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The σC Gene Characterization of Seven Turkey Arthritis Reovirus Field Isolates in Pennsylvania during 2011-2014

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Abstract

Seven turkey arthritis reovirus (TARV) field isolates made in our laboratory during 2011-2014 were characterized by sequencing of reovirus σC genes. Of the seven TARV field isolates, six were isolated from 3-to-17-week-old turkeys with tenosynovitis in six Pennsylvania farms and one was isolated from an Indiana turkey case submitted to our lab in 2014. This report describes the amplification and sequencing of the σC genes for genetic characterization studies on the seven TARV field isolates. Phylogenetic analysis of the sequence data of the seven TARV isolates together with recent published five TARVs detected in Minnesota (MN) and 25 avian reovirus (ARV) strains retrieved from GenBank revealed that all the seven PA TARV field isolates and five MN TARVs fit into genotyping cluster two when compared with a total of five different genotyping clusters (cluster 1-5) generated by the TARV and ARV reference strains. Comparison of amino acidsequences of the seven TARV isolates in cluster two with ARV vaccine strains (S1133, 1733, and 2048) in cluster one revealed that there were less than 60% similarity in nucleotide sequence and less than 56% in amino acid sequence between the two clusters. However the seven PA TARV isolates shared greater than 99% similarity with each other. Our research findings have indicated that the seven PA TARV field isolates and the five MN TARVs are grouped in the same genotype two, a separate genotype or virus species within the *Orthoreovirus* genus.

Abbreviations

aa: Amino acids; nt: Nucleotide; ARV: Avian Reoviruses; bp: Base Pair; CPE: Cytopathic Effects; LMH: Male-Chicken Hepatocellular-Carcinoma; ORF: Open Reading Frame; RT: Reverse Transcriptase; TARV: Turkey Arthritis Reovirus

Introduction

Avian reoviruses (ARV) belong to the genus *Orthoreovirus* in the family *Reoviridae*. They are non-enveloped viruses and contain a double-stranded RNA genome with ten segments. The viral genome is enclosed within a double protein capsid shell with a diameter of 70-80 nm [1,2]. Based on migration pattern on polyacrylamide gel electrophoresis, the ten genomic segments can be separated into three large segments (L1, L2, L3), three medium segments (M1, M2, M3), and four small segments (S1, S2, S3, S4) [3,4]. The segmented genome encodes for at least eight structural proteins (λA, λB, λC, μA, μB, σA, σB and σC) and four non-structural proteins (μNS, P10, P17 and σNS) [5].

The ARV σC protein is a minor outer-capsid protein and is encoded by the largest open reading frame (ORF) of the S1 genomic segment. Although the S1 genomic segment is relatively small protein of 327 aa, it plays an important role for virus attachment [6] and acts as apoptosis inducer [7]. Thenucleotide (nt) homology and aa homology of σC have been found suitable for comparison among different strains; firstly, the σC protein is the most variable protein in the ARV [8,9] for both very hypervariable aa regions one to 122 and 196 to 326 [10]; and secondly, it induces the production of neutralizing antibodies [11].

ARV strains have been associated with disease conditions such as viral arthritis/tenosynovitis [12] and can also cause damage to liver, heart and intestine [13]. All avian species of domestic poultry and wild birds are very susceptible to ARV infections, especially when they are young [14]. ARV infections have been reported in various avian species including chickens [15], geese [16,17], turkeys [18,19], ducks [20-22], pigeons [23], quail [24-26], and psittacine birds [27]. However, meat-type chickens have been shown to bemore susceptible to ARV infection than other avian species (De Gussem et al., 2010; Jones and El-Taher, 1985). ARV infections in turkeys are less well understoodwhen compared to ARV infections in chickens.

Turkey arthritis reovirus (TARV) infections were initially reported in 1980's in the United States [28,29]. Thereafter, no TARV case reports until the recent TARV out breaks occurred in commercial turkeys in the Midwest of the United States in 2009 and 2010 [30]. In Pennsylvania (PA), there are about four commercial companies producing several hundred flocks of turkeys, with average flock size around 12,000 birds per flock. TARV infections in turkeys were diagnosed the first time in PA in June 2011. Two commercial turkey flocks from one company were initially affected with severe lameness and swollen leg joins or entire legs. The causative agent of TARV was isolated from tendon and synovial tissues in the two affected turkey flocks, which represented the first confirmed TARV infections in the East of the United States. Similar TARV infections with various morbidities (30-40%) and mortalities (5-10%) were diagnosed continuously in PA turkey flocks till the present time. A total of 62 TARV field isolates were obtained during 2011 and 2014, and a turkey company had an estimated $3 million dollar of losses in 2014. In the present study, we report our research findings on

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genetic characterizations of seven TARV field isolates obtained from six PA turkey flocks/farms and one Indiana (IN) turkey flock during 2011-2014, and comparisons of the σC gene nt sequences of the seven TARV field isolates with other reported TARVs and ARV reference strains.

Materials and Methods

TARV field isolates

A total of seven TARVs isolated from clinical cases in our laboratory during 2011 to 2014 were selected for genetic characterization studies. Of the seven TARV isolates, six were isolated from six different turkey farms in Pennsylvania and one was isolated from samples submitted from an IN turkey flock. All seven turkey flocks had clinical signs of lameness and tenosynovitis (Table 1) and the TARVs were isolated from tendons. Virus isolation and propagation were conducted in Leghorn Male-chicken Hepatocellular-carcinoma (LMH) (CRL-2113, ATCC, Manassas, VA) cell cultures as per routine cell culture procedures [31]. The TRAV infected LMH cell cultures showed giant or bloom-like cytopathic effects (CPE). When 70-100% CPEs were observed, the LMH cell culture flask was frozen-thawed 2-3 times, and then the cell culture materials were transferred to a 15 ml sterile centrifuge tube for centrifugation at 2000 g for 10 min, and thereafter the supernatant was collected for this research study.

RNA extraction and RT-PCR

The RNA extraction was carried out with an RNeasy Mini Kit (Cat. No.74106, QIAGEN, Valencia, CA) following the manufacturer's instructions. Ten microliters of RNA solution were used for synthesis of viral cDNA using the One Step RT-PCR Kit (Cat. No. 210212, QIAGEN, Valencia, CA) with two degenerate primers P1 (5'-AGTATTTGTGAGTACGATTG-3') and P4 (5'-GGCGCCACACCTTAGGT-3') which corresponding to σC gene of ARV [32]. The RT-PCR reaction master mix per reaction consists of 25 μl of RNase-free water, 10 μl of 5× Buffer, 2 μl of dNTP mix (10 mM each dNTP), 1 μl Enzyme mix, and 1 μl of each of the two primers (20 pMol/μl), which brings a total volume of 40 μl of the RT-PCR master mix. The thermal cycling parameters are set as, one cycle for RT step at 50 °C for 30 min, initial PCR activation step at 95 °C for 15 min, and then followed by 38 cycles for PCR step at 94 °C for 30s for denaturation, 50 °C for 30s for annealing, 72 °C for 90s for extension, 72 °C for 5 min for final extension.

RT-PCR product purification and sequencing

RT-PCR products were isolated by 1% agarose gel electrophoresis and purified by gel extraction kit (Lot No. 04113KE1, Axygen, Tewksbury, MA) following the manufacturer's instructions. The DNA concentration of the purified PCR product was measured using a NanoDrop™1000 (Thermo Scientific, Waltham, MA) spectrophotometer and then submitted to Penn State Genomics Core Facility for Sanger sequencing.

Phylogenetic analysis

Phylogenetic analysis was performed on nt of the σC gene (981 bases) in S1 segment (525–1613, 1088 bases). Sequence data from all isolates were edited using DNAMAN software (version 7.212, Lynnon Corp., San Ramon, CA). Prediction of the ORFs and translation of ORF into aa sequences were also conducted by using the DNAMAN software. Sequence similarities of the seven TARV field isolates with other avian reovirus reference strains were analyzed by BLAST search in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and their sequences were aligned by using the ClustalW 1.83 program (http://align.genome.jp/). The aa and nt identities were obtained using ClustalW matrix as the comparison scoring tables for aa and nt comparisons, respectively. Phylogenetic trees were constructed using neighbor-joining method implemented in MEGA4.0 [33]. Bootstrap analysis was performed with 1000 pseudoreplicates.

Results

Sequence of σC protein alignment

Prediction and comparison of aa (1 to 286) sequences of σC segments from the seven PA TARV field isolates, five MN TARV strains, and standard ARV vaccine strains (Figure 1) revealed that aa residues are highly conserved between the seven PA TARVs and five MN TARVs (aa identity>98.6%). The TARVs and ARV vaccine strains shared two high genetic variability sites in the N-terminal portion (aa 41 to 120) and the C-terminus portions (aa 233 to 266). The C-terminal portions of σC monomers of the seven PA TARVs and five MN TARVs contained a number of universally conserved aromatic aa residues (aa 267 to 286). The single aa variation among PA TARVs and MN TARVs were found in residues, 13, 24, 26, 30, 33, 38, 77, 105, 137, 212, 238 and 239.

Comparison analyses of σC gene

BLAST results showed that all the seven PA TARV shared high sequences similarities with each other (nt: 98.8–99.8%; aa: 97.6-99.7%), but from 47.4% to 99.8% nt similarities with other reference ARV sequences published in GenBank. Pairwise comparison indicated that the nt sequences of the σC protein of the seven PA

Table 1: A list of the seven turkey reovirus field isolates and related case information.

Name of turkey reovirus field isolate	Date of case submission	Age of sick birds (weeks)	Clinical symptoms	Case Origin
Reo/PA/Turkey/12883/11	05/27/2011	9	Tenosynovitis	PA
Reo/PA/Turkey/13417/11	06/03/2011	17	Tenosynovitis	PA
Reo/PA/Turkey/27011/13	07/30/2013	15-17(Adult)	Tenosynovitis	PA
Reo/PA/Turkey/22342/13	10/01/2013	14	Tenosynovitis	PA
Reo/PA/Turkey/17010/13	11/27/2013	17	Tenosynovitis	PA
Reo/PA/Turkey/00659/14	01/10/2014	3-5(Juvenile)	Tenosynovitis	PA
Reo/PA/Turkey/01769/14	01/28/2014	19	Stunting Feed refusal	IN

Note: PA: Pennsylvania; IN: Indiana

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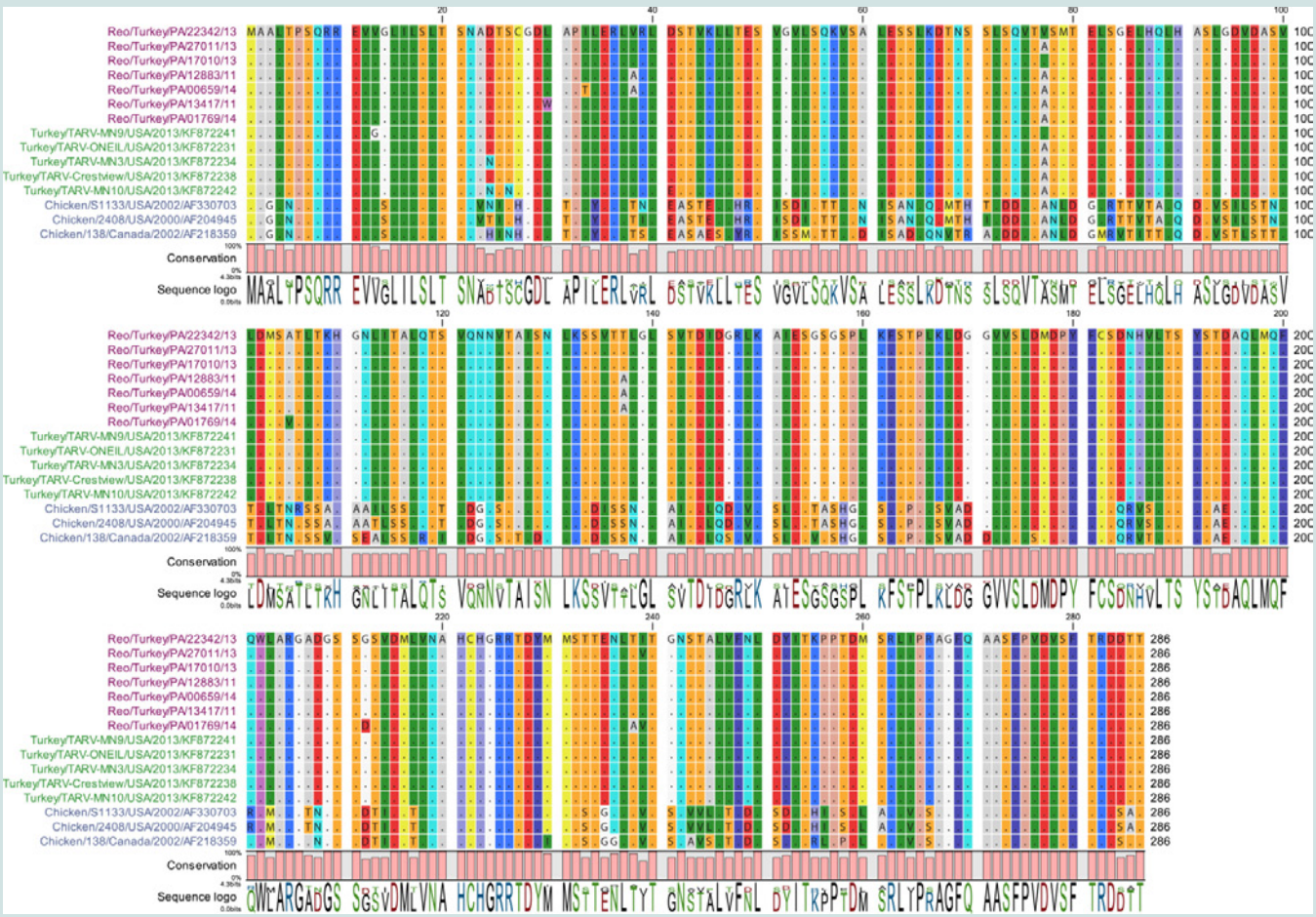

Figure 1: Amino acid alignment of σC protein (286 aa). Only residues differing from the consensus are shown. The last three sequences are the vaccine strains (S1133, 2408, 138).

Table 2: GenBank accession numbers of the 7 turkey arthritis reovirus (TARV) field isolates (#1-7) detected in Pennsylvania (PA), other TARVs (#9-12) and avian reovirus (ARV) reference strains (#13-37) used in this study.

Serial No.	Name of ARV or TARV strains	Avian species	Origin of country or region	Year	GenBank accession number
	PA TARV field isolates in this study				
1	Reo/PA/Turkey/12883/11	Turkey	PA, USA	2011	KM116023
2	Reo/PA/Turkey/13417/11	Turkey	PA, USA	2011	KM116022
3	Reo/PA/Turkey/27011/13	Turkey	PA, USA	2011	KM116019
4	Reo/PA/Turkey/22342/13	Turkey	PA, USA	2013	KM116020
5	Reo/PA/Turkey/17010/13	Turkey	PA, USA	2013	KM116021
6	Reo/PA/Turkey/00659/14	Turkey	IN, USA	2014	KM116024
7	Reo/PA/Turkey/01769/14	Turkey	PA, USA	2014	KM116025
8	MN3 (TARV)	Turkey	MN, USA	2011	KF872234
9	O'NEIL (TARV)	Turkey	MN, USA	2011	KF872231
10	Crestview (TARV)	Turkey	MN, USA	2011	KF872238
11	MN9 (TARV)	Turkey	MN, USA	2011	KF872241
12	MN10 (TARV)	Turkey	MN, USA	2011	KF872242
13	ISR5225	Chicken	Israel	2006	FJ793546
14	ISR5215	Chicken	Israel	2007	FJ793531

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15	ISR5226	Chicken	Israel	2007	FJ793547
16	ISR5220	Chicken	Israel	2007	FJ793532
17	ISR528	Chicken	Israel	2005	FJ793523
18	ISR5217	Chicken	Israel	2007	FJ793535
19	ISR5223	Chicken	Israel	2007	FJ793549
20	ISR525	Chicken	Israel	2005	FJ793539
21	GEL12 98M	Chicken	Germany	1998	AF354225
22	GEI10 97M	Chicken	Germany	1997	AF354219
23	GEL13A 98M	Chicken	Germany	1998	AF354226
24	GEL13B 98M	Chicken	Germany	1998	AF354227
25	NLI12 96M	Chicken	Netherland	1996	AF354230
26	601G	Chicken	Taiwan	1992	AF297217
27	R2-TW	Chicken	Taiwan	1992	AF297213
28	601SI	Chicken	Taiwan	1992	AF204947
29	916	Chicken	Taiwan	1992	AF297214
30	918	Chicken	Taiwan	1992	AF297215
31	1017-1	Chicken	Taiwan	1992	AF297216
32	JR1	Chicken	USA	2006	EF122836
33	2048	Chicken	USA	1983	AF204945
34	1733	Chicken	USA	1983	AF330703
35	S1133	Chicken	USA	1973	AF330703
36	AVS-B	Chicken	USA	2005	FR694197
37	42563-4/2005	Chicken	USA	2005	DQ872801

TARV isolates exhibited high identity with those of the MN TARV field strains (nt: 98.5–99.7%; aa: 98.3–99.7%) that were isolated in MN in 2011 [30]. When compared with ARV S1133, 1733, 2048, and AVS-B strains, the σC sequences of the seven PA TARV isolates shared low similarity to the ARV reference strains (nt, 55.0–59.9%; aa, 48.3–55.2%) (Table 3). Since the TRAVs are in genotyping cluster two, and the three vaccine stains of S1133, 2408 and 138 in genotyping cluster one are very similar (nt>95%) each other (Figure 1), thus we used two (S1133 and 2408) of the three vaccine strains in Table 3 and Figure 2 for aa comparisons.

Division of σC sequences

Construction of phylogenetic-tree analysis for conservation of the seven TARV σC sequences with other 30 ARV sequences retrieved from GenBank, resulted in five clusters based on their σC sequences (Figure 2), which showed more than 70% identity within each cluster. The ARV vaccine strains (S1133, 1733 and 2048) grouped into cluster onewere very diverse from all of the seven PA TARVs and 5 MN TARVs in cluster two (Figure 2). There are three chicken-origin ARV reference strains (916, ISR528, GEL13A 98M) were felled into the cluster two, only one (GEL13a98M) [32] showed a close relationship to TARVs but with more than 14% nt and 13% aa divergence.

GenBank accession numbers

Sequences of the seven TARV field isolates were submitted to GenBank and published recently, and their assigned GenBank accession numbers are: KM116023 for Reo/PA/Turkey/12883/11, KM116022 for Reo/PA/Turkey/13417/11, KM116019 for Reo/PA/Turkey/27011/13, KM116020 for Reo/PA/Turkey/22342/13, KM116021 for Reo/PA/Turkey/17010/13, KM116024 for Reo/PA/Turkey/00659/14, and KM116025 for Reo/Turkey/PA/01769/14 (Table 2).

Discussion

Research studies have suggested that the σC gene diverges more quickly than the other S-class genes due to the selection pressure placed on a cell-attachment protein [34]. ARV σC protein plays an important role in virus attachment to host cells and as apoptosis inducer. Exchange of σC protein gene segments would enable the virus to continue circulating [9]. In this study, the alignment of σC gene sequences showed that the seven TARV field strains were evolutionarily distant from the three traditional reference ARV strains of S1133, 1733 and 138 as they shared only about 61% nt identity and 54% aa identity. The seven TARV sequences examined in this study had high nt and aa identities not only with each other but also with the five MN turkey reovirus field strains isolated in 2011. This finding suggests that the recent PA TARV may have originated from the Midwest.

Among all 12 single aa variation sites of the seven PA and five MN TARVs, only four out of the 12 sites were displayed by two or multiple strains, but the other eight sites were limited to each site by a single strain, which suggests that the aa substitutions may be continuing in the σC gene of the re-emerging TARVs. Continued investigation may reveal more changes in the σC encoding gene that help understand the epidemiology of these infections. Traditionally, ARV strains

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Table 3: Comparison of nucleotide and deduced amino acid sequences of σC gene among the 7 turkey arthritis reovirus (TARV) isolates (#10-16, highlighted origin) detected in Pennsylvania (PA), 5 other TARVs (#5-9, highlighted pink) and 4 avian reovirus (ARV) (#1-4, highlighted blue) vaccine strains retrieved from GenBank.

		% Amino acid identity															
% Nucleotide identity		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	S1133 (ARV)	**100**	98.3	97.9	46.5	54.2	54.2	54.5	53.5	54.5	54.2	53.8	54.5	53.8	53.8	53.8	54.5
2	1733 (ARV)	99.1	**100**	99.7	47.2	54.9	54.9	54.9	54.2	54.9	54.9	54.5	55.2	54.5	54.5	54.5	55.2
3	2408 (ARV)	98.7	99.7	**100**	47.2	54.9	54.9	54.9	54.2	54.9	54.9	54.5	55.2	54.5	54.5	54.5	55.2
4	AVS-B (ARV)	53.3	53.5	53.7	**100**	48.3	48.3	48.3	47.9	49.0	48.3	47.9	48.6	48.3	48.3	48.3	48.6
5	O'NEIL (TARV)	59.3	59.5	59.4	55.3	**100**	100	99.7	99.3	99.0	99.3	99.3	99.7	99.7	99.7	99.0	98.6
6	Crestview (TARV)	59.3	59.5	59.4	55.3	100	**100**	99.7	99.3	99.0	99.3	99.3	99.7	99.7	99.7	99.0	98.6
7	MN3 (TARV)	59.4	59.7	59.5	55.3	99.5	99.5	**100**	99.0	99.3	99.0	99.0	99.3	99.3	99.3	98.6	98.3
8	MN9 (TARV)	59.2	59.4	59.3	54.9	98.7	98.7	99.0	**100**	98.3	98.3	98.6	99.0	99.7	99.7	98.3	97.9
9	MN10 (TARV)	59.7	59.9	59.8	55.3	98.8	98.8	99.3	99.2	**100**	98.3	98.3	98.6	98.6	98.6	97.9	97.6
10	Reo/PA/Turkey/12883/11	59.7	59.9	59.8	55.2	99.1	99.1	99.3	99.0	99.1	**100**	99.3	99.0	99.0	99.0	99.7	97.9
11	Reo/PA/Turkey/13417/11	59.3	59.5	59.4	55.0	99.2	99.2	99.4	99.1	99.2	99.7	**100**	99.0	99.0	99.0	99.0	97.9
12	Reo/PA/Turkey/27011/13	59.5	59.8	59.7	55.3	98.8	98.8	99.1	98.7	98.8	99.1	99.2	**100**	99.3	99.3	98.6	99.0
13	Reo/PA/Turkey/22342/13	59.5	59.8	59.7	55.1	99.1	99.1	99.3	99.7	99.5	99.3	99.4	99.1	**100**	100	98.6	98.3
14	Reo/PA/Turkey/17010/13	59.5	59.8	59.7	55.1	99.1	99.1	99.3	99.7	99.5	99.3	99.4	99.1	100	**100**	98.6	98.3
15	Reo/PA/Turkey/00659/14	59.4	59.7	59.5	55.1	99.1	99.1	99.3	99.0	99.1	99.8	99.7	99.1	99.3	99.3	**100**	97.6
16	Reo/PA/Turkey/01769/14	59.5	59.8	59.7	55.3	98.6	98.6	99.8	98.5	98.6	99.8	99.0	99.5	98.8	98.8	98.8	**100**

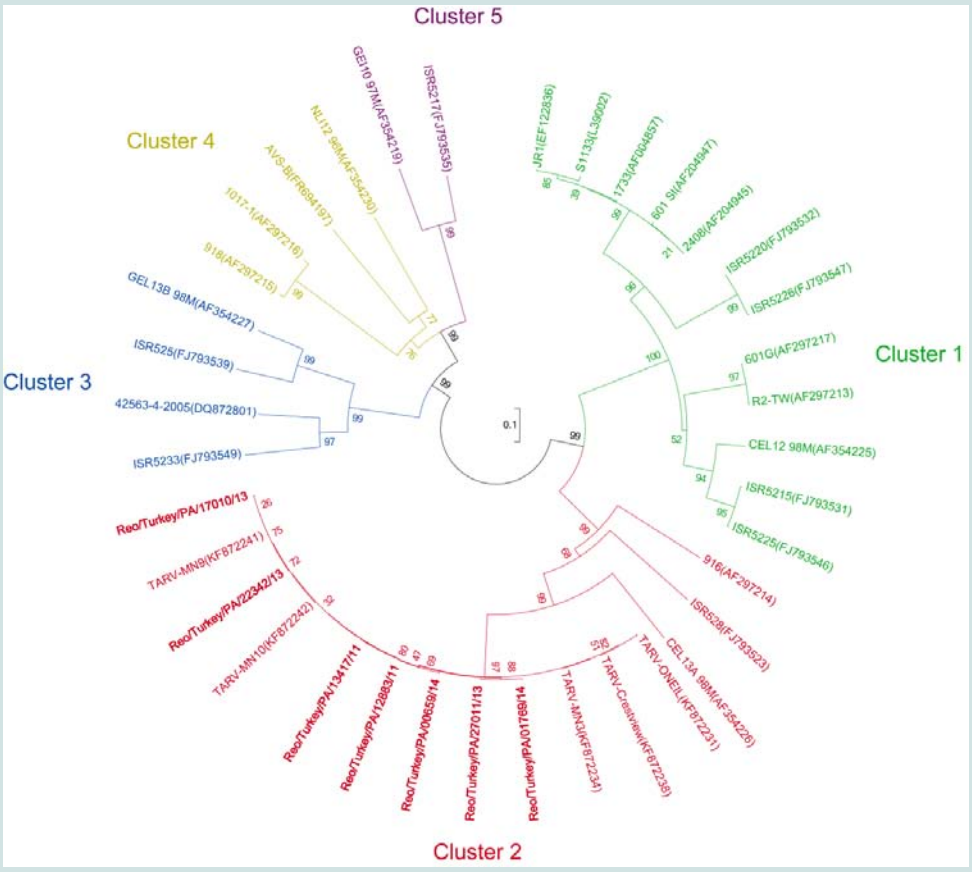

Figure 2: Phylogenetic tree showing the five clusters of ARV isolates. The analysis was based on the sequence of σC protein. Branch lengths are proportional to the evolutionary distances between sequences. The scale representing nt substitutions perposition is shown. The sequences were either retrieved from GenBank or sequenced in this study. The accession numbers are given in the "Material and methods" section.

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were classified by virus neutralization tests and the conventional RT-PCR in combination with sequencing or other molecular techniques [35,36]. The ARV σC protein is an outer-capsid, mediating virus attachment to cells, and containing antigenic epitopes that can induce neutralizing antibodies [6]. The σC gene displayed the highest level of sequence divergence and rapid evolution; therefore, the gene could be used as a genetic marker for rapid differentiation and classification of TARV field strains or isolates. By working on the full-length σC encoding gene of ARV, which is responsible for serotype specificity in ARV, it is possible to obtain a better correlation between genetic and serologic classification since no correlative relationship has been found between genotypes, serotypes, and pathotypes [32].

Phylogenetic analysis of the seven PA TARV sequences was compared with those of other 30 ARV strains or isolates documented in publications on the basis of the σC gene. The results revealed that the nt of the σC gene had variability, with a maximum divergence of 45% at nt level and 53% at deduced aa level. The σC gene of the seven PA TARV field strains and reference strains had formed five distinct genotyping clusters, while the other S-class genes were reported to have diverged into two or three distinct lineages [9]. The seven PA TARVs in this study and five MN TARVs were all categorized in one group at genotyping cluster two. Despite the close relationship with the five MN TARVs belonging to the same cluster; 916, ISR528, and GEL13A98M strains isolated from chickens with malabsorption syndrome were also in the same cluster. The GEL13A98M shared 76% nt similarity with turkey isolates of this study, which is in agreement with previous studies reporting 73.6%–83.1% nt similarity between enteric reoviruses of chickens and turkeys [37]. This finding suggests that enteric reovirus and tenosynovitis reoviruses in turkeys may have originated from a common ancestor. To better understand the relationship between these groups of reoviruses, full genomic sequencing of TARV along with pathogenicity studies should be conducted to obtain more detail information.

Further studies are needed to investigate relationships between genetic and serologic characteristics among TARVs in combination with pathogenicity studies to understand TARV transmission. This may lead to improved strategies for prevention and control of these pathogens.

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