RESEARCH ARTICLE

Phylogenetic characterization of circulating Dengue and Alkhumra Hemorrhagic Fever viruses in western Saudi Arabia and lack of evidence of Zika virus in the region: A retrospective study, 2010-2015

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Flaviviruses represent a global public health concern. They consist of ∼70 viruses with almost half of them causing human diseases with unspecified febrile illnesses. Cities in western Saudi Arabia are endemic for viruses (DENV) with sporadic infections due to Alkhumra hemorrhagic fever virus (AHFV). They also represent a major destination for travelers coming for annual religious pilgrimages (Hajj and Umrah) from all over the world. However, whether other flaviviruses are circulating is not known because of the limited number of surveillance studies. Here, we retrospectively screened 690 samples for flaviviruses in samples from patients with unexplained febrile illnesses between 2010 and 2015 in western Saudi Arabia using a pan-flaviviruses RT-PCR assay. Despite Zika virus RNA was not detected, this study confirms circulation and/or sporadic spread of DENV-2, DENV-3, and AHFV, higher prevalence of DENV-2, and a role for visitors from DENV endemic countries in DENV importation into the Kingdom. Further analysis also showed very low genetic diversity of AHFV confirming its slow microevolution. Accordingly, continuous and prospective surveillance for flaviviruses using such assay are warranted in Saudi Arabia which receives millions of Muslims annually to implement effective control measures in light of the global widespread and outbreaks of several flaviviruses.

KEYWORDS
Alkhumra hemorrhagic fever, dengue, flaviviruses, Saudi Arabia

1 | INTRODUCTION

Clinical manifestations of viral febrile illness include a wide range of non-specific symptoms which makes it almost impossible to determine the etiological agents based on the clinical picture. Accurate and timely diagnosis of these viral pathogens is critical because of the measures that have to be implemented for the monitoring and management of the patient and for the prevention of secondary cases or nosocomial spread. Early identification of viral species also has a great impact on public health initiatives, as we experienced with severe acute respiratory syndrome-coronavirus (SARS-CoV) or currently experiencing with recently emerging or re-emerging viruses such as the Middle East respiratory syndrome coronavirus (MERS-CoV) and Zika virus (ZIKAV). Most of these viral pathogens are of zoonotic origin and can be transmitted to humans either directly such as filoviruses and arenaviruses or via arthropod

vectors (mosquitoes, ticks, midges, sandflies) such as flaviviruses and bunyaviruses (arboviruses).

The genus *Flavivirus* in the family *Flaviviridae* comprises at least 70 known and tentative viruses.[1] More than half of these viral species are considered human pathogens causing encephalitis, hemorrhagic fever, or biphasic fever.[2] While most flaviviruses are vector (mosquito or tick)-borne viruses, some species are of unknown vector.[3,4] Flaviviruses include major vertebrate pathogens such as West Nile virus (WNV), Dengue viruses (DENV 1-4), Yellow fever virus (YFV), ZIKAV, Alkhumra hemorrhagic fever virus (AHFV), Tick-borne encephalitis virus (TBEV), and Japanese encephalitis virus (JEV).[2] Furthermore, several studies suggest that this genus may contain larger number of viral species yet to be discovered. This is supported by the discovery of new arboviruses[5–12] and novel and highly divergent flaviviruses such as Tamana bat virus,[13] Ngoye virus,[14] Nounané virus (NOUV),[15] and Lammi virus (LAMV).[16]

In Saudi Arabia, at least five vector-borne flaviviruses are circulating including DENV-1, DENV-2, DENV-3, DENV-4, and AHFV in addition to two other arboviruses (Rift Valley fever virus [RVFV] and Crimean-Congo haemorrhagic fever virus [CCHF]).[17–25] These viruses are known to be endemic and causing outbreaks or sporadic infections in several regions in Saudi Arabia. Cities such as Jeddah, Makkah, and Al-madinah in western Saudi Arabia are endemic for DENV and AHFV, and represent a major destination for pilgrims coming for annual Hajj and/or Umrah pilgrimages from all over the world. This clearly poses a great concern about the possible introduction of new viruses into the Kingdom [Azhar et al., 2016].[24] Although there have been no reports of Zika infection in Saudi up to now, the current outbreak in South and Central America as well as the heavy travel to Saudi from all over the world may put the Kingdom at the front-line in the fight against ZIKAV particularly with the high prevalence of *Aedes aegypti* which is the vector for both Zika and dengue viruses.[26] Furthermore, it is not clear whether other flaviviruses do exist in Saudi Arabia or not mostly because of the limited number of surveillance studies and the lack of systematic screening of patients with unexplained febrile illnesses, thus substantial number of cases may remain undiscovered or unrecognized.

Most of the currently available viral diagnostic tests are based on specific target testing for a single viral type. Therefore, hypothesis-driven diagnostic approaches are usually undertaken when a particular virus is suspected. However, these conventional methods have some drawbacks in identifying novel, altered or unpredictable viruses which represent a big challenge in diagnostic virology. This is further complicated when broad range of viruses are expected as in the case of viral febrile illness. Therefore, genus-specific diagnostic approaches can be used because they are less sensitive to failure compared to targeted molecular approaches. Several assays based on molecular amplification of highly conserved fragments in the NS3 and NS5 regions of flaviviruses have been developed[27–37] and proven to be a valuable tool in the discovery of new classical and atypical flaviviruses. However, most of these techniques depend on the use of conventional RT-PCR format, nested PCR, and agarose gel electrophoresis which could hamper the sensitivity and specificity of these assays due to PCR contamination and false-positive results. Moureau et al developed a single-step universal flaviviruses (pan-flaviviruses) real-time RT-PCR which was able to identify at least 54 different known or tentative flaviviruses.[38] This assay combines the SybrGreen format of the real-time PCR which facilitate large-scale testing without PCR contamination risk, and the universal detection of known and tentative flavivirus species. The sensitivity of this assay was found to be high (>50 copies/reaction) which permitted the detection of several flaviviruses from field and clinical samples. Most importantly, sequencing of the short NS5 fragment amplified by this assay (~232 nts) is suitable for reliable identification and taxonomic characterization of flaviviruses as well as the discovery of novel viral species. Therefore, this study was designed not only to screen for possible circulation and/or introduction of new or previously uncharacterized flaviviruses such as ZIKAV but also to phylogenetically characterize circulating DENV in the western region of Saudi Arabia using this pan-flaviviruses assay.[38]

2 | MATERIALS AND METHODS

2.1 | Samples

A total of 690 human plasma samples collected over a period of 6 years between January 2010 and December 2015 were retrieved from the Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. All samples were from patients with undiagnosed acute viral febrile illness. All samples were anonymized before the study, checked and confirmed retrospectively from hospital records to be negative for DENV by RT-PCR (Table 1). Ethical approval was obtained from the biomedical ethics unit at King Abdulaziz University Hospital.

TABLE 1 Serological details of tested samples against dengue virus

Test positivity	2010	2011	2012	2013	2014	2015	Total
IgM	1	7	6	52	24	5	1
IgM + IgG	5	3	2	29	14	1	5
IgG	0	0	0	1	1	0	0
Negative	16	84	78	130	149[a]	82	16
Total no of tested samples	22	94	86	212	188	88	690

[a]AHFV was detected in one sample.

2.2 | Pan-flaviviruses real-time RT-PCR

RNA was extracted from samples using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions and as previously described.[39] Eluted viral RNA was used in a one-step real-time RT-PCR as previously described.[38] In brief, viral RNA was amplified with the following NS5-specific primers: PF1-S 5′-TGYRTBTAYAACAT-GATGGG-3′ and reverse primer: PFR2-BIS 5′-GTGTCCCAICCNGC NGTRTC-3′ using QuantiTect SYBR Green RT-PCR (Qiagen) on a RotorGene Q real time PCR machine (Qiagen). Positive samples were re-screened with AHFV and DENV-specific real-time RT-PCR assays using primers and probes as previously described.[40,41]

2.3 | NS5 and full genome sequencing

NS5 regions were re-amplified using conventional RT-PCR with PF1 and PFR2 primers using one-step RT-PCR kit (Qiagen). Products were separated on 1.5% agarose gel, and bands (∼232 nts) were excised from the gel and purified using QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions. Cycle sequencing was performed on an ABI 3500 Automatic Sequencer (Applied Biosystems, Foster City, CA) using PF1 and PFR2 primers and Bigdye Terminator V3.1 Reaction Cycle Kit (Applied Biosystems) according to manufacturer's instructions. Complete genome of AHFV was sequenced as previously described.[20] All generated sequences were deposited in GenBank database with the following accession numbers for DENV-2 (KU886699, KU886700, KU886701, KU886702, KU886703, KU886704, KU886705, KU886706, KU886707, KU886708, KU886709, KU886710, KU886712, and KU886713), for DENV-3 (KU886711) and for AHFV (KU884971).

2.4 | Phylogenetic and genetic analysis

Phylogeny reconstruction was performed by using the Maximum Likelihood statistical method based on Kimura 2-parameter substitution model or Tamura-Nei model. The trees with the highest log likelihood are shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the NJ/BioNJ method to a matrix of pairwise distances estimated using the Nearest-Neighbor-Interchange (NNI) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated. Evolutionary analyses were conducted in MEGA6.[42] Complete AHFV sequences were downloaded from the GenBank and multiply aligned using ClustalW integrated in Geneious R7 software[43] and nucleotide sequences identity matrix as well as amino acid substitutions were analyzed.

3 | RESULTS

3.1 | Circulating DENV-2, DENV-3, and AHFV viruses in western Saudi Arabia

All tested samples were retrieved from archived samples collected between January 2010 and December 2015 from patients with dengue-like symptoms or acute febrile illnesses of unknown source (Table 1). As shown in Table 1, the pan-flaviviruses assay showed that only one sample was positive for flaviviruses. Sequencing of the obtained ∼232 nts region in the NS5 gene of this sample confirmed these results and showed that it was AHFV. It is of note that this sample was only tested for DNEV previously and retesting it with AHFV-specific RT-PCR assay confirmed our results. To further confirm the usefulness of this pan-flaviviruses RT-PCR assay as a diagnostic and screening tool in epidemiological studies, we tested and sequenced 15 known DENV positive samples collected during the same period. Blasting of the obtained NS5 sequences showed that 14 out of the 15 sequences belong to DENV-2 and the remaining sequence belong to DENV-3, suggesting that using this pan-flaviviruses RT-PCR assay could represent a very useful screening and surveillance molecular tool for new or under-characterized flaviviruses. These data also indicate the high prevalence of DENV-2 and sporadic transmission of AHFV in western Saudi Arabia.

3.2 | Cosmopolitan DENV-2 genotype is widespread in western Saudi Arabia

In order to test the suitability of the amplified amplicon of NS5 region (∼232 nts) in phylogenetic and evolutionary analyses and to investigate the relationship between circulating DENVs in western Saudi Arabia and those circulating elsewhere, we performed phylogenetic analysis using obtained DENV sequences and corresponding regions from representative NS5 sequences in the GenBank database. Analysis of all DENV-2 NS5 sequences showed that all the identified viruses between 2010 and 2015 in this study are from the currently circulating Indian subcontinent lineage of the Cosmopolitan genotype in Saudi Arabia in which they clustered with strains isolated between 2011 and 2013 from Pakistan (Fig. 1). Similarly, the detected DENV-3 virus clustered with isolates from India with close proximity to other stains from Sri Lanka and Singapore (Fig. 2) in genotype GIII.

3.3 | Low diversity of circulating AHFV in Saudi Arabia

Upon identification of AHFV from one archived sample collected in 2014 by sequencing of the NS5 fragment, full genome sequence of this isolate (2014-KU884971-Saudi-Human) was obtained. Multiple alignment of this 2014 strain with other available complete AHFV sequences in the GenBank database showed high homology with 99.7% identity with the last isolate of AHFV obtained in 2011 in Saudi Arabia (2011-JX271893-Saudi-Human). Although these two most recent isolates showed several similar but unique synonymous mutations scattered throughout the genome compared to other AHFV sequences (Table 2), only four unique non-synonymous mutations in the coding region resulted in distinctive amino acid substitutions in these two isolates that were not observed in other isolates (Table 3). Interestingly, 3 out of these four changes were in the NS5 protein. Nonetheless, the 2014-KU884971-Saudi-Human isolate also had several scattered unique mutations compared to its closest match (Table 2) but most of these changes were synonymous with five
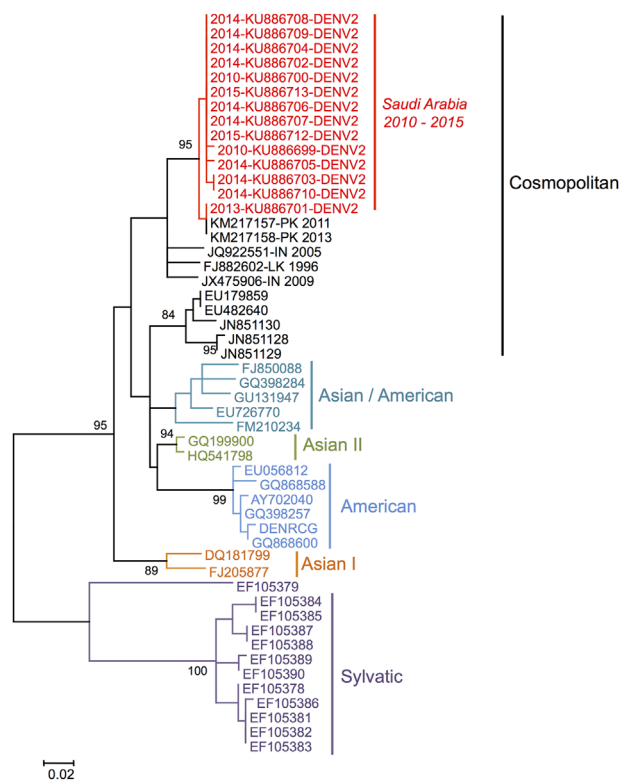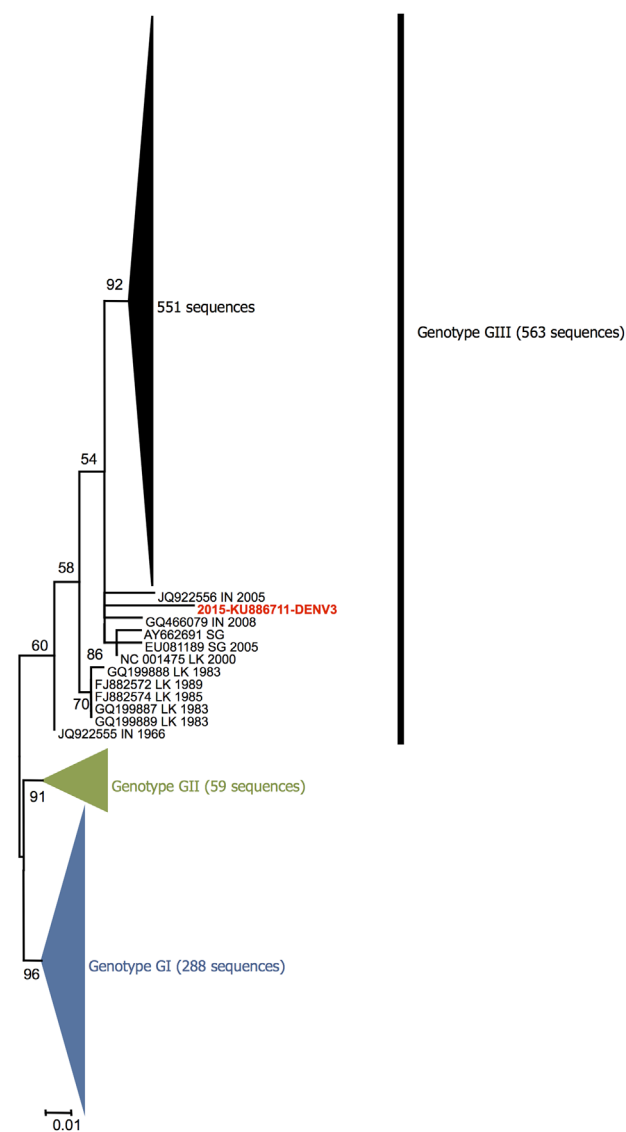
FIGURE 1 Phylogenetic analysis of DENV-2 viruses. Phylogenetic tree was constructed using Maximum Likelihood statistical method based on Kimura 2-parameter substitution model. A Gamma distribution with Invariant sites (G + I) was used to model evolutionary rate differences among sites (5 categories). Codon positions included were 1st + 2nd + 3rd + Noncoding. Tree was based on sequences of the amplicon obtained from NS5 gene and involved 51 nucleotide sequences. There were a total of 208 positions in the final dataset. Number of bootstrap replicates was 500. Related sequences are identified by GenBank accession number, country of isolation and the year of isolation

FIGURE 2 Phylogenetic analysis of DENV-3 viruses. Phylogenetic tree was constructed using Maximum Likelihood statistical method based on Kimura 2-parameter substitution model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories). Codon positions included were 1st + 2nd + 3rd + Noncoding. Tree was based on sequences of the amplicon obtained from NS5 gene and involved 910 nucleotide sequences. There were a total of 204 positions in the final dataset. Number of bootstrap replicates was 500. Sequences are identified by GenBank accession number, country of isolation and the year of isolation

unique amino acid changes only that have never been observed before (Table 3). Phylogenetic and multiple alignment analysis of all available AHFV sequences including the current isolate (24 sequences) showed close clustering of all sequences with nucleotide and amino acid identity matrixes ranging from 98.48% to 99.70% and 99.21% to 99.82%, respectively (Fig. 3), suggesting low diversity of circulating AHFV in the region and confirming its slow microevolution.

4 | DISCUSSION

Flaviviruses are highly diverse with ~70 known and tentative species.[1] Many of these viruses are human pathogens causing a wide range of clinical symptoms.[2] In Saudi Arabia, at least five flaviviruses are circulating including DENV-1, DENV-2, DENV-3, and AHFV[18-20,22-25] in addition to recent detection of DENV-4 in Jeddah (personal communication). However, whether other flaviviral species are circulating in the Kingdom is not known because of the few number of surveillance studies. Several emerging and re-emerging flaviviruses

such as ZIKAV are being reported and characterized globally. This is of great concern especially for countries such as Saudi Arabia where the largest annual mass-gathering event (Hajj) may exacerbate the spread of these viruses all over the world. Moreover, the similarity in clinical signs and symptoms of viral febrile illnesses and the use of species-specific diagnostic assays add several limitations to the ability to differentiate between causative viruses especially when several viruses are co-circulating. Therefore, accurate and timely identification of causative viruses is critically important in order to minimize the risk of human-to-human transmission, introduction of new viruses and potential outbreaks or epidemics.

TABLE 2 Nucleotide and amino acid identity of the AHFV 2014 isolate full genome compared to other known isolates

	Nucleotide		Amino Acid	
Strains	Identity (%)	Differences (#)	Identity (%)	Differences (#)
2011-JX271893-Saudi-Human	99.70	31	99.82	6
1994-JF416957-Saudi-Human	99.10	92	99.44	19
1995-AF331718-Saudi-Human	99.05	97	99.42	20
1995-NC_004355-Saudi-Human	99.05	97	99.42	20
2000-JX271892-Saudi-Human	99.01	102	99.36	22
2002-JF416952-Saudi-Human	99.00	103	99.42	20
2002-JF416953-Saudi-Human	98.97	106	99.33	23
2002-JF416950-Saudi-Human	98.94	109	99.36	22
1995-JF416956-Saudi-Human	98.90	113	99.30	24
2002-JF416954-Saudi-Human	98.89	114	99.27	25
2002-JF416951-Saudi-Human	98.89	114	99.27	25
2003-JF416955-Saudi-Human	98.86	117	99.30	24
2003-JN860200-Saudi-Human	98.85	118	99.27	25
2002-JF416949-Saudi-Human	98.84	119	99.27	25
2009-JF416961-Saudi-*Hyalomma dromedarii*	98.75	128	99.21	27
2009-JF416962-Saudi-*Ornithodoros savignyi*	98.75	128	99.21	27
2009-JF416963-Saudi-*Ornithodoros savignyi*	98.71	132	99.27	25
2009-JF416966-Saudi-*Ornithodoros savignyi*	98.65	138	99.24	26
2009-JF416964-Saudi-*Ornithodoros savignyi*	98.65	138	99.24	26
2009-JF416965-Saudi-*Ornithodoros savignyi*	98.65	138	99.24	26
2009-JF416967-Saudi-*Ornithodoros savignyi*	98.60	144	99.27	25
2010-JX914664-Egypt-Human	98.54	150	99.24	26
2010-JX914663-Egypt-Human	98.48	156	99.30	24

In this study we sought to utilize a pan-flaviviruses real-time RT-PCR assay[38] to screen for flaviviruses in samples collected retrospectively from undiagnosed patients with suspected viral febrile illnesses. While we did not find any evidence of ZIKAV nor any unexpected flaviviruses in the current study, the potential introduction of such viruses into Saudi is highly possible during Hajj and Umrah pilgrimages especially with the rapidly increasing number of Zika cases. Therefore, further surveillance studies are necessary to confirm the absence of ZIKAV in Saudi Arabia. This is of utmost importance considering the Hajj and Umrah mass gatherings and the continuous travelling to and from Saudi Arabia all year around.

Our findings showed that DENV-2, DENV-3, and AHFV are circulating or sporadically being transmitted during the 6-year period of the study between 2010 and 2015 in western Saudi Arabia. DENV-2 was the most detected virus during this period confirming its higher prevalence as previously reported.[18,44,45] The close clustering of DENV-2 and DENV-3 isolates reported in the current study with strains from Pakistan and India demonstrate that several DENV viruses might have been imported into Saudi Arabia from hyper-endemic regions with travelers and pilgrims which provide further evidence to support our previous conclusions.[24,25] In addition, our data show that the DENV-2 Cosmopolitan genotype and DENV-3 genotype GIII are the only reported genotypes from Saudi Arabia so far since their first isolation in 1994.[24] DENV-1 is known to be one of the circulating viruses in Saudi Arabia,[18,25,44,45] and few cases of DENV-4 infection have been detected in Jeddah in 2015 (personal communication).

TABLE 3 Amino acid substitutions observed in the AHFV 2014 isolate compared to other isolates

Gene	CP	preM	NS1	NS1	NS5	NS5	NS5	NS5	NS5
Position	20	224	785	1126	2644	2664	2669	2962	3323
2014-KU884971	R	R	T	A	N	S	A	G	T
2011-JX271893[a]	K	K	T	S	N	S	V	G	M
Other strains[b]	K	K	A	S	D	N	V	A	M

[a]Most related strain.
[b]Includes all available full genome sequences from the GenBank.
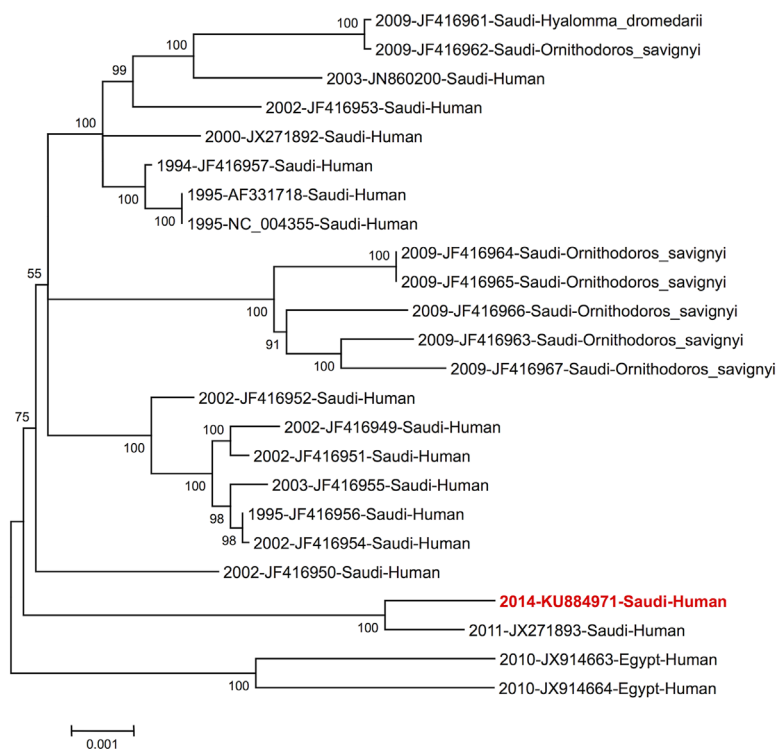
FIGURE 3 Phylogenetic analysis of AHFV viruses. Phylogenetic tree was constructed using Maximum Likelihood statistical method based on Tamura-Nei model. Uniform rates were used to model evolutionary rate differences among sites. Codon positions included were 1st + 2nd + 3rd + Noncoding. Tree was based on full genome sequences and involved 24 nucleotide sequences. There were a total of 10249 positions in the final dataset. Number of bootstrap replicates was 500. Sequences are identified by year of isolation, GenBank accession number, country of isolation and species

However, we did not detect any DENV-1 or DENV-4 in the tested samples. While these results may suggest low circulation of these viruses, more samples should be tested to draw such conclusion. Nonetheless, our results are consistent with the known endemicity of DENV in western region of Saudi Arabia.

AHFV is a newly characterized flavivirus first isolated in Jeddah, Saudi Arabia, in 1995.[46] Until now, AHFV has been isolated sporadically from humans and ticks in Saudi Arabia and Egypt and shown to have high mortality rate (~25%).[20,47,48] Whether the reported numbers of cases reflect the actual incidence of AHFV infections is not known mostly due to the limited epidemiological studies and/or misdiagnosis. The isolation of AHFV in the current study from one of the samples which was originally examined for DENV only clearly provides an evidence that many cases of AHFV are being missed during clinical testing. Thus, implementation of assays such as the one used in this study could be an important diagnostic and research tool. The observed low diversity and slow microevolution of AHFV is consistent with the sporadic nature of the infection and the limited number of reported cases, most probably due to the less mobile vector (ticks). Furthermore, while this low genetic diversity suggests that current molecular tests are still suitable for AHFV diagnosis, it is critical to carry out more genomic studies not only to enhance diagnostic tools but also to better understand the replication and pathogenesis of AHFV and to develop vaccines and therapeutics.

Compared to other assays,[27–37] the assay used here has been experimentally tested against all recognized flaviviruses at the time of

its development and proven to be suitable not only for the detection of ZIKAV but also all dengue serotypes in clinical samples from suspected patients.[49,50] Therefore, implementation of such assay in diagnostic laboratories could provide a great tool to detect any unexpected flaviviruses or when several viruses are expected with very short turnaround time compared to using several species-specific assays. Furthermore, larger prospective and continuous studies using such assay are also required to understand and examine the true diversity of circulating flaviviruses in Saudi Arabia and other regions in order to implement and develop public health measures and disease control strategies.

CONFLICTS OF INTEREST

None.

AUTHORS' CONTRIBUTION

SAE, RNC, EIA, and AMHash conceived and designed the experiments. MSA, SAF, TLA, NAO, HGBH, MMA and AMHass performed the

experiments. MSA, SAE, AA, RNC, and AMHash analyzed the data. SAE, RNC, EIA and AMHash wrote the paper.

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