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MOLECULAR ANALYSIS OF HEXON AND FIBER GENES OF FOWL AVIADENOVIRUSES ISOLATED FROM FIELD CASES OF INCLUSION BODY HEPATITIS

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ABSTRACT

Inclusion body hepatitis (IBH) is an infectious viral disease caused by fowl aviadenoviruses (FAdVs) leading to a significant economic losses in broilers in Saudi Arabia and worldwide. In this study, the FAdVs causing IBH were identified using the polymerase chain reaction (PCR) with primers directing the hexon gene. For a detailed molecular characterization, the complete open reading frames (ORFs) of hexon and fiber 2 genes were amplified and sequenced. The generated sequences were aligned and analyzed with certain FAdV reference strains. Five FAdV-D were detected had an average percentage of identity of approximately 96.80%. The detailed molecular analysis of the hyper-variable regions (HVRs) in the hexon loop 1 region revealed several amino acid substitutions that might alter the antigenicity as shown by an antigenic index analysis. The comparison of the genomic sequences of the fiber genes confirmed a 17–amino acid deletion in the shafts of three investigated strains isolated during 2017-18. Several amino acid substitutions were reported in the fiber gene knob and tail regions. Based on fiber gene sequence analysis, the average percentage of identity of the investigated strains and certain FAdV-D reference strains was 94.24%. The frequent isolation and identification of FAdV-Ds in IBH clinical cases with an average genetic diversity of 56.10% when compared with reference strains of FAdV-C emphasize that current preventive measures should be reviewed and that a new homologous vaccine may be needed.

Keywords: IBH, Hexon gene, Fiber gene, Phylogenetic analysis, Fowl aviadenoviruses

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INTRODUCTION

The fowl adenovirus (FAdV) is the etiology of IBH in poultry. It has a significant economic influence because it leads to dramatic increase in mortalities and reduced productivity (Alvarado *et al*., 2007). The disease is found around the world and causes significant losses among broiler flocks in Saudi Arabia (Mohamed *et al.,* 2018). The virus affects mainly broiler chickens aged 3-6 weeks, causing mortalities ranged from 10-30% or up to 80% in more complicated cases. Lesions include an enlarged, discolored or necrotic liver with hemorrhages (Ren *et al*., 2019).

FAdVs are none enveloped, dsDNA linear viruses of 43 to 46 kb in length, belonging to the genus Aviadenovirus of the family Adenoviridae (Adair *et al.,* 2008). FAdVs are assembled into 5 species (FAdV-A to FAdV-E) based on genomic restriction enzyme digest patterns, with up to 12 serotypes identified as: FAdV-A (serotype FAdV-1); FAdV-B (serotype FAdV-5); FAdV-C (serotypes FAdV-4 and -10); FAdV-D (serotypes FAdV-2, -3, -9, and -11); and FAdV-E (serotypes FAdV-6, -7, -8a, and -8b) (McFerran & Smyth, 2000). The 12 FAdVs serotypes were recognized and isolated from IBH worldwide, which has a mortality range from 10% up to 30% (Mase *et al.,* 2012). The infection mainly affects broilers between 3 and 4 weeks old and is characterized by a friable, congested, swollen livers with hepatic necrosis with ecchymotic hemorrhage in most cases (Mohamed *et al.,* 2018, Steer *et al.,* 2015, Nakamura *et al.,* 1999).

The viral genome encodes 3 main proteins, the hexon, fiber, and penton base proteins. Hexon is the abundant capsid protein, and it has a region associated with virus neutralization and serotyping (Shah *et al.,* 2017). Four hypervariable regions (HVRs 1 to 4) located in loop 1 of the hexon gene are strictly responsible for antibody binding (Niczyporuk, 2018). The fiber protein is the second major protein of the viral capsid is the fiiber protein, it is divided into a tail, shaft, and knob; it has been involved in virus entry and implicated in the variation of pathogenicity of FAdV species and virus–host interactions (Grgic *et al.,* 2014). Recent conclusions showed that both hexon and fiber 2 genes are involved in the virulence determination of FAdVs (Zhang *et al*., 2018).

To date, vaccination policies are based mainly on the usage of inactivated whole virus vaccine, the prevention of inclusion body hepatitis using vaccination is a complex process due to the diverse variety of viral species and serotypes (Schachner *et al*., 2016). Birds are still susceptible to heterologous FAdVs infections regardless of the presence of antibdies against other FAdVs species, and likely represents the molecular basis for the newly stated natural recombination of FAdVs (Schachner *et al*., 2019). The cross protection between different FAdVs species was reported between FAdV-C and FAdV-E strains as inactivated vaccines (Steer *et al*., 2019).

The aim of this study was to identify the molecular characteristics of FAdVs from existing IBH cases by combining the data from complete hexon and fiber2 gene sequences.

MATERIALS AND METHODS

Clinical inspection and specimen: The clinical histories of 15 broiler flocks were presented to the avian clinic of the VTH, College of Veterinary Medicine, King Faisal University, Saudi Arabia, during the period from 2015-18. The main clinical signs and/or lesions were reported in livers from broilers showing characteristic clinical signs and lesions of suspected IBH infection. Collected samples were preserved and submitted to the Central Biotechnology Laboratory. The virus detection was achieved using primers and PCR conditions described by (Meulemans *et al*., 2004) for the conserved hexon loop-1 gene amplification. All the animal-related experiments were performed following the guidelines of the laboratory safety and experimental animal rights committee of the College of Veterinary Medicine, King Faisal University, Saudi Arabia.

Sequencing: The complete open reading frame (ORF) of the hexon gene was amplified and the generated product were sequenced using primer sets HexA, HexB, and HexC as previously defined by (Changjing *et al*., 2016). The whole ORF of the fiber 2 gene was amplified using primer sets FibA15/-GACGTAACCGGGGTTCAGGG-3/ and FibA25/-AYGGTKCCKGGGTTGTAGG-3 designed using the primer designing tool NCBI (https://www.ncbi.nlm.nih.gov /tools/primer-blast/) based on the whole genomic sequences retrieved from the GenBank databases KY012057 and KT862805.

Sequence analysis: The specific products of the target gene DNA were purified from the excised gel slices with the Montage DNA gel ext. kits (Millipore, Burlington, MA, USA) and sequenced in both directions with specific forward and reverse primers in an automated ABI3730 DNA-sequencer (Macrogen, Seoul, Korea). The generated sequences were assembled, aligned, and analyzed by DNASTAR-Lasergene 11 software (DNASTAR, Madison, WI, USA). The obtained sequences of each hexon and fiber gene were submitted to the GenBank database under accession numbers MK995481 to MK995490. The multiple-sequences alignment of the full hexon and fiber nucleotides and derived amino acid sequences were generated using ClustalW. Evolutionary trees based on the nucleotides and amino acid sequences of the five field strains and certain reference strains were constructed using the neighbor-joining method, and the reliability of the trees was tested by bootstrapping with 1000 replicates by MEGAX software.

RESULTS

Detection and genotyping of FAdVs based on hexon gene: Clinical samples from 15 broiler flocks were submitted to the clinic from broilers showed signs and lesions were suggestive for IBH including, mortalities 10-30%, hemorrhagic and swollen livers with or without hepatic necrosis. The presence of the virus's nucleic acid was confirmed using PCR based on the amplification of 890 bp representing the L1 hexon gene. The FAdVs were successfully detected in 5 out of the 15 investigated broiler flocks, followed by the full gene sequencing of both investigated genes.

Thirty-eight sequences of the hexon gene were used in this study. For molecular analysis, the 38-nucleotide sequences was divided, and the obtained strain sequences were constructed for the five major FAdV species from A to E representing the 12 aviadenoviruses serotypes (Figure 1). Based on the entire hexon gene nucleotide sequence, the five Saudi field FAdV strains were grouped into FAdV-D species serotype 2, with sequences from reference strains (Fig 1). In all of the investigated field and reference strains of the same species, the lowest nucleotide identity in the Hex L1 gene region was 96.80% (FAdV-D2). In the examined field strains only, the genetic variance was significantly low, with greater than 98.2% nucleotide identity. The comparison of the hexon gene nucleotide sequences revealed that the highest identity was 98.30% between strain SAC104 and the FAdV-D2 reference strains (KT862805 and KT862806).

The entire sequence of the hexon genes was translated into amino-acid sequences (aa), and the resulting 800 amino acids were analyzed. HVRs 1 to 4 in loop L1 (amino acids 130 to 290) were defined by searching the conserved and variable sequences (Fig 2). HVR-1 was the longest of the compared regions, spanning 64 amino acids. The average similarity of the aa in the HVR loop 1 hexon gene of the Saudi strains and FAdV-A to FAdV-E were as follows; 58.58%, 68.90%, 56.10%, 92.90%, and 78.50%, respectively. The amino

Table 1. Representing Amino-acids substitutions among investigated *hexon* genes.

	Amino acids substitutions (aa300-800)																									
Position	307	329	338	351	363	364	369	373	400	418	419	428	439	443	446	538	551	553	554	555	564	585	588	590	616	63
Majority	D	Q	Q	Y	H	D	N	Y	A	S	G	N	L	Q	F	H	L	L	L	P	L	A	V	S	A	T
D2_KT862805	.	.	.	.	.	.	.	.	I	N	A	Y	I	.	.	.	.	.	.	S	.	.	I	N	.	.
C4_HE608152	.	F	.	F	P	E	S	.	T	N	S	H	H	P	L	.	.	.	.	.	.	.	.	.	.	.
MK995481	.	.	.	.	.	.	.	F	.	.	S	H	.	.	.	.	.	.	.	.	.	.	.	F	.	.
MK995482	.	.	.	.	.	.	.	.	T	N	S	H	.	.	.	Q	W	R	V	A	F	.	.	.	.	R
MK995483	.	.	.	.	.	.	.	.	.	.	S	H	.	.	.	.	.	.	.	.	.	.	G	.	.	.
MK995484	.	.	.	.	.	.	.	.	.	.	S	H	.	.	.	.	.	.	.	.	.	T	.	.	G	.
MK995485	G	.	.	.	.	.	T	.	.	.	.	H	Q	.	.	.	.	.	.	.	.	.	.	.	.	.
MG029109	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MG029110	.	.	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MG029111	.	.	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MG029113	.	.	H	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MG029114	.	.	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SA15_1965	.	.	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Dots indicate conserved residues

Table 2. Amino-acids substitutions among investigated *fiber* genes shaft regions.

	Amino acids substitutions in the Shaft region																													
Position	110	118	131	136	138	139	149	191	203	227	230	240	241	242	250	255	257	260	265	266	267	268	279	282	283	284	288	289	290	2
Majority	A	D	A	R	S	P	S	T	P	T	A	K	P	S	Q	-	-	-	-	-	-	-	I	S	S	N	V	V	T	
D2_KT862805	T	A	T	.	.	.	.	V	S	N	D	.	.	.	.	A	A	L	N	A	-	-	.	.	.	.	.	T	.	
MK995481	.	.	.	K	.	.	.	.	.	.	.	R	A	T	H	D	P	L	S	A	G	T	V	A	T	A	I	A	K	
MK995482	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	-	-	-					.	.	.	.	.	.	.	
MK995483	.	.	.	.	A	R	A	.	.	.	.	.	.	.	.	A	A	L	N	T	G	T	.	.	.	.	.	.	.	
MK995484	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.					.	.	.	.	.	.	.	
MK995485	.	.	.	.	.	.	.	.	.	.	.	.	Q	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	

Dots indicate conserved residues; dashes indicate deleted aa

Table 3. Amino-acids substitutions among investigated *fiber* genes Knob.

		Amino acids substitutions in the knob region																					
	Position	424	425	432	447	454	458	459	465	475	481	482	483	484	485	487	488	489	490	492	493	505	5
	Majority	Q	L	F	V	P	P	W	A	K	P	P	S	I	Y	E	P	E	S	S	V	D	
Strains	D2_KT862805	T	F	.	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	L	N	
	MK995481	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
	MK995482	.	.	L	A	Q	.	C	S	.	.	.	.	.	.	.	.	.	.	P	.	.	
	MK995483	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	

	MK995484	.	.	L	.	H	.	.	.	.	L	A	K	H	V	P	A	R	T	.	.	.
	MK995485	.	.	.	.	.	.	.	.	.	.	.	.		.	Q	-	.	.	.	.	.

Dots indicate conserved residues

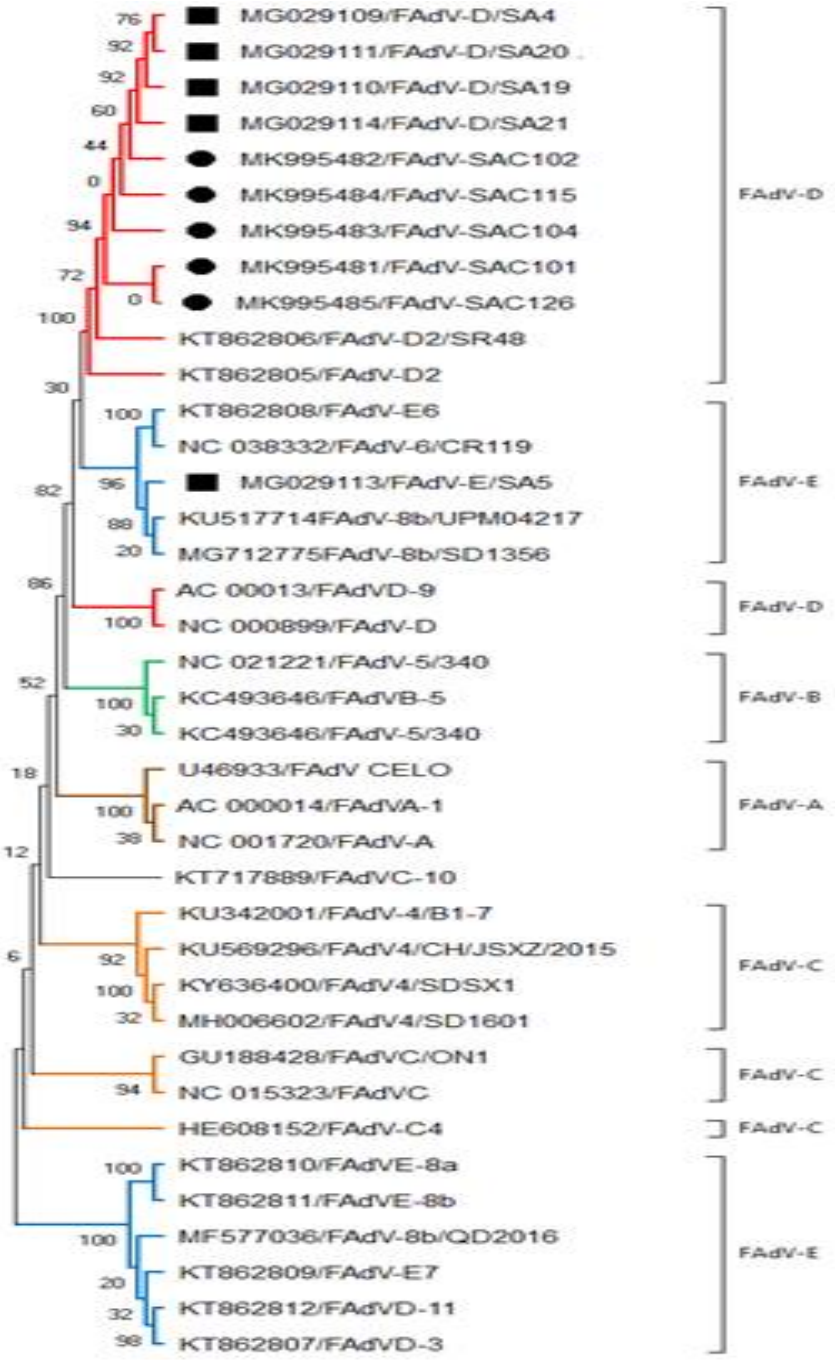

Fig 1. The phylogenetic analysis of the field strains were constructed based on the complete Hexon gene sequences along with certain reference strain. The phylogenetic tree was generated using NJ methods as implemented by MEGAX software. The investigated strains "black hexagon" while other strain from previous study "black circle".

acid substitutions from amino acids 300 to 800 are presented in Table 1. The antigenic index illustrated peaks of different shapes among the Saudi strains and reference strains KT862805 (FAdV-D2) and HE608152 (FAdV-C4) (Fig 3).

Detailed molecular characteristics of fiber gene: Fiber gene nucleotides and aa sequences of the Saudi field strains were compared with the reference FAdVs (Fig 4). The fiber genes of all of the investigated sequences were clustered within FAdV-D serotype 2. The average amino acid identity between the Saudi strains and D2_KT862805 representing FAdV species D was 94.24%. The lowest amino acid identity was between the investigated strains and FAdV species A. The average

amino acid identity among the investigated strains was 95.43%. The fiber gene comprising 572 amino acids was divided into tail, shaft, and knob. In the tail, comprising the first 63 amino acids, 15 amino acid substitutions were reported (Fig 5) between the investigated strains and the D2_KT862805 isolate. The conserved motif (nuclear localization motif) RKRP (amino acids 17 to 20) and the penton base interaction motif VYPF (amino acids 53 to 56) were present in the investigated strains. Six poly-G stretches were conserved among the investigated strains between amino acids 64 to 70. The fiber shaft located between amino acids 75 to 416 was conserved with 31 substitutions compared with the D2_KT862805 summarized in Table 2. In three investigated strains, a gap of 17 amino acids between amino acids 252 to 270 was present (Fig 6). The knob comprised the region from amino acids 417 to 572 next to the last pseudo-repeat in the shaft region (RDN). Sixteen amino acid substitutions were present among the investigated strains when compared with the D2_KT862805 isolate (Table 3).

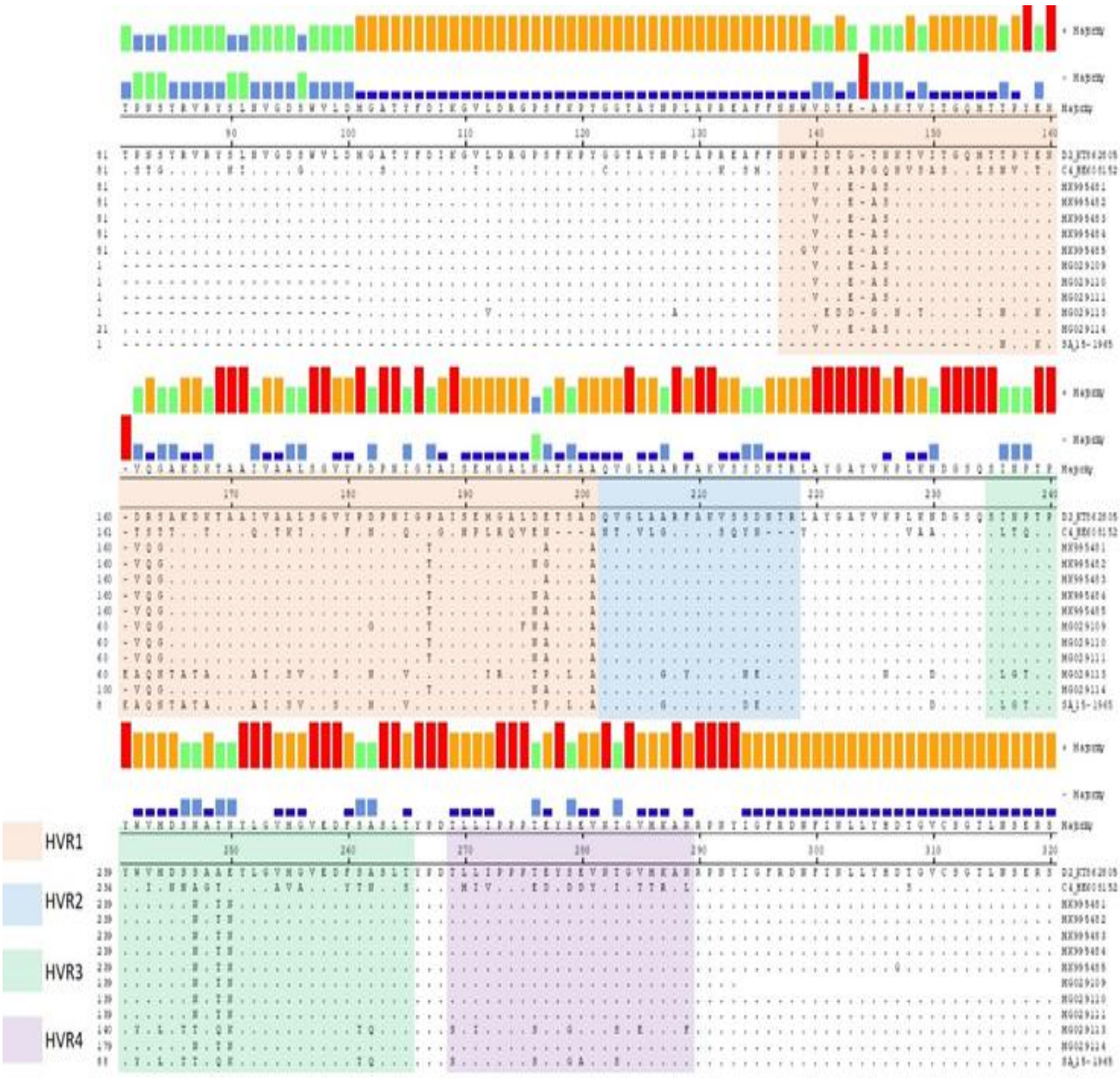

Fig 2. Alignment of amino acids residues of Hexon gene HVRs 1-4 of investigated strains certain field and reference FAdV-D and FAdV-C strains. Dots indicate conserved residues.

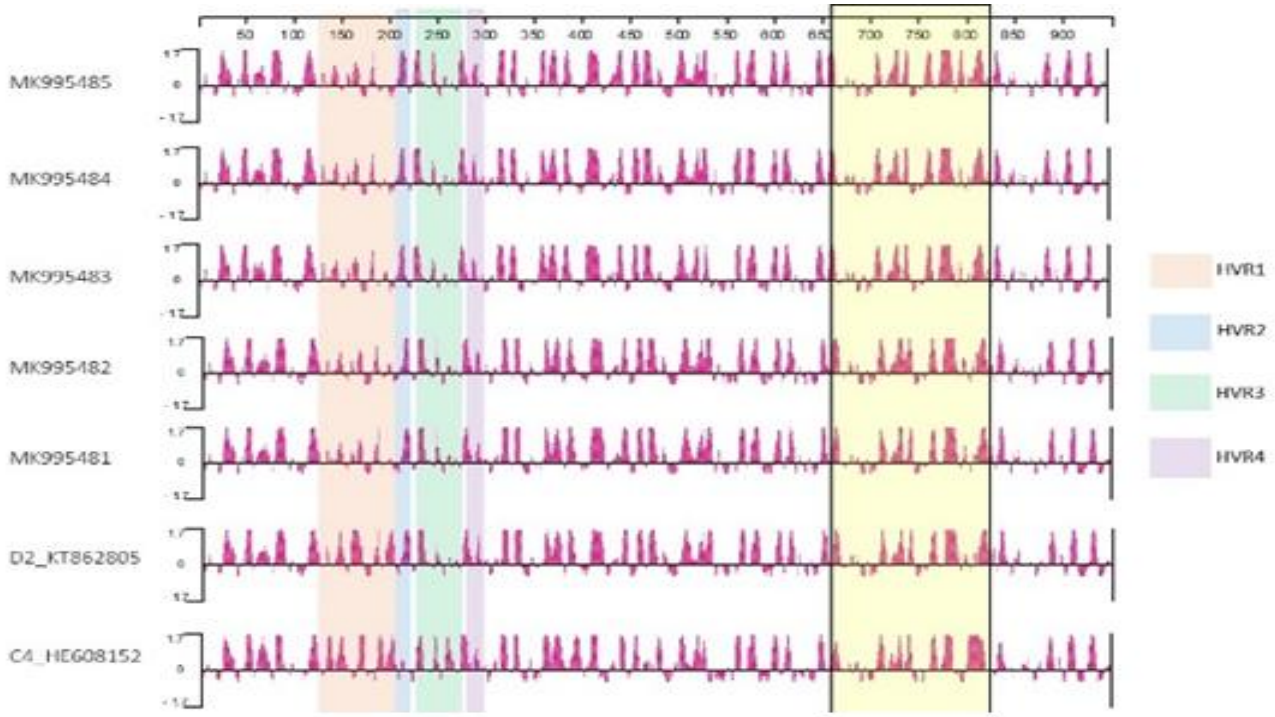

Fig 3. Protean analysis of Hexon gene amino acids of investigated strains certain field and reference FAdV-D and FAdV-C strains showing differences in antigenic peaks.

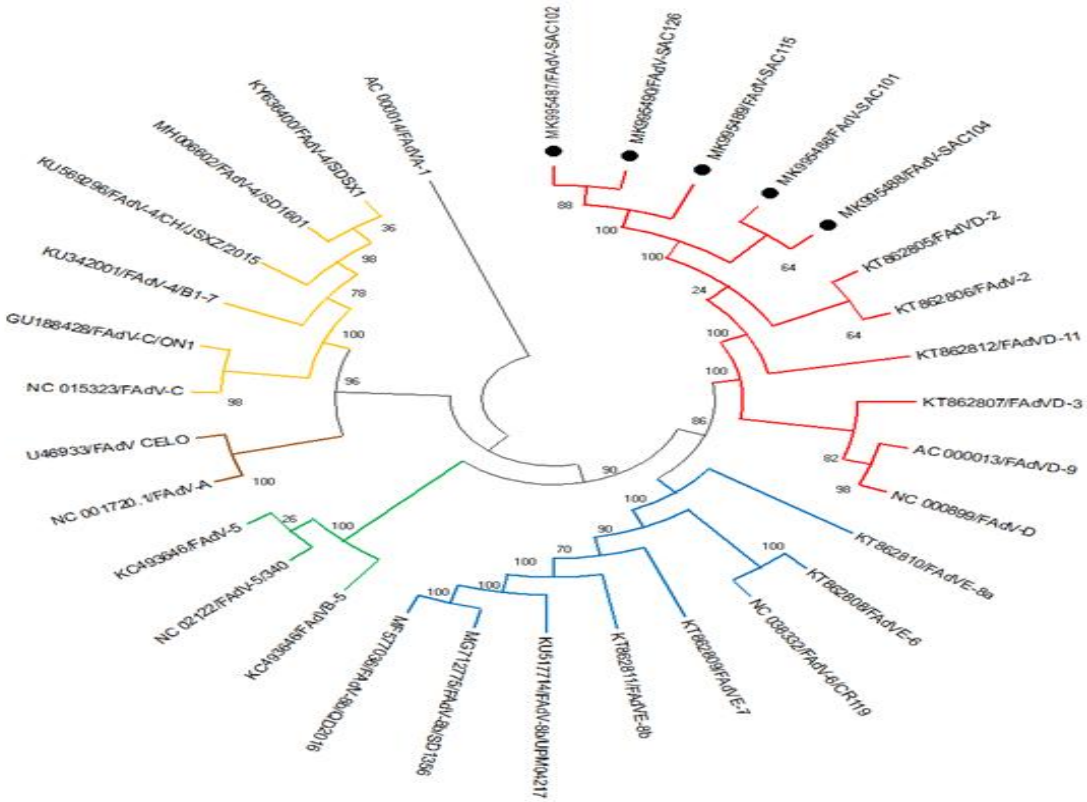

Fig 4. The phylogenetic analysis of the field strains were constructed based on the complete Fiber amino acids sequences along with certain reference strain. The phylogenetic tree was generated using NJ methods as implemented by MEGAX software. The investigated strains "black circle".

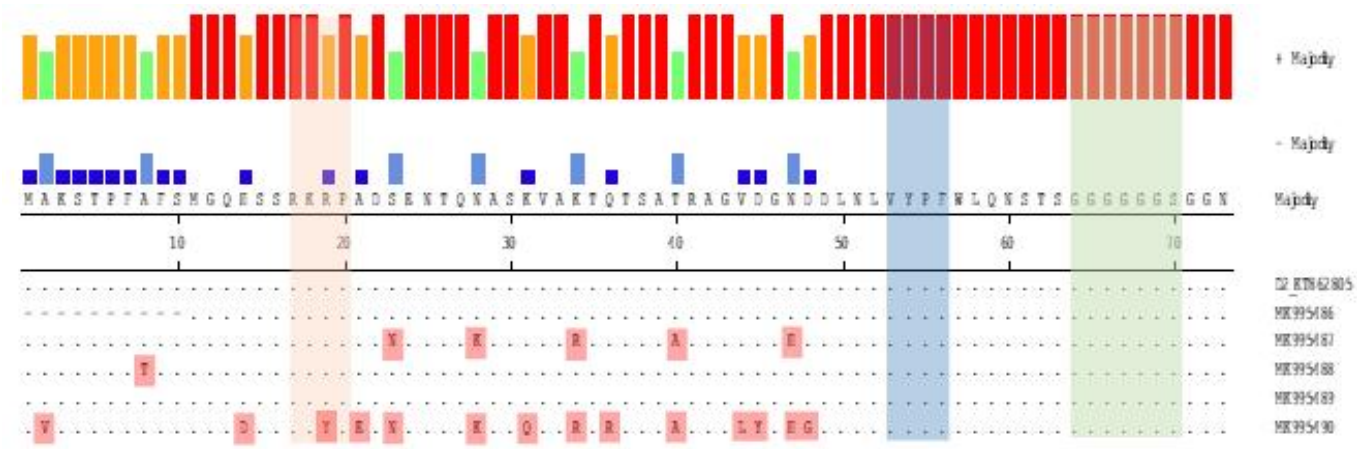

Fig 5. Alignment of amino acids residues of fiber gene tail region of investigated strains with reference FAdVs-D strain. Dots indicate conserved residues.

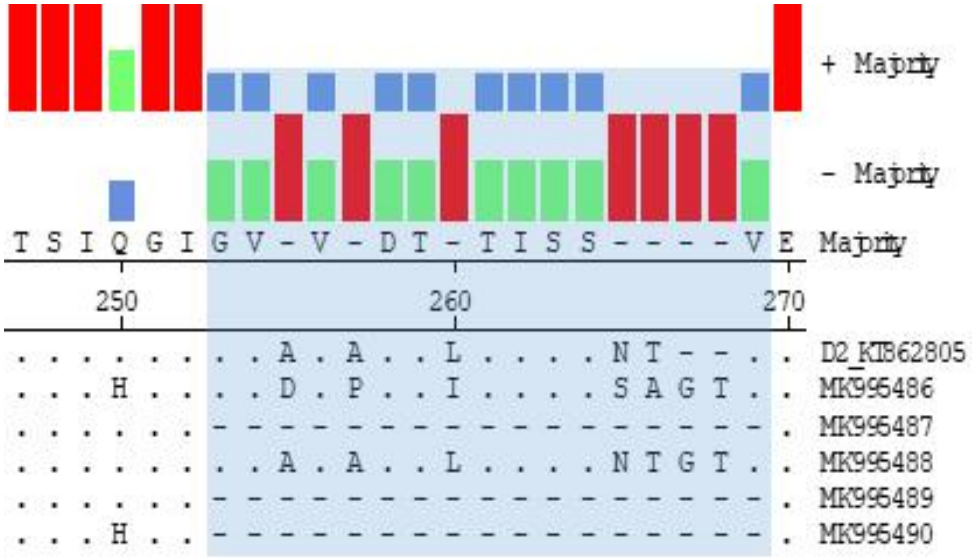

Fig 6. Alignment of amino acids residues of fiber gene shaft region of investigated strains with reference FAdVs-D strain. Dots indicate conserved residues; dashes indicate deleted 17 amino acids between residues 252 to270.

DISCUSSION

Inclusion bodies hepatitis (IBH) outbreaks have been reported periodically and have had a large impact on the poultry industry (Schachner *et al*, 2018). FAdVs cause different disease conditions in poultry, especially in broilers. In Saudi Arabia since 2015, FAdV infections have led to high mortality rates among 3- to 5-weeks old broilers (Mohamed *et al*, 2018). We reported that most FAdV infections in such broilers were characterized by hepatitis, had high mortality rates, and continued for at least a week (Li *et al*, 2018). Hexon and fiber2 proteins encoded by the FAdV genome all contain dominant antigenic sites (Shao *et al*, 2019). The hexon protein is a major surface-exposed capsid structure containing groups, types, and subtypes of specific antigenic determinants (Russel, 2009). The fiber protein is different in different FAdVs, is responsible for the primary cell attachment, and might also be involved in pathogenicity (Russel, 2009); it might be a target for the development of serotype-specific diagnostics and vaccines for FAdVs (Feichtner *et al,* 2017; He *et al,* 2018; Shao *et al*, 2019). In this study, five FAdVs were isolated from clinically infected broiler chickens aged 22 to 30 days. The complete gene sequences of both investigated genes were successfully generated for all investigated FAdVs. The genomic analysis was based on the genetic constitution of the hexon gene, which is the largest gene in the FAdV genome. The hexon gene has four HVRs and is the target of most studies focusing on antigenic properties (Johnson *et al,* 2005; Harrach *et al,* 2011). The investigation of the full hexon gene nucleotides in investigated strains confirmed that all strains belonged to FAdV species D type 2 and showed an average percentage of identity of

96.80% D2_KT862805 (Mohamed *et al,* 2018). The 4 hypervariable regions in loop L1 of the investigated strains were recognized as the regions of the highest sequence variability: HVR-1 was approximately 64 amino acids long, HVR-2 approximately 17 amino acids long, HVR-3 approximately 31 amino acids long, and HVR-4 approximately 20 amino acids long. The lengths of the HVRs were different from those described by Raue (2005) and Niczyporuk *et al* (2018). The nucleotide sequences of HVRs are constant for every species, although there are a major variances between FAdV types (Niczyporuk *et al,* 2018). We reported several amino acid substitutions among the investigated strains compared with strain D2_KT862805. The effect of these sequence differences on virus replication and pathogenicity remains to be investigated. Mutations in gene qualities and locations can influence the protein structure and subsequently may affect the antigenicity (Niczyporuk *et al,* 2018). The average percentage of identity between the consensus sequence of the investigated strains and FAdV-D with consensus sequence FAdV-C was 56.10%; FAdV-D was the most distant from FAdV-C and indicated multiple differences (Niczyporuk *et al,* 2018). The nucleotide sequences of all of the investigated strains were translated into amino acids and analyzed, and the antigenic peaks were compared with the corresponding peaks of references FAdV-C and FAdV-D. The differences in peaks were focused in the HVRs and area between amino acids 650 to 850 that may interfere with the cross antigenicity between FAdV types C and D.

The fiber gene sequence could be divided into main domains; tail, shaft, and head and had some definite features associated to virus neutralization, virus pathogenicity, tropism, cell receptor binding, and pathogenicity (Russel, 2009; Liu *et al,* 2016). The fiber tail region was 73 amino acids in length and located between amino acids 1 to 73, and the length of the tail region was conserved in FAdV type D (Grgic *et al,* 2014). The investigated strains shared a conserved motif, RKRP, which was similar to the reference FAdV type D (Grgic *et al,* 2014); the RKRP motif was semiconserved in the MK995490 isolate with a single amino acid substitution, R19Y. The other motifs, RKRP and VYPY, were similar in all investigated strains compared with the reference FAdV type D (Hess *et al,* 1995). A poly-G stretch consisting of six Gs was present in the investigated strains and FAdV type D (Sheppard *et al*, 1995), but the number of poly-Gs differed among FAdV species (Hess *et al,* 1995). The fiber shaft was widely conserved in the FAdV-Ds (Schachner *et al,* 2018) of our investigated strains compared with the KT862805 FAdV-D strain, which showed some substitutions across the whole shaft. A gap of 17 amino acids between amino acids 252 to 270 was present in the shaft of three of the investigated strains, possibly altering the fiber length in these strains. The conserved motif TLWT at the start of the head region was absent in all of the investigated strains and Fowl Aviadenoviruses species A, C, D, and E (Mase *et al,* 2010). The amino acid sequences were conserved in three of the investigated strains with a single amino acid substitution, whereas strains SA102 and SA115 showed 5 and 11 amino acid substitutions, respectively. Schachner *et al* (2018) reported a scarcity of amino acid substitutions among the FAdV-D strains.

Conclusion: In summary, our data demonstrated that FAdV-D type 2 was the predominant type of FAdV in Eastern region of Saudi Arabia, isolated from the livers of clinically infected broiler chickens. On the basis of the phylogenetic analysis of both nucleotides and aa sequences, we highlighted the differences between the field strains and types D and C. The genetic diversity among the isolated viruses can support epidemiological studies and help in the understanding of the antigenic relation between different FAdVs and field strains. These findings support the use of homologous vaccines as new preventive strategies.

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Author contribution statement: Mahmoud Mohamed: Data collection, analysis, Methodology, Funding Resources, Writing and editing. Ibrahim El-Sabagh: Data curation, reviewing, editing, Methodology, Funding acquisition and Resources.

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