of swollen hock joints of 2- to-18-week-old tom turkeys from 2011- 2014 were used in this study. These isolates were named as TARV-MN2, TARV-MN4, TARV-MN9, TARV-MN10, TARV-MN11, TARV-MN12 and TARV-MN13. Three TERVs named as TERV-MN1, TERV-MN2 and TERV-MN6 were isolated from cases of turkey viral enteritis (PES and LTS) for comparison. The TARVs were isolated from gastrocnemius and digital flexor tendons of lameness affected commercial turkeys and TERVs were isolated from fecal samples of enteritis affected commercial turkeys. All viruses were isolated and propagated in QT-35 cell line as described previously (Mor et al., 2013a; 2013b; Mor et al., 2014b).

2.2. Illumina sequencing, sequence analysis and phylogeny

RNA was extracted from the viral isolates using Trizol LS reagent (Invitrogen, NY, USA) and extracted RNA was submitted to University of Minnesota Genomics Center (UMGC) for library preparation and 250 cycles paired end reads on the Illumina MiSeq next generation sequencing. The obtained sequence reads were analyzed by CLC Genomics Workbench 6.0 (www.clcbio.com). After trimming and sequence quality testing, contigs were prepared by *de novo* assembly. Extracted contigs were analyzed by BLAST (tBLASTx) analysis on NCBI.

The nucleotide sequences were generated from this study (n=10) and the sequences generated available from GenBank (n=31) were aligned using Clustal W method in MEGA 6.06 software (Tamura et al., 2013). The best substitution model for analysis of DNA sequences of each segment was selected on the basis of the lowest BIC score (Bayesian Information Criterion) in MEGA 6.06. We used GTR (General Time Reversible) +G (Gamma distribution with 5 rate categories) for all three (L1, L2 and L3) gene segments. The phylogenetic trees were constructed with selected model in MEGA 6.06 software and further statistical analysis was done by using 1000 bootstrap replicate values. The percent identities and amino acid histograms were constructed using Geneious Pro (Drummond et al., 2011). Chicken arthritis reovirus S1133 was used as a reference strain throughout this study.

2.3. GenBank accession numbers

The nucleotide sequences of all seven TARV and three TERV isolates were submitted to GenBank with accession numbers KJ865902-KJ865912, KJ865893-KJ865902, KJ865883-KJ865892 for L1, L2 and L3 genes, respectively.

3. Results

3.1. Sequence comparison of L class gene and proteins

3.1.1. L1 gene and λA protein

The L1 gene segment of TARVs and TERVs was of the same size (3959bp) as that of the CRV reference strain S1133 (AY641735). This gene segment encodes an ORF of 3882bp from nucleotide (nt) position 22-3903, which in turn encodes for λA protein of 1293 amino acid (aa). The 5' and 3' untranslated regions (UTR) were 21bp and 56bp in size. A total of 16 conserved C residues were observed in λA protein. A variable region was present in the first 120 aa of hydrophilic area of TARVs and TERVs (Fig. 5.1). The predicted C2H2 zinc-binding motif was present in λA protein of all TARVs and TERVs from position 177 to 207 and had four amino acid substitutions (N178S, N179S/G, T196T/A, and N207N/D) when compared to CRV. The unique motif 698-ELHKGRILQ-706 was observed in Taiwanese strain 750505 (DQ238093) different from reference strain (698-QNIDRRQCP-706). There were a total of five substitutions (T15A, D/N22T, V400I, A1070S, and I1261V) that differentiated TARVs and TERVs from CRVs.

3.1.2. L2 gene and λB protein

The L2 gene segment of TARVs and TERVs was also of the same size (3830bp) as that of CRV (DQ534201). This gene segment encodes an ORF of 3780bp from 15-3794nt position, which then encodes for λB protein of 1259aa in size. The 5' and 3' UTR were of 14bp and 36bp in size. Eight aa substitutions (D246N, S345A, S351A, T362I, A391S, K764R, N953S, A1150T) were observed in TARVs and TERVs that differentiated them from CRV. Similar to CRV reference strain, TARVs and TERVs also had conserved polymerase region from position 516-GLRNQVQ**RR**P**R**TIMP-529, 583-IDIK**A**C-588; bold and underlined functional residues are predicted to be responsible for proper positioning of incoming NTP triphosphate and of template nucleosides. In addition, residues 557-TSGSAVIEKVVP-568 were also observed; the underlined residues are responsible for maintaining NTP priming. The functional residues responsible for specific ribonucleotide activities (underlined) were observed at positions 583-IDIK**A**C-588 and 678-TFPSGS-683. The residues responsible for RNA polymerase activity (underlined) were at position 728-YVCQGDDG-735.

3.1.3. L3 gene and λC protein

The L3 gene and its encoded protein matched in size (3907bp) with those of CRV reference strain (DQ300175) and encoded an ORF of 3858bp in size (12-3870 nt position), which encoded a 1285 aa protein. The 5' and 3' UTR were 12bp and 37bp in size. Conserved functional motifs and residues were also observed in TARVs and TERVs that were similar to the two conserved K residues in CRV at 169 and 188 positions. These residues are considered responsible for guanylytransferase activity of λC protein

(Breun et al., 2001; Shen et al., 2007). A conserved ATP/GTP-binding site motif A (Hsiao et al., 2002; Shen et al., 2007) was also observed at position 379 to 386 in TARVs and TERVs with some aa substitutions as compared to CRV. The residues G and K at positions 384 and 385, respectively, were also conserved in TARVs and TERVs where λC protein is predicted to be cleaved into 42kDa and 100kDa. The λC protein also had S-adenosyl-L-methionine (SAM)-binding pocket for methyltransferase at position 822-LDLGAGPEA-830 in all TARVs and TERVs with one substitution (T826A) when compared to CRV (822-LDLGTGPEA-830).

3.2. Sequence identities and phylogenetic analysis of L genes and proteins

3.2.1. Sequence analysis of LI gene and λA protein

Using the seven TARV and three TERV sequences generated from this study and the reovirus L1 gene segment sequences from GenBank, a pairwise identity chart and phylogenetic tree was constructed on the nt and aa level (Fig. 5.2, 5.3). Based the nt phylogenetic tree and pairwise chart, an 88% cut off value was suitable. Sequence comparison and phylogenetic analysis of L1 gene divided all ARVs in to six genotypes (Fig. 5.3). Genotype L1-I included all TARVs and TERVs and three CRVs (Taiwan/601G, Taiwan/750505, Japan/OS161). Genotype L1-II included 15 CRVs and Genotype L1-III included one CRV from Hungary (Hungary/T1781). The DRVs and GRVs were divided into two separate genotypes: genotype L1-IV and genotype L1-V. Reovirus isolated from a wild crow (Tvarminne avian virus or TVAV) was included in genotype L1-VI. The nt identity within genotypes L1-I and L1-II was 89-100% and 88%-100%, respectively. Genotype L1-I sequences had 83%-88% and 83%-84% nt identity

with CRVs of genotypes L1-II and L1-III, respectively. The divergence at nt level was higher in all genotypes but they were closely related to each other at the aa level (Table 5.1). All ARVs (except the TVAV) grouped differently from MRVs with only 49%-69% nt and 42%-78% aa identity. The genotype L1-VI TVAV strain had 69% nt and 81% aa identity with Steller sea lion strain of reovirus.

3.2.2. Sequence analysis of L2 gene and λB protein

Using the seven TARV and three TERV sequences generated from this study and the reovirus L2 gene segment sequences from GenBank, a pairwise identity chart and phylogenetic tree was constructed on the nt and aa level (Fig. 5.4, 5.5). Based the nt phylogenetic tree and pairwise chart, an 90% cut off value was suitable. Based on phylogenetic analysis of L2 gene and its encoded protein all ARVs were divided into seven genotypes (Fig. 5.5). Genotype L2-I included all TARVs, and TERVs and genotype L2-II included two CRV strains: one arthritic strain from Canada (ARV/138) and one enteritic strain from USA (AVS-B). Genotypes L2-III and L2-IV included one Hungary strain (T1781) and five CRVs strains, respectively (Fig. 5.5). All DRVs and GRVs were divided into genotypes L2-V and L2-VI while the reovirus from crow (TVAV) constituted genotype L2-VII. The nt identity was 91%-100% within genotype L2-I as well as genotype L2-II sequences and 99%-100% in genotype L2-IV sequences. Similar to L1 gene, the divergence at nt level was higher within genotypes I and II but they were closely related to each other at the aa level (Table 5.1). The MRVs were grouped differently from ARVs (except TVAV) with 53%-66% nt and 42%-78% aa

identity. The genotype III TVAV strain was related to Steller sea lion strains with 71% nt and 82% aa identity.

3.2.3. Sequence analysis of L3 gene and λC protein

A pairwise identity chart and phylogenetic tree was constructed on the nt and aa level, using the seven TARV and three TERV sequences generated from this study and the reovirus L3 gene segment sequences from GenBank, (Fig. 5.6, 5.7). Based the nt phylogenetic tree and pairwise chart, an 87% cut off value was suitable. Sequence analysis of L3 gene divided all ARVs in to eight genotypes (Fig. 5.7). All TARVs and TERVs were grouped together in genotype L3-I with 89%-100% nt and 95%-100%aa identity with each other. Genotype L3-II had four CRVs (ARV138, AVS-B, R2 and 1017-1) with 89%-94% nt and 96%-98% aa identities. Genotype L3-III included 13 CRVs reported from Asia and North America with 95%-100% nt and 98%-100% aa identity with each other. Genotype L3-IV had two CRVs: one from Hungary (T1781) and one from Taiwan (916SI) with 90%-100% nt and 97%-100% aa identity with each other. The DRVs and GRVs were divided into three genotypes: L3-V, L3-VI and L3-VII with high nt and aa identities between sequences of each genotypes (Table 5.1). The wild bird TVAV strain formed genotype L3-VIII (Fig. 5.7). The nt and aa identities were higher between sequences of different genotypes as compared to L1 and L2 gene segments (Table 5.1). As in L1 and L2 genes, the L3 sequences of all ARVs (except TVAV) grouped differently from MRVs with only 36%-49% nt and 11%-41% aa identity. The genotype VIII wild crow TVAV strain was related to Steller sea lion strain with 47% nt and 53% aa identity.

3.3. Reassortments

Phylogenetic trees were constructed to discover possible reassortments. All ARVs (except TVAV) formed a group different from MRVs, indicating that there was no possibility of reassortment between ARVs and MRVs. The TVAV, DRVs and GRVs formed separate genotypes according to all three gene segment sequences indicating that they also do not reassort with CRVs, TARVs and TERVs (Fig. 5.3, 5.5, 5.7). The TARVs and TERVs grouped together in genotype L1-I with two arthritic CRV strains from Taiwan and on enteritic CRV strain from Japan but formed a separate genotypes L2-I and L3-I in L2 and L3 genes, respectively (Table 5.2). All CRVs were divided into three different genotypes in L1, L2 and L3 genes in different combinations which indicate reassortment between different strains of CRVs (Table 5.2). When compared both M and L class genotypes we found that three types of genotype constellations (GC) of TARVs and TERVs in M class formed one type of GC in L class and seven GCs of CRVs in M class formed three different GCs in L class indicating reassortments of M and L class segments between TARVs, TERVs and CRVs.

We observed some aa substitutions in TARVs and TERVs that were similar to DRVs and GRVs but were different from CRVs. On the other hand, some aa substitutions matched with CRVs but were different from DRVs and GRVs. The CRV sequences also had some aa substitutions specifically that matched with DRVs but were different from TARVs and TERVs. When we observed aa substitutions in relation to host species we found three different aa at one position representing host species specific substitutions in all three L class genes.

4. Discussion

In late 2009, the problem of turkey arthritis/lameness surfaced in market age tom turkeys in upper Midwest area of U.S., which continues until today. Flock histories of the such cases suggested that clinical lameness might occur as early as 10 weeks of age and affected at least 3-5% of the flock with >1% mortality or culling per week due to poor performance starting at 15 weeks of age. However, in severely affected flocks up to 25% of birds were affected (Trites *et al.,* 2012). This indicates the importance of understanding the ecology of this newly isolated virus so steps can be taken to mitigate its devastating effects. In a previous study, we reported on molecular characterization of these isolates based on complete S and M class gene sequences (Mor et al., 2014a; 2014b). In this study, TARVs were further characterized based on their major core proteins (λA, λC) and the polymerase protein (λB). Since there were no sequences available for TERVs in GenBank, we sequenced three isolates of TERV and used it for comparison with those of TARVs.

The L class genes and proteins of all TARVs and TERVs had the same size as the CRV reference strain S1133. Sequence alignments confirmed the presence of conserved residues and motifs throughout the L class as in CRVs with some substitutions. The presence of conserved motifs such as C2H2 zinc-binding motif in λA and conserved polymerase region in λB were consistent with findings in CRVs, DRVs and GRVs (Dandar et al., 2014a; Shen et al., 2007; Wang et al., 2013; Xu et al., 2008; 2009; Yun et al., 2014). The conserved K residue characteristic of guanylytransferase activity, ATP/GTP-binding site motif, predicted cleavage residues G and K at positions 384 and 385 and methyltransferase residues were all consistent with findings in other ARVs but

with some aa substitutions (Breun et al., 2001; Hsiao et al., 2002; Shen et al., 2007; Xu et al., 2008; 2009). This indicates that reovirus has beauty to maintain functional residues conserved inspite of divergence in different host species.

The criteria established by the International Committee on Taxonomy of Viruses (ICTV) for species demarcation include nucleotide identity between homologous genome segments of >75% within species versus <60% between species for most of the segments. The amino acid identity for species demarcation for conserved core proteins is>85% identity within species and<65% between species and for more divergent outer capsid proteins, >55% within species and <35% between species (Attoui et al., 2011). However ARVs have been divided into different lineages with a criterion of less than or equal to 10% divergence within a lineage (Liu et al., 2003; Shen et al., 2007; Wang et al., 2013). This criterion of lineage separation does not seem appropriate because each gene and its encoded proteins are different and hence separate cut off values for each segment should be defined. We here propose cut off values of nt identity for dividing ARVs into different genotypes. In addition, this classification method has been used to classify other viruses including samporviruses, noroviruses, hantaviruses, papillomaviruses, and rotaviruses (de Villiers et al., 2004; Maes et al., 2009; Marthaler et al., 2012; Schuffenecker et al., 2001; Zheng et al., 2006)

Based on our criteria, all ARVs were divided into six, seven and eight genotypes in L1, L2 and L3 genes, respectively. In L1 and L2 genes the divergence was higher at the nt level but not at the aa level due to synonymous substitutions but nonsynonymous substitutions predominate in L3 gene resulting into more divergence at the aa level also. These findings of more divergence at nt level in L1 and L2 genes and at both nt and aa

levels in L3 gene correlate with previous studies on CRVs, DRVs and GRVs (Shen et al., 2007; Wang et al, 2013; Xu et al., 2008; 2009). Interestingly TARV and TERVs grouped with two arthritic strains from Taiwan and one enteritic strain from Japan in genotype L1-I of L1 gene. TARVs and TERVs formed separate genotypes L2-I and L3-I in L2 and L3 genes, respectively and point mutation were observed within the TARVs and TERVs indicating the possibility of finding additional genotypes of TARVs and TERVs in the future. When compared L class genotypes and M class genotypes we found that three and seven GCs of TARVs, TERVs and CRVs in M class formed one and three GCs in L class, respectively indicating reassortment of M and L class gene segments between TARVs, TERVs, and CRVs. In consistence with our previous studies (Mor et al., 2014a; 2014b) on M and S classes, we did not find any motif that could differentiate TARVs from TERVs and only random point mutations were observed. There was no differentiation observed based on pathotypes, geographical area and time of isolation which correlates with previous studies (Mor et al., 2014a; 2014b; Shen et al., 2007; Wang et al., 2013)

In experimental studies, we have shown that only TARV is able to reproduce lameness and tenosynovitis in turkeys but not TERV or CRV (Sharafeldin et al., 2014a; 2014b). When TARV and CRV were inoculated into broiler chickens, only CRV was able to reproduce the disease but not TARV (Sharafeldin et al., 2014b). However, TARV and CRV did multiply in chickens and turkey poults, respectively, indicating the possibility of exchange of genetic material between TARVs and CRVs after infecting a common source. Phylogenetic analysis also indicates the possibility of reassortment among TARVs, TERVs and CRVs based on L class (this study), M class (Mor et al.,

2014b), and S class sequences (Mor et al., 2014a). Mixed infection/coinfection is one method by which new strains emerge as demonstrated in an experimental study by Ni and Kemp (1992) in which they coinfected chicken embryo fibroblast cells with CRV strain 883 and one of the other three CRV strains (176, S-1133, or 81-5) and found that the selection of genome segments in coinfection was virus strain specific. In future it will be interesting to see how reassortments occur between TARVs, TERVs and CRVs.

The TERVs have been isolated from apparently healthy and enteritis affected turkeys (Jindal et al., 2010a; 2010b; Pantin-Jackwood et al., 2008) for years but the problem of turkey lameness surfaced recently in late 2009 after a hiatus of >20 (al Afaleq and Jones, 1989; Page et al., 1982). New variants of CRVs causing lameness and arthritis in commercial broilers at the age of 2.5 to 8 weeks have also been reported in Europe and North America (Rosenberger et al., 2013a; Sellers et al., 2013; Troxler et al., 2013). This begs the question whether turkey lameness or arthritis is a re-emerging disease and if so then what is the source of this reovirus. Did TARVs and new variants of CRVs emerge simultaneously from the same source or from different sources? We are not able to answer this question at this time because complete genome sequences of new variants of CRVs are not available at this time in GenBank. Based on virus neutralization test, it has been reported that new variants of CRVs and TARVs are antigenically different and do not cross neutralize (Rosenberger et al., 2013b). But in this study we did observe point mutations and reassortments between TARVs and TERVs as well as among TARVs, TERVs and CRVs. At this point TERVs seem to be source of these newly isolated TARVs due to point mutations and reassortments. During reassortment between two strains that infect the same cell and undergo for a completely random assortment, then

resulting progeny would have 2^n (where n is the number of gene segments) possible gene combinations of genome segments from two parents. Theoretically, with 10 gene segments of reoviruses, there will be 2^{10} (1024) different gene combinations, of which two are parental and the rest are reassortants. However, in real practical conditions, only a total of 3-25% progeny reassortants are produced from co-infection (Fields, 1971). The complete genome sequences of TARVs and TERVs reported back in 1980s are not available which unable us to come with any conclusive reason for sudden appearance of TARVs after over more than 20 years. Breeder companies keep continuing improvement in their breeder flocks and it might be possible that change in breed characters makes them more susceptible to TARVs. Jones and Kibenge (1984) studied effect of breed of chicken on infection with an arthrotropic CRV strain R2 by oral and footpad inoculation. The 1-day-old chicks of three different breeds: i) SPF light-hybrid, ii) commercial white leghorn egg-layer and iii) commercial Ross-1 broiler were inoculated and observed for 12 weeks of age. Birds of all three breed developed swelling of hock joint at 3-4 weeks of age but lesions of tenosynovitis observed in broiler which become progressively more severe. Hence, it seems that point mutations, reassortment and change in breed characters together may lead to sudden appearance of TARVs.

Recently, Troxler et al. (2013) analyzed 21 CRVs based on sigma C protein from 17 broiler farms and five hatcheries in France. They succeeded in predicting a common origin of the virus as well as its horizontal and vertical transmission on the basis of genetic mutations and possible recombination events. Future studies on screening of breeder and commercial turkey flocks for TARVs and TERVs and their complete genome sequencing may help predict the source of these viruses. In a recent study, we detected

reovirus associated with enteritis in chickadee (*Poecileatricapillus*); this virus had 89.4% -98.3% nt identity with TARVs and TERVs based on the S4 gene (Mor et al., 2014c). In contrast, the TVAV virus from wild crow was very divergent from ARVs, which is not surprising because the authors of that report did suspect that the crow was probably an accidental host for this virus (Dander et al., 2014b). Studies are indicated on complete genome analysis of reoviruses associated with enteritis and other diseases in wild birds which should be helpful in better understanding the ecology of ARVs.

To the best of our knowledge, this is the first report on characterization of TARVs and TERV based on complete L class genome segments. The results indicate the presence of point mutations at nucleotide and amino acid levels as well as possible reassortments. We have suggested criteria to define genotypes of ARVs. Future studies should be conducted on co-infection and reassortment between TARVS, TERVs and CRVs to better understand the possibility of genetic exchange among these viruses. Recent reports on new variants of CRVs, newly isolated TARVs, and closely related enteric reovirus from chickadee indicate a strong need for continued surveillance, epidemiological, and genetic studies on these viruses.

Table 5.1. Nucleotide and amino acid identities of different avian reovirus genotypes. Upper part represents per cent nucleotide identity and lower highlighted part represents amino acids identities.

L1 gene Genotypes	**L1-I**	**L1-II**	**L1-III**	**L1-IV**	**L1-V**	**L1-VI**
L1-I	89-100 97-100	83-88	83-84	76-78	77-78	69-71
L1-II	96-98	88-100 97-100	82-84	77-78	77-78	69-70
L1-III	96-97	96-97	NA NA	77-78	77-78	71
L1-IV	93-95	94-95	94-95	88-97 97-99	86-87	70-71
L1-V	93-96	94-96	94-95	97-98	97-98 99-100	71-72
L1-VI	81-82	81-82	82	81-82	81-82	NA NA

L2 gene Genotypes	**L2-I**	**L2-II**	**L2-III**	**L2-IV**	**L2-V**	**L2-VI**	**L2-VII**
L2-I	91-100 97-100	83-85	82-83	83-85	75-76	76-77	63-64
L2-II	95-96	91-100 98-100	83-84	84-85	76-77	76-77	64-65
L2-III	94-95	97	NA NA	86-87	75-76	76-77	63
L2-IV	95-96	96-98	96-97	99-100 99-100	75-76	75-77	63-64
L2-V	90-92	91-92	91-92	91-92	97-100 99-100	87-88	64
L2-VI	91-92	91-92	91-92	91-92	97-98	91-99 97-99	64-65
L2-VII	70-71	72	72	71-72	71-72	71-72	NA NA

L3 gene Genotype	**L3-I**	**L3-II**	**L3-III**	**L3-IV**	**L3-V**	**L3-VI**	**L3-VII**	**L3-VIII**
L3-I	89-100 95-100	83-86	72-73	72-73	69-70	69-70.0	69-70	48-49
L3-II	90-92	89-94 96-98	72-73	73-74	69-70	70-71	70-71	48-49
L3-III	81-83	83-84	95-100 98-100	82-87	70-70	69-70	70-71	47-48
L3-IV	82-83	83-85	93-95	90-100 97-100	69-70	69-70	69-70	48-49
L3-V	78-79	79-80	78	79	100 100	82-83	79	48
L3-VI	78-80	79-80	79-80	79-80	92	98.8-100 98-100	79-80	48-49
L3-VII	78-79	79-80	78-79	78-79	92	92-93	97-100 99-100	47-48
L3-VIII	38-39	38-39	39-39	39-40	40	40-41	40	100 100

Table 5.2. Genotypes of ARVs indicating possible reassortment

Virus strain	Origin	Source	Pathotype (*)	Year of isolation	Genotype					
					M1	M2	M3	L1	L2	L3
TARVs										
TARV-MN2	USA/MN	Tendon	VA	2011	I	II	I	I	I	I
TARV-MN4	USA/MN	Tendon	VA	2011	I	III	I	I	I	I
TARV-MN9	USA/MN	Tendon	VA	2013	I	II	I	I	I	I
TARV-MN10	USA/IA	Tendon	VA	2013	I	II	I	I	I	I
TARV-MN11	USA/MN	Tendon	VA	2013	I	II	I	I	I	I
TARV-MN12	USA/MI	Tendon	VA	2013	I	I	I	I	I	I
TARV-MN13	USA/MN	Tendon	VA	2014	I	II	I	I	I	I
TERVs										
TERV-MN1	USA/MN	Feces	PES	2011	I	II	I	I	I	I
TERV-MN2	USA/MN	Feces	PES	2010	I	II	I	I	I	I
TERV-MN6	USA/MN	Feces	LTS	2011	I	I	I	I	I	I
Chicken reoviruses										
S1133	USA	Tendon	VA	1971	I	I	I	II	IV	III
1733	USA	Feces	MAL	1983	I	I	I	II	-	III
2408	USA	Feces	MAL	1983	I	I	I	II	-	III
OS161	Japan	Feces	MAL	1970	I	IV	I	I	-	III
601SI	Taiwan	Tendon	VA	1992	I	I	I	II	-	III
T6	Taiwan	Lung	RES	1970	I	I	I	II	-	III
750505	Taiwan	Tendon	VA	1986	I	III	I	I	-	III
916SI	Taiwan	Feces	MAL	1992	I	III	II	II	-	IV
918	Taiwan	Feces	MAL	1992	I	V	II	II	-	III
919	Taiwan	-	Normal	1992	I	I	I	II	-	III
R2	Taiwan	Tendon	VA	1992	I	I	II	II	-	II
601G	Taiwan	Tendon	VA	1992	I	III	I	I	-	III
1017-1	Taiwan	Feces	MAL	1992	I	I	II	II	-	II
C98	China	Tendon	VA	2006	I	I	I	II	IV	III
T98	China	Tendon	VA	2006	I	I	I	II	IV	III
GuangxiR1	China	-	-	2000	I	I	I	II	IV	III
AVS-B	USA	Feces	RSS	2006	I	III	I	II	II	II
T1781	Hungary	Brain	CNS	2012	II	V	II	III	III	IV
ARV138	Canada	Tendon	VA	-	I	I	I	II	II	II
ARV176	USA	Tendon	VA	-	I	I	I	II	IV	III
Duck reoviruses										
NP03	China	Liver	HNH	2003	V	VI	III	IV	V	VII
J18	China	Liver	HNH	2008	V	VI	III	V	V	VII
091	China	Liver	LNS	2009	V	VI	III	V	V	VII
TH11	China	Liver	LNS	2011	V	VI	III	V	V	VII
MW9710	China	Liver	MDA	1997	III	VI	III	-	VI	-
815-12	China	Liver	MDA	2010	V	VII	III	IV	VI	VI
ZJ2000M	China	Liver	-	2011	III	VII	III	IV	VI	VI
ZJOOM	China	Liver	-	2000	V	VII	III	V	V	VII
Goose reoviruses										
D20	Hungary	Liver	GOA	1999	IV	VII	IV	IV	VI	V
03G	China	Liver	HNH	2003	V	VI	III	IV	VI	VII
Wild bird										
TVAV	Finland	Brain	CNS	2002	VI	VIII	V	VI	VII	VIII

*VA= viral arthritis; MAS= malabsorption syndrome, RSS= runting-stunting syndrome, PES= poult enteritis syndrome, LTS= light turkey syndrome, RES= respiratory disease, CNS= central nervous system disease, HNH= hemorrhagic-necrotic hepatitis, LNS= large necrotic foci in spleen, MDA= ARV-Md-associated lesions, including necrotic foci in liver and spleen, GOA= ARV-Go-associated lesions, including necrotic foci in liver and spleen

Figure 5.1. The 1-120 amino acids hydrophilic region of lambda A protein representing variable region.

Figure 5.1

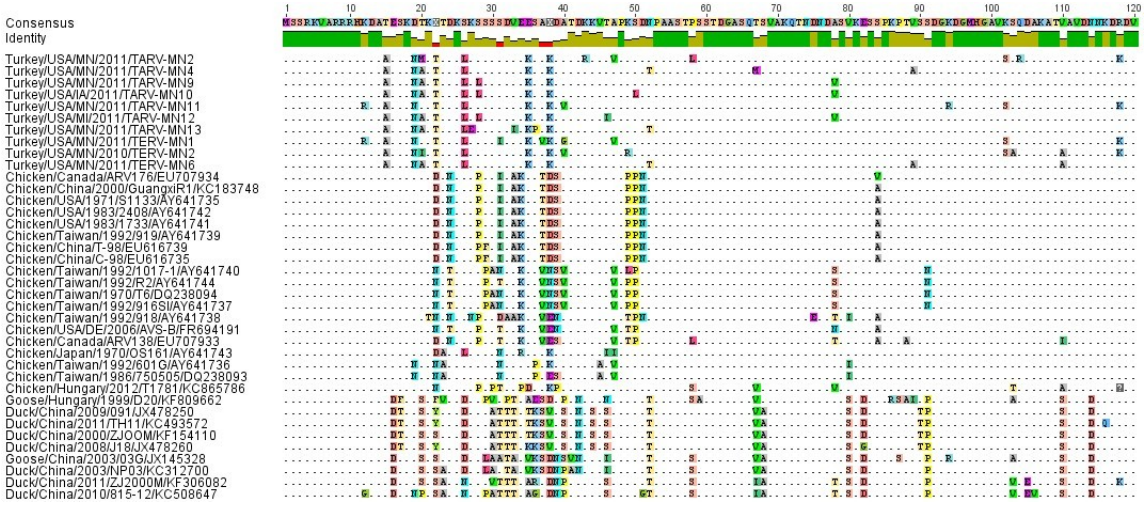

Consensus
Identity
Turkey/USA/MN/2011/TARV-MN2
Turkey/USA/MN/2011/TARV-MN4
Turkey/USA/MN/2011/TARV-MN9
Turkey/USA/IA/2011/TARV-MN10
Turkey/USA/MN/2011/TARV-MN11
Turkey/USA/MI/2011/TARV-MN12
Turkey/USA/MN/2011/TARV-MN13
Turkey/USA/MN/2011/TERV-MN1
Turkey/USA/MN/2010/TERV-MN2
Turkey/USA/MN/2011/TERV-MN6
Chicken/Canada/ARV176/EU707934
Chicken/China/2000/GuangxiR1/KC183748
Chicken/USA/1971/S1133/AY641735
Chicken/USA/1983/2408/AY641742
Chicken/USA/1983/1733/AY641741
Chicken/Taiwan/1992/919/AY641739
Chicken/China/T-98/EU616739
Chicken/China/C-98/EU616735
Chicken/Taiwan/1992/1017-1/AY641740
Chicken/Taiwan/1992/R2/AY641744
Chicken/Taiwan/1970/T6/DQ238094
Chicken/Taiwan/1992/916SI/AY641737
Chicken/Taiwan/1992/918/AY641738
Chicken/USA/DE/2006/AVS-B/FR694191
Chicken/Canada/ARV138/EU707933
Chicken/Japan/1970/OS161/AY641743
Chicken/Taiwan/1992/601G/AY641736
Chicken/Taiwan/1986/750505/DQ238093
Chicken/Hungary/2012/T1781/KC865786
Goose/Hungary/1999/D20/KF809662
Duck/China/2009/091/JX478250
Duck/China/2011/TH11/KC493572
Duck/China/2000/ZJOOM/KF154110
Duck/China/2008/J18/JX478260
Goose/China/2003/03G/JX145328
Duck/China/2003/NP03/KC312700
Duck/China/2011/ZJ2000M/KF306082
Duck/China/2010/815-12/KC508647

Figure 5.2. Pairwise identity frequency graph using complete ORF nucleotide sequences of L1 gene of seven TARVs, three TERVs and available ARV sequences in GenBank. The suitable nucleotide cut-off value (82%) is depicted by vertical solid line.

Figure 5.2

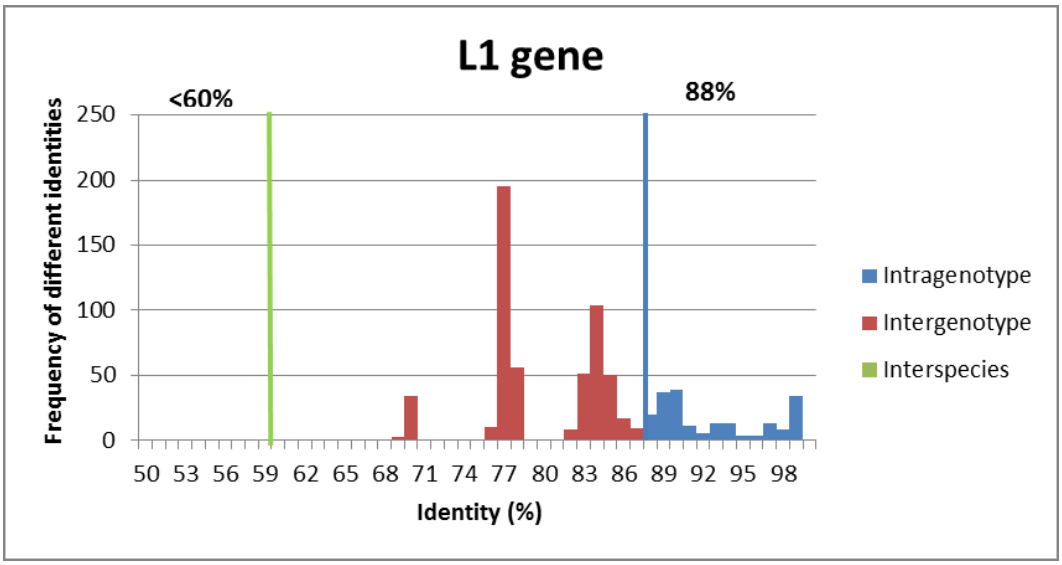

L1 gene
<60%
88%
Frequency of different identities
250
200
150
100
50
0
50 53 56 59 62 65 68 71 74 77 80 83 86 89 92 95 98
Identity (%)
Intragenotype
Intergenotype
Interspecies

Figure 5.3. Phylogenetic tree constructed based on complete ORF nucleotide sequences of L1 gene of ARV sequences. Tree was constructed in MEGA 6.06 using GTR+G model with Maximum Likelihood method and 100 bootstrap replicates.

Figure 5.3

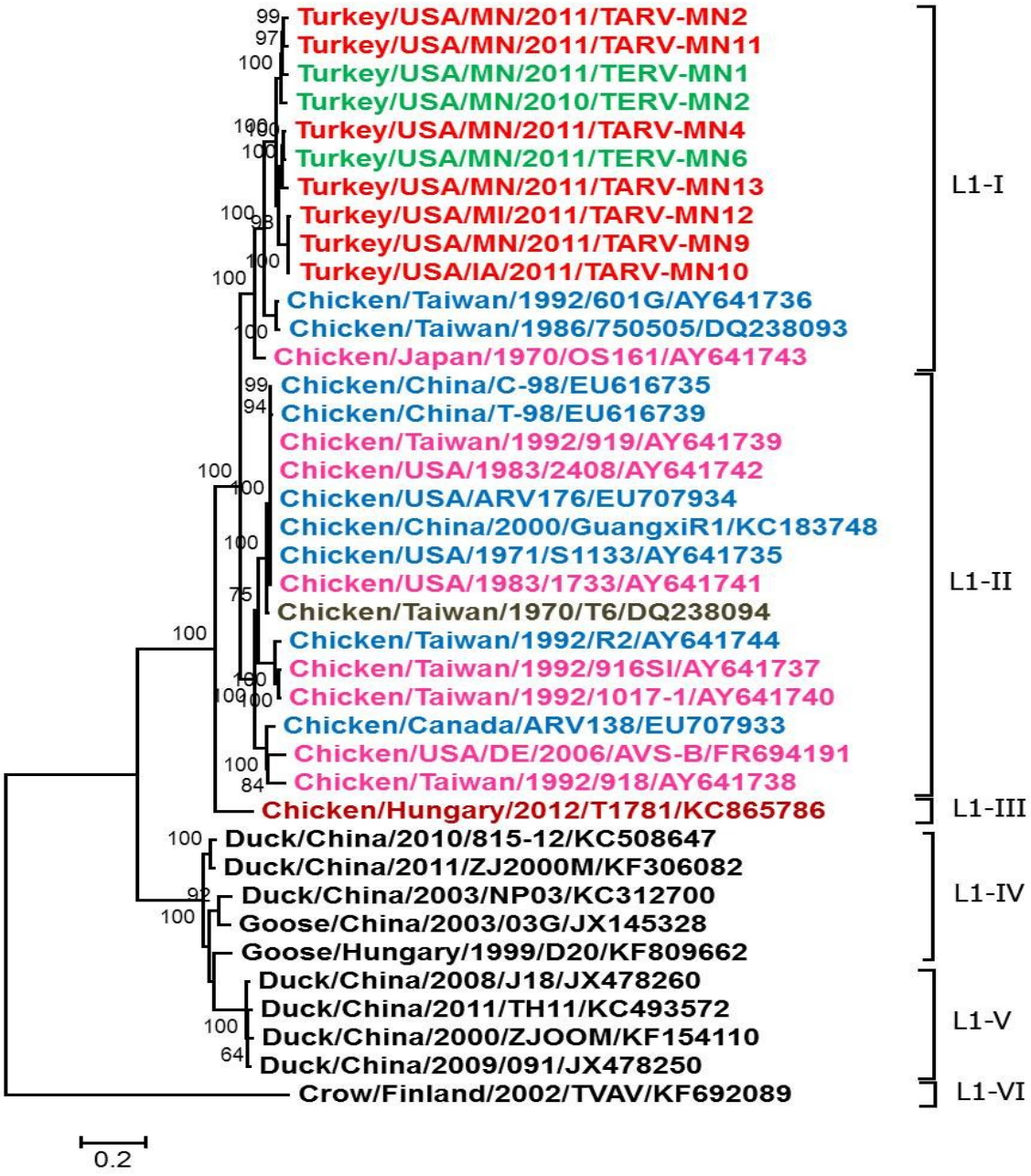

Turkey/USA/MN/2011/TARV-MN2
Turkey/USA/MN/2011/TARV-MN11
Turkey/USA/MN/2011/TERV-MN1
Turkey/USA/MN/2010/TERV-MN2
Turkey/USA/MN/2011/TARV-MN4
Turkey/USA/MN/2011/TERV-MN6
Turkey/USA/MN/2011/TARV-MN13
Turkey/USA/MI/2011/TARV-MN12
Turkey/USA/MN/2011/TARV-MN9
Turkey/USA/IA/2011/TARV-MN10
Chicken/Taiwan/1992/601G/AY641736
Chicken/Taiwan/1986/750505/DQ238093
Chicken/Japan/1970/OS161/AY641743
Chicken/China/C-98/EU616735
Chicken/China/T-98/EU616739
Chicken/Taiwan/1992/919/AY641739
Chicken/USA/1983/2408/AY641742
Chicken/USA/ARV176/EU707934
Chicken/China/2000/GuangxiR1/KC183748
Chicken/USA/1971/S1133/AY641735
Chicken/USA/1983/1733/AY641741
Chicken/Taiwan/1970/T6/DQ238094
Chicken/Taiwan/1992/R2/AY641744
Chicken/Taiwan/1992/916SI/AY641737
Chicken/Taiwan/1992/1017-1/AY641740
Chicken/Canada/ARV138/EU707933
Chicken/USA/DE/2006/AVS-B/FR694191
Chicken/Taiwan/1992/918/AY641738
Chicken/Hungary/2012/T1781/KC865786
Duck/China/2010/815-12/KC508647
Duck/China/2011/ZJ2000M/KF306082
Duck/China/2003/NP03/KC312700
Goose/China/2003/03G/JX145328
Goose/Hungary/1999/D20/KF809662
Duck/China/2008/J18/JX478260
Duck/China/2011/TH11/KC493572
Duck/China/2000/ZJOOM/KF154110
Duck/China/2009/091/JX478250
Crow/Finland/2002/TVAV/KF692089
L1-I
L1-II
L1-III
L1-IV
L1-V
L1-VI
0.2

Figure 5.4. Pairwise identity frequency graph using complete ORF nucleotide sequences of L2 gene of seven TARVs, three TERVs and available ARV sequences in GenBank. The suitable nucleotide cut-off value (82%) is depicted by vertical solid line.

Figure 5.4

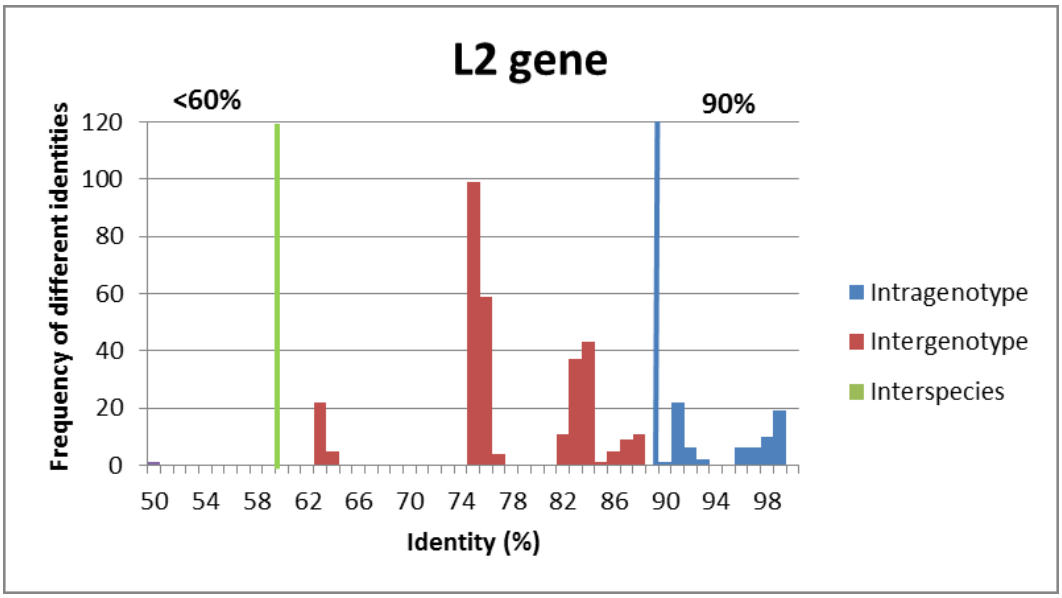

L2 gene
<60%
90%
Frequency of different identities
120
100
80
60
40
20
0
50 54 58 62 66 70 74 78 82 86 90 94 98
Identity (%)
Intragenotype
Intergenotype
Interspecies

Figure 5.5. Phylogenetic tree constructed based on complete ORF nucleotide sequences of L2 gene of ARV sequences. Tree was constructed in MEGA 6.06 using GTR+G model with Maximum Likelihood method and 100 bootstrap replicates.

Figure 5.5

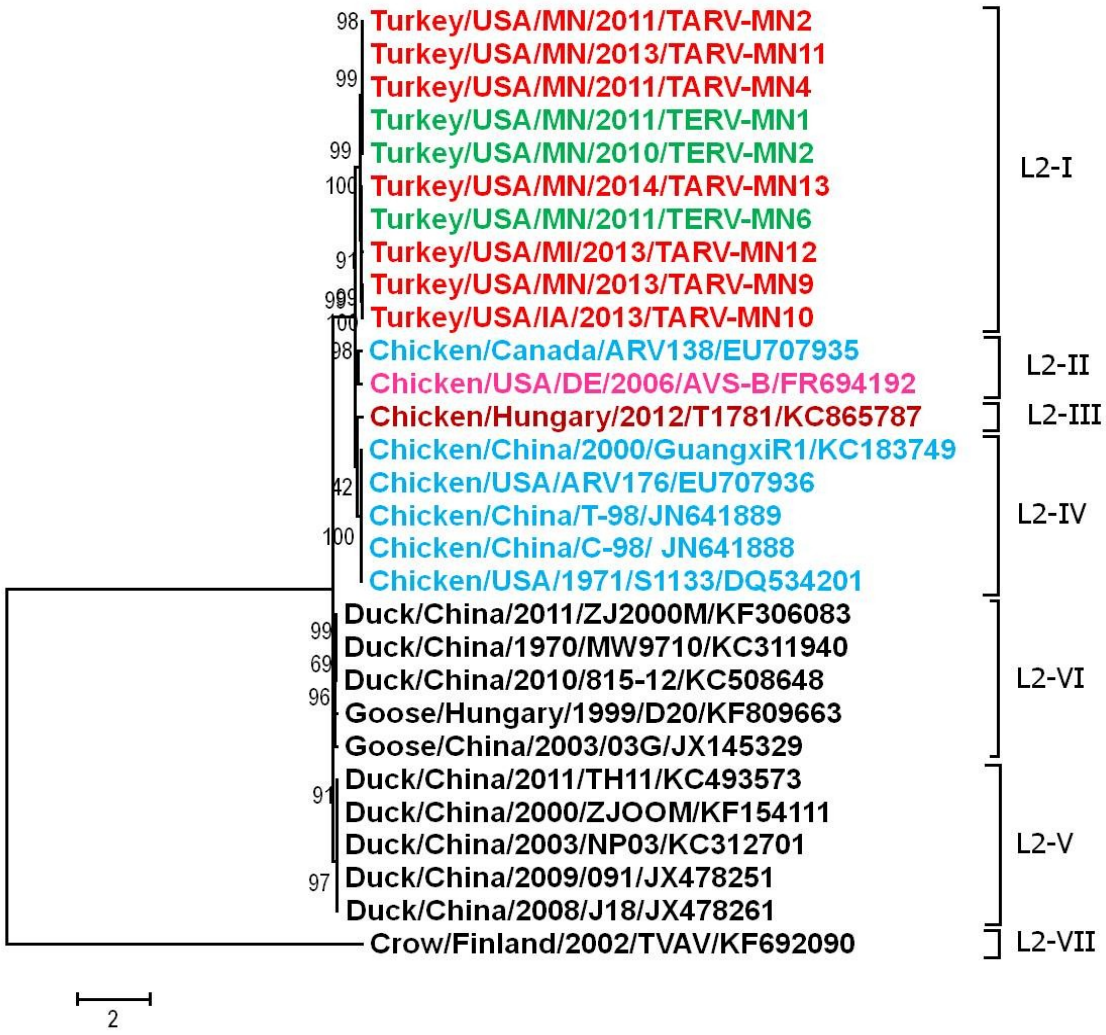

Turkey/USA/MN/2011/TARV-MN2
Turkey/USA/MN/2013/TARV-MN11
Turkey/USA/MN/2011/TARV-MN4
Turkey/USA/MN/2011/TERV-MN1
Turkey/USA/MN/2010/TERV-MN2
Turkey/USA/MN/2014/TARV-MN13
Turkey/USA/MN/2011/TERV-MN6
Turkey/USA/MI/2013/TARV-MN12
Turkey/USA/MN/2013/TARV-MN9
Turkey/USA/IA/2013/TARV-MN10
Chicken/Canada/ARV138/EU707935
Chicken/USA/DE/2006/AVS-B/FR694192
Chicken/Hungary/2012/T1781/KC865787
Chicken/China/2000/GuangxiR1/KC183749
Chicken/USA/ARV176/EU707936
Chicken/China/T-98/JN641889
Chicken/China/C-98/ JN641888
Chicken/USA/1971/S1133/DQ534201
Duck/China/2011/ZJ2000M/KF306083
Duck/China/1970/MW9710/KC311940
Duck/China/2010/815-12/KC508648
Goose/Hungary/1999/D20/KF809663
Goose/China/2003/03G/JX145329
Duck/China/2011/TH11/KC493573
Duck/China/2000/ZJOOM/KF154111
Duck/China/2003/NP03/KC312701
Duck/China/2009/091/JX478251
Duck/China/2008/J18/JX478261
Crow/Finland/2002/TVAV/KF692090
L2-I
L2-II
L2-III
L2-IV
L2-VI
L2-V
L2-VII
2

Figure 5.6. Pairwise identity frequency graph using complete ORF nucleotide sequences of L3 gene of seven TARVs, three TERVs and available ARV sequences in GenBank. The suitable nucleotide cut-off value (87%) is depicted by vertical solid line.

Figure 5.6

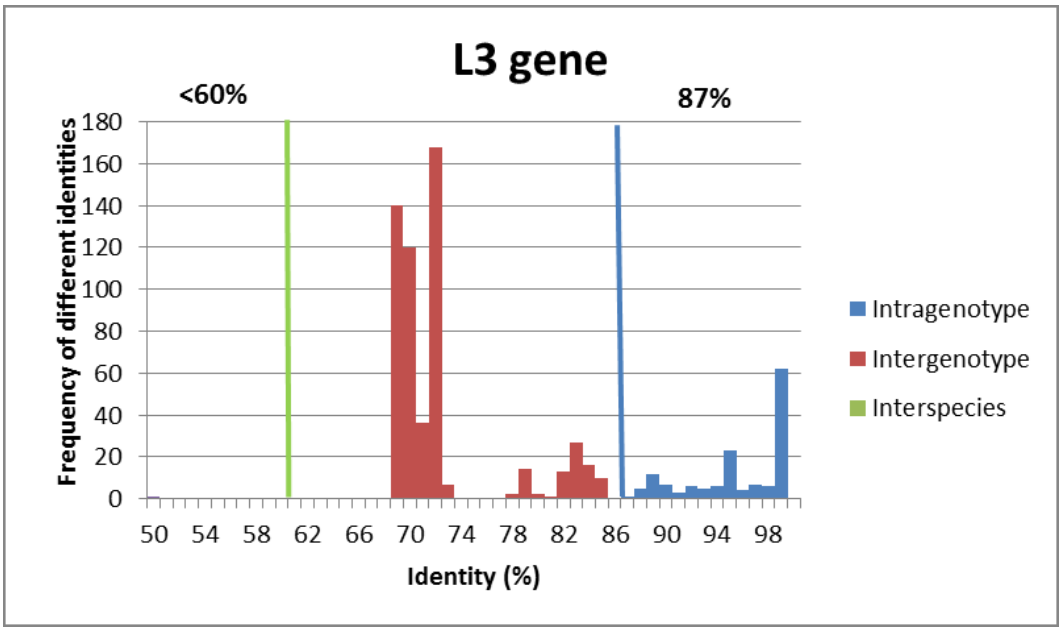
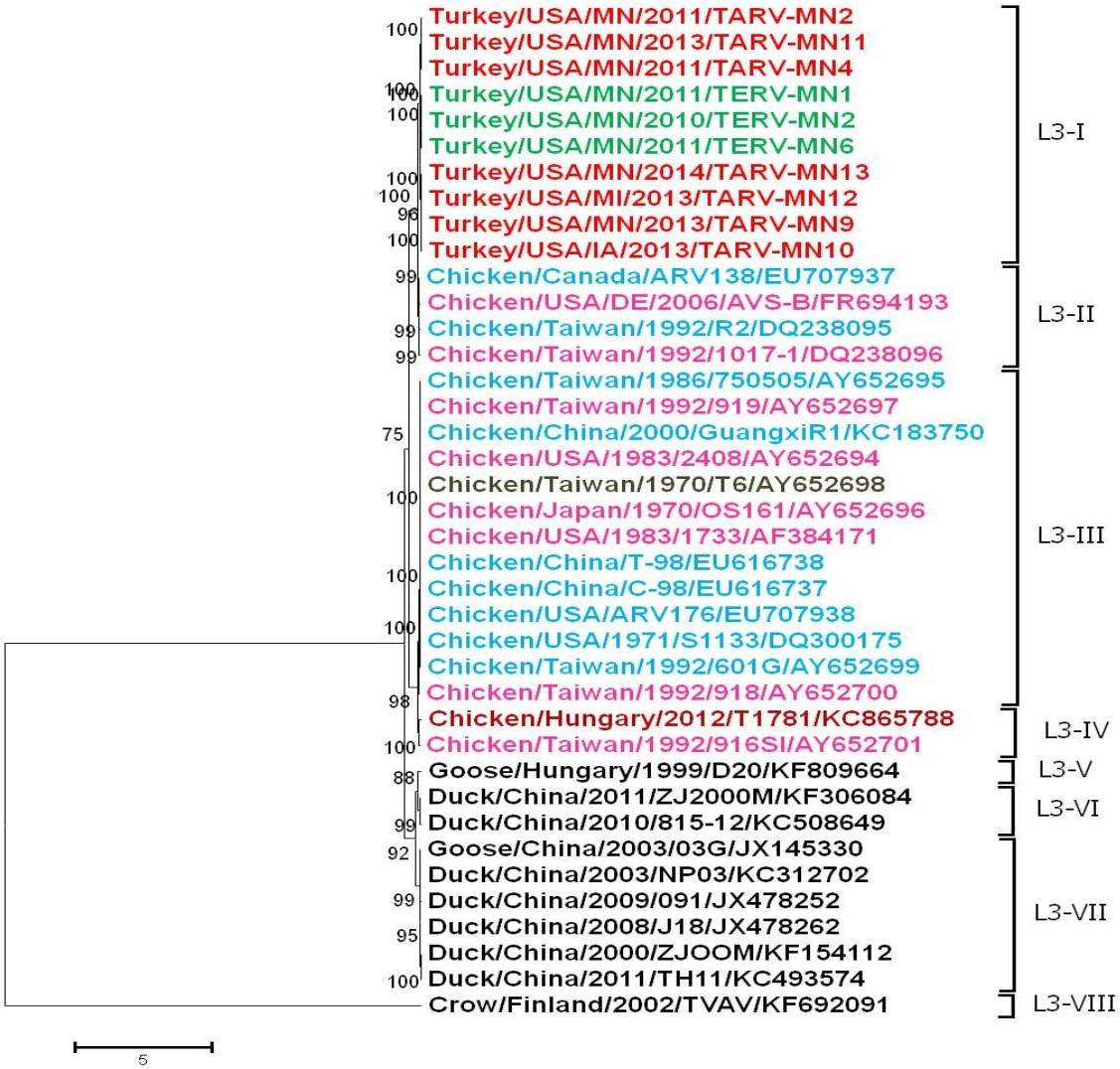

L3 gene
<60%
87%
Frequency of different identities
180
160
140
120
100
80
60
40
20
0
50 54 58 62 66 70 74 78 82 86 90 94 98
Identity (%)
Intragenotype
Intergenotype
Interspecies

Figure 5.7. Phylogenetic tree constructed based on complete ORF nucleotide sequences of L3 gene of ARV sequences. Tree was constructed in MEGA 6.06 using GTR+G model with Maximum Likelihood method and 100 bootstrap replicates.

Figure 5.7


Turkey/USA/MN/2011/TARV-MN2
Turkey/USA/MN/2013/TARV-MN11
Turkey/USA/MN/2011/TARV-MN4
Turkey/USA/MN/2011/TERV-MN1
Turkey/USA/MN/2010/TERV-MN2
Turkey/USA/MN/2011/TERV-MN6
Turkey/USA/MN/2014/TARV-MN13
Turkey/USA/MI/2013/TARV-MN12
Turkey/USA/MN/2013/TARV-MN9
Turkey/USA/IA/2013/TARV-MN10
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Chicken/China/C-98/EU616737
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Chicken/USA/1971/S1133/DQ300175
Chicken/Taiwan/1992/601G/AY652699
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Chicken/Taiwan/1992/916SI/AY652701
Goose/Hungary/1999/D20/KF809664
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Goose/China/2003/03G/JX145330
Duck/China/2003/NP03/KC312702
Duck/China/2009/091/JX478252
Duck/China/2008/J18/JX478262
Duck/China/2000/ZJOOM/KF154112
Duck/China/2011/TH11/KC493574
Crow/Finland/2002/TVAV/KF692091
L3-I
L3-II
L3-III
L3-IV
L3-V
L3-VI
L3-VII
L3-VIII
5

Chapter 6: A one step real-time RT-PCR for the detection of turkey reoviruses

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1. Introduction

Diseases associated with avian reovirus (ARV) are common in commercial chicken flocks and are often manifested by lameness and swelling of joints and feet (Rosenberger, 2003). The ARV belongs to family *reoviridae* and genus *orthoreovirus* (Attoui et al., 2011). The virus particle is non-enveloped, icosahedral, and multilayered, with a diameter of 70–80 nm. The genome contains 10 segments of double-stranded RNA, each of which is classified into three major classes; large (L1–L3), medium (M1–M3), and small (S1–S4) based on their migration patterns in polyacrylamide gels. The genome segments consist of a single open reading frame (ORF) and conserved untranslated regions at the 5' and 3' ends (Day, 2009; Duncan, 1999).

Virus isolation in embryonated chicken eggs, continuous cell lines, or primary cells is used for virus detection for diagnostic purposes. Serological methods such as virus neutralization, agar-gel precipitin assay, and enzyme-linked immunosorbent assay are often used for the detection of anti-ARV antibodies (Adair et al., 1987; Menendz et al., 1975; Robertson and Willcox, 1986; Slaght et al.,1978). More sensitive molecular diagnostic techniques e.g., reverse transcription-polymerase chain reaction (RT-PCR), nested PCR, and multiplex PCR (Bruhn et al., 2005; Caterina et al., 2004; Lee et al., 1998; Liu et al., 1997; Liu et al., 2004; Rosenberger et al., 1989) have also been developed and used for the detection of ARVs in clinical samples (Guo et al., 2011; Ke et al., 2006; Mackay et al., 2002). Real time RT-PCR assays using TaqMan technology have also been developed (Guo et al., 2011; Orru et al., 2004).

In addition to causing disease in chickens, the ARVs are known to be associated with turkey enteritis (Dutta and Pomeroy, 1969; Jindal et al., 2010; Ritter et al., 1986).

The isolates of ARVs from turkey enteritis are often called turkey enteric reoviruses (TERV). The clinical signs of enteritis in poults include diarrhea, litter eating, decreased feed efficiency, decreased weight gain, and uneven flock growth. A few reports in the 1980s suggested that ARVs are also a cause of lameness in turkeys (Levisohn et al., 1980; Page et al., 1982). In late 2010 and early 2011, a problem of lameness and swollen hock joints in 14-16 week old tom turkeys was observed by multiple producers in the upper Midwest area. We were successful in isolating reovirus strains from tendons and joint fluids of these cases, which were negative for bacterial pathogens such as mycoplasma and staphylococcus (Mor et al., 2013). For the sake of clarity, we call these isolates TARVs (turkey arthritis reoviruses) as opposed to TERVs. This study was undertaken to develop a real-time RT-PCR (rRT-PCR) for the selective detection of both TERV and TARV. Primers and probes for this study were designed from the conserved region of the S4 segment of the TARV and TERV genome.

2. Materials and Methods

2.1. Extraction of viral RNA

TARV-MN4 strain (Mor et al., 2013) was propagated and titrated in QT-35 cells (Japenese quail fibrosarcoma). After the appearance of cytopathic effects, the infected cells were frozen and thawed three times followed by centrifugation at 4^0C at 2500×g for 15 min. Viral RNA was extracted from 140-µl aliquots of the supernatant using QIAamp viral RNA mini kit (Qiagen, Valencia, CA). RNA was eluted in 40 µl of buffer AVE.

2.2. Oligonucleotide primers and TaqMan MGB probes for real time PCR

Primers and probes were designed from conserved region of S4 gene based on a multiple sequence alignment (MEGA version 5.1) (Tamura et al., 2011) to ensure that they would only amplify RNA of TARV and TERV. The S4 gene was selected, because of smallest in size and it codes for the antigenic and pathogenic protein of the virus (Guo et al., 2011). Forward, reverse primers and a TaqMan TAMRA probe were designed to amplify 99 bp fragments from S4 gene (Table 6.1). The probe had reporter dye FAM (6-carboxy-fluorescein) attached to the 5'-end and a non-fluorescent quencher (NFQ) and minor groove binder (MGB) attached to the 3'-end. Published primers that encompass the S4 genome segment were selected for obtaining the full-length S4 gene segment (23). All probes and primers were manufactured by Integrated DNA Technologies (Coralville, IA).

2.3. Construction of in vitro transcribed (IVT) RNA

Viral RNA was extracted from 140 µl aliquots of virus stock (TARV-MN4) using QIAamp viral RNA mini kit. RNA was eluted in 40 µl of buffer AVE. A 310 bp fragment from S4 gene was amplified using forward primer F 5'- TCGATC GAAT TAAGCARCCCCG -3' and reverse primer R 5'- CGCGTTTGGTAGCTCAAGTTT- 3'. This amplified product was cloned into the plasmid vector (Zero-Blunt® PCR cloning kit; Invitrogen, NY, USA) according to the manufacturer's instructions. The plasmid was cut with EcoRI (New England Biolabs, MA, USA) and run off transcription was done using MAXIscript® Kit with T7 polymerase (Invitrogen). DNA template was removed by adding Turbo DNase (Invitrogen) at 1U/µg of template DNA. RNA was purified using ammonium acetate precipitation method (Osterberg et al., 1975). RNA pellet was

dissolved in nuclease-free water and concentration was estimated by spectrophotometric analysis. This quantified RNA was serially diluted and used as IVT RNA to generate standard curves and determine the viral RNA copies in the samples.

2.4. Real-time RT-PCR

The rRT-PCR reactions were set up in 96-well format and carried out in Mastercycler® ep realplex2 (Eppendorf, Hamburg, Germany) using One-step RT -PCR Kit (Qiagen, Valencia, CA). Each 25 µl of reaction mixture contained 5µl, 5X reaction buffer, 0.8 µl enzyme mix, 0.2 µl RNase inhibitor (Promega, Madison, USA), 300nM of each primer and 200 nM of TaqMan probe and 2 µl of RNA. Two no template control (NTC) wells were included in each run. Wells were sealed with optical lids. The PCR cycling conditions were 30 min at 50ºC, 15 min at 95ºC and then 45 cycles each of denaturation at 95ºC for 15s and annealing and extension at 56ºC for 45s. Amplification data were analyzed by realplex software version 2.2. The software generated standard quantitation curves by plotting the threshold cycle (Ct) values against the logarithm of the input copy number. The copy number of S4 RNA was calculated as per formula described by previously (Guo et al., 2011, Ke et al., 2006).

2.5. Sensitivity

The RNA copy number and titer ($TCID_{50}$ /mL) of TARV-MN4 were compared to determine assay sensitivity (Karber, 1931). Eight, 10-fold serial dilutions of virus stock were prepared and tested to determine the sensitivity of the rRT-PCR to detect virus.

2.6. Specificity

Specificity of rRT-PCR assay was evaluated by testing turkey astrovirus -2 (TAstV-2), avian influenza virus (AIV), New castle disease virus (NDV), avian rotavirus, chicken arthritis reovirus (CARV) and fish reovirus.

2.7. Reproducibility

To assess the intra- and interassay reproducibility, serial ten-fold dilutions of TARV-MN4 IVT RNA containing 10^8 to 10^1copies/reaction were tested in triplicate on three different days. Coefficients of variation (CV) were tested to see any statistical correlation.

2.8. Comparison with gel based RT-PCR

Serial 10-fold dilutions of TARV-MN4 virus stock were prepared and viral RNA was extracted from 140-µl volume of each dilution by QIAamp viral RNA mini kit (Qaigen). RNA was eluted in 40 µl of buffer AVE. Two µl of the extracted RNA was subjected to rRT-PCR reaction and conventional RT-PCR in parallel. Amplicon size for RT-PCR product (1,100 bp) was confirmed by electrophoresis in 1.2% agarose gel.

2.9. Validation using samples from experimentally infected birds

Intestinal contents and tendon samples from turkeys experimentally inoculated with TARV-MN4 and other strains of TARVs were tested with the new assay. Samples from control, uninoculated birds were also tested (Mor et al., 2013; Sharafeldin et al., 2014a).

3. Results

3.1. Standardization

Optimum amplification (lowest threshold cycle and highest fluorescent signal) for various copies of IVT RNA (10^8 through 10^1) was obtained at primer concentration of 300 nM for each primer and 400 nM for the probe. Standard curve with IVT RNA was generated using 10^8 through 10^1copies/reaction. The threshold was adjusted manually in the exponential region of the amplification curves and the data collected were processed automatically to generate a log-linear regression plot. A linear relationship was obtained from 10^8 through 10^1 starting copies/reaction (r^2 =0.9995). The detection limit of the assay with TARV-MN4, IVT RNA was 10 copies per reaction (Fig. 6.1).

3.2. Sensitivity

Results of sensitivity testing indicated that one $TCID_{50}$ (1.3 x 10^3 copies/mL of viral RNA) of TARV-MN4 was equivalent to 11.6$\pm$0.2 genome copies.

3.3. Specificity

The assay was able to specifically amplify TARV viral RNA and there was no amplification with TAstV-2, AIV, NDV, avian rotavirus, CARV and fish reovirus.

3.4. Reproducibility

Intra-assay variability (the CV between ct values of triplicate reactions) was 0.08 for 10^8 copies and 0.00 for 10 copies of TARV-MN4 IVT RNA. Inter-assay variability was 0.06 for 10^8 copies and 0.02 for 10 copies. No statistical difference was found between the

intra- and the inter-assay CV, confirming the reproducibility of the assay (Tables 6.2 and 6.3).

3.5. Comparison of real-time RT-PCR with gel based RT-PCR

The gel based RT-PCR was able to detect 5.8 x 10^3 TARV-MN4 genome copies (-1 dilution) while the rRT-PCR assay detected 26 TARV-MN4 (virus stock) genome copies (-4 dilution), thus the gel based PCR was 223 times (5.8 x 10^3/26) less sensitive than the rRT-PCR assay.

3.6. Validation using experimental samples

All samples collected from infected birds were found to be positive by this rRT-PCR and the Ct values ranged between 18 and 32. Samples from control birds did not show any amplification signal.

4. Discussion

The present work describes the development of a rapid, sensitive, and accurate quantitative rRT-PCR assay for the detection of TARV and TERV. Conventional RT-PCR, multiplex PCR and nested PCR detection methods targeting regions of ARV genome segments have previously been described (Bruhn et al., 2005; Lee et al., 1998; Page et al., 1982). A rRT-PCR has also been described for the detection of ARVs based on the conserved S4 gene which is useful in detecting chicken reoviruses (chicken enteric and chicken arthritis reoviruses) (Guo et al., 2011; Ke et al., 2006; Meckay et al., 2002). However, a specific method for the detection and quantification of TARV and TERV is not available. Our previous study based on isolation and characterization of TARV

strains suggests that chicken reoviruses grouped differently from TARV and TERVs with 78.0% nucleotide identity based on S4 gene sequence analysis (Mor et al., 2013). Hence, we decided to develop a rRT-PCR specific for turkey reoviruses based on the conserved S4 region of TRV genome.

The choice of the molecule to be used for generation of the standard curve for the target quantification plays a critical role in accurate quantitation. It is well known that the most critical step in an RT-PCR is the RT reaction rather than the PCR; hence the use of in vitro transcribed ssRNA was preferred over cloned ds DNA molecule. Sensitivity and specificity are important measures for the evaluation of a real-time TaqMan RT-PCR assay. The gel based RT-PCR was 223 times less sensitive than the rRT-PCR assay which is not surprising (Guo et al., 2011, Ke et al., 2006). Absence of amplification signals with non-TRV RNA, confirmed the specificity of the assay. The range of detection for the assay was 10^8 to 10^1 copies/reaction which shows remarkable sensitivity of the assay. The highest values of coefficient of variation for intra-experimental and inter-experimental variability were 0.08 and 0.06 respectively, indicating reproducibility of the assay. Multiple repeats resulted in nearly identical standard curves with small variations in the intercept (34.94±0.37) and slope (−3.55±0.08) of the regression equation ($R^2 = 0.999$). For TARV-MN4 strain a 50% tissue culture infectious dose ($TCID_{50}$; 1.3 x 10^3 copies/mL of viral RNA) is equivalent to 11.6 + 0.2; TARV genome copies.

In conclusion, the newly developed rRT-PCR in this study promises to provide a rapid and sensitive method for the detection of TARV and TERV and may be useful in screening and early detection of virus load of TARV and TERV. The assay should also be useful in the detection of turkey reoviruses during disease outbreaks and for routine

monitoring of turkeys. We are now in early stages of designing an assay that can differentiate between TARV and TERV.

Table.6.1. Sequences of real time RT-PCR primers and probe.

Primers and probe	Sequence (5' to 3')	Length
Forward primer	ATCATGGCTGGGTTTGTGCC	20
Reverse primer	AGAACGAATTTGTARGCGACCA	22
Probe	FAM-TGAGMGTGATGACTTTACYCC-TAMRA	21

Table 6.2. Intra-experimental variability in the Ct values of TARV-MN4, IVT RNA.

Copies of TARV RNA	Threshold cycle (Ct) value of replicates 1 to 3			Mean	SD*	CV**
	1	2	3			
10^8 copies	7.40	8.41	8.68	8.14	0.67	0.08
10^7 copies	11.14	10.83	10.87	10.95	0.17	0.02
10^6 copies	13.90	14.11	14.22	14.08	0.16	0.01
10^5 copies	17.68	17.91	17.45	17.68	0.23	0.01
10^4 copies	21.00	21.01	21.13	21.05	0.07	0.00
10^3 copies	24.71	25.05	24.73	24.83	0.19	0.01
10^2 copies	28.52	28.00	28.37	28.30	0.27	0.01
10 copies	32.05	32.24	31.96	32.08	0.14	0.00

* SD= standard deviation
** CV= coefficients of variation

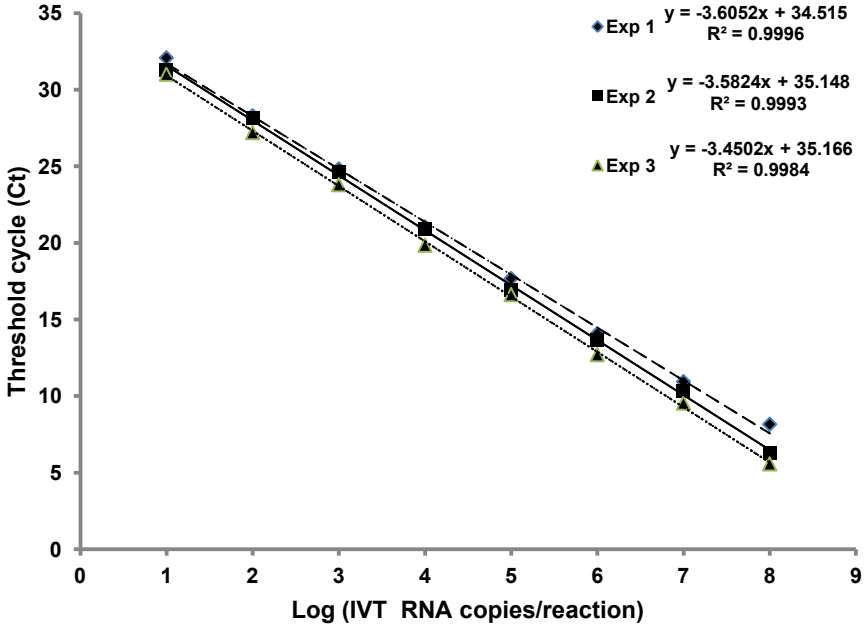
Table 6.3. Inter-experimental variability in the Ct values of TARV-MN4, IVT RNA.

Copies of TARV RNA	Threshold cycle (CT) values of replicates 1 to 3			Mean	SD*	CV**
	1	2	3			
10^8 copies	6.14	6.28	5.57	5.99	0.38	0.06
10^7 copies	10.95	10.36	9.55	10.27	0.70	0.07
10^6 copies	14.08	13.67	12.70	13.47	0.70	0.05
10^5 copies	17.68	16.90	16.63	17.06	0.55	0.03
10^4 copies	21.05	20.92	19.85	20.60	0.66	0.03
10^3 copies	24.83	24.62	23.80	24.41	0.54	0.02
10^2 copies	28.30	28.14	27.20	27.87	0.59	0.02
10 copies	32.08	31.32	31.01	31.47	0.55	0.02

* SD= standard deviation
** CV= coefficients of variation

Figure 6.1. Standard curve plots of tenfold serially diluted TARV-MN4, IVT RNA.

Figure 6.1


Exp 1
y = -3.6052x + 34.515
R² = 0.9996
Exp 2
y = -3.5824x + 35.148
R² = 0.9993
Exp 3
y = -3.4502x + 35.166
R² = 0.9984
Threshold cycle (Ct)
Log (IVT RNA copies/reaction)
0
5
10
15
20
25
30
35
0
1
2
3
4
5
6
7
8
9

Chapter 7: Survival of newly isolated turkey arthritis reovirus in poultry litter and drinking water

This work has been submitted for publication as:

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1. Introduction

Avian reoviruses (ARVs) are ubiquitous in domestic poultry, with 85-90% of them being non-pathogenic (Jones, 2000). Of the diseases caused by ARVs, viral arthritis and runting-stunting syndrome (RSS) in chickens is the most common but respiratory and enteric diseases and immunosuppression can also occur (Jones, 2008). Reovirus is a non-enveloped, double-stranded RNA virus with icosahedral symmetry and a particle size of 70-80nm (Varela and Benavente, 1994). In addition to chickens, ARVs can also infect turkeys, geese, ducks, and wild birds. Turkey reovirus (also called turkey enteric reovirus or TERV) form a distinct group within the *Reoviridae* family based on the genetic analysis of S class genome segments (Day et al., 2007; Pantin-Jackwood et al., 2008; Jindal et al., 2010a; Mor et al., 2014) and has been detected in the gastrointestinal tracts of both apparently healthy and enteritic turkeys (Pantin-Jackwood et al., 2008; Jindal et al., 2010a; 2010b; Mor et al., 2013).

In late 2009, the problem of lameness and arthritis was observed in market age tom turkeys (Mor et al., 2013; Sharafeldin et al., 2014) in the Upper Midwest area of the U.S. Reoviruses, tentatively named as turkey arthritis reoviruses (TARVs) were isolated from gastrocnemius and digital flexor tendons of lame turkeys (Mor et al., 2013). In addition, the disease was experimentally reproduced by oral inoculation of turkey poults (Sharafeldin et al., 2014a, 2014b). The problem of lameness and arthritis caused by TARVs appears to be re-emerging in U.S. turkeys because the disease was been seen for over 20 years after it was initially reported in late 1980s and early 1990s (al Afaleq et al., 1989; 1991).

We have genetically characterized TARVs based on S class gene segments and found that TARVs grouped differently from chicken arthritis reoviruses (CARVs; Mor et al., 2014). Since fecal-oral route appears to be the most common route of infection for both TARVs and TERVs, it is important to determine environmental stability of these viruses. Hence we conducted this study to determine the comparative survival of TARVs, TERV, and CARV in poultry litter and drinking water.

2. Materials and Methods

2.2. Source of virus strains

Three TARVs isolates (TARV-O'Neil, TARV-MN2, TARV-MN4), one TERV (TERV-MN1) and one CARV isolates were used in this study. All five isolates were grown in QT-35 cells as described previously (Mor et al., 2013). Virus titers were: $10^{5.8}$, $10^{4.5}$, $10^{6.2}$, $10^{3.8}$, and $10^{5.5}$ ($TCID_{50}$/mL) for TARV-MN2, TARV-MN4, TARV-O'Neil, TERV-MN1, and CARV-1, respectively.

2.3. Survival in drinking water

Two experiments were conducted using autoclaved water in experiment 1 and non-autoclaved water in experiment 2. In experiment 1, tap water was de-chlorinated followed by sterilization by autoclaving for 15 min. After cooling, five 50 mL aliquots of water were prepared in sterile vials followed by the addition of 2.5mL of appropriate virus. After mixing well, an aliquot was removed from all five vials to determine baseline virus titers. The vials were stored at room temperature (~25^0C) for 14 weeks. Aliquots (2mL amounts) were removed from all vials weekly. Serial 10-fold dilutions of the aliquots

were inoculated in QT-35 cells followed by incubation at 37^0C. Viral titers were calculated by the method of Reed and Muench (1938). In experiment 2, to simulate field conditions, non-autoclaved water collected from drinkers of a turkey farm was used. Aliquots of water (5mL amounts) were spiked with 200 µL of appropriate virus. Samples were collected at 0 time and daily thereafter for 10 days followed by virus titration in QT-35 cells. The experiment was done in triplicate.

2.4. Survival in poultry litter

Again, two experiments were conducted using autoclaved and non-autoclaved litter. Litter samples were collected from six different commercial turkey farms. Equal portions of the six litter samples were mixed together and used as a pool. In experiemnt1, the pooled litter was autoclaved followed by distribution in several vials @ 2g per vial. All aliquots were spiked with 100µL of the appropriate virus. After mixing well, the vials were stored at room temperature for eight weeks. Three vials per virus were removed at 0 time (as a baseline) and then weekly thereafter. Viruses present in these samples were eluted by adding 5 mL of an eluent solution (3% beef extract-0.05M glycine solution, pH 7.2) followed by vortexing and centrifugation at 2500xg for 10 min. Serial 10-fold dilutions of the supernatants were used for virus titration in QT-35 cells. In experiment 2, non-autoclaved litter was tested and found to be negative for reoviruses. Aliquots of litter were distributed in several vials @2g per vial followed by spiking with 100µL of the appropriate viruses. Three vials from each virus group were removed at 0 time and daily thereafter for 10 days. Titers of surviving virus in these vials were determined as done for autoclaved litter samples.

3. Results

3.1. Survival of virus in drinking water

In autoclaved de-chlorinated tap water all five reoviruses (CARV, TARV and TERV) were able to survive for 9 to 13 weeks with minor differences (Figure 7.1); TERV-MN1 and TARV-MN4 survived for 9 and 10 weeks, respectively, while TARV-O'Neil and CARV survived for 12 and 13 weeks, respectively. For up to 4 weeks, there was no reduction in virus titers; gradual decrease was seen thereafter (Figure 7.1). In non-autoclaved water, all five viruses were able to survive for up to 10 days although there was a gradual decrease in virus titers after 4 days of incubation at room temperature (Figure 7.2).

3.2. Survival of virus in litter

In autoclaved litter, TARV-O'Neil, TARV-MN2 and CARV were able to survive for seven weeks but not for eight weeks. TARV-MN4 and TERV-MN1 were the least resistant being inactivated within 6 and 7 weeks, respectively (Figure 7.3). In non-autoclaved litter, TARV-O'Neil, TARV-MN2, and CARV-1 survived for 8 days while TERV-MN1 and TARV-MN4 survived for 6 and 7 days, respectively (Figure 7.4). In all experiments, the viral titers decreased gradually over time.

4. Discussion

Savage and Jones (2003) have reported that CARV strains were capable of surviving for at least for 10 weeks in drinking water. However, no such information is available on survival of TARVs and TERVs in either water or litter. All five viruses in our study were

able to survive for 9-12 weeks in autoclaved water and none survived at 13 and 14 weeks. These results are in contrast to those of Savage and Jones (2003), who reported little decrease in titer of CARV-R2 after 10 weeks. There were minor differences in survival of different viruses; The TERV-MN1 and TARV-MN4 survived for relatively less time than TARV-O'Neil, TARV-MN2 and CARV-1. These results are interesting since the latter three viruses are more pathogenic than TERV-MN1 and TARV-MN4 (Sharafeldin et al., 2014).

In non-autoclaved water, the virus titers continued to decrease and live viruses were still detected after 10 days of incubation. Unfortunately, this experiment was not continued beyond 10 days. However, the trend is clear; the viruses survive for less time in non-autoclaved water than in sterile water. This is not surprising because the flora present in non-autoclaved water may present competition for these viruses. Savage and Jones (2003) have also reported that addition of fecal material to water was helpful in decreasing the survival of CARV from 10 to 5 weeks.

In autoclaved poultry litter, the viruses were able to survive for five to seven weeks. Again, TERV-MN1 and TARV-MN4 survived for less time than the other three viruses. This could also be due to lower starting titer of these two viruses. No study is available on survival of avian reoviruses in poultry litter and hence a direct comparison with the results of this study is not possible.

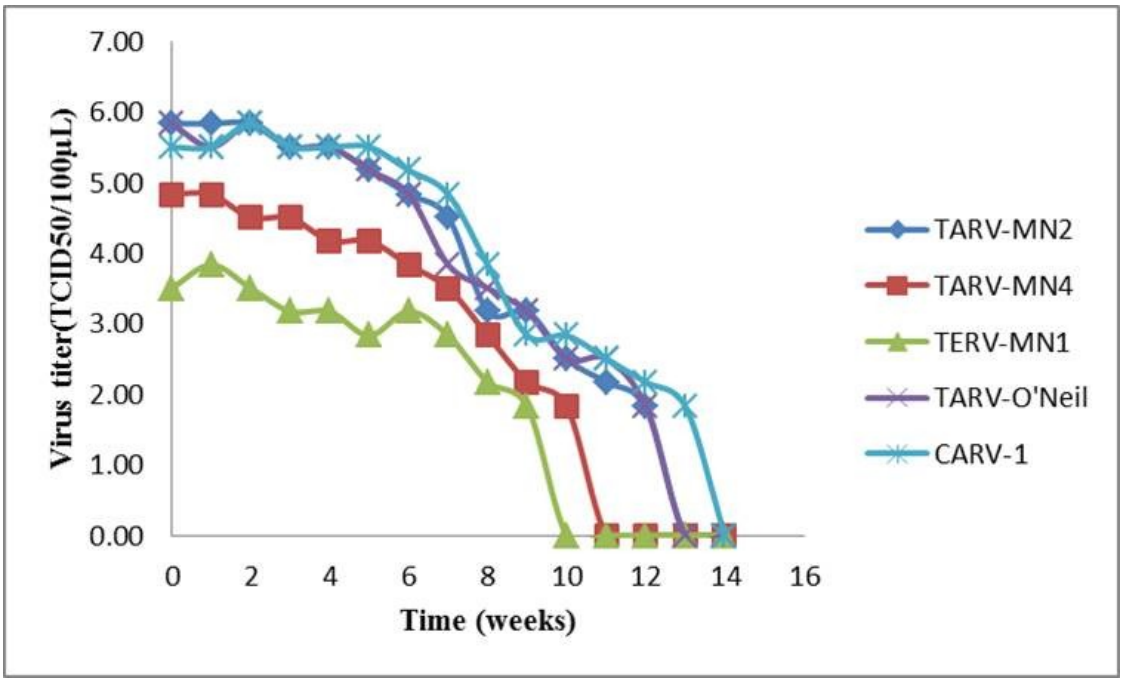
It should be noted that the results obtained in laboratory may not be fully applicable in field situations; in laboratory experiment, the virus was added to water only once while under field conditions the infected birds may keep shedding virus continuously and hence the virus may survive for the whole life of a particular flock. al

Afaleq and Jones (1994) reported prolonged persistence of disease in repeated exposure to reovirus as compared to a single infection. It should also be noted that even a small amount of surviving virus under field conditions is important because the ingestion of a small amount of virus by the bird may lead to replication in their gut to the point that it may cause disease.

To the best of our knowledge, this is first study on survival of TARVs and TERVs in drinking water and poultry litter. The results should be helpful in proper planning of preventive and control measures against these viruses.

Figure 7.1. Survival of reoviruses in sterile drinking water at room temperature (~25^{0}C).

Figure 7.1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2.00
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Virus titer(TCID50/100µL)
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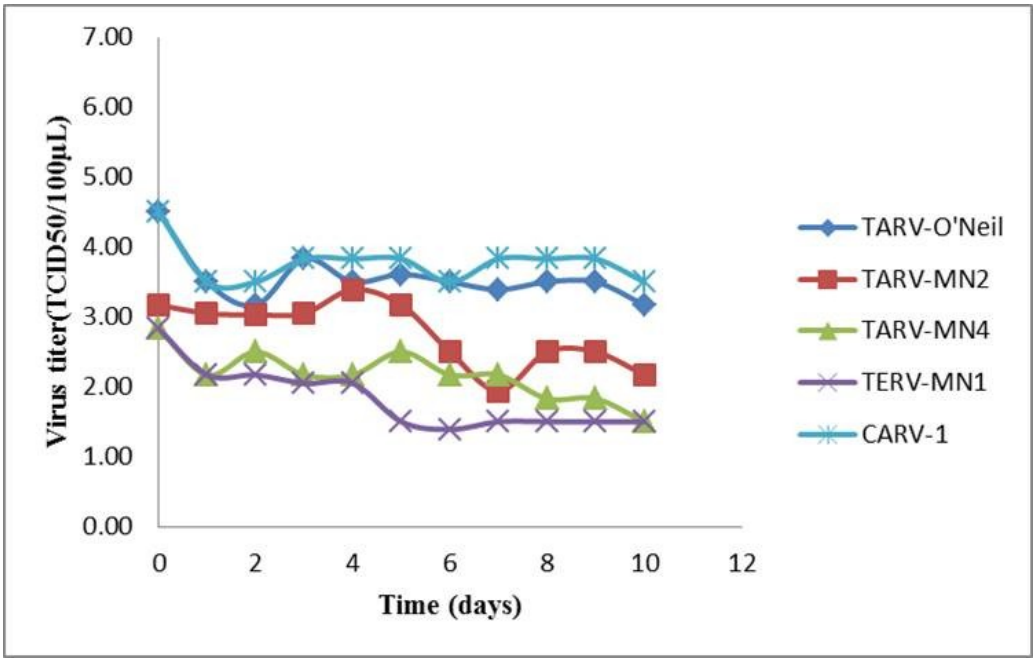
2
4
6
8
10
12
14
16
Time (weeks)
TARV-MN2
TARV-MN4
TERV-MN1
TARV-O'Neil
CARV-1

Figure 7.2. Survival of reoviruses in non-sterile drinking water at room temperature (~25^{0}C).

Figure 7.2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Virus titer(TCID50/100µL)
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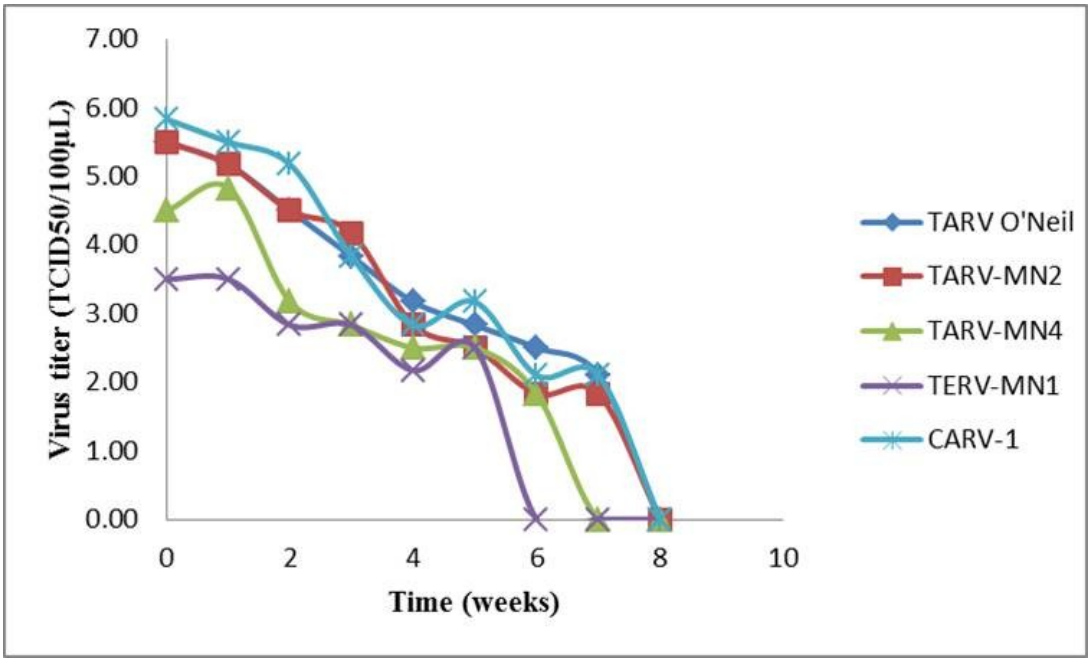
2
4
6
8
10
12
Time (days)
TARV-O'Neil
TARV-MN2
TARV-MN4
TERV-MN1
CARV-1

Figure 7.3. Survival of reoviruses in sterile litter at room temperature (~25^{0}C).

Figure 7.3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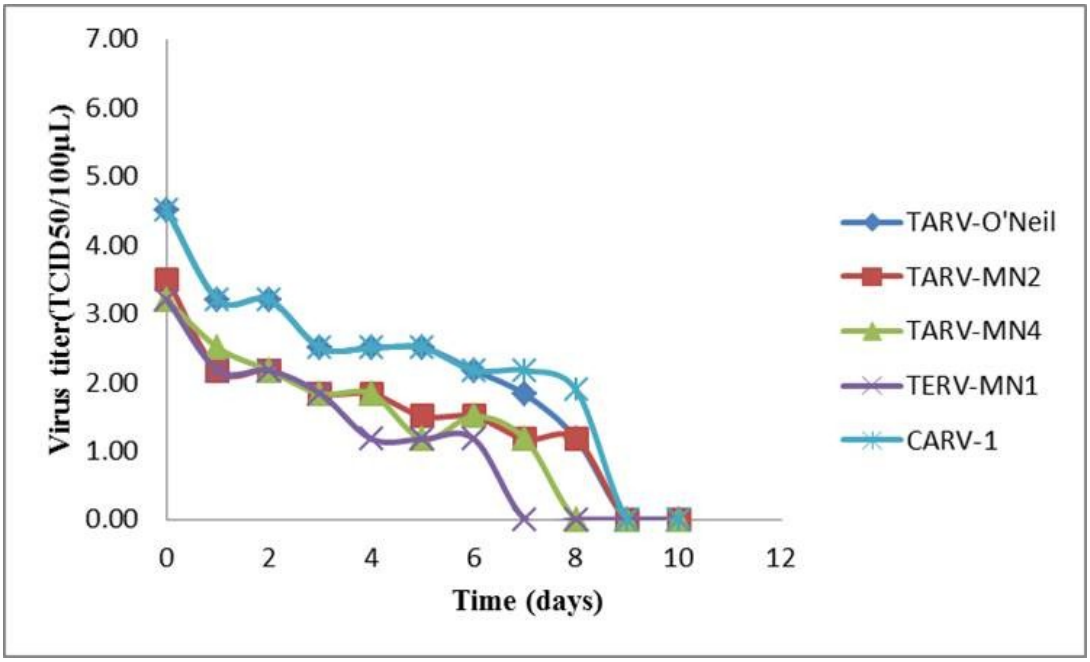
Virus titer (TCID50/100µL)
0
2
4
6
8
10
Time (weeks)
TARV O'Neil
TARV-MN2
TARV-MN4
TERV-MN1
CARV-1

Figure 7.4. Survival of reoviruses in non-sterile litter at room temperature (~25^{o}C).

Figure 7.4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Virus titer(TCID50/100µL)
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2
4
6
8
10
12
Time (days)
TARV-O'Neil
TARV-MN2
TARV-MN4
TERV-MN1
CARV-1

Chapter 8: Efficacy of five commonly used disinfectants against turkey arthritis reovirus

This work has been submitted for publication as:

Mor, S.K., Bekele, A. Z., Sharafeldin, T.A., Porter, R.E., Goyal, S.M., 2014. **Avian Diseases**

1. Introduction

In late 2009, the problem of turkey arthritis reovirus (TARV)-associated lameness and swollen hock joints was observed in market age tom turkeys throughout the upper Midwest area of the United States (Mor *et al.,* 2013b). The problem appears to be re-emerging after it was first reported in the late 1980s and early 1990s (Al Afaleq and Jones., 1989; 1991; Levisohn *et al.,* 1980). Molecular characterization of these newly isolated TARVs indicated that they are closely related to turkey enteric reoviruses (TERV), one of the causative agents of poult enteritis complex (Jindal *et al.,* 2010a; 2010b, Mor *et al.,* 2013a; Pantin-Jackwood *et al.,* 2008; Mor *et al.,* 2013b; 2014a). When inoculated in turkey poults, TARVs, but not TERVs, were able to reproduce the disease (Sharafeldin *et al.,* 2014a).

Re-emergence of reovirus-associated lameness in turkeys and recent detection of new variants of chicken arthritis reovirus (CARVs) in Europe and North America (Rosenberger *et al.,* 2013; Sellers *et al.,* 2013; Troxler *et al.,* 2013) indicate the importance of control measures to minimize the impact of these viruses. Biosecurity, which includes regular cleaning and disinfection, is a cost-effective way to control and prevent viral diseases. Disinfectants are commonly used in breeder and commercial turkey farms to inactivate pathogens if present. This study was undertaken to determine if five of the commonly used disinfectants in turkey farms are effective against reoviruses.

2. Materials and Methods

2.1. Virus propagation

Three TARV isolates (TARV-O'Neil, TARV-MN2 and TARV-MN4) were tested in this study (Mor *et al.,* 2013). We selected these three isolates because in an experimental study we observed differences in their pathogenicity (Sharafeldin *et al.,* 2014). For comparison, we used one isolate each of TERV (TERV-MN1) and CARV (CARV-1). All five isolates were propagated and titrated in QT-35 cells. The virus titers were $10^{5.8}$, $10^{5.5}$, $10^{4.2}$, $10^{4.2}$, and $10^{6.2}$ $TCID_{50}$/mL for TARV- O'Neil, TARV-MN2, TARV- MN4, TERV-MN1, and CARV-1, respectively.

2.2. Disinfectants

Five disinfectants (Virocid, Keno X5, Synergize, One Stroke, and Tek Trol) commonly used on Minnesota turkey farms, based on information provided by poultry veterinarians and production managers, were used in this study (Table 8.1). All disinfectants were used at their recommended dilutions (Table 8.1).

2.3. Procedure

All experiments were done at room temperature (~25C). The bottom of a sterile, 24-well tissue culture plate was used as a non-porous contact surface for testing the disinfectant efficacy. To each well was applied 20µl of appropriate virus followed by drying for approximately 30 min in a laminar flow hood. Disinfectant to be tested was then applied to the dried virus layer at 40µl per well. For negative control, 40µl of PBS (phosphate buffered saline at pH 7.2) was used. After contact times of 0.5, 1, 2, 5 and 10 min, 400µl

of an eluent (3% beef extract-0.05M glycine solution, pH 7.5) was added to all wells. The eluent was pipetted 10 times back and forth in each well to facilitate virus elution. Serial 10-fold dilutions of elutes were prepared and inoculated in monolayers of QT-35 cells contained in 96-well microtiter plates using three wells per dilution. Inoculated plates were incubated at 37^0C and observed daily for up to six days for the appearance of cytopathic effects (CPE). Virus titers were calculated using the method of Reed and Muench (1938). Virus titers in treated and control wells were compared to determine the amount of virus reduction by the disinfectant. Each virus was tested three times and average titer value of three experiments was used for further analysis. Efficacy of each disinfect was analyzed in terms of per cent reduction of virus titer at each time point.

3. Results

In general, all disinfectants were found effective against all five viruses. There were some minor differences. For example, Tek Trol and One Stroke took longer time to kill viruses than the other disinfectants (Table 8.2). Among viruses, TARV-O'Neil and CARV-1 were more resistant than the other three viruses; it took 5 to 10 minutes to inactivate them as compared to 2 to 5 minutes for the other three viruses (Table 8.2).

4. Discussion

Effective vaccines and strict biosecurity measures including the use of disinfectants are considered the only options to prevent and control viral infections. Biosecurity measures are advocated and used in breeder as well as commercial poultry units. Even then, the re-emergence of TARV-associated lameness in turkeys (Mor *et al.,* 2013b,

Sharafeldin *et al.,* 2014) and the emergence of new variants of CARVs in broiler chickens (Rosenberger *et al.,* 2013; Sellers *et al.,* 2013; Troxler *et al.,* 2013) point to the need of continued research in these areas. This current study was undertaken to determine if commonly used disinfectants are effective against TARVs, TERVs, and CARVs.

Three of the five disinfectants were able to kill 4 log10 of all viruses within 2 to 5 minutes of contact time except Tek Trol and One Stroke, which took 10 min indicating that contact time is an important factor in using a particular disinfectant. Both Tek Trol and One Stroke belong to the phenolic category of disinfectants. This is in contrast to Patnayak *et al.* (2008) who reported that phenolic and glutaraldehyde compounds were very effective against avian influenza virus (AIV), Newcastle disease virus (NDV) and avian metapneumovirus (aMPV). A direct comparison with our study results is not possible because of lack of published research on the effect of disinfectants on avian reoviruses.

In an experimental study, we found TARV-O'Neil to be the most pathogenic to turkey poults followed by TARV-MN2 and TARV-MN4 (Sharafeldin *et al.,* 2014). In this study also we observed some correlation between pathogenicity and efficacy of disinfectant as TARV-O'Neil took a longer time to be inactivated as compared to the other viruses. For example, Tek Trol took longer (5-10 min) to inactivate TARV-O'Neil, TARV-MN2 and CARV-1 but was able to kill TARV-MN4 and TERV-MN1 within 2-5 min.

Pantin-Jackwood *et al.* (2008) conducted a study of enteric viruses (astrovirus, rotavirus and reovirus) in commercial broiler and turkey farms. Environmental samples collected before the placement of chicks or poults were found positive for enteric viruses

by reverse transcription polymerase chain reaction (RT-PCR). In normal practice, farms are properly cleaned and disinfected before the placement of new flocks. The detection of enteric viruses in environmental samples before placement was believed to be due to these possibilities: (i) inactivated viruses were detected by RT-PCR; (ii) disinfectants were not properly used e.g., they were used at a higher dilution than prescribed or were stored for a long time after being diluted; (iii) the presence of organic matter because of improper cleaning may have been responsible for ineffectiveness of disinfectants; or (iv) the disinfectants used were not effective.

Although the disinfectants were found to be effective under laboratory conditions, several factors can influence their efficacy in the field. For example, disinfectants in general are more effective in the absence of organic material (Patnayak *et al.,* 2008; Ruano *et al.,* 2001; Stringfellow *et al.,* 2009); the presence of organic material has negative effect on their efficacy often requiring higher concentration or longer contact time or both (Ruano *et al.,* 2001; Stringfellow *et al.,* 2009). Shelf life of the diluted disinfectant should also be considered; freshly prepared dilutions of disinfectants are significantly more effective than older ones (Stringfellow *et al.,* 2009). Temperature may also affect the efficacy of disinfectants as has been reported by Jang *et al.* (2014). The efficacy of phenolic disinfectants was significantly reduced against *Salmonella* Typhimurium after storage for 6 weeks at 42^0C and after 16 weeks at 32^0C (Stringfellow *et al.,* 2009).

In conclusion, commonly used disinfectants were found effective against avian reoviruses (TARV, TERV, and CARV) with minor differences amongst them. As has been reported in previous studies, it is important that disinfectants are properly stored and

used. Future studies with several different strains of TERV and CARV (including the new variants) are indicated to determine if all strains can be equally disinfected. Studies on the shelf life of various diluted disinfectants should be of practical value for the poultry industry.

Table 8.1. List of disinfectants and dilutions used.

No.	Name of disinfectant	Disinfectant category	Recommended dilution used
1	Virocid	Aldehyde	1:256
2	Keno X5	Oxidizing agent	1:400
3	Synergize	Aldehyde	1:200
4	One Stroke	Phenol	1:250
5	Tek Trol	Phenol	1:200

Table 8.2. Efficacy of commercial disinfectants against reoviruses.

Disinfectant	Contact time (min)	Per cent inactivation of indicated virus:[a]				
		TARV O'Neil	TARV MN2	TARV MN4	TERV MN1	CARV-1
Virocid	0.5	95.83	96.01	98.41	97.11	92.5
	1	98.41	98.41	98.41	98.41	95.83
	2	99.91	99.99	99.99	99.99	98.41
	5	>99.99	>99.99	>99.99	>99.99	99.99
	10	>99.99	>99.99	>99.99	>99.99	>99.99
Keno X5	0.5	95.83	96.01	94.98	96.01	92.5
	1	98.41	98.41	98.41	98.41	95.83
	2	99.91	99.99	99.99	99.99	98.41
	5	>99.99	>99.99	>99.99	>99.99	99.99
	10	>99.99	>99.99	>99.99	>99.99	>99.99
Synergize	0.5	94.98	96.01	94.98	96.01	91.68
	1	97.11	98.41	98.41	98.41	94.98
	2	99.91	99.99	99.99	99.99	98.34
	5	>99.99	>99.99	>99.99	>99.99	99.99
	10	>99.99	>99.99	>99.99	>99.99	>99.99
One Stroke	0.5	94.98	96.01	94.98	97.11	92.5
	1	96.01	98.41	98.41	98.41	95.83
	2	99.91	97.11	99.99	99.99	98.34
	5	>99.99	>99.99	>99.99	>99.99	99.81
	10	>99.99	>99.99	>99.99	>99.99	>99.99
Tek Trol	0.5	89.28	96.01	94.98	96.01	89.01
	1	92.5	96.01	96.01	98.41	91.68
	2	94.98	97.11	99.99	99.99	97.11
	5	99.49	99.99	>99.99	>99.99	98.41
	10	>99.99	>99.99	>99.99	>99.99	>99.99

[a] Turkey arthritis reoviruses (TARV O'Neil, TARV MN2, TARV MN4); turkey enteric reovirus (TERV MN1); chicken arthritis reovirus (CARV-1).

Chapter 9: General discussion and conclusions

1. General discussion

Reoviruses are distributed worldwide and are associated with different disease conditions such as enteritis, viral arthritis, and respiratory, neurological, hepatic and cardiovascular diseases in chickens, ducks, geese, turkeys and wild birds. In late 2009, the problem of turkey lameness re-emerged after a hiatus of more than 20 years and continues till today causing huge economic losses to the turkey industry. Flock histories suggest that lameness occurrs as early as ten weeks of age and affects at least 3-5% of the flock with >1% mortality or culling per week. In severely affected flocks, up to 25% of birds are affected (Trites et al., 2012). Turkey arthritis reovirus (TARV) was isolated from tendons and/or joint fluids of several cases and no other pathogen was isolated. The objective of this thesis work was to do complete genome characterization of these newly isolated TARVs in comparison with TERV, CRV, DRV and GRV. In addition, we were interested in developing a sensitive and specific test for the rapid detection of turkey reoviruses (TARV and TERV). Another objective was to test the comparative survival of TARV, TERV, and CARV in drinking water and poultry litter as well as to test the antiviral efficacy of commonly used disinfectants.

In chapter 2, we introduced the re-emerging problem of lameness and arthritis in turkeys. The samples from these cases were tested for aerobic bacteria by culture, for Mycoplasma by culture and real time PCR, and for viruses by inoculation in embryonated eggs from specific-pathogen-free (SPF) chickens and QT-35 cells. Inoculation in yolk sac and QT-35 cells was found to be an effective method for reovirus isolation. The TARV isolates were further confirmed as reovirus by sequencing of the S4 gene. This preliminary study raised several questions: what was the source of these TARVs and

were they different from TERVs and CARVs. Other questions raised were: how can these viruses be detected in the early stages of infection or before the onset of clinical signs; are they hardy in the environment as has been shown with CARVs; are commonly used disinfectants effective against them?

In chapter 3, we characterized TARVs based on their complete S class gene segments because these segments are small in size and encode both structural (σC, σA, σB) and non-structural (σNS) proteins. Also in GenBank only S class gene segment sequences of TERVs are available for comparison. A total of 12 TARVs isolated from 2011 to 2013 were sequenced for complete S class segments and compared with almost complete segment sequences of CRV, DRV and GRV available in GenBank. Based on phylogenetic analysis all TARVs were clustered in lineage I in S1 gene but were divided into two lineages in S2, S3 and S4 gene segments. Point mutations and reassortments were also observed within TARVs and between TARVs and TERVs.

The CARVs were divergent from TARVs based on S1, S3 and S4 gene sequence analysis but were related in the S2 gene. The divergence among TARVs was comparatively less than the divergence reported in CARVs, which could be due to one of three possibilities: (i) eight of the 12 TARVs were isolated during the onset of disease problem in 2011, (ii) All TARVs were isolated from different commercial turkey flocks, however these commercial flocks were related to two different hatcheries (hatchery A and hatchery B). The10 of 12 were isolated from different commercial turkey flocks from 2011 to 2013 in which the poults originated from a single hatchery (hatchery A) making it possible that we isolated similar types of strains at that time point. However, the 2012 and 2013 isolates were from different breeder flocks of hatchery A and (iii) the virus is a

newly emerging pathogen and has not yet gone through selective pressure. To know more in detail about TARVs, M and L class gene segments were also analyzed as given below.

In chapter 4, we characterized the M class gene segments of TARVs to compare then with those of TERVs. Unfortunately, there are no sequences available in GenBank for complete M class of TERVs; hence we sequenced eight TERVs isolated in our laboratory. In addition to 12 TARVs studied in the S class study, we included another three recent TARV isolates from 2014 for comparison. At this point we realized that criteria of assigning lineages of CRVs and DRVs based on >10% nt divergence between lineages is not appropriate and hence we proposed a new criteria of genotype classification of ARVs based on nt and aa cut-off values for each M class segment. With these new criteria TARVs and TERVs grouped with CRVs in genotype M1-I and M3-I in M1 and M3 gene segments, respectively, but grouped differently from DRVs and GRVs. An interesting finding was that TARVs and TERVs were divided into three genotypes in M2 gene segment with genotype M2-II being novel and specific for TRVs. Another interesting finding was that genotype M2-III TARV and TERV grouped with AVS-B strain of CRV reported from USA and with three strains of CRV (two arthritic and one enteric) reported from Taiwan in 1980s and 1990.

In this chapter 4, we also proposed a novel approach of assigning genotype constellations (GCs) and used a method that has been used to classify other viruses including sapporo-like-viruses, noroviruses, hantaviruses, papillomaviruses, and rotaviruses. Using the proposed genotypes for the three M gene segments, an M class GC was formed, yielding 12 different GCs. The TARV and TERV strains consisted of 3 GC (GC1-GC3), the CRV strains consisted of 8 GC (GC1-GC9, excluding GC2), the DRV

strains consisted of 3 GC (GC9-GC11), and the GRV strains consisted of 2 GC (GC11 and GC12). The GC2 was unique to TARV and TERV strains while GC1 and GC3 were shared among TARV, TERV and CRV strains. The maximum number of sequences of TARVs, TERVs and CRVs formed GC1 which indicates the possibility that a universal type vaccine can be potentially produced against chicken and turkey reoviruses. However, this may be a premature prediction at this time because of very low number of complete M class sequences available in GenBank. Hence, there is a strong need for conducting molecular epidemiology studies based on complete genome analysis of enteric and arthritis strains circulating in chicken and turkey population of breeder and commercial flocks.

Based on M class gene segment analysis we found interesting results especially novel genotype of TARVs and TERVs in M2 gene. Hence, we did further L class gene segments analysis of seven strains of TARVs and three strains of TERVs in chapter 5. Based on this analysis, TARVs and TERVs were similar in size and function to the CRVs. We also proposed new genotype criteria for each L class and based on these criteria TARVs and TERVs grouped together with CRVs in one genotype L1-I and L2-I in L1 and L2 gene segments, respectively. The maximum divergence was observed in L3 gene and TARVs and TERVs formed a separate genotype (L3-III) and CRVs were also divided into three genotypes. The random point mutations were observed in all L class segments. The different grouping of L1 and L2 genotypes into L3 gene indicates reassortments among TARVs, TERVs and CRVs.

At about the same time, there was a great demand by the turkey industry to develop a sensitive and specific test for early detection of TARVs. Hence, we developed

a TaqMan based rRT-PCR test for quantification of the virus in field samples as discussed in chapter 6. The detection limit of this assay is 10 genome copies per reaction. The gel based RT-PCR was found to be 223 times less sensitive than rRT-PCR assay.

In chapter 7, we studied the environmental stability of TARVs. The survival in drinking water and poultry litterof three TARVs (TARV-O'Neil, TARV-MN2 and TARV-MN4), one TERV (TERV-MN1) and one CRV (CARV-1) were studied. All five isolates were able to survive for 9-12 weeks in autoclaved drinking tap water. The TERV-MN1 and TARV-MN4 isolates survived for a comparatively smaller time. All viruses were able to survive in non-autoclaved drinking water for $\geq$10 days indicating a potential concern in implementing preventive and control measures.

The TARVs were able to survive for five to seven weeks in autoclaved poultry litter and for five days in non-autoclaved litter. In this study we tried our best to mimic the field conditions by using water from drinkers as well as litter without autoclaving, but the situation is different in the field as population of infected birds continuously shed virus into the litter and drinking water. Hence virus might be present for the whole life of a particular flock. When we used litter without autoclaving and kept it in tubes without a lid, the litter dried after few days which is again at odds with field conditions where litter remains moist and hence may affect virus survivability.

The last objective of this was to test the efficacy of five commonly used disinfectants against TARVs and TERVs. For this objective (chapter 8), we studied five disinfectants against three TARVs, one TERV and one CARV. All disinfectants were effective in killing virus in 2-5 min except Tek Trol which took 5-10 minutes to kill them. This study concluded that commonly used disinfectants were effective in killing

viruses. However, factors such as improper use of disinfectants, improper cleaning, and presence of organic matter may protect viruses against disinfectants.

2. Limitations of the study

There are some limitations of this study that need mentioning here: (i) Most of the isolates studied were isolated in 2011 although we did include a few 2014 isolates, which could be one reason for high similarity among these isolates; (ii) Although these TARVs were isolated from commercial turkey flocks from six different states in the US, the source of these poults was only two breeder companies. Of the 15 TARVs, 12 were from hatchery A and three were related to hatchery B. All TERVs were from hatchery B; (iii) Almost all of the isolates used in this study were from cases received at the MVDL, which may have biased strain selection. The possibility of more divergent strains circulating in the field should not be ignored in future studies; (iv) We were unable to study TARVs from apparently healthy and lame breeder flocks. The screening of breeder flocks may help us determine the source of TARVs; (v) Recently, new variants of CRVs have been reported from North America and Europe but no complete genome sequence is available and hence we were unable to determine any relationship between TARVs and the new variants of CRVs; (vi) The diagnosis and quantification of rRT-PCR will depend on proper sampling and processing of tendons. In our experience, improper homogenization of tendons affected the results of virus isolation and RT-PCR; (vii) In the virus survival study, it was hard to correlate with the situation in the field. For example, in the field all affected birds keep on shedding virus and add more and more virus to the litter, which is harder to simulate under laboratory conditions; (viii) In the disinfectant

study we found that all five disinfectants were effective in killing the virus. However, this testing was done under ideal laboratory conditions of direct contact of disinfectant with the virus. In the field, the situation might be different because of the presence of organic material, which may protect viruses against disinfection.

3. Future directions

To the best of our knowledge this is the first study on complete genome analysis of TARVs and TERVs hence it should serve as a baseline for future studies on molecular characterization of new variants circulating in field. In this study, we isolated TARVs and TERVs from cases of turkey lameness submitted to the MVDL but there is a strong need to conduct a complete molecular epidemiological study of TARVs and TERVs in breeder flocks especially during the time of lay. This should then be followed up by testing their respective commercial flocks (progeny) after placement. This will help shed light on vertical as well as horizontal transmission of TARVs and TERVs.

In this study we were not able to differentiate between TARVs and TERVs but observed random point mutations. In the future, complete genome analysis of TARVs and TERVs from breeder and commercial flocks may be helpful in finding markers that can differentiate these two pathotypes of turkey reoviruses. Most of the isolates of TARVs were from samples of frozen legs received at the MVDL. In future, it is important to analyze not only on tendon samples but also on the affected birds' intestine, liver, heart, spleen, bursa of Fabricius, kidney and trachea to characterize virus dissemination. If viruses are isolated from these organs, it will be interesting to find if unique mutations (if any) are present in these isolates as compared to those in TARVs

isolated from tendons. This should be beneficial in finding markers defining tissue tropism of turkey reoviruses.

We have shown that there is a possibility of reassortment among TARVs, TERVs and CRVs. Reassortment events allow emergence of new variants that are potentially more pathogenic for either one or both avian species (chicken and turkey). Hence, it is important to understand reassortment events between TARVs and TERVs as well as among TARVs, TERVs and CRVs.

The most important future aspect of this study will be to develop an effective vaccine against TARVs. The selection of best candidate for an effective vaccine is a big challenge. TARV-Crestview is being used currently as an autogenous killed vaccine but still new cases continue to arise indicating that this killed vaccine is not completely effective. We have proposed a new genotyping system for classification of ARVs and further analysis of genomic constellations. Based on this new classification system in M class we found that CRVs and TRVs formed five and three GCs out of which GC1 included maximum number of sequences. Based on the GC pattern, there is a possibility of developing universal type vaccine which is effective against both chicken and turkey reoviruses. This type of approach has been successfully used in developing Rota Teq and Rotarix vaccines which are now being used effectively in areas from where complete genome sequences are available and GCs are known. But these vaccines are less effective in some Asian and South African countries from where sufficient information about circulating rotaviruses is lacking. A similar approach might be helpful in developing an effective universal type vaccine against chicken and turkey reoviruses.

Biosecurity measures that include proper cleaning and disinfection are known to help control and prevent viral infections. On one hand, we found that CRVs and TRVs can survive for a long time in the environment and on the other hand commonly used disinfectants were effective in killing these viruses. The next step is to conduct such studies in commercial and breeder farms to test virus load in the farm environment before and after applying disinfectants. Farms should be tested for the presence of live viruses before and after placement of new flocks. This will give a clear picture about the real situation in the field.

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