

RNA Deep-Sequencing Analyses for Detection and Characterization of Avian Orthoreovirus and Fowl Adenovirus Co-Infections in Layer Chickens

Keywords: Avian orthoreovirus; Fowl adenovirus; Layer chicken; Co-infection; Genome; Next-generation sequencing (NGS)

Abstract

Avian orthoreovirus (ARV) and Fowl Adenovirus (FAdV) infections are pervasive in domestic poultry species, especially in chickens. Co-infections of the two viral pathogens could cause much severer symptoms on infected birds. In our recent research studies on application of Next-Generation Sequencing (NGS) techniques, we have identified two co-infection viruses of ARV (Reo/PA/Layer/27614/13 or Reo/PA27614) and FAdV (FAdV/PA/Layer/27614/13 or FAdV/PA27614) from one isolation from tendon tissue of 35-week-old commercial layer chickens. Among a total of 831,429 RNA-seq reads, 40,954 reads (4.92%) were confirmed to be ARV genome sequence, whereas an extremely small number of 566 reads (0.06%) were confirmed to be FAdV mRNA which transcribed by viral genome DNA. The de novo assembly of two types of viral reads generated 10 ARV contigs and 23 FAdV contigs, which according to 10 genome segments of ARV full genome and 14 mRNAs of partial FAdV transcriptome, respectively. Sequence comparison of nucleotide (nt) and amino acid (aa) sequences of Reo/PA27614 genome and FAdV/PA27614 hexon gene revealed that the Reo/PA27614 field variant had 40.0-94.1% nt and 27.4-98.8% aa identities in comparison with ARV reference strains, and the FAdV/PA27614 variant had 73.6-98.2% nt and 83.1-98.8% aa identities in comparison with FAdV reference strains. Genome alignment and phylogenetic analysis revealed that the Reo/PA27614 evolved distant from most ARV reference strains in three major outer capsid proteins, whereas the FAdV/PA27614 showed a close relationship with pathogenic reference strains of FAdV group C. Taken together, the NGS-based deep RNA sequencing techniques allowed us to identify the RNA virus and DNA virus co-infections at the same time and provided important epidemiological insights into ARV and FAdV co-infections in chickens.

Introduction

As a segmented double-stranded RNA (dsRNA) virus, avian orthoreovirus (ARV) is the important species in the Orthoreovirus, one of the 11 genera in the Reoviridae family [1-4]. The full genome of ARV is comprised by 10 dsRNA segments which are clustered into three major groups according to mobility in polyacrylamide gel electrophoresis, namely, large segments (L1, L2, and L3), medium segments (M1, M2, and M3), and small segments (S1, S2, S3, and S4) [5-7]. Each genome segment of ARV is not used directly for viral protein synthesis, but is transcribed to form functional mRNA which is identical to the positive strand of dsRNA [5]. The expression products of ARV mRNA are 8 structural proteins (λ A, λ B, λ C, μ A, μ B, σ A, σ B and σ C) and 4 nonstructural proteins (μ NS, p10, p17 and σ NS) [4]. Three of them are major outer capsid proteins (μ B, σ B and σ C) associated with host cell attachment and induction of virus-neutralizing



Journal of Veterinary Science & Medicine

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Submission: 19-October, 2019

Accepted: 15-November, 2019

Published: 18-November, 2019

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antibodies [9-12]. The transcription and translation ARV mRNA are occurred in the cytoplasm of infected cells and the mature virion is 70-80 nm in size without the lipid envelope [13]. ARVs are usually associated with a variety of clinical diseases in poultry but the viral arthritis/tenosynovitis, enteric disease, and immunosuppression have been considered as the primary [14-16].

Members of genus Adenoviridae are medium-sized (90-100 nm), non-enveloped viruses with an icosahedral nucleocapsid containing a double stranded DNA genomes, which belong to the Adenoviridae family [17,18]. Based on the isolated species and the serological differences, avian or fowl adenoviruses (FAdVs) are currently divided into three groups including conventional FAdV of group I; Haemorrhagic Enteritis Virus (HEV) and Avian Adenovirus Splenomegaly Virus (AASV) of group II; and Egg Drop Syndrome Virus (EDSV) of group III [19,20]. Although chickens are susceptible to all of the three group viruses, but group I FAdV infections occur most commonly in commercial chickens worldwide [21]. The group I FAdVs are sub typed into 12 serotypes in five different subgroups (A-E) [22]. Because of the great diversities among the 12 serotypes, different clinical symptoms and pathological lesions associated with FAdV infections are often observed, including Inclusion Body Hepatitis (IBH), hydropericardium disease, proventriculitis, tracheitis and pneumonias [21,23].

Experimental co-infections of ARV and FAdV were reported in specific-pathogen-free Leghorn chickens for evaluation studies of gastrointestinal and arthrotropic activity by these two pathogens [24,25]. However, there was no report for genomic characterization studies on the ARV and FAdV co-infections naturally occurred in field chickens. From 2011 to present, the newly emerging ARV variants have become a major problem in causing severe lameness and arthritis diseases in Pennsylvania (PA) poultry [26-28]. Additionally, as one of the most common avian viral disease pathogens, FAdVs were isolated periodically from our diagnostic broiler and layer cases which were clinically suspicious to ARV infections. Considering the highly contagious and pathogenic features of ARV and FAdV in poultry, their co-infections can cause much severer clinical diseases as our observations of clinical symptoms during ARV outbreaks occurred in

PA in recent years. However, simultaneous virus isolations for both ARV and FAdV in co-infections of field cases is not easy, traditionally or commonly, only one type virus (ARV or FAdV) can be isolated or detected, which could be due to the difference of nucleotide (nt) types and viral growth kinetics in cell cultures or chicken embryo [29,30].

By using the most advanced Next Generation Sequencing (NGS) technologies, it has become available to generate large amounts of sequence data of any virus genome sequences and thus to discover co-infections of RNA and DNA viruses by RNA deep-sequencing of the viral genome and transcriptome at the same time [31-33]. In the present study, we describe our NGS genomic characterization studies for detection of ARV and FAdV variant co-infections on one viral isolation made from tendon tissue of field layer chickens, which provide detail genomic data for the confirmation of naturally occurring co-infections of ARV and FAdV strains in layer chickens.

Materials and Methods

Virus and virus isolation

Isolations of various avian viruses from clinical specimens of diagnostic avian species are routinely conducted at our laboratory. The diagnostic isolation of ARV field variant strain (Reo/PA/Layer/27614/13, or Reo/PA27614) used in this study was isolated from tendon tissue of 35 weeks-old layer chickens from a flock experienced feather loss and egg production drop. The ARV isolation and identification tests were conducted per procedures described in our previous publications [26-28]. Briefly, 1) tendon tissue of the layer chickens showed symptoms of ARV infections was processed for virus isolation in LMH cell (CRL-2113, ATCC) cultures for 2-3 serial cell passages; 2) ARV-infected LMH cells, which were characterized by “bloom-like” giant Cytopathic Effect (CPE) cells, were harvested and prepared on a glass slide for ARV identification test; and 3) ARV positive isolates were confirmed by ARV Fluorescent Antibody (FA) (Ref No. 680, VDL 9501, NVSL, Ames, IA) staining the ARV-infected CPE cells.

RT-PCR and σC gene sequencing of ARV

Total RNA was extracted from the ARV isolate (Reo/PA27614) using an RNeasy Mini Kit (Cat. No. Z74106, QIAGEN, Valencia, CA, USA). The RT-PCR amplification of σC gene was carried out using P1 and P4 primers with a One Step RT-PCR Kit (Cat. No.210212, QIAGEN, Valencia, CA, USA) [34]. The RT-PCR products, obtained through 1% agarose gel electrophoresis, were purified using a gel extraction kit (Cat. No.04113KE1, Axygen, Tewksbury, MA, USA) per the manufacturer’s protocol and then were directly submitted to Penn State Genomics Core Facility at University Park campus for Sanger sequencing.

PCR amplification of FAdV hexon gene from the ARV isolate

Total DNA from the ARV isolate (Reo/PA27614) described above was extracted using a DNeasy Blood & Tissue Kit (Cat. No. 69504, QIAGEN, Valencia, CA, USA) according to manufacturer instruction. The PCR amplification of hexon gene was carried out using H1 and H2 primers with a Taq PCR Master Mix Kit (Cat. No. 201443, QIAGEN, Valencia, CA, USA) [35]. The PCR products were isolated through electrophoresis using 1% agarose gel with ethidium bromide and visualized under UV light, and then were purified and

submitted to Penn State Genomics Core Facility at University Park campus for Sanger sequencing.

Next-generation sequencing

RNA libraries were constructed from 1 μg of DNase-treated total RNA samples using the TruSeq Stranded Total RNA Sample Prep Kit (Cat. No. RS-122-2201, Illumina, San Diego, CA, USA) according the manufacturer’s protocol but without the initial poly-A enrichment step. Briefly, the total RNA was fragmented into small pieces using 5x fragmentation buffer under elevated temperature [36]. First strand cDNA was synthesized using random hexamer primer and SuperScript® II reverse transcriptase (Cat. No. 18064-014, Invitrogen, Grand Island, NY, USA). The second-strand cDNA was synthesized using RNase H (Cat. No. 18021-071, Invitrogen, Grand Island, NY, USA) and DNA polymerase I (Cat. No. M0209S, New England BioLabs, Ipswich, MA, USA). The double-stranded cDNA was purified by a QIAquick PCR extraction kit (Cat. No. 28104, Invitrogen, Grand Island, NY, USA), and end repair were performed before the ligation of sequencing adaptors. The library size and quality were checked by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The library product was directly sequenced via Illumina MiSeq using 150-nt single-read sequencing according to the manufacturer’s protocol.

De novo assembly of viral genome

De novo assembly and analyzing of NGS raw data were carried out by different modules in “NGS Core Tools” and “De Novo Sequencing” main tools of CLC Genomics Workbench V7.5.2 software (QIAGEN, Boston, MA, USA). Briefly, sequencing adaptors, reads mapping to chicken rRNA or mRNA reads, and low-quality reads were trimmed off by “Trim Sequences” module before further

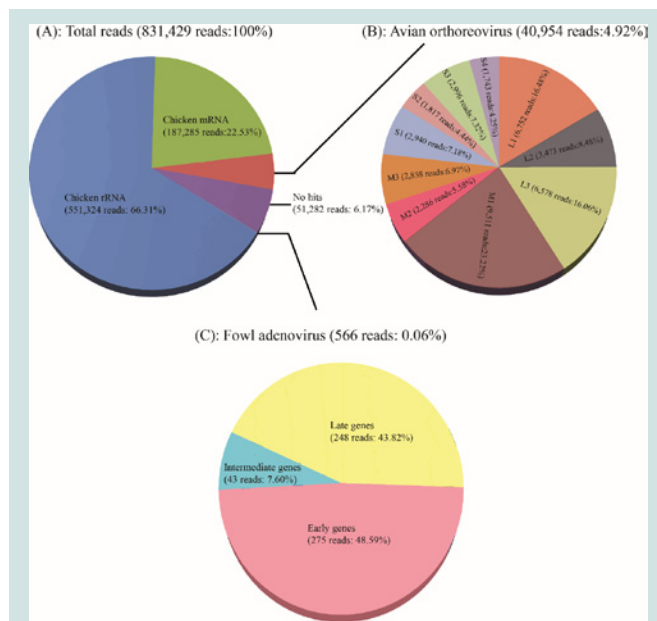


Figure 1: Illustrations of the homology search results for NGS reads and the sequencing coverage analysis. (A): Total NGS reads homology search result; (B): The mapping reads of 10 segments of Reo/PA/Layer/27614/13; (C): The mapping reads to the hexon gene of FAdV/PA/Layer/27614/13.

Table 1: De novo assembly and general genome features of the layer-origin avian orthoreovirus (ARV) strain (Reo/PA/Layer/27614/13).

Contig Length(bp)	Target Segment	Highest similarity ARV strain in GenBank	Identities (%)	Mapped reads	Average coverage	Encoded protein
3958	L1	AVS-B, lambda A gene (FR694191)	92.86	6752	213.15	λA(core shell)
3829	L2	AVS-B, lambda B gene (FR694192)	92.59	3473	114.40	λB(core RdRp)
3907	L3	Reo/PA/Broiler/15511/13, lambda C gene (KP731613)	94.29	6578	217.85	λC(core turret)
2283	M1	GuangxiR2, muA gene (KF741729)	95.83	9511	261.78	μA(core NTPase)
2158	M2	526, muB gene (KF741700)	95.83	2286	128.05	μB(outer shell)
1996	M3	Reo/PA/Broiler/05682/12, muNS gene (KM877330)	100.00	2858	178.51	μNS(NS factory)
1643	S1	526, p10, p17 and sigma C genes (KF741702)	91.38	2940	224.52	p10(NS FAST)
						p17(NS other)
						σC(outer fiber)
1324	S2	AVS-B, sigma A gene (FR694198)	93.55	1817	166.30	σA(core clamp)
1202	S3	526, sigma B gene (KF741704)	91.76	2996	309.14	σB(outer clamp)
1192	S4	AVS-B, sigma NS gene (FR694200)	94.26	1743	181.27	σNS(NS RNAb)

Table 2: De novo assembly of the layer-origin fowl adenovirus (FAdV) strains (FAdV/PA/Layer/27614/13) Complete genome of ARV and partial FAdV transcriptome.

Contig Length(bp)	Genes type	Highest similarity FAaV Strains from GenBank	Identities (%)	Mapped reads	Average coverage	Located gene
239	Early	C-2B (EF458162)	100	5	2.41	ORF43
289	Early	KR5 (HE608152)	98.61	5	2.04	DBP
275	Early	KR5 (HE608152)	98.53	5	2.25	DBP
485	Early	JSJ13 (KM096544)	100	8	2.04	100K
236	Early	JSJ13 (KM096544)	99.58	9	4.72	100K
386	Early	YN08 (KF234781)	100	8	2.57	100K
3514	Early	KR5 (HE608152)	100	216	7.39	pol
328	Early	MX-SHP95 (KP295475)	99.39	19	6.05	pVIII
228	Intermediate	ON1 (GU188428)	100	25	10.18	22K
246	Intermediate	MX-SHP95 (KP295475)	99.15	13	5.46	33K
208	Intermediate	MX-SHP95 (KP295475)	100	5	3.16	33K
313	Late	Krasnodar (KJ207053)	99.36	6	2.53	fiber-2
995	Late	SA 2 (M87008)	100	56	6.97	pVII/pX
409	Late	KR5 (HE608152)	100	13	3.53	protease
255	Late	MX-SHP95 (KP295475)	100	12	4.44	protease
569	Late	KR5 (HE608152)	98.59	22	4.23	hexon
1084	Late	C-2B (AF339923)	97.64	64	7.09	hexon
245	Late	MX-SHP95 (KP295475)	99.59	11	5.04	hexon
399	Late	FAV 4 (AY683545)	98.26	18	4.83	hexon
335	Late	MX-SHP95 (KP295475)	100	8	2.07	hexon
380	Late	MX-SHP95 (KP295475)	100	11	3.24	pIIIa
440	Late	FAV 4 (AJ554049)	100	18	4.82	pVI
317	Late	MX-SHP95 (KP295475)	100	9	2.92	pVI

processing. The clean reads were assessed through “De Novo Assembly” module to get assembled contiguous sequences (contigs). To identify the origin of the assembled contigs, the sequence of the contigs were extracted and submitted to “BLAST at NCBI” module. Based on the BLASTN searching results, all ARV-homologous and FAdV homologous contigs were selected as target sequences to build the full-genome of ARV and the transcriptome of FAdV. By re-mapping the NGS raw reads to the viral contigs of two viruses using

“Map Reads to Reference” module, the target contigs were further improved in length and sequencing coverage. Finally, the consensus sequences were obtained and considered as the final assembly of ARV genome and FAdV transcriptome.

Sequence analyses

To predict the viral Open Reading Frames (ORFs), align the homologous segments of genes, and identify the sequence similarities

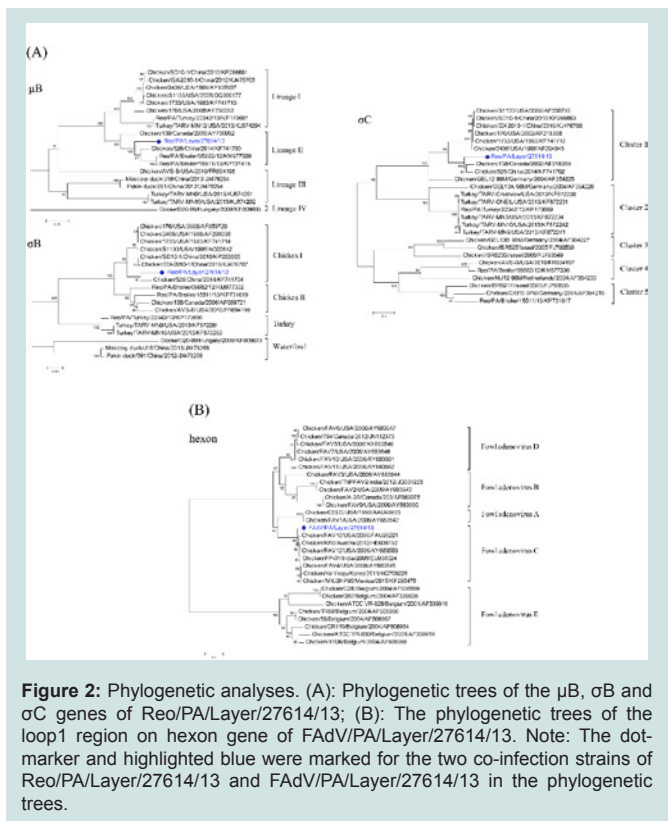


Figure 2: Phylogenetic analyses. (A): Phylogenetic trees of the μB, σB and σC genes of Reo/PA/Layer/27614/13; (B): The phylogenetic trees of the loop1 region on hexon gene of FAdV/PA/Layer/27614/13. Note: The dot-marker and highlighted blue were marked for the two co-infection strains of Reo/PA/Layer/27614/13 and FAdV/PA/Layer/27614/13 in the phylogenetic trees.

in pairwise, the tools of EditSeq and MegAlign of DNASTAR Lasergene 12 Core Suite (DNASTAR, Inc. Madison, WI, USA) were employed. The highest sequence identities searching in Genbank were carried out by BLASTN online program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequencing coverage and reads mapping of each assembled viral contigs were calculated by “Map Reads to Reference” module of CLC Genomic Workbench V7.5.2 software (QIAGEN, Boston, MA, USA) and visualized in pie chart using PowerPoint 2010 (Microsoft, Redmond, WA, USA). Phylogenetic trees of viral genes were estimated in MEGA 6 program using a neighbor-joining model and the bootstrap validation method with 1000 replications [37]. Visualized whole-genome or gene alignment results were generated by mVISTA online program (<http://genome.lbl.gov/vista/mvista/submit.shtml>) and the studied ARV genome and FAdV hexon gene were set as scales.

Genbank accession numbers

The ARV full genome sequence and FAdV hexon gene sequence obtained in this study have been deposited in the Genbank under the accession numbers of KU169288 - KU169297 and KT428298. The ARV reference strains Reo/PA/Broiler/05682/12 (or PA05682), Reo/PA/Broiler/15511/13 (or PA15511), S1133, AVS-B, 526, Reo/PA/Turkey/22342/13 (or PA22342) and J18 were listed in (Supplementary Table S1). The full-length of hexon gene sequences of FAdV reference strains, CELO, MX-SHP95, KR5, 764 and A-2A were listed in (Supplementary Table S2).

Results

RT-PCR, PCR and Sanger sequencing

The S1-based one-step RT-PCR using P1/P4 primers successfully amplified viral RNA of the ARV field variant strain (Reo/PA27614) at the 1088bp position. Sanger sequencing results of the ARV variant’s PCR product (KP727769) revealed about 91% nt identities with the most similarity ARV strain in GenBank (KF741702). Unfortunately, our attempt to obtain the FAdV hexon gene was not successful in amplifying the estimate 1219bp PCR product.

NGS raw data processing

After removing low-quality reads and trimming sequencing adapter through the Quality Control (QC) filters of the Illumina Miseq sequencer, a total of 831,429 sequencing reads were outputted in a 238Mb fast q format file. By using BLASTN searching, the reads mapped to the mRNA and rRNA of chicken or other origins were finally confirmed and considered as the contamination or non-target reads. As a result among the 831,429 reads, 551,324 reads (66.31%) were identified to be the chicken rRNA source and 187,285 reads (22.53%) to be the chicken mRNA source (Figure 1A). The remaining 92,792 reads (11.16%) were identified as the clean reads that consisted of ARV genome group (40,954 reads, 4.92%), no hits group (51,282 reads, 6.17%), and FAdV transcriptome group (566 reads, 0.06%) (Figure 1A).

De novo assembly

The total of 92,792 clean reads described above were subject to de novo assembly of viral contigs. After processing through the “De Novo Assembly” module of CLC Genomics Workbench software, a total of 131 contigs were generated with length from 50nt to 3958nt. The mapped reads of assembled contigs were calculated at various numbers (2 reads to 613 reads), which resulted the average coverage ranging from 2.04x to 309x. BLASTN online searching results revealed the existing ten ARV associated contigs with length from 1192nt to 3958nt and 23 FAdV associated contigs with length from 208nt to 3514nt among total assembled contigs (Table 1 and 2). After the most homology sequence searching in Genbank, ten ARV contigs showed high nt similarities (91.8%-100%) with the published strains and FAdV contigs showed higher nt similarities (97.64%-100%) with the published strains (Table 1 and 2). The initial alignment of ARV and FAdV contigs with the most homology reference sequences indicated that the size of the ARV contigs exactly matched the full-length of 10 ARV genome segments, respectively, whereas most FAdV contigs were only partial sequences of the target mRNAs.

By mapping back NGS raw reads to the 10 ARV contigs, the length and sequencing coverage of assembled contigs were further improved to yield the consensus sequences as final ARV genome segments. The mapped reads of each segment were summarized in (Table 1 and Figure 1B). The full-genome of this ARV variant, Reo/PA27614, was 23,495 bp in size contained 10 genome segments ranged from 1192 bp (S4) to 3958 bp (L1). Nine out of ten segments are monocistronic and only S1 segment is tricistronic. These segments encoded 12 viral proteins and the length of ORFs ranged from 297 bp (p10) to 3882 bp (λA), which were identical to published strains of these general ARV features. At the 5’ and 3’ termini of the each genome segment of the Reo/PA27614 variant strain located between 12bp to 98bp of the Untranslated Regions (UTRs). By aligning the 5’ UTRs and 3’ UTRs, respectively, the highly conserved terminal sequence was confirmed

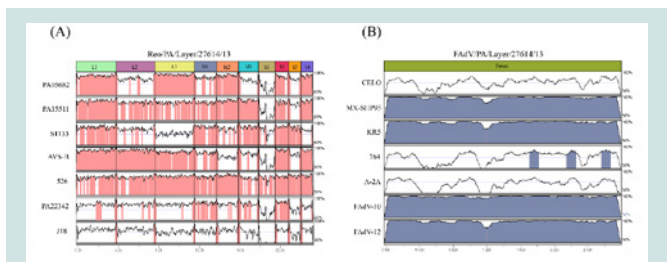


Figure 3: The mVISTA method for whole genome or gene nucleotide alignment. (A) Alignment result of the Reo/PA/Layer/27614/13 in comparisons with the Reo/PA/Broiler/05682/12 (PA05682), Reo/PA/Broiler/15511/13 (PA15511), S1133, AVS-B, 526, Reo/PA/Turkey/22342/13 (PA22342) and J18 strains was illustrated; (B) Alignment result of the FAdV/PA/Layer/27614/13 in comparisons with the 7 reference strains of the CELO, MX-SHP95, KR5, 764, A-2A, FAaV10 and FAaV12. Note: (1) Areas mapped in pink (A) and blue (B) represent $\geq 90\%$ similarities; (2) Areas unmapped in white represent $< 90\%$ similarities; (3) The scale bar measures approximate length of the concatenated genome.

at 5' UTR (5'-GCUUUU-3') and 3' UTR (5'-UCAUC-3'). These common genome features of the Reo/PA27614 variant strain were similar to other published ARV reference strains.

By using the same contig improvement method as ARV, the partial transcriptome and mapped reads of FAdV strain, FAdV/PA27614, were finally obtained (Table 2 and Figure 1C). The resulting FAdV/PA27614 mRNA clusters were divided into three main groups. Group 1 was early genes group included 8 contigs corresponding to 5 viral genes that were expressed immediately after the infection. Group

2 was intermediate gene group included only 3 contigs corresponding to 2 viral genes (22K and 33K) that located proximal to the terminus of the upper and lower strands of the genome. The late gene group (group 3) was the largest group included 12 contigs corresponding to 6 viral that mainly located in the central region of the genome. Five out of twelve contigs is targeted on the most important FAdV structural protein of hexon. By further aligning five hexon gene contigs to reference sequence (KP295475), the full hexon gene of the FAdV/PA27614 strain was successfully assembled at length of 2,814 bp.

Sequence comparisons

The nucleotide (nt) and amino acid (aa) of ten genes of Reo/PA27614 strain were compared in their homologs with seven ARV strains, including two PA broiler ARV strains (PA15511 and PA05682), three broiler ARV reference strains (S1133, AVS-B and 526), one PA turkey ARV strain (PA22342), and one duck ARV reference strain (J18) (Supplementary Table S1). The pairwise nt and aa comparisons between the Reo/PA27614 strain and chicken origin ARV strains revealed low to high similarities (nt: 53.7-94.1%; aa: 51.1-98.8%), which were higher than those of turkey origin strains PA22342 (nt: 59.0-89.0%; aa: 54.7-97.7%) and duck origin strains J18 (nt: 40.0-77.7%; aa: 27.4-94.7%). The highest identities for individual genes between the Reo/PA27614 strain and reference strains were showing heterogeneities (Table 3), meaning that the most homologous strain for each gene is different. In general, the AVS-B strain was considered as owning the largest number (total 4 genes) of highest identity (nt: 91.9-92.8%; aa: 98.3-98.9%) segments with Reo/PA27614 including

Table 3: Sequence identities of genome segments between the Reo/PA/Layer/27614/13 (Reo/PA27614) strain and orthoreoviruses.

Genes	PA15511		PA05682		S1133		AVS-B		526		PA22342		J18	
	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt
λA	91	98.5	90.9	97.8	88.2	97.5	91.9	98.8	87.9	98.2	83.2	96.3	77.4	94.7
λB	83	96.1	87.5	97.5	83.7	96.5	92.5	98.7	82.2	97.8	83.4	95.1	76.6	91.3
λC	94.1	97.7	87.6	94.9	72.7	84.5	88.4	95.3	90.4	96	83.5	90.5	70.6	79.6
μA	85.9	96.2	87.5	97.7	89.8	98	86.4	97.5	88.9	97.3	87.5	97.7	73.4	86.5
μB	91.2	97.5	91.4	97.6	84.1	94.1	76.1	88.6	92.6	96.5	83.6	93.5	76.5	89.5
μNS	80.5	91.7	92.5	96.1	89	95.6	85	93.2	86.5	94.8	85.5	92.3	71.3	79.7
σA	88.8	97.4	88.2	98.1	90.2	95.9	93.1	98.3	89.9	95.7	89	96.2	76.7	91.1
σB	83.1	93.8	82.8	92.9	88.4	95.1	82.2	94.3	91.1	95.9	70	75.5	64.3	67.4
σC	54.7	51.4	53.7	51.1	78.6	80.1	54.2	51.4	88.2	87.5	59	54.7	40	27.4
σNS	90.8	98.4	90.7	98.1	82.2	93.2	92.8	98.9	92.2	97.8	79.1	91.3	77.7	91

Table 4: Sequence identities of L1 loop of hexon gene between the FAdV/PA/Layer/27614/13 (FAdV/PA27614) strain and fowl adenoviruses.

% Amino acid identity		% Nucleotide identity												
		1	2	3	4	5	6	7	8	9	10	11	12	13
1	FAdV/PA27614	100	78.6	76.6	78.2	98.2	81.5	81.5	81.1	81.7	73.6	97.5	80.3	98.1
2	FAdV1(AAU46933)	86.6	100	75.7	77.8	78.6	80.8	80.7	80.8	80.8	74.3	80.1	80.1	78.5
3	FAdV2(JQ034223)	85.3	87.4	100	79.3	75.9	80.9	80.7	80.2	80.8	80.7	77.9	79.8	73.3
4	FAdV3(AY683544)	85.3	87.1	93.2	100	78	83	82.8	82.5	83.1	78.2	82.3	82.1	77.8
5	FAdV4(EU938324)	98.8	86.6	85.7	85.7	100	81.2	81	80.7	81.2	72.8	79.8	79.9	99.4
6	FAdV5(AY683546)	85.5	88.3	95.8	93.2	85.9	100	99.1	98	98.9	77.8	97.4	94.9	81.1
7	FAdV6(AY683547)	83.1	85.9	92.9	90.6	83.6	97.1	100	98	99.5	77.6	97.1	95.1	80.4
8	FAdV7(AY683548)	85	87.8	95.6	92.5	85.4	98.8	96	100	98.1	77.6	99.2	96.6	80.5
9	FAdV8(JN112373)	86.1	88.3	95.6	93.4	86.6	99.3	96.4	98.1	100	78	97.4	95	81.1
10	FAdV9(AY683550)	89.5	87.1	93.4	92.7	84.7	94.1	91.3	93.7	94.4	100	77.3	77.5	71.8
11	FAdV10(FAU26221)	97.3	84	82.9	82.9	96	83.1	80.8	82.7	83.8	81.9	100	96.1	79.9
12	FAdV11(AY683552)	84.3	87.1	94.2	91.5	84.7	97.4	95.1	98.4	97.2	92.7	81.9	100	79.5
13	FAdV12(AY683553)	93	86.6	85.7	85.7	99.5	85.9	83.6	85.5	86.7	84.7	96.4	84.8	100

the in λ A-, λ B-, σ A- and σ NS-encoding genes among all compared strains. For other six genes, the Reo/PA27614 strain showed highest identity with the 526 strain in μ B-, σ B- and σ C-encoding genes (nt: 88.2-92.6%; aa: 87.5-96.5%), the PA15511 in λ C-encoding gene (nt: 94.1%; aa: 97.7%), the PA05682 strain in μ NS-encoding gene (nt: 92.5%; aa: 96.1%), and the S1133 strain in μ A-encoding gene (nt: 89.8%; aa: 98.0%).

The pairwise nt and aa comparisons of the loop 1 region (residues 101 to 298) on hexon gene were carried out between the FAdV/PA27614 strain and 12 serotypes of FAdV reference strains (Table 3). Overall, the FAdV/PA27614 strain showed highest identity with the FAdV-4 in the homologous gene region (nt: 98.2%; aa: 98.8%) belonging to subgroup C of FAdVs. For the nt sequence comparison results between FAdV/PA27614 and other 11 FAdV serotypes, the relatively high identities were observed in FAdV10 and FAdV12 (nt: >97.5%), but lower in compared with FAdV1, FAdV2, FAdV3 and FAdV9 (nt: <81.7%). When compared sequence similarities in aa with non-FAdV4 strains, FAdV/PA27614 showed high similarities with FAdV9, FAdV10 and FAdV12 (aa: >89.5%) which belonged to the same group as FAdV4 (subgroup C). Interestingly, as the typically representative serotype of FAdV subgroup A, FAdV1 also showed relatively high identity with FAdV/PA27614 (aa: 86.6%), indicating the next closest relationship of the studied strain to subgroup A of FAdVs. However, the other 7 serotypes of FAdVs form subgroup B, D and E showed lower identities with FAdV/PA27614 (aa: <86.1%) and the lowest identity (aa: 83.1%) were found between FAdV/PA27614 and FAdV6, which belonged to the subgroup D of FAdVs.

Phylogenetic analysis of the Reo/PA27614 and FAdV/PA27614

To study the evolutionary relationships of the Reo/PA27614 strain with other ARV reference strains, the nt sequence of three major outer capsid encoding genes proteins (μ B, σ B and σ C) were subjected to phylogenetic-tree analysis using rooted maximum likelihood method (Figure 2A, μ B). For μ B gene analysis, four genotyping lineages were formed by the Reo/PA27614 strain and reference strains and no specific host-associated relationships were identified between these lineages. The Reo/PA27614 strain together with two PA broiler ARV field strains (PA05682 and P15511) and one classic ARV strain 138, formed the lineage II group, and the studied strain showed closer relationship with two PA field strains than 138 strain. In contrast with μ B gene, the phylogenetic tree of σ B gene revealed four host-associated groups which formed by Reo/PA27614 and reference strains (Figure 2A, σ B). Although Reo/PA27614 strain was located at chicken I group with most classic ARV reference strains, but only showing distant relatedness. As the most diverse gene of ARV, σ C phylogenetic analysis using the Reo/PA27614 strain and reference strains generated five genotyping clusters which showing less than 70% nt identity between any two clusters (Figure 2A, σ C). The Reo/PA27614 was classified as a member of cluster 1 (PA01224a), exhibiting significant divergence with most included strains, even the reference strains in the same cluster, which confirmed the sequence comparison results as described above.

The evolutionary relationships between the FAdV/PA27614 strain and other FAdVs were shown in (Figure 2B). All analyzed FAdV strains were clustered into five major groups (A-E). Although the FAdV/PA27614 was clustered into the C group with the FAdV

reference strains isolated in different countries, it also closely related to two FAdV1 strains of A group which consistent with pairwise comparison results as described above.

The visualized genome or gene alignments

The mVISTA online program aligned whole genomes of Reo/PA27614 and reference ARV strains and visualized the sequence identities of individual genome segments between them (Figure 3A). The classic ARV reference strain 526 showed a continuous high genetic relatedness (nt: >90%) with Reo/PA27614 throughout whole genome. The highest related segments between the study strain and reference strains were found at L1, L2 segments of AVS-B and L3 segment of PA05682 with more than 95% nt identities in most regions of these segments. The turkey-origin PA22342 strain shared moderate sequence identities with Reo/PA27614 of the study strain throughout most whole genomes, only M1 and S2 segments showed higher similarity between them. The duck-origin J18 strain shared low genetic relatedness with Reo/PA27614 throughout whole genomes, and an even lower identity was observed in S1 segment (nt: <50%), only showing high identities in the 5' and 3' termini of each segment.

The visualized hexon gene alignments of FAdVs revealed wide-ranging genetic relatedness between FAdV/PA27614 strain and FAdV4 reference strains (MX-SHP95 and KR5) (Figure 3B). The FAdV10 and FAdV12 were also showed high identities with FAdV/PA27614 throughout the whole hexon gene and FAdV10 was consider as the closest strain to FAdV/PA27614 among all reference strains. The CELO strain shared moderate sequence identities with FAdV/PA27614, whereas the 764 and A-2A strains only showed shared low sequence identities with FAdV/PA27614, especially from 303nt-894nt which corresponding to region of L1 loop (nt: <50%).

Discussion

Many research studies have indicated that ARV-infections in poultry can cause various clinical symptoms [2,38], particularly severe viral arthritis or tenosynovitis, runting-stunting syndrome, enteric disease and malabsorption syndromes [2,32-34]. The newly emerged/emerging ARV field variant strains have been detected in various poultry species including broilers, broiler breeders, layers, turkeys, chukar partridges, guinea fowls, pheasants and quails in PA during the last several years, and severe viral arthritis or tenosynovitis are the most common symptoms seen in ARV-affected poultry [26-28,39-41].

In addition to ARV infections, FAdV is another ubiquitous pathogen in poultry farms and pathogenic FAdV strains may cause clinical diseases but their pathogenic roles were not well studied or remained questionable in the past [21]. As published studies indicated that only FAdV4 was confirmed as a causative agent of broiler disease called infectious hydropericardium, Angara disease or hepatitis and Hydropericardium Syndrome (HHS) [42]. The HHS affected broiler flocks were seen mainly at 3 to 5 weeks of age and the mortality rate could be up to 75%. Research findings showed that the precondition of immunosuppression in chickens could lead to an increased intensity and severity of HHS by synergistic effect under experimental conditions [43]. In recent years in China, FAdV4 and FAdV8 have been confirmed the severely pathogenic strains which caused significant losses in broiler chickens and ducks [44-46]. ARV

as an immunosuppressive agent, it could be accompanying initial infections or secondary infections during FAdV epidemic outbreaks. Thus in field conditions, ARVs can not only induce primary tenosynovitis in chickens, but also aggravate symptoms of FAdV-associated HHS.

Genomic characterization finding of the ARV and FAdV strains in one isolation described in the present study is the first report of these two viruses' co-infections naturally occurred and detected in commercial layer chickens, which provides scientific methodology and important epidemiological insights for detection of co-infections and genomic characterization of RNA and DNA viruses from virus isolations or clinically infected animals. This specific layer chicken isolate was one of more than 20 other layer and broiler ARV isolates we obtained from diseased flocks and selected for full genome sequencing characterization studies. By using pairwise nt and aa sequence comparisons, we found the AVS-B strain had the largest number of highest identity segment with the ARV variant Reo/PA27614 described in this study, and we also found that at least one highest identity segment existed in each of other reference ARV strains. Indeed, segments 3 was the most homologous segment numbers of the ARV 526 strain, indicating the AVS-B and 526 strains may mainly contribute to the origin of Reo/PA27614 variant by terming reassortment. Each of the PA broiler ARV field strains of PA05682 and PA15511 also shared most homology L3 and M3 segments with Reo/PA27614, respectively, indicating further reassortments may occur between the original reassortant strain and ARV field strains during infections in poultry.

Sequence homology and phylogenetic analysis of the major outer capsid proteins (μ B, σ B and σ C) of the newly isolated ARV revealed that these proteins were originated from ARV 526 strain. As the important structural proteins, μ B was involved in virus entry and transcriptase activation [47]; σ B was responsible for inducing group-specific neutralizing antibodies [48]; and σ C played an important role for virus attachment and acted as an apoptosis inducer [9,49]. Therefore, the Reo/PA27614 variant strain in present study may have the same serological and infection features with the ARV 526 strain. In addition, the mVISTA alignment results of ARVs also revealed that the ARV 526 strain shared continually high sequences of identities with Reo/PA27614 variant strain throughout the whole genome, whereas other ARV reference strains only shared high sequences of identities with the Reo/PA27614 variant strain in some non-continually segments. In this case, we can further speculate that there may be a series of reassortments and mutations on ARV 526 strain and lead to the generation or reassortments for the Reo/PA27614 variant strain, which was the major co-infection virus we described in this study.

Because the transcriptome of the FAdV/PA27614 strain was partially identified in the present study, the sequence comparison and phylogenetic analysis of FAdV/PA27614 were carried out using only hexon gene which fully obtained through the RNA-seq. The hexon protein of FAdV is the major capsid protein which contains type-, group-, and subgroup-specific antigenic determinants [21], thus most detection, differentiation, comparison and phylogenetic analysis of FAdVs were conducted based on the most variable region of loop 1 on hexon [50]. The comparison of aa and nt sequence of

the hexon loop 1 region revealed that the FAdV/PA27614 strain was belonging to FAdV4 serotypes and also shared high identities with FAdV9, FAdV10 and FAdV12 strains. Phylogenetic analysis indicated the FAdV/PA27614 together with the FAdV9, FAdV10 and FAdV12 reference strains were clustered into genotype C group. The members of this group also included most pathogenic strains of FAdV4 which isolated worldwide in recent years and some of them associated with HHS. The close relationship between FAdV/PA27614 and FAdV4 pathogenic strains was not only showed at loop 1 region, but also showed at full-length of hexon which confirmed by mVISA alignment. Base on the above sequence comparison and analysis, FAdV/PA27614 was likely to be a pathogenic strain which could cause the HHS in broiler chickens.

In this study, our routine virus isolation tests showed that the Reo/PA27614 variant caused the significant CPEs of cell fusion on LMH cells, whereas the formation of FAdV CPE was not observed or occurred in this case, which was possibly due to the very low population of FAdV/PA27614 in the sample and also the dominated fast growth of the ARV, thus PCR or traditional immunoassays failed in detection of FAdV/PA27614 in this co-infection case. Fortunately, the most advanced NGS technology for metagenomics studies provides a powerful tool for the conduct of a fast and high-throughput sequencing of genomes in a wide range of organisms from viruses to mammalian genomes [51,52]. By employing a deep RNA sequencing technique, we successfully identified ARV genome and FAdV transcriptome from a single isolate. The mapping reads of ARV genome is 40,954 (4.92% of total reads) which was much higher than that of FAdV transcriptome 566 (0.06% of total reads), indicating that there was a huge difference between the amount of ARV viral RNA and FAdV mRNA in the sequencing sample. Such difference may associated with the numbers of the viruses in the original tissue specimen or the viral characteristic of growth kinetics in LMH cell culture [53]. Although the transcriptome of the FAdV/PA27614 strain was partial, we made successful in assembling the full-length of the hexon gene and carrying out the sufficient sequence analyses for the characterization of the FAdV/PA27614 strain.

In summary, we obtained the detailed genomic information of naturally occurred co-infections of ARV and FAdV variant strains in one isolation from layer chickens using NGS deep-sequencing analyses, providing a research methodology for genomic characterizing the co-infections of RNA and DNA viruses. By using the comprehensive sequence analyses, we identified that the Reo/PA27614 variant strain was a reassortant virus with its genome segments from both historical ARV strains and the newly emerged ARV field variant strains; the FAdV/PA27614 strain was closely related with FAdV4 pathogenic strain and could be associated with HHS disease. The findings of this study indicate that one virus isolate could contain both detectable and undetectable viruses by traditional virus identification tests. Thus, genomic characterizations provide the most advanced technique in detecting all viruses by their genome sequences, which is particularly useful in correct selections of autogenous vaccine candidates from field virus isolations.

Supplementary Materials

Author Contributions: Project conductors: T.Y. and H.L.; whole viral genome sequencing and NGS data analysis: T.Y.; manuscript

draft: T.Y.; manuscript review and edit: H.L..

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Acknowledgment

The avian reovirus research projects were funded by The Pennsylvania Poultry Industry Broiler/Egg Check-Off Research Program in 2016/2018, and The Pennsylvania Soybean Board Research Program in 2018, Pennsylvania, USA.