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Prevalence and molecular characteristics of fowl adenovirus serotype 4 in eastern Saudi Arabia

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Abstract: Fowl adenovirus serotype 4 (FAdV-4) is a new emerging viral disease of chickens worldwide. It causes inclusion body hepatitis and hepatitis-hydropericardium syndrome. Little is known about its prevalence in the Middle East. Here we report the prevalence of FAdV-4 in five chicken farms in eastern Saudi Arabia. High mortality rates were reported from birds from those five farms at 15 weeks of age. Gross examination revealed typical hydropericardium syndrome and accumulation of jelly-like materials in the pericardial cavities. We isolated FAdV-4 by using embryonated chicken egg inoculation. The inoculated embryos showed dwarfing, deformities, hemorrhage, and death after 3–5 days of inoculations. Detection of FAdV-4 in the heart and liver tissues was achieved by polymerase chain reaction (PCR) and real-time PCR using the primers targeted to the partial hexon gene. Further confirmation was done by restriction fragment length polymorphism and the digestion patterns of the isolated FAdV-4 DNAs were close to those of other known FAdV-4 strains. The average genome size of the virus was ~43 kb. Phylogenetic analysis of the partial hexon gene sequences confirmed that these strains were closely related to other Asian strains from Kuwait, India, and Pakistan reported to GenBank. To our knowledge, this is the first study that reports the isolation and molecular characterization of FAdV-4 in chickens in Saudi Arabia.

Key words: Fowl adenovirus, hydropericardium, hexon gene, polymerase chain reaction, restriction enzyme digestion, phylogenetic analysis

1. Introduction

Fowl adenoviruses are a diverse group of viruses affecting chickens. They belong to the genus *Aviadenovirus* in the family Adenoviridae (1). Currently, there are 12 known serotypes of fowl adenoviruses (2). Fowl adenovirus serotype 4 (FAdV-4) belongs to group I and is classified as a member of group C fowl adenoviruses according to restriction fragment length polymorphism (RFLP) (3). FAdV-4 is one of the newly emergent serotypes in many regions worldwide. It was reported in Angora Goth close to Karachi in Pakistan for the first time (4). Clinically, FAdV-4 causes several syndromes in the affected chicken populations such as inclusion body hepatitis and hepatitis-hydropericardium syndrome (5,6). The affected chickens show depression, ruffled feathers, and resting on the ground with their chest close to death (5). Necropsy examination reveals several changes in the liver, such as mottling and being large and pale. Sometimes ecchymotic hemorrhages and multifocal necrotic areas in the parenchyma of the liver are found (7). The clinical picture of FAdV-4-induced hydropericardium syndrome is characterized by the accumulation of jelly-like amber viscous fluid in the pericardial cavities of the affected birds. The infected hearts appear large, flappy, and rounded (7). It is not an easy task to diagnose FAdVs due to the existence of a large number of serotypes. This is in addition to the presence of a heterogeneous population of antibodies against different adenoviruses in the sera of apparently healthy birds. However, the combination of polymerase chain reaction (PCR) and RFLP patterns is one of the most recent trends for the identification of FAdV-4 (8). According to the International Committee of Taxonomy and Nomenclature of Viruses (ICTV), FAdVs were classified into 12 serotypes. Further classification based on RFLP digestion patterns classified them into five groups (A–E) (8,9). Several outbreaks were reported in many Asian countries such as Iraq, India, Japan, and many other countries (10–12). Despite the detection of FAdV-4 in Kuwait (10), nothing is known about its prevalence in the other Gulf countries, especially Saudi Arabia.

Several breeder farms reported high morbidity and mortality rates in chickens around 15 weeks old. Birds in these farms showed typical hydropericardium syndrome. Necropsy examination showed that the affected hearts were

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pale yellow and flappy, with rounded edges, and had a large amount of viscous yellow fluid in the pericardial cavities. The main goal of the current study was to test birds from these farms for the presence of FAdV-4. We isolated the circulating FAdV-4 strains and then we achieved further identification by PCR using the partial hexon gene.

2. Materials and methods

2.1. Sampling and collection of tissue specimens

Eighty-five birds were selected for the collection of tissue specimens from the FAdV-4-suspected chicken farms in the eastern region of Saudi Arabia. Several pieces of different body organs of the diseased chickens, especially the hearts and livers, were collected under complete aseptic conditions. These tissue specimens were transported to the laboratory on viral transport medium (Dulbecco modified Eagle's medium, penicillin, and streptomycin cocktail) in ice tanks. Processing of these specimens was done by preparing 10% tissue suspensions as previously described (6). The prepared tissue suspensions were stored at –80 °C for further use.

2.2. Statement of animal ethics

All animal experiments and sample collections were conducted as per King Abdul-Aziz City of Science and Technology Royal Decree No. M/59 (http://www.kfsh.med.sa/KFSH_WebSite/usersuploadedfiles%5CNCBE%20Regulations%20ENGLISH.pdf). This animal utilization protocol was amended by the King Faisal University Animal Ethics Committee and the National Committee of Bioethics (NCBE).

2.3. Isolation of FAdV-4

We used 9-day embryonated chicken eggs (ECEs) of the local breed to isolate FAdV-4. This procedure was done as previously described (12). Briefly, 100 µL of the prepared 10% tissue suspensions was inoculated into the chorioallantoic membrane of five ECEs per sample. Harvesting of the inoculated egg materials and examination of the embryos was done 5 days after inoculation. Sham inoculated eggs with phosphate buffer saline (PBS) were used in parallel as a negative control.

2.4. Extraction of the viral DNA

The total viral DNA was extracted from 255 suspected tissue specimens using DNA extraction kits (QIAamp DNA Mini Kit, QIAGEN, Hilden, Germany, Cat No./ID: 51306) as per the kit instructions. The DNA concentration was measured with a NanoDrop machine (Thermo Scientific NanoDrop 2000, Applied Biosystems).

2.5. Primers and probes

We used the partial hexon gene as a target for testing the presence of FAdV-4 in the tested tissues. The primers and probes used for amplification of the FAdV-4 hexon gene are listed in Table 1.

2.6. Real-time PCR

One set of degenerative primers (Table 1) was used to amplify the partial FAdV-4 hexon gene from the suspected tissues by real-time PCR as previously described (13). This technique was performed as previously described with some modification (13). Briefly, we used the Applied Biosystem 7500 thermal cycler machine to amplify the partial FAdV-4 hexon gene. A total reaction volume of 30 µL was prepared per sample. The reaction mixture included 2 µL of template DNA, 2 µL of each primer, 4 µL of dNTPs, 1.5 U of GoTaq DNA Flexi Polymerase (Promega, Cat No. M8295), 3 µL of $MgCl_2$, and water up to 30 µL. We used chicken GAPDH (Bio-Rad, qGgaCED0029996) as a positive control.

2.7. Polymerase chain reaction

The partial hexon gene of the FAdV-4 isolate was amplified by PCR as previously described (9) with minor modifications. The extracted viral DNAs were used as a template for these PCR detection reactions. The indicated primers in Table 1 were used to amplify the partial hexon gene. The amplification conditions were denaturation at 94 °C for 40 s, then annealing at 56 °C for 35 s and extension at 72 °C for 40 s with a final elongation step for 5 min. These cycles were repeated 35 times. The amplified PCR products were separated by 1% agarose gel electrophoresis and then viewed by UV light. Purification of the amplified PCR products was done with the QIAquick Gel extraction kit (QIAGEN, Cat No./ID: 28704) according to the instructions of the kits. The purified PCR products were stored at –20 °C for further use.

2.8. RFLP pattern

Several restriction endonuclease enzymes (*APaI*, *BamHI*, *CLaI*, *ECoRI*, *KpnI*, and *XbaI*) (New England Biolabs, Cat Nos (R0114S, R0136S, R0197S, R0101S, R0142S, and R0145T, respectively) were used for the digestion of the extracted FAdV-4 DNAs. The digestion was carried out as previously described with some modification (14). We repeated the process of digestion at least three times per enzyme to make sure the results were consistent. Briefly, we prepared reaction volumes of 100 µL including 1 g of FAdV-4 genome plus 2 U of each enzyme in a separate tube, then added the recommended buffer solution for each enzyme as per the company instructions. The digestion reactions were incubated overnight at 37 °C except for *APaI*, which was incubated at 25 °C. Ten microliters of each digestion mixture was loaded into 1% agarose gel. The obtained gels were visualized under the UV light of the agarose gel documentation system (Gel Doc XR, Bio-Rad).

2.9. FAdV-4 genome size determination

We used Image Lab software to estimate the genome size of FAdV-4 isolates. Briefly, this involved quantitative estimation of each generated band after the restriction enzyme digestion was done. The total size of the FAdV-

4 isolate was reported as the sum of all the separated fragments per enzyme (15).

2.10. DNA sequencing and sequencing analysis

Five of the purified partial hexon gene PCR products were subjected to sequencing using the original primers listed in Table 1. Sequencing was done at the King Saud University, Date Palm Research Laboratory, Riyadh, Saudi Arabia. The BLAST of the obtained sequences was checked by BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

2.11. Phylogenetic analysis

We developed the phylogram based on the obtained partial FAdV-4 hexon gene sequences. The GenBank accession numbers of the used sequences are as follows: FN869970.1, AY581275.1, AY581274.1 AY581298.1, AY581297.1, AY581297.1, AY581296.1, EU177544.1, AJ459805.1, AF508951.1, AF339917.1, AJ431719.1, AJ554049.1, AF154246.1, AF339920.1, AF508954.2, AF339921.1, AF508953.1, AF339923.1 , AF508955.1, and KC750800.1. Multiple alignment was performed with the MEGA 7 package by the neighbor-joining approach using 1000 bootstrap replicates as previously described (16).

3. Results

3.1. Clinical and pathological findings

The current study was mainly focused on five chicken farms in eastern Saudi Arabia. Birds in these farms showed high morbidity and mortality rates. The affected birds showed ruffled feathers, huddling together, and inappetence. Gross pathological examination during the necropsy inspection revealed enlarged and pale hearts; the bases of the hearts were rounded and the presence of jelly-like substances in the precordial sacs was noted (Figure 1). Nine-day-old ECEs were used to isolate the FAdV-4 strain. The inoculated embryos showed dwarfing, deformities, hemorrhage, and death after 4–5 days following inoculation (Figure 2).

Table 1. Oligonucleotides and probes used for amplification of the partial FAdV-4 hexon gene.

N	Primer name	Sequence 5'----------3'	Size, bp	Position	Ref.
1	FAdV-4-HF1	ATGGGGTCGACCTATTTCGAC	310	20659	This study*
2	FAdV-4-HR1	CGACCGAGCACGCCGGTGTTGG		20969	
3	FAdV-4-HF2	GCGCCBACYCGVAAYGTCA	191	166–184	(13)
4	FAdV-4-HR2	TTGAARGAVGGHCCBCKGTC		356–337	

*Primers designed based on fowl *Aviadenovirus* 4 strain HLJFAd15 and CELO genes for hexon proteins, complete genome.

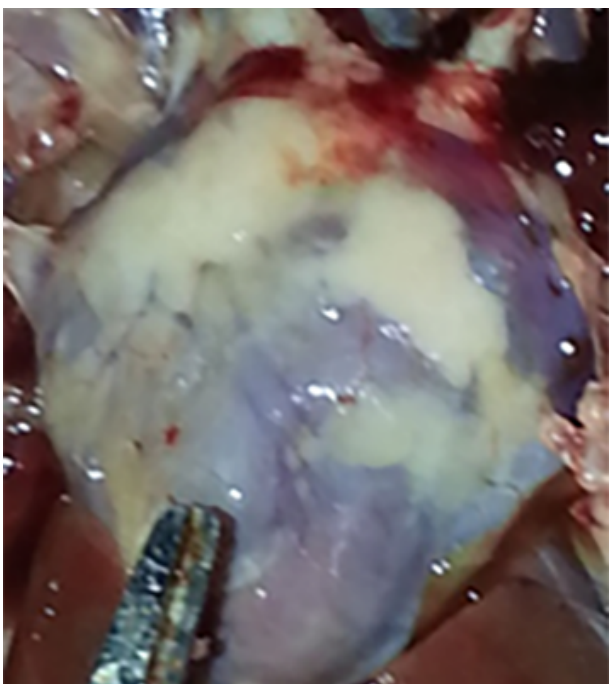

Figure 1. Gross pathological profile of birds infected with FAdV-4. Postmortem inspection of FAdV-4-infected bird. The affected heart is large, pale, with rounded apex and jelly-like fluid in the pericardial sac.

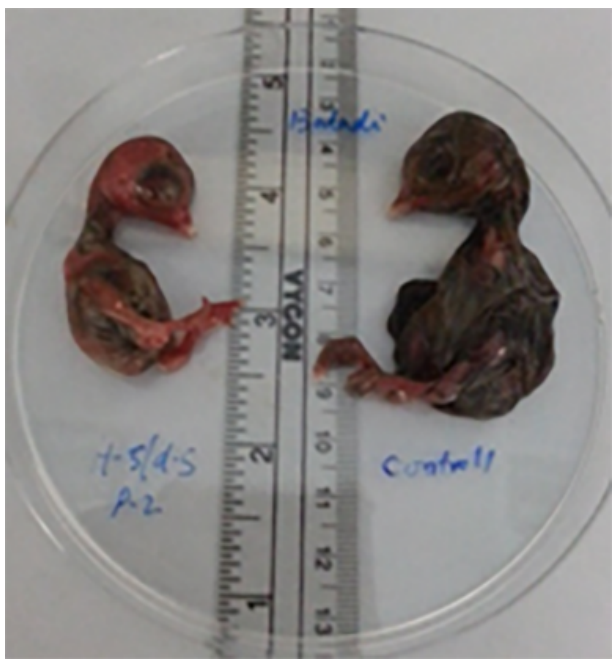

Figure 2. Isolation of FAdV-4 using embryonated chicken eggs. The infected embryo shows curling and dwarfing 4 days after inoculation in comparison to the sham (PBS) inoculated embryo.

3.2 Real-time PCR

The results of testing different organs of the suspected FAdV-4-infected birds (liver and heart) are listed in Table 2 according to real-time PCR. Briefly, 47 (55%) heart samples were found positive out of 85 tested samples. Meanwhile, 24 (28%) liver samples were positive by real-time PCR testing (Table 2).

We used the primers listed in Table 1 to amplify the partial hexon gene in selected real-time PCR-positive tissues (Figure 3). The highest rate of FAdV-4 was detected in the hearts. Fifty-five hearts out of the 85 tested were found positive by real-time PCR (Table 2; Figure 3). The Ct value of the positive control sample was about 15 while the test sample Ct values ranged from 22 to 35. Samples with Ct values above 35 were considered negative. The negative samples did not show any peaks of amplification (Figure 3; Table 2).

3.3. RFLP pattern

Digestion of the extracted FAdV-4-DNAs by different restriction endonucleases was done. Each enzyme digested the FAdV-4 genome into several fragments under gel electrophoresis (data not shown). Each enzyme resulted in a unique restriction pattern (Table 3). The restriction enzyme profiles of the digested FAdV-4 DNA are listed in

Table 2. Summary of the real-time PCR results of suspected organs.

Farm	Location	No. of tested birds	Heart		Liver	
			+	-	+	-
H1	Al-Ahsa	15	10	5	4	11
H2	Al-Ahsa	20	12	8	5	15
D1	Dammam	15	6	9	3	12
D2	Dammam	15	8	7	9	6
A1	Abqaiq	20	11	9	3	17
Total		85	47	38	24	61

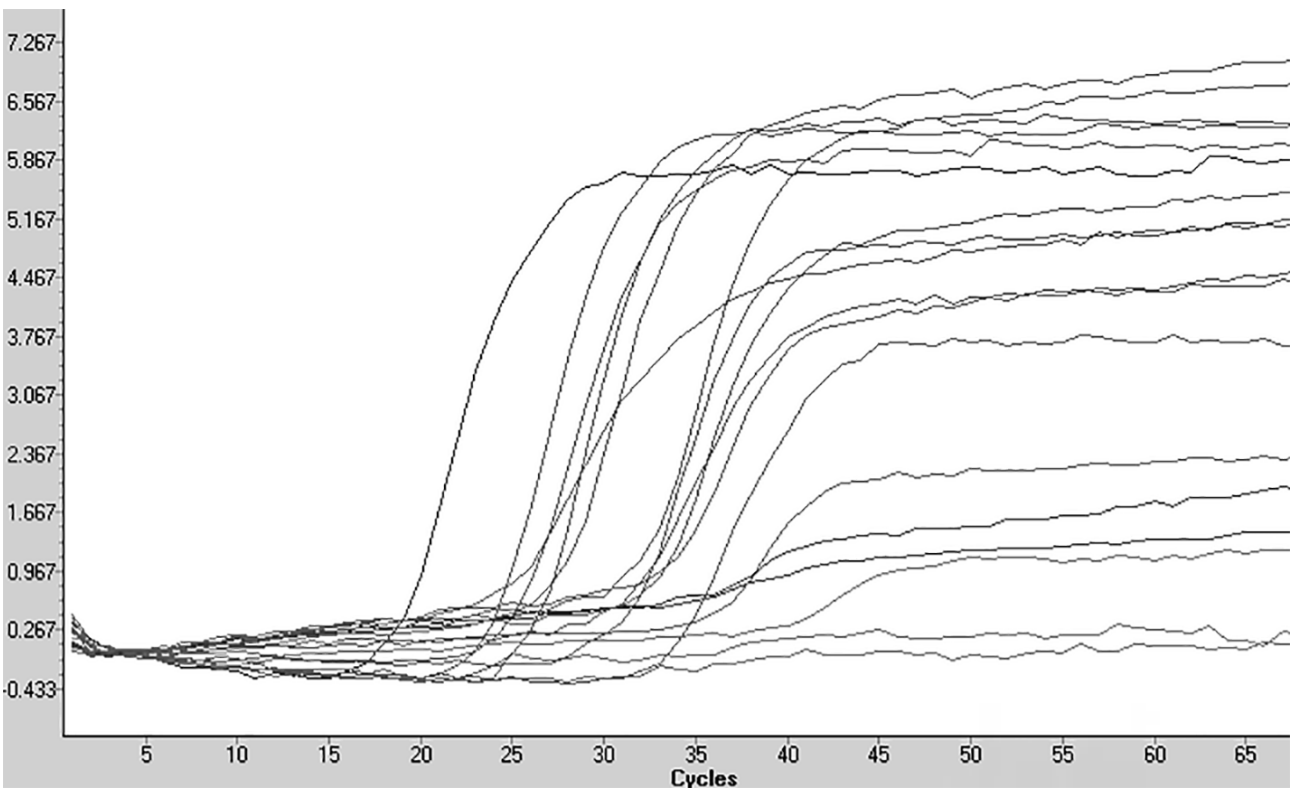

Figure 3. Real-time PCR amplification curves. Amplification curve of the amplified FAdV-4 hexon gene from the tested tissue specimens (heart and liver) of the infected chickens. The positive control (+Ve Cl) and the negative control (-Ve CL) are indicated by arrows.

Table 3. These restriction enzymes separated the FAdV-4 genome into 8, 6, 9, 6, 9, and 11 fragments in the case of *BamHI*, *ECoRI*, *APAI*, *XbaI*, *KPn1*, and *ClaI*, respectively (Table 3). The average FAdV-4 genome size was 43.5 kb, as shown in Table 3.

3.4. Sequencing and phylogenetic analysis

We amplified the partial hexon gene from the isolated FAdV-4 strains in eastern Saudi Arabia. We used the listed primers in Table 1 to amplify 310 nucleotides out of the FAdV-4 genome (Figure 4). Sequencing analysis of the obtained FAdV-4 partial hexon gene revealed that the isolated FAdV-4 strain (accession no: KY606586) shared high nucleotide similarity with the FAdV-4 serotype previously reported to GenBank. This FAdV-4 isolate was clustered together with other FAdV-4 strains reported from Kuwait, India, and Pakistan (Figure 5). It was also found to be closely related to other fowl adenoviruses such as FAdV-7 (accession no: AF508955.1), FAdV-9 (accession no: AF339923.1), and FAdV-10 (accession no: KC750800.1) (Figure 5).

Table 3. Summary of the RFLP of FAdV-4.

Restriction enzyme	*BamHI*	*ECoRI*	*APAI*	*XbaI*	*KpnI*	*ClaI*
No. of DNA fragments	8	6	9	6	9	11
Total size, bp	43.496	43.890	43.720	43.839	43.788	43.890

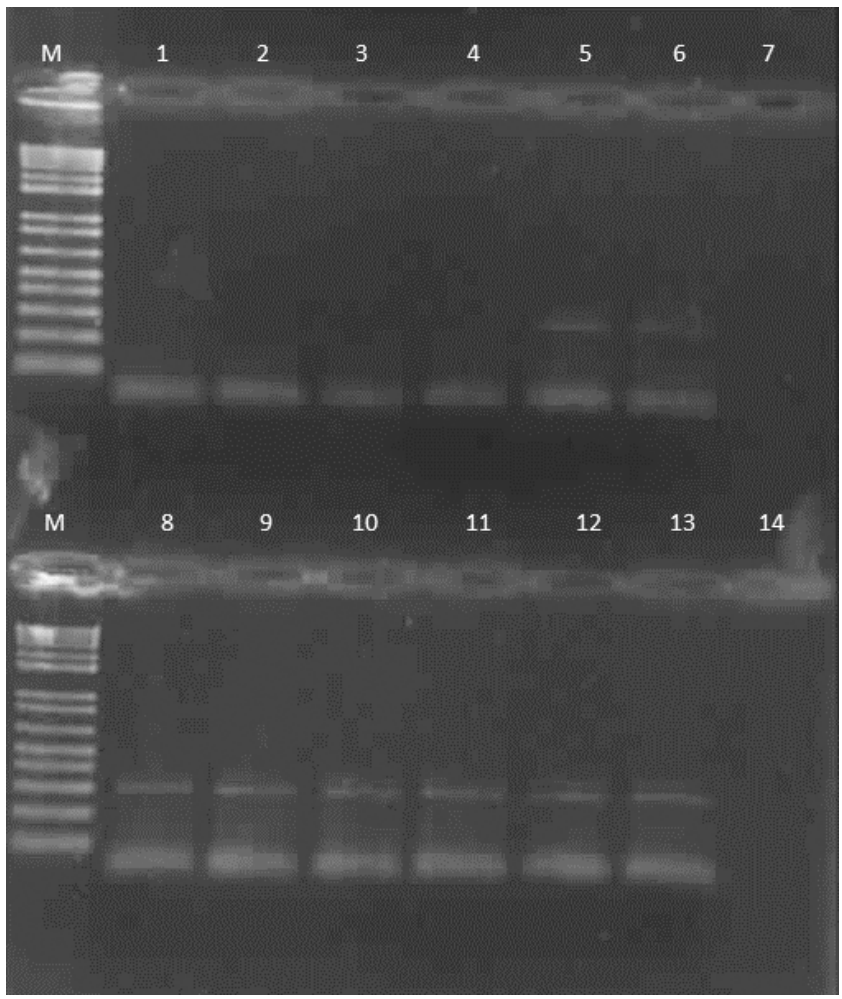

Figure 4. PCR amplification of the FAdV-4 hexon gene. Agarose gel electrophoresis picture for the FAdV-4 partial hexon gene from tested organs. Top panel: lane 1, 1-kb ladder; lanes 2–7, liver samples; lane 8, negative control. Bottom panel: lane 1, 1-kb ladder; lanes 2–7, heart samples; lane 8, negative control.

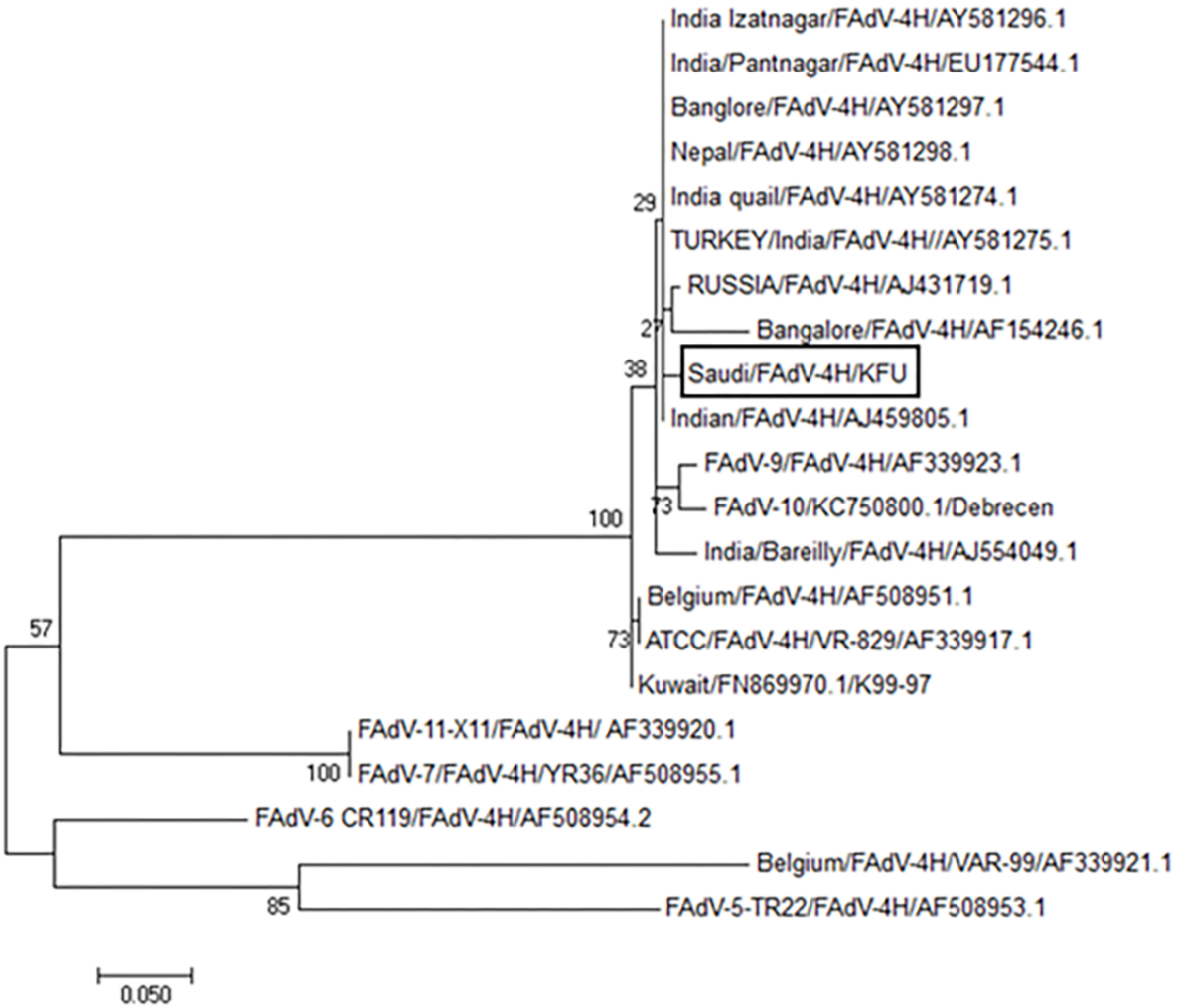

Figure 5. Phylogenetic analysis of FAdV-4 sequences. Maximum likelihood phylogenetic tree based on the partial FAdV-4 hexon gene isolated from eastern Saudi Arabia. The FAdV-4 strain clustered with other isolates from India, Bangalore, Nepal, and Russia.

4. Discussion

Fowl adenoviruses are dangerous viral threats for the chicken industry in many regions in the world such as North America, Europe, Asia, and the Middle East (5,6,10,17,18). FAdV-4 was previously reported in some Gulf countries such as Kuwait and Iraq, but it had never been reported in Saudi Arabia (5,10). The major goal of the current study was to perform isolation and molecular characterization of FAdV-4 strain that spread in some chicken farms in eastern Saudi Arabia in 2015. Chickens from five layer farms were showing high morbidity rates of up to 70% and variable mortality that ranged from 20% to 40%. Necropsy examination of the affected chicken revealed large rounded hearts, pericardium filled with jelly-like materials, and the presence of pinpoint foci in the livers of some chickens. A similar clinical picture was reported in chickens in many FAdV-4 outbreaks in India, Pakistan, Iraq, and Kuwait (5,6,10,19–21). Several systems were used for the isolation of fowl adenovirus in laboratories, such as ECEs and cell cultures (21).

In the present study, we used ECEs of 9–11 days old to isolate the circulated FAdV-4 strain in eastern Saudi Arabia and our results showed dwarfing, curling, hemorrhage, and death of the inoculated eggs 4 days after inoculation. Several techniques were used to detect FAdV-4 in different tissues specimens, such as PCR and real-time PCR. Several primers were designed to amplify several fragments of the FAdV-4 genome, especially hexon and fiber genes (8,9,17). We used a similar approach to monitor the presence of FAdV-4 DNA in the samples of some organs of the infected birds. We designed one pair of primers to amplify the partial hexon gene (Table 1; Figure 3) in addition to using the previously designed real-time PCR probes and primers to confirm our PCR results (13).

Our results clearly showed that the most affected organs are the hearts and livers of the affected chickens (Table 2). These results match well with the typical clinical picture of FAdV-4 in affected birds that have been reported elsewhere (2,5,11,12). Restriction endonuclease digestion of fowl adenoviruses is a new tool for the classification of different FAdVs. Furthermore, the combination of PCR and the RFLP of the obtained PCR products is another new tool for FAdV classification (11,22). The RFLP pattern of the reported strain suggests that the circulating strain belongs to the FAdV-4 genotypes (data not shown). Meanwhile, RFLP patterns have also been recently used to estimate viral genome length and size (23). The RFLP pattern of the isolated Saudi strain is closely related to previously reported ones from India (17). However, the average genome size of the FAdV-4 isolate from North America was 46 kb (17). Meanwhile, the genome size of our Saudi isolate of FAdV-4 is about 3 kb shorter than that of the North American isolate. Furthermore, the isolate of this study seems to be very close to the newly reported FAdV-4 from China (22,24). The phylogram clearly shows that the reported isolates from eastern Saudi Arabia were clustered together with other Asian strains. This variation among the FAdV-4 strains may contribute to the viral virulence. The Asian strains seem to be more virulent than the other strains of FAdV-4 reported from North America (3,17,22). Furthermore, the sequencing analysis of the partial hexon gene revealed the high similarity of the FAdV-4 strain isolated in this study to those from Kuwait, India, Pakistan, and China (data not shown). To the best of our knowledge, this is the first study to report the detection, isolation, and molecular characterization of FAdV-4 in Saudi Arabia. Further molecular studies are necessary to understand the characteristics of these FAdV-4 isolates.

In conclusion, this is the first report of fowl adenoviruses in this region. Furthermore, we performed some molecular characterization of this FAdV-4 isolate. This study clearly shows that the isolated strain is closely related to the strains circulating in other Asian countries such as Kuwait, India, and Pakistan. We also reported the genome size of this FAdV-4 isolate(43.5 kb) for the first time. We think that this study will have a positive impact on our understanding of not only fowl adenoviruses in general but also of FAdV-4, with particular emphasis on the Middle East.

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