http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2018.38.50

Research Article
Molecular Characterization of Dengue E/NS1 Junction Genotype 2 Isolated From Saudi Patients, Jeddah Province

[1,2]Sahar EL Hadad, [1]Alawiah Alhebshi and [1]Haifa Al Amri

[1]Department of Biological Science, Faculty of Science, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia
[2]Research Center of Genetic Engineering and Bioinformatics, VACSERA, Cairo, Egypt

Abstract

Background and Objective: Dengue is the most important arthropod-borne viral disease which caused by the four-dengue virus serotypes (1-4) and its incidence has grown dramatically around the world in the recent decades. This study was conducted to determine the molecular characterization of dengue virus genotypes spreading in Jeddah province. **Methodology:** To distinguish dengue virus genotypes, serum samples from 13 infected patients were subjected to envelop and non-structural 1 (*E/NS1*) gene amplification and sequence analysis at the nucleotide and amino acid levels. **Results:** The present partial dengue virus phylogenetic analysis announced the domination of dengue virus 2 genotype among the current dengue virus samples circulating in Jeddah province. Dengue virus 2 current isolates were grouped in one branch and seemed to be more closely related to various strains isolated from Sri-Lanka, Australia and Singapore and confirmed by internucleotide distance average ranged +/-0.01. Interestingly, sequences analysis of amino acids confirmed substitution of 8 amino acid residue (Ser729Gua, Ser729Arg, Val762Gau, Val780phe, Val781Leu, Val781Ala, Glu858Asp and Gln873His) among the present isolates comparing with previous references strains isolated from different countries. Remarkably, one unique amino acid residue Ala741Val was verified in the 10 present isolates compared to the reference sequence previously isolated from Jeddah. **Conclusion:** Notably, the present study demonstrated the sequencing analysis of the dengue virus 2-*E/NS1* on both nucleotide and amino acid levels and confirmed its endogenously prevalence in Jeddah.

Key words: Dengue virus, DENV genotype, DENV-1, E/NS1 region, DENV/ENS1 region, viral isolates, population studies

Citation: Sahar EL Hadad, Alawiah Alhebshi and Haifa Al Amri, 2018. Molecular characterization of dengue E/NS1 junction genotype 2 isolated from Saudi patients, Jeddah province. Pak. J. Biol. Sci., 21: 38-50.

Corresponding Author: Sahar EL Hadad, Department of Biological Science, Faculty of Science, King Abdulaziz University, Old Airport St., 80400 Mailbox, Jeddah, Kingdom of Saudi Arabia Tel: 00966533450633

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Dengue is the most common arboviral (arthropod-transmitted) disease and is currently considered as the most important arthropod-borne viral disease in the world[1]. The incidence of dengue has grown dramatically around the world in recent decades. Over 2.5 billion people-over 40% of the world's population are now at risk from dengue. WHO currently estimates there may be 50-100 million dengue infections worldwide every year[2]. Dengue virus (DENV) is a member of the taxonomic family Flaviviridae, genus *Flavivirus* and it is responsible for a wide range of clinical manifestations in humans, including dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) with circulatory failure[3,4].

DENV enveloped genome consists of a single-stranded, positive-sense RNA molecule roughly 10.7 kb in size. It contains a single translated open reading frame (ORF) for three structural proteins, core (C), membrane (M) and envelope (E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5)[5]. Dengue has four serotypes and designated DEN/1, 2, 3 and 4, that are different based on 6% sequence divergence within a 240 nucleotide region of the DENV-E/NS1 junction[6-8]. Each serotype of the dengue virus can be further classified into several genetic groups called genotypes based on sequence diversity[9]. The DENV-1 genotypes are primarily predominant in Japan, China, Southeast Asia, Thailand and Malaysia[10]. The DENV-2 is distributed in Latin America, Australia and India[11], while DENV-3 and 4 are predominant in Indonesia, Sri Lanka, Singapore, Bangladesh and Vietnam[12,13]. The infection with one serotype does not protect against the others[7,8]. However, it is still inconclusive whether any of these DENV serotypes and genotypes can be consistently associated with causing more severe dengue[14].

Since the first case of DHF died in Jeddah in 1993, WHO reported three major epidemics in Saudi Arabia: A DENV-2 epidemic in 1994 with 469 cases of dengue, 23 cases of DHF, two cases of DSS and two deaths; a DENV-1 epidemic in 2006 with 1269 cases of dengue, 27 cases of DHF, 12 cases of DSS and six deaths and a DENV-3 epidemic in 2008 with 775 cases of dengue, nine cases of DHF, four cases of DSS and four deaths[2]. In addition, Saudi Ministry of Health (SMOH) issued a report that Saudi Arabia total infection with DENV up to 3526 cases in 2010, including 2244 cases in Jeddah. Factors associated with the spread of DF in Jeddah have included the presence of stagnant water in indoor drainage holes, indoor larvae, nearby construction sites and older age[15]. Clinical profile of DF infection in Saudi Arabia were demonstrated significant differences in the clinical presentation of DENV infection, indicative of a variation in disease severity from DF to DHF and DSS over the years[16].

The *E/NS1* gene junction is one of the most widely studied for DENV comparative sequence analyses due to its uniform rate of random mutation with minimal selective advantages[9]. Firstly, this area showed the uniform rate of random mutation, with no hyper variable regions that might affect the expression of epitopes. Secondly, the majorities of the mutations in this region occurred in the third position of the codon and are mostly silent, probably random mutations which provide selective advantages[17].The occurrence of DENV infection is usually high in Asian nations[9-13]. Furthermore, DENV infection considered as one of the most prevalent disease in Jeddah province of Saudi Arabia because Jeddah is a main entry point as well as being the largest commercial port in the country. For these reasons and because little is known regarding the DENV-E/NS1 junction sequences either on nucleotide and amino acid levels circulating in Saudi Arabia, the present study intended to determine the molecular characterization of the most prevalent DENV-E/NS1 genotype circulating in Jeddah province. Also, evolutionary and epidemiologic relationships between different isolates from worldwide countries and strains were isolated from Jeddah in order to investigate whether DENV activity in Jeddah was because of endogenously established circulating dengue strains or to strains newly introduced.

MATERIALS AND METHODS

Patient samples: This study was included 51 DENV suspected patient's plasma samples, which were collected from Saudi patients from 2016-2017. All plasma samples were randomly collected as samples became available and were divided into aliquots and stored at -80°C until use. The study protocol was reviewed and approved by the Deanship of Scientific Research Ethical Committee of King Abdulaziz University and the King Abdulaziz Hospital Ethical Committee. Written consent was obtained from all patients after full explanation of the purpose of the study.

Cell line and dengue virus propagation: The "standard" methods of isolating DENV utilize the various type of cell lines include *Aedes albopictus* cells C6/36, Vero cells and baby hamster kidney (BHK-21) cells[18]. All the suspected DENV patient's samples were inoculated to C6/36 cells culture and kept at 37°C for 1 h to allow for virus adsorption. The infected tissue culture supernatant was harvested 4-5 days after incubating at 28°C. The extract supernatant was transferred from culture tube without disturbing the cell pellet and stored at -70°C[19].

DENV-RNA extraction and DENV-E/NS1 junction amplification: The RT-PCR reaction has ascertained the presence of DENV in the C6/36 cells culture supernatants, where viral RNA was extracted from the infected cells using QIAamp Viral RNA mini kit (Qiagen, Germany) in accordance with the manufacturer's instructions. The obtained DENV-RNA samples were stored at -70°C until use. The DENV-E/NS1 gene was amplified using both DVF1 (134-161) 5'-GAGAGGAGCGAAGAGAATGG-3' and DVR2 (616-644) 5'-TCAGCTCAGTGGGTTGAGG-3' primers, which had been used previously for detection of four DENV serotype[20]. The RT-PCR was performed using One-step RT-PCR Master Mix kit (QIAGEN) in accordance with the manufacturer's instructions and the reactions were assayed in a 25 µL reaction mixture including 10 µL DENV-RNA and 50 pmol each of primers DVF1 and DVF2. Thermal cycling profile involved of reverse transcription step at 50°C for 30 min, *Taq* polymerase activation at 95°C for 10 min, followed by 35 cycles of PCR at 94°C for 30 sec, 60°C for 40 sec and 72°C for 50 sec. All the PCR contamination precautions were taken to ensure specificity of the reaction. Each test included negative control containing distilled water in place of sample[21,22].

Interpretation of DENV-E/NS1 partial nucleotide sequences and phylogenetic analysis: Partial sequence of both directions was accomplished using purified PCR products and Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems (ABI), Foster City, CA, USA). Both sequence electrophoresis and data collection were completed using the ABI Prism 310 genetic analyzer consistent with the manufacturer's protocol. All isolates sequences were assembled using SeqMan II software (DNAStar Inc., Madison, WI, USA) and multiple alignments with the reference sequences of DENV genotypes were confirmed using CLUSTAL W and MEGA 6 software.

Phylogenetic analysis was constructed using the nucleotide sequence of DENV/ENS1 amplified regions of the present isolates and reference sequences of DENV isolates retrieved from the DDBJ/EMBL/GenBank (Table 1). The phylogenetic tree was drawn by MEGA 6 software using neighbor-joining method and the Tamura-Nei model of evolutionary distance and the topology evaluated by bootstrap analysis (1,000 replicates)[23,24].

E/NS1 amino acid sequence analysis: The obtained sequences were edited and BLAST search was conducted to confirm the identity of the sequences. Protein-coding regions of DENV-*E/NS1* gene were translated into amino acid sequences using Mega version 6 software and was compared to E/NS1 amino acids sequences of other DENV strains which were retrieved from DDBJ/EMBL/GenBank database[25].

Table 1: Complete genome of DENV genotypes and DENV-E/NS1 partial reference sequences retrieved from DDBJ/EMBL/GenBank database. All References sequences were defined by their accession numbers and the country of the origin

	E/NS1 references sequences	
DENV complete genome	Global references	Previous Jeddah references
DENV-1 KJ649286 Jeddah	AB194883.1 Sri Lanka	AM748169
DENV-2 AB189123.1 Indonesia	JQ955624.1 Australia	AM748168
DENV-3 AB214880 East Timor	GQ252676.1 Sri Lanka	AM748166
DENV-4 KF55510 Cambodia	Eu081180.1 Singapore	AM748156
	L10052.1 Taiwan	AM748167
	AF410357.1 Vietnam	AM748160
	EU482779.1 Vietnam	AM748164
	AF119661.1 China	AM748163
	FJ639697.1 Cambodia	AM748155
	GQ398314.1 Puerto-Rico	AM748123
	M20558.1Jamaica	AM748162
	AB189123.1 Indonesia	AM748161
	AB122022.1 Dominican	AM748165
	AF208496.1 Martinique	AM748153
	AY702034.1 Cuba	AM748159
	GU131883.1 Brazil	AM748152
	AF038403.1 New Guinea	AM748146
	FJ390389.1 Colombia	AM748145
	AF204178.1 China	AM748144
	XI5433.1 Malaysia	AM748143
	DQ518652.1 Myanmar	AM748151
	DQ181858.1 Thailand	AM748150
	DQ18652.1 Manila	AM748154
	DQ518651.1 Myanmar	
	M24445.1 Thailand	
	DQ181858.1 Thailand	
	AF100464.1 Thailand	
	EU687248.1 Vietnam	
	FJ744725.1 Thailand	

Nucleotide sequence submission: The base sequence data reported in this study has been submitted to DDBJ/EMBL/GenBank and assigned accession numbers KY263628- KY263644 partial sequences.

RESULTS

DENV propagation using continuous C6/36 cell line: The C6/36 cell culture confirmed the presence of specific DENV-CPE in 13 samples (25.5%) out of 51 suspected patient's plasma samples, while total absences of any DENV-CPE was verified in 38 (74.5%) samples. The CPE of DENV was detected in between 1-4 days' post inoculation, while uninfected C6/36 cells showed no CPE and no observable decrease in cell

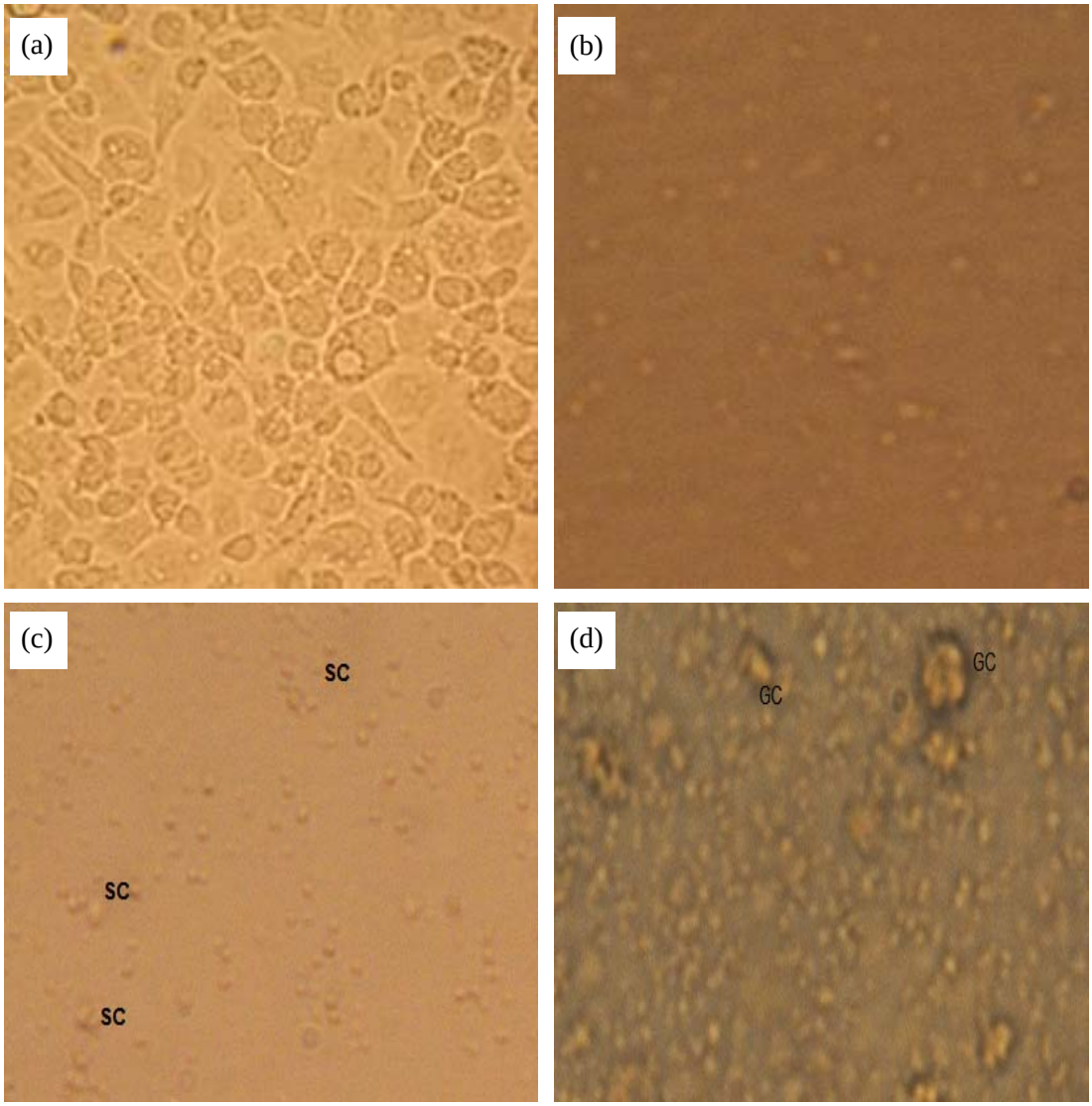

Fig. 1(a-d): Phase contrast photomicrographs of C6/36 cells at 4 days post-challenge with DENV. (a) Unchallenged naive control cells where no any CPE was observed during the 4 days, (b) CPE after the first day of C6/36 cells challenged with DENV patients' serum showed round and swollen, (c) CPE after 2 days of C6/36 cells challenged with DENV patients' plasma showed small aggregates (SC) between the cells and (d) CPE after 4 days of C6/36 cells challenged with DENV patients' plasma showed multinucleated giant cells (GC), many degenerated cells and cell debris

number (Fig. 1a). The CPE findings in C6/36 cells post the first day of DENV inoculation were characterized as the inoculated cells turned to round and swollen (Fig. 1b), a small aggregate between the cells was observed post second day of inoculation (Fig. 1c). Finally, day four of DENV inoculation multinucleated giant cells, many degenerated cells and cell debris were illustrated. Some cells showed necrosis and become detached from the tubes at later stages of infection (Fig. 1d).

Amplification of the DENV-E/NS1 region using nested PCR: One step RT-PCR confirmed the outcomes of DENV-CPE, wherever the presence of DENV-cDNA in 13 samples (25.5%) was verified and 38 samples (74.5%) showed total absences of DENV-cDNA. The band size assessed by direct comparison with a 100 bp step ladder DNA marker ranged from 100-1000 bp. All the obtained E/NS1 PCR products were at the expected size of approximately >600 bp (Fig. 2). Gel extraction of specific amplified PCR products confirmed

Fig. 2: PCR amplification of DENV-*E/NS1* gene using one-step RT-PCR. Where (L) represents the 100 step DNA ladder marker that ranged from 100-1000 bp. Lane 1, 2, 3, 4, 7 and 8, represented specific cDNA fragments of *E/NS1* gene which approximately ranged approximately 600 bp. Lane 5 and 6 represented negative control samples
The amplified products were resolved on a 2% agarose gel and stained with ethidium bromide prior to visualization under UV

the presence of cDNA of *E/NS1* gene in all the 13 PCR products (100%) and was of the expected size of approximately >600 bp (Fig. 2).

Identification of DENV genotypes based on the partial nucleotide sequence of the *E/NS1* gene: The present DENV-*E/NS1* genes were directly amplified, sequenced and aligned to establish the similarities among the 13 sequenced fragments and reference sequences represented all DENV genotypes (1-4) that were retrieved from DDBJ/EMBL/GenBank database. The created phylogenetic tree was demonstrated four distinct clusters comparable to four DENV genotypes where each cluster represents one of DENV genotype. All the 13 current isolates were joined to the cluster belonging to DENV-2 genotype with a distance average ranged +/-0.01, while no isolate was grouped with either DENV-1, DENV-3 or DENV-4 (Fig. 3).

Another phylogenetic tree was provided more analysis to DENV-E/NS1 genotype 2, based on the partial nucleotide sequence of 42 isolates of DENV-E/NS1 region including the 13 nucleotide sequences obtained in the present study. All 29 references isolates were grouped into four clusters that represented the different strains of DENV-2. An exclusive subset of all the 13 current isolates was observed to be closest to references samples isolated from Sri Lanka (AB194885, GQ252676), Australia (JQ955624) and Singapore (EU081190) (Fig. 4).

Analysis of the amino acids residues specific to the existing isolates: In the present study, all current isolates exhibited common sequences of amino acid in the E/NS1 region consistent with the categorization into DENV genotype 2. The amino acid sequence of all the present isolates was compared against those of DENV-2 reference sequences that seemed more related to them and representing strains from different countries such as Sri Lanka, Australia and Singapore.

As indicated in Table 2, the identification of substitutions was established across the partial sequencing of E/NS1 region and particularly within the E region, where two isolates (S1315 and S915) demonstrated two different amino acids substitutions Ser729Gua and Ser729Arg, respectively, isolates S1915, S1415, S4715 and S915 verified one unique amino acids substitution Val762Gau, Val780phe, Val781Leu and Val781Ala, respectively. Ten current isolates (S1315, S5515, S115, S315, S1615, S4715, S1415, S4515, S1515 and S3315) confirmed the presence of one amino acid substitution Glu858Asp. Otherwise, both S1315, S5515, S115, S315, S4715, S1415, S4515, S1515 and S3315 verified one different amino acid substitution Gln873His (Table 2).

Sequencing analysis comparison between the present isolates and previous DENV-2 strains isolated from Jeddah (1994-2006): A further sequencing analysis was carried out between the present 13 isolates and 23 E/NS1 sequence references of DENV-2 strains isolated from Jeddah. The constructed phylogenetic tree was established two distinct clusters comparable to DENV-2 isolates where the first cluster joined branches included the 13 present isolates (S115, S1115, S1415, S1515, S1315, S1615, S1915, S315, S3315, S4515, S4715, S5515 and S915) as well as the 22 reference strains previously isolated from Jeddah province (1994-2006). The second cluster included the sequence of DENV-2 reference isolate (AB189123) which isolated from Indonesia (Fig. 5).

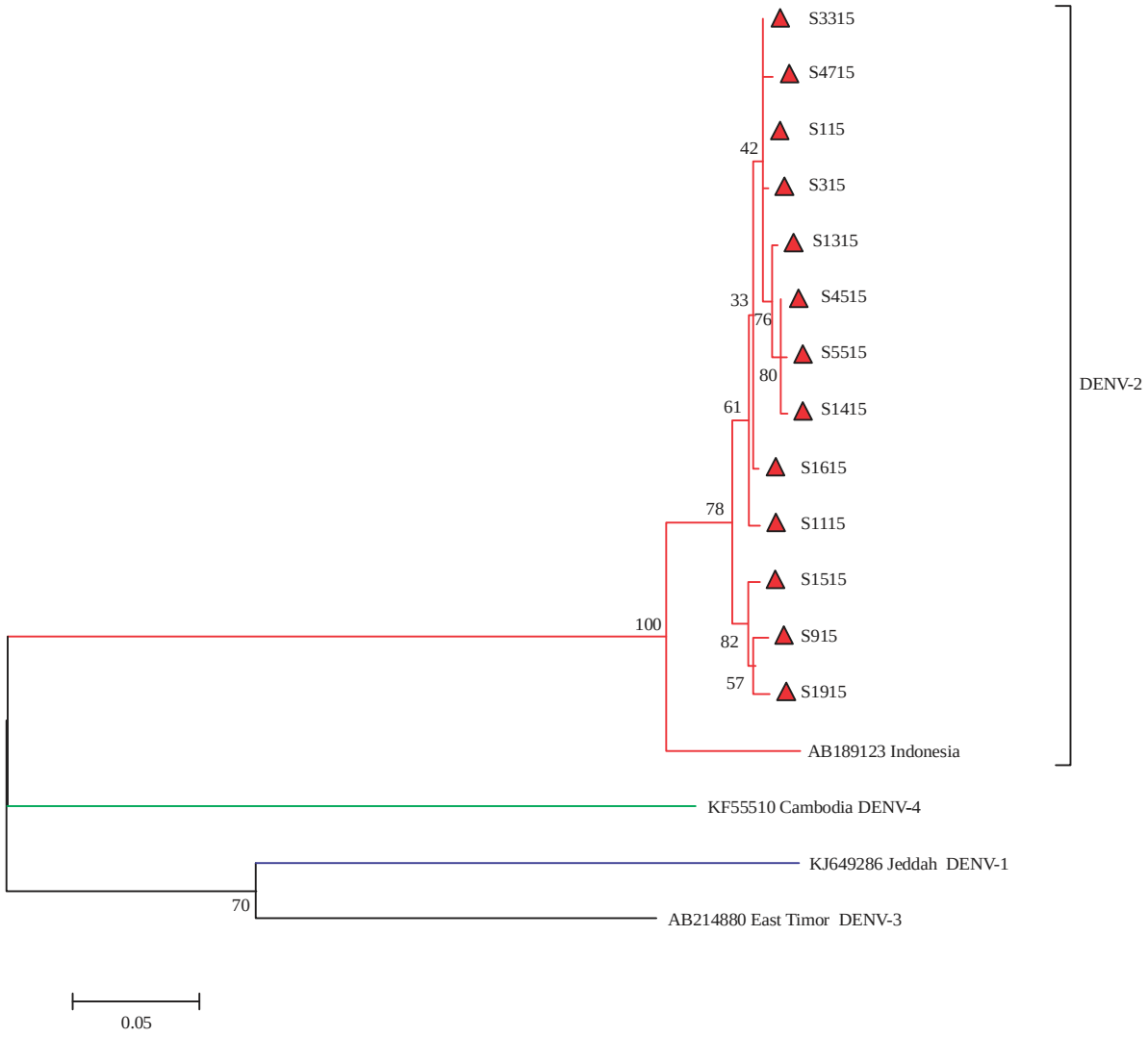

Fig. 3: Phylogenetic tree constructed by the neighbor-joining method (NJ), based on the approximately 600 bp of DENV-E/NS1 belonged to the current isolates and DENV four genotypes reference sequences retrieved from GenBank database. All DENV genotypes references represented by different colored branches and indicated by blue closed circles. The 16 Saudi DENV-E/NS1 partial sequences were determined in the present study indicated by the closed red triangle

Bootstrap values indicate the major nodes as a percentage of the data obtained from 1000 resampling

Table 2: Conserved amino acid residues in the present 13 DENV-2 strains and three reference sequences (Sri Lanka GQ252676, Australia JQ955624 and Singapore EU081190) retrieved from the DDBJ/EMBL/GenBank database

	Nucleotides															
E/NS1	GQ252676	JQ955624	EU081180													
positions	Sir Lanka	Australia	Singapore	S1315	S5515	S1115	S115	S315	S1615	S4715	S1415	S4515	S1915	S1515	S915	S3315
E/729	S	S	S	G	S	S	S	S	S	S	S	S	S	S	R	S
E/762	V	V	V	V	V	V	V	V	V	V	V	V	G	V	V	V
NS1/780	V	V	V	V	V	V	V	V	V	V	F	V	V	V	V	V
NS1/781	V	V	V	V	V	V	V	V	V	L	V	V	V	V	A	V
NS1/858	E	E	E	D	D	E	D	D	D	D	D	D	E	D	E	D
NS1/873	Q	Q	Q	H	H	Q	H	H	Q	H	H	H	Q	H	Q	H

E: Glu, V: Val, Q: Gln, H: His, D: Asp, R: Arg, A: Ala, F: Phe, L: Leu, S: Ser, G: Gua

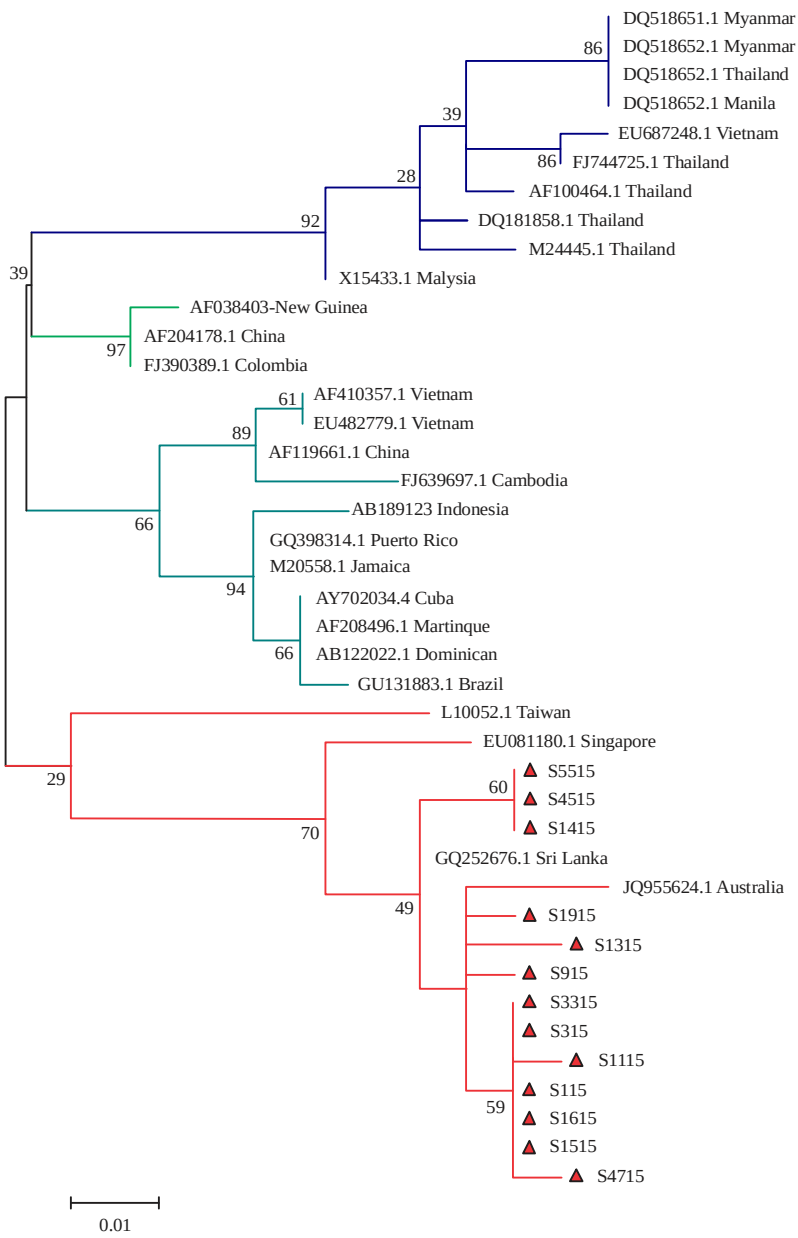

Fig. 4: Phylogenetic tree constructed by the neighbor-joining method (NJ), based on the approximately 600 bp of the existing 13 isolates and DENV E/NS1 reference sequences retrieved from GenBank database which represented different strains. The cluster that grouped the present isolates indicated with red color branch and red closed triangle

Bootstrap values indicate the major nodes as a percentage of the data obtained from 1000 resampling

The pairwise analysis was determined to estimate the nucleotide distance average between the current DENV-2 isolates and 22 DENV-2 strains previously isolated from Jeddah province from 1994-2006. Ten (76.9 %) present isolates sequences (S1315, S1115, S115, S315, S1615, S4715, S1915, S1515, S915 and S3315) seem more related to AM748163 Jeddah strain and was verified by nucleotide distance identity, average +/-0.02. Otherwise, three (23.07%) present isolates (S1415, S4515 and S5515) seems more belonged to three previous isolates AM748155, AM748160 as well as AM748161 and was verified by nucleotide distance identity, average +/-0.021, +/-0.017 and +/-0.013, respectively (Table 3).

Amino acids mutations of the current DENV-2 isolates comparable to previous Jeddah strains according to partial sequence of *E/NS1* gene: Amino acids residues mutation has been detected by comparing of the amino acid sequences alignment of Sri Lanka GQ252676 reference sequence, four

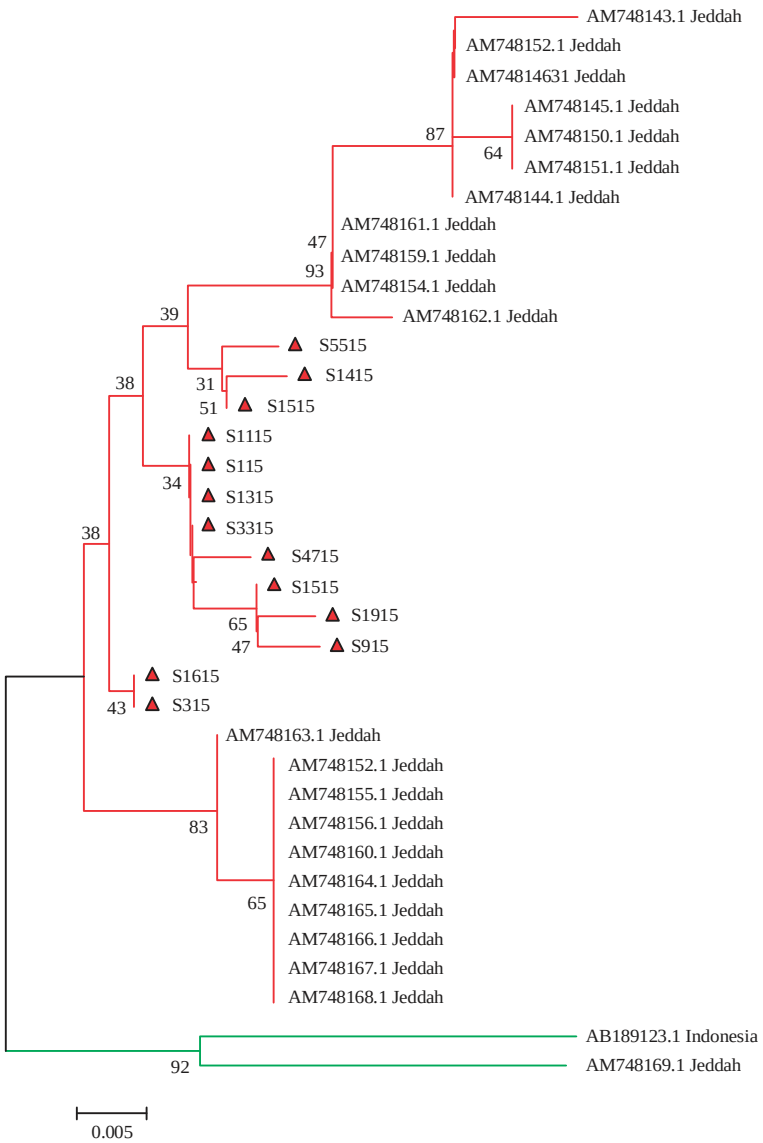

Fig. 5: Phylogenetic tree constructed by the maximum Likehood method based on the 600 bp of 22 partial E/NS1 reference sequences retrieved from GenBank database and represented available DENV isolates previously isolated from Jeddah. International DENV-2 reference isolate (AB189123 Indonesia) was represented by Blue colored branches and indicated by its accession number and country of origin. In addition to 16 partial E/NS1 present sequences that indicated by the closed red triangle

Bootstrap values indicate the major nodes as a percentage of the data obtained from 1000 resampling

references isolates (AM748154, AM748159, AM748161 and AM748163) belonging to DENV-2 strains isolated previously from Jeddah and the present 13 DENV-2 isolates.

Amino acid sequences comparison was performed between three references isolates (AM748154, AM748159 and AM748161 with four obtained isolates S1415, S515 and S4515, which that previously showed the low pairwise distance. One specific amino acid substitutions (Val780Phe) were verified in S1415 isolate. On the other hand, no amino acid mutation was recorded in either S5515 or S4515 isolates (Fig. 6).

Although, one unique amino acid residue Ala741Val was verified in all the 10 present isolates (S1315, S1115, S115, S315, S1615, S4715, S1915, S1515, S915 and S3315) compared with AM748163 reference sequence of Jeddah isolate, three isolates (S1915, S915 and S1415) showed more one unique amino acids substitution resides (Val762Gly), (Val781Ala) and (Val781Leu) (Fig. 7), respectively.

Table 3: Pairwise distances between the entire nucleotide sequence of E/NS1 region of previous DENV isolated from Jeddah (1994-2006) and 13 present isolates generated by MEGA6.05 software

AM748123	AM748143	AM748144	AM748145	AM748146	AM748150	AM748151	AM748152	AM748153	AM748154	AM748155	AM748156	AM748159	AM748160	AM748161	AM748162	AM748163	AM748164	AM748165	AM748166	AM748167	AM748168	AM748169
S1115	0.059	0.039	0.030	0.035	0.030	0.035	0.035	0.030	0.022	0.022	0.022	0.022	0.022	0.022	0.026	0.017	0.022	0.022	0.022	0.022	0.022	0.022
S115	0.059	0.039	0.030	0.035	0.030	0.035	0.035	0.030	0.022	0.022	0.022	0.022	0.022	0.022	0.026	0.017	0.022	0.022	0.022	0.022	0.022	0.022
S1315	0.059	0.039	0.030	0.035	0.030	0.035	0.035	0.030	0.022	0.022	0.022	0.022	0.022	0.022	0.026	0.017	0.022	0.022	0.022	0.022	0.022	0.022
S1415	0.069	0.039	0.030	0.034	0.030	0.034	0.034	0.030	0.031	0.021	0.031	0.031	0.021	0.031	0.026	0.026	0.031	0.031	0.031	0.031	0.031	0.031
S1515	0.065	0.044	0.035	0.039	0.035	0.039	0.039	0.035	0.027	0.026	0.027	0.027	0.026	0.027	0.030	0.022	0.027	0.027	0.027	0.027	0.027	0.027
S1615	0.054	0.044	0.035	0.039	0.035	0.039	0.039	0.035	0.017	0.026	0.017	0.017	0.026	0.017	0.026	0.030	0.013	0.017	0.017	0.017	0.017	0.017
S1915	0.069	0.048	0.039	0.043	0.039	0.043	0.043	0.039	0.031	0.030	0.031	0.031	0.030	0.031	0.030	0.035	0.026	0.031	0.031	0.031	0.031	0.031
S315	0.054	0.044	0.035	0.039	0.035	0.039	0.039	0.035	0.017	0.026	0.017	0.017	0.026	0.017	0.026	0.030	0.013	0.017	0.017	0.017	0.017	0.017
S3315	0.059	0.039	0.030	0.035	0.030	0.035	0.035	0.030	0.022	0.022	0.022	0.022	0.022	0.022	0.026	0.017	0.022	0.022	0.022	0.022	0.022	0.022
S4515	0.064	0.035	0.026	0.030	0.026	0.030	0.030	0.026	0.027	0.017	0.027	0.027	0.017	0.027	0.017	0.021	0.022	0.027	0.027	0.027	0.027	0.027
S4715	0.064	0.043	0.034	0.039	0.034	0.039	0.039	0.034	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.030	0.022	0.026	0.026	0.026	0.026	0.026
S5515	0.059	0.030	0.021	0.026	0.021	0.026	0.026	0.021	0.031	0.013	0.031	0.031	0.013	0.031	0.013	0.017	0.027	0.031	0.031	0.031	0.031	0.031
S915	0.070	0.048	0.039	0.044	0.039	0.044	0.044	0.039	0.031	0.031	0.031	0.031	0.031	0.031	0.031	0.035	0.027	0.031	0.031	0.031	0.031	0.031

Values represent the mean distances within each genotype and the present isolates

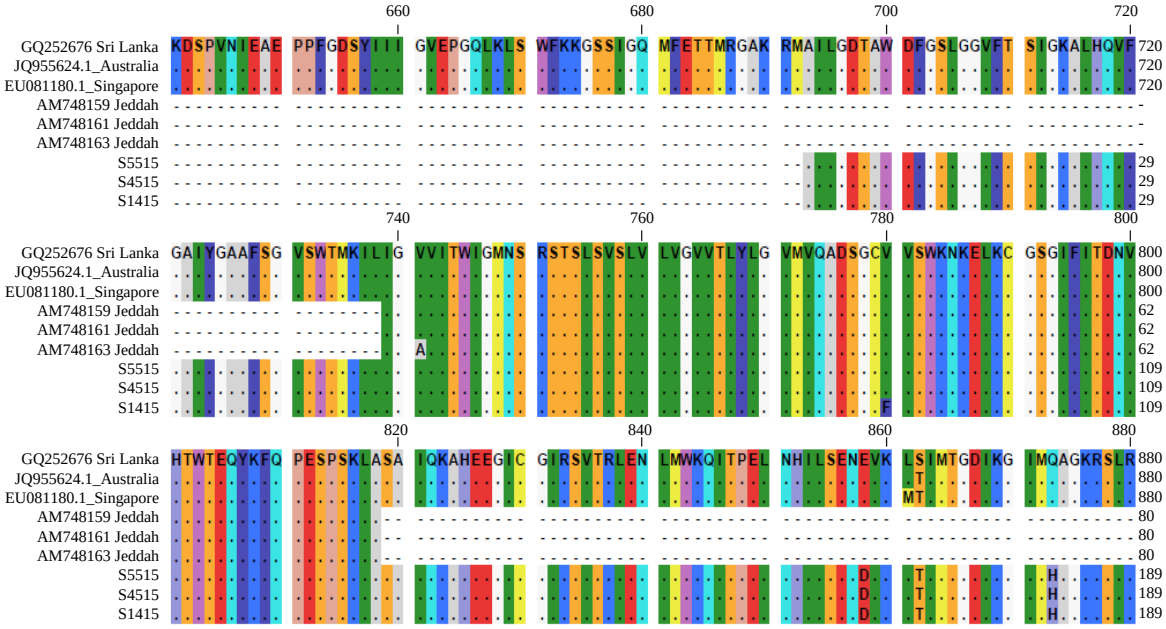

Fig. 6: Multiple alignments of amino acids sequences of E/NS1 (approximately 600 bp) of current four DENV strains and four reference sequences include one complete DENV-2 genome (GQ252676 Sri Lanka) and three E/NS1 partial references sequences of Jeddah previous isolates (AM748159.1, AM748154, AM748161) retrieved from the DDBJ/EMBL/GenBank database.

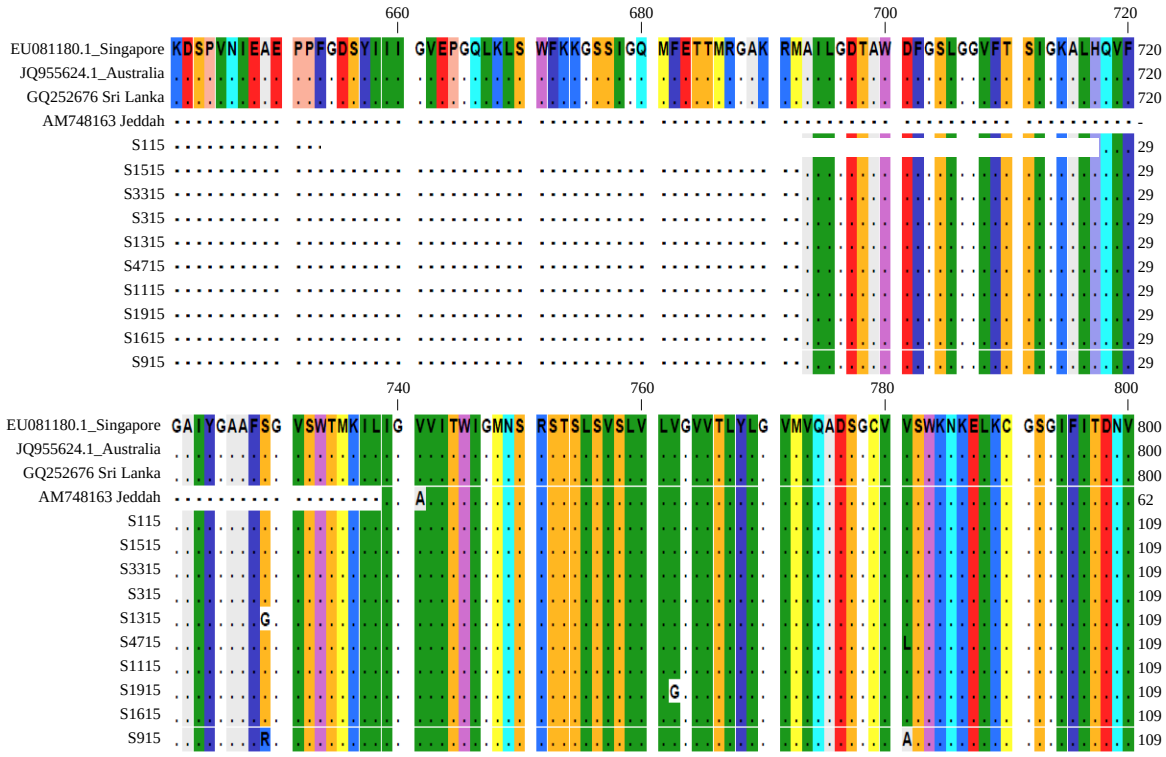

Fig. 7: Multiple alignments of amino acids sequences of the current E/NS1 (approximately 600 bp) belonged to 10 DENV strains, one complete DENV genome reference sequences (GQ252676 Sri Lanka) and E/NS1 partial reference sequences of Jeddah previous isolates (AM748163) retrieved from the DDBJ/EMBL/GenBank database

DISCUSSION

Although cell culture and molecular methods remain the "gold standard "for detection of DENV in samples, DENV isolation on cell culture is still difficult due to several reasons[26] while amplification of E/NS1 region has been reported as one of the best for molecular epidemiology and phylogenetic studies of DENV due to the high variation that exists in this region[9,10]. In the present study, both inoculation of the suspected DENV samples in the specific C6/36 cells as well as amplification of *E/NS1* gene have demonstrated the presence of specific DENV in 25.5% of the suspected samples, while total absence of any DENV were verified in 74.5%. The CPE of the current positive DENV isolates in the C6/36 cells were detected between 1-4 days post-inoculation, the current result agreed with Azhar *et al*.[26] Although cell line culture has been used as a typical method for DENV isolation over its ancestor and till now, the CPE produced in mosquito cell culture by DENV is difficult to detect because of its morphological variability[26], it may lack a DENV receptor in particularly, known as dendritic cell-specific ICAM-3[18], or may be due to the isolation of DENV from clinical specimens is frequently possible only during the viraemic-phase which occurs in the first 5 days after onset of the symptoms either on the cell line isolation[27] or molecular levels[28]. This may result in less efficient virus isolation and propagation[18].

The sequence spanning the E/NS1 junction of the present DENV isolates were determined and analyzed at the levels of nucleotide sequences and amino acid residues. The present DENV-E/NS1 genotyping phylogenetic tree succeeded to classify all of the present isolates and illustrated the dominance of DENV-2 genotype which may reach (100%) among current DENV samples with inter nucleotide distance average ranged +/-0.01, without any traces for either DENV-1, 3 and 4 genotypes in the current isolates. In harmonization to the current findings, many previous studies were demonstrated the dominance of both DENV-2 and by DENV-2 in Saudi patients, suggesting that DENV-2 was the most prevalent among Saudi patients with ratio 66-70% compared to 30% for DENV-1, while none were DENV-3 or DENV-4 positive[29,30]. It is evidenced that genotype distribution of DENV-2 remained stable in Jeddah for the last 24 years. Further phylogenetic tree has been constructed to realize more information about the origin of the present DENV strains circulating in Jeddah. All the current DENV- E/NS1 sequences clustered in one branch and seemed to be closest to DENV references samples isolated from Sri Lanka (AB194885, GQ252676), Australia (JQ955624) and Singapore (EU081190) and confirmed by nucleotide distance average +/-0.011. Though, very limited previous study had been recorded sequences similarity of the DENV genotypes in Saudi Arabia, in particular, Jeddah province[31].

Also, sequencing analysis comparison was carried out between the present isolates and previous sequence references of DENV-2 strains isolated from Jeddah (1994-2006). The current constructed phylogenetic tree was established two distinct clusters comparable to DENV-2 isolates where one cluster joined branches included the present and the previous DENV strains isolated from Jeddah province, while the other cluster included an international reference (AB189123 Indonesia). This result was supported by the pair wise analysis where 10 present isolates sequences seem more related to AM748163 Jeddah reference strain, proving by nucleotide distance identity, average +/-0.03, while three of the present isolates seem more belonged to three references isolates AM748154, AM748159 and AM748161 and verifying by nucleotide distance identity, average +/-0.021, +/-0.017 and +/-0.013, respectively. The present DENV strains that have been subjected to nucleotide analysis established these Saudi patients probably acquired their infection in their own country[32].

The DENV-E protein considered as the initial attachment of the viral particle to the host cell whereas, several molecules which interact with the viral E protein and the mannose receptor have been shown to be important factors mediating attachment and viral entry. Therefore, DENV/E confers a major target for humoral immunity[33-35]. The existing partial E/NS1 amino acid sequencing analysis compared to three references strains (Sri Lanka GQ252676, Australia JQ955624 and Singapore EU081190) demonstrated six different amino acids substitutions Ser729SGua, Ser729Arg, Val762Gau, Val780Phe, Val781Leu and Val781Ala in five current isolates (S1315, S1915, S1415, S4715 and S915). Two isolates (S315 and S1615) verified one amino acid substitution Glu858Asp, while eight current isolates (S1315, S5515, S115, S4715, S1415, S4515, S1515 and S3315) confirmed the presence of two amino acid substitution Glu858Asp and Gln873His. Interestingly, ten isolates verified the presence of unique amino acid residue Ala741Val compared to the deduced amino acid sequences of Jeddah AM748163 reference sequence, while three current isolates (S1415, S515 and S4515) showed one specific amino acid substitutions (Val780Phe) compared to previous Jeddah references isolates (AM748154, AM748159 and AM748161). There are no previous studies have been analyzed the sequence of E/NS1 amino acids of DENV strains isolated from Saudi Arabia but most of the researchers corresponded to the isolation, molecular and serological diagnosis of DENV[26,29,30,36].

The DENV E protein is a viral receptor for cell binding and fusion in various type of immune cells[37,38], which affect virulence of the DENV as well as the DENV pathogenesis *in vivo*, consistent with observations in DENV patients' immune responses[39], virus evolution via translational selection of specific sites in the DENV genome[40] or may cause variation on the immunoglobulin responses to DENV-2 infection, where antibody populations targeting broadly cross-reactive epitopes centered on the fusion peptide were large, highly variable and greater in primary than in secondary DENV-2 infected sera[41].

CONCLUSION

The results of DENV-E/NS1 sequence analysis showed the change in nucleotide and amino acid sequence in present isolates taken from the Jeddah patients and also confirmed its endogenously prevalence in Jeddah, Saudi Arabia.

SIGNIFICANCE STATEMENT

The present study confirmed the mutation in nucleotide sequence during the analysis of dengue isolates which is not explored by the previous findings and this genotype shift may help the researchers to improve the understanding of viral genotype shifts locally and their relationship with worldwide epidemiology.

ACKNOWLEDGMENTS

The authors are grateful to the King Abdulaziz City for Science and Technology (KACST), Jeddah, KSA, for their technical and financial support (Fund Number 120029).

REFERENCES

1. Huhtamo, E., E. Hasu, N.Y. Uzcategui, E. Erra and S. Nikkari *et al*., 2010. Early diagnosis of dengue in travelers: Comparison of a novel real-time RT-PCR, NS1 antigen detection and serology. J. Clin. Virol., 47: 49-53.
2. WHO., 2011. Comprehensive Guidelines for Prevention and Control of Dengue and Dengue Haemorrhagic Fever. Revised and Expanded Edition. World Health Organization, Geneva, ISBN-13: 978-9290223948, Pages: 340.
3. Gubler, D.J., 1998. Dengue and dengue hemorrhagic fever. Clin. Microbiol. Rev., 11: 480-496.
4. McBride, W.J.H. and H. Bielefeldt-Ohmann, 2000. Dengue viral infections; pathogenesis and epidemiology. Microbes Infect., 2: 1041-1050.
5. Mendez, J.A., J.A. Usme-Ciro, C. Domingo, G.J. Rey, J.A. Sanchez, A. Tenorio and J.C. Gallego-Gomez, 2012. Phylogenetic reconstruction of dengue virus type 2 in Colombia. Virol. J., Vol. 9. 10.1186/1743-422X-9-64.
6. Chambers, T.J., C.S. Hahn, R. Galler and C.M. Rice, 1990. *Flavivirus* genome organization, expression and replication. Annu. Rev. Microbiol., 44: 649-688.
7. Kuno, G., G.J.J. Chang, K.R. Tsuchiya, N. Karabatsos and C.B. Cropp, 1998. Phylogeny of the genus *Flavivirus*. J. Virol., 72: 73-83.
8. Holmes, E.C. and S.S. Burch, 2000. The causes and consequences of genetic variation in dengue virus. Trends Microbiol., 8: 74-77.
9. Rico-Hesse, R., 1990. Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. Virology, 174: 479-493.
10. Lanciotti, R.S., J.G. Lewis, D.J. Gubler and D.W. Trent, 1994. Molecular evolution and epidemiology of dengue-3 viruses. J. Gen. Virol., 75: 65-75.
11. Twiddy, S.S., J.J. Farrar, N.V. Chau, B. Wills and E.A. Gould *et al*., 2002. Phylogenetic relationships and differential selection pressures among genotypes of dengue-2 virus. Virology, 298: 63-72.
12. Aviles, G., J. Meissner, R. Mantovani an S.S. Jeor, 2003. Complete coding sequences of dengue-1 viruses from Paraguay and Argentina. Virus Res., 98: 75-82.
13. A-Nuegoonpipat, A., A. Berlioz-Arthaud, V. Chow, T. Endy and K. Lowry *et al*., 2004. Sustained transmission of dengue virus type 1 in the Pacific due to repeated introductions of different Asian strains. Virology, 329: 505-512.
14. Rico-Hesse, R., 2003. Microevolution and virulence of dengue viruses. Adv. Virus Res., 59: 315-341.
15. Kholedi, A.A.N., O. Balubaid, W. Milaat, I.A. Kabbash and A. Ibrahim, 2012. Factors associated with the spread of dengue fever in Jeddah Governorate, Saudi Arabia. East. Mediterr. Health J., 18: 15-23.
16. Alhaeli, A., S. Bahkali, A. Ali, M.S. Househ and A.A. El-Metwally, 2016. The epidemiology of dengue fever in Saudi Arabia: A systematic review. J. Infect. Public Health, 9: 117-124.
17. Thant, K.Z., K.M. Aye, S. Thein, T. Swe, F. Hasebe, K. Morita and A. Igarashi, 1995. Genotype determination of three dengue type 2 virus strains from Myanmar by sequencing *E/NS1* gene junction. Southeast Asian J. Trop. Med. Public Health, 26: 664-668.
18. Phanthanawiboon, S., A. A-Nuegoonpipat, N. Panngarm, K. Limkittikul, K. Ikuta, S. Anantapreecha and T. Kurosu, 2014. Isolation and propagation of dengue virus in Vero and BHK-21 cells expressing human DC-SIGN stably. J. Virol. Methods, 209: 55-61.
19. Ammerman, N.C., M. Beier Sexton and A.F. Azad, 2008. Growth and maintenance of Vero cell lines. Curr. Protoc. Microbiol., 11: A.4E.1-A.4E.7.

20. Lanciotti, R.S., C.H. Calisher, D.J. Gubler, G.J. Chang and A.V. Vorndam, 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J. Clin. Microbiol., 30: 545-551.
21. Fakhr, A.E., M.R. Pourkarim, P. Maes, A.H. Atta, A. Marei, M. Azab and M. Van Ranst, 2013. Hepatitis C virus NS5B sequence-based genotyping analysis of patients from the Sharkia Governorate, Egypt. Hepatitis Mon., Vol. 13, No. 12. 10.5812/hepatmon.12706.
22. El Hadad, S., S. Alakilli, S. Rabah and J. Sabir, 2015. Identification of novel D11 hepatitis B surface antigen subgenotype in Jeddah, Kingdom of Saudi Arabia. Biotechnology, 14: 119-128.
23. Saitou, N. and M. Nei, 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4: 406-425.
24. Tamura, K. and M. Nei, 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol., 10: 512-526.
25. Tallo, V.L., H. Carabin, P.P. Alday, E.Jr. Balolong, R.M. Olveda and S.T. McGarvey, 2008. Is mass treatment the appropriate schistosomiasis elimination strategy? Bull. World Health Organ., 86: 765-771.
26. Azhar, E., M. Kao, M. Niedrig, B. Masri and A. Godus *et al*., 2010. Virological diagnosis of dengue fever in Jeddah, Saudi Arabia: Comparison between RT-PCR and virus isolation in cell culture. J. Infect. Dis. Immunity, 2: 24-29.
27. Kong, Y.Y., C.H. Thay, T.C. Tin and S. Devi, 2006. Rapid detection, serotyping and quantitation of dengue viruses by TaqMan real-time one-step RT-PCR. J. Virol. Methods, 138: 123-130.
28. Fatima, F., N. Pathak and S.R. Verma, 2014. An improved method for soil DNA extraction to study the microbial assortment within rhizospheric region. Mol. Biol. Int., Vol. 2014. 10.1155/2014/518960.
29. Ashshi, A.M., 2017. The prevalence of dengue virus serotypes in asymptomatic blood donors reveals the emergence of serotype 4 in Saudi Arabia. Virol. J., Vol. 14, No. 1. 10.1186/s12985-017-0768-7.
30. Fakeeh, M. and A.M. Zaki, 2001. Virologic and serologic surveillance for dengue fever in Jeddah, Saudi Arabia, 1994-1999. Am. J. Trop. Med. Hyg., 65: 764-767.
31. Darwish, M.A., T.A. Raouf, P. Rushdy, N.T. Constantine, M.R. Rao and R. Edelman, 1993. Risk factors associated with a high seroprevalence of hepatitis C virus infection in Egyptian blood donors. Am. J. Trop. Med. Hyg., 49: 440-447.
32. Zaki, A., D. Perera, S.S. Jahan and M.J. Cardosa, 2008. Phylogeny of dengue viruses circulating in Jeddah, Saudi Arabia: 1994 to 2006. Trop. Med. Int. Health, 13: 584-592.
33. Guirakhoo, F., A.R. Hunt, J.G. Lewis and J.T. Roehrig, 1993. Selection and partial characterization of dengue 2 virus mutants that induce fusion at elevated pH. Virology, 194: 219-223.
34. Lee, E., R.C. Weir and L. Dalgarno, 1997. Changes in the dengue virus major envelope protein on passaging and their localization on the three-dimensional structure of the protein. Virology, 232: 281-290.
35. Lozach, P.Y., L. Burleigh, I. Staropoh, E. Navarro-Sanchez and J. Harriague *et al*., 2005. Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN)-mediated enhancement of dengue virus infection is independent of DC-SIGN internalization signals. J. Biol. Chem., 280: 23698-23708.
36. Shahina, W., A. Nassara, M. Kalkattawia and H. Bokharia, 2009. Dengue fever in a tertiary hospital in Makkah, Saudi Arabia. Deng. Bull., 33: 34-44.
37. Clyde, K., J.L. Kyle and E. Harris, 2006. Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. J. Virol., 80: 11418-11431.
38. Rodenhuis-Zybert, I.A., H.M. van der Schaar, J.M. da Silva Voorham, H. van der Ende-Metselaar, H.Y. Lei, J. Wilschut and J.M. Smit, 2010. Immature dengue virus: A veiled pathogen? PLoS Pathog., Vol. 6, No. 1. 10.1371/journal.ppat.1000718.
39. Grant, D., G.K. Tan, M. Qing, J.K. Ng and A. Yip *et al*., 2011. A single amino acid in nonstructural protein NS4B confers virulence to dengue virus in AG129 mice through enhancement of viral RNA synthesis. J. Virol., 85: 7775-7787.
40. Behura, S.K. and D.W. Severson, 2013. Nucleotide substitutions in dengue virus serotypes from Asian and American countries: Insights into intracodon recombination and purifying selection. BMC Microbiol., Vol. 13, No. 1. 10.1186/1471-2180-13-37.
41. Netsawang, J., S. Noisakran, C. Puttikhunt, W. Kasinrerk and W. Wongwiwat *et al*., 2010. Nuclear localization of dengue virus capsid protein is required for DAXX interaction and apoptosis. Virus Res., 147: 275-283.