

Genetic diversity of Dengue virus serotypes circulating among Aedes mosquitoes in selected regions of Nigeria

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Abstract

The DENV mosquito vector is endemic to tropical and subtropical climates, placing ~40% of the world's population at direct risk of dengue infection. Currently, in Nigeria the status of DENV serotypes circulating among mosquitoes' vectors is unknown. Our study was designed to identify and characterize the DENV serotypes circulating in *Aedes* mosquito populations collected in selected sites in Nigeria. The mosquitoes were collected, identified morphologically to species level using colored identification keys of Rueda. Generally, each species identified was tested in pools of 20 individuals of each *Aedes* species. RT-PCR and nested PCR were used to detect DENV serotypes in mosquitoes and characterized using Sanger sequencing methods. The results show that DENV serotypes were detected in 58.54% of the pools of mosquitoes screened. This corroborates with our unpublished report on the presence of DENV IgM antibodies in humans in the same study area. All DENV1-4 serotypes were detected in *Ae. aegypti*, *Ae. albopictus* and *Ae. gaillosi* with DENV4 serotype being reported for the first time in Nigeria. DENV2 (37.8%) was the most detected serotypes, while double and triple co-infections of serotypes were detected in 24.4% of the pools. Phylogenetic analysis revealed a strong evolutionary relatedness of DENV serotypes in our study with that of South and Southeast Asia, North America, and other African countries. This is the first reports on the co-infection of natural DENV in *Aedes* species pools in Nigeria. Thus, our study speculates a possible linkage between DENV serotypes and febrile flu-like disease burden being experienced by host communities in northern Nigeria.

Introduction

Dengue is a mosquito-borne viral disease that has spread to almost all the regions of the world in recent years. Climate change and globalization has firmly established Dengue as threat to public health systems (1). Dengue virus (DENV) is transmitted by infected female mosquitoes mainly of the species *Aedes aegypti* and to a lesser extent, *Aedes albopictus*. These are also vectors of Chikungunya, Yellow fever and Zika viruses (1). The *Ae. aegypti* is recognized as the main vector of DENVs worldwide, largely attributable to its vector competency and strong host preference for humans compared to *Ae. Albopictus* (2). However, *Ae. albopictus* has been a driving force in the worldwide emergence of Chikungunya virus since 2004 (3).

The African *Aedes* mosquito breeds in both the domestic environment and ancestral sylvatic habitat. Whereas domestic *Aedes* mosquito larvae develop in artificial containers (tires, cans, jars, flowerpots, plastic/metal drums) within or proximity to human habitation, while larvae of the sylvatic ecotype are found in natural breeding sites such as rock pools, phytotelmata in forested areas (4). High populations of *Aedes* mosquitoes were reported in Adamawa state (5).

It has been estimated that 3.9 billion people in 129 countries are at risk of DENV infection and 390 million dengue infections occur per year of which 96 million manifests clinically. In addition, the largest number of dengue cases was recorded in 2019 that affected most regions of the globe, and dengue transmission was recorded in some countries for the first time (1). Despite its global significance, little attention has

been given to *Aedes*-borne arboviruses in Africa. These posed a greater public health threat for immunologically naive non-African human populations. The neglect of these viruses could be due to the high burden of malaria and other neglected tropical diseases in Africa, in addition to poor-resource settings for accurate identification of arboviral infections (6). In Nigeria, most cases of dengue are undiagnosed or misdiagnosed as malaria or referred to as fever of unknown cause (7). Nigeria's urban ecology, with limited sanitation infrastructure, multiple rainy seasons, pervasive household water storage, and virtually no public awareness of dengue transmission, presents a similarly prime environment for breeding of *Aedes* mosquitoes. The vectorial capacity of *Aedes* species in Nigeria is still poorly studied (8). A study conducted in Zaria, Kaduna Nigeria reported co-circulation of dengue and yellow fever viruses in the *Aedes* populations (9). A prevalence rate of 51.9% of IgM antibodies to dengue fever virus was reported in the northwest zone of Nigeria (10).

DENV is a positive sense, single-stranded RNA virus of the family Flaviviridae which comprised of more than 70 viruses. The viruses have four antigenically distinct serotypes, DENV 1–4 (11). These viruses have a broad geographic range, circulating on every continent except Antarctica (12). The virus circulates in two distinct transmission cycles; the sylvatic cycle between the mosquito vector and non-human primates and the human transmission cycle which includes domestic and peri domestic *Aedes* mosquitoes (13). Limited available evidence suggests only *Ae. aegypti* is involved in transmission of dengue in Nigeria, as several isolates of DENV were obtained from this mosquito species and none from *Ae. albopictus* and *Ae. gaillosi* (14). Also, despite the growing effect of dengue infections amongst human populations, there was virtually no information on the natural presence of DENV serotypes circulating in adult field *Aedes* mosquitoes' populations in Adamawa region of Nigeria. Therefore, this study was designed to evaluate and characterized DENV serotypes circulating in wild caught *Aedes* mosquitoes in this area.

Materials And Methods

Study Area

The study was carried out in three purposively selected Local Government Area (LGAs) of Adamawa State, Nigeria that is located between latitude 9^o 14'N and longitude 12^o 28'E (Fig. 1). It has an estimated area of 39,742,12sq. Km which accounts for 4.4% of the total landmass of Nigeria. The projected population as per 2006 population figure stands at 3,737,223 and it is traversed by mountainous landforms like the Mandara Mountains, Cameroon Mountains and Adamawa hills and large rivers: Benue, Gongola and Yadzarem. The vegetation in Yola and environs is secondary type due to human activities through construction, farming, wood gathering for fuel and grazing which have altered the natural vegetation. Adamawa state is prone to flood water and have swampy terrain especially during the rainy season. The mean annual rainfall ranges from 700 mm in the northeastern and of the 1600 mm in the southern part (15). The study areas are also characterized with high population movement due upheavals in the northeast with many people storing water around their homes and dumping of solid waste in open

gutter and close to water bodies, which provide suitable environment for breeding of *Aedes* species and interaction with humans (16).

***Aedes* mosquitos' collection and preparation for detection of DENV serotypes**

Aedes species were collected both indoors and outdoors from Mubi, Yola and Numan LGAs. By volunteers for four months (July to October). Collection was in the morning between 6:00–10 am); while the evening collection was from 3:00 to 7:00pm. Collection consisted of a combination of aspiration of indoor resting adults, human-landing techniques that attracted mosquitoes on exposed parts of human-bait and sweep nets (17, 18). All mosquitoes collected were sorted and *Aedes* mosquitoes were identified using colored identification /taxonomic keys by Rueda (19). The *Aedes* species identified morphologically were segregated generally, in pools of 20 females labeled according to species, LGAs and communities. A total of 41 pools were used. Sixteen (16) pools from 3 communities in Numan, 17 pools from 3 communities in Yola and 8 samples pools from 3 communities in Mubi were analyzed (Fig. 1). Samples were stored in -80°C for RNA extraction.

RNA extraction and cDNA synthesis from pools of *Aedes* species

DENV RNA was extracted with All Prep DNA/RNA Mini extraction kit (Qiagen, Germany, Cat. No. 80204) in a Level 3 (L3) Safety Laboratories for Viral Research, University of Bremen, Germany, following manufacturer's instruction with little modification (www.qiagen.com). Each pool of *Aedes* species was thoroughly homogenized with a mini handheld homogenizer and single-use microfuge pestle (Sigma Aldrich, Munich, Germany) in 350 μL buffer RLT (lysis buffer). The homogenate was centrifuged for 3 mins at 13000 rpm. The homogenized lysate was transferred to an AllPrep RNA spin column in a 2 mL collection tube and one volume (350 μL) of 70% ethanol was added to the flow-through and mixed thoroughly by pipetting repeatedly. Seven hundred microliter (700 μL) of the lysate was transferred to an RNAeasy spin column placed in a 2 mL collection tube and was centrifuged for 15 secs at 13000 rpm. The filtrate from the centrifuge was discarded and 700 μL of buffer RW1 (washing membrane-bound RNA) was added to the RNeasy spin column, centrifuged, and discarded. Five hundred microliter (500 μL) of buffer RPE was added twice to the column, centrifuged for 2 mins at 13000 rpm and the flow-through was discarded. The column was dried centrifuging for 1 min at 13000 rpm after which 30 μL of RNA free water was used to elute the RNA into a 1.5 mL collection tube by centrifuging for 1 min at 13000 rpm. The RNA was quantified using Nanodrop 1000 apparatus (Thermo Scientific, Dreieich, Germany) at a wavelength of 260 nm. The RNA extracted was stored at -80°C until used. RNA quality and integrity were determined using cytochrome oxidase 1 (COI) gene forward and reverse primers (Table 1). RNA was amplified in a 25 μL reaction mixture containing 2.5 μL (10x dreamTaq buffer containing 20 MgCl_2), 0.5 μL (dNTPMix, 10 mM), 1.0 μL (25mM of MgCl_2), 0.5 μL (500U, 5U/ μL green dream Taq polymerase) and 5 μL of RNA template. The temperature cycling conditions are as follows: initial denaturation at (95°C , 5 min), 35 cycles of denaturation (94°C , 1 sec), primers annealing (55°C , 1 min), primer extension (72°C , 2 mins) and final extension at 72°C for 10 mins.

Table 1

Primer sequences used for Cytochrome Oxidase 1, dengue consensus and serotype-specific RT-PCR reactions.

Primers	Oligonucleotide Sequences	Amplified size (bp)	References
CO1	5'-TTGATTTTTTGGTCATCCAGAAGT - 3' 5'-TGAAGCTTAAATTCATTGCACTAATC - 3'	930	(Dyer <i>et.al.</i> , 2008)
D1 D2	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' 5'-TTGCACCAACAGTCAATGTCTTCAGGTTTC-3'	511 Dengue consensus sequence	(Lanciotti <i>et.al.</i> , 1992)
D1 TS1	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' 5'-CGTCTCAGTGATCCGGGGG-3'	482 (DI and TS1) DENV1	(Lanciotti <i>et.al.</i> , 1992)
D1 TS3	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' 5'-TAACATCATCATGAGACAGAGC-3'	290 (TS3 and DI) DENV3	(Lanciotti <i>et.al.</i> , 1992)
D1 TS4	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' 5'-CTCTGTTGTCTTAAACAAGAGA-3'	392 (DI and TS4) DENV4	(Lanciotti <i>et.al.</i> , 1992)
DV1 DSP2	5'-GGRACKTCAGGWTCTCC-3' 5'-CCG GTGTGCTCRGCYCTGAT-3'	362(DV1 and DSP2) DENV2	(Hakami <i>et.al.</i> , 2018)

For cDNA syntheses, 80ng of RNA extract were mixed with 1µL (0.2µg/µL) random hexamer primer and 1µL (10 mM) dNTP mix in a 0.2 mL PCR tube. The mixture was made up to 15 µL with nuclease-free water on ice. The following component was then added to the PCR tube: 4 µL of 5X reaction buffer, 0.5 µL of Ribolock RNase inhibitor (40U/µL) and 0.5µL of Maxima H minus Reverse Transcriptase (10000U, 100 U/µL) making up to a total volume mixture of 20 µL. The reaction was vortexed, centrifuged and incubated in a thermocycler with the following conditions: 25⁰C for 5 mins, 50⁰C for 30 mins. The reaction was then terminated by heating at 85⁰C for 5 mins (Thermo scientific).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) amplification

The cDNA was amplified using DENV serotypes -specific consensus primers D1 and D2 that target the envelope and non-structural protein 1 (E/NS1) junction of the virus genome (Table 1). The RT-PCR and Multiplex- nested PCR were carried out according to a previous protocol (20) with some adjustment. In 25

μL of final volume reaction mixture containing 2.5 μL of *Aedes* species cDNA product, 2.5 μL (10 x dreamTaq buffer containing 20 MgCl₂), 0.5 μL of dNTP Mix (10 mM, final concentration of 0.2 mM), 1 μL of 25 mM MgSO₄ (final concentration 1.0 mM), 0.5 μL of dream Taq polymerase (final concentration 5U/μL), 0.875μL of forward D1 and reverse D2 primers (100 μM; final concentration 0.7 μM), which was designed against the consensus cDNA sequence of DENV1-4 serotypes that amplify a fragments of 511 base pair. The reaction volume was made up to 25 μL with double-distilled water (ddH₂O). The thermo cycling conditions are as follows: Initial denaturation at (94°C, 3 mins), 35 cycles of denaturation (94°C, 30 secs), primers annealing (55°C, 1 min), primer extension (72°C, 2 mins) and final extension at 72°C for 5 mins.

Serotyping Of Denv Serotypes

The PCR product obtained from the RT-PCR amplification using D1 and D2 as primers was further used as the template for DENV1-4 different serotypes. Based on the above temperature conditions and reagent volumes, 0.5 μL and 1μL of the Nested 1 RT-PCR product (1:10 and 1:1000 in ddH₂O for DENV serotypes 2 and DENV serotypes 1, 3 and 4 respectively) was used as template in the subsequent nested PCR reaction. A 25 μL reaction volume containing forward primer D1 and type-specific (TS) reverse primers: TS1, TS3 and TS4 (reverse primers for serotype 1, 3 and 4), and forward primer DV1 and DSP2 reverse primers for serotype 2 (Table 1) was further amplified by nested PCR step II. The entire PCR product was resolved on a 3% agarose gel containing 0.5 μg/mL ethidium bromide (SERVA, Heidelberg, Germany). The expected sizes of the amplified products were 482 for DENV1, 362 for DENV2, 290 for DENV3 and 392 for DENV4 (20).

Sequencing And Phylogenetic Analysis

The PCR amplicons were purified using GeneJet DNA purification kit (Thermo Scientific, Dreieich, Germany) following manufacturer's protocols and sequenced at Microsynth Sequencing Laboratories Göttingen, Germany. Forward and reverse strands were sequenced, and amplified target bands of Sanger sequence chromatograms were assembled and visualized using Geneious Pro Version 5.5.9. The sequences were compared with available sequences using Basic Local Alignment Search Tool (BLAST) for Virus Pathogen Resources (ViPR) (<https://www.viprbrc.org/brc/blast.spg>) and the Genbank data base to validate the identity of the virus isolate. Phylogenetic analysis was inferred using MEGAX (43). Consensus sequences were aligned using Muscles alignment tool. The phylogenetic tree was reconstructed using a Maximum Likelihood (ML) method and estimated using best-fit General Time Reversible (GTR) model with gamma-distributed rate variation among sites. Other sequences like the study sequences in GenBank were obtained using the BLAST algorithm were also included in the analysis. Bootstrap replicates of 1000 were employed to assess the robustness of individual nodes of phylogeny. Complete gap deletion was employed for all the E gene sequences.

Data Analyses

Descriptive statistics was used to analyse the distribution of DENV serotypes in *Aedes* species in Adamawa state. The proportion of DENV positive individuals True Infection Rate per 100 mosquitoes was calculated and the minimum infection rate (MIR) was also estimated per 1000 *Aedes* mosquitoes.

Results

Detection of DENV serotypes in *Aedes* species

A total of 706 *Aedes* mosquitoes were collected from the study locations. Samples were identified morphologically and confirmed molecularly targeting CO1 gene. Overall, 58.5 % (24/41) pools were positive for DENV in the study locations (Table 2). Mubi had the highest 100% (08/08) pools positivity rate while Numan 43.75% (07/16) with the lowest DENV pools positivity rate.

Table 2
Dengue Virus positive pools of *Aedes* species collected in Adamawa state.

Study locations	No. of pools screened	DENV positive pools	Percentage
Numan	16	7	43.75
Yola	17	9	52.94
Mubi	8	8	100
Total	41	24	58.54

The proportion of DENV positive individual's true infection rate (TIR: estimated number of positive mosquitoes per 100 mosquitoes tested) determined to 5.24% (37/706 x100), corresponding to presumed infected *Aedes* species (9,12 and 16 detected serotypes from Numan, Yola, Mubi respectively) per total of 706 *Aedes* mosquitoes processed in all the pools as some of the pools could not reach 20 mosquitoes. The Minimum Infection Rate (MIR) was 33.99% (24 positive pools/706 total processed individual mosquitoes × 1,000). There was a strong indication from the estimation that several individual mosquitoes were infected with the same serotypes thereby increasing the proportion of infection in individual's mosquitoes. However, since our study design did not permit us to separate the mosquitoes from each other, we decided to estimate the proportion as simply as possible.

Specifically, the Agarose gels electrophoregrams images of the PCR products shown in Fig. 2A, 2B and 2C. Lane 5 is amplicon for DENV2 infection in *Ae. galloisi* pool (Fig. 2C) while lanes 14, 15, 23, 27, 37 and 38 show DENV1, 2, 3 and 4 serotypes infection in *Ae. albopictus* pools (Fig. 2A, B and C). Concurrent infection of *Ae. albopictus* pools with DENV1 and 2 amplicons in lanes 37(Fig. 2B and 2C). Four species was identified including *Ae. aegypti*, *Ae. albopictus*, *Ae. galloisi* and *Ochlerotatus vigilax* (Table 3). DENV1-4 serotypes were positive in 24 pools of *Aedes* species. Seventeen (17) of the positive pools was *Aedes aegypti*, 6 of the positive pools was *Ae. albopictus* while one (1) positive pool was *Ae. galloisi* and

none for *Ochlerotatus vigilax* (Table 3). All the four DENV1-4 serotypes were detected in Yola and Mubi while DENV3 was (0.0 %0.0/09) not detected in Numan (Fig. 3).

Table 3

Aedes species identified and dengue virus serotypes detected in Adamawa State.

Study Location	Pools no.	Mosquito Species	Positive DENV	DENV serotypes			
				1(482bp)	2(362bp)	3(290bp)	4(392bp)
Numan	1	<i>Aedes aegypti</i>	+	+	-	-	-
	2	<i>Aedes aegypti</i>	-	-	-	-	-
	3	<i>Aedes aegypti</i>	-	-	-	-	-
	4	<i>Aedes aegypti</i>	+	+	-	-	+
	5	<i>Aedes galloisi</i>	+	-	+	-	-
	6	<i>Aedes aegypti</i>	-	-	-	-	-
	7	<i>Aedes aegypti</i>	+	-	+	-	-
	8	<i>Aedes aegypti</i>	-	-	-	-	-
	9	<i>Aedes aegypti</i>	-	-	-	-	-
	10	<i>Aedes aegypti</i>	-	-	-	-	-
	11	<i>Aedes aegypti</i>	-	-	-	-	-
	12	<i>Aedes albopictus</i>	-	-	-	-	-
	13	<i>Aedes aegypti</i>	-	-	-	-	-
	14	<i>Aedes albopictus</i>	+	+	+	-	-
	15	<i>Aedes albopictus</i>	+	-	+	-	-
	16	<i>Aedes aegypti</i>	+	+	-	-	-
Yola	17	<i>Ochlerotatus vigilax</i>	-	-	-	-	-
	18	<i>Aedes aegypti</i>	+	-	-	-	+
	19	<i>Aedes aegypti</i>	+	-	-	-	+
	20	<i>Aedes aegypti</i>	+	-	+	-	-
	21	<i>Aedes albopictus</i>	-	-	-	-	-

Key: DENV = Dengue virus, bp = base pair, + = positive, - = negative,

Study Location	Pools no.	Mosquito Species	Positive DENV	DENV serotypes			
	22	<i>Aedes aegypti</i>	+	-	-	-	+
	23	<i>Aedes albopictus</i>	+	-	-	-	+
	24	<i>Aedes aegypti</i>	+	+	-	-	-
	25	<i>Aedes aegypti</i>	-	-	-	-	-
	26	<i>Aedes aegypti</i>	-	-	-	-	-
	27	<i>Aedes albopictus</i>	+	-	-	-	+
	28	<i>Aedes aegypti formosus</i>	+	-	+	+	+
	29	<i>Aedes aegypti</i>	+	-	+	-	+
	30	<i>Aedes aegypti</i>	-	-	-	-	-
	31	<i>Aedes aegypti</i>	-	-	-	-	-
	32	<i>Aedes aegypti formosus</i>	-	-	-	-	-
	33	<i>Aedes aegypti</i>	-	-	-	-	-
Mubi	34	<i>Aedes aegypti</i>	+	+	+	-	+
	35	<i>Aedes aegypti</i>	+	+	+	-	+
	36	<i>Aedes aegypti</i>	+	+	+	-	-
	37	<i>Aedes albopictus</i>	+	+	+	-	-
	38	<i>Aedes albopictus</i>	+	-	+	-	-
	39	<i>Aedes aegypti</i>	+	+	+	-	-
	40	<i>Aedes aegypti</i>	+	+	+	-	-
	41	<i>Aedes aegypti</i>	+	-	-	+	-
Key: DENV = Dengue virus, bp = base pair, + = positive, - = negative,							

The occurrence of DENV serotypes in the pools of species in the locations, shows that DENV2 (37.8%;14/37) was the most occurring serotypes circulating than DENV-1, (29.7%; 11/37) and DENV4, (27.0%; 10/37) while DENV3 (5.4%; 02/37) was the least in Adamawa during the study (Fig. 4A).

Accordingly, the distribution of DENV serotypes circulating in *Aedes* species collected from the pools in the locations indicated DENV4 (58.3%;07/12) as the highest DENV serotype circulating in Yola, while DENV1 and DENV2 serotypes (44.4%, 04/09) in Numan and DENV2 (43.75%) in Mubi.

Categorically, Co-infections of DENV serotypes where two serotypes were detected in a pool of *Aedes* species in Adamawa state, with the highest double occurrence of DENV1/DENV2 (66.7%; 04/06) in Mubi. Furthermore, triple DENV2/DENV3/DENV4 was detected in a pool from Yola 50.0 % (01/02). Similarly, triple occurrence DENV1/DENV2 and DENV4 was detected in Mubi (33.3%; 02/06). Both DENV1/DENV4 was detected in the same pool in Numan 50% (01/02) but no similar was detected in Yola and Mubi (Fig. 4B).

Phylogenetic analysis of DENV serotypes distribution in *Aedes* species

Out of the 27 samples purified and sequenced, only 11 samples had good quality reads, which was used for the phylogenetic analysis for DENV1-4. The genetic relationship of DENV strain was analyzed targeting the E gene sequences of the virus. The tree was generated using E gene sequences of only one (1) DENV1 serotype and 7 reference strains (Fig. 5A). Notably, DENV1 from the study location form clade with that of South East Asia, West Africa, and North America. This study indicated diverse geographical distributions of DENV1 serotype in different continents. Also, 7 DENV2 E gene sequences were aligned with 7 reference strains from Genbank (Fig. 5B). The DENV2 from Mubi in Adamawa, Nigeria clustered together with that of Cameroon, Ghana, Senegal, and Kenya (Africa continent) and Philippines in Asia continents. While four of the DENV2 from Yola, Numan, and Mubi formed a separate clade.

Furthermore, one (1) DENV3 serotype from Yola, Adamawa Nigeria was determined with that of 10 reference serotypes from Genbank (Fig. 5C). DENV3 serotype from Adamawa, Nigeria formed a separate cluster which is distantly related with the referenced DENV3 serotype. However, DENV3 serotype from Kenya (East Africa) is closely related with DENV3 from India (South Asia). Only 2 DENV4 serotype were used from the study location with 9 referenced DENV4 (Fig. 5D). The result of the Phylogenetic analysis revealed that DENV4 from Yola appears to be more closely related to viruses from Asia continents. While DENV4 from Mubi is distantly related to the reference viruses.

Discussion

Dengue virus (DENV) is considered endemic in Africa (21), but there has been limited information documenting active DENV transmission in Nigeria with few of the available information relying hugely on serological surveys and has not fully described the circulating serotypes in field caught *Aedes* mosquitoes. Our study indicated that *Aedes aegypti*, *Ae. galloisi*, *Ae. albopictus*, *Ochlerotatus vigilax* and subspecies of *Ae. aegypti formosus* occurred with variable abundance in Adamawa State, Nigeria with the four DENV serotypes circulating in the study area (Table 3).

The circulating DENV serotypes were detected in 58.54% of the *Aedes* mosquito pools (Table 2). This rate is lower than 62% detected in *Ae. aegypti* pools collected at rural areas in Colombia (22). This is high in

comparison to 33% and 11% detected in adult mosquito pools in Armero and Brazil respectively (23, 24). The high pools positivity rate obtained in our study could be due to an increased interaction between *Aedes* species and humans, because the *Aedes* mosquitoes were collected at the peak period of mosquitoes' activities during the raining season, July-October. This agrees with Halstead, (44) who stated that DENV peak transmission is usually associated with periods of higher rainfall in most dengue-endemic countries. Also, it might have been exacerbated due to changes in human behavior that favored the *Aedes* mosquitoes in the study locations, which may not be unconnected to the protracted humanitarian crisis in the northeast that lasted for over 10 years displacing millions of people from the neighboring state who settles in the study areas thereby overstressing the infrastructure and increasing interaction between *Aedes* species and humans. Moreover, it could also be speculated due to increase in the cross-border socioeconomic activities between the state and Cameroon since they share borders. Thus, highlighting the possible cross-border importation of the vectors and DENV. This is a latent potential reservoir which can spill over to other parts of the country if left unchecked.

Virtually, all the *Aedes* mosquitoes' pools in Mubi were positive 100 % (08/08) for DENV (Table 2). This agrees with one of our studies, where we detected highest DENV IgM in serum samples from Mubi (unpublished). This further confirms the unique position of Mubi in Adamawa state to the other study locations. Mubi is known as the commercial hub of Adamawa state, educational center as well as harbor good numbers of Internally Display Persons (IDPs) from Borno state which could aid the spread of DENV.

The estimated *Aedes* mosquito's individual's true infection rate in our study was calculated at 5.24% and the MIR was 33.99/1000 mosquitoes. This is high when compared to studies conducted in 2016 at Anapoima and La Mesa municipalities (Colombia) who found estimated *Ae. aegypti* infection rate of 4.12% and MIR of 33.3/1000 (22). Also, it contrasts individuals TIR of 0.78% and 7.75% MIR of DENV infection estimated in *M. Africana* mosquitoes collected in Bayelsa, Nigeria (25). However, the high infection rate reported in our study may not be due to carry-over contamination but rather an attestation to a possible and continual transmission of DENV in the study area.

Our study detected DENV2 in *Ae. galloisi* and DENV1-4 serotypes circulating in *Ae. albopictus* (Fig. 3). This is the first time we are reporting this occurrence in Nigeria. However, DENV2 has been proven experimentally in *Ae. galloisi* and *Ae. albopictus* in Japan (26). Similarly, Paupy (27), reported *Ae. albopictus* as being susceptible to DENV2 and highly competent vector for CHIKV in Cameroon. Thus, our study here confirmed the susceptibility of *Ae. albopictus* infection to all the four DENV serotypes. *Ae. albopictus* (Asian Tiger mosquito) is an invasive species in Nigeria and can easily spread through international trade especially of used tires and transportation. It is known to exhibit strong physiological and ecological adaptability. There are several reports of epidemics of CHIKV and DENV infection coinciding with *Ae. albopictus* spread in Cameroon (28).

Also, *Ae. albopictus* was reported as the main vector of dual outbreaks of CHIKV and DENV in Gabon (29). Changes usually occur in the epidemiology of arboviruses after an introduction of invasive species (30). *Ae. albopictus* is competent enough to propagate zoonotic pathogens across humans and animal

species. In 2014, part of the study areas was captured by the insurgents who led to the displacement of thousands of residents who took refuge in Cameroon. Thus, our results further confirm the possibility of cross border transmission since there are reports of outbreaks associated with *Ae. albopictus* in Cameroon. Therefore, we have an emergency in our hands to curtail before it gets out of control as the risk of infection with DENV serotypes could increase in the area.

We detected all the four DENV serotypes circulating in the pools of *Aedes* mosquitoes from Mubi and Yola, while DENV3 was not detected in Numan (Fig. 3). This is not surprising because Mubi and Yola share similar characteristics as the main entry points into the state for travelers and visitors from Cameroon and different parts of the world because of international airport in Yola. In addition, many IDPs camps and humanitarian partners are situated in Yola and Mubi, accommodating thousands of refugees from Borno and different parts of Adamawa state, affected by the insurgency. Besides, dengue control activities are not practiced in the study areas. The sites are characterized with large river Yedzaram and river Benue, the people engaged in fishing and irrigation farming around the rivers, household water storage, digging of earth for clay, damaged septic tanks, indiscriminate discarded used food containers such as cans and old tire and presence of solid waste blocking water channels and around water bodies (32, 5), such practices provides good and abundant breeding sites for *Aedes* species and increase DENV serotypes susceptibility in the study areas.

All the four serotypes of DENV have been detected in Africa. However, in Nigeria, Ayolabi (33) reported DENV1 and DENV3 as the active circulating serotypes in Lagos, Nigeria from serum samples taken from febrile patients. Similarly, DENV1 and DENV2 serotypes were found circulating in serum samples collected in Cross River, Nigeria (34). In addition, DENV3 was amplified in *Mansonia africana* in Bayelsa, Nigeria (25).

Interestingly, our study detected DENV4 serotype, which has not been previously reported in Nigeria. This may be due to vector evolutionary competence or from the invasive arthropods' species found in the area since we detected DENV4 also in *Ae. albopictus*. This is a serious public health concern since mosquitoes are usually infected for life and infections in humans sometimes can be asymptomatic (35). Therefore, the circulation of DENV in humans and mosquito populations could be maintained for an exceptionally long periods and portend a public health hazard. Also, being infected with one serotype does not make someone immune from being infected with the other serotypes. Circulation of multiple serotypes could also lead to increased cases of severe form of dengue such as Dengue Shock Syndrome (DSS) and Dengue Hemorrhagic Fever (DHF) (36).

Furthermore, DENV2 was the most frequent serotypes detected in our study followed by DENV1 (Fig. 4A). This agrees with previous research by Amarasinghe (21) that reported DENV2 as the cosmopolitan serotypes and cause most epidemics in the Africa continent. However, in contrast Konongoi (31) who reported DENV1 as the most common serotypes circulating in serum samples collected in selected regions of Kenya.

The study showed concurrent detection of more than one DENV serotypes in single pools of *Aedes* species (Fig. 4B). Co-infection of individuals' mosquitoes is not common; however, Thavara (37) reported the first case of dual infections in adult *Ae. aegypti* and *Ae. albopictus* in Southern Thailand. Our study detected double DENV serotypes in five (5) *Aedes aegypti* pools, two (2) *Ae. albopictus* pools and triple infection of DENV serotypes in three (3) *Ae. aegypti* pools. Highest Co-infection (66.7%) occurred in Mubi. This corroborated the report of Perez-Castro (22) on the detection of double and triple co-infection of DENV in one pool of *Ae. aegypti*. Similarly, Johari (38) reported co-infection of double DENV2/DENV4 and DENV3/DENV4 in *Ae. albopictus* from Kuala Lumpur, Malaysia.

Infection of larvae with up to three DENV serotypes is possible experimentally (39). Although there is paucity of information on natural infections of four serotypes in an individual *Aedes* or humans, that narrative also could be possible during intense transmission in hyperendemic areas, due to transovarial transmission. However, the concurrent infections detected in this study may likely reflect single infections per *Aedes* mosquito. The serotypes can remain in the vector for transmission which could cause future severe dengue related cases with sequential secondary infection in the same individuals (22).

Our study investigated, variation in DENV serotypes using sequence analysis. DENV 1, 2 and 4 serotypes (Fig. 5A, B and C) respectively from the study locations formed clades together with that of South and Southeast Asia, North America and other African countries indicating strong evolutionary relatedness of the virus, signifying a common ancestor. Our study corroborates with that of Konongo (31) who reported relatedness of DENV serotypes in Kenya (East Africa) with that of South and Southeast Asia. Therefore, these countries portend high-risk areas for DENV because of the possibility of importation and exportation of DENV serotypes from any of these continents. Thus, our study reveals continued spread and wide geographic range of DENV serotypes.

However, DENV2 (Fig. 5A) in our study showed different level of relatedness. Some of the DENV 2 serotypes from the study location showed close relationship while others are distantly related with the referenced DENV sequences used. The differences observed could be due to possible genetic distances since evolution is a continuous process and geographic variations are inevitable. Point mutation could occur in lineages over time which could lead to divergence (40). The study also indicated DENV3 as distantly related with serotypes from Asia and African countries suggesting possible variation from the other serotypes.

Our study further confirms the reports that the major epidemics thought to be dengue, emanated from three continents: Asia, Africa, and North America (42). There is a sudden increase in the number of imported DENV cases worldwide due to globalization (41). Adamawa is a state in Nigeria, a country located in West Africa region and shares a porous border with many African countries and accelerated in global trade and bilateral exchange program with many Asian countries such as China, India, Philippines, Malaysia, and Dubai. It is imperative to note that travelers may serve as vehicles for DENV transmission, since 50% of the world's populations are living in dengue endemic countries (31).

In conclusion, our study detected all the DENV1-4 serotypes circulating in *Aedes* species in Adamawa state and reporting for the first time the occurrence of DENV4 serotype, as new serotype, which has not been previously described in Nigeria. Also, the isolation of DENV in *Ae. albopictus* and *Ae. galloisi* present a new threat of increase in vector competency in the transmission of DENV across animal species and to humans. And may likely be capable of transmitting other arboviruses. There was strong evolutionary relatedness of DENV serotypes in our study with that of South and Southeast Asia, North America, and other African countries. Our study also speculates possible linkage between the humanitarian crisis in the northeast and sudden surge of DENV serotypes infection in *Aedes* species. Therefore, it identified potential virus reservoir which may likely spread in humans during populations' movement. This is a wakeup call for prevention of future epidemics.

Declarations

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Authors' contributions

II, ISN, MA and AYS conceived and designed the study. II conducted the field study. II performed field data collection under ISN and AYS supervision. II performed the laboratory and data analysis under AD's and GC supervision. II drafted the manuscript. GC, MA, ISN, AD, and AYS critically revised the manuscript. II and AYS wrote the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data supporting the conclusions of this article are included within the article.

Declarations

Ethics approval and consent to participate

Ethical approval for the study was obtained from the Ethical Committee of the Adamawa State ministry of Health and from University Ethical Committee on the use of Human and Animal Subject for Research, ABU, Zaria (Ref. NO. S/MoH/1131/1).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

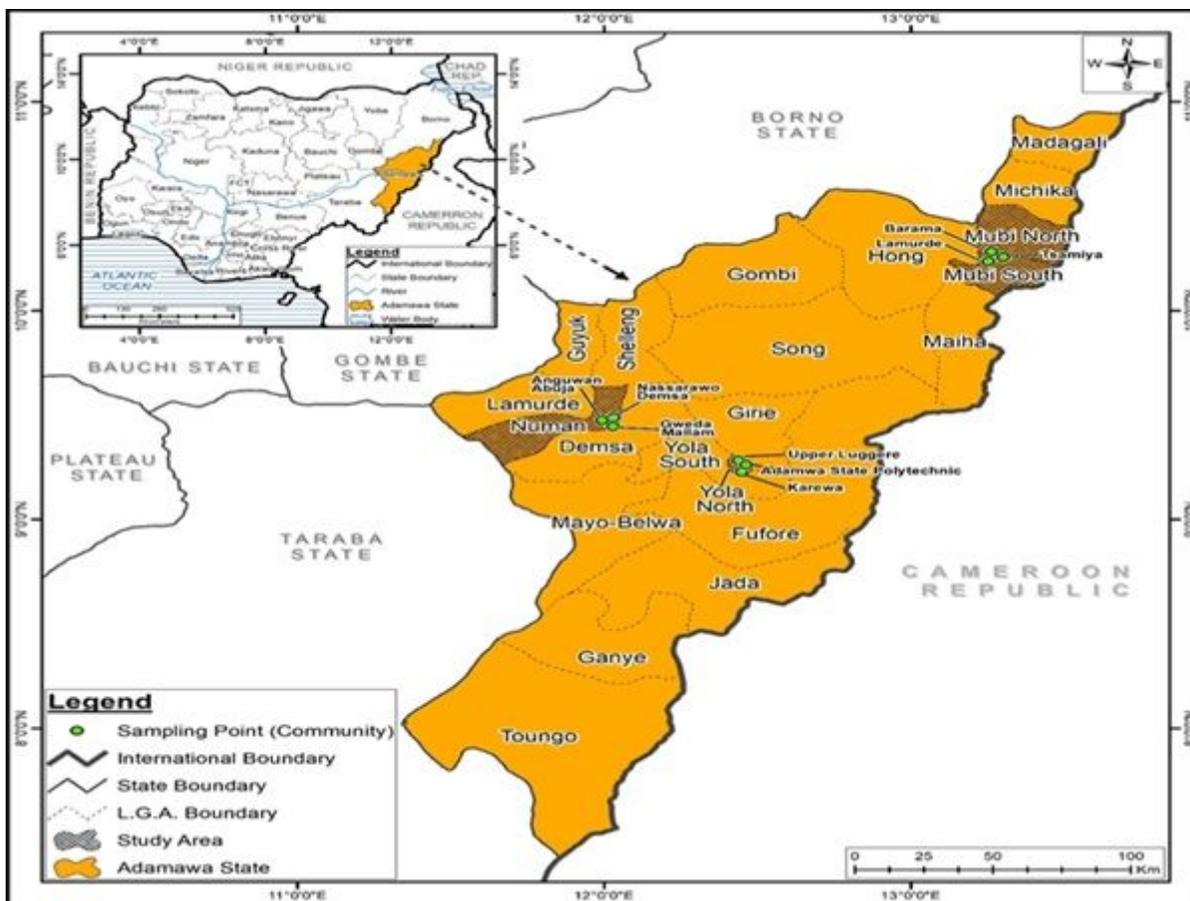


Figure 1

Map of Adamawa State showing study area. (Source: Modified from the administrative map of Adamawa State). Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Fig 2A

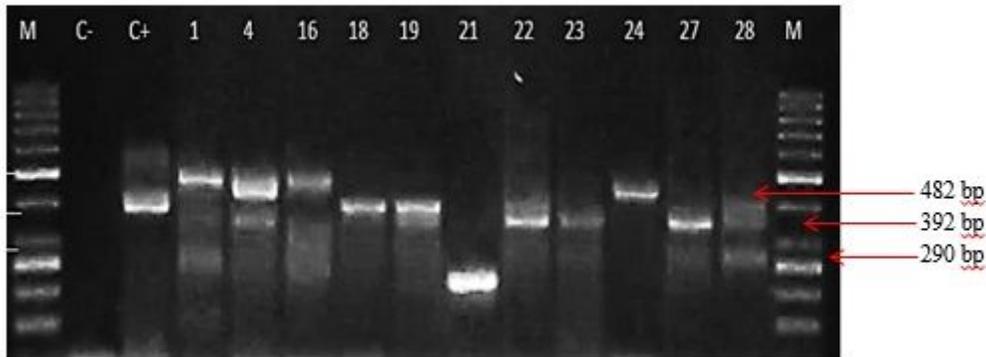


Fig 2B

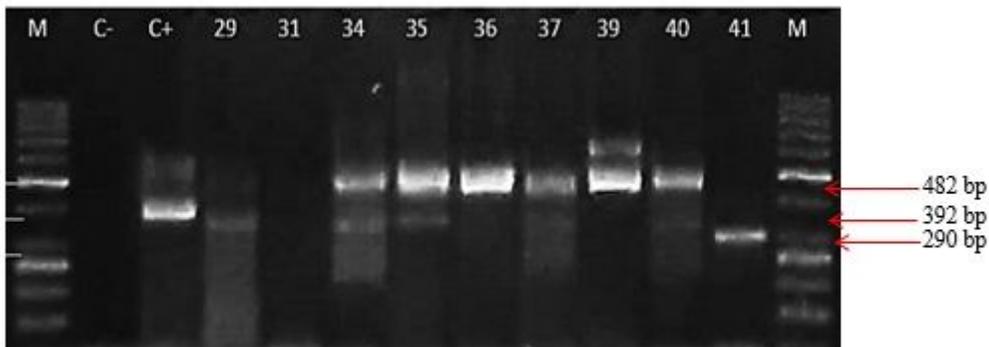


Fig 2C

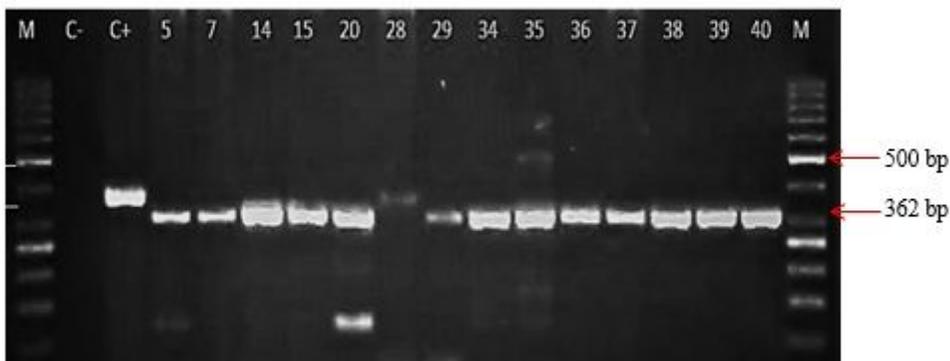


Figure 2

A, B, C: Electrophoregram of amplified cDNA by RT-PCR. DENV 1, 2, 3 and 4 serotypes specific primer sets were used with specific amplicons of 482 bp, 290 bp, 392 bp and 362 bp respectively. Lane M: Standard size marker of 1000bp, C- = negative control, C+ = positive control of DENV4 (392bp) and DENV2 (362) from cell culture propagation in Vero cells. Lane 1-41 Test samples

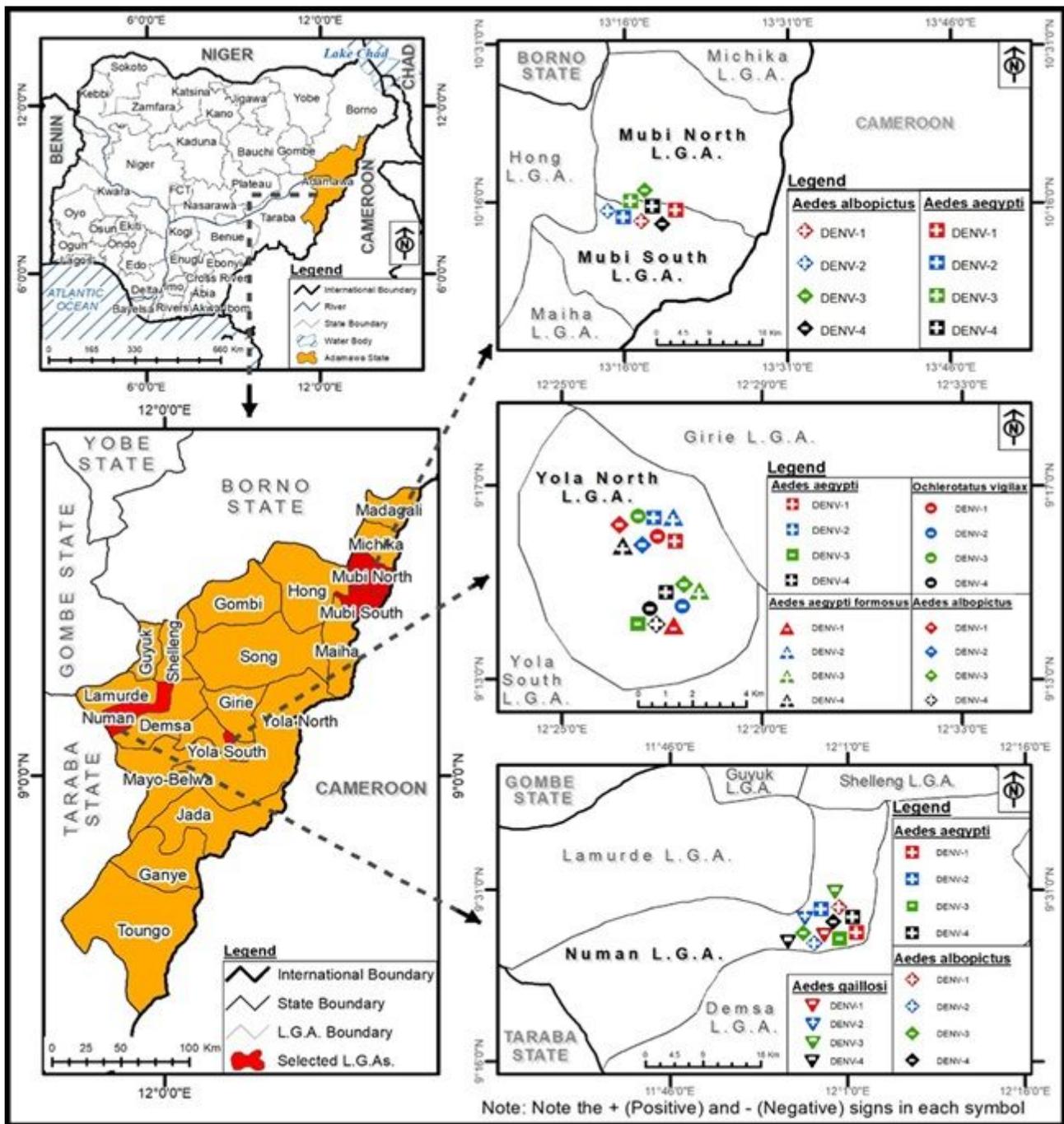


Figure 3

Map of Adamawa State showing Local Government Areas where Dengue virus serotypes were detected in *Aedes* species. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Fig 4A

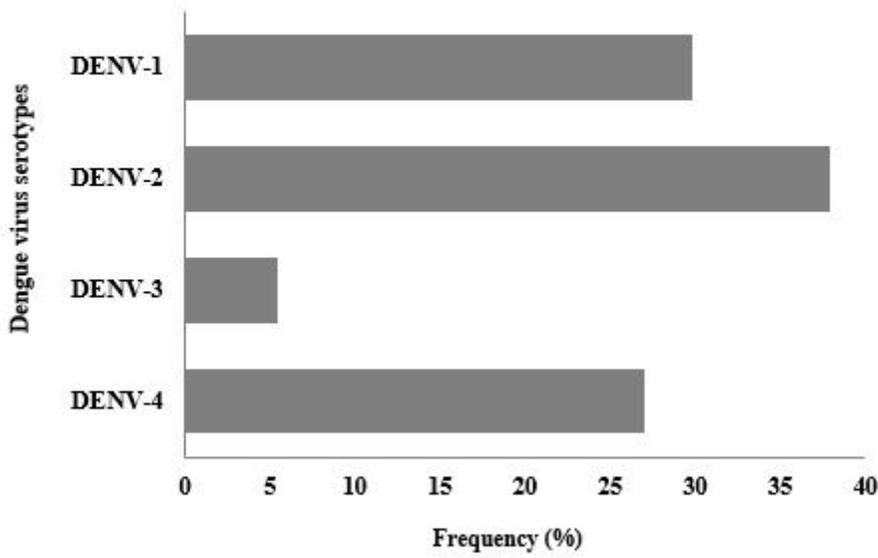


Fig 4B

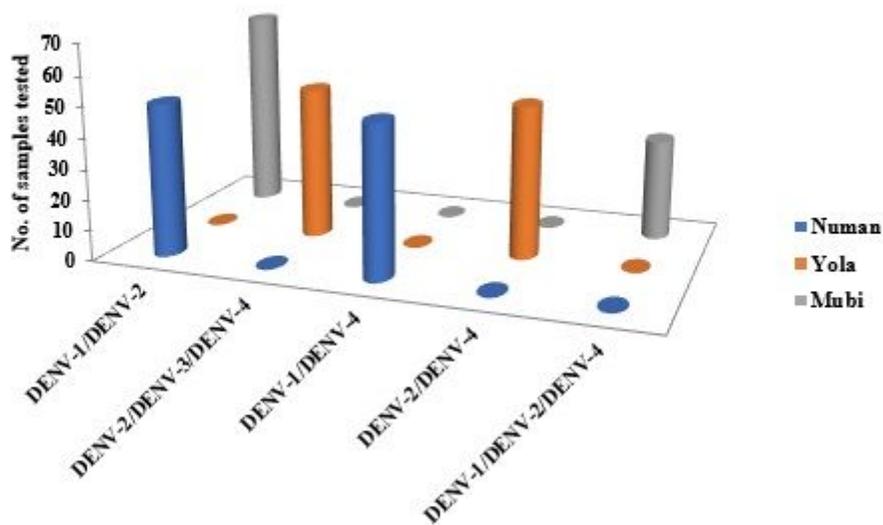


Figure 4

Occurrence of DENV serotypes and Fig 4B, co-infections of DENV serotypes in pools of Aedes mosquitoes in Adamawa state.

Fig 5A

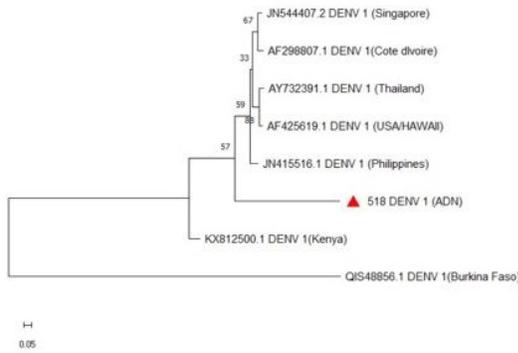


Fig 5C

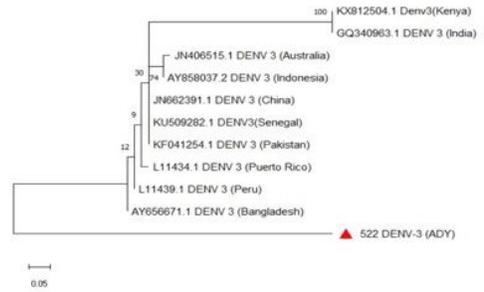


Fig 5B

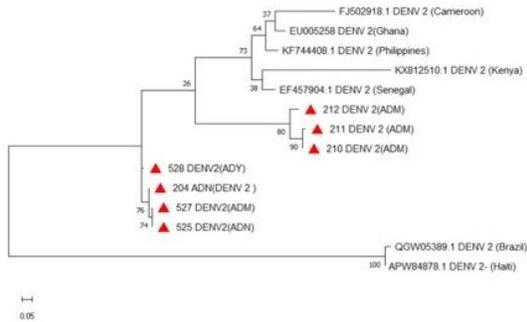


Fig 5D

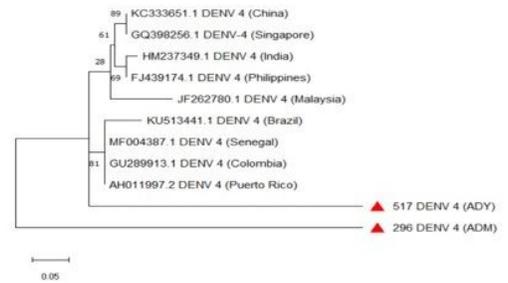


Figure 5

A, B, C and D. Phylogenetic analysis tree of DENV1-4 Serotypes isolated from *Aedes* species in the study area with other referenced DENV1-4 serotypes. The evolutionary analyses are based on the partial E gene sequences of DENV serotypes and reconstructed using Maximum Likelihood method (MEGA X). Value at nodes indicates bootstrap support. Study samples are designated in solid triangle. Abbreviations: ADN, Adamawa Nigeria while taxon names for references are labelled by their Genbank accession number, strains, and country.

Supplementary Files

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