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 73.4-98.2% 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 (μA, μB, μC, μA4, μB1, μC1, μBand μC) and 4 nonstructural proteins (μNS1, p10, p17 and eNS) [4]. Three of them are major outer capsid proteins (μB, μB1 and μC) associated with host cell attachment and induction of virus-neutralizing 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 aicosahedral 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 Splenomegalia 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), hyperoercardium 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 layer field 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 cDNA 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 cDNA 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.

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 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 layer field chickens, which provide detail genomic data for the confirmation of naturally occurring co-infections of ARV and FAdV strains in layer chickens.

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Figure 1: Illustrations of the homology search results for NGS reads and the sequencing coverage analysis. (A): Total NGS reads homology search result; (B): Avian orthoreovirus (49,594 reads 4.92%); (C): Fowl adenovirus (506 reads 0.00%)
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

**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)  |
|                  | 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  | 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(out outer shell) |
| 1996             | M3             | Reo/PA/Broiler/05862/12, muNS gene     | 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)   |
|                  |                |                                        |                |             |                 | μC(out outer shell) |
| 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(out outer clamp) |
| 1192             | S4             | AVS-B, sigma NS gene (FR694200)         | 94.26          | 1743        | 181.27          | σNS(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 FAdV 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          |
| 287              | Early      | JSJ13 (KM096544)                              | 99.58          | 9           | 4.72            | 100K         |
| 386              | Early      | YN08 (KF234781)                               | 99.58          | 9           | 4.72            | 100K         |
| 3514             | Early      | KR5 (HE608152)                                | 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)                  | 99.15          | 13          | 5.46            | 33K          |
| 246              | 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)                                | 99.58          | 9           | 4.72            | 100K         |
| 490              | Late       | KR5 (HE608152)                                | 99.58          | 9           | 4.72            | 100K         |
| 255              | Late       | MX-SHP95 (KP295475)                          | 99.58          | 9           | 4.72            | 100K         |
| 569              | Late       | KR5 (HE608152)                                | 99.58          | 9           | 4.72            | 100K         |
| 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)                          | 98.26          | 18          | 4.83            | hexon        |
| 380              | Late       | MX-SHP95 (KP295475)                          | 98.26          | 18          | 4.83            | hexon        |
| 440              | Late       | FAV 4 (AJ554049)                              | 98.26          | 18          | 4.83            | hexon        |
| 317              | Late       | MX-SHP95 (KP295475)                          | 98.26          | 18          | 4.83            | hexon        |
RT-PCR, PCR and Sanger sequencing

Results

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. These segments encoded 12 viral proteins and the length of ORFs ranged from 297 bp (p10) to 3882 bp (AA), 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.
Table 3: Sequence identities of genome segments between the Reo/PA/Layer/27614/13 (Reo/PA27614) strain and orthoreoviruses.

| Genes    | AA1551 | AA05682 | S1133 | AVS-B | 526 | PA22342 | J18 |
|----------|--------|---------|-------|-------|-----|---------|-----|
| 1A       | 91.8   | 98.4   | 79.3  | 97.8  | 98.3 | 97.5    | 91.0 |
| 2B       | 91.0   | 96.0   | 86.2  | 91.4  | 97.8 | 74.4    | 55.5 |
| 3C       | 85.7   | 87.5   | 75.0  | 93.1  | 94.9 | 95.9    | 94.7 |
| 4D       | 85.3   | 87.1   | 90.2  | 99.6  | 97.6 | 96.9    | 98.1 |
| 5E       | 85.0   | 86.5   | 91.4  | 94.2  | 97.8 | 98.3    | 97.1 |
| 6F       | 85.5   | 88.3   | 95.8  | 93.6  | 93.8 | 94.9    | 94.9 |
| 7G       | 83.1   | 85.9   | 92.9  | 90.5  | 87.7 | 97.1    | 95.6 |
| 8H       | 85.4   | 87.8   | 95.6  | 92.5  | 95.6 | 97.5    | 95.1 |
| 9I       | 85.4   | 87.8   | 95.6  | 92.5  | 95.6 | 97.5    | 95.1 |
| 10J      | 85.4   | 87.8   | 95.6  | 92.5  | 95.6 | 97.5    | 95.1 |
| 11K      | 85.4   | 87.8   | 95.6  | 92.5  | 95.6 | 97.5    | 95.1 |
| 12L      | 85.4   | 87.8   | 95.6  | 92.5  | 95.6 | 97.5    | 95.1 |

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 | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 |
|-----------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 1                     | 82.8 | 81.5 | 81.5 | 81.5 | 81.7 | 73.6 | 97.5 | 80.3 | 98.1 |
| 2                     | 86.6 | 80.7 | 84.0 | 87.0 | 80.8 | 80.8 | 80.8 | 74.0 | 80.1 | 80.1 | 78.5 |
| 3                     | 85.3 | 84.0 | 92.0 | 90.0 | 90.0 | 90.0 | 90.0 | 90.0 | 80.9 | 79.9 | 79.7 | 83.3 |
| 4                     | 85.3 | 84.0 | 92.0 | 90.0 | 90.0 | 90.0 | 90.0 | 90.0 | 80.9 | 79.9 | 79.7 | 83.3 |
| 5                     | 85.3 | 84.0 | 92.0 | 90.0 | 90.0 | 90.0 | 90.0 | 90.0 | 80.9 | 79.9 | 79.7 | 83.3 |
| 6                     | 85.3 | 84.0 | 92.0 | 90.0 | 90.0 | 90.0 | 90.0 | 90.0 | 80.9 | 79.9 | 79.7 | 83.3 |
| 7                     | 85.3 | 84.0 | 92.0 | 90.0 | 90.0 | 90.0 | 90.0 | 90.0 | 80.9 | 79.9 | 79.7 | 83.3 |
| 8                     | 85.3 | 84.0 | 92.0 | 90.0 | 90.0 | 90.0 | 90.0 | 90.0 | 80.9 | 79.9 | 79.7 | 83.3 |
| 9                     | 85.3 | 84.0 | 92.0 | 90.0 | 90.0 | 90.0 | 90.0 | 90.0 | 80.9 | 79.9 | 79.7 | 83.3 |

Figure 3: The mViSTAMethod 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/L13 (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, KRS, 764, A-2A, FAdV10 and FAdV12. Note: (1) Arg; mapped in pink (A) and blue (B) represent ≥ 90% similarities; (2) Areas unmapped in white represent < 90% similarities; (3) The scale bar measures approximate length of the concatenated genome.
FAdV strains were clustered into five major groups (A-E). Although exhibiting significant divergence with most included strains, even the less than 70% nt identity between any two clusters (Figure 2A, σC). reference strains generated five genotyping clusters which showing ARV, σC phylogenetic analysis using the Reo/PA27614 strain and but only showing distant relatedness. As the most diverse gene of located at chicken I group with most classic ARV reference strains, (Figure 2A, σB). Although Reo/PA27614 strain was four host-associated groups which formed by Reo/PA27614 and showed closer relationship with two PA field strains than 138 strain. ARV strain 138, formed the lineage II group, and the studied strain PA broiler ARV field strains (PA05682 and P15511) and one classic reference strains (Figure 2A, σB). For μB gene analysis, four genotyping to phylogenetic-tree analysis using rooted maximum likelihood method (Figure 2A, μB). For μ 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 gene, the phylogenetic tree of eB gene revealed four host-associated groups which formed by Reo/PA27614 and reference strains (Figure 2A, eB). 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, eC 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, eC). The Reo/PA27614 was classified as a member of cluster I (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.

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 (μB, eB and eC) were subjected to phylogenetic-tree analysis using rooted maximum likelihood method (Figure 2A, μB). For μ 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 gene, the phylogenetic tree of eB gene revealed four host-associated groups which formed by Reo/PA27614 and reference strains (Figure 2A, eB). 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, eC 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, eC). The Reo/PA27614 was classified as a member of cluster I (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 KRS5) (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 2-2A 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, peafowls 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 (μBoB and oC) of the newly isolated ARV revealed that these proteins were originated from ARV 526 strain. As the important structural proteins, μB was involved in virus entry and transcriptase activation [47]; oB was responsible for inducing group-specific neutralizing antibodies [48]; and oC 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 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 FAV4 pathogenic strains was not only showed at loop 1 region, but also showed at full-length of hexon which confirmed by mVISTA 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 ressortant 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...
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