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MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF DIFFERENT DENGUE VIRUS SEROTYPES REPORTED IN JAZAN AREA, KINGDOM OF SAUDI ARABIA

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KEYWORDS

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ABSTRACT

Dengue virus (DENV) is the public health threat worldwide. In fact, since 1994, Kingdome of Saudi Arabia (KSA) was reported dengue-free country. Later, outbreaks have been reported in Jeddah and Makkah and the dissemination of new DENV strains and serotypes into different localities could be attributed to the annual displacement of pilgrims from dengue endemic regions. Therefore, early detection of multiple infections is a crucial factor for patient's hospitalization and symptomatic treatment. The objective of the current study was to detect, characterize and to analyse the magnitude of concurrent DENV infection in Jazan area as base data for future control strategies. A total of 123 blood samples from febrile patients at Jazan area were collected. Viral RNA was subjected to Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and nested PCR. Dengue virus RNA from 12 RT-PCR positive blood samples was extracted, purified, the all viral genome sequenced, and their nucleotide sequences were searched for similarity in the GenBank. The RT-PCR results of 123 tested samples, showed 79 (64.2%) DENV positive while 44 (35.8%) are negative. DENV1 was detected in 63 out of 79 samples (79.7%) followed by DENV2 (13/79 =16.5%), and concurrent mixed infection with both DENV1 and DENV2 (3/79 = 3.8%), but DENV3 and DENV4 were not detected in all tested samples. Concurrent mixed infection with multiple DENV serotypes (DENV1, DENV2) was detected for the first time in Jazan area, KSA.

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1 Introduction

Dengue virus (DENV) is the most common arthropod-borne viral disease, causes a substantial public health burden (Guzman et al., 2010). Disease has different forms including dengue fever, dengue hemorrhagic fever and dengue shock syndrome (Whitehead et al., 2007; Varatharaj, 2010). The disease severities are range from undifferentiated acute febrile illness to the life-threatening conditions (Gubler, 2002). The virus is a single strand RNA virus with 4 (DENV 1 to 4) genetically distinct groups known as serotypes (de Alwis et al., 2011) and is transmitted to human by *Aedes aegypti* mosquitoes (Lambrechts et al., 2010). Many factors (temperature, rainfall, humidity, air travel, topographic societal, insecticides resistance and impact of ecology) (Alto & Juliano, 2001; Armstrong & Rico-Hesse, 2003; Simard et al., 2005; Chan & Johansson, 2012) are responsible for increased disease incidence and sustainable transmission in many countries including Kingdom of Saudi Arabia (KSA). In fact, since 1994, KSA was reported dengue-free country (Ashshi, 2015). Later, outbreaks have been reported in Jeddah and Makkah and the dissemination of new DENV strains and serotypes into different localities could be attributed to the annual displacement of pilgrims from dengue endemic regions (Ashshi, 2015; Azhar et al., 2015; Ashshi, 2017; Organji et al., 2017). Worldwide, concurrent mixed infection with multiple DENV serotypes is considered as alarm signs of the large dispersal of different DENV serotypes resulting in dengue complicated cases (WHO, 2014), and co-infected patients are skewed towards more severe clinical manifestations compared to mono-infected patients (Dhanoa et al., 2016) and the occurrence of co-infections rates are ranging from 5-30 % (Martins et al., 2014), from 40-50 % (Vinodkumar et al., 2013), and recently very high up to 100% mixed infections were reported in India (Reddy et al., 2017). To date, DENV severities, detection and characterization were not extensively studied in Jazan area, KSA. In this area, the virus was routinely diagnosed by serology; the results of these diagnostic techniques are less sensitive and non-specific to differentiate between the four viral serotypes infections. However, diagnosis of DENV concurrent mixed infections using molecular species-specific primers was effective diagnostic tool for serotyping and for early detection at field levels has a significant impact on the prediction and characterization for future control strategies. The objective of the current study was to detect, characterize and to analyse the magnitude of concurrent DENV infection in Jazan area as base data for future control strategies and to determine their genetic relatedness with other published sequences in the GenBank.

2 Materials and methods

2.1 Study area

Jazan Region, KSA is stretching out 300 km along the Southern Red Sea coast, covering an area of 11,671 km² and is populated by 1533,680. The area is characterized by a distinctive hot and humid climate, inadequate sewage and waste management systems provide important sides for mosquito breeding. In addition, the area attracts large number of visitors, and migrants, thus the dissemination of DENV in the area is expected.

2.2 Ethical approval and informed consent:

The research proposal was reviewed and accepted by the Research Ethic Committee, King Abdulaziz University. Ethical approval was obtained from Ministry of Health, Saudi Arabia. Before blood samples collection, the patient, parent, or guardian provided a written informed consent.

2.3 Blood samples

Five ml venous blood samples from 123 patients were aseptically taken from febrile patients admitted to 13 hospitals at Jazan area, KSA. Sera samples were processed by divided into two aliquots in screw-capped cryotubes (Greiner Bio-One, Germany) and were kept at −80 °C until use.

2.4 RNA isolation

High Pure Viral Nucleic Acid Kit (Roche Applied Science, Germany) was used for the extraction of RNA from all sera samples, following the manufacturer procedures with minor modifications. Briefly, in a nuclease-free 1.5 ml microcentrifuge tube added 200 µl of binding buffer, 50 µl Proteinase K and 200 µl of serum sample, immediately mixed and were incubated for 10 minutes at 72°C. To the mixture, extra 100 µl Proteinase K was added, mixed and was 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 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, centrifuged at 10000 rpm for 1 minute and the eluted viral RNA was kept at −80 °C until use.

Table 1 RT-PCR and Nested-PCR oligonucleotide primers

Serotype	primer	Sequence 5' – 3'	Size in bp
1, 3 and 4	D1	TCAATATGCTGAAACGCGCGAGAAACCG	511
	D2	TTGCACCAACAGTCAATGTCTTCAGGTTC	511
	TS1	CGTCTCAGTGATCCGGGGG	482 (Dl and TS1)
	TS3	TAACATCATCATGAGACAGAGC	290 (Dl and TS3)
	TS4	CTCTGTTGTCTTAAACAAGAGA	392 (Dl and TS4)
2	DV1	GGRACKTCAGGWTCTCC	362
	DSP2	CCG GTGTGCTCRGCYCTGAT	

2.5 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

In the current study, all the sera samples were screened by RT-PCR with dengue virus group-specific consensus primer pairs for the presence of dengue virus RNA. RT-PCR and the conventional nested PCR were performed according to the previous protocol (Lanciotti et al., 1992) with some modifications. The viral RNA was amplified using DENV consensus primers and serotype-specific primers (Table 1). Briefly, in 50 µl final volume reaction mixture containing 10 µl of AMV/Tfl 5X Reaction Buffer, 1 µl of dNTP Mix (10mM each dNTP, final concentration 0.2 mM), 2 µl of 25 mM $MgSO_4$ (final concentration 1 mM), 1 µl of AMV Reverse Transcriptase 5 U/µl (final concentration 0.1 U/µl), 1 µl of Tfl DNA Polymerase (final concentration 5 U/µl), 50 pmol (final concentration 1 µM) of each forward (D1) and reverse (D2) primers (Table 1), 5 µl of purified viral RNA and nuclease free water was added to make total volume 50 µl. The RT-PCR temperature cycling conditions involved: Incubation for 1 hour at 42°C, initial denaturation at (94°C, 3 min), 35 cycle of denaturation (94°C, 30 second), primers annealing (55°C, 1 minute), primer extension (72°C, 2 minutes) and final extension at 72°C for 5 minutes.

2.6 Serotyping

Under the same above mentioned temperature cycling condition, 1.0 µl of the above RT-PCR product (1:10 in sterile distilled water) was added as a template in the subsequent nested PCR reaction to 50 µl reaction volume mixture containing forward primer D1 and type-specific (TS) reverse primers: TS1, TS3 and TS4 reverse primers for serotype 1, 3 and 4, and forward primer DV1 and DSP2 reveres primers for serotype 2 (Table 1) and was further amplified by nested PCR step (second round). The PCR products amplification was analysed on gel electrophoresis (1.5 agarose in 1X Tris-Acetate EDTA buffer) and gel was stained with 1% ethidium bromide and the size compared with a 1 Kb plus DNA ladder (Invitrogen ™, catalogue number 10787018) using Gel Doc XR Imaging System (Bio-Rad).

2.7 Sequencing and bioinformatics analysis

RT-PCR products (480, 362 bp) of 12 DENV positive samples were purified and were sequenced by the Macrogen Company (Seoul, Korea). The sequences similarities were done following the previous method (Altschul 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/).

3 Results

3.1 RT-PCR

RT-PCR detected 79 (64.2%) DENV positive samples and 44 (35.8%) negative out of 123 febrile illness human subjects tested samples. The results of 1.5% agarose electrophoresis analysis of RT-PCR and nested PCR products were demonstrated in the figure (1, 2) respectively. The RT-PCR detected 79 positive samples, 63 out of these samples are DENV1, 13 DENV2 and 3 concurrent mixed infections with (DENV1, DENV2), on the other hand DENV3 and DENV4 were not detected in all tested samples.

3.2 Sequencing

Dengue virus RNA from 12 RT-PCR positive blood samples was extracted, purified, its concentration was tested using Nano photometer (2000C; Thermo Scientific, Waltham, MA, USA), sequenced by Macrogen Company (Seoul, Korea) and the sequencing reactions were performed using MJ Research PTC-225 Peltier Thermal Cycler. The two DENV serotypes (DENV1 and DENV2) detected by RT-PCR were further sequenced and their partial E gene sequences were directly determined from the selected samples in the current study.

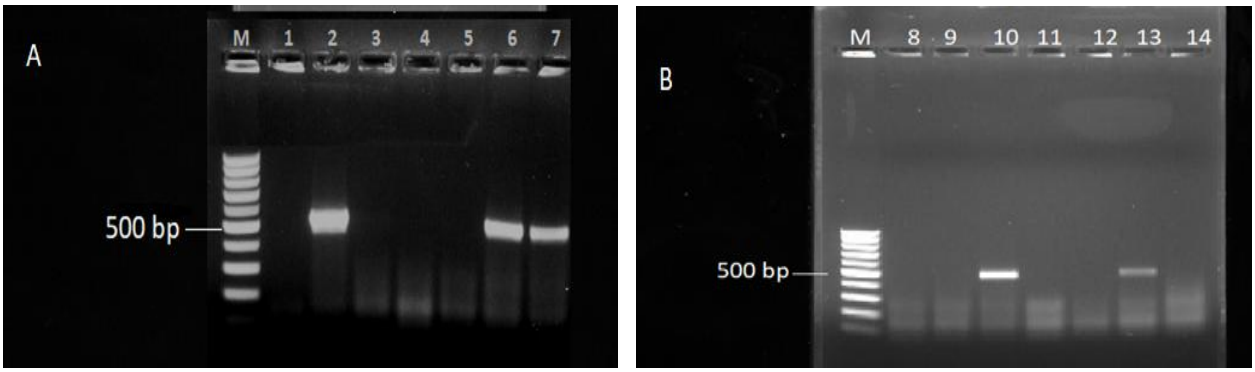

Figure 1 (A, B) Agarose gel images showing the separation of amplified DNA by RT-PCR using DENV1 specific primer sets. Lane M, standard size marker, L1 negative control, L2 positive control (DENV1/500 bp), L 3-14 test samples

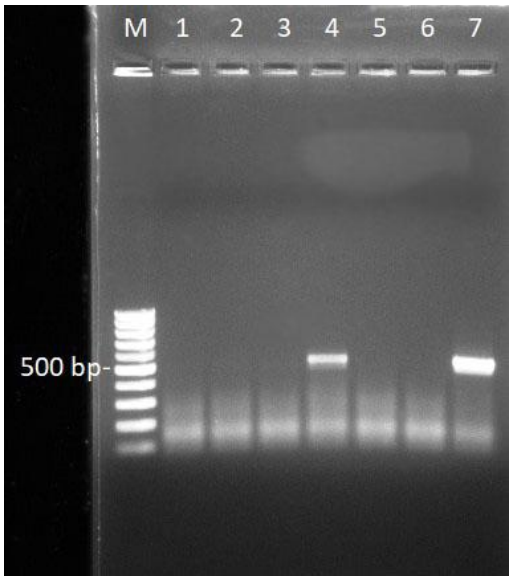

Figure 2 Agarose gel images showing the separation of amplified DNA by nested PCR using DENV1 specific primer sets. Lane $_{M,}$ standard size marker, L_6 negative control, L_7 positive control (DENV1/500 bp), L_{1-5} test samples

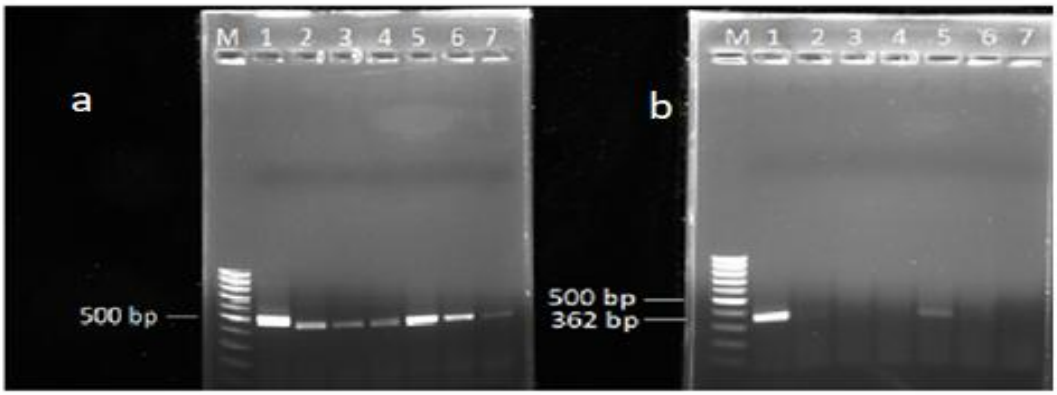

Figure 3 Agarose gel images showing the separation of amplified DNA (a) dengue virus serotype1 (500 bp) and (b) dengue serotype2 (362 bp) by RT-PCR using dengue serotype specific primer sets. Lane $_{M,}$ standard size marker, L_1 positive control, L_{2-7} test samples

3.3 Phylogenetic analysis

Complete and partial envelope genome sequences from DENV1 and DENV2 were isolated and identified in this study. The sequences were searched for sequence similarity through BLAST (www.ncbi.nlm.nih.gov/BLAST/) and compared to reference sequences of Dengue serotypes detected in BLAST and downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/). Complete genome sequences of DENV1 was 97% (462/476) similar to isolate from Eritrea and Taiwan (sequence ID: KU509258. 1Length: 10377 Number of Matches) and DENV2 sequence was 99% (460/464) similar to isolate from Sudan and India sequence ID: JN935394. 1Length: 486 number of Matches. From dengue serotype 1 and serotype 2 sequences were correlated to the sequences available in GenBank and their generated phylogenetic similarity trees were respectively shown in figure (4, 5).

4. Discussion

Dengue is an important emerging disease of the tropical and sub-tropical regions (Bhatt et al., 2013). It is a complex disease whose symptoms are difficult to distinguish from other common febrile illnesses and can progress from a mild, non-specific viral disease to irreversible shock and death within a few hours. This makes the differential diagnosis problematic especially in the Jazan area, where there is a high incidence of febrile illnesses. Total of 123 blood samples from acute febrile illness subjects all (age, sex and nationality) during their first hospital admission to 13 hospitals at Jazan area were collected. All sera were subjected to reverse transcriptase polymerase chain reaction (RT-PCR) and nested PCR. Dengue virus RNA from 12 RT-PCR positive blood samples was extracted, purified, sequenced and their nucleotide sequences were searched for similarity in the gen-bank. The molecular serotyping of RT-PCR positive samples (n = 79) revealed DENV1 (63/79), DENV2 (13/79) and (3/79) concurrent mixed infection with (DENV1, DENV2) using dengue serotype1 (480 bp) and serotype 2 (362 bp) specific primer sets, on the other hand DENV3 and DENV4 were not detected in all collected blood samples. During 2008, the same dengue serotypes (serotype 1 and 2) was first reported from Al-Madinah, Saudi Arabia (El-Badry et al., 2014) But Fakeeh & Zaki (2003) and Organji et al. (2017) reported three dengue serotypes (DEN 1, 2 and 3) in Jeddah.

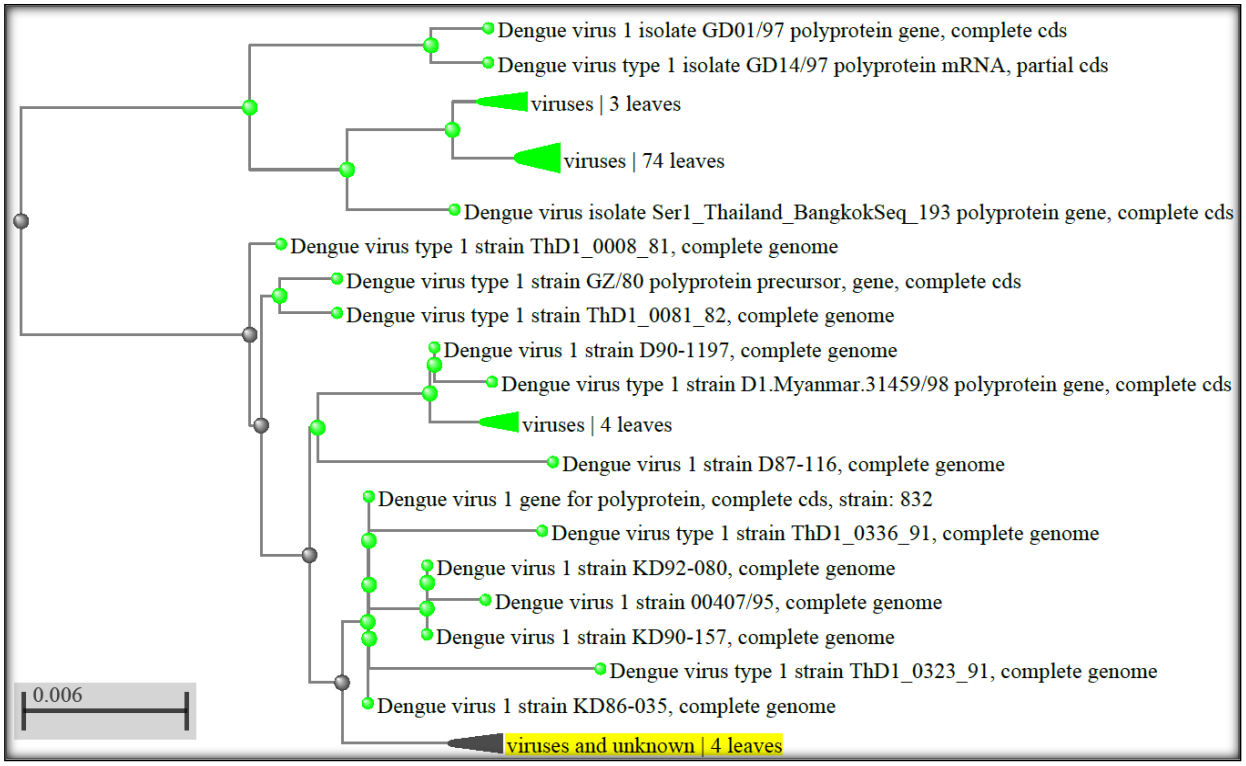

Figure 4 Phylogenic relationships between DENV1 isolated from febrile illness patient at Jazan area, KSA with other established DENV1 relevant serotypes. The MrBayes V.3.2 software is used to analyse the phylogenetic similarity tree. Only values above 90% of similarities were shown in this illustrated figure.

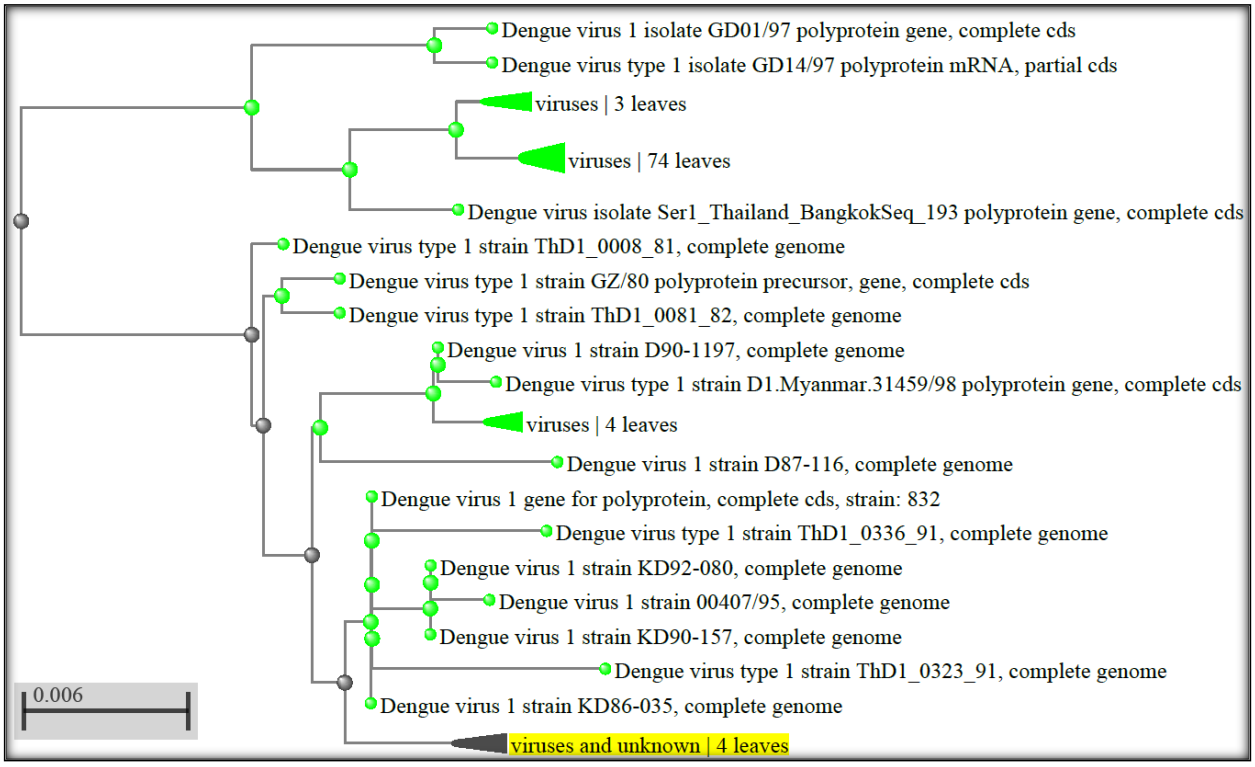

Figure 4 Phylogenic relationships between DENV1 isolated from febrile illness patient at Jazan area, KSA with other established DENV1 relevant serotypes. The MrBayes V.3.2 software is used to analyse the phylogenetic similarity tree. Only values above 90% of similarities were shown in this illustrated figure.

Therefore, we established dengue serotype1 and serotype2 as the dominant serotypes currently circulating in Jazan area for the first time. Globally, over the last five decades, dengue has emerged as a real critical threat to population health in most country included Saudi Arabia (El-Badry et al., 2014). The World Health Organization estimates that 50 to 100 million dengue infections occur annually half the world's population lives in countries where dengue is endemic (Guzman et al., 2010). The four dengue virus types (1- 4), called dengue virus serotypes, form a phylogenetic group and differ in nucleotide sequence from each other. These are closely related to one another rather than to other flaviviruses and form an antigenic complex of their own.

Complete and partial envelope genome sequences from serotype 1 and serotype 2 were isolated and identified. The sequences was searched for sequence similarity through BLAST (www.ncbi.nlm.nih.gov/BLAST/) and compared to reference sequences of Dengue serotypes detected in BLAST and downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/). Complete genome sequences of DENV1 was 97% (462/476) similar to isolate from Eritrea and Taiwan (Shihada et al., 2017) and DENV2 sequence was 99% (460/464) similar to isolate from Sudan and India (Kumar et al., 2013). From dengue serotype 1 and serotype 2 sequences were correlated to the sequences available in GenBank and their generated phylogenetic similarity trees were shown. Concurrent mixed infection with multiple DENV serotypes (DENV1, DENV2 was detected in three patients; one of them expired during hospitalization and treatment. Our result was consistent with a prospective observational study that conducted among adults dengue patients hospitalized in Bangkok (Thanachartwet et al., 2016).

Conclusion

Concurrent mixed infection with multiple DENV serotypes (DENV1, DENV2 was detected in three patients; one of them was expired during the hospitalization and treatment. This finding is considered as alarm signs of the large dispersal of different DENV serotypes. Therefore, prevising a disaster situation could be expected in Jazan area. However, this observation should be warning to health authorities for control measures. We recommended that all febrile illness patients should be tested for dengue antibodies and clinicians/physicians consider the possibility of dengue cases when dealing with febrile patients. Epidemiological studies of dengue infections in Jazan areas are required.

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Conflict of interests

The authors declare no competing interests and contribute equally.

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