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Detection of Dengue Viral Infections in *Aedes aegypti* in Kassala City, Sudan 2014 - 2015

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Abstract

Dengue burden has been rising in the recent years and became a public health problem of significant importance especially in Kassala City, Sudan. This study was conducted in Kassala City, Sudan, during 2014 and 2015, to carry out molecular detection of dengue virus in Aedes aegypti. Aedes aegypti samples were collected from 20 sites of Kassala City in summer, winter and autumn seasons. Indoor and outdoor resting Ae. aegypti adults were collected from residential houses using knockdown technique, light traps and mouth aspirators. The samples of Ae. aegypti were preserved in RNAlater for molecular detection of dengue virus. The randomly captured females were pooled in five pools 4 to 5 sites in one pool and each pool was crushed ten mosquitoes per pool, and using RT-PCR test. The results revealed that 68/100 (68%) according to pooling system were positive for dengue virus. It is concluded that dengue virus is endemic in Kassala City of Eastern Sudan. Control of Ae. aegypti is recommended targeting

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water holding containers and to increase people's awareness and medical intervention for treatment of dengue disease.

Key words: Dengue virus, *Aedes aegypti*, Sudan

1. INTRODUCTION

Dengue fever (DF) is the most important disease caused by dengue (DEN) virus transmitted by the *Aedes aegypti* (Liu *et al*., 2016). Some *Aedes* species are also vectors of some filariasis and viral disease (Beerntsen *et al*., 2000). There are four antigenically related, but distinct, dengue virus serotypes; DEN-1, DEN-2, DEN-3 and DEN-4, all of which can cause dengue fever and dengue haemorrhagic fever (Gurugama *et al*., 2010), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) have emerged as a global public health problem in recent decades. DHF and DSS are most commonly observed in children under 15 years, but they also occur in adults (Sam *et al*., 2013). Infection with one serotype gives life-long immunity for that serotype but not to the others. Secondary infection with a heterologus serotype from the primary infection enhances the risk of developing DHF/DSS (Wikramaratna *et al*., 2010).

The first documented epidemic of DENV in Africa refers to South Africa (1927) (Franco *et al*., 2010) being the first isolate, of DENV-1, obtained from Nigeria (1964) (Konongoi *et al*., 2016). Other cases and/or outbreaks of DENV-1 were reported in many parts of Africa including the Sudan. The detection of DENV-specific antibodies and circulation of the virus in infected people is well documented along the Red Sea coast of Eastern Sudan. Recently, Kordofan region in western Sudan has witnessed several dengue fever outbreaks due to social unrest and war in the region. In the Sudan, the first outbreak of DENV was reported in 1986 among febrile patients

in the Red Sea State (Eldigail *et al*., 2018), and DENV serotypes 1 and 2 were identified as the causative agents of the outbreak (Seidahmed *et al*., 2012). An outbreak of acute febrile illness occurred later in 1989 in the Northern Province of Sudan and the prevalence of DEN-2 antibody was 24% Additional serological evidence of DEN-2 infection in Sudan was reported 1995 (Malik *et al*., 2011). Port Sudan city is Sudan's main seaport on the Red Sea. The main dengue vector *Ae. aegypti* has been reported in the area since the 1930s (Seidahmed *et al*., 2012). The dengue virus serotypes DEN1 and DEN2 were first detected in the 1980s in Port Sudan, while DEN-3 was recently identified in an outbreak (Malik *et al*., 2011) including eastern parts (Adam *et al*., 2010; Abdallah, 2014) particularly Kassala, Eastern Sudan (Abdallah *et al*., 2012). A recent Seroepidemiology survey reported high prevalence of DENV-specific IgG among febrile patients in Kassala State in Eastern Sudan (Himatt *et al*., 2015). The aim of this study was to carry out molecular detection of dengue virus in *Ae. aegypti* in Kassala City of Eastern Sudan.

2. MATERIALS AND METHODS

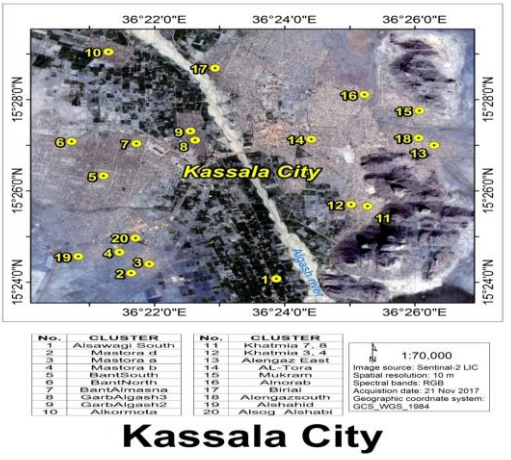
2.1. Study design: A cross sectional longitudinal entomological study was performed during the three seasons; dry (winter), hot (summer) and wet (autumn) for two consecutive years (2014 and 2015).

2.2. Study area and experimental design

Adults female *Aedes aegypti* were collected in 20 sites of Kassala City in three seasons dry (winter), hot (summer) and wet (autumn) on a monthly basis for 24 months in 2014 and 2015, using Knockdown technique, mouth aspirators and light traps. Collections were made from 0600 hrs to 1800 hrs in all rooms within the homestead. Indoor collections involved

inspections of every room, and any resting mosquitoes were aspirated. Each five females were stored in RNAlater in -20°C in eppendorf tubes until used for RNA extraction of dengue virus.

Map 1: Map of Sudan – Kassala State



Map 2: Kassala City where sites (o) of sampling are numbered
The Source: National Center for Research

2.3. Samples preparation

Using NS1 primer for detection of dengue RNA virus in pooled samples *Ae. aegypti* were used in five groups of localities. These groups were Alkormota GarbAlgash2 and Garb Algash3 in one group, BantAlmasna, BantNorth and BantSouth, in another group, Khatmia3,4, Khatmia7,8 and Mukram in the third group, Alsawagi South, Alshahid and Alnorab in the fourth group and Biriai, Alengaz East, Alengaz south and Altora (Table 1). *Ae. Aegypti* females preserved in RNAlater were divided into pools (ten females per pool), according to seasons and place of collection. Each ten females were washed by 500 µl phosphate buffered saline (PBS) pH (7.5) to remove RNAlater. A volume of 500 µl PBS was added to crush each ten females in a sterile mortar and pestle until it was liquefied. This was resuspended in 250 µl PBS and then centrifuged shortly for three minutes at 10,000 rpm in order to remove particles which were discarded. The crushed samples were taken using a pipette and kept in a separate sterile 1.5 ml eppendorf and kept at -20^{o}C for further use.

2.4. RNA extraction:

Total RNA was extracted by using the InnuPREP Virus DNA/ RNA kit according to the protocol of the manufacturer (AnalytikaJena, Germany). Briefly, 400 µl of lysis solution containing carrier Mix were added into a 1.5 µl reaction tube into 400 µl of the sample and 20 µl proteins K, mixed vigorously by vortexing for ten sec, and then incubated for ten minutes at 70^{o}C. Subsequently, 400 µl of binding solution was added to the lysed samples and mixed by vortexing. Then, 650 µl of the resulting solution were applied to a column, and centrifuge at 12.000 rpm for one minute, 500 µl Washing Solution were added to the sample and centrifuge at 12.000 rpm for one minute, then, centrifuged a column at 14.000 rpm for three minutes to remove all traces. Then, 60 µl pre-heat RNase–free

water were added to the column and incubated at room temperature for two minutes, then centrifuged at 10.000 rpm for one minute. Finally the collected RNA was stored at -80°C for further analysis.

2.5. cDNA synthesis:

Target of the virus RNA was converted to a complementary DNA copy (cDNA) using reverse transcriptase (RT) on extracted RNA by using Maxime RT PreMix Reverse Transcriptase kit (Intron, Korea). Template RNA (5 µl) were added into Maxime RT premix tubes, then 15 µl RNase –free water (Oligo dTor random primer) were added to a total volume of 20 µl, and dissolved the clear pellet by pipetting and placed in a thermal cycler programmed as follows: 45°C for 60 minutes to cDNA synthesis, 95°C for five minutes to RTase inactivation step, and then stored RT product frozen at −20°C.

2.6. Polymerase Chain Reaction (PCR) for dengue amplification:

The PCR was performed by processing the synthesized cDNA with prime sequences (Forward: 5-ACAGGTTCTTTTAG GGAG-3, reverse: 5-TGCCATCGTCGTCAC -3) were selected from a conserved region of the fourth exon of the dengue (NS1) gene, located in the Hind III-X fragment of the AD-169 strain. Optimized PCR reaction for dengue amplification was performed according to Bonilauri *et al*. (2008). Briefly, PCR was carried out in a total volume of 25µl ready master mix (MaximPCR PreMix kit(i-taq), koria) 5 µl Master Mix (dNTPs, MgCl2, i-taq polymerase, Reaction Buffer, Gel Loading buffer). Two µl of primers (1 µl included of each specific primers), 13 µl of distilled water, 5 µl of cDNA, then dissolved the tube pellet by pipetting. Finally, The mixture amplified in thermo-cycling condition using PCR machine (TECHNE, JAPAN) as follows95°C for 5 minutes, followed by 35 cycles of 95°C for 30

sec, 55°C for 30 sec and 72°C for 60 sec. The PCR was carried out in a total volume of 25µl and the amplified PCR product was detected by agarose gel electrophoresis.

2.7. Agarose gel-electrophoresis:

2.7.1. Preparation of Gel:

Sufficient electrophoresis buffer were prepared (1X Tris Borate EDTA buffer) to fill the electrophoresis tank and to prepare the gel, then added the correct amount of powdered agarose (1.5%) to a measured quantity of electrophoresis buffer in an Erlenmeyer flask, heated the slurry in a microwave oven until the agarose dissolves. Cooled the solution to 60°C and ethidium bromide to a final concentration of 2 µg/ml added and mix thoroughly. Horizontal slab gels were poured on a glass plate or plastic tray that can be installed on a platform in the electrophoresis tank, then the gel was allowed to set.

2.7.2. Electrophoresis procedure:

Electrophoresis was carried out with the gel submerged just beneath the surface of the buffer. 10 µl of the PCR product samples were loaded into the slots of the submerged gel using a disposable micro pipette. DNA Marker of known size was loaded into slots on the left sides of the gel. The lid of gel tank was closed and attached the electrical leads so that the DNA migrated towards the anode (red lead). To obtain maximum resolution of DNA fragments agarose gels should was run at 120 volts/cm^2 and 35A, and was run for 1hur at room temperature. Turned off the electric current and removed the lid from the gel tank. The presence of ethidium bromide allowed the gel to be examined by ultraviolet illumination at any stage during electrophoresis. The gel was examined by gel documentation system INGeNius (Germany). The expected size of immediate early (NS1) gene amplicon was 1,013 bp.

3. RESULTS

The results showed that out of 68% samples females *Ae. aegypti* were positive (68%/100%) using NS1 primer. All dengue total positive mosquito pools were found highest positivity in winter (72.2%), followed by autumn season (64.7%), and the lowest in summer season (66.7%). The total positivity of dengue virus was the highest in pools of BantAlmsna, BantNorth and BantSouth (78.6%), followed by pools of Alkrmota, Garb Algash2, and Garb Algash 3(70%), compared with other pools. It was highly negative in autumn (17.6%) than winter and summer (14.7% for both). The total positive percent was high in winter season (38.2%), followed by autumn season (32.4%) (Table 1).

Table 1: Detection of dengue viral RNA in *Ae.aegypti* females collected pooled clusters in Kassala City during 2015

Pooled Clusters	Winter	Summer	Autumn	Total Positive/total samples (%)
	+ve/total	+ve/total	+ve/total	
Alkrmota GarbAlgash2 Garb Algash3	4/6	6/8	4/6	14/20(70)
BantAlmasna BantNorth BantSouth	8/10	6/8	8/10	22/28(78.6)
Khatmia3,4 Khatmia7,8 Mukram	4/6	2/4	6/8	12/18 (66.8)
Alsawagi South Alshahid Alnorab	6/8	4/6	2/4	12/18 (66.8)
Biriai Alengaz East Alengaz south Altora	4/6	2/4	2/6	8/16(50)
Total (%)	26/36 (72.2%)	20/30(66.7%)	22/34(64.7%)	68/100(68)
Out of total +ve	26/68 (38.2%)	20/68(29.4%)	22/68 (32.4%)	68/100(68)

+ve = positive dengue RN

Representative photographs of agarose gel analysis of RT-PCR for detection and typing of dengue virus in 50 field caught *Ae. aegypti* pools are shown in (Fig.). Lines 3, 5, 6, 9, and 13) represent negative while (4, 7, 8, 10, 11, and 12) positive. Prototype of DEN band visible in 1.013bp, and the DNA size marker (100 bp).

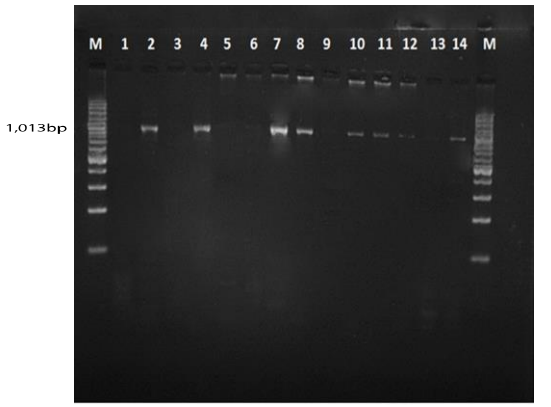


Fig.1: Dengue RT-PCR results (1,013 bp)on 2 % agarose gel. Lanes 1 shows negative control; lane 2 shows positive control, lanes 3,5,6,9 and 13 show negative samples; lanes 4, 7, 8,10,11,12, and 1show positive samples; M: 100 bp DNA size marker.

4. DISCUSSION

RT-PCR is known to be of high sensitivity for detection dengue virus. De Paula and Fonseca. (2004) found that RT-PCR is a rapid, sensitive and simple method used for genome diagnosis in human clinical samples, biopsies and autopsy tissues and mosquitoes and the results showed that out of 68% samples mosquito pools were positive for dengue RNA, this seems to be unrealistic result due to the sampling followed by the pooling of samples from different clusters, these may be found at least one of sample per pool could be positive .Then for the prevalence may vary from 3-68%.In this study the RT-PCR should be taken as a quantitative outcome indicate in this

study. This indicate the Kassala locality is high prevalence of dengue fever and this agrees with Eldigail *et al.* (2018) who found high prevalence of DENV-specific IgG among febrile patients in Kassala State in Eastern Sudan. Himatt *et al.* (2015) reported the dengue is endemic in Kassala locality with wide spread of the dengue vector, and Secondary infection is a risk factor for DHF and DSS. Also suspected cases were reported from Kassala and the Red sea States Khair (2015). Total positive *Ae. aegypti* pools were found high positivity in winter (26%) than summer (20%) and autumn (22%).It can be explained that a moderately humidity in the environment may influence vector efficiency and dengue virus transmission. This finding agrees with Ooi and Gubler, (2009) who reported that during the appearance of the disease epidemics, the environmental factors such as temperature, rainfall and humidity affecting dengue transmission should be considered as well. It was concluded that the information of the seasonal field surveys showed that *Ae. aegypti* was found in very high densities in some residential areas of Kassala City. Dengue virus was detected in *Ae. aegypti* population in 68% of the specimens examined**.** It is important to increase people's awareness and medical intervention for control of dengue fever in Kassala City is recommended**.**

Some a group of pooled like BantAlmasna, Bant North, and Bant South were recorded high prevalence of dengue virus (78.6%) this referred to the habits of the different ethnic groups in the some residential areas are heavily populated and they store plenty of water in different types of containers, some keep animals and store water for them.

5. CONCLUSIONS

Ae. aegypti populations were found in very high densities in some residential areas of Kassala City**.** Dengue virus was

detected in *Ae. aegypti* in pooled samples in 68%of the five pools**.**

6. RECOMMENDATIONS

In the present study RT-PCR based molecular method was developed and tested for detection of dengue virus in *Ae. aegypti*. Use of this method as a tool for vector surveillance can serve as an early warning monitoring system of dengue outbreak in Kassala City.

Conflict of Interest

The authors declare no conflict of interest in relation to this work.

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