

Pathogenicity of newly emergent turkey arthritis reoviruses in chickens

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ABSTRACT Turkey arthritis reoviruses (TARVs) were isolated recently from gastrocnemius and digital flexor tendons of lame turkeys with swollen joints and tenosynovitis. These TARVs were genetically different from chicken arthritis reoviruses (CARVs) and produced gastrocnemius tenosynovitis when inoculated into turkey poults. The purpose of this study was to determine the pathogenicity of TARVs in chickens. One-week-old, specific-pathogen-free chicks were inoculated with either a TARV (TARV-MN2 or TARV-O'Neil) or CARV via oral, intratracheal, or footpad routes. At 2 and 3 weeks post inoculation (PI), a subset of chicks from each group was euthanized followed by collection of tissues for real-time RT-PCR (rRT-PCR), virus isolation, and histopathology. Chickens inoculated with

CARV via intratracheal and footpad routes developed gastrocnemius lymphocytic tenosynovitis at 2 and 3 weeks PI. Both TARV-MN2 and TARV-O'Neil induced gastrocnemius lymphocytic tenosynovitis in chicks inoculated only via the footpad route at 2 and 3 weeks PI. Although there was no evidence of clinical lameness, the virus was present in leg tendons, internal organs, and intestines of all TARV-inoculated chicks regardless of route of inoculation, as indicated by rRT-PCR and virus isolation. These results indicate that TARVs do not produce gastrocnemius tenosynovitis in chicks by 3 weeks PI when administered via the most probable natural route (e.g., oral and intratracheal). Further studies are needed to determine the long term effects these viruses might play in inducing lameness in chickens.

Key words: reovirus, chickens, tenosynovitis, arthritis, lameness

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INTRODUCTION

The first clinical case of viral tenosynovitis/arthritis in *Mycoplasma synoviae*-negative chickens in the United States was reported by Olson and Solomon (1968). Affected chickens had swelling and edema of intertarsal joints, wing joints, and digital flexor tendons. Inflammation in the gastrocnemius tendon sheath of chickens at one week post inoculation (PI) with chicken arthritis reovirus (CARV) via the footpad route was described by Kerr and Olson (1969). Both naturally and experimentally infected birds showed heart lesions. Reovirus was then isolated from tendons of 28-day-old broiler chickens with tenosynovitis/arthritis and ruptured tendons (Jones et al., 1975). Experimental inoculation of one-day-old, specific-pathogen-free chickens with chicken reovirus via the oral and footpad routes produced necrosis and congestion of liver, spleen, kidney, and bursa of Fabricius, in addition to causing tenosynovitis, myocarditis and pericarditis (Jones and Georgiou, 1984).

In the 1980's, reoviruses were identified in gastrocnemius tendon of turkeys with tenosynovitis and arthritis (Levisohn et al., 1980; Page et al., 1982). For nearly 30 years there were no further reports on reovirus-induced lameness in turkeys until we isolated turkey arthritis reoviruses (TARV) from gastrocnemius and digital flexor tendons of lame commercial turkeys with tenosynovitis, and these TARVs were found to be genetically different from chicken reoviruses (Mor et al., 2013). In an experimental study, 3 different TARVs produced lymphocytic tenosynovitis in turkey poults within 4 weeks PI through oral, intratracheal, and footpad routes at one week of age (Sharafeldin et al., 2014). Inoculation with TARVs did not cause clinical lameness in turkey poults until 4 weeks PI, although they developed high histologic scores for tenosynovitis. Clinical lameness appeared at 7 weeks PI and the percentage of lame turkeys increased at 11 and 15 weeks PI in another experiment in which turkey poults were inoculated at one week of age (Sharafeldin et al., 2015).

With the close proximity of turkey and broiler production in the United States there are concerns about the risk that TARVs may pose to chickens. It was previously demonstrated that 3 reoviruses isolated from turkeys with tenosynovitis could produce erosive

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arthritis and tenosynovitis at 3 weeks PI when inoculated into the footpad of one-day-old chicks (Al-Afalet and Jones, 1989); however, other more natural routes of inoculation (oral and intratracheal) were not investigated in that study. In another study, oral inoculation of turkey enteric reovirus in specific-pathogen-free chickens did not produce clinical illness (Nersessian et al., 1986; Spackman et al., 2005). The aim of the present study was to determine the pathogenicity of 2 newly isolated TARVs, previously shown to be pathogenic in turkeys, when inoculated in chickens and to compare the results with that of a pathogenic CARV.

MATERIALS AND METHODS

Viruses

The isolation and characterization of TARVs and their pathogenicity in turkeys have been described (Menendez et al., 1975; Rosenberger et al., 1989). In this study, we used 2 different strains of TARV; TARV-MN2 and TARV-O'Neil. For comparison, a pathogenic CARV (strain 2048) (Rosenberger et al., 1989) kindly supplied by Dr. Jack Rosenberger (AviServe LLC, Newark, DE) was used. All viruses were grown and titrated on QT-35 cells and had an average titer of $10^{5.5}$ Tissue Culture Infective Dose₅₀/mL.

Birds

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

Experimental Design

Each group consisting of ten 6-day-old chicks was placed in separate isolators. The birds in these groups were inoculated with a virus (TARV-MN2, TARV-O'Neil, or CARV) via one of the 3 routes (oral, intratracheal, or footpad) (Table 1). The chicks were inoculated in a blind fashion with 0.1 mL of the inoculum. Control chicks were inoculated with virus-free Minimum Essential Medium. Two individuals, who were blinded to the type of virus and route of inoculation for each isolator, observed the chicks daily for clinical signs (lameness and swollen red joints) and mortality until the termination of the experiment. At 2 and 3 weeks PI, 5 birds from each isolator were removed and euthanized by exposure to CO₂ gas. The birds were necropsied, gross lesions were recorded, and tissues (right leg gastrocnemius and digital flexor tendons; center leg gastrocnemius and digital flexor tendons; a pool of liver, spleen, heart, bursa of Fabricius; and intestinal contents) were collected from each bird for rRT-PCR, histopathology and virus isolation. Procedures for housing, inoculation, and euthanasia of birds were approved by the Institutional Animal Care and Use Committee, University of Minnesota.

Virus Detection

For initial detection of the virus in tendons of birds inoculated with TARVs, a TARV-specific rRT-PCR was used (Mor et al., 2014). Samples found positive by TARV-specific rRT-PCR were further confirmed by virus isolation in QT-35 cells (Mor et al., 2013). Tendons from CARV-inoculated birds were tested by a chicken reovirus specific rRT-PCR (Guo et al., 2011) and by virus isolation. Forward primer 5'- AT-CATGGCT GGGTTTGTGCC-3' and reverse primer 5'- AGAACGAATTTGTARGCGACCA-3' were used

Table 1. Medians of gastrocnemius tendons histologic inflammation scores in different experimental groups of chickens.¹

Treatment	Route	Oral		Intratracheal		Footpad	
		No.	Median score at: 2 wks 3 wks	No.	Median score at: 2wks 3wks	No.	Median score at: 2wks 3wks
TARV-MN2		10	0 0	10	0 0	10	9 0 ^{*,+}
TARV-O'Neil		10	0 0	10	0 0	10	13 13 ⁺
CARV		10	0 0	10	27 ^a 23 ^a	10	26 ^a 24 [*]
Control (MEM)		10	0 0	10	0 0	10	0 0 ^{*,+}

¹12 groups (3 routes and 4 treatments) in different separated isolators.

^aSignificantly higher than other values in the same column ($P < 0.05$) Mann Whitney U test.

MEM = Minimum Essential Medium.

No. = Number of birds; Weeks: weeks post inoculation.

^{*,+}Significant difference between the values carrying the same symbol ($P < 0.05$) Mann Whitney U test. At 2 weeks PI, groups that were inoculated with TARV-MN2 and TARV-O'Neil via the footpad route had median tenosynovitis scores that were significantly lower than those of the positive control group, which was inoculated with CARV via the footpad route. At 3 weeks PI, the median histologic inflammation score of the TARV-O'Neil footpad group had a median histologic inflammation score that was similar to the CARV footpad group. TARV-MN2 and TARV-O'Neil groups inoculated via oral and intratracheal routes had no histological tendon lesions at 2 and 3 weeks PI.

Table 2. Virus detection in the inoculated birds.

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

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

Positive results are bold and underlined.

*Virus was detected in these tendon samples by virus isolation only and confirmation of virus was done by S4 gene sequencing.

to amplify a 99 bp fragment from the S4 gene. A TaqMan probe, 5'-FAM-TGAG MGTGATGACTTTA-CYCC -TAMRA-3' was similarly selected. The rRT-PCR reactions were carried using One-step RT-PCR kit (Qiagen, Valencia, CA). Each reaction mixture (25 μ L) had 23 μ L of reagent mix (5 μ L, 5X reaction buffer, 1.0 μ L enzyme mix, 0.2 μ L RNase inhibitor, 300 nM of each primer and 200 nM of TaqMan probe) and 2 μ L of RNA. The PCR cycling conditions started with 50°C for 30 minutes, 95°C for 15 minutes and then 45 cycles of a 2-step cycle (denaturation at 95°C for 15 s; annealing and extension at 56°C for 45 s). The cycle threshold value threshold for TARV detection in chickens was 33. Any value of 33 or more was considered negative.

Histopathology

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

trocnemius tendon were added together as a total score for one leg. Scores of right and left legs were added to arrive at the final score for each chicken.

Statistical Analysis

Mann Whitney U test was used to detect the significance of difference among histologic inflammation scores of different groups (NCSS 9 Statistical Software, NCSS LLC., Kaysville, UT).

RESULTS

Clinical Signs and Mortality

No clinical signs or gross lesions were observed in any group except the CARV-footpad group in which chickens were often recumbent and developed swollen and reddened right shanks, hocks, and feet by 4 days PI. Two birds inoculated with CARV died 4 days PI due to non-specific causes; one was inoculated by the intratracheal route and the other by the footpad route. No gross lesions were observed in the 2 birds that died during the study.

Virus Detection

TARV was detected by TARV-specific rRT-PCR in tendons, internal organs and intestinal contents of the chicks inoculated with TARV-MN2 via oral and footpad routes at 2 weeks PI, while those inoculated with intratracheal route became positive by 3 weeks PI. The tendons, internal organs, and intestinal contents of chicks inoculated with TARV-O'Neil through oral, intratracheal and footpad routes were positive by TARV-specific rRT-PCR at both 2 and 3 weeks PI (Table 2). None of the CARV-inoculated birds were positive by TARV-specific rRT-PCR or chicken reovirus-specific rRT-PCR (Guo et al., 2011). CARV was isolated on QT-35 cells from tendons of CARV-inoculated

chickens. Virus isolation on QT-35 cells from tendon samples followed by S4 gene sequencing confirmed that the isolated virus was either TARV or CARV (Table 2).

Histopathology

Internal organs showed no histologic lesions except lymphocytic epicarditis and myocarditis in the CARV group at 2 and 3 weeks PI in all routes. Mild heterophilic enteritis in duodenum, jejunum, and cecum was seen at 2 weeks PI in the CARV group inoculated by oral and intratracheal routes (Figure 1). The CARV-inoculated groups had significantly higher median histologic gastrocnemius tendon inflammation scores (all routes together) than those inoculated with any of the two TARVs at 2 and 3 weeks PI ($P < 0.05$). By 3 weeks PI chicks inoculated with CARV by both footpad and intratracheal routes showed gastrocnemius lymphocytic tenosynovitis with high inflammation scores while those inoculated with the oral route showed minimal inflammation scores (Table 1).

The birds inoculated with TARV-MN2 and TARV-O'Neil had numerically higher histologic inflammation scores (all routes together) than the negative controls, but the difference was not significant ($P > 0.05$). At 2 and 3 weeks PI, no histologic lesions were observed in gastrocnemius tendon and intertarsal joints of chicks inoculated with TARV-MN2 or TARV-O'Neil by the oral and intratracheal routes. However, chicks inoculated in the footpad with TARV-O'Neil developed gastrocnemius lymphocytic tenosynovitis (Table 1) with high median inflammation scores by 2 and 3 weeks PI. At 2 weeks PI, the TARV-O'Neil-footpad group had a median histologic inflammation score that was significantly lower than that of the CARV-footpad group (positive control) ($P < 0.05$) (Table 1). At 3 weeks PI, the TARV-O'Neil-footpad group had a median histologic inflammation score that was significantly higher than that of the negative control, but was similar to that seen in the CARV-footpad group (positive control) ($P < 0.05$) (Table 1).

DISCUSSION

This study evaluated the pathogenic effects of 2 TARVs in chickens for up to 3 weeks PI. A 3-week testing period was chosen because TARV has previously been shown to cause lymphocytic tenosynovitis in turkey poults within 2 to 3 weeks PI and CARV has been shown to cause tenosynovitis in chicks within one week PI. We inoculated chickens at one week of age because of demonstrated age resistance to CARV infection by 2- to 3 weeks of age (Jones et al., 1975). We inoculated chicks via oral and intratracheal routes because these are the likely natural routes of infection. Previous work has shown that turkey poults infected with TARV can shed the virus in their feces and transmit it laterally to pen mates (Sharafeldin et al., 2014). Additionally,

we inoculated chicks via the footpad route, which is a classical challenge route and has been proposed to be a possible route for reovirus infection through broken skin (Al-Afaleq and Jones, 1990).

TARV-MN2 and TARV-O'Neil produced no clinical signs, no gross lesions, and no microscopic lesions in internal organs of chickens, although the respective viruses were detected in all tissues at 3 weeks PI. Only the footpad route of inoculation with TARV-MN2 and TARV-O'Neil produced histologic gastrocnemius lymphocytic tenosynovitis although there was no clinical lameness by 2 and 3 weeks PI. In a previous study, footpad route inoculation of one-day-old chicks with reoviruses isolated from turkeys with tenosynovitis/arthritis produced mortality with hock joint swelling and erosive arthritis by 3 weeks after inoculation (Al-Afaleq and Jones, 1989). Difference in inoculated strains and age of inoculation might be the reason behind the variation of results between the previously mentioned study and our present study. In addition, the present study investigated 2 additional inoculation routes (intratracheal and oral), which are likely natural routes of infection.

The results of rRT-PCR and virus isolation indicated that TARVs can infect and multiply in chickens but do not produce clinical disease for up to 3 weeks PI (4 weeks of age). These 2 viruses (TARV-MN2 and TARV-O'Neil) were previously shown to produce histologic lymphocytic tenosynovitis in one-week-old turkeys within 2 to 3 weeks PI (Sharafeldin et al., 2014, 2015) and clinical lameness was not observed until 7 weeks PI of TARV-O'Neil (Sharafeldin et al., 2015). It is possible, therefore, that chickens might show clinical lameness later in life but this point was not investigated in this preliminary study.

TARV-MN2 and TARV-O'Neil inoculated via oral and intratracheal routes did not produce histological lesions of tenosynovitis by 3 weeks PI, although the viruses were detected in tendons, intestines, and internal organs of inoculated chickens. The TARV-O'Neil footpad group showed a mild lymphocytic tenosynovitis at 2 weeks PI and the median inflammation score was significantly lower than that of the CARV footpad group. However, at 3 weeks PI, the median histologic inflammation scores of TARV-O'Neil and CARV footpad groups were similar and greater than the median score of the control group ($P < 0.05$), indicating that TARV-induced inflammation in chicks became progressively worse from 2 to 3 weeks PI (Table 1). In turkeys, TARV-MN2 and TARV-O'Neil induced tenosynovitis in one-week-old turkeys within 2 to 3 weeks PI via oral, intratracheal and footpad routes (Sharafeldin et al., 2014, 2015).

The long term clinical effects of TARV-induced tenosynovitis in chicks are not known. It should be mentioned here that in turkeys, TARV inoculation via the oral route at one week of age displayed clinical lameness at 7 weeks PI and later (Sharafeldin et al., 2015) and that histologic tenosynovitis associated with TARV

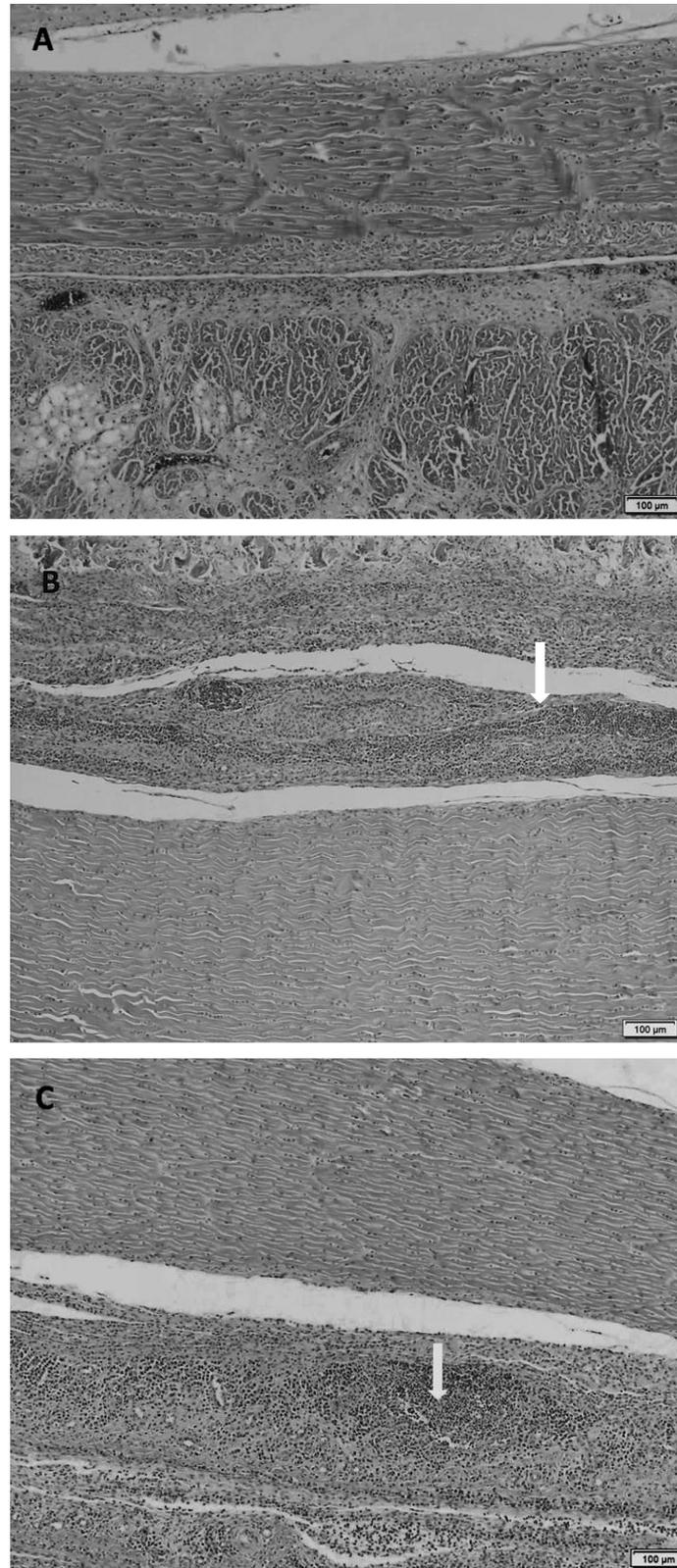


Figure 1. Tendons and tendon sheath at 3 weeks PI A) Non-infected control with no inflammation; B) Lymphocytic tenosynovitis (White arrow) induced by TARV-O'Neil which is as severe as lesions in C) CARV-infected gastrocnemius tendon and tendon sheath with lymphocytic tenosynovitis (white arrow).

infection was shown to be an early endpoint (indicator) for future clinical disease in turkeys (Sharafeldin et al., 2015). In chickens, oral and intratracheal TARV inoculation did not reach the early endpoint (histologic tenosynovitis) at 3 weeks PI while TARV administered via the footpad route reached the inflammation endpoint at two weeks PI. The findings in our study indicate that TARV is capable of infecting chickens via multiple routes, but the full consequences (e.g., tenosynovitis, lameness) of that infection have yet to be determined. Perhaps after 3 weeks PI, TARV-O'Neil footpad inoculation could have induced gross lesions and a clinical disease in chickens and reovirus-positive tissues in oral and intratracheal-challenged chicks may have progressed to documentable tenosynovitis and eventual lameness via a delayed-type immune response. However, this has not been determined under the parameters of this preliminary study. Further research is indicated to prove or disprove this point.

In conclusion, TARV-MN2 and TARV-O'Neil, 2 reoviruses that induce tenosynovitis and lameness in turkeys, could infect chickens via multiple routes and multiply in internal organs and tendons. Chicks inoculated at one week of age had reovirus detectable by rRT-PCR and virus isolation in tendon samples at 2 and 3 weeks PI. Only chickens inoculated with TARV-O'Neil by the footpad route showed gastrocnemius tenosynovitis as severe as those of CARV-inoculated by the footpad at 3 weeks PI. The possible natural routes (oral and intratracheal) are not producing a disease while experimental footpad route is. These findings indicate that TARV is infectious for chickens by various routes, but does not cause clinical signs in the short term. A planned long term (10 to 20 weeks) study will further define the progression of TARV infection in chickens.

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