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Title:

Dengue Virus and Malaria Concurrent Infection among Febrile Subjects within Ilorin Metropolis, Nigeria

Running Head:

Dengue and Malaria Concurrent Infection in Ilorin, Nigeria

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ABSTRACT

Dengue is a mosquito-borne disease of public health importance. It is caused by four serotypes of Dengue virus (i.e. DENV-1, -2, -3 and -4). As a result of practices that are conducive for mosquito breading, its vector is widespread in Nigeria and this could result to possible DENV outbreaks in Nigeria and beyond. This study aimed to assess the recency of DENV infection as well as occurrence of DENV and Malaria co-infections within Ilorin, Nigeria. Blood samples were obtained from 176 febrile subjects and analyzed using Enzyme Linked Immunosorbent Assay (ELISA) for the presence of DENV antibodies. Malaria infection was detected using a rapid diagnostic test kit for malaria parasites. Malaria and DENV (IgM positive) co-infected samples were further subjected to RT-qPCR analysis. A seroprevalence of 46.0% was recorded for anti-DENV IgM antibodies and 2.84% for concurrent dengue and malaria infections. Out of 95 IgM negative samples, 48 were found to be positive for DENV IgG antibodies. Eleven (6.25%) samples were confirmed DENV positive following RT-qPCR. The CT values of the amplicons were between 19.0 and 20.0. DENV serotype 2 dominated the study, while serotype 3 and 4 were equally distributed. Based on the high seroprevalence of DENV obtained in this study, there is a high possibility of experiencing Dengue virus outbreak in Ilorin, Nigeria, not neglecting the fast geographical spread of the vector. Therefore, surveillance and intensive vector control program should be instituted

KEYWORDS

Flavivirus; Immunoglobulin; RNA Extraction; Seroprevalence

TEXT

1. INTRODUCTION

Dengue was classified by the World Health Organization (WHO) in 2012 as the most important mosquito-borne viral disease in the world [WHO, 2012]. Aedes aegypti is the principal vector of Dengue. Dengue virus (DENV) belongs to the genus Flavivirus within the Flaviviridae family. It is circular in shape and contains an envelope covered with two viral proteins (E and M). These proteins

enable viral attachment and establishment of infection [Perera and Kuhn, 2008].

Dengue virus has four serotypes (DEN 1- 4); infection with one doesn't provide immunity against others. Each DENV serotypes has about 65% similarity in their viral genome, which is approximately the same degree of genetic relatedness Japanese encephalitis virus has in common with West Nile virus. Each DENV serotype causes similar symptoms in humans and circulates in the same ecological niche [Walts et al., 1987]. Dengue hemorrhagic fever (DHF) and Dengue Shock Syndrome (DSS-Complicated case of DENV) were first noted among some Americans in 1981 when DENV-2 was contracted by people who had previously been infected with DENV-1 some years earlier [Gould et al., 2008]. Several observations by scholars have led to a conclusion that subsequent infection of preimmune individuals with a different DENV serotype could exacerbate rather than mitigate disease, a process caused by antibodies and termed antibody- dependent enhancement (ADE) of disease [Halstead, 1970].

The first documented report of DENV in Africa was an isolate from Ibadan, Nigeria around 1960. There are few countries with no reported cases of dengue virus in Africa [Amarasinghe et al., 2011]. For several decades, DENV outbreaks have persistently occurred in some part of

Africa (Nigeria inclusive), but possibly go unreported due to diagnostic inadequacies and/ or underreporting [Baba et al., 2016].

Malaria is the most prevalent infectious disease in the tropical and subtropical regions of the world, and has a high morbidity especially in tropical countries [Mishra et al., 2003]. In those who have recently survived an infection, re-infection typically causes milder symptoms. This partial resistance disappears over months to years if the person has no continuing exposure to malaria [Caraballo and King, 2014].

Although infection with dengue or malaria can be life-threatening, co-infections with DENV and malaria could be more severe (Epelboin et al., 2012). Owing to the similar symptoms common to DENV- and malaria-infected individuals, misdiagnosis of either, and/ or co-infections have informed the need for a differential diagnostic approach especially in DENV-/ malaria-endemic regions (Magalhaes et al., 2014). This study sought to investigate the incidence of co-infections with DENV and malaria parasites in Ilorin, Nigeria, as well as the immunological status of febrile subjects against DENV infections in Ilorin, Nigeria.

2. MATERIALS AND METHODS

2.1 STUDY DESIGN

This study adopted a cross-sectional design that included prospective hospital-based surveillance of cases among febrile participants attending three major health facilities within Ilorin metropolis, Kwara State, Nigeria. This study was conducted within the peak period of the dry season (January - February, 2016).

2.2 STUDY AREA

Kwara state occupies a key position in North-central Nigeria. It borders Benin Republic to the West as well as five Nigerian states, to the North (i.e. Niger state), East (i.e. Kogi state) and South (i.e. Ekiti, Osun and Oyo states). These borders make the efflux and influx of people easy. This study was conducted within the state capital which covers two (2) Local Government Areas of Kwara state [Ilorin East ($8^0$23'N, $4^0$75'E) and Ilorin South ($8^0$30'N, $5^0$00'E)], Nigeria. The climatic condition of the North-central region of Nigeria (including Kwara state) is dominated by the influence of three major wind currents, namely: the maritime tropical (mT) air mass, the continental tropical (cT) air mass and the equatorial easterlies. The rainy seasons begin in March and end in October. The population of Ilorin East and Ilorin South was estimated to be 207,462 and 209,251 respectively [NPC, 2006].

2.3 STUDY POPULATION

A total of 176 participants were recruited for this study at three health centers within Ilorin metropolis. The inclusion criteria, which was based on their medical history and malaria symptomatology, included ongoing febrile illness, suspected to be malaria, typhoid or pyrexia of unknown origin at three health centers within Ilorin metropolis. This category of subjects was selected because the prodrome stage of Dengue and these diseases is similar. The study population included males and females from all age group.

2.4 SAMPLE COLLECTION

Blood sample of about 5ml was collected intravenously using sterile syringes from febrile patients into Ethylene Diamine Tetra-acetic Acid (EDTA) anti-coagulant bottles. The samples

collected were centrifuged at 3000 r.p.m. for 5 minutes and the plasma was carefully harvested into sterile tubes. The plasma was stored at -80^{0}C until needed for assay. A closed-ended questionnaire was administered to participants in order to harvest socio-demographic data, as well as DENV/malaria predisposing risk factors.

2.5 DETECTION OF IgM AND IgG ANTIBODIES AGAINST DENGUE VIRUS

In order to diagnose recent cases of dengue, plasma samples of study participants were screened for the presence of anti-DENV IgM antibodies using a commercial Human Dengue IgM ELISA kit (WKEA MED SUPPLIES CORP., China) according to the manufacturer's instructions. In order to detect past infections, plasma samples that tested negative for anti-DENV IgM were screened for anti-DENV IgG using a commercial Human Dengue fever IgG (DF-IgG) ELISA Kit [Antibodies-online GmbH, Germany] according to the manufacturer's instructions.

2.6 MALARIA PARASITE SCREENING

In other to investigate the occurrence of concurrent infections, subjects that tested positive for recent DENV infection were screened for malaria parasite (MP). The MP screening was conducted using a CareStart™ Malaria HRP2 (PF) rapid diagnostic kits (New Jersey, USA) following the manufacturer's instruction.

2.7 MOLECULAR CHARACTERIZATION OF DENGUE VIRUS

Subjects' samples collected within the first 5 days of illness were subjected to molecular diagnosis of DENV infections by RT-qPCR. RNA extraction was carried out using RNeasy mini

kits (Qiagen, Maryland, USA) according to the specifications of the manufacturer. The extracted RNA was preserved at -80°C until needed for further assay.

RT- qPCR FOR DETECTION AND SEROTYPING OF DENGUE VIRUS

The Dengue virus primers (DENV_F- GCATATTGACGCTGGGARAGAC, DENV_R1-3-TTCTGTGCCTGGAATGATGCTG, DENV_R4- YTCTGTGCCTGGATWGATGTTG) and probe (DENV_P- CAGAGATCCTGCTGTC) used were designed to target the 3' untranslated region, and was obtained from INQABA BIOTECH, South Africa. The PCR reaction mixture contained 12.5 pL of Maxima SYBR Green qPCR master mix (2.5mM MgCh, dNTP, dUNTP, Maxima Hot Start Taq DNA polymerase, SYBR Green I dye), 0.3 pM of the sense and antisense primers, 500 ng of the extracted RNA and 4.5pL of PCR grade water to make a final volume of 25 pL. The PCR mixture was introduced into a thermocycler (iCycler) (Bio-Rad, USA) programmed to incubate for 60 minutes at 37^{o}C and 10 minutes at 95^{o}C to allow cDNA production and initial denaturation and then proceed with 40 cycles of denaturation (95^{o}C for 30s), primer annealing (65^{o}C for 60s), primer extension (72^{o}C for 7 mins) and the amplicon maintained at 4^{o}C. The four dengue virus serotypes was differentiated as illustrated by (Rahman et al., 2013).

2.8 DATA ANALYSIS

Data generated in this study were entered and analyzed in Microsoft Excel 2007 and Statistical Package for the Social Sciences (SPSS) version 21.0. Descriptive statistics including average and proportion was used to summarize the data. Pearson Chi-square test of significance was applied. A 95% confidence interval ($P< 0.05$) was considered statistically significant.

2.9 ETHICAL CLEARANCE

An ethical clearance for the study was obtained from the Ethical Review Committee (ERC) of Kwara state Ministry of Health, Nigeria, and from the respective hospitals included in this study. Informed consent was obtained from study participants on voluntary basis.

3. RESULTS

In this study, 76 (43.2%) subjects tested positive to anti-DENV IgM only, while 5 (2.8%) subjects tested positive to anti-DENV IgM and MP (Figure I). Out of the 95 IgM negative samples, 48 were found to be positive for dengue virus IgG antibodies (Figure II). However, four samples were not assayed for dengue virus IgG antibodies due to insufficient plasma. Out of 18 samples selected for RT-qPCR, 6 (five of which were IgM positive) had insufficient plasma and were thus excluded from the RT-qPCR assay.

Females were more seropositive (54.3%) to recent dengue mono-infection while the males accounted for 45.6% (Table I). The total number of females who were screened for Dengue virus infection was slightly higher than the males. There was no statistical association between Dengue virus IgM antibodies serostatus and subjects' sex (X^2= 0.150, P=0.698).

The seroprevalence of recent Dengue virus infection with respect to age showed that all age groups had individuals who were seropositive for the virus. Subjects in the age group 31-40 had the highest seropositivity when compared to others. Married subjects were more seropositive (38.6%) and there was a statistical association between the subjects' marital status and the acquisition of Dengue virus infection (X2 =29.461, P < 0.001) (Table I). Among the predisposing

risk factors, rash history had a significant association with the acquisition of Dengue virus ($P<0.05$) (Table II).

Female subjects (1.7%) predominated the observed concurrent infections (Table III). Surprisingly, the highest optical density (OD) value obtained from the Dengue virus IgM antibodies assay was 2.086nm and was from a female with a concurrent infection.

4. DISCUSSION

Dengue virus outbreaks have persistently occurred in some part of Africa (Nigeria inclusive) for several decades, but many go unreported due to lack of diagnostic facilities [Baba et al., 2016]. The high prevalence of recent DENV infection (46.0%) recorded in this study which was conducted towards the decline of dry season when temperature was high could be linked to the effect warm temperature has on Aedes species [Reuda et al., 1990]. Warm temperature affects Aedes aegypti (the principle vector of Dengue virus) larval, thereby resulting in the development of smaller adult size. The smaller adult size females in turn tend to feed frequently on blood meal in order to nourish their developing eggs [Reita, 1988] which increase the tendency of Dengue virus transmission. However, Mohapatra et al. [2012] and Idris et al. [2013] recorded high prevalence in studies conducted during the mid-rainy season.

The high prevalence of Dengue virus reported in this study is closely related to the studies of Adesina and Adeniji [2016] and Bello et al. [2016] that reported 25.7% and 51.9% respectively. The reason for the variation in the prevalence rate could be due to researchers' interest in some specific serotypes. While this study investigated all serotypes, researchers like Idris et al. [2015] were interested in serotype III which could have influenced the prevalence rate obtained. It could

also be due to variation in climatic condition [Baba and Talle, 2011] and the different geographical locations where the studies were conducted [Baba et al., 2009].

Females were more seropositive for Dengue virus infection than males, but the acquisition of Dengue virus infection was not dependent on the sex of the subject. This finding is similar to other studies [Dawurung et al., 2010; Idris et al., 2013] that reported higher prevalence in females.

In this study, it was noticed that some age groups seemed to be more exposed to the vector of Dengue virus than others. Adults within the age group 31-40 were the most infected (49.38%) when compared to other age groups. This is in line with the findings of Idris et al. [2013] where subjects seropositive to Dengue type III neutralizing antibodies were highest in age group 30-39. The reason for the high seropositivity in adults could be due to more exposure of the young adults to the vector in course of searching for means of livelihood. More than half of the married subjects enrolled in this study were seropositive. This negates the report of Adesina and Adeniji [2016] where the single individuals were more seropositive to Dengue IgM antibodies.

Subjects with tertiary education dominated this study and it could be due to the presence of tertiary institutions like polytechnics, universities, college of education, etc. within the study area. More than half of the subjects who had no form of education were seropositive and it corresponds to the findings of Adesina and Adeniji [2016] where individuals with no form of education had Dengue infection the most.

In this study, the type of house subjects live in seemed to influence the rate of Dengue virus acquisition. Subjects staying in flats had a significantly high number of seropositivity for Dengue IgM antibodies compared to subjects who live in other house types. The reason for the high

seroprevalence of this group is unclear, but it could be attributed to the sanitary conditions of the houses. This finding contradicts an earlier study [Awando et al., 2013] that reported the acquisition of Dengue virus to be independent of house type. Majority of the subjects that indicated regular usage of mosquito nets were less seropositive to Dengue virus infection. The nets may have effectively interrupted the disease transmission cycle by restricting interaction between the vectors and the subjects [Onwujekwe et al., 2004].

Surprisingly, most of the subjects who claimed not to be exposed to certain risk factors (presence of stagnant water around homes, staying outdoors during the day, and rash history) were more seropositive to the virus compared to those exposed, which is similar to the report of Adesina and Adeniji [2016]. Apart from exposure to the virus, a number of reasons could lead to the establishment of the virus in the subjects. Some of these reasons include: level of immunity of the subjects, viral load infected with, previous exposure to the virus, as well as the nutritional level of the subjects.

In this study, subjects who were not on any medication were more seropositive compared to those on medication. However, Idoko et al. [2015] reported seropositivity in all the subjects under antimalaria medication as compared to those who were not under medication.

The use of Dengue virus universal primer and probe which targets the 3'UTR (untranslated region) of Dengue virus serotype 1-4 is important because it enables the detection of all Dengue virus serotypes. The 3' UTR plays a crucial role in Dengue virus gene expression by influencing the localization and translation efficiency of Dengue virus messenger RNA (mRNA) [Alm et al., 2014]. The RT-qPCR confirmed the presence of Dengue virus in Eleven (6.3%) of the twelve subjects who were positive for MAC-ELISA. The only negative subject observed following RT-

qPCR could have been positive for MAC-ELISA due to cross reactivity from other species of the genus Flavivirus. This cross reactivity could easily occur when assaying for Dengue virus using Enzyme-Linked Immunosorbent Assay (ELISA). Previous studies [Baba and Talle, 2011; Baba et al., 2013] suggest that Flaviviruses such as West Nile virus and yellow fever virus are in circulation in Nigeria.

Out of the 11 samples confirmed, Dengue serotype 2 dominated and is related to an earlier study [Baba and Talle, 2011] that reported four out of five Dengue positive samples as Dengue serotype 2. Dengue serotype 3 and 4 were equally distributed, suggesting that the two serotypes are also in circulation in the study area, although there is paucity of data detailing Dengue serotypes in infected subjects.

4.1 LIMITATION OF STUDY

This study was conducted in a particular season. Inclusion of other periods of the season (e.g. early and mid-rainy season) could have significant impact on the prevalence of Dengue and malaria distribution. Furthermore, cross-reaction with other members of the Genus Flavivirus wasn't sufficiently ascertained because the RT-qPCR was not conducted for all the IgM positive Dengue samples.

4.2 CONCLUSION

The study has revealed a high prevalence of recent Dengue infection and low prevalence of malaria infection within Ilorin metropolis, Nigeria. Evidence of concurrent malaria and DENV infections were also presented. Infections with DENV serotype 2 was most predominant within the study area.

4.3 RECOMMENDATION

The government should prioritize surveillance and mosquito control programs. Advanced research laboratories should be made available so as to aid adequate screening exercise and avoid misdiagnosis. In addition, medical practitioners should not be hasty in considering only malaria or typhoid when addressing febrile cases.

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COMPETING INTEREST

Authors declare no conflicts of interest.

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Table I: Seroprevalence of Dengue virus IgM antibodies among the febrile subjects with respect to socio-demographic factors (n=176).

Parameters	IgM positive (%)	IgM negative (%)	Total (%)	X^2	df	P Value
Sex:				**.150**	**1**	**.698**
Male	36 (20.5)	45 (25.6)	**81 (46.0)**			
Female	45 (25.6)	50 (28.4)	**95 (54.0)**			
Total	**81 (46.02)**	**95 (53.95)**	**176 (100)**			
Age:				**91.907**	**7**	**< 0.001**
0-10	3 (1.70)	10(5.68)	**13 (7.38)**			
11-20	2 (1.1)	21 (11.93	**23 (13.03)**			
21-30	3 (1.70)	46 (26.1)	**49 (27.3)**			
31-40	40 (22.72)	10 (5.7)	**50 (28.4)**			
41-50	18 (10.2)	7 (3.97)	**25 (14. 2)**			
51-60	9(5.1)	1 (0.56)	**10 (5.7)**			
61-70	4 (2.3)	0	**4 (2.3)**			
>70	2 (1.1)	0	**2 (1.1)**			
Total	**81 (45. 9)**	**95 (53. 1)**	**176 (100)**			
Marital Status:				**29.461**	**1**	**< 0.001**
Married	68 (38. 6)	42 (23.9)	**110 (62.5)**			
Single	13 (7.4)	53 (30.1)	**66 (37.5)**			
Total	**81 (46)**	**95 (54)**	**176 (100)**			
Occupation:				**19.963**	**3**	**< 0.001**
Farmer	10(5.7)	7 (4.0)	**17 (9.7)**			
Civil Servant	31(17.6)	26 (14.8)	**57 (32.4)**			
Business	31 (17.6)	23 (13.1)	**54 (30.7)**			
Student	9(5.1)	39 (22.2)	**48 (27.3)**			
Total	**81 (46.0)**	**95 (54.0)**	**176 (100)**			

X^2-chi square, df - degree of freedom, $P < 0.05$ - statistical significance at 95% confidence level

Table II: Seroprevalence of Dengue virus IgM antibodies among subjects with respect to some predisposing risk factors.

Predisposing Factor	Risk	IgM Positive (%)	IgM Negative (%)	Total (%)	X^2	P3 Value
Usage of mosquito net					**.001**	**.982**
	Yes	24(13.6)	28(15.9)	52 (29.5)		
	No	57 (32.4)	67 (38.1)	124 (70.5)		
	Total	**81 (46.0)**	**95 (54.0)**	**176 (100)**		
Presence of stagnant water around residence					**3.450**	**.063**
	Yes	8 (4.5)	19(10.8)	27(15.3)		
	No	73 (41.5)	76 (43.2)	149 (84.7)		
	Total	**81 (46.0)**	**95 (54.0)**	**176 (100)**		
Staying outdoors during the day					**.038**	**.845**
	Yes	44 (25.0)	53(30.1)	97 (55.1)		
	No	37(21.0)	42 (23.9)	79 (44.9)		
	Total	**81 (46.0)**	**95 (54.0)**	**176 (100)**		
Travel within the last 1 month					**.572**	**.450**
	Yes	38(21.6)	50 (28.4)	88 (50.0)		
	No	43 (24.4)	45 (25.6)	88 (50.0)		
	Total	**81 (46.0)**	**95 (54.0)**	**176 (100)**		
History of rash					**6.805**	**.009**
	Yes	10(5.7)	27(15.3)	37 (21.0)		
	No	71 (40.3)	68 (38.6)	139 (79.0)		
	Total	**81 (46.0)**	**95 (54.0)**	**176 (100)**		
Usage of medication					**.780**	**.377**
	Yes	28(15.9)	39 (22.2)	67 (38.1)		
	No	53 (30.1)	56(31.8)	109 (61.9)		
	Total	**81 (46.0)**	**95 (54.0)**	**176 (100)**		

X^2-chi square, $P < 0.05$ - statistical significance at 95% confidence level

Table III: Serotyping of Dengue Viral RNA using RT-qPCR

S/N	Patient Code	Sex	Age Group	Day(s) Since Onset of Illness	Threshold Cycle (CT Value)	Dengue Virus IgM status	Malaria status	Dengue Virus Amplicons	Dengue Serotype
01	40	F	31-40	4	19.3	+ve	+ve	+	2
02	23	F	31-40	2	19.7	+ve	+ve	+	4
03	T57	F	41-50	3	19.7	+ve	+ve	+	4
04	O44	M	51-60	3	No Ct	+ve	+ve	-	Nil
05	O8	M	>70	2	19.5	+ve	+ve	+	3
06	O31	M	31-40	3	19.4	+ve	- ve	+	3
07	4	F	21-30	1	19.5	+ve	-ve	+	2
08	20	F	51-60	2	19.5	+ve	-ve	+	2
09	O25	F	>70	4	19.6	+ve	-ve	+	2
10	T76	F	31-40	3	19.6	+ve	-ve	+	2
11	24	M	0-10	2	19.6	+ve	-ve	+	2
12	O16	F	0-10	2	19.5	+ve	-ve	+	2

KEY: Positive = +ve Present = + Female = F
Negative = -ve Absent = - Male = M

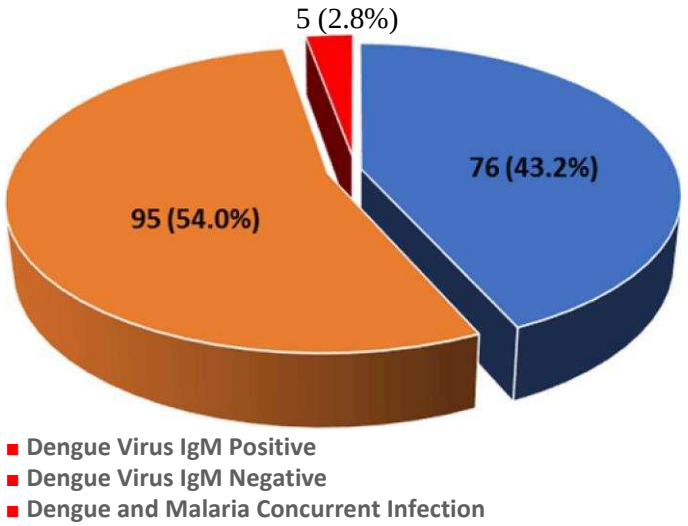

Figure I: Seroprevalence of Dengue virus IgM antibodies and concurrent infection among subjects

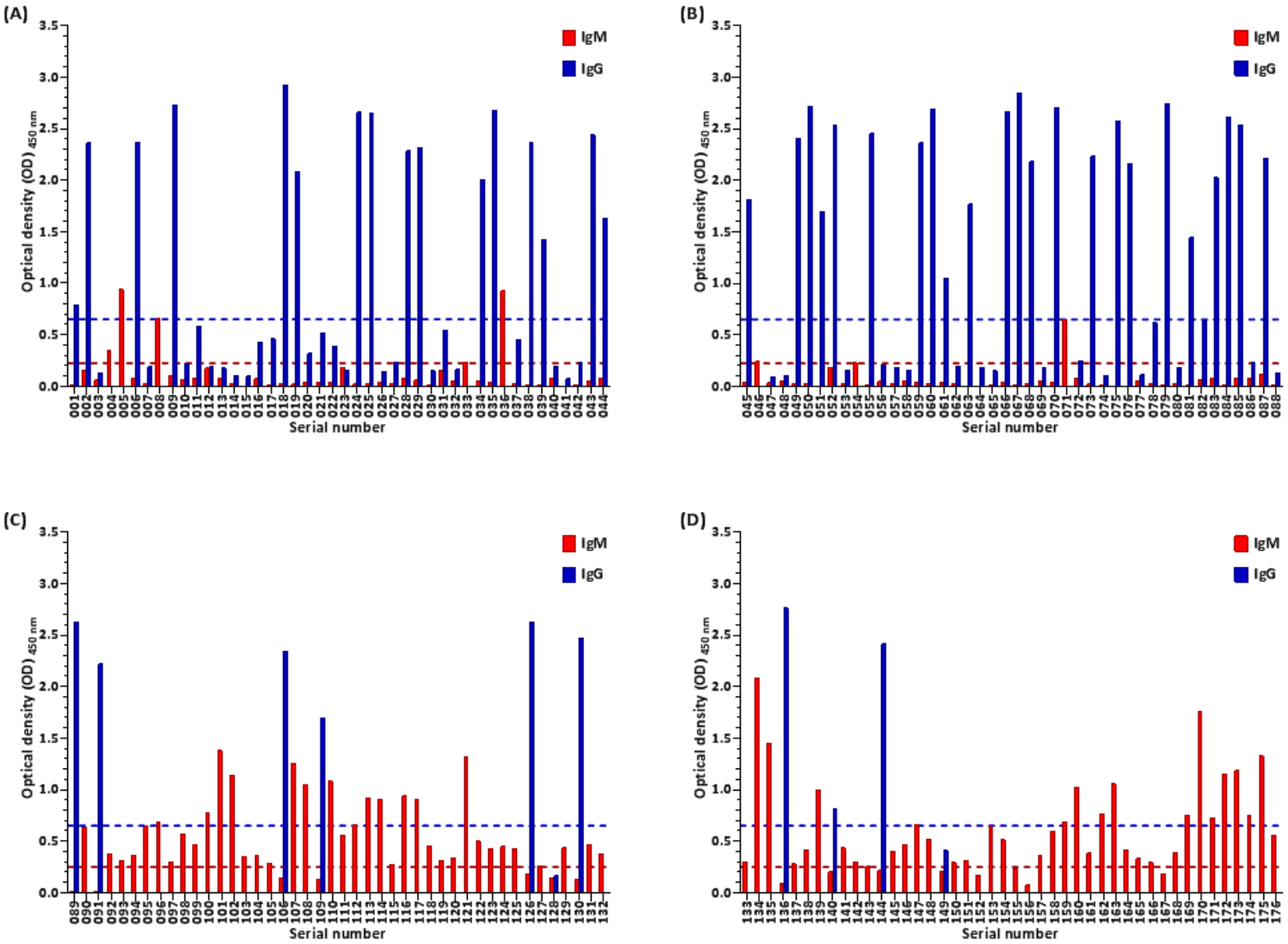
(A)
(B)
(C)
(D)
IgM
IgG
Optical density (OD) 450 nm
Serial number

Figure 2. History of DENV infection in participants. In order to determine the history of DENV infection in participants, the presence of anti-DENV IgM (red bars) and IgG (blue bars) in participant's serum samples were detected by sandwich ELISA. Participant's serum that tested negative for anti-DENV IgM were further tested for anti-DENV IgG. Figure shows ELISA test results for participants (A) 001 - 044, (B) 045 - 088, (C) 089 - 132 and (D) 133 - 176. Optical density (OD) values were taken at 450 nm and are shown on the y-axes; assigned serial numbers for respective participant is shown on the x-axes. Positive cut-off values for IgM (red broken lines) and IgG (blue broken lines) are also shown. IgG data are omitted for participants 152, 155, 156 and 167 owing to technical glitches with serum samples.