Detection and molecular typing of dengue virus circulating among febrile patients in a private tertiary institution in North Central Nigeria

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Abstract- There is still paucity of published data on dengue virus particularly its circulating serotypes in Nigeria and Africa at large. This study was however conducted to detect and type dengue virus (DENV) serotype(s) circulating among febrile patients in a private tertiary institution in North Central Nigeria. After informed consent, 400 blood samples were collected from febrile patients at the University Health Centre. The resulting sera were screened for DENV seromarkers (IgM, IgG and NS1) using Aria Dou DENV RDT kits (CTK Biotech, Inc, San Diego, USA) while all positive samples were serotyped by PCR using type specific primers. Data obtained were analyzed using Smith's Statistical Package (Version 2.8, California, USA) and p value of ≤0.05 was considered statistically significant. Of the 400 patients screened, 12(3.0%) were positive for acute and recent DENV infection. Age and gender of the patients were not associated with the infection (p>0.05). However, it was higher among female subjects of age ≤20 years. Of the 12 positive samples serotyped, 7(58.3%) were of DENV-3 while the remaining 5(41.7%) were not-typable. Our findings confirmed the circulation of DENV-3 in the study area. Hence, there is need for the inclusion of dengue virus test in the routine diagnosis of febrile illnesses to avoid misdiagnosis and mistreatment.

Index Terms- Dengue, Febrile illness, Infection, Nigeria, Serotypes

I. INTRODUCTION

Dengue, also known as 'breakbone fever', is the fastest spreading vector-borne viral infection transmitted between humans by the day biting vector mosquitoes; *Aedes aegypti* and *Aedes albopictus* [1]. The infection is caused by one of four dengue virus serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) belonging to the genus flavivirus within the *Flaviviridae* family and each serotype has different interactions with the antibodies in human serum [2]. Dengue virus contains a positive strand RNA with a spherical lipid envelope. The RNA genome codes for envelope, capsid and membrane structural proteins in addition to seven non-structural proteins. Infection with any of the dengue virus sub-types may result either in an asymptomatic infection or a febrile illness of varying severity ranging from mild illness to more severe forms such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [3, 4].

The disease is endemic to more than 100 countries in the tropical and subtropical regions of the world especially tropical Asia, Central and South America, Africa and the Caribbean [5, 6, 7]. This infection is rapidly expanding and its global footprint is a public health challenge with an economic burden [7, 8].

Early signs and symptoms of dengue virus infection are indistinguishable from those of other tropical disease such as malaria and typhoid. However, infected individuals may be asymptomatic but sometimes they may present with dengue fever (DF), dengue hemorrhagic fever (DHF), or Dengue shock syndrome (DSS) [4, 7].

A vaccine for dengue fever has been approved and is commercially available in a number of countries [7]. Other methods of prevention include reducing mosquito habitat and limiting exposure to bites [7] while treatment of acute dengue is supportive and includes giving fluid either by mouth or intravenously for mild or moderate disease. For more severe cases, blood transfusion may be required [9].

The surveillance of dengue virus infection in Nigeria is affected by the lack of routine laboratory diagnosis which may include culture, polymerase chain reaction (PCR), and serological assays [10]. The true incidence and impact of dengue fever in Nigeria is unknown and this could be attributed to the fact that it is not a reportable disease in Nigeria. Most cases are often, misdiagnosed as malaria, typhoid or referred to as pyrexia of unknown origin [11]. In Nigeria where malaria and typhoid are highly endemic, most cases of febrile illness are likely to be treated as presumptive malaria or typhoid some of which often resist anti-malaria and antibiotic treatment [12].

Dengue and malaria infections have similar geographical areas of distribution, and similar factors encourage the spread of both infections [13, 14]. For instance, due to poor drainage system, poor environmental sanitation of the villages surrounding the University, the University is in its self-surrounded by bush and streams which may result in infestation of the day-biting mosquitoes that transmit dengue infection and night biting mosquitoes that spread Malaria. Thus an existence of high dengue burden where malaria and typhoid are endemic may be expected. Therefore, this study was conducted to detect and type dengue virus circulating among febrile patients in a tertiary institution in North Central Nigeria.

II.   MATERIALS AND METHODS

2.1 Study area

The study was conducted among patients that visited Bingham University Health Centre, Karu, Nigeria. This is a facility that provides health care services majorly to students and staff of the University and also to people living in the neighboring communities.

Bingham University is located around kilometer 25 Keffi-Abuja express way, Auta-Baleifi in Kodape District, Karu Local Government Area of Nasarawa State [15].

2.2 Ethical clearance

The ethical clearance for this study was obtained from Research and Ethical Committee of Bingham University Teaching Hospital Jos, Plateau State, Nigeria.

2.2 Study population

The study population consisted of male and female adults Bingham University students, staff and members of the neighboring communities that accessed the University Health Centre from February to July, 2017. Each participant was consented and socio-demographic information was obtained from them by the use of a designed questionnaire.

2.3 Sample size determination

The sample size was determined using the formula by Naing *et al.* [16] for sample size calculation a 0.05 level of precision:

$$n = \frac{Z^2 pq}{d^2}$$

Where:

n = derived minimum sample size if the target population is > 10, 000.

Z = standard normal deviation at the required confidence interval (1.96) which corresponds to 95% confidence interval.

p = Expected prevalence rate (6.0%) (0.1) [11].

q = 1 − p = 0.9

d = degree of accuracy/precision expected i.e. 0.05

$$n = \frac{(1.96)^2 (0.1)(0.9)}{(0.05)^2}$$
$$= \frac{3.8416 \times 0.09}{0.0025}$$
$$= \frac{0.3457}{0.0025}$$
$$= 138.29$$
$$n = 138$$

This was however rounded up to 400 samples.

2.4 Sample collection, processing and storage

A total of 400 blood samples were collected from febrile in and out patients seeking medical care at Bingham University Health Centre, from February through July, 2015. About 3 ml of venous blood sample was collected from each participant aseptically into an EDTA container. The serum was obtained after centrifugation at 1,200 revolutions per minute for 5minutes [17]. The plasma was stored at -20°C until ready for use.

2.5 Laboratory analysis

2.5.1 Detection of dengue virus seromarkers

All samples were screened for the presence of dengue virus Immunoglobulin G (IgG), Immunoglobulin M (IgM) and dengue virus antigen (NS1 Ag) using Aria Dou dengue virus rapid diagnostic test kit (CTK Biotech, Inc, San Diego, USA). The test was conducted and interpreted according to manufacturer's instructions.

2.5.2 Molecular characterization of dengue virus

A molecular typing system based on reverse transcriptase polymerase chain reaction (RT-PCR) using type-specific primers was used for the detection of the four dengue virus serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) according to previously described methods [18].

2.5.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was carried out in two rounds with MJ Research PTC-100 programmable thermal cycler (MJ Research Inc., Watertown, USA), using oligonucleotide primers that were adopted from the work of Lanciotti *et al.* [19]

2.5.3 Dengue virus RNA extraction

The viral RNA was extracted from dengue virus positive samples using QIAamp Viral RNA isolation kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

2.5.4 RT-PCR procedure

In a single tube, viral RNA was converted to a DNA copy (cDNA) prior to enzymatic DNA amplification by the use of reverse transcriptase (RT) and the DENV downstream consensus primer D2-5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3' homologous to the genomic RNA of the four serotypes (DENV-1, DENV-2, DENV-3 and DENV-4). Subsequent Taq polymerase amplification was performed on the resulting cDNA with the upstream dengue virus consensus primer D1-5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'. Target RNA was amplified in 25 µL volumes containing the following components: 800mMdeoxynucleotide triphosphates (dNTPs), 8mM dithiothreitol, 0.24 µM each of primers D1and D2, 0.5U of AMV RT (Promega, Madison, WI, USA), and 0.625U of Dreamtaq DNA polymerase (Fermentas Inc., USA). The reactions were allowed to proceed for 1 h at 42^{0}C and then to proceed with 95^{0}C for 3 minutes for initial denaturation followed by 35 cycles of denaturation (95^{0}C for 30 sec), primer annealing (55^{0}C for 1min), and primer extension (72^{0}C for 2min) along with final extension (72^{0}C for 5 min).

DENV serotyping was conducted by second-round amplification (nested PCR) initiated with 10 µ of diluted material (1:100 in sterile distilled water) from the initial amplification reaction. The total 20 µL of reaction mixture was prepared using 2 µL of diluted first PCR products, 0.8mM dNTPs, 0.5U of Dreamtaq DNA Polymerase and 0.3 µM of primer D1 and 0.3µM of dengue virus typespecific primers: TS1 5'-CGTCTCAGTGATCCGGGGG-3', TS2 5'-CGCCACAAGGGCCATGAACAG-3', TS3 5'-TAACATCATCATGAGACAGAGC-3', and TS4 5'-CTCTGTTGTCTTAAACAAGAGA-3'. Dithiothreitol and AMV RT were eliminated. The samples were subjected to initial denaturation (95^{0}C for 3min) followed by 20 cycles of

denaturation (95^0C for 30 s), primer annealing (55^0C for 1min), and primer extension (72^0C for 1min) along with final extension (72^0C for 5min). The PCR products were analyzed by running a 1.5% agarose gel stained with ethidium bromide.

The sizes of PCR products were estimated in relation to the migration pattern of a 100bp to 1000bp increments plus DNA molecular marker (BIONEER Daejeon, North Korea). The results were interpreted considering the specific size of each serotype as follows; 482bp for serotype 1, 389bp for serotype 2, 290bp for serotype 3 and 114bp for serotype 4.

2.6 Data analysis

The information obtained from the questionaires and results of laboratory tests were analysed using Smith's Statistical Package (version 2.8, California, USA). Descriptive Statistics were presented in table and figure. Chi-square test was used to determine the relationships between the socio-demographic data and the prevalence of dengue virus infection. P value of ≤ 0.05 was considered statistically significant at 95% confidence interval.

## III.   RESULTS AND DISCUSSION

In Nigeria, most cases of febrile illnesses are often misdiagnosed and/or mistreated as presumptive malaria or typhoid some of which often resist antimalaria and antibiotic treatment [11, 12]. In this study, 400 patients with febrile illnesses attending Bingham University Health Centre were screened for dengue virus infection. Dengue virus IgM, IgG and NS1 were used as surrogates for the detection of dengue virus infection. Of the 400 patients screened in this study, 12(3.0%) were positive for both NS1 antigen and IgM antibody while none (0%) of the patient was positive for IgG antibody (Figure 1). This is an indication that there was no past exposure to the virus in the study population (since IgG antibody was negative) rather positive cases (3.0%) were of acute and recent infection [11].

The 3.0% prevalence of dengue virus infection recorded in this current study is higher than the 2.3% reported by Onyedibe *et al.* [11] in Maiduguri and Jos, 2.2% by Dawurung *et al.* [20] Jos and 1.8% by Idoko *et al.* [10] in Kaduna. However, much higher prevalence were reported especially from the Southern region of the country. These include the 17.2% in Ogbomosho [21], 25.7% Ile-Ife [22] and 35.0% in Ibadan [23]. Studies from other parts of Sub-Saharan Africa also show higher prevalence between 21-26.3% of the viral infection [24, 25, 26]. The climatic conditions in the rainforest region of Southern Nigeria which support increased mosquito breeding than the dry Sahel region of Northern Nigeria may possibly account for the higher prevalence reported in Southern Nigeria and other parts of Africa [27].

There is no significant association between prevalence of dengue virus infection with age and gender in this study ($P > 0.05$). This is an indication that regardless of age and gender, all patients are equally susceptible to the virus (Table 1). However, the higher prevalence recorded among females of age ≤20 years in this study is consistent with the r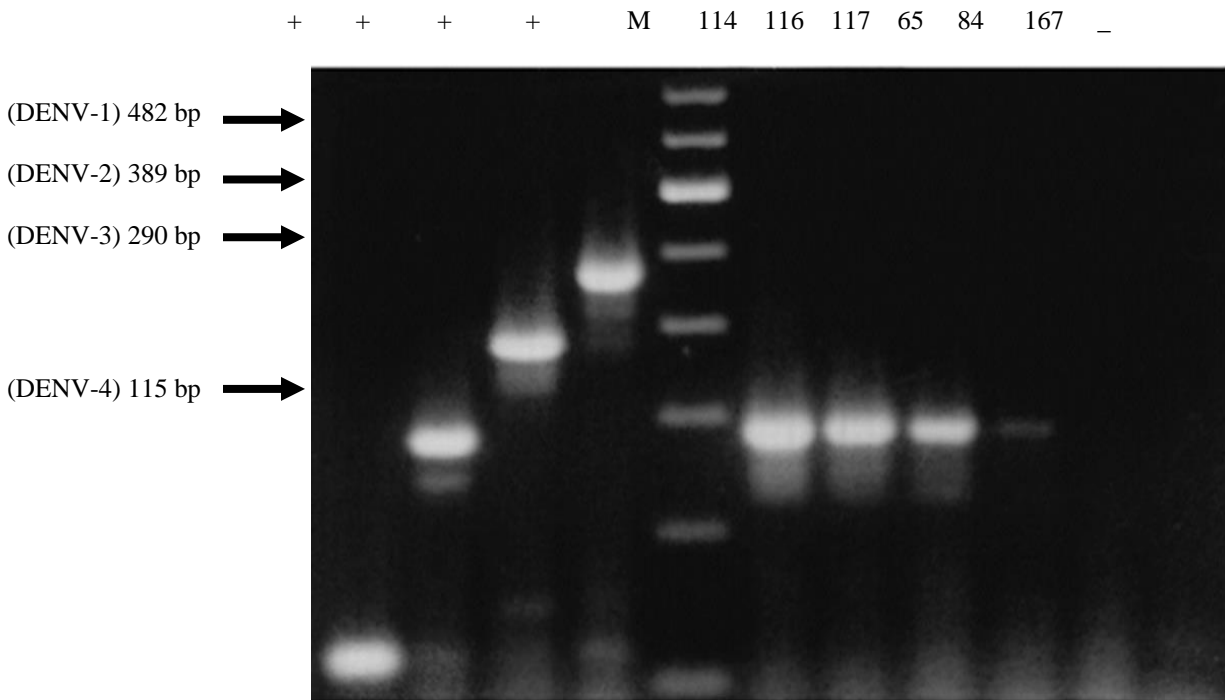eports of other previous studies [22, 28]. There is still paucity of published data on DENV particularly its circulating serotypes in Nigeria and Africa at large. In this study however, all the 12(3.0%) samples positive for DENV were serotyped by a molecular typing system based on RT-PCR using type-specific primers for the detection of the four DENV serotypes (DENV-1, DENV-2, DENV-3 and DENV-4). Of the 12 samples serotyped, 7(58.3%) were of DENV-3 while the remaining 5(41.7%) samples were not-typable (Figures 2 and 3).
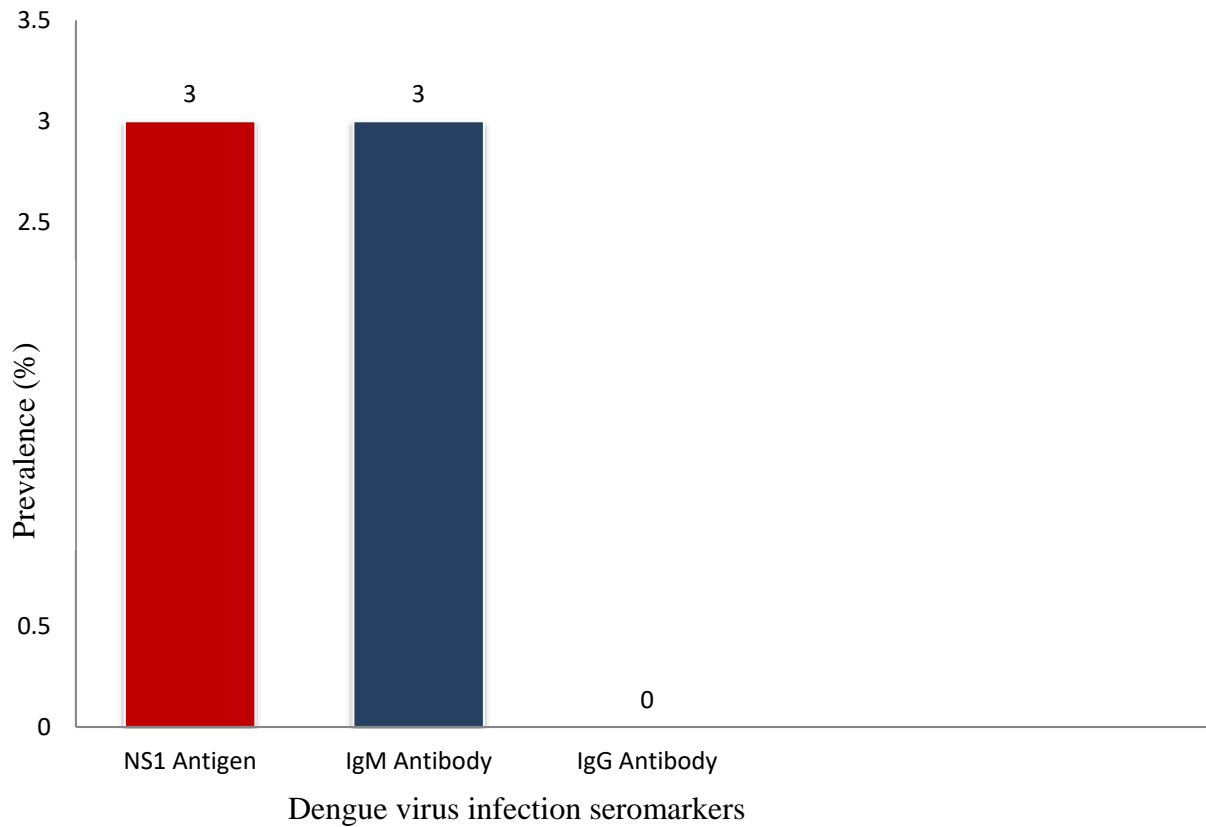
Figure 1: Prevalence of dengue virus infection seromarkers among febrile patients in a private tertiary institution in North Central Nigeria.

Table 1: Prevalence and distribution of dengue virus infection among febrile patients in a private tertiary institution in North Central Nigeria in relation to age and gender

Parameter	No. Examined (N=400)	No. Positive (N=12)	Prevalence (%) (Overall=3.0%)	P-value
Age (Years)				
≤20	242	9	3.7	
21-30	116	3	2.6	
≥30	42	0	0.0	0.1480
Gender				
Male	204	3	1.8	
Female	194	9	4.6	0.0506

Figure 2: Results of dengue-specific RT-PCR followed by second round nested PCR of RNA samples, showing band in 1.5% agarose gel electrophoresis stained with ethidium bromide. Lane M: 100 bp DNA ladder, 482 bp for DENV-1, 389 bp for DENV-2, 290 bp for DEN-3, 114 bp for DENV-4, + for positve controls and – for negative control.
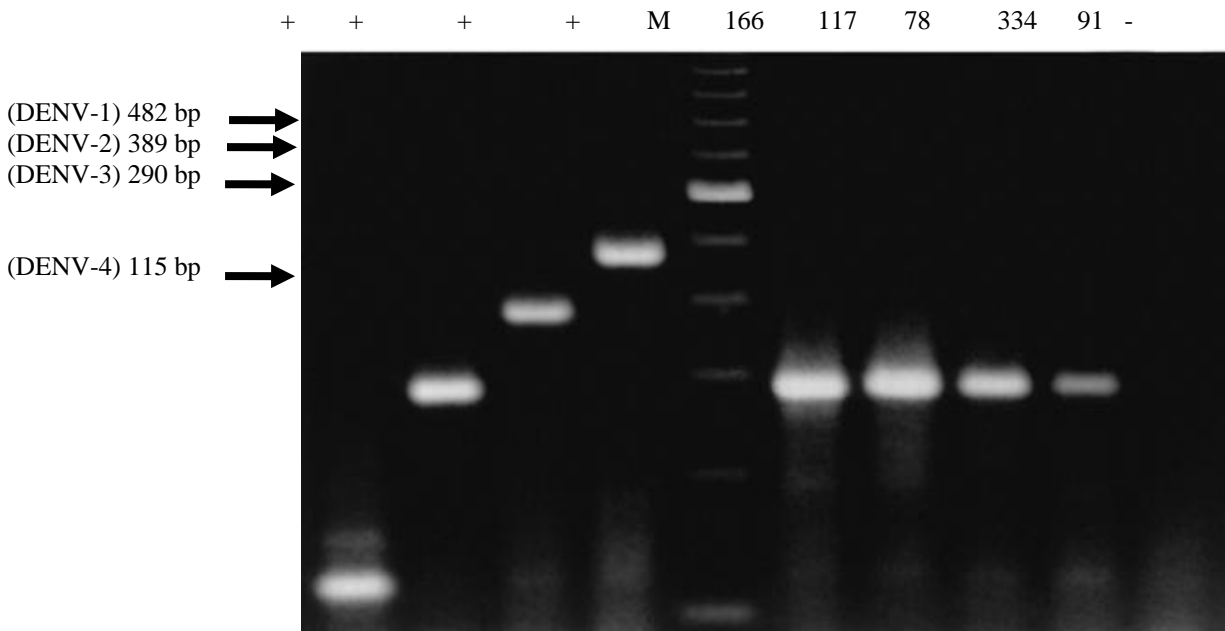
Figure 3: Results of dengue-specific RT-PCR followed by second round nested PCR of RNA samples, showing band in 1.5% agarose gel electrophoresis stained with ethidium bromide. Lane M: 100 bp DNA ladder, 482 bp for DENV-1, 389 bp for DENV-2, 290 bp for DEN-3, 114 bp for DENV-4, + for positve controls and – for negative control.

The detection of only DENV-3 in this study implies that the infection in the study population is monotypic with a single serotype. Furthermore, 5(41.7%) DENV positive samples were not-typable in this study and this might probably be due to mutation in the viral genome, errors in DNA extraction protocols or PCR procedures.

All the four DENV serotypes (DENV-1, 2, 3 and 4) have been detected in Nigeria [6, 29, 30] with predominance of DENV-3 [29, 30, 31, 32]. DENV serotyping is of medical importance because each serotype has different interactions with the

antibodies in human serum [2]. That is, infection with any serotype produces antibody which does not give long-lasting homologous immunity which does not confer protection against other serotypes [33].

IV. CONCLUSION

This study recorded 3.0% prevalence of acute and recent DENV-3 infection among patients with febrile illnesses at Bingham University Health Centre, Nigeria. The present of the infection in the study area which was found to be higher among female subjects of age ≤ 20 years calls for alarm particularly in Nigeria where it may be misdiagnosed and mistreated as malaria or typhoid.

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