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Serotypes of dengue viruses circulating in Jazan region, Saudi Arabia

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ABSTRACT

Dengue fever is considered to be the most important mosquito-borne disease and considered as endemic disease in Jazan region, Saudi Arabia. The present study aimed to analyze the prevailing dengue virus serotypes for the first time in the region. Serum samples of 220 suspected dengue cases were collected throughout 2016 and tested by one step Reverse Transcription Polymerase Chain Reaction (RT-PCR) with a set of specific primers for detection of four dengue virus serotypes followed by sequencing the PCR products to confirm the results. Out of the 220 serum samples, 124 were found positive for dengue infection (56.4%). Three dengue virus serotypes were detected; DEN-1, DEN-2 and DEN-3. DEN-2 is the most common and predominant type in the region rating 83.9% (104/124), followed by DEN-1 8.9% (11/124), and then DEN-3 7.2% (9/124). The high seroprevalence of dengue virus infections in Jazan region indicates its endemicity. The present study highlights the importance of tracking the spread of dengue virus types and its implication for analyzing changes in dengue endemicity in specified areas over time. Complete genome sequencing is required for the three detected dengue virus serotypes circulating in the region (DEN-1, DEN-2, and DEN-3) to serve as references for any future epidemiological researches and/or outbreaks.

KEY WORDS: DENGUE FEVER, SEROTYPES, 1, 2, AND 3, JAZAN REGION, SAUDI ARABIA

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INTRODUCTION

Dengue fever is considered to be the most important re-emerging vector-borne disease worldwide and is endemic in more than 125 countries (Murray *et al.*, 2013). Four hundred million of cases are estimated to occur annually (CDC, 2016). Dengue is a viral disease transmitted to humans by the bite of infected females of the main vector *Aedes aegypti* and to a lesser extent by *Aedes albopictus* mosquitoes (WHO, 2009).

There are five genetically related but antigenically distinct single-stranded RNA serotypes belonging to *Flaviviridae* family and genus *Flavivirus*; DEN-1, DEN-2, DEN-3, DEN-4 (WHO, 1997), and DEN-5 (Mustafa *et al.*, 2015).

However, no cross protection occurred between the dengue serotypes, the immunity is serotype specific.

According to disease severity, the World Health Organization has classified dengue into three categories; Dengue Fever (DF), Dengue Hemorrhagic Fever (DHF), and Dengue Shock Syndrome (DSS) (WHO, 1997).

Unplanned urbanization and climatic factors, including high temperatures and rainfall, might contribute to epidemics of dengue (Mackenzie *et al.*, 2004; Crowell *et al.*, 2011; Banu *et al.*, 2011).*Aedes* mosquito is found in the urban settings, especially in tropical areas, where it maintains a sustainable relationship with humans leading to reemergence of dengue infections and creating a public health threat (Glenn and Sia, 2008).Spatial patterns in the recent and sequential circulation of DEN1-5, along with the host and virus genetics, should be regarded as potential population risk factors for sever forms of dengue fever (Guilarde *et al.*, 2008; Chaturvedi 2006) because most secondary infection bearing heterologous dengue virus type may lead to severe disease complications (Green and Rothman, 2006; Vaughn *et al.*, 2000; Gibbons and Vaughn, 2002; Rico-Hesse, 2003).

In Saudi Arabia, the first dengue outbreak has been reported in 1994 in Jeddah with 289 confirmed cases, and DEN-1 and DEN-2 were circulating dengue virus serotypes (Fakeeh and Zaki, 2001). Since then, several dengue fever outbreaks have been recorded in Saudi Arabia (Fakeeh and Zaki, 2003; Ahmed, 2010; Khan *et al*, 2008; Ayyub *et al.*, 2006; El-Badry *et al.*, 2013; Zaki *et al.*, 2008) and Yemen (Madani *et al.*, 2013). The case fatality rate was 4.6 per thousand in 2007 (Saudi Ministry of health, 2007).The incidence of dengue fever has increased in Saudi Arabia during the past few years; 6512 cases in 2013; 2081 cases in 2014; and 4312 cases in 2015 (Saudi Ministry of health, 2016).

The emergence of DEN-3 in Jeddah was in 1997 (Fakeeh and Zaki, 2001), and since then all the 3 dengue serotypes (DEN 1–3) were being circulated in the city (Azhar *et al.*, 2015). Recently, Organji *et al* (2017) have reported DEN-1, DEN-2, and DEN-3 to be circulated in Makkah city. In Jazan region, there were 1790 confirmed dengue cases between 2005 and 2016 with highest outbreaks in 2016 (555 cases), followed by 2010 (290 cases), and 2012 (289 cases) (Dengue control program in Jazan). Al-Arzaqi *et al* (2013) reported dengue prevalence of up to 26.5% in Jazan region, while Gamil *et al* (2014) noted 47.74% dengue positivity rate in the area.To the best of our knowledge, no data has been published on the circulation of dengue virus serotypes in Jazan region, thus our present study is the first of its own and aimed to analyze the prevalence of dengue virus serotypes circulating in Jazan region, southwest of Saudi Arabia.

MATERIAL AND METHODS

Study area: Jazan Region in Southwest Saudi Arabia lies between 16°-12, and 18°-25, latitude north. It is bordered in the South by Arabic republic of Yemen with total area of about 22,000 km² and 1.3 million populations (census 2011). Thirty percent of the population concentrated in six major cities, and the remainders living in over 3500 villages (Alsheikh, 2011). Jazan region is situated in the subtropical zone and has average monthly temperatures ranging between 25.8°C in January to 33.4°C in July. The average relative humidity ranges between 55% and 72.5%. The rainy season is started at August through October with a monthly average of 77 and 56.7 mm, respectively (Alsheikh, 2011). Jazan is divided into eleven small Governates (Al-Aridah, Damad, Twal, Al-Ahad, Jazan , Al-Khobah, Samttah, Abuareesh, Sabyah, Beash and Al-Darb), these locations (Fig.1) although with different altitudes and geographical Characteristics, they are almost share the same demographical, agricultural, educational, cultural, housing, health system, and environmental characteristics.

Sampling: During 2016 about 220 suspected dengue fever patients serum samples included in this study were collected from five different hospitals in Jazan region and stored at -80° till further use.

RNA isolation: High Pure Viral Nucleic Acid Kit from Roche applied science (Germany) used for extraction of RNA follow the manufacture procedure; 200 µl of binding buffer supplemented with poly (A) and 50 µl Proteinase K added to 200 µl of serum sample then mixed immediately and incubated for 10 minutes at 72°C. Addition of 100 µl Proteinase K was mixed with sample and transferred to High Filter Tube inserted into Collection Tube. After centrifugation for 1 minute at 10000 rpm, the collection tube was discarded. The filter tube combined with new collection tube and 500 µl of inhibitor removal buffer was added and centrifuged for 1 minute at 10000 rpm. After changing collection

FIGURE 1. Map of Jazan region showing the different Governates.

tube, the high filter tube washed twice by adding 450 µl of wash buffer at the same condition of centrifugation, followed by centrifugation for 15 seconds at 13000 rpm to remove any residual wash buffer. Then the high filter tube was inserted into nuclease free, sterile 1.5 ml centrifuge tube and 50 µl of elution buffer was added to elute the viral nucleic acid by centrifugation at 10000 rpm for 1 minute.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

One step RT- PCR is a rapid, sensitive, and simple for dengue serotype-specific diagnosis method. The test was performed according to the protocol of Lanciotti *et al* (1992) with some modification; DEN consensus primers and serotype-specific primers (Table 1) were used to amplify the viral genome in this study and synthesized in Integrated DNA Technology (Belgium). The one step RT-PCR reactions were performed according to access RT-PCR–system protocol (Promega-USA) in total volume of 50 µl containing 10 µl of AMV/Tfl 5X Reaction Buffer, 1 µl of dNTP Mix (10mM each dNTP, final concentration 0.2mM), 2 µl of 25mM MgSO4 (final concentration 1mM), 1 µl of AMV Reverse Transcriptase 5u/µl (final concentration 0.1u/µl), 1 µl of Tfl DNA Polymerase 5u/µl (final concentration 0.1u/µl), 50pmol (final concentration 1µM) of each forward (D1) and reverse (D2) primers, 5 µl of RNA virus and nuclease free water to total volume 50 µl. The thermal cycling incubations temperatures programmed as follows: incubation for 1 hour at 42°C (to convert the RNA to cDNA) then initial denaturation for 3 minutes at 94°C followed by 35 cycle of denaturation (94°C, for 30 second), primers annealing (55°C for 1 minute), primer extension (72°C for 2 minutes) and final extension for 5 minutes.

Nested-PCR

Nested PCR was performed in 2 tubes for each sample in 50 µl reaction mixture containing 25 µl GoTag®G2 green master mix ready to use from Promega, 10 µl of the diluted (1:100) RT-PCR product, 50 pmol (final concentration 1µM) of each forward primer D1 and TS1, TS3 as reverse primers for the first tube and TS2, TS4 as reveres primers for another tube. The samples were subjected to initial denaturation at 94°C for 3 minutes, 30 cycles of denaturation (94°C, 30 s), primer annealing (55°C, 1 min), primer extension (72°C, 2 min) and final extension for 5 minutes. In each run negative and positive controls were included. The PCR products of nested amplification were analyzed by gel electrophoresis (1.5 agarose in Tris-Acetate EDTA buffer) staining with ethidium promide. The visualization was carried out using Gel Doc XR Imaging System (Bio-Rad).

Sequencing and bioinformatics analysis

Purification and standard sequencing for RT- PCR products were performed by Macrogen Company (Seoul, Korea). Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using D1 (forward) primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with Big Dye®X Terminator™ purification protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). The sequences were searched for sequence similarity through BLAST

Table 1. oligonucleotide primers used in RT-PCR and Nested-PCR			
primer	**Sequence 5 - 3**	**Genome position**	**Size in bp**
D1	TCAATATGCTGAAACGCGCGAGAAACCG	134-161	511
D2	TTGCACCAACAGTCAATGTCTTCAGGTTC	616-644	511
TS1	CGTCTCAGTGATCCGGGGG	568-586	482 (Dl and TS1)
TS2	CGCCACAAGGGCCATGAACAG	232-252	119 (Dl and TS2)
TS3	TAACATCATCATGAGACAGAGC	400-421	290 (Dl and TS3)
TS4	CTCTGTTGTCTTAAACAAGAGA	506-527	392 (Dl and TS4)

Table 2. Results of dengue virus serotyping using RT-PCR and nested-PCR					
No of samples	**+ve DEN**	**+ve DEN-1**	**+ve DEN-2**	**+ve DEN-3**	**+ve DEN-4**
220	124 (56.4%)	11 (8.9%)	104 (83.9%)	9 (7.2 %)	0 (0%)

(www.ncbi.nlm.nih.gov/BLAST/) (Atschul *et al.*, 1997) and compared to reference sequences of Dengue serotypes detected in BLAST and downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/).

Similarity tree was obtained from database online by phylogeny.fr (http://www.phylogeny.fr/).

RESULTS

RT-PCR and Nested-PCR: One hundred twenty four samples out of 220 (56.4%) suspected patient serum samples tested by RT-PCR were confirmed positive for dengue virus when using D1 and D2 primers (511bp) for all serotypes, and the RT-PCR product was used as a sample for the nested-PCR using a set of serotype-specific primers pair as described in the methodology. Three dengue virus types (DEN-1, DEN-2 and DEN-3) were detected and the results showed that DEN-2 is the most common and predominant type in Jazan region rating one hundred four out of one hundred twenty four (83.9%), followed by DEN-1 (eleven out of one hundred twenty four, 8.9%), and then DEN-3 (nine of one hundred twenty four, 7.2%) and serotype 4 was not detected (Table 2 and Fig. 2).

Sequencing: To confirm the serotype-specific results, the partial sequencing was done for nineteen RT-PCR product samples represent the three serotypes (DEN-1, DEN-2, and DEN-3). The Blast search showed that the sequences of our samples aligned along with many published sequences of dengue virus serotypes as shown in Table 3 and Fig.3, Fig.4 and Fig.5, and similarity tree (Fig.6, Fig.7 and Fig.8) which illustrates the Gen bank accession numbers and the country of isolates.

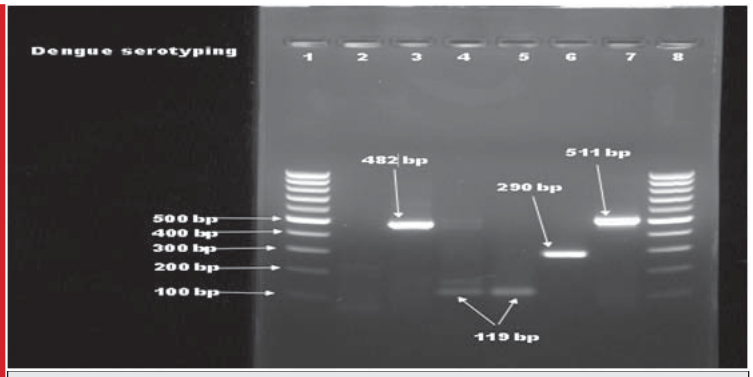

FIGURE 2. Agarose gel electrophoresis of RT-PCR (D1, D2 primers) and nested-PCR by the specific primers. Lane 1 and 8 DNA 100bp marker, lane (2) negative control, lane (3) positive sample DEN-1, lane (4,5) positive samples DEN-2, lane (6) positive sample DEN-3 and lane (7) positive RT-PCR product sample (D1 and D2 primers for all serotypes).

Table 3. Results of RT-PCR and nested-PCR

DEN-1		DEN-2		DEN-3	
Gen bank accession No	Country	Gen bank accession No	Country	Gen bank accession No	Country
AB608788	Taiwan	JN935383	India	KM097092	India
KJ649286	Saudi Arabia	KU351296	India	KM097092	Singapore
JN638338	Thailand	GU968539	India	KF954949	China
AF298808	Djibouti	KX577706	China	GQ466079	India
Z74047	Vietnam	KT180256	India	FJ644564	India
AF538024	Cambodia	KU351306	India	DQ317393	India
KU509258	Eritrea	JQ639472	India	KU216208	India

Sequencing of DEN-1 in this study revealed that it is in close similarity to some Asian (Taiwan, Saudi Arabia, Thailand, Vietnam, and Cambodia) and African (Djibouti and Eritrea) types (Table 3, Fig.3, and Fig.6). DEN-2 on other hand, is similar to varies Indian types (Table 3, Fig.4, and Fig.7), while DEN-3 is in similarity to some Asian types including India, China, and Singapore (Table 3, Fig.5, and Fig.8).

DISCUSSION

Jazan region has witnessed several outbreaks during the recent decade (290 cases in 2010, 289 cases in 2012, and 555 cases in 2016 – Dengue control program in Jazan). The current available data on dengue in Jazan has concentrated mainly on serological surveys (Al-Arzaqi *et al.*, 2013; Gamil *et al.*, 2014) and has not analyzed the circulating serotypes in the region.

FIGURE 3. Identities between DEN-1 from Jazan and DEN-1 of Taiwan (dbj|AB608788.1) Dengue virus 1 gene for polyprotein, complete cds, strain: 832, Length=10693, Score = 778 bits (421), Expect = 0.0, Identities = 459/477 (96%), Gaps = 4/477 (1%), Strand= Plus/Plus.

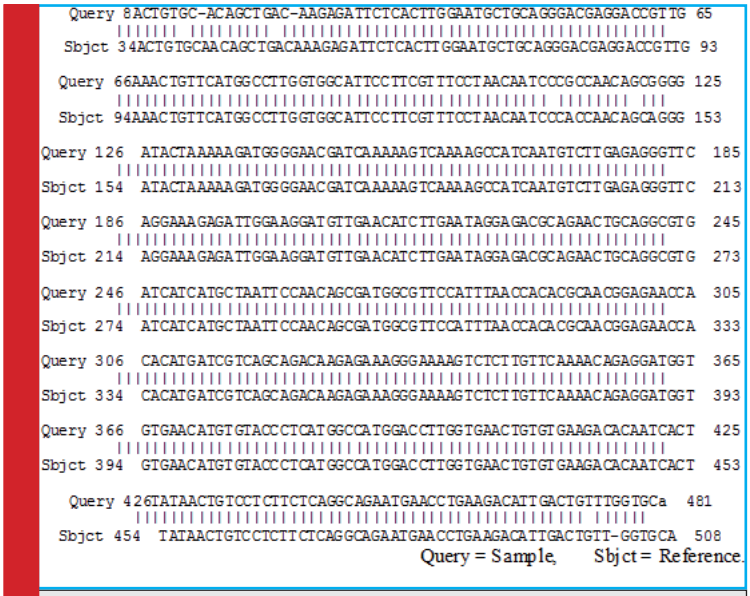

FIGURE 4. Identities between DEN-2 from Jazan and DEN-2 of India(gb|JN935383.1) Dengue virus strain VCRC/DENV2/03/10 polyprotein gene, partial cds Length=508, Score = 848 bits (459), Expect = 0.0, Identities = 471/476 (99%), Gaps = 3/476 (1%), Strand=Plus/Plus

Our results indicated that dengue fever is becoming highly prevalent in Jazan region (56.4 %) compared to the previous reports of Al-Arzaqi *et al* (2013) and Gamil et el (2014) who reported dengue prevalence of 26.5% and 47.74% , respectively, in the region. In this study, three dengue virus types (DEN-1,DEN-2 and DEN-3) were found circulating in Jazan region with the predominance of DEN-2 scoring 104 out of 124 dengue

FIGURE 5. Identities between DEN-3 from Jazan and DEN-3 of India (gb|KF954949.1) Dengue virus 3 isolates 13GDZDVS30E, complete genomeLength=10677 Score = 861 bits (466), Expect = 0.0, Identities = 473/476 (99%), Gaps = 1/476 (0%, Strand=Plus/Plus

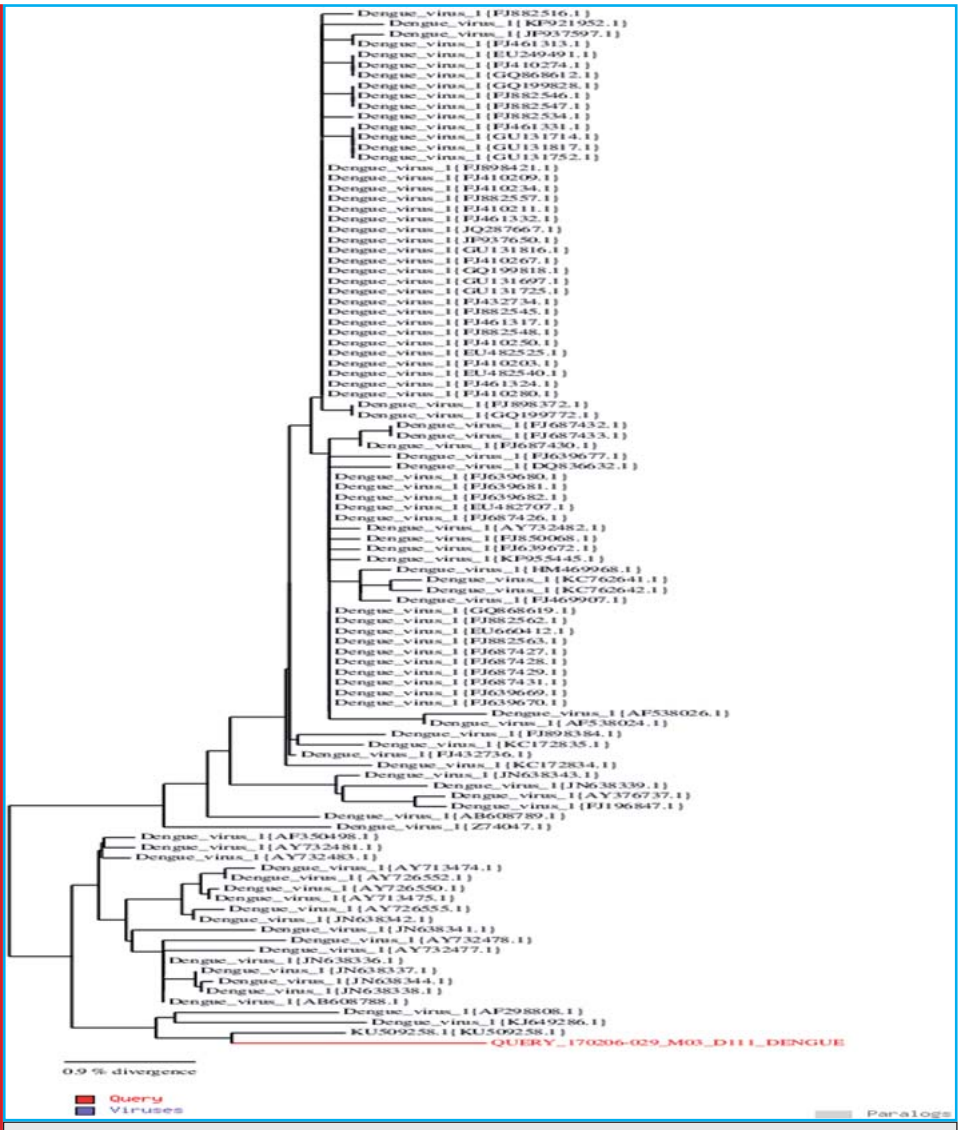

FIGURE 6. DEN-1 serotype similarity tree.

positive samples (83.9%), followed by DEN-1 (11 out of 124 - 8.9%), and then DEN-3 (9 of 124 - 7.2%), however serotype 4 was not detected in any of the 124 dengue cases. This finding is in complete accordance with the work of Fakeeh and Zaki (2001) who reported that DEN-2 was the predominant serotype, followed by DEN-1, and DEN-3 in Jeddah, Saudi Arabia. Whereas Organji *et al* (2017) in Makkah city, showed that DEN-1 was the predominant dengue virus type, followed by DEN-2 and then DEN-3, although the positive blood samples they used were only six.

The results also coincide partially with the findings of Khan *et al* (2008) who reported high prevalence of the DEN-2 in contrast to the prevalence of DEN-1 found by Organji *et al* (2017) in Makkah city.In Jeddah, Zaki *et al* (2008) revealed that DEN-1 and DEN-2 caused the major outbreak in 1994, while DEN-3 emerged in 1997. Moreover, they indicated two genotypes for DEN-1 (America-Africa genotype, and Asia-2 genotype), DEN-2 genotype clustered within Cosmopolitan genotype, and DEN-3 clustered within genotype III.

In the present study, we found DEN-2 to be the predominant dengue virus type, a result which is in line with the reports of Fakeeh and Zaki (2001, 2003) and Zaki *et al.* (2008) who stated that DENV-2 virus is the predominant serotype in Saudi Arabia particularly in western Saudi Arabia since 1992.El-Kafrawy *et al.* (2016) showed that DEN-2 isolate from Jeddah belongs to the Cosmopolitan genotype was most genetically related to isolates from Pakistan circulating from 2008 to 2013.The

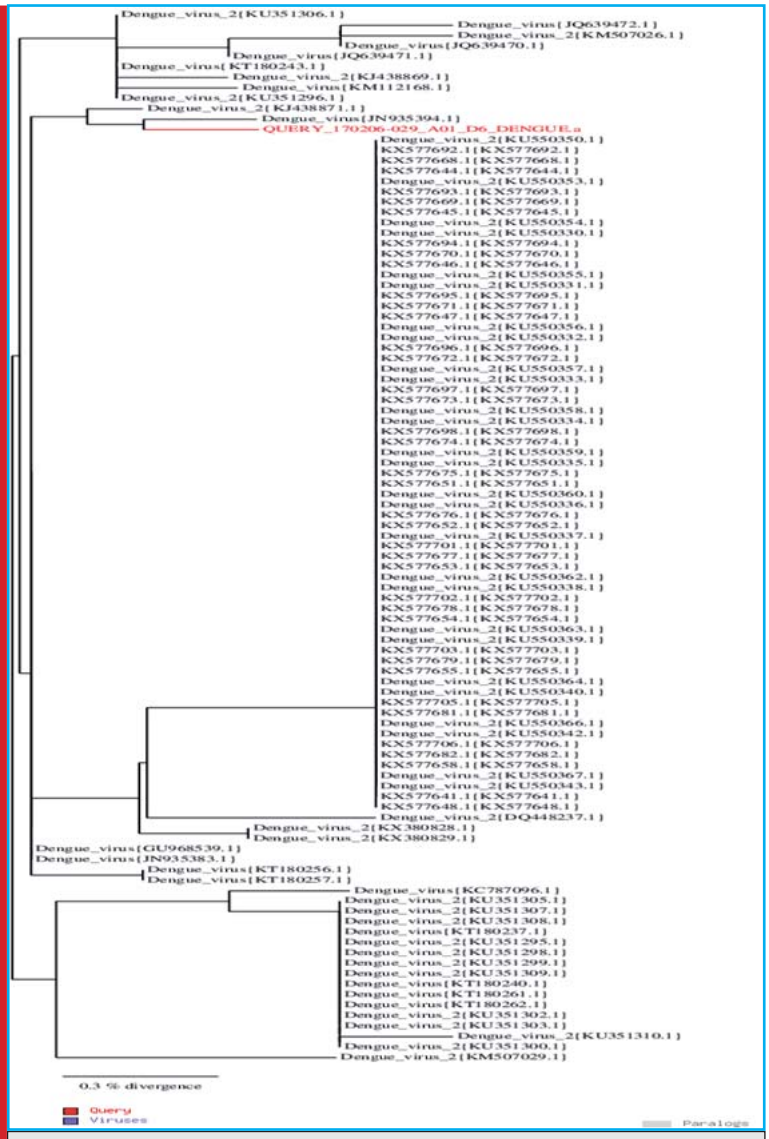

FIGURE 7. DEN-2 serotype similarity tree.

three dengue virus serotypes DEN-1, DEN-2, and DEN-3 are thought to be predominant in the Middle East, especially in Yemen and Saudi Arabia (Nedjadi *et al.*, 2015).

Dengue viruses circulating locally in Saudi Arabia are likely to have been imported into Saudi Arabia by Saudi traveling abroad to dengue endemic countries, or during Hajj and Umrrah seasons, or by migrant labour (Zaki *et al.*, 2008).The introduction of the three dengue virus types in Jazan region may be resulted from several factors; traveling of the Jazan citizens for Hajj and Umrrah or for trade or other purposes, or by traveling abroad to dengue endemic countries, or by migrant labour, or due to the proximity of Jazan region to Yemen where the disease is endemic and the three dengue virus serotypes DEN-1, DEN-2, and DEN-3 are circulating.

The close similarity of DEN-1 in this study to some Asian (Taiwan, Thailand, Vietnam, and Cambodia) and African (Djibouti and Eritrea) types, the similarity of DEN-2 to varies Indian types, in addition to, the similarity of DEN-3 to some Asian types including India, China, and Singapore suggested the likelihood of introduction of theses serotypes to Jazan region either by travelling from and to those countries especially the migrant labours (DEN-1,DEN-2, DEN3,), or through direct introduction from Jeddah (DEN-1 Jeddah genotype) and Yemen which is closet to Djibouti and Eritrea (DEN-1 African origin).

It is stated that shifts in circulating dengue virus type or introduction of new dengue virus type in endemic areas have shown to be related with incidence of severe

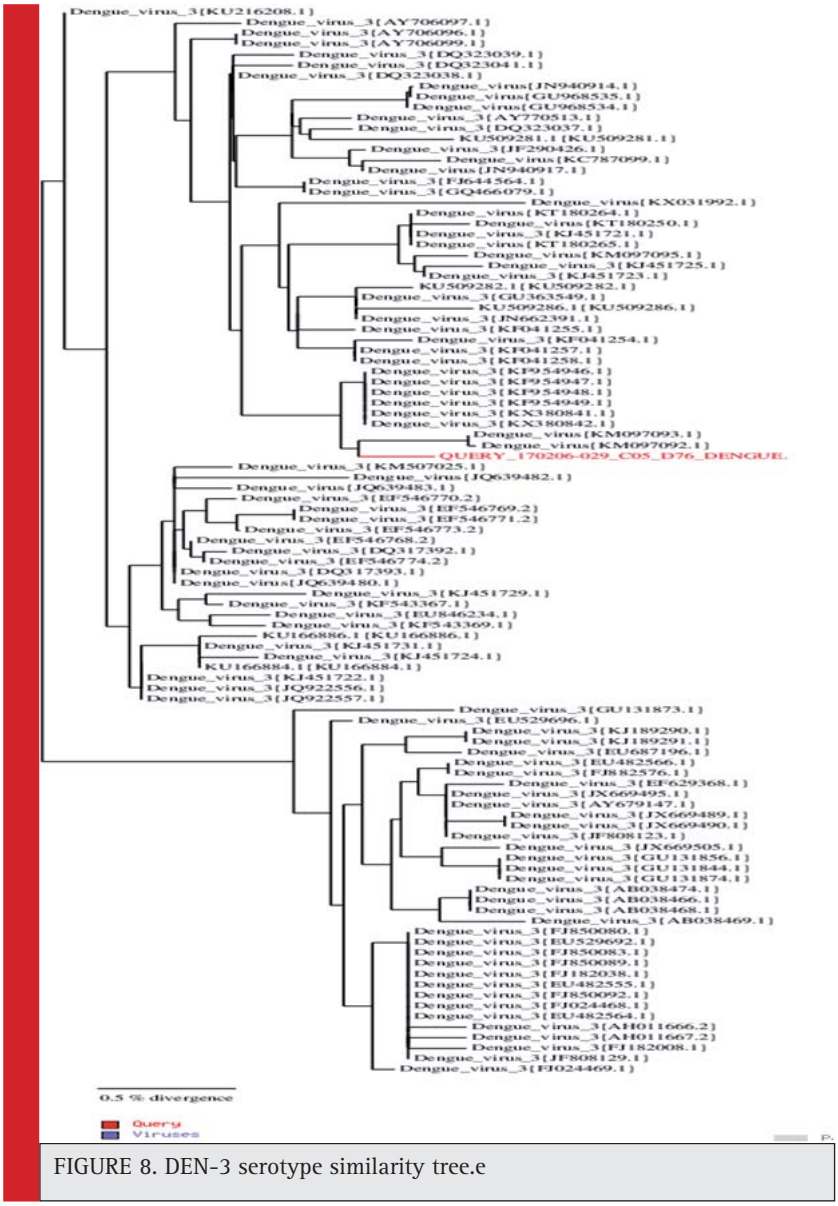

FIGURE 8. DEN-3 serotype similarity tree.e

dengue infections; DHF and DSS (Messer *et al*., 2003; Rico-Hesse *et al*., 1997). Moreover, it is worthy to note that the primary infections by DEN-1 and DEN-3 are related with more dengue sever infections, whereas infections with DEN-2 and DEN-4 are associated with increased dengue severity when they present as secondary infections (Balmaseda *et al*, 2006). Results of our study have analysed multiple dengue serotypes which would help in providing clear evidence of current active dengue transmission and endemicity in Jazan region.

CONCLUSION

The results of this study reported for the first time the dengue virus types DEN-1, DEN-2, and DEN-3 circulating in Jazan region with the DEN-2 being the predominant one. The high seroprevalence of dengue virus infection in Jazan region indicates its endemicity. The present study highlights the importance of tracking the spread of dengue virus types and its implication for analyzing changes in dengue endemicity in specified areas over time. Continuous surveillance of dengue virus serotypes in the region to detect as earlier the local origin circulating serotypes from the imported ones especially new types DEN-4 and DEN-5, for which continued surveillance is imperative. Complete genome sequencing is required for the three detected dengue virus serotypes circulating in the region (DEN-1, DEN-2, and DEN-3) to serve as references for any future epidemiological researches and outbreaks.

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