American Journal of Epidemiology and Infectious Disease, 2022, Vol. 10, No. 1, 7-18
Available online at http://pubs.sciepub.com/ajeid/10/1/2
Published by Science and Education Publishing
DOI:10.12691/ajeid-10-1-2

Multiple Serotypes of Dengue Unmasked from Suspected Malaria Patients are Significantly Associated with Yellow Fever Vaccination in Northeastern Nigeria

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Received March 13, 2022; Revised April 17, 2022; Accepted April 25, 2022

Abstract In Nigeria, greater than 70% of febrile illnesses are treated presumptively as malaria, without a laboratory evaluation. This study aimed at providing evidence-based information on the burden, classification of dengue and determination of the circulating Dengue virus (DENV) serotypes in Nigeria. Serum samples from suspected malaria patients who visited randomly selected health institutions in three northeastern states were analyzed using Rapid NS1 antigen detection, IgM and IgG ELISA, NS1 IgG ELISA and plaque reduction neutralization tests ($PRNT_{90}$). Cumulative results of the study detected 21.3% confirmed, 77.6% highly suggestive and 34% probable dengue. In Bauchi State, the IgG: IgM and IgM: IgG ratio indicated 93.5 % recent secondary and 6.5% primary dengue virus infections respectively. In Borno state, 86.3% had recent secondary dengue by NS1IgG. Five of seven NS1 were single infections, one multiple infection while one was likely to be serotype 1 which was not tested. Serotype 2 was the commonest in the three states but 2 and 4 were significantly higher in Adamawa than Bauchi and Borno. Multiple dengue serotypes obtained including 2 and 3, 3 and 4, 2 and 4 and 2 3, 4 were significantly higher in Adamawa than in Bauchi and Borno states. Antibody to serotype 2 was observed in all the ages studied especially 0-9 and 30-39 years. Numerically, samples collected 1-7 days after onset of symptoms had more DENV NS1 and antibodies than 7-10 days. Patients who received treatment with either or combination of antibiotics/antimalarial are more likely to be protected against dengue than untreated. Similarly, patients who were vaccinated against YF had more neutralizing antibodies to DENV than the unvaccinated and NS1 was more in unvaccinated than the vaccinated. Antibiotics/ antimalarial may ameliorate recovery from acute dengue and Yellow Fever vaccination may reduce dengue burden and its associated complications in resource-constrained countries.

Keywords: *dengue virus, malaria, antigen, antibody, serotypes, Nigeria*

Cite This Article: Bamidele Soji Oderinde, Zakari Adamu Shuaibu, Chiamaka Precious Okoye, Musa Idowu Audu, Erick Mora-Cárdenas, Tea Carletti, Alessandro Marcello, Thlama Gulanda Awi, Jesse Romeo Buba, and Marycelin Mandu Baba, "Multiple Serotypes of Dengue Unmasked from Suspected Malaria Patients are Significantly Associated with Yellow Fever Vaccination in Northeastern Nigeria." *American Journal of Epidemiology and Infectious Disease*, vol. 10, no. 1 (2022): 7-18. doi: 10.12691/ajeid-10-1-2.

1. Introduction

Dengue, which is also known as 'break-bone fever'' is caused by the Dengue virus which belongs to the genus *Flavivirus* and family *Flaviviridae*. DENV consists of four closely related but antigenically distinct serotypes (DENV 1-4), which cross-react but do not cross-protect. Thus, infection with one serotype provides lifelong immunity to that infecting serotype only [1,2] though reinfection by the same serotype could occur [3]. The clinical outcomes of dengue with any of the serotypes vary from asymptomatic to symptomatic illness, which can be non-specific (mild illness) or malaria-like, to more severe forms (Dengue hemorrhagic fever (DHF) or Dengue shock syndrome (DSS) with concrete risk of fatal outcome [4]. Acute DENV infection is detectable through RT-PCR, NS1 antigen, or virus isolation and a four-fold increase in the antibody titers between acute and convalescent samples in serological assays (Haemagglutination inhibition test (HI), ELISA IgM / IgG and the plaque reduction neutralization test (PRNT)).

Dengue virus (DENV) incidence has more than doubled every decade from 1990 to 2013 compared to other communicable diseases [5]. Over the last two decades, dengue cases have alarmingly increased more than 8-fold ranging from 505,430 cases in 2000, to over 2.4 million in 2010, and 4.2 million in 2019, with deaths from 960 to 4032 [6]. World Health Organization (WHO) has estimated 390 million DENV infections per year (95% credible interval 284–528 million), of which 96 million (67–136 million) are symptomatic [4]. Similarly, the report of severe dengue epidemics has also increased from 9 cases before 1970 to 100, with Asia bearing 70% of the global burden. Interestingly, dengue is the second most diagnosed cause of febrile illness after malaria among residents of low- and middle-income countries as well as travelers returning from these countries. Negligence of DENVs and other arboviruses poses a great public health threat to immunologically naïve populations in Africa. It is eminent that the hyper-endemicity of malaria, its atypical signs with arbovirus infections at its prodromal phase and the lack of appropriate diagnostic facilities for differential diagnosis of febrile illness are major confounding factors. Presently, there is no specific therapeutics for dengue and effective supportive management requires appropriate diagnosis at the prodromal phase (non-specific symptoms). Dengue vaccine (Dengvaxia (CYD-TDV) has been licensed in 20 countries where it is limited to seropositive individuals and those aged 9-45 years for safety reasons [7,8] but is not yet available in many African countries. The climatic conditions, vegetation, and high immunological naïve population in Nigeria favor the breeding of vectors, the emergence and re-emergence of the pathogens they transmit as exemplified in previous reports [9,10,11]. The first documented DENV outbreak in Abeokuta, Nigeria occurred in 1977 during which six strains of DENV 1 were isolated [12]. Between 1980 and 2008, surveillance for DENV was completely absent till 2009 when it was detected in suspected malaria cases [9]. Several studies have reported the endemicity of DENVs in different parts of Nigeria [9,13,14,15,16]. In 2014, a 15-year-old female died of DHF caused by co-infections with the four serotypes of DENV in Nasarawa State, Nigeria [17]. Nevertheless, DENV infections are still neglected, under-recognized, under-reported and under-estimated in the country [9,18]. Whilst systemic surveillance for dengue in Nigeria is still lacking, malaria remains the primary suspect in every febrile illness. Co-infection of DENV and malaria parasites, which have been reported in many countries could further complicate the timely diagnosis and proper management of dengue cases [18,19,20,21]. Therefore, estimating the dengue burden in the country will remain a mirage in the absence of differential diagnoses of febrile illness.

This study aimed at providing evidence-based information on the current status of DENV infections masked by malaria suspected cases in northeastern Nigeria. To achieve this objective, the burden of dengue, its classification and circulating serotypes were determined. The use of three diagnostic assays with varied specificity and sensitivity produced findings that necessitate the need for the establishment of the appropriate diagnosis of dengue with the view to reducing its burden, malaria misdiagnosis and wrong treatment. Effect of other variables such as the interval between onset of symptoms and sample collection, treatment with antibiotics/antimalarial and Yellow Fever vaccination is discussed.

2. Materials and Methods

2.1. Study Area

Nigeria, which is a West African country and most populous in Africa is located at latitude 9.081999 and longitude 8.67527 (https://www.geodatos.net/en/coordinates/nigeria). It covers an area of 923,769 square kilometers (356,669 square mi). The current population of Nigeria based on Worldometer elaboration of the latest United Nations data was estimated at 212,277,004 on 16 September 2021. Nigeria shares a border with the Nigeria Republic in the north, Chad in the northeast, Cameroon in the east and Benin in the west and comprises 36 states and Federal Capital Territory, Abuja. The southern part of the country is defined by its tropical rainforest climate with an annual rainfall of 1,500 to 2,00 millimeters (60 to 80 in) per year [22]. The mean annual temperature in Nigeria is 26.9°C with average monthly temperatures ranging between 24°C (December, January) and 30°C (April). North-eastern Nigeria is one of the six geopolitical zones and comprises six states: Adamawa, Bauchi, Borno, Gombe, Taraba and Yobe (Figure 1). The northeast is marked by Sudan and Sahel savanna ecology with a population of 18,984,299 (according to the 2006 Nigerian population census) and is characterized by relatively high temperatures throughout the year with the annual average varying from 28.32°C in Yola to 25.92°C in Bauchi while rainfall ranges between 467 mm at Nguru to 1091 mm at Ibi [23]. The randomly selected health institutions located in the three states studied were Federal Medical Centre (FMC), Adamawa state, Abubakar Tafawa Balewa University Teaching Hospital (ATBUTH), Bauchi State and Specialist Hospital, Borno State (Figure 1).

2.2. Study Population

Only patients with febrile illness who visited the selected hospitals for a malaria test were recruited. 316 females and 284 males whose ages ranged between 2 and 86 years were considered (Table 1). Formal approval for this study was obtained from the Research and Ethics Committee of Abubakar Tafawa Balewa University Teaching Hospital, Bauchi (ATBUTH), ref ATBUTH/ADM/42/Vol.1, dated 25 May 2017, State Specialist Hospital (SSH), Maiduguri, Borno State, with ref no. SSH/GEN/641/Vol.1 dated 14 February 2018 and Federal Medical Centre, Yola with ref no FMC/YO/001/Vol.1 dated May 2018. Written informed consent was obtained from each patient and from the parents of children under five years of age before sample collection.

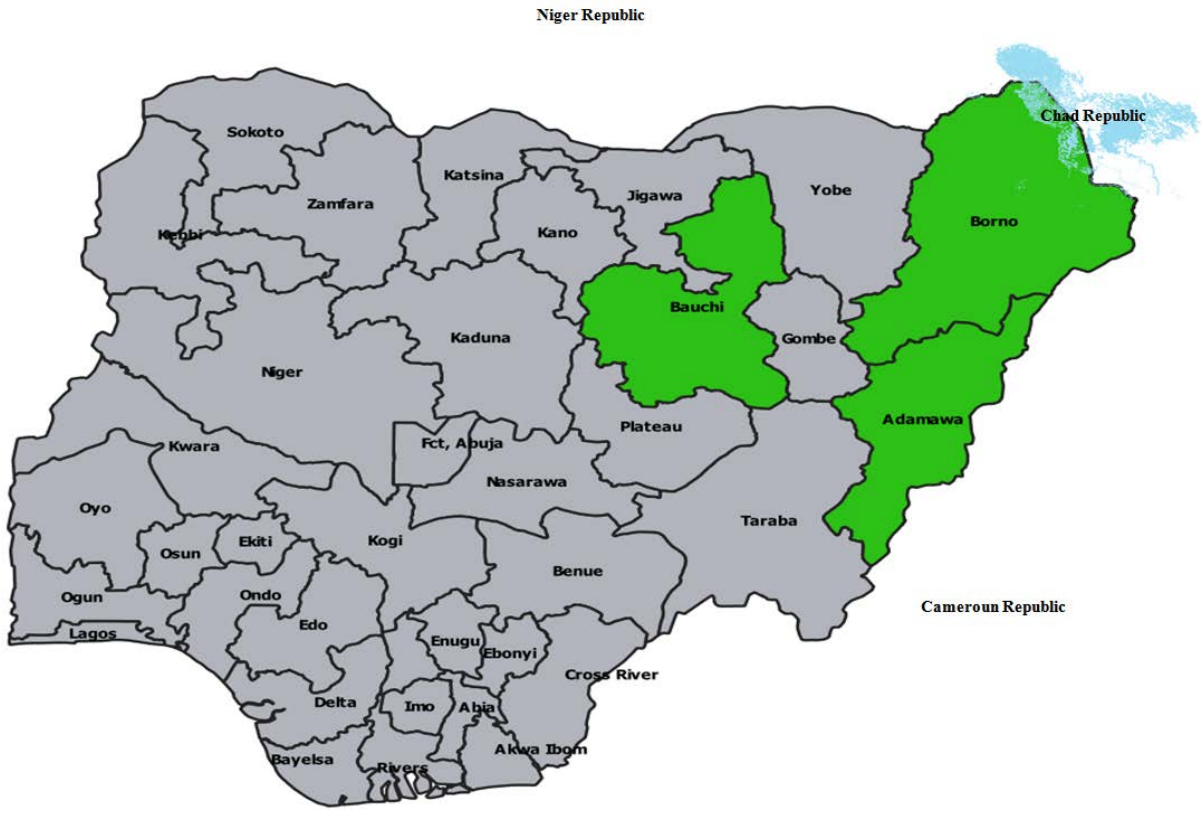

Figure 1. Map of Nigeria with indicated states and collection sites (green)

Table 1. DEMOGRAPHIC CHARACTERISTICS OF THE PATIENTS ENROLLED IN THE STUDY

Items	States			Total
	Adamawa	Bauchi	Borno	
Gender				
Male	104	104	108	316
Female	96	96	92	284
Total	200	200	200	600
Age Groups (years)				
0-9	10	25	6	41
10-19	40	31	28	99
20-29	52	39	60	151
30-39	50	48	52	150
40-49	26	32	26	84
50-59	12	19	14	45
60-69	6	6	9	21
70-79	3	0	3	6
80-89	1	0	2	3
Total	200	200	200	600
Interval between symptoms onset and sample collection (days)				
1-7	147	132	169	448
7-10	52	68	31	152
Total	200	200	200	600
Treatment with antibiotics/antimalarial before malaria test				
Treated	139	104	112	355
Not treated	59	86	88	233
Not specified	2	10	0	12
Total	200	200	200	600
Yellow Fever Vaccination status				
Vaccinated	107	12	15	134
Not vaccinated	93	61	183	337
Not specified	0	127	2	129
Total	200	200	200	600

2.3. Sample Collection

About 3 ml of each patient's whole blood was collected by venepuncture into sterile glass plain bottles, allowed to clot at room temperature for 20 minutes and centrifuged at 3000rpm for 5 minutes. The serum was aspirated into the sterile cryovials and stored at – 20°C until it was transported from Adamawa and Bauchi States to the Virology Laboratory, University of Maiduguri Teaching Hospital (UMTH) using a Thermobox.

2.4. Plaque Reduction Neutralization Test (PRNT)

The PRNT was performed as described previously with few modifications [28]. Each serum sample was inactivated at 56°C for 30 minutes and stored at 180°C. Vero E6 cells were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 2% Penicillin/Streptomycin and 1 % HEPES and 2.5 % sodium bicarbonate. The cells were seeded in 24-well plates at a density of 1 x 10^5 cells/ well and incubated at 37°C for 24-48 hours or until it reached 70-80% confluence. Each serum sample was diluted 1:8 using in-house diluent (IHD) prepared using PBS supplemented with Penicillin/Streptomycin (22%), Gentamycin (0.2%) and Fungizone (0.02%). An equal volume of DENV virus stock (obtained from Bernhard Nocht Institute for Tropical Medicine (BNITM), Hamburg, Germany in 2009) at a concentration of 100 PFU/ml was added to the diluted serum and incubated at 37°C for 1 hour. Each serum dilution–virus mixture was prepared in duplicate and three controls including a virus dose control (100 PFU virus plus diluent only), a cell control (diluent and cell only)

and negative virus control were tested simultaneously. After incubation at 37°C for 1 hour, the virus-serum mixture was added to Vero cells and incubated for 1 hour at 37°C. The plates were rocked gently every 15 minutes for uniform distribution of the inoculum. The overlay medium was prepared by mixing equal volume of 12% Carboxyl methylcellulose sodium salt (Low viscosity) (Sigma) with EMEM supplemented with 4% foetal bovine serum. 1 ml of the overlay medium was added to each of the 24 wells and incubated at 37°C for 10-14 days. Thereafter, each well was fixed with 500 µl of 10% formaldehyde solution for 30 minutes after removing the overlay and stained with 500 µl of 0.5% crystal violet (Sigma) for 20-30 minutes. The percentage of plaque reduction by specific antibody was calculated using the formula: 100-(Number of plaques in sample/Number of plaques in control) x 100. The validity of the test was determined by the virus control having a minimum of 50 plaques and cell control and positive sera having no plaques at all. The PRNT titre was defined as the reciprocal of the serum dilution that reduced the number of plaques by 90% ($PRNT_{90}$) [24]. Modification in this study includes non-removal of inoculum before the addition of the overlay medium unlike the previous report [25].

2.5. Qualitative DENV 1-4 Antibody IgM ELISA (Performed on Samples from Adamawa, Bauchi and Borno)

Sera samples were analyzed using Sandwich Complex ELISA kits (Diagnostic Automation/Cortez Diagnostics, Inc., USA, code: 8117-35) and the protocol described by the manufacturer was used. Briefly, each serum was diluted at 1:40 (positive, negative controls and patients' sera) and treated with 40 µl of Rheumatoid factor at room temperature (RT) for 10 minutes. 140 µl of the treated sera were incubated in microtiter wells coated with DENV 1-4 antigens for 10 minutes at RT. After appropriate washing, the wells were incubated with the enzyme-conjugated antibodies for 10 minutes at room temperature. The wells were treated with the chromogen and incubated for 10 minutes at 37°C before the reaction was stopped. The plate was read at an optical density (OD) of 450 nm within one hour after the reaction was stopped. The average OD units of the positive control wells were ≥ 0.5 while the average OD units of the negative control wells were 0.0-0.3. The cut-off values of ≥ 0.5 and ≤ 0.3 were used to determine the positive and negative samples respectively. Samples with OD units of > 1.0 were considered strong reactive. According to the manufacturer, the kit has a specificity of 100% and a sensitivity of 85.9%.

2.6. Qualitative DENV 1-4 Antibody IgG ELISA (Performed only on Bauchi Samples)

Sera samples were analyzed using Sandwich Complex ELISA kits (Diagnostic Automation/Cortez Diagnostics, Inc., USA, code: 811-35) according to the manufacturer's instructions. Briefly, each diluted serum (1:40), including positive, negative controls and the patient's serum was incubated with the DENV 1-4 antigen in a coated microtitre plate. The plate was incubated at RT for 10 minutes. Enzyme conjugate was added to the wells after appropriate washings, incubated at RT for 10 minutes. The plates were washed and incubated with the chromogen at RT for 5 minutes before the reaction was stopped. The plate was read at an optical density (OD) of 450 nm within one hour after the reaction was stopped. The cut-off OD units of ≥ 0.5 and ≤ 0.3 were used to determine the positive and negative samples respectively.

2.7. NS1 detection by Rapid Test (Performed on Samples from Adamawa, Bauchi and Borno)

The Dengue NS1 Detect Rapid Test (InBios, USA, code: DNS1-RD) uses immunochromatography technique for the qualitative presumptive detection of non-structural protein 1 (NS1) in human serum. It is a membrane-based immunoassay that aids early detection of DENV infections even prior to the presence of IgM or IgG. The Dengue NS1 Detect Rapid Test membrane is pre-coated with NS1 specific antibody on the test line region. The test strip has a separate control and sample region to assure assay flow and performance. During testing, the strip was placed in the well containing three drops of the buffer. 50ul of the sample was dropped into the sample pad. The sample side of the rapid test was made to face downward into the well. Within 20 seconds, the red colour was expected to move up the membrane. However, when this aspect was not observed, the arrows above the sample pad were touched gently to permit the flow of the conjugate and sample up the membrane. The result was read after 30 but before 45 minutes. Each batch of the samples was tested with positive and negative control sera. The result was considered negative when only the control line appeared but positive when both the control and test lines were observed on the strip. According to the Manufacturer's instruction, the presence of weak but distinctively red line was considered positive because the intensity of the red colour depends on the concentration of NS1 present in the sample. The test was considered invalid if no lines appeared at the control region. According to manufacturer, the sensitivity and specificity of the test was 76.5% and 96.7% respectively.

2.8. NS1-based Enzyme-linked Immunosorbent Assay (rNS1-based ELISA)

Recombinant rNS1-based ELISA was produced and supplied by the Laboratory of Molecular Virology, International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy. The test was performed as previously described by Mora et al [26]. Briefly, ELISA plates coated with DENV rNS1 were incubated with serum diluted 1/20 in PBS and probed with anti-human IgG antibodies. The OD 450 cut-off values of ≥ 0.5 and ≤ 0.3 were used to determine the positive and negative samples, respectively.

2.9. Statistical Analysis

Statistical analysis was done on an MS Windows-based PC computer. The data were first keyed into a Microsoft

Excel spreadsheet and then analyzed by Statistical Package for the Social Sciences (SPSS), Version 20.0, from SPSS incorporation Chicago IL. We used the mean standard deviation/standard error of the mean, and percentage when appropriate for the patient's characteristic description. Age groups and Gender were compared using the Pearson $\chi 2$ or Fisher's exact test for categorical variables. Epi-info version 7.0 which was designed and distributed by CDC was used to obtain odd ratio and Confidence intervals for DENV serotype distribution in the three states, the association between dengue and treatment with antibiotics as well as Yellow Fever vaccinations. No odd ratio was calculated for variables with zero values and P-values of 0.05 or less were considered statistically significant. Likelihood ratios were determined for different IgG/IgM ratios and the best value was determined by looking at the combination of these parameters. The sensitivity and specificity of IgM ELISA and Rapid DENV NS1 detection were computed using PRNT (positive for any of the serotypes tested) as reference.

3. Results

The presence of DENV neutralizing antibodies (nAb), confirmed by $PRNT_{90}$, and NS1 antigen in the serum samples tested indicated undetected dengue outbreaks.

3.1. The Classification of DENV Infections Based on Serological Findings

Dengue virus infections were confirmed, highly suggestive and probable among 21.3% (106/496), 77.6% (385/496) and 6.8 % (34/496) patients who had a febrile illness and were suspected of malaria. In Bauchi State, 50% (100/200) of the patients simultaneously had DENV IgM and IgG which indicated recent secondary DENV infections. The IgG: IgM ratio on these Bauchi patients also indicated 93.5% (187 /200) of recent secondary DENV infections while IgM: IgG ratio of 6.5% (13/200) indicated primary infections at the time of sample collection. Similarly, 86.3% (170/197) of Borno patients had DENV NS1 IgG which indicated recent secondary DENV infections. Only a few patients (30.8%;153/496) had no dengue in the three states studied.

3.2. The Distribution of DENV Antigen and Antibodies in Adamawa, Bauchi and Borno States

1.4% (7/496) of the patients in the three states had acute dengue represented by the NS1. Only one of the seven NS1 neutralized serotypes 2, 3 and 4 simultaneously, two neutralized DENV-3, two DEN-4 and one DENV-2 while one failed to neutralize serotypes 2, 3 and 4 with a high probability of being DENV-1 which was not included in this study. More patients in Borno (4 (0.8%) had DENV NS1 than Adamawa with 2 (0.4%) and Bauchi with only 1 (0.2%). The presence of NS1 was not significantly different in the three states but it is more likely to occur in Borno (OR=2.701) than Bauchi (OR= 0.33) and Adamawa (OR=0,798). DENV neutralizing antibodies to one or more serotypes were detected among 404 (71.9%) of 562 patients while 19% (114/600) had IgM.

Table 2. Classification of dengue virus serotypes based on serology

S/No	Status of DENV infections	Serologic findings	No. Positive			Total positive (%)
			Adamawa	Bauchi	Borno	
1	Confirmed dengue	Positive IgM plus PositiveNS1 Ag	0	0	0	0
		Positive IgM plus Positive NS1 Ag plus positive PRNT for one or more DENV serotypes	0	0	0	0
		Positive NS1 plus positive PRNT for one or more serotypes	2	0	3	5
		Negative IgM plus Positive NS1 Ag	2	1	4	7
		**Positive NS1 plus positive NS1 IgG	-	-	4	4
		Positive IgM plus positive IgG plus positive PRNT for one or more serotypes	-	90	-	90 (54)
	Total		**4**	**1**	**11**	**106 (21.3)**
2	Highly suggestive of dengue	Positive IgM plus Negative NS1 Ag plus positive PRNT for one or more DENV serotype	10	18	0	28 (5.6)
		Negative IgM plus Negative NS1 plus Positive PRNT for one or more serotypes	165	79	113	357 (72)
	Total		**175**	**97**	**113**	**385 (77.6)**
3	Probable dengue	Positive IgM plus Negative NS1 Ag	10	20	4	34 (6.8%)
4	[a]Anti-flavirus antibody	Positive IgG only	-	200	-	200 (100)
5	[a]Anti-flavivirus antibody	Positive IgM and IgG	-	100	-	100 (50)
6	Secondary DENV infections	DENV IgG: IgM ratio (>1.1)	-	187	-	187 (93.5)
	Primary DENV infections	DENV IgM:IgG ratio (>1.4)	-	13	-	13 (6.5)
7	[b]Secondary DENV infections	DENV NS1 IgG	-	-	170	170 (86.3)
	No Dengue Infection	Negative IgM, NS1, PRNT	23	16	114	153 (31)

[a]Performed only on Bauchi samples
[b]Performed only for Borno samples.

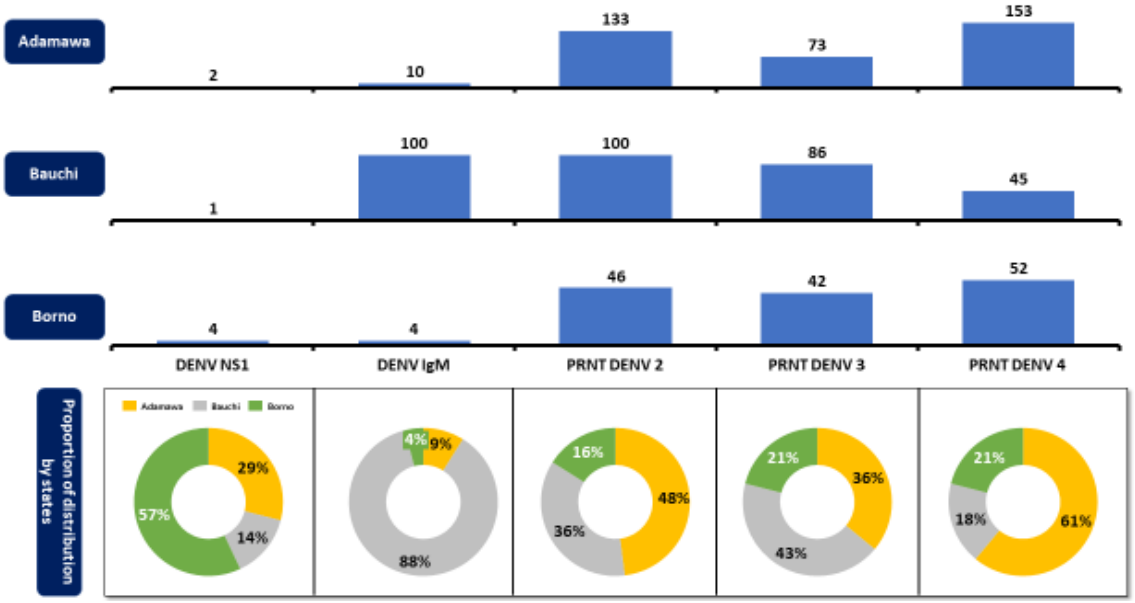

Figure 2. Distribution of DENV antigen and antibody in three Northeastern States, Nigeria

3.3. Circulating DENV Serotypes in Three Northeastern States Based on PRNT$_{90}$ Results

DENV 2 was the most predominant serotype with a 51.5% prevalence rate in the three states, followed by DENV 4 (48.4%) and DENV 3 (33.7%). DENV 2 was significantly higher (χ^2= 47.037, p=0.000, cl=2.416-4.937) in Adamawa and Bauchi than Borno. Similarly, DENV 4 was significantly higher (χ^2=147.62, p=0.000, cl=6.834-15.15) in Adamawa (29.7%) compared to Borno and Bauchi. DENV 3 was significantly higher (χ^2=11.522, p=0.000, Cl=1.312-2.663) in Bauchi compared to Adamawa and Borno.

3.4. Multiple DENV Infections in Three Northern States of Nigeria Based on PRNT Results

Adamawa had more DENV serotype co-infection: DENV 2 and 3 (30.2%), DENV 2 and 4 (57.8%), DENV 3 and 4 (33.7%) and DENV 2, 3, 4 (28.6%) than Bauchi and Borno states. Co-infections of DENV 2, 3, 4 were least in Bauchi (4.0%) compared to Adamawa (28.6%) and Borno (9.0%). Overall, multiple DENV infections were significantly higher (χ2= 59.632, p=0.000, cl=3.093-7.753) in Adamawa compared to Bauchi and Borno.

3.5. Age Distribution of DENV NS1 Antigen and Antibodies

A significant association (χ^2= 47.142, df=8, p=0.000) between the ages of the patients and the distribution of DENV antigen and nAb was observed with the Fisher's Exact Test. NS1 was only detected among those aged 20-69 years. DENV IgM was highest (41%) among patients aged 0-9 years and least (15%) in 20-29 years old. Patients aged 0-9 years had the highest prevalence rate of DENV-2 (63%) while 70-79 years had the least (33%). DENV-3 was highest (67%) among those aged 80-89 years and least (14%) at 60-69 years. 50% and 27% of the patients aged 70-79 years and 0-9 years respectively had DENV-4 infections. Overall, all the age groups studied had antibodies against DENV 2, 3, and 4 but from ages 60-89 years, the number of patients with DENV nAb decreased significantly. In comparison with other serotypes, antibody to DENV-2 was more represented in all the age groups studied especially in patients aged 0-9 and 30-39 years. These data show that DENV-2 remains the predominant serotype in Nigeria.

Table 3. CIRCULATING DENGUE VIRUS SEROTYPES BASED ON PRNT$_{90}$ RESULTS

S/No	Serologic findings (PRNT$_{90}$)	Total tested	Dengue serotype	No. Positive (%)			
				Adamawa	Bauchi	Borno	Total
1	Positive DENV 2 plus negative DENV 3 & 4	542	DENV 2 only	133 (24.5)	100 (18.5)	46 (8.5)	279 (51.5)
2	Positive DENV 3 plus negative DENV 2 & 4	595	DENV 3 only	73 (12.3)	86 (14.5)	42 (7.1)	201 (33.8)
3	Positive DENV 4 plus negative DENV 2 & 3	516	DENV 4 only	153(29.7)	45 (8.7)	52(10.1)	250 (48.4)

Table 4. MULTIPLE DENV INFECTIONS IN THREE NORTHERN STATES OF NIGERIA BASED ON PRNT RESULTS

Serologic findings (PRNT$_{90}$)	No. positive (%)			Total
Co-infection of dengue serotypes	Adamawa N=199	Bauchi N= 162	Borno N=200	
Positive DENV 2 and 3	60 (30.2)	46 (28..4)	21 (10.5)	127 (22.6)
Positive DENV 2 and 4	115 (57.8)	23 (14.2)	35 (17.5)	173 (30.8)
Positive DENV 3 and 4	67 (33.7)	13 (8.0)	21 (10.5)	101 (18.0)
Positive DENV 2, 3, 4	57 (28.6)	6 (4.0)	18 (9.0)	81(14.4)

Table 5. AGE DISTRIBUTION OF DENV NS1 ANTIGEN AND ANTIBODIES

Age Groups	Number Tested	DENV NS1	DENV IgM	PRNT DENV 2	PRNT DENV 3	PRNT DENV 4
0-9	41	0	17	26	11	11
10-19	99	0	22	48	29	39
20-29	151	2	23	60	56	64
30-39	150	1	23	75	52	67
40-49	84	2	14	35	25	41
50-59	45	0	11	20	21	16
60-69	21	2	4	11	3	8
70-79	6	0	0	2	2	3
80-89	3	0	0	2	2	1
Total	600	7	114	279	201	250

3.6. Distribution of DENV NS1 Antigen and Antibodies at Different Intervals between Onset of Symptoms and Sample Collection

Numerically, 17.6 % (79/448) of samples collected at 1-7 and 23.2% (35/151) from 7-10 days after onset of symptoms had DENV IgM. Similarly, 45% (202/448), 34.2% (153/448), 41.5% (186/448) of samples collected 1-7 days and 50.3% (76/151), 31.8% (48/151), 41.2% (63/151) at 7-10 days neutralized serotypes 2, 3 and 4 respectively. Overall, neutralizing antibody to DENV 4 was significantly higher (χ^2 = 120.904, p= = 0.000, Cl=10.219-38.897) with samples collected within 7-10 days after onset of symptoms than 1- 7. Also, neutralizing antibodies to DENV-2 (OD= 1.239) and 4 (OD= 19.837) are more likely to be detected by $PRNT_{90}$ with samples collected 7-10 days after onset of symptoms compared to 1-7 days. However, nAb to DENV-3 is more likely to be detected with samples collected 1-7 days (OD=1.124) than 7-10 days (OD=0.902). 100% (7/7) of 496 patients whose samples were collected at 1-7 days had DENV NS1 antigen while none of the samples collected at 7-10 had it.

3.7. Yellow Fever Vaccination Status of Patients and the Distribution of DENV Antigen and Antibodies

5.2% (7/134) of the patients who were not vaccinated with the YF vaccine had NS1 while the vaccinated had none. DENV IgM was more among patients (13.1%; 44/337) who were not vaccinated against YF than the vaccinated (6.0%; 8/134). Nevertheless, DENV neutralizing antibodies were more among the vaccinated (53% (71/134), 35.1% (47/134), 59.7% (80/134) than unvaccinated (41.2% (139/337), 28.8% (97/337) and 41.8% (141/337 for serotypes 2, 3 and 4 respectively. Overall, the Yellow Fever (YF) vaccination of the patients and the presence of DENV neutralizing antibodies were significantly different (χ^2 =22.143, p= 0.000, Cl= 1.741-3.823). People who were vaccinated against YFV are 1.4, 1.1, 2.5 (OR values) times more likely to be protected against DENV 2, 3 and 4 than those unvaccinated (OR= 0.617, 0.618, 1.064). However, acute DENV infections as evidenced by NS1 were significantly higher (χ^2= 3.873, df=8, p=049) among the patients who were not vaccinated against YFV than those vaccinated.

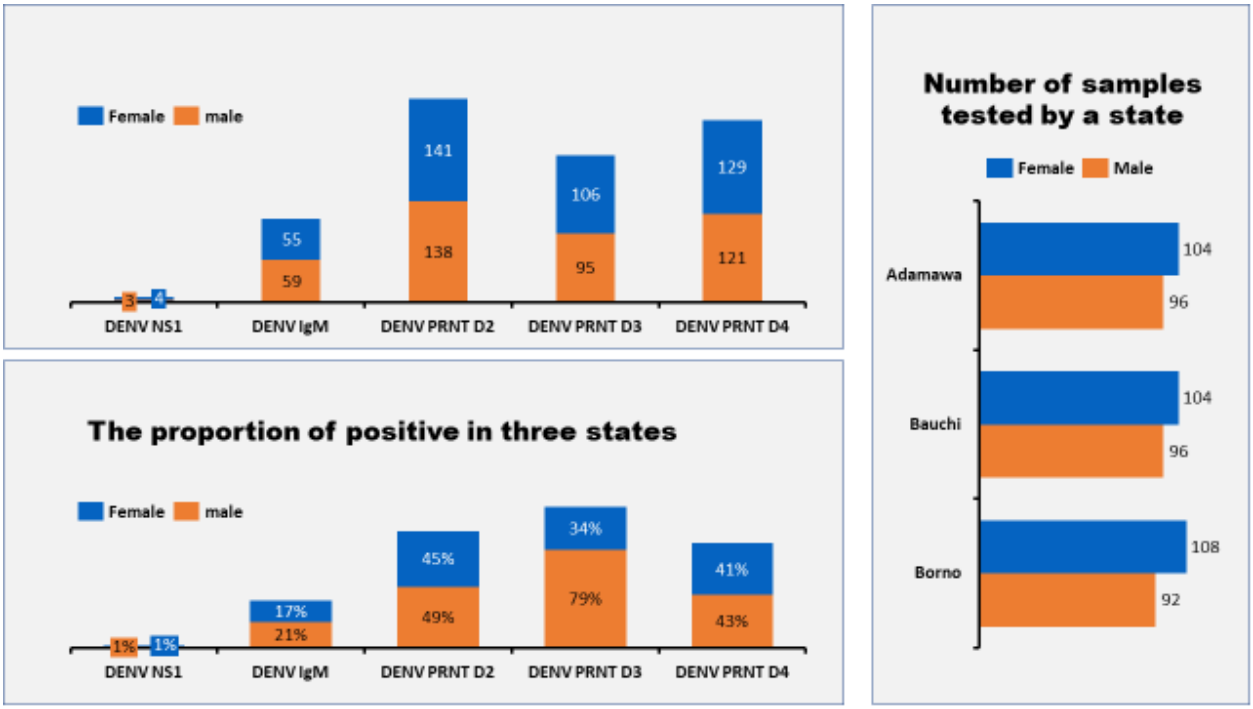

Figure 3. Gender distribution of DEV NS1 antigen and antibodies

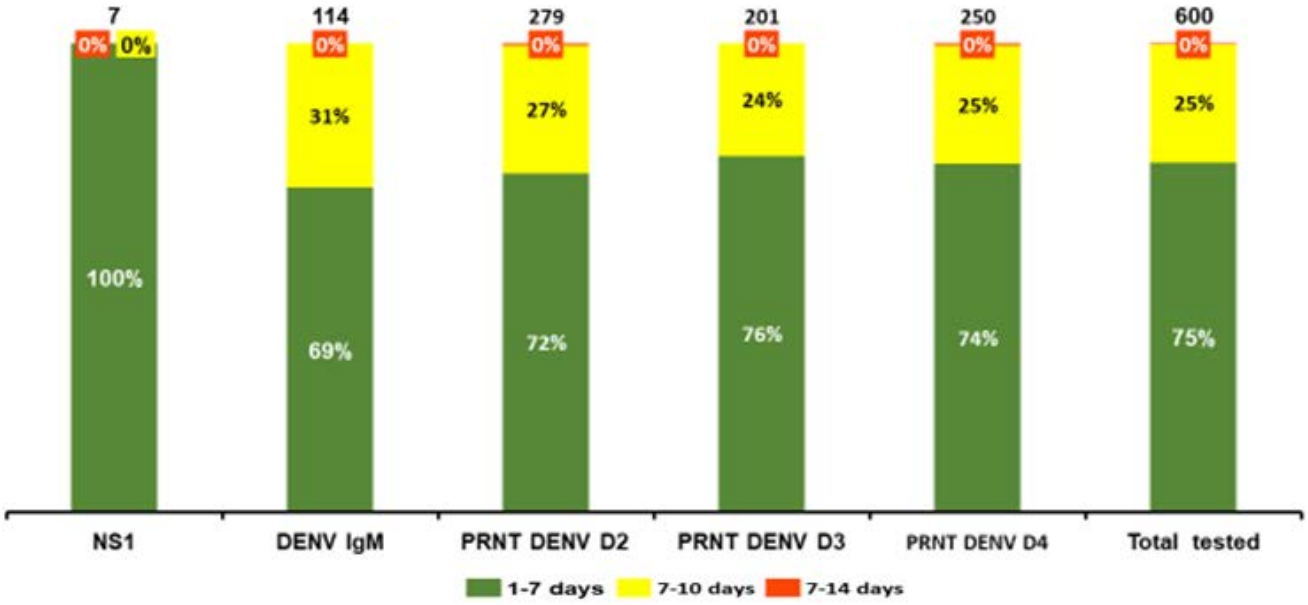

Figure 4. Distribution of DENV NS1 antigen and antibodies at different intervals between onset of symptoms and sample collection

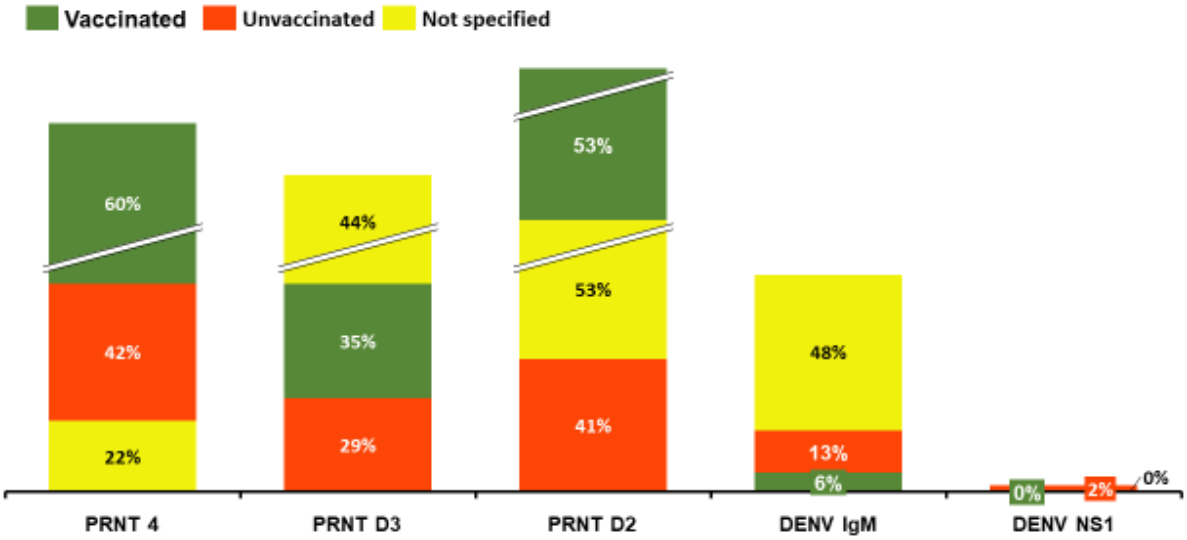

Figure 5. Yellow fever vaccination status of patients and the distribution of DENV antigen and antibodies

3.8. Distribution of DENV Infections among Patients who Received Antimalaria/Antibiotic Treatment before Performing Laboratory Tests for Malaria Parasites

A significant number of patients tested (59.2%) received treatment with antibiotics/anti-malaria before performing laboratory tests for malaria parasites. Among the treated, 4 (1.1%) had NS1 while 15 % (53/355) of the treated and 24% (58/233) of the untreated had DENV IgM. Patients with nAb to DENV-2, 3 and 4 among the treated were 47% (167/355), 36% (126/355), 44% (156/355) but untreated were 45% (105/233), 32% (74/233) and 38% (88/233) respectively. DENV infections among patients with febrile illness who received treatment with antibiotics/antimalaria were not significantly different (χ^2= 12.629, df=8, P= 0.128) from those who did not. However, patients who received treatment with antibiotics/antimalarial are more likely to be protected against DENV 2, 3, and 4 (OR= 1.005, 1.247, 1.259 respectively) than those untreated. Similarly, the untreated patients (OR=1.83) are 1.2 times more prone to acute dengue (as evidenced by NS1) than the treated (OR=0.919).

Table 6. DISTRIBUTION OF DENV ANTIGEN AND ANTIBODY AMONG PATIENTS WHO RECEIVED ANTI-MALARIA/ANTIBIOTIC TREATMENT BEFORE SAMPLE COLLECTION

Treatment status	Total tested (%)	DENV NS1 (%)	DENV IgM (%)	$PRNT_{90}$ DENV 2 (%)	$PRNT_{90}$ DENV 3 (%)	$PRNT_{90}$ DENV 4 (%)
Treated	355 (59.2)	4 (1.1)	53 (14.9)	167 (47)	126 (35.5)	156(44)
Not treated	233 (38.8)	3 (1.3)	58 (24.9)	105 (45.1)	74 (31.8)	88 (37.8)
Not specified	12 (2)	0	3 (25)	7 (58.3)	1(8.3)	6(50)
Total	600	7 (1.2)	114 (19.0)	279 (46.5)	201 (33.5)	250 (41.7)

4. Discussion

Three different diagnostic assays (DENV NS1, ELISA IgM and $PRNT_{90}$) with varied sensitivity and specificity were used to determine the precise status of DENV infections in three northeastern states. PRNT which is a gold standard assay for serotype specificity [25,28] was used as a reference to obtain specificity and sensitivity of 94% and 25% in ELISA IgM and 98.8% and 2% in Rapid NS1 assay respectively in agreement with previous reports [29,30]. Unlike IgM, DENV NS1 denotes acute DENV infection, confirms the diagnosis even in a single sample and informs clinical management of cases [31] but does not differentiate serotypes. Cross-reactions may occur with PRNT but the use of a higher stringent endpoint titer of 90% provides greater species specificity than IgM [25].

In this study, a significant number of patients with febrile illness had confirmed, highly suggestive and probable dengue (Table 2). Fifty percent of the patients in Bauchi had recent secondary dengue as evidenced by a simultaneous increase in IgM/ IgG antibody [32,33] which neutralized 90% of one or more serotypes of the DENV. In Borno state, 86.3% of patients with DENV NS1 IgG also denoted recent secondary dengue in agreement with previous reports [26]. The sera of four NS1 positive patients neutralized a single serotype of DENV once or twice (two neutralized serotype 3, one serotype 2 and 1 serotype 4) indicating single infections, one neutralized the three serotypes simultaneously (possible multiple infections) while 2 failed to neutralize serotypes 2,3 and 4 suggesting DENV-1 which was not included in the study. Additionally, with the cut-off points of ≥1.0 as reported in previous studies [30,34,35], IgG: IgM and IgM:IgG ratio on Bauchi samples indicated 93.5% and 6.5 recent secondary and primary DENV infections respectively. Secondary dengue with different serotypes poses the risk of DHF/DSS and other possible complications [36,37] (Table 2).

In this study, DENV 1-4 were detected among febrile patients in Adamawa, Bauchi, and Borno using the $PRNT_{90}$ results (Table 3). Previous studies in different parts of Nigeria have consistently reported antibodies to different serotypes of DENV in many parts of the country [10,12,38,39]. The unique feature of this study is the similarity of our findings on febrile patients by PRNT 90 with that of a previous report [38] where nested RT-PCR was used to analyze mosquito vectors (Aedes aegypti) for DENV serotypes in the same environment. In both studies, DENV 1-4 with 2 and 4 as predominant serotypes and co-infections of serotypes 2 and 4 and 2,3, and 4 were detected in the Adamawa state. Notably, whilst this study covered three northeastern states (Adamawa, Bauchi and Borno), the previous study concentrated on only one state (Adamawa state). Additionally, we detected co-infections of 2 and 3 and 3 and 4 that were not reported in the Aedes aegypti in the previous study. Overall, DENV co-infections were significantly higher ($\chi 2$= 59.632, p=0.000, cl=3. 093-7.753) in Adamawa than in Bauchi and Borno states (Figure 2) in agreement with Isa et al [38] (Table 3 and Table 4). The common factors in the three states studied include high population movement due to persistent terrorist attacks with many people storing water in their homes, dumping of solid waste in the open gutter and close to water bodies, which provide breeding sites for Aedes species and allow interaction with human's populations [39]. However, the three states differ with respect to vegetation and climatic conditions: Adamawa spans between sub-Sudan vegetation marked by short grasses interspersed with short trees in the north and Guinea savannah in the north. The state is very warm with an annual average temperature of 35°C [40]. Bauchi is a Sudan savanna with an oppressive wet season and partly cloudy overcast dry season. It is hot year-round with temperatures ranging from 57°F to 100°F Sahel [41]. Borno is a Sahel savanna and is one of the warmest regions in Nigeria with an average daily high temperature of 37°C [42]. It is possible that climatic, vegetation, and possibly other variables such as genetic differences in the vectors and the human hosts in Adamawa favor the transmission of DENV serotypes 4 and 2 compared to 3. Our speculation is supported by previous reports that the vegetation [9,11] and climatic conditions [41,42] have an effect on the competence of Aedes aegypti populations for DENV transmission. A similar trend was also observed in Indonesia where differed DENV serotypes were distributed in the three regions having different vegetations and climatic conditions [43,44].

DENV infections detected from samples collected between 1-7 days and 7-10 days after onset of symptoms were not significantly different (Figure 4). Nevertheless, more dengue including the 7 DENV NS1were obtained from samples collected within 1-7 days than 7-10 days. (Figure 4). The timing of sample collection and the purpose of the testing depends on the choice of the assay. However, a wider interval than this study should be investigated for a better insight into the diagnostic outcomes during sero-epidemiological studies of DENV infections. A significant number of patients tested (59.2%) received treatment with antibiotics/anti-malaria before performing laboratory tests for malaria parasites presumed to be the etiologic agents of the illness (Table 6). Notably, treatment with either or a combination of both drugs is a common practice in the country because every febrile illness is presumed and immediately treated as malaria and or bacterial infection. In this study, those who received these treatments (OR=1.259) are more likely to be protected against DENV infections than the untreated (OR=0.768). Similarly, the untreated (OR=1.183) are more likely to have acute dengue than the treated (0.919). This observation is supported by a report that some antibiotics and antimalarial are effective treatments for many viral diseases [45]. For instance, teicoplanin, ivermectin, abamectin tetracyclines, quinolones, aminoglycosides, glycopeptides antibiotics [46,47], erythromycin, tetracycline, azithromycin are active against Yellow Fever and DENV [45]. Similarly, Antimalaria such as mefloquine [48,49], Chloroquine [50,51], atovaquone [52] can inhibit DENV replication. We speculate that the inhibiting effect of these drugs on dengue progression is contributory to the absence of DHF/DSS despite the co-infections of DENV serotype in the environment. Further studies are necessary to gain more insights into the effect of these drugs on DENV, especially in the face of the absence of an effective DENV vaccine and therapeutics.

Overall, all the age groups studied had antibodies against DENV 2, 3, and 4 but from ages 60 to 89 years,

the number of patients with DENV neutralizing antibodies decreased significantly (Table 5). In comparison with other serotypes, antibody to DENV-2 was more represented in all the age groups studied especially in patients aged 0-9 and 30-39 years. The commonality of serotype 2 in all the age groups further confirms the long duration of its circulation in the environment (Figure 3). The gender of the patients and the distribution of DENV infections were not significantly different (Figure 3). In contrast to our findings, the risk of dengue in males was much higher than in females in Asian countries [53,54]. In agreement with our finding, the gender distribution of DENV infections was not significantly different in South America (P> 0.05) [55,56]. Probably hungry female mosquitoes in Nigeria and South America do not discriminate between males and females in their quest for blood meals.

DENV neutralizing antibodies were significantly higher among patients who received Yellow Fever (YF) vaccination in consistence with previous reports [57,58,59]. According to these authors, YF vaccination actually increased the cross-neutralizing antibody response to the four dengue serotypes. For instance, in a previous study, 42% of sera collected after YF vaccination were DENV IgM positive despite none of them being positive before vaccination [60]. Acute dengue observed among the unvaccinated as opposed to YF vaccinated patients in this study (Figure 5) compares favorably with that report. Notably, YF immunity could have been acquired through vaccination (incorporated in National Program on Immunization (NPI) in Nigeria since 2004) or natural infections as previously reported [61]. This information is very useful and may constitute a more cost-effective means of protection against DENV and its associated complications in the absence of an established vaccine. There is a need for further studies to establish the significance of YF vaccination in DENV immunity.

The limitations of this study include our inability to test all the samples for DENV 1 due primarily to the unavailability of appropriate reagents. Also, PRNT positive sera were not further titrated beyond 1:8 to determine the highest dilution of the serum that neutralized 90-100% of the virus infectivity due to insufficient quantity of the samples. Additionally, we were unable to test all the samples from the three states for NS1 IgG and IgG due to the high cost of these kits. Therefore, limited testing for DENV IgG ELISA and NS1 IgG to samples collected from Borno and Bauchi respectively revealed the status (Primary / secondary) of dengue cases in Nigeria.

5. Conclusion

Three diagnostic assays including NS1, IgM and PRNT were used to unmask DENV infections from malaria suspected febrile patients. Both IgM ELISA and Rapid DENV NS1 assays had high specificity but low sensitivity. Serologically defined confirmed, highly suggestive and probable dengue caused by serotypes 2, 3 and 4 were detected in Adamawa, Bauchi and Borno states. DENV 2 was the most predominant, followed by DEN-4 and DENV-3 in the three states but serotype 4 was significantly higher in Adamawa than in the other two states. DENV 2 and 4 as well as multiple serotype infections were significantly higher in Adamawa than in Bauchi and Borno states. In Bauchi, only 6.5% were primary while 93.5% were recent secondary and in Borno, 85% were recent secondary DENV infections. Whilst the ages of the patients and DENV infections were significantly different, gender was not. Treatment with anti-malaria and antibiotics may ameliorate recovery from dengue and the use of these therapeutics without appropriate laboratory diagnosis could contribute to the burden of antimicrobial resistance. Although dengue and the interval between onset of symptoms and sample collection were not significantly different, more infections were detected when samples were collected within 1-7 than 7-10 days. Both acute DENV infections and neutralizing antibodies were significantly associated with YF vaccination. Persistent misdiagnosis and improper management of dengue outbreaks could cause serious socioeconomic impact and impose a very heavy burden on the already overstretched health care systems in Nigeria. This study has revealed that dengue outbreaks, which were masked by malaria, had occurred due to monotypic or multiple DENV serotypes in Nigeria. Since dengue has no cure and no available vaccine in resource-constrained countries, YF vaccination may serve as a useful tool to reduce the dengue burden and its associated.

Conflict of Interest

The authors do not have a commercial or other association that might pose a conflict of interest.

Funding

This project was funded by International Centre for Genetic Engineering and Biotechnology (ICGEB) -CRP: Contract No: CRP/16/010, Reference No. CRP/NGA 16-03.

Acknowledgments

We are indeed grateful to all the staff of the WHO National Polio laboratory, Maiduguri for their moral and technical assistance. We sincerely thank the Collaborative Research Program (CRP) arm of the International Centre for Genetic Engineering and Biotechnology (ICGEB) for making this work feasible and affordable. We thank World Health Organization for supplying the InBios Dengue NS1 Detect Rapid Test and the University of Maiduguri Teaching Hospital (UMTH) for hosting the Laboratory facility where the test was performed. We also thank Mr. Kunle Ijaya for producing all the charts and maps of Nigeria which highlight the areas where samples were collected . The technical support of Mr. Ayodele Isreal Ajayi is also appreciated.

Abbreviations

DENV: Dengue virus, **DHF:** Dengue Haemorrhagic Fever, **DSS:** Dengue shock syndrome, **HI:** Haemagglutination

inhibition, **PRNT:** Plaque reduction neutralization test, **WHO**: World Health Organization, **ATBUTH:** Abubakar Tafawa Balewa University Teaching Hospital, Bauchi, **FMC:** Federal Medical Centre, **SSH:** State Specialist Hospital, **UMTH:** University of Maiduguri Teaching Hospital**, BNITH**: Bernard Notch Institute for Tropical Medicine and Hygiene, **EMEM:** Eagles Minimum Essential Medium, **OD:** Optical density, **NS1:**Non-structural protein1, **rNS1**: Recombinant Non-structural protein 1, **CDC:** Centre for Disease Control, **nAb:** neutralizing antibody**, CI**: Confidence interval, **YF**: Yellow Fever, **OR**: Odd ratio, **ELISA:** Enzyme-linked immunosorbent assay.

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