JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Emerging Problems in Infectious Diseases

Molecular cloning, sequencing, expression and purification of Alkhumra hemorrhagic fever virus capsid protein in Saudi Arabia

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Abstract

Introduction: Alkhumra hemorrhagic fever virus (AHFV) is a newly discovered virus in the *Flaviviridae* family. It was discovered in 1995 among animal handlers in Saudi Arabia. AHFV spreads through close contact with infected animals and tick bites. Symptoms range from fever and flu-like symptoms to hemorrhagic manifestations, and rarely, encephalitis. The Saudi Arabian Ministry of Health has reported 604 cases so far. There are no approved vaccines, antiviral therapies, or routine screening systems for AHFV. This lack of preventive measures makes it challenging to predict future outbreaks or re-emergence in endemic regions of Saudi Arabia.

Methodology: We cloned, sequenced, analyzed, expressed, and purified the recombinant AHFV capsid protein (CP) using the PET-28a (+) vector. The *CP* gene was amplified through reverse transcriptase polymerase chain reaction (RT-PCR) and cloned into a vector. The expression and purification processes were carried out in *E. coli*.

Results: The sequence of the *CP* gene was deposited in GenBank (Accession number OR785375) and designated as AHFV-SIAU-1-KSA. Sequence analysis revealed similarities with other AHFV isolates obtained from humans, animals, and ticks. Phylogenetic analysis showed that the AHFV *CP* gene formed distinct clusters with other AHFV genomes collected at different time intervals. The expressed protein was successfully purified and analyzed by SDS-PAGE.

Conclusions: This study is the first to document the cloning, expression, and purification of the recombinant AHFV *CP* gene from Saudi Arabia. The purified protein will be used to develop a serological assay for routine screening of AHFV samples in humans, animals, and ticks in Saudi Arabia.

Key words: Alkhumra hemorrhagic fever virus; capsid protein; cloning; sequencing; expression; purification.

J Infect Dev Ctries 2024; 18(12):1806-1814. doi:10.3855/jidc.19854

(Received 15 January 2024 – Accepted 27 May 2024)

Introduction

The Alkhumra hemorrhagic fever virus (AHFV) was discovered in 1995 from the Alkhumra District in Jeddah, Saudi Arabia as the cause of hemorrhagic fever among butchers and animal handlers [1,2]. The infected individuals develop a range of symptoms that includes fever, headache, retro-orbital pain, arthralgia, myalgia, anorexia, vomiting, leukopenia, thrombocytopenia, rhabdomyolysis, severe muscle weakness, and gastroenteritis-like illness [3]. Based on the diagnosis, characterization, and close similarities with the Kyasanur forest disease virus (KFDV), this virus was designated as Alkhumra hemorrhagic fever virus (AHFV). It consists of an ssRNA genome and belongs to the Family *Flaviviridae*, and genus *Flavivirus* [4]. Since then, AHFV has been reported from many regions of Saudi Arabia, Tihama region of western Arabian Peninsula, as well as regions outside Saudi Arabia including Egypt, Djibouti, and Sudan with case fatality rates of up to 30% [3,5-9]. Tick bites play an important role in virus transmission and the presence of AHFV in *Orinthodorus* ticks has been confirmed from Jeddah [10], and *Orinthodorus savignyi* and *Hyaloma dromedarii* ticks in Najran [11]. Transmission of the virus also occurs by close contact with domestic animals like sheep and camels. The viral RNA has also been identified in *Hyalomma rufipes* ticks infesting migratory birds in Europe and Asia Minor [12].

The full genome of AHFV has been sequenced and analyzed. The viral genome contains 10,546 nucleotides encoding for a single polyprotein with 3,416-amino acid, which has 99% (JN860200) to 99.7% (KU884971) similarity with previously reported AHFV strains [13-14]. Overall, 604 confirmed cases of AHFV

have been reported in Saudi Arabia since 1995. Based on the current status, a major outbreak of AHFV can be anticipated in the high-risk communities. Currently, there is no specific treatment or vaccine available against this virus, and only supportive care are suggested for infected patients. Full genome sequence information of AHFV can provide significant targets to design and develop vaccines and antivirals against this virus. Currently, viral diagnosis is being performed by both serological and molecular assays. The sero-surveillance data on AHFV antibodies among humans in Saudi Arabia or elsewhere are scarce. Therefore, continuous serological surveillance is required. The main objective of this study was to clone, sequence and express the recombinant capsid protein (CP) of AHFV and to develop in-house serological ELISA for the continuous and routine screening of AHFV in Saudi Arabia.

Methodology

Sample collection and virus isolation

The blood samples were collected from the suspected patients from January 2010 to December 2015 and transported on dry ice to the Special Infectious Agents Unit, (SIAU), King Fahd Medical Research Center (KFMRC), King Abdulaziz University (KAU), Jeddah, Saudi Arabia. Ethical approval was obtained from the Biomedical Ethics Unit at King Abdulaziz University Hospital. The samples were further screened by serological and molecular assay. The positive serum samples were used for inoculation of LLC-MC2 cell line for virus culture. The cells were inoculated with 100 µL of patient serum in tissue culture flasks and incubated at 37 °C for 1 h to allow for virus growth and multiplication. The grown virus was isolated from the infected cells supernatant and further confirmed by laboratory tests. The cell supernatant was used for viral RNA isolation [14].

Viral RNA extraction and real time-polymerase chain reaction (RT-PCR)

The viral RNA was extracted and purified from the cell supernatant by using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The eluted and purified RNA was used for the detection of viruses by using the QuantiFast Probe RT-PCR Kit (Qiagen, Hilden, Germany) following the previously published protocol [14].

PCR amplification, cloning, sequencing and analysis

The purified viral RNA was used for PCR amplification of capsid protein (CP) gene. The cDNA synthesis and amplification of the target CP gene were carried out by using the One-Step RT-PCR Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, the reaction was completed in a total volume of 50 µL. The mixture consisted of 5x One Step RT-PCR buffer, 200 µM dNTP Mix, 2.0 µL One Step RT-PCR enzyme mix, 0.5 µM of forward and reverse primers, 5µL of the viral RNA as template, and the final volume was made up to 50 µL by adding double distilled water. The sequences of the primers were: forward primer 5'CACTCGAGATGGCCAAAGGAGCCGTCC 3'; and reverse primer 5' TGAAGCTTGGAGATCACCAGTGTCGCCA3'. The PCR was performed in a thermal cycler (Eppendorf, Hamburg, Germany), under specific cycling conditions to amplify the CP gene. The cycling conditions were: 50 °C for 30 min for cDNA synthesis; followed by 1 cycle at 94°C for 1 min for initial denaturation; and then 35 cycles at 94°C for 1 min for denaturation, 1 min at 58°C for annealing, and 1 min at 72°C for extension; and 10 min for the final extension as last cycle. The PCR product was separated by gel electrophoresis with 1% agarose gel which was stained with ethidium bromide and visualized under the Gel Doc System (IN GENIUS, Syngene Bio Imaging, UK) to observe and confirm the size of the PCR amplified product by comparing with a 1 kb DNA ladder (ThermoScientific, Waltham, MA USA).

The AHFV-CP gene (350 bp) was cloned in pET-28a (+) vector (5601bp) (GenScript, New Jersey, USA). The PCR amplified product was eluted and purified from the gel by using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The purity and quantity of purified DNA was determined by using a nanodrop spectrophotometer (DeNovix-DS-11FX+, Wilmington, DE, USA). The purified DNA was digested with selected restriction enzymes (*XhoI* and *HindIII*; ThermoScientific, Waltham, MA, USA). Similarly, the vector DNA was purified by using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and digested with the same restriction enzymes. The digested and purified DNA (insert/vector) were ligated by using the T4DNA ligase enzyme. Briefly, insert DNA (100 ng) and vector DNA (300 ng) were mixed for ligation in a tube, and 1 µL of T4DNA ligase (ThermoScientific, Walthman, MA, USA) and 2 µL of 10x buffer were added. The final volume was made up to 20 µL by

adding double distilled water. The ligation mixture was incubated overnight at 22°C for successful ligation of the insert into the vector. The ligation mixture was used to deliver the DNA fragments into competent *E. coli* (DH5α) cells by the heat shock transformation method. The transformed cells were spread on Luria Broth Agar (LBA) plates with Kanamycin (50 mg/L) and incubated at 37°C overnight. The putative recombinant clones were confirmed by using colony PCR, plasmid DNA-PCR, and restriction enzyme digestion. Briefly, the putative recombinant colonies were grown in 5 mL Luria Broth (LB) media at 37°C overnight. The plasmid DNA was purified from the overnight grown culture by using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and restricted with the same restriction enzymes used for digestion of insert and vector. The restricted DNA was visualized on a 1% agarose gel, and recombinant clones were confirmed by determining the size of insert and vector by comparing with 1 kb DNA ladder. Additionally, the purified plasmid DNA was used for clone confirmation. The purified plasmid DNA from the positive clones were used for sequencing.

Sequencing was performed at the Special infectious agents unit (SIAU) using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Waltham, MA, USA) by the Sanger dideoxy sequencing method, following the manufacturer's instructions. The capsid protein gene forward and reverse primers were used for sequencing. The sequencing products were purified by using the ethanol/EDTA precipitation method and analyzed with the ABI Prism 3500 genetic analyzer (Applied Biosystems, Waltham, MA, USA). The resultant raw sequences were assembled and analyzed by BioEdit (v.7.2), and the final sequences were selected for further analysis. The sequence identity matrix of generated sequences was determined with submitted AHFV genome sequences in NCBI-GenBank database. The generated nucleotide sequences were aligned together and analyzed by using multiple sequence alignments tools in CLUSTAL-W programme [15].

Phylogenetic analysis

The comparisons were made with sequences obtained from the GenBank nucleotide sequence database. The sequence file was imported into Molecular Evolutionary Genetic Analysis (MEGA X) [16]. Phylogenetic analysis was performed and the relationship dendrogram was constructed from the aligned nucleotide sequences using neighbor joining and maximum parsimony methods through MEGA X [16].

Expression and purification of recombinant AHFV capsid protein

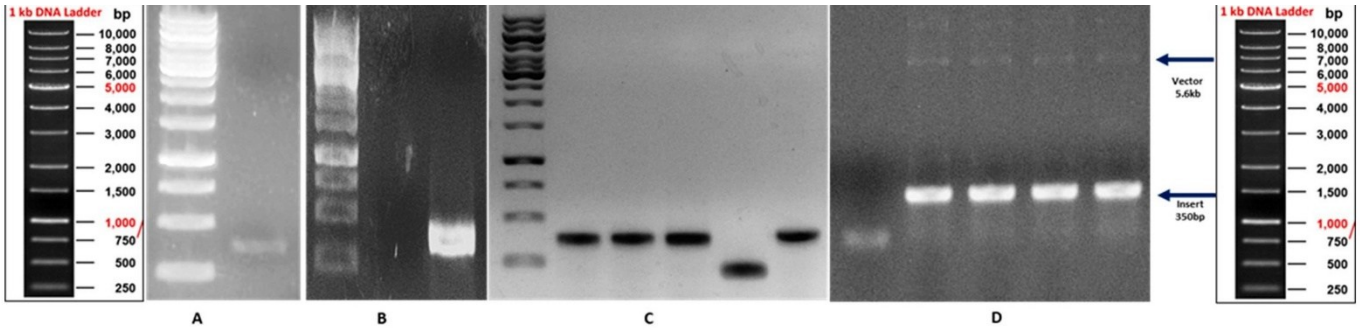
The recombinant AHFV capsid protein (rAHFV-C) was expressed in *E. coli* BL21(DE3) cells with pET-28a (+) plasmid vector. Recombinant plasmid DNA (2 µL) was mixed with 100 µL of competent *E. coli* BL21(DE3) cells and incubated on ice for 15 min, followed by heat shock at 42 °C for 50 sec, and immediately cooled down on ice for 10 min. The transformed cells were further grown at 37 °C for 2 hrs with shaking (200 rpm) and fresh LB media was added. The cells were spread onto an LBA plate containing Kanamycin (50 mg/L) and incubated overnight at 37 °C. The recombinant colony was used for further confirmation by colony PCR. The positive recombinant clone was initially inoculated into LB media (5 mL) containing Kanamycin (50 mg/L) to prepare a primary starter culture by incubating at 37 °C overnight on a shaker incubator at 200 rpm. Then, fresh LB media (100 mL) was inoculated by using 100 µL overnight grown starter culture and further incubated at 37°C with 200 rpm shaker incubator until it reached $O.D_{600}$ of 0.5-0.7. Initially, 1 mL bacterial culture was collected as un-induced control. The remaining culture was used for induction and expression of rAHFV-C. The induction of protein expression was initiated by using an inducer; isopropyl-β-D galactopyranoside (IPTG) (1mM) at 37°C for 6 hrs at 200 rpm in a shaker incubator. The cells were centrifuged at 5000 rpm for 10 minutes at room temperature and cell pellets from both un-induced and induced cultures were further used for cell lysis and protein purification. The expressed recombinant protein was purified by using the Ni-NTA agarose purification system (Ni-NTA Super Flow Kit, Qiagen, Hilden, Germany) following the manufacturer's instructions. The quantity of purified recombinant protein was measured by a nanodrop spectrophotometer as mentioned above, and the quality was determined by analyzing and visualizing on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Results

Sample collection and virus isolation

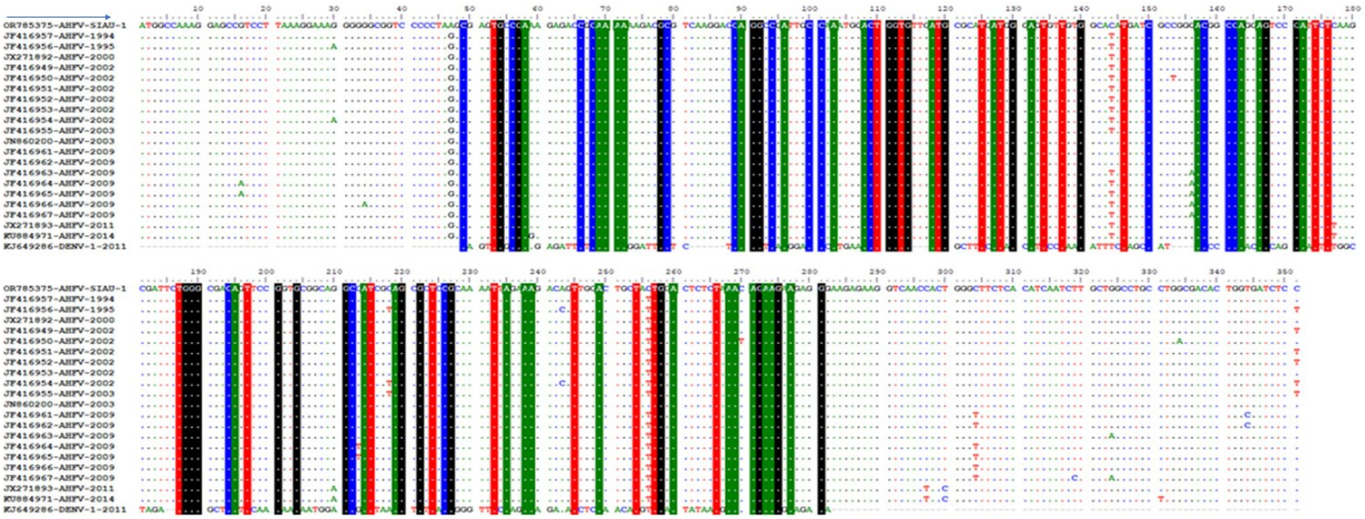
Samples were collected from January 2010 to December 2015. A total of 690 samples were collected and only one case was found that was positive for AHFV infection, which was confirmed by the AHFV-specific RT-PCR assay. The other samples were found to be dengue virus (DENV) positive. Sequencing of the amplified ~232 bp region in the *NS5* gene confirmed the AHFV infection. The positive sample was used for virus inoculation and growth in cells.

Figure 1. RT-PCR amplification of AHFV-Capsid Protein gene and confirmation of clones in pET-28a (+) vector by plasmid DNA PCR, colony PCR, and restriction enzyme digestion. **A:** RT-PCR amplification: M; 1Kb ladder, 1: Amplicon (~350 bp); **B:** Clone confirmation by plasmid DNA PCR; M: 1 Kb ladder, 1: Negative control; 2: Amplicon (~350 bp); **C:** Colony PCR confirmation: M: 1 Kb ladder; 1-5 Recombinant clones (~350 bp); **D:** 1: Negative control; 2-5: Recombinant clones confirmed by restriction enzyme digestion.

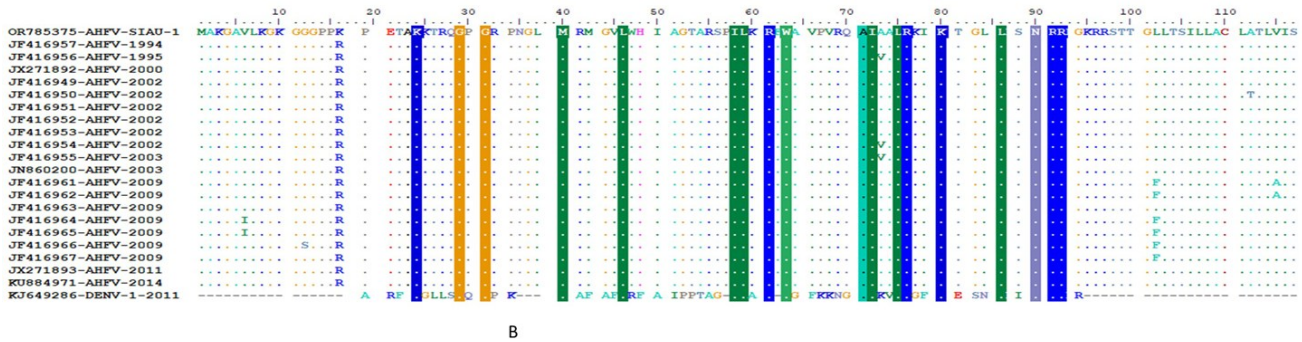


RT-PCR: real time polymerase chain reaction; PCR: polymerase chain reaction; AHFV: Alkhumra hemorrhagic fever virus.

Figure 2. Multiple Sequence Alignment of Capsid Protein gene of AHFV-SIAU-1-KSA (OR785375) with selected AHFV strains. (**A:** Nucleotide Sequences); **B:** Amino acid sequences. The shadings with different colors indicate the homology in all the analyzed sequences and dots in multiple colors denote the similarities and dissimilarities in the sequences of AHFV in both nucleotides and amino acids.



A



B

AHFV: Alkhumra hemorrhagic fever virus.

The inoculated virus which grew successfully in the cells was isolated from the cells and used for RNA isolation.

Viral RNA extraction and real time-PCR

The viral RNA was successfully isolated and purified by using a QIAamp Viral RNA Mini Kit (Hilden, Germany) Qiagen kit following the manufacturer's protocol. Real time PCR using specific primers confirmed the presence of virus.

PCR amplification, cloning, and sequence analysis

The purified RNA was used to amplify the AHFV-CP gene by RT-PCR using specific primers and an amplicon of ~350 bp was obtained and visualized on 1% agarose gel (Figure 1A). The PCR amplified product was cloned into the pET-28a (+) vector. A total of 6 colonies were grown on the LBA plate which contained Kanamycin. The putative recombinant colonies (only five) were used for screening by colony PCR with only one for plasmid DNA PCR confirmation and 4 colonies by restriction enzyme digestion with negative control. Colony PCR confirmed the amplification of AHFV-CP gene (~ 350 bp) in only 4 screened colonies (Figure 1C). Only one plasmid DNA PCR confirmed the amplification of the ~ 350 bp amplicon (Figure 1B) and 4 clones by restriction enzyme digestion (Figure 1D). The positive clone was sequenced, and resultant sequences were initially used for sequence analysis by BioEdit and BLAST. Based on the homology with the AHFV strains submitted in the GenBank, we designated our clone as AHFV-SIAU-1-KSA sequence and deposited it in GenBank (National Center for Biotechnology Information, NCBI) with the accession number OR785375. Multiple sequence alignment analysis of both nucleotide and amino acids sequences of AHFV-SIAU-1-KSA (OR785375) showed very good homology with the selected AHFV isolates from human, animal, and ticks collected in the period 1994–2014. A total of 24 nucleotides and 8 amino acids variations scattered throughout the entire capsid protein (*CP*) gene were observed in the 20 years (1994–2014) (Figure 2A and B).

The nucleotide sequence identity matrix of AHFV-SIAU-1-KSA (OR785375) with other strains showed a variation of sequence identity ranging from 97.0-99.7% sequence similarity. The highest similarity (99.7%) was observed with a AHFV strain reported from Saudi Arabia in the 2003 collection (JN860200) and the lowest (KU884971) was with AHFV-KSA reported in 2014 (KU884971). The Amino acid sequence of AHFV-SIAU-1-KSA (OR785375) strain showed the highest similarity (99.1%) with many strains collected during different periods from various hosts. The similarity ranged from 97.4%-991% with other AHFV strains (Table 1).

The phylogenetic tree analysis based on nucleotide sequence results showed that AHFV-SIAU-1-KSA (OR785375) formed a closed cluster with the AHFV-SCVHF001strain (JN860200), and other strains formed separate clusters with different AHFV strains collected from humans, and *Hyalomma dromedarii* (JF416961) and *Ornithodoros savignyi* (JF416962) ticks (Figure 3A). The amino acid sequences were used to generate a phylogenetic tree of AHFV-SIAU-1-KSA (OR785375) which formed a closed cluster with AHFV (JX271892

Table1. Sequence identity matrix of AHFV-SIAU-1-KSA (OR785375) with other AHFV strains.

S.No	Accession Nos.	Acronym	Hosts	Country	Collection Date	% Identity (NT)	% Identity (AA)
1	JF416957	AHFV	Homo sapiens	KSA	1994	99.1	99.1
2	JF416956	AHFV	Homo sapiens	KSA	1995	98.0	98.2
3	JX271892	AHFV	Homo sapiens	KSA	2000	99.1	99.1
4	JF416949	AHFV	Homo sapiens	KSA	2002	98.8	99.1
5	JF416950	AHFV	Homo sapiens	KSA	2002	98.2	98.2
6	JF416951	AHFV	Homo sapiens	KSA	2002	98.8	99.1
7	JF416952	AHFV	Homo sapiens	KSA	2002	98.8	99.1
8	JF416953	AHFV	Homo sapiens	KSA	2002	99.1	99.1
9	JF416954	AHFV	Homo sapiens	KSA	2002	98.0	98.2
10	JF416955	AHFV	Homo sapiens	KSA	2003	98.5	98.2
11	JN860200	AHFV	Homo sapiens	KSA	2003	99.7	98.2
12	JF416961	AHFV	*Hyalomma dromedarii*	KSA	2009	98.8	97.4
13	JF416962	AHFV	*Ornithodoros savignyi*	KSA	2009	98.8	97.4
14	JF416963	AHFV	*Ornithodoros savignyi*	KSA	2009	98.5	99.1
15	JF416964	AHFV	*Ornithodoros savignyi*	KSA	2009	98.0	97.4
16	JF416965	AHFV	*Ornithodoros savignyi*	KSA	2009	98.0	97.4
17	JF416966	AHFV	*Ornithodoros savignyi*	KSA	2009	98.2	97.4
18	JF416967	AHFV	*Ornithodoros savignyi*	KSA	2009	98.0	98.2
19	JX271893	AHFV	Homo sapiens	KSA	2011	98.0	99.1
20	KU884971	AHFV	Homo sapiens	KSA	2014	97.4	98.2

AHFV: Alkhumra hemorrhagic fever virus; KSA: Kingdom of Saudi Arabia.

and JF416957) strains collected at different collection periods from human samples and all the strains from ticks formed a separate cluster. Interestingly, the amino acids phylogenetic tree analysis showed that the AHFV strains collected in the year 2009 formed separate closed clusters except for only one strain (JF416963) which clustered with other strains collected during 2003 and 2011. Additionally, a separate cluster was observed among human and tick samples collected during different periods. A mixed clustering was observed among the various strains collected during the 1994, 2002 and 2003 collection periods (Figure 3B).

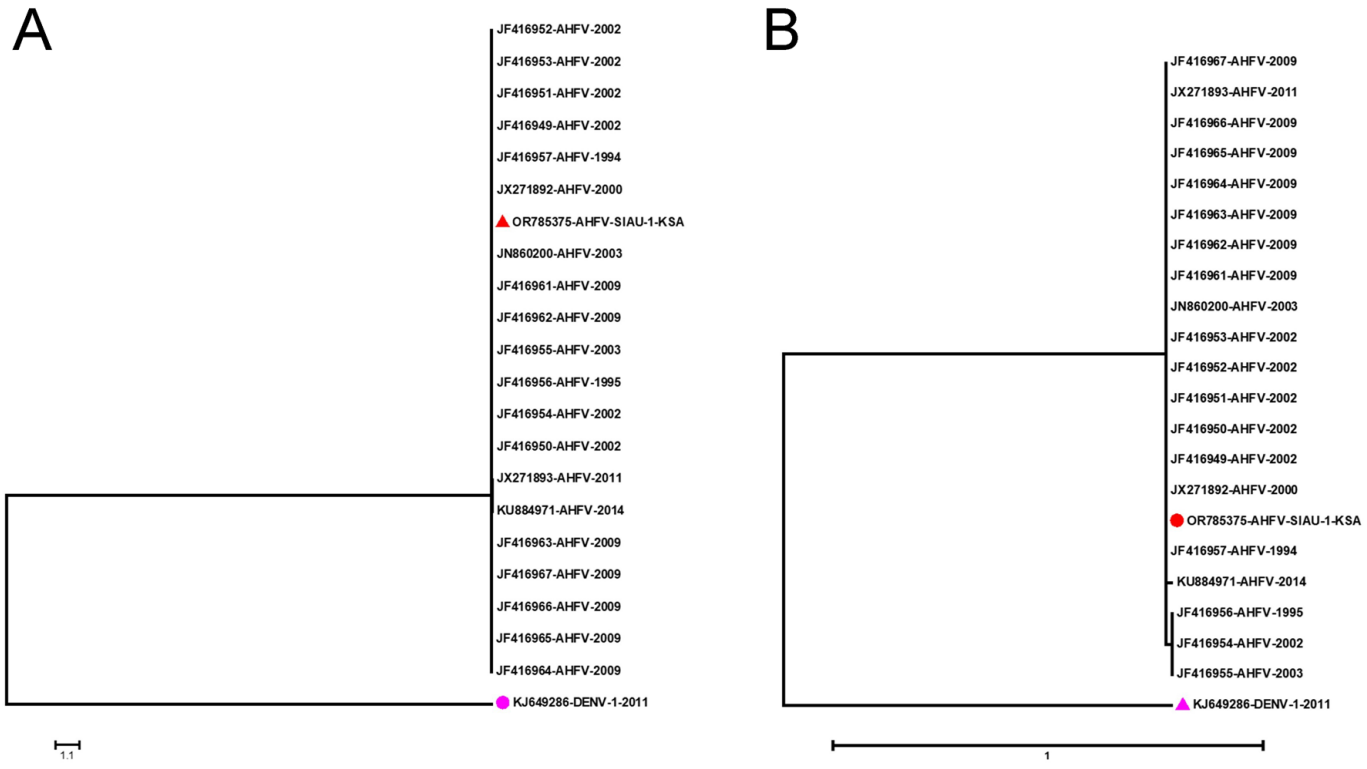
Expression and purification of rAHFV-C protein

The rAHFV-C protein was cloned and expressed in pET28a (+) vector using BL21(DE3) cells. In this work, we successfully expressed and purified the rAHFV-C protein by using the Qiagen-Ni-NTA agarose kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The recombinant protein was visualized and analyzed by SDS-PAGE. Figure 4A shows un-induced and induced proteins and Figure 4B shows the purification of rAHFV-C proteins (size 12.4 kDa), with the highest concentration achieved during elution 4. The purified protein was measured by a nanodrop spectrophotometer and found to be 0.287 ng/mL. The purified protein was used in further experiments for mice immunization to develop antibodies against rAHFV-C protein. The antibodies will be purified and used in serological assay validation and development for the routine screening of AHFV samples collected from humans, animals, and ticks in the near future.

Discussion

AHFV is a distinctive member of the tick-borne *Flavivirus* group. It was first identified in Saudi Arabia and is the first tick-borne *Flavivirus* known to cause hemorrhagic fever in humans [17]. Among the other tick-borne Flaviviruses, Crimean Congo hemorrhagic fever, dengue fever, and Rift Valley fever have been reported in the Makkah and Jeddah regions [18]. In this study, we present the cloning, sequencing, expression, and purification of the recombinant AHFV capsid protein (rAHFV-C) using the pET-28a (+) vector. This study is the first report of cloning, expression, and purification of the AHFV capsid protein in Saudi Arabia. The results demonstrate a close resemblance to previously reported AHFV genome sequences from various hosts and collection periods, with sequence homology ranging from 97.4% to 99.8% with other AHFV isolates. Only minor variations, consisting of 24 nucleotide and 8 amino acid sequence differences, were observed in the capsid protein (CP) gene. However, limited information is available regarding sequence

Figure 3 A. Phylogenetic tree based on nucleotide sequences of AHFV-SIAU-1-KSA (OR785375) and other strains; **B.** Phylogenetic tree based on amino acid sequences of AHFV-SIAU-1-KSA (OR785375) and other strains.

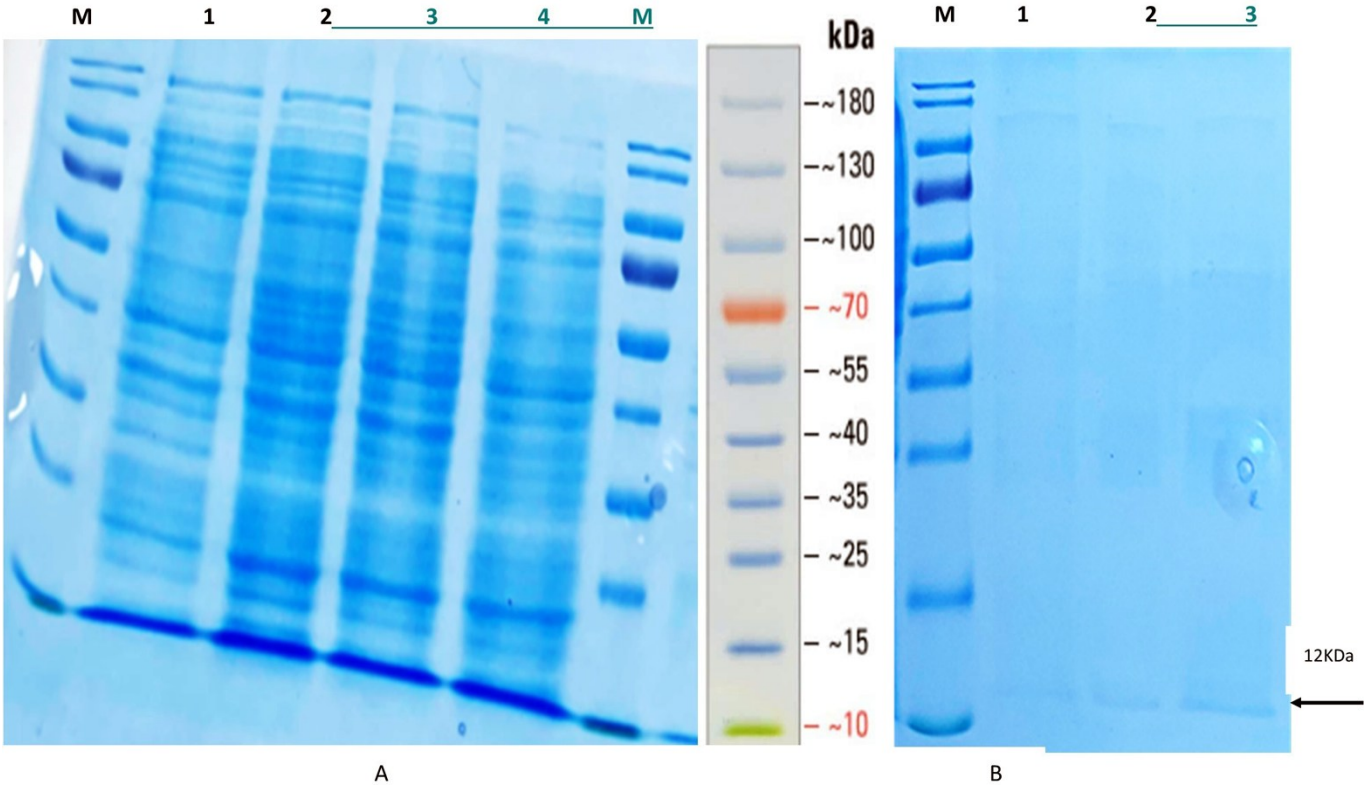


variations in other regions of the genome in AHFV cases reported after 2014. It is crucial to investigate these newly reported AHFV infections and identify potential variations in the full genome sequence, as this information is vital for understanding the emergence of new strains or isolates with altered host range and viral properties. Such sequence variation data will provide insights for the design and development of vaccines, antiviral therapies, and advanced diagnostic technologies. Phylogenetic analysis of both nucleotide and amino acid sequences revealed close clustering with recently sequenced isolates from the SIAU. While complete genome sequences of other AHFV isolates from Saudi Arabia have been previously reported [13,14], the cloning, expression, and purification of the capsid protein gene had not been documented in this region. The relation between Alkhumra virus and other AHFV strains/isolates was confirmed through analysis of the capsid protein gene (CP) sequences. Our findings are consistent with the observations of Dodd *et al.* [19], who reported a deeper evolutionary history for AHFV and Kyasanur Forest disease virus (KFDV) based on previous partial genome analysis. The occurrence of AHFV in Makkah and Jeddah is likely associated with the introduction of a large number of livestock during the Haj season. Genetic diversity, epidemiological data, clinical observations, and laboratory investigations suggest that direct contact with livestock or mosquito bites are the primary risk factors for AHFV infection, and it is anticipated that the pattern and epidemiology of AHFV infection are changing [20-21]. The close phylogenetic relationship between AHFV and KFDV indicates that ticks play a significant role in AHFV transmission. The emergence of AHFV in the mid-1990s and its close genetic similarity to KFDV suggest a recent introduction of KFDV from India as the source of AHFV [22-23]. The changing epidemiological pattern of AHFV may lead to its wider spread and the occurrence of new outbreaks in Saudi Arabia and globally [24].

Conclusions

Based on the findings in this study, it was determined that the currently circulating strains of AHFV in humans, animals, and ticks have the potential to undergo significant mutations in the near future, leading to the emergence of a severe infection and the possibility of an epidemic outbreak in Saudi Arabia.

Figure 4. Expression and purification of rAHFV-C protein in pET-28a(+) vector.



A: Expressed protein. M, protein ladder (10-180kD); 1, un-induced protein; 2, induced protein; **B:** Purified protein. M, protein ladder; 1–4, elution 1, 2, 3, 4 respectively.

Consequently, there is an immediate need for a comprehensive investigation, focusing on the complete genome of the virus. This detailed examination will provide vital insights into its genetic composition, enabling a better understanding of its pathogenicity and potential for transmission. Moreover, the development and utilization of an expressed and purified protein derived from AHFV could prove instrumental in validating and establishing a routine serological in-house assay for the detection of AHFV infection in humans, animals, and ticks. Such an assay would enable effective surveillance and monitoring efforts, facilitating early detection and prompt response to potential outbreaks. Given the urgency and significance of these findings, immediate action is warranted to address these research gaps and prepare the healthcare system for any future AHFV-related challenges.

Acknowledgements

The authors are grateful for the facilities provided by Special Infectious Agents Unit, King Fahd Medical Research Centre, King Abdulaziz University, Jeddah, Saudi Arabia. The authors thank Norah A Othman and Tagreed AL-Subhi for sequencing of the viral genome.

Authors' contributions

AMH, sample collection and processing; NMA, methodology, validation, data curation; writing and editing; MAG, review and editing; SSS, software, validation, data curation, formal analysis, writing—original draft and final editing; EIA, supervision, project administration; investigation, resources, funding acquisition. All authors have read and agreed to publish this manuscript.

Funding

The authors acknowledge the generous charitable donation from the Late Sheikh Ibraheem Ahmed Azhar in the form of reagents and supplies as a contribution to the scientific research community.

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Conflict of interests: No conflict of interests is declared.