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Molecular Investigation of Dengue virus serotype 2 Circulation in Kassala State, Sudan

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Summary

The tropical nature of Sudan promotes the spread of mosquito-transmitted diseases such as dengue virus (DENV) infection. The current knowledge about the geographical distribution of DENV serotypes and genotypes in Sudan is limited. In this study, molecular techniques (Reverse Transcriptase -PCR and sequencing) followed by phylogenetic analysis were used to characterize DENV isolated from blood samples of suspected dengue patients admitted to Kassala Hospital, Kassala state, Sudan, in 2016/2017. We identified DENV infection in 4 patients by RT-PCR. Phylogenetic analysis demonstrated that the isolated virus sequences belong to the Cosmopolitan genotype of DENV serotype 2. This is the first study to confirm the presence of DENV serotype 2 in Kassala state, Sudan. This study urges the need for a wider investigation of the DENV serotypes composition and estimating their contribution to the ongoing transmission.

Dengue fever is an infection caused by the arthropod-borne dengue virus (DENV), a Flavivirus, family Flaviviridae. The virus is transmitted to humans through the bites of infected Aedes mosquitoes (Ae. aegypti). This mosquito is widely distributed throughout the tropical and subtropical regions (1). Dengue symptoms in humans range from mild self-limiting fever to a severe and sometimes fatal hemorrhagic illness (2). There are four serotypes of the virus: DENV-1, DENV-2, DENV-3, and DENV-4. Studies have shown that secondary infection with a different serotype of DENV within 6 months to 1 year of primary infection can result in a more severe form of dengue disease, “antibody-dependent enhancement” phenomenon (3).

Furthermore, the evolution of new genotypes within the serotype may have a great impact on the infectivity and pathogenicity of the virus (4). Therefore it is highly important to have a continuous update of the virus status in endemic areas. In Sudan, the most affected areas by
repeated DENV outbreaks are the eastern part of Sudan (Port Sudan and Kassala states) (5).
There is insufficient information about the serotypes of DENV circulating in the region. In this study we aimed to identify the causative DENV for the dengue fever outbreak in Kassala state, using molecular techniques (RT-PCR, sequencing) and phylogenetic analysis.

Samples for this study were obtained from patients admitted to Kassala hospital, Kassala state, Sudan in the time between October 2016 and January 2017. A total of one hundred and six blood samples were collected one sample from each patient. Patients complained of fever (3-5 days) and were malaria negative. Whole blood was collected in EDTA (ethylenediaminetetraacetic acid) vacutainers from each of the 106 patients. Blood collection tubes were centrifuged at 1500 rpm for 10 minutes at room temperature to separate the blood into plasma, red blood cells and the buffy coat. Manual buffy coat extraction was performed by slowly aspirating the buffy coat with a pipette and transfer into separate tubes. RNA was extracted from the buffy coat (6) using Easy-blue total RNA extraction kit and as instructed by the manufacturer (Intron Biotechnology, Inc., Korea). Extracted RNA was kept at -80°C for further analysis.

Ethical clearance was obtained from Ahfad University for Women’s Ethics Board. Consent was taken prior to study enrolment from adult participants or in the case of children, consent was taken from their guardians.

Maxime one-step RT-PCR premix kit (Intron Biotechnology, Inc., Korea) was used for reverse transcription reaction and amplification of target region using consensus sense and antisense primers to amplify the capsid pre-membrane (C-PrM) region. Template RNA and specific primers were added into the Maxime RT-PCR premix tubes as follows: 5µl RNA template, 1µl forward primer DC1: (5’-TCAATATGCTGAAACGCGCGAGAAACCG-3’), 1µl reverse primer DC2: (5’-TTGCACCAACAGTCAA TGTCTTCAGGTTC-3’) (7). The PCR mixtures
were conducted for 35 cycles of denaturation (94°C for 30 seconds), primer annealing (55°C for 60 seconds), and extension (72°C for 120 seconds) in the Thermal Cycler (Sensoquest, Germany). Final extension step was performed at 72°C for 10 minutes. A 5μl of the reaction product was electrophoresed on a 1.5% agarose gel in 0.5X TBE buffer (pH 8.3). PCR products were then purified and sequenced. Sequencing reactions were prepared according to Applied Biosystems standard protocol. BLAST search was conducted to confirm the identity of the obtained sequences and identify similarity with other GenBank sequences. The evolutionary history was inferred using the Neighbor-Joining method based on the Tamura-Nei model (8) with a bootstrap value of 1000, the analysis involved 26 nucleotide sequences for serotype analysis and 27 nucleotide sequences for genotype analysis. The phylogenetic tree and molecular evolutionary analyses were conducted in MEGA 7 (9).

DENV was confirmed in four out of 106 of the samples using RT-PCR, showing a band size of 511bp. DNA sequences of C-PrM region were successfully obtained from the 4 samples. NCBI accession numbers of the four sequences are MF574722, MF574723, MF574724, and MF574725. The sequences determined clustered with DENV-2 when aligned with sequences representing the 4 DENV serotypes (Fig.1) and with the Cosmopolitan genotype in the same lineage with sequences from India, Pakistan, China and Jeddah-Saudi Arabia (Fig.2).

This study revealed that DENV-2 was causing a febrile illness in Kassala state. In Sudan, most researchers investigating DENV infections used serology to explore the presence of the virus, with no indication of the serotype or the genotype of the virus (10, 11, 12). Of the four DENV serotypes, only DENV-3 has been reported to have caused a major outbreak in PortSudan city, Sudan, in 2005 (13). DENV-2 has been recently reported in Jeddah, Saudi Arabia, DENV-2 isolated in Saudi Arabia belonged to the Indian lineage of the Cosmopolitan genotype with close
similarity to other strains from South Asian countries and they attributed these findings to pilgrims coming from South Asia during the pilgrimage season (14). Interestingly, the phylogeny of DENV-2 genotyped in this study also showed that it belongs to the same lineage of the Indian isolates of the Cosmopolitan genotype and isolates from China, Pakistan and Jeddah, Saudi Arabia (Figure 2). The fact that dengue in Sudan is a public health issue confined to the coastal and subcostal parts of Sudan at the Red Sea and the phylogenetic similarity of the detected virus with the Asian strains suggest that the virus has been introduced through the Sea-shipping movement and international trading with dengue endemic Asian countries, either through infected ship-workers or introducing infected mosquito (\textit{Ae. aegypti}) into the area. DENV-2 has been associated with a more severe form of dengue upon secondary infection (15). The detection of DENV-2 among humans in Kassala state warns of the possibility of a future outbreak of the disease and the emergence of severe dengue cases. These findings may also suggest that DENV-2 might have co-existed and contributed along with DENV-3 in the previous outbreaks of DENV infection in this area without being confirmed. Further studies are needed to investigate the current epidemiology of DENV serotypes circulating in the Sudanese population, and also to evaluate the contribution of the entomological and socioeconomic factors.

We conclude from these findings, that eastern parts of Sudan are currently affected by the DENV-2, this is not only risking the lives of the Sudanese population in these areas but also there is a great risk of exporting this virus to the neighbour countries which we have open borders and intensive human and trading movements including particularly Eretria and Ethiopia.
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Conflict of interest

The authors declare no conflict of interest.

References


Figure legends

**Figure 1.** Phylogenetic tree of DENV serotypes: Sequence dataset was analysed using MEGA7, the neighbour-joining (NJ) method, and bootstrap analysis (1000 replicates) based on the ClustalW algorithm. The scale bar indicate 0.1 nucleotide substitutions per site. Reference sequences shown as: country/year__accession number. Sequences isolated in this study are designated by black circles. The tree was rooted with out-group yellow fever virus sequence.

**Figure 2.** Phylogenetic tree of DENV-2 Genotypes: Sequence dataset was analysed using MEGA7, the neighbour-joining (NJ) method, and bootstrap analysis (1000 replicates) based on the ClustalW algorithm. The scale bar indicate 0.05 nucleotide substitutions per site. Each Reference sequence of DENV-2 is abbreviated by its accession number followed by country and year of isolation. Sequences isolated from Sudan are designated by black circles. The tree was rooted with out-group DENV-3 sequence.