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Original Article

Complete Genome Sequencing of an Embryonated Chicken Egg-Adapted *Duck atadenovirus A*

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Abstract

Egg drop syndrome (EDS) is prevalent in industrial poultry globally. This disease is caused by *Duck atadenovirus A* or EDS virus (EDSV), a member of the genus *Atadenovirus* under the family *Adenoviridae*. The disease is attributed to significant economic losses in the poultry industry worldwide due to a drop in egg production, reduction in egg quality, and failure to reach maximum egg production. Oil-adjuvant inactivated vaccines, which are widely used in the poultry industry, provide good protection for immunized chickens against EDS. This study aimed to genetically and phylogenetically analyze the full-length genome of an embryonated chicken egg-adapted EDSV strain 127. After extraction of viral DNA from the allantoic fluid, overlapping fragments of the viral genome sequence were generated by polymerase chain reaction (PCR) using 25 pairs of primers. Purified PCR products were subjected to complete genome sequencing by the next-generation sequencing (NGS) approach. The nucleotide homology observed between genomes of the studied strain and that of the original strain 127 (NC_001813) of laying chickens was 99.9%. Its genome was 33,213 bp in length, with a G + C content of 43.01%. A comparison of the genome sequence of the egg-adapted virus with strain 127 revealed only three non-synonymous single-nucleotide polymorphisms (SNPs) between these viral genome sequences. Two mutations of S320G and I62K out of these SNPs were found within the coding regions of fiber and hypothetical proteins which may play a role in the adaptation of EDSV in the embryonated chicken eggs. The full genome sequencing of EDSV using NGS techniques provides insights into the discovery of genetic variants. Moreover, the genome sequence information of the EDSV provides valuable data for vaccine development in near future.

Keywords: *Duck atadenovirus A*, Egg drop syndrome, Egg-adapted virus, Molecular characterization, Next generation sequencing

1. Introduction

After the first description of egg drop syndrome (EDS) in 1976, the causative agent has been isolated from chickens and ducks in many countries (1). In chicken flocks, EDS outbreaks could be categorized into classical, enzootic, and sporadic forms. The classical pattern results from infected breeders and vertical transmission to their progeny. The appearance of the enzootic outbreak in some geographic areas and the persistence of EDS virus (EDSV) in commercial egg-laying flocks could be mostly due to EDSV contamination in the egg-packing station. The third form (the sporadic outbreak) can arise from the spread of EDSV most particularly from aquatic wild birds to chickens through contaminated water by droppings. The first clinical sign includes loss of eggshell pigmentation followed by the production of thin-shelled, soft-shelled, or shell-less eggs. According to the antibody level, two conditions of reduced egg production occur in EDSV-infected flocks. A sudden

apparent fall in egg production near peak production is manifested in flocks without antibodies. In flocks with a detectable antibody in a small percentage of individuals, the symptoms include failure to achieve or hold the predicted egg production (2). Endemic EDS is mainly controlled by using inactivated oil-adjuvanted vaccines between 14 and 18 weeks of age (3).

According to the International Committee on Taxonomy of Viruses, the most recent classification of *Adenoviridae* includes *Atadenovirus*, *Aviadenovirus*, *Ichtadenovirus*, *Mastadenovirus*, *Siadenovirus*, and *Testadenovirus*. The causative agent of EDS belongs to a member of the genus *Atadenovirus* and is classified as *Duck atadenovirus A* or EDSV (4). The disease was initially reported in laying hens in the late 1970s and became often referred to as EDS76 (1, 5).

The EDSV possesses a genome size of approximately 33 kb and is composed of non-enveloped icosahedral viruses with a double-stranded DNA genome (6). Although all EDSVs belong to one serotype, four genotypes of EDSV have been recognized in different regions (Europe, United Kingdom, Australia, and India) until now based on restriction endonuclease analysis (7, 8). The icosahedral capsid is built up of 252 capsomers: 240 nonvertex capsomers (hexons) and 12 vertex capsomers (pentons). Pentons of EDSV virion carry a single fiber in contrast to aviadenoviruses which have two fibers in each vertex (6).

For the propagation of EDSV, cell systems derived from duck should be used. This virus is readily replicated in duck kidney, duck embryo liver, and duck embryo fibroblast cell cultures and grows to high titers in chick embryo liver cells and goose cell cultures. Embryonated duck or goose eggs are also recommended for virus isolation (9, 10). Despite the fact that previous studies showed no growth detection of EDSV in embryonated chicken eggs, we propagated EDSV strain 127 in high titers after seven serial passages in this laboratory host. In order to determine genetic divergence between egg-adapted EDSV and the reference strain (NC_001813), the studied virus was completely sequenced and compared with the parental and other representative viruses phylogenetically and molecularly.

2. Materials and Methods

2.1. Virus

Strain 127 of *Duck atadenovirus A* was obtained from the depository at the Department of Avian Disease Research and Diagnostics, Razi Vaccine and Serum Research Institute, Karaj, Iran. This virus was originally isolated from pooled nasal and pharyngeal mucosa of a broiler breeder in Northern Ireland in the autumn of 1976 (11). This virus was propagated in an allantoic sac of 10-day-old specific-pathogen-free embryonated chicken eggs (200 µl per embryo). The embryos were incubated at 37 °C and examined daily for their viability for seven days.

After harvesting the allantoic fluid aseptically from eggs, six blind passages were performed through the allantoic cavity into another fresh set of embryonated eggs until the high hemagglutination (HA) titer was observed. The HA assay was performed using 1% chicken erythrocytes for the titration of the EDSV. The allantoic fluid was harvested six days post-inoculation, centrifuged at 2500 rpm for 15 min, and stored at −70 °C for viral DNA extraction.

2.2. Viral DNA Extraction

The genomic viral DNA was extracted from the virus-positive allantoic fluid using the NucleoSpin Dx Virus kit (MACHEREY-NAGEL, Düren, Germany) according to the instructions of the manufacturer. Briefly, 150 µl of allantoic fluid was added to the 600 µL buffer RAV1 containing carrier RNA and proteinase K and incubated at 70 °C for 5 min. After incubation, 700 µl of ethanol was added to the lysate. Subsequently, the sample was washed following the user manual. Finally, viral nucleic acid was eluted with 50 µl of pre-heated buffer RE provided in the kit.

2.3. Egg Drop Syndrome Virus Confirmation

The allantoic fluid was confirmed to contain EDSV by primer described previously targeting the hexon gene (12). The polymerase chain reaction (PCR) was carried out in a 50 µl reaction volume containing 37 µl

of nuclease-free water, 5 µl of 10X Ampliqon Ammonium Buffer, 1 µl of 25mM $MgCl_2$, 2 µl 10 mM of dNTP mix, 1 µl of each primer (10 µM), 1 µl of 2.5 units/µl Ampliqon AccuPOL™ DNA Polymerase (AMPLIQON, Odense M, Denmark), and 2 µl of extracted viral DNA. The thermal cycling condition was initiated at 94 °C/3 min followed by 35 cycles of denaturation at 94 °C/15 s, annealing at 58 °C/30 s, and elongation at 72 °C/2 min. The PCR was terminated by a final extension of 72 °C/5 min.

The PCR products were purified using a High Pure PCR Product Purification kit (Roche Life Science, Mannheim, Germany) and cloned into the pJET1.2/blunt vector (CloneJET PCR Cloning kit, Thermo Fisher Scientific, MA, USA) following the recommendations of the manufacturer. Finally, the plasmid was extracted from the transformed *Escherichia coli* strain Top10 by a High Pure Plasmid Isolation kit (Roche Life Science, Mannheim, Germany). Confirmation of recombinant plasmids was performed by PCR screening with the aforesaid specific primers. It should be mentioned that sequencing was conducted by a DNA service company (Macrogen, Seoul, South Korea).

2.4. Complete Genome Sequencing

In total, 25 pairs of overlapping oligonucleotides were designed with the software Primer 3 (version 4) based on the complete sequence of strain 127 of EDSV (Accession number at GenBank: NC_001813). The sequence of primers and their expected PCR product size are presented in table 1. *In silico* analysis was performed using the National Center for Biotechnology Information (NCBI) Primer-Basic Local Alignment Search Tool (BLAST) module to verify the specificity of the primers.

Similar PCR amplification was conducted for all sets of primers in a 50 µl reaction volume. The reaction mixture contained 1.5 µl of genomic DNA, 0.4 µM of each primer, 0.5 mM $MgCl_2$, 400 µM of dNTP mix, 1× PCR buffer with 15 mM $MgCl_2$, and 2 U of Ampliqon AccuPOL™ DNA Polymerase (AMPLIQON, Odense M, Denmark). The PCR condition was one cycle of 94 °C for 2 min, 35 cycles at 94 °C for 30 s, 52 °C for 30 s, 72 °C for 4 min, and a final cycle at 72 °C for 7 min.

After the detection of PCR products by electrophoresis on a 1.0% (w/v) agarose gel, 4 µl of them were pooled and purified using a High Pure PCR Product Purification kit (Roche Life Science, Mannheim, Germany). The pooled PCR products were sequenced by Macrogen (Seoul, South Korea) with the HiSeq 4000 platform (Illumina, San Diego, CA, USA) using the TruSeq Nano DNA Library Prep kit (Illumina, San Diego, CA, USA) with a read length of 151 bp paired ends (Illumina). Sanger sequencing was used to confirm mutations in corresponding PCR-amplified segments detected by next-generation sequencing (NGS).

2.5. Quality Trimming, Assembly, and Single-Nucleotide Polymorphism Detection

The raw reads from Illumina HiSeq were evaluated using FastQC (version 0.11.9) to control the quality of raw data. Low-quality reads (Phred score <30) were removed from the total reads in FASTX-Toolkit (version 0.0.13) and identical reads were collapsed to obtain high-quality reads. A combination of reference-assisted (Burrows-Wheeler Aligner software, version 0.7.17) and *de novo* (SPAdes, version 3.15.4) assemblies was used to obtain a full-length genome sequence of EDSV similar to the reference genome.

2.6. Nucleotide Sequence Analysis

The complete genome of egg-adapted EDSV was subjected to BLAST analysis to retrieve the closest complete genomes of these viruses for phylogenetic analysis. Nucleotide and amino acid sequences were aligned using the ClustalW algorithm in the Molecular Evolutionary Genetics Analysis X (MEGA X) suit (13). A phylogenetic tree was generated using the neighbor-joining method in MEGA X with 1,000 bootstrap replications to check the robustness of the tree. Genome annotation was performed based on the reference genome of strain 127 using the Genome Annotation Transfer Utility software (14).

2.7. GenBank Accession Number

The complete genomic sequence of the egg-adapted EDSV in this study was deposited in GenBank under accession number OP712666 and raw FASTQ data are available in the NCBI Sequence Read Archive under project PRJNA861084.

Table 1. Primers used for amplification of whole genome sequencing of EDSV

Primer name	Primer Sequence	PCR Product (bp)
MB-Seg-1-F	5′-CTCATGTCATTAATAAGACCATGCAGA-3′	1970
MB-Seg-1-R	5′-TGATGGTCAAACTCGGTCCA-3′	
MB-Seg-2-F	5′-TTGTGTGATTCAGTTGCCGG-3′	1871
MB-Seg-2-R	5′-ACGGGCCTGTATGCATCATA-3′	
MB-Seg-3-F	5′-TGCGGGCATGGTCTACAATA-3′	1568
MB-Seg-3-R	5′-CTCAGATCGCCTCGTTTGTG-3′	
MB-Seg-4-F	5′-TTTGGCGCTAGGTACACAGA-3′	1687
MB-Seg-4-R	5′-CGTCTCTTAGAAACGCAGCC-3′	
MB-Seg-5-F	5′-TCACTTTGACGTCGGCAATG-3′	1643
MB-Seg-5-R	5′-CCACTCAACATGCAGACACC-3′	
MB-Seg-6-F	5′-TGCCGCAGATGTATTTGGTG-3′	1639
MB-Seg-6-R	5′-CAATCAGCCTCCATTCCGTG-3′	
MB-Seg-7-F	5′-TGATGCGCTAAGGAGGTAGG-3′	1529
MB-Seg-7-R	5′-CACCGCTCTCTACCATCCTT-3′	
MB-Seg-8-F	5′-TCGGTGCTCAGACGTTTACT-3′	1750
MB-Seg-8-R	5′-CACAGACGGAGCAACAGAAG-3′	
MB-Seg-9-F	5′-CAAGAGTCGCCATCTGATGC-3′	1739
MB-Seg-9-R	5′-TAGCTGTCCCAATCCACCAG-3′	
MB-Seg-10-F	5′-CTTGGCATTCGGAAGCGTAA-3′	1598
MB-Seg-10-R	5′-AAAACCTCCACGCATGTCAC-3′	
MB-Seg-11-F	5′-GTCAAATAGGAAACGGGGCC-3′	1509
MB-Seg-11-R	5′-GCTGTTTTGCCTCTGTGACA-3′	
MB-Seg-12-F	5′-AACTTCATCTGCGCATCGTC-3′	1584
MB-Seg-12-R	5′-TACGCAGCATCAATTCCAGC-3′	
MB-Seg-13-F	5′-CTTGAGAGCAGATGGAGCCT-3′	1619
MB-Seg-13-R	5′-GAACTGTGCGAGCGAAGAAA-3′	
MB-Seg-14-F	5′-AGTGTACTTGCGCTGGTAGT-3′	1782
MB-Seg-14-R	5′-TGCAAAGCTAAACACTCCGG-3′	
MB-Seg-15-F	5′-AATGGTTGTCGCCTTTTCCC-3′	1791
MB-Seg-15-R	5′-TTCCTTTTCCCGCCTGATCT-3′	
MB-Seg-16-F	5′-TGGGGCCATGTGTTACTTCT-3′	1583
MB-Seg-16-R	5′-TCCCACCCAGATTTACCCAC-3′	
MB-Seg-17-F	5′-AGACTATGGCGCGGTTATCA-3′	1798
MB-Seg-17-R	5′-GAATAGTCAACCCCTGCCCT-3′	
MB-Seg-18-F	5′-GAACCTGCCAAGCCGTTAAA-3′	1627
MB-Seg-18-R	5′-AGTGGACGCCTTGGTTATCA-3′	
MB-Seg-19-F	5′-CTTTCGAGACGGCCATCAAG-3′	1501
MB-Seg-19-R	5′-GGCGGGAAGTGAATGTGTTT-3′	
MB-Seg-20-F	5′-GCCGTATGAATTGCAGGAGG-3′	1508
MB-Seg-20-R	5′-GCTCCATCTACCTAGCCGAT-3′	
MB-Seg-21-F	5′-CCCTGCAAGCCACAAATGAT-3′	1773
MB-Seg-21-R	5′-TGAAAAGCGCGTCTCTCAAC-3′	
MB-Seg-22-F	5′-ACCTCCGGCTATTAACGCTT-3′	1502
MB-Seg-22-R	5′-GGTGATGCCCAGTTTCGATC-3′	
MB-Seg-23-F	5′-ACGGTTGGTGGTGTAGCTTA-3′	1537
MB-Seg-23-R	5′-CCTGTGACATTGAGCTGAGC-3′	
MB-Seg-24-F	5′-GCAGCATACAGACTTCACCG-3′	1547
MB-Seg-24-R	5′-AGAAATTGGAGGACGGCTCA-3′	
MB-Seg-25-F	5′-ATCCGCGTCACACCGTATAT-3′	702
MB-Seg-25-R	5′-TCCATGTCATTAATAAGACC-3′	

3. Results

3.1. Hemagglutination Assay

The HA activity test was used for the titration of EDSV in the allantoic fluid. After seven passages in embryonated chicken eggs, the highest virus yields were obtained with titers of 12 log10. This process was continued up to five subsequent passages in order to confirm that the virus titers did not show higher HA than the seventh passage.

3.2. Polymerase Chain Reaction Amplification Results of 25 Segments

The PCR protocols for 25 segments were optimized and all segments were identified. The agarose gel electrophoresis results for PCR-amplified 25 segments of studied EDSV are illustrated in figure 1. The amplicon sizes ranged from 702 to 1970 bp.

3.3. Quality Assessment of Raw Next-Generation Sequencing Reads

The raw NGS reads were assessed using FastQC software (version 0.11.9) to evaluate sequencing quality control (Table 2). In total, 3,518,854,774 bases from 23,303,674 reads were sequenced that had an average read length of 150 nucleotides. Moreover, 93.11% of nucleotide bases had a Phred score of 30 (Q30) in which the chances of a base being called incorrectly are 1 in 1,000. The depth of coverage using the Illumina technology was greater than 100,000 fold.

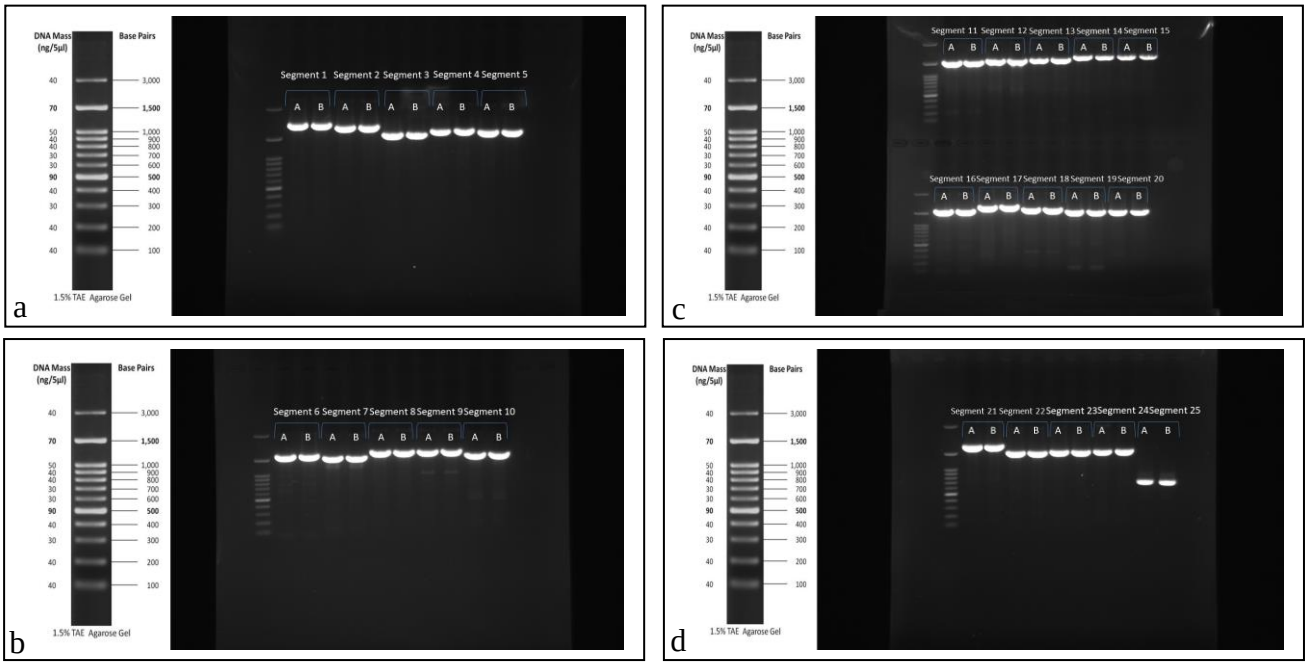

Figure 1. PCR amplification products corresponding to 25 segments of EDSV genome

Table 2. Sequencing statistics

Sample ID	Total read bases (bp)	Total Reads	Q30 (%)	Coverage
Egg-adapted EDSV strain	3,518,854,774	23,303,674	93.11	> 100,000 X

3.4. Homology Analysis and Phylogenetic Relationships

The BLAST search was performed to retrieve the complete genome of EDSVs. Since there were few complete genome sequences of this virus available in GenBank, comparative analysis of the nucleotide sequences was limited to eight reference and field strains (Table 3). The genome showed 99.9% identity to the strain 127 genome sequence. It is noteworthy that only about 2% divergence has been observed for this virus over the 50-year period of the first description of EDSV, compared to a recent isolate. In the phylogenetic tree, the available EDSV nucleotide sequences were grouped into three major clades suggesting that the studied EDSV and strain 127 form a distinct evolutionary group (Figure 2).

3.5. Molecular characterization

The genome length of egg-adapted EDSV was found to be 33,213 base pairs with a base composition of 27.20% A, 29.79% T, 20.68% C, and 22.33% G. The G + C content of the examined virus was 43.01% which is a hallmark of *Atadenovirus*. Based on genome annotation, the genome has the typical genetic structure of all EDSV strains with 29 open reading frames (Table 4). In comparison with typical EDSV, three single nucleotide polymorphisms (SNPs) were found in the complete genome of the studied strain. Furthermore, two SNPs were located in the protein-coding regions of the studied strain and the remaining one was in the non-coding region.

In the studied EDSV, adenosine at the nucleotide position of 23,642 had changed to guanosine, resulting in an amino acid change (serine to glycine) at residue 320 in the fiber. In addition, at the nucleotide position of 32,214, adenosine was changed to thymidine, causing an amino acid change (isoleucine to lysine) at amino acid residue 62 in the coding sequence of the hypothetical protein.

Table 3. Pairwise comparison of the egg-adapted EDSV strain with the other representative EDSV sequences

#	Strain	1	3	4	5	6	7	8	9	10
1	Egg-adapted EDSV strain		99.9	99.9	99.7	99.7	99.9	99.8	99.7	98.3
3	Strain 127	99.9		99.9	99.7	99.7	99.9	99.8	99.7	98.3
4	C10-GY-001/South Korea/2010	99.9	99.9		99.7	99.8	100	99.8	99.7	98.3
5	D11-JW-012/South Korea/2011	99.7	99.7	99.7		99.9	99.7	99.7	99.7	98.2
6	D11-JW-017/South Korea/2011	99.7	99.9	99.8	99.9		99.8	99.7	99.7	98.2
7	D11-JW-032/South Korea/2011	99.9	99.9	100	99.7	99.8		99.8	99.7	98.3
8	FJ12025/China/2012	99.8	99.8	99.8	99.7	99.7	99.8		99.7	98.2
9	JL-EDSV-629/China/2018	99.7	99.7	99.7	99.7	99.7	99.7	99.7		98.2
10	FMV-19-2234581/Canada/2019	98.3	98.3	98.3	98.2	98.2	98.3	98.2	98.2	

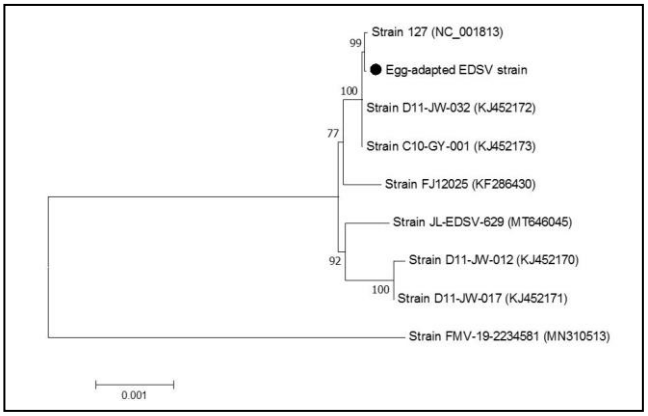

Figure 2. Phylogenetic relationship based on the complete genome of EDSV strains. The sample from this study is shown with black circle. The numbers indicate the bootstrap values calculated from 1000 replicates

Table 4. Genetic structure of egg-adapted EDSV strain compared to strain 127

Protein	Start	Stop	+/- strand	Coding sequences of 127 strain	Coding sequences of studied EDSV strain	Identity (%)
hypothetical protein	250	1110	-	861	861	100
E1B protein, small T-antigen	1174	1650	+	477	477	100
E1B protein, large T-antigen	1675	2856	+	1182	1182	100
IVa2 protein	2906	4111	-	1206	1206	100
DNA polymerase	3979	7218	-	3240	3240	100
terminal protein	7197	8942	-	1746	1746	100
52K protein	9034	10050	+	1017	1017	100
IIIa protein	10016	11743	+	1728	1728	100
penton protein	11773	13131	+	1359	1359	100
core protein 1	13181	13663	+	483	483	100
core protein 2 (mu protein)	13682	13885	+	204	204	100
pVI protein	13941	14633	+	693	693	100
hexon protein	14655	17387	+	2733	2733	100
endopeptidase	17384	17992	+	609	609	100
DNA-binding protein	18004	19167	-	1164	1164	100
100K protein	19216	21345	+	2130	2130	100
pVIII protein	21686	22438	+	753	753	100
fiber protein	22684	24618	+	1935	1935	99.8
E4 protein	24677	25564	-	888	888	100
hypothetical protein	25512	26240	-	729	729	100
hypothetical protein	27133	27636	-	504	504	100
hypothetical protein	27714	28226	-	513	513	100
hypothetical protein	28349	28807	+	459	459	100
hypothetical protein	28845	29159	+	315	315	100
hypothetical protein	29144	29521	+	378	378	100
hypothetical protein	29824	30402	+	579	579	100
hypothetical protein	30814	31854	-	1041	1041	100
hypothetical protein	31936	32397	-	462	462	99.3
hypothetical protein	32614	32985	-	372	372	100

4. Discussion

The EDS has been recognized widely all over the world since the first discovery of EDSV in the late 1970s. Inactivated oil-emulsion vaccines have proven to induce the highest levels of antibodies and effective protection against the disease (2, 15). Although cell cultures of duck and goose embryo origin are still the most appropriate propagation systems for EDSV, in an attempt to propagate EDSV in embryonated chicken eggs, seven serial passages were performed as an alternative production system.

Inoculation of strain 127 into the allantoic cavity did not result in embryo mortality after seven passages. In the present research, the highest EDS HA titer obtained from allantoic fluid was 12 log10. Seven consecutive passages may be required to achieve high titers of this virus in the embryonated chicken eggs. In this study, the complete genomic sequence of chicken embryo-adapted strain 127 was determined and analyzed genetically.

The full nucleotide sequence of the egg-adapted EDSV consists of 33,213 bp whose length is similar to that of the original version of the virus, strain 127. The studied virus shared a close phylogenetic relationship with its origin since they showed about 100% nucleotide sequence identity among themselves. A comparison of the full-length genome sequences revealed that the nucleotide identities ranged from 98.3% to 99.9% between egg-adapted EDSV and other representatives. Phylogenetic analysis of the complete genome of EDSVs indicated that the egg-adapted strain

was closely related to EDSV strains, including strain 127 and Asian strains, especially two Korean field isolates, which most likely originated from the same ancestor.

Comparison to the composite 127 genome sequence revealed that three SNPs were detected at the genomic level in the egg-adapted EDSV. The two SNPs, located in coding regions of the fiber and hypothetical protein, were confirmed using Sanger sequencing. These two SNPs lead to S320G and I62K mutations in the fiber and hypothetical proteins of the egg-adapted virus, respectively; however, no such mutations were found in strain 127.

The hexon protein constitutes the main capsid component on which serotype-specific epitopes are located (6). The hexon protein structure contains two functional components, including pedestal regions and loops. Variable loops 1-4 project outward from the surface of the virus and contain hypervariable regions of the hexon gene (16). The pentons hold a morphologically prominent position at the vertex capsomer in the adenovirus particle (2).

In *Duck atadenovirus A*, there is only a single fiber projecting from each penton base whereas aviadenoviruses have two fibers. The penton base proteins also bind to cellular receptors after initial attachment by the fiber protein (2, 17). The hexon and penton genes have shown complete amino acid conservation across strain 127 and egg-adapted virus. The fiber protein is composed of 644 amino acid residues with a molecular weight of 68 kDa (6). The tropism of adenoviruses depends on the fiber protein as specific attachment is achieved through interactions of host-cell surface receptors and adenoviral fiber knob. Since the main function of fiber protein is to mediate the recognition and attachment of the EDSV to the host cells, this protein is the major contributor to the spread of EDS from ducks to chickens.

The trimeric fiber protein of EDSV can be divided into the following three domains: i) a short N-terminal tail that interacts with the penton base protein of the capsid and contains potent nuclear localization signal, ii) a shaft consisting of repeating sequence motifs, and iii) a C-terminal knob or head that is responsible for cell attachment (18). Two non-synonymous mutations were identified in the coding sequences of fiber and hypothetical genes. Mutations in EDSV fiber protein may result in the adaptation of this virus in embryonated chicken eggs. The other 27 protein sequences did not exhibit any mutations, compared to strain 127.

It is known that fiber protein determines viral tropism to a large extent through interaction with specific host cellular receptors. However, the mechanism by which a mutation in this protein affects the pathogenicity needs to be evaluated. Additionally, the effects of other mutations on the hypothetical protein in terms of adaptation and enhanced virulence of the egg-adapted virus are not known yet.

In our previous study, we developed a laboratory-scale production of Newcastle disease + infectious bronchitis + EDS multivalent vaccine based on V4, M41, and egg-adapted EDSV strains. This vaccine elicited a robust humoral immune response similar to the other commercial trivalent vaccines (19). Although serial passages in embryonated chicken eggs might lead to a point mutation in the fiber and hypothetical proteins, they did not affect inactivated EDS vaccine immunogenicity in poultry.

The results of this study provide a rationale for further investigation of these SNPs as potential causes of adaptation of EDSV in embryonated chicken eggs. The full genome sequencing of EDSV strains using NGS techniques provides insights into the sequence variations and genetic features in addition to the prediction of the functional impact of mutations.

Authors' Contribution

M. B. and M. BN. conceptualized and designed the study. M. B. and B. H. A. performed the experiment and carried out all the analyses. M. B. and F. S. constructed the phylogenetic trees. M. B. and F. S. interpreted the results and drafted the manuscript. M. B. supervised the study and reviewed and edited the

manuscript. All authors read and approved the final manuscript.

Ethics

This study was approved by the Ethics Committee of Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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