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The Editor,
Trends in Medical Research
Dear Sir,

RE: SUBMISSIONOF MANUSCRIPT FOR PUBLICATION

I wish to submit an article titled **'DENGUE VIRUS INFECTIONS IN PATIENTS SUSPECTED OF MALARIA/TYPHOID IN NIGERIA'** for publication in your journal. I wish to affirm the fact that this article has not been published in any other journal and there is no competing interest among the authors. This project was sponsored by Third World Organization for Women in Science (TWOWS) and Institute Pasteur de Dakar, Senegal. The information generated in this manuscript is to improve the strategy of clinical investigation in health care delivery in Nigeria. This implies that if an illness is properly investigated and managed/treated, the health status of Nigerians will be improved. I cannot find another journal that is more suitable for the dissemination of this information than yours. I obtained permission from the Head of Department of Immunology, University of Maiduguri Teaching Hospital, Nigeria to use stored patients' samples. All the authors of this article are actively involved in health research generating information that would promote the health of people in their respective countries.

Thank you.

Dr. Marycelin Baba

TITLE: DENGUE VIRUS INFECTIONS IN PATIENTS SUSPECTED OF MALARIA/TYPHOID IN NIGERIA

Short title: Dengue virus infections in Nigeria

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ABSTRACT

Introduction: Dengue fever and hemorrhagic fever/shock syndrome are clinically difficult to diagnose and could easily be mistaken for malaria, typhoid etc. This study was designed to determine the significance of these viruses in febrile illnesses.

Materials: About 1948 serum samples from suspected cases of malaria and typhoid were collected in June 2001 to July 2002 from six ecological zones in Nigeria. Also 59 pools of *Aedes Spp* from Rain forest were tested by RT-PCR and for virus isolation MAC-ELISA was used to test all the sera for IgM and IgG antibodies. All IgM positive sera were further analyzed by RT-PCR and Plague reduction neutralization test.

Results: Thirteen (0.67%) of the1948 sera were positive for DEN 1and 2 IgM from 4 zones. Mixed infections of DEN-2 and WN virus observed in two samples, eventually had neutralizing antibody for WNV. Overall, PRNT and ELISA results for DEN were in concordance. Dengue IgG antibodies in Sahel savanna (81.7%), Rain forest (69.0%), and Wooded savanna (69.0%) were significantly different from Grass (38.15) and Sudan (32.6%) savanna. One IgM positive serum had detectable RNA to DENs. Fourteen of 59 pools of Aedes sp showed viral RNA to DEN 1-4. The prevalence of the antibodies to these viruses and the ages as well as the gender of the patients was not significantly different

Conclusion:DEN infections could be mistaken for malaria/typhoid. There is need to include DENs and other endemic arboviruses routinely in the differential diagnosis of febrile illness in Nigeria.

Keywords: Dengue, virus, febrile illness, malaria, typhoid and Nigeria

INTRODUCTION

The clinical outcomes of DEN virus infection can vary from asymptomatic infection to mild febrile dengue fever (DF) to severe and life threatening dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) (**Gunther et. al. 2007**). The four closely related, but antigenically distinct, serotypes of DENs (DEN-1, DEN-2, DEN-3, and DEN-4) do not cross-protect but cross react. Infection with

one of these serotypes provides lifelong immunity to the infecting serotype only. Therefore, persons can acquire a second dengue infection from a different serotype, and second infections place them at greater risk for dengue hemorrhagic fever (DHF), the more severe form of the disease (*MMWR Morb Morta Wkly Rep*. 2007). These viruses are transmitted between human and monkey hosts by the mosquitoes of the genus *Aedes*, and principally *Aedes aegypti* (Holmes et. Al. 1999) popularly known as the 'tiger mosquito'.

The early symptoms of arbovirus infections mimic malaria, typhoid, measles and influenza which are hyper endemic in the environment, thereby rendering the diagnosis of these cases (arboviral infections) very confusing. In such situations, these infections are quite often misdiagnosed and so, inappropriately treated. Consequently these cases often result in high rate of morbidity, complications and mortality. Surprisingly, most (if not all) health Institutions in Nigeria lack appropriate diagnostic facilities for this group of viruses even with the common existence of factors (human populations, increased urbanization, incursion of human activity into the new ecosystems, increased global travel, climatic changes, and collapse of vector control and public health programs (Gubler 1988), which favor the emergence of arboviruses globally. This study was designed to survey the epidemiology of arboviral infections, with particular reference to Dengue viruses (DENs) in febrile patients suspected of malaria/typhoid Nigeria.

MATERIALS AND METHODS

Study population:

Patients with febrile illness sent to the laboratory for either malaria or Widal tests were used for the study. The common clinical manifestations on these patients by the time of sample collection include: fever, headache, and abdominal discomfort, and diarrhea, gastroenteritis while enteric fever, hepatitis, and HIV were less common. The commonest of all was fever either intermittent or recurrent.

Study areas:

Six ecological zones in Nigeria were randomly selected for the study. The zones include. Guinea/ Grass savanna (Abuja), Rain forest (Ibadan), Wooded / Guinea savanna (Gombe), Deltaic / Swan savanna (Calabar), Sudan savanna (Kano) and Sudan / Sahel savanna (Maiduguri). A brief closed-ended questionnaire was designed to collect demographic data of most of these patients: Such data collected were: age, sex and the clinical history.

Samples Collection A total of 1948 serum samples were collected in June 2001 and July 2002 from febrile patients. About 5ml of blood was collected by venu puncture from febrile patients. The blood was allowed to clot at room temperature

and the serum was carefully collected after centrifugation at 2,000rpm for 10 minutes and stored at –20^{o}C until tested. Human baits and scoop nets were used in catching mosquitoes from the field. The mosquitoes were caught alive and stored at -20^{0}C in Nigeria and were eventually transported with cold ice pack to Dakar for analysis.

SEROLOGY

Stock antigens were prepared in mouse brain from viruses supplied by WHO Collaborating Centre for Reference and Research on Arboviruses (CRORA), IPD, Senegal. All reactants were appropriately standardized.

Detection of IgM antibodies:

An IgM capture ELISA (MAC– ELISA) as previously described by Vorndam and Kuno (5) was used for the detection of IgM antibodies against DENs. The virus with a higher Optical density (OD) was considered the infectious agent as reported by Vorndam and Kuno (Vorndam and Kuno 1997). IgM positive samples were further subjected to Plague Reduction Neutralization Test (PRNT) as described by Mangiafico et al (1988).

Detection of IgG antibodies. For the detection of IgG antibodies against DENs, an IgG capture ELISA was used as previously described by Chunge et al. (1989). Binding of the IgG antibodies was detected using goat anti-human IgG antibodies labeled horseradish peroxidase. Unfortunately these samples were not confirmed by Plaque reduction neutralization technique (PRNT) because of the large sample size and the cost of the reagents.

Interpretation of results: The standard deviation of a battery of negative sera was calculated. A value of three standard deviations from the mean was used as the cut -off value to minimize false results as suggested by Innis et al (Innis et. al. 1989)

Mosquito processing

The field- caught mosquitoes were identified to the species level when possible. The identified mosquitoes were placed in 12x 75mm tubes in pools of 50. Each pool was tested by RT-PCR assay using a set of primer and with a cell culture assay. The cell culture assay was conducted by inoculating 100µl aliquot of clarified supernatant from the mosquito pool onto sub confluent AP-61 cell and incubated for 8-10 days. The presence of the virus was determined by the use of indirect Immunofluorescence assay as described by Beckwith et. al (2000)

RT - PCR IN SERA AND MOSQUITOES

THE EXTRACTION OF RNA FROM SERUM/MOSQUITO SUSPENSION/TISSUE CULTURE EXTRACT

RNA extraction was carried out according to the specifications of the kit's (Q1 a Amp viral RNA Mini Kit) manufacturer. For each batch of mosquito suspension/serum/

extracted, positive controls (cell culture of the seed virus concerned) and uninoculated cell as negative control were included.

RT- PCR for detection of DEN virus

THE FIRST ROUND OF AMPLIFICATION IN PCR FOR DENGUEVIRUSES .

[The method previously described for dengue (Lanciotti 1992) was adopted]

A semi-nested RT-PCR was carried out. All relevant aspects of the RT-PCR (Mgcl2, primers, RT, Taq polymerase, number of cycles, and annealing temperatures) were initially optimized by using quantitated purified DEN virus RNA to achieve a maximum level of sensitivity before testing the field samples. The reaction product was electrophoresed on a 1% composite agarose gel in 0.4M Tris- 0.05 M sodium acetate-0.01 M EDTA buffer. The gel was stained with ethidium bromide. The resulting DNA band was visualized on a UV transilluminator. The target viral RNA was converted to a DNA copy (cDNA) prior to enzymatic DNA amplification by using RT and the dengue virus downstream consensus primer (D2), homologous to the genomic RNA of the four serotypes. Subsequently, Taq polymerase amplification was performed on the resulting cDNA with the upstream dengue virus consensus primer (DS1).

DENGUE VIRUS TYPING BY SECOND-ROUND AMPLIFICATION WITH TYPE SPECIFIC PRIMERS (nested PCR) as previously described by Lanciotti et al (1992)

In this method, type-specific primers replaced dengue virus downstream consensus primer, while dengue upstream primer was retained. The Tag DNA dependent DNA polymerase amplified the products of the first amplification to generate a DNA strand of different length, which was identified by gel electrophoresis. Thus the second amplification differentiated dengue species into different serotypes.

RESULTS

PATTERN OF DENGUE VIRUS INFECTIONS IN NIGERIA

IgM CAPTURE ELISA FOR DENGUE VIRUSES

Figure 1 shows the IgM and its corresponding IgG antibodies to the different serotypes of DEN in the four ecological zones. For example in Figure 1, sample 6 appeared to be a recent infection with DEN-2 with high OD value (1.151) while sample 4 seemed to be a case of anamnestic response.
. Thirteen (0.6%) of the 1948 sera were positive for DEN1 and 2 IgM antibodies from 4 of the 6 ecological zones in Nigeria studied. The zones with positive cases were Rain forest (DEN-2), Grass savanna (DEN-2), Deltaic savanna (DEN2), and Sahel Savanna (DEN1 and 2). (Table1).

Plaque reduction neutralization test on DEN IgM positive sera:
All the sera that were DEN IgM positive by MAC-ELISA were found positive by PRNT. (Data not included). Two sera which showed mixed infections of WNV and dengue by MAC-ELISA were later confirmed to be positive for WNV by PRNT. Failure to carry out PRNT for all the samples limits this study from giving the precise status of these patients with regards to dengue virus infections in Nigeria. This is because a negative acute-phase specimen is inadequate for ruling out such an infection underscoring confirmation by demonstrating virus-specific serum IgG antibodies in the same or later specimen.

THE PREVALENCE OF DEN IgG ANTIBODIES IN NIGERIA
The zonal distribution of DEN IgG antibodies is displayed on Table 2. The prevalence of DEN IgG antibodies and the zones were significantly different with the highest in Sahel savanna (81.7%), followed by Rain forest (69.0%) and Wooded (69.0%) and the least in Sudan savanna (32.6%) and Grass savanna (38.1%). DEN

AGE AND GENDER DISTRIBUTION OF PATIENTS WITH WNV IgM ANTIBODIES
The prevalence of these antibodies and the ages as well as the gender of the patients were not significantly different (X^2=P>0.05).

VIRUS ISOLATION FROM MOSQUITOES

No dengue virus was isolated from *Aedes* mosquitoes (59 pools) tested.

RT-PCR ON *AEDES* MOSQUITOES/ IgM POSITIVE SERA FOR WNV

The results of RT-PCR on Aedes species are presented on table 2. Fourteen of 59 pools of mosquitoes (*Aedes spp*) tested showed DEN viral RNA and these include one DEN-1, 4 DEN-2, 5 DEN-3 and 4 DEN-4. However, one DEN IgM negative serum was positive by RT-PCR. Samples that showed non-specific bands were not considered. TI TAN (Combination of reverse- transcription of viral RNA and subsequent Taq polymerase amplification in a single reaction vessel) seemed to exhibit higher degree of sensitivity and specificity compared with separate reverse-transcription and PCRs . Sequencing of RT-PCR results was beyond the scope of this study.

DISCUSSION

In Nigeria most cases of PUOs are routinely investigated for malaria and /or typhoid and not viruses. This study has revealed that, 13 (0.67%) of 1948 febrile patients in four of six ecological zones in Nigeria had DEN IgM antibodies.. Though the prevalence rate of DEN infection as revealed in this study is low but it confirms the activities of this virus in Nigeria. Like Yellow Fever, a positive case of DEN virus infection in a community, is of epidemiological importance, probably because of its mode of transmission. The common feature shared by a previous (Fagbami et. al. 1977) and the present studies is that DEN antibody was higher in Rain forest while the Guinea savanna had the lowest. DEN IgM measures evidence of recent infection because, although IgM positive by MAC-ELISA on acute serum samples are only provisional, but certainly that infection would have occurred sometime in the previous one or two months before sample collection (World Health Organization, 2001**).** However, PRNT is more specific than ELISA because it shows a monotypic reaction to the infecting virus through the late convalescent phase of illness (Vorndam and Kuno 1997).

The prevalence rate of DEN IgG antibodies and the ecological zones were significantly different with the highest in Sahel savanna (81.7%), followed by wooded savanna (69.2%), and Rain forest (69.0%). The least among them were Grass and Sudan savanna with 32.6% and 38.1% respectively. The low percentage of people with DEN IgG antibodies in the two zones is of epidemiological importance. This is because any introduction of an epidemic strain or serotypes of any of the Flaviviruses in these zones could result in epidemic due to the presence of high proportion of susceptible host.

Thein (2003) observed that levels of anti dengue IgG in acute phase sera collected during a period of high dengue activity correlated with disease severity but low dengue activity showed no association. In this study, because there is no active surveillance for dengue or other arbovirus activities in Nigeria, it is

difficult to differentiate periods of high and low virus activities. Also, because there was no follow-up on these cases, correlation of levels of IgG and diseases severity was not applicable in this study. Nevertheless the clinical importance of IgG in diagnosis of DEN infections is its usefulness in distinguishing between primary and secondary dengue infections with 100% primary and 96% of secondary infections being correctly classified (Innis et. al. 1989, Vauhgn et. al. 1999). Based on these reports, from figure 1 of this study, samples 1, 4, 9, 12 and 13 could be described as some suspected cases of anamnestic response to DEN infections. Moreover, these patients could be assumed to be at the risk of developing DSS because the risk of developing DSS following an anamnestic infection was from 82-103 times greater than that of developing DSS following a primary dengue infection (Thein 2003)**.** In addition this author observed a significantly higher rate of anamnestic infections with DEN-2 (which is the most prevalent serotype of DEN in Nigeria) in DSS compared with other serotypes. Lack of surveillance activities for these viruses in the country poses constrain to the precise status of these infections in the community. For instance, in 2003 two suspected cases of viral hemorrhagic fever were reported in University of Maiduguri Teaching Hospital, Maiduguri (a Tertiary Health Institution in Northeastern Nigeria. (Personal communication). These patients died within few hours on the same day they visited the hospital for treatment. Probably when the infection was at the prodromal phase (the phase at which the symptoms and signs mimic malaria or typhoid), the patients were receiving different malaria treatments (with the assumption that the drugs were resistant to the infecting parasites) till symptoms of haemorrhages appeared. Therefore, since the patients tested in this study were not followed up, associating these cases with DSS was beyond the scope of this study. The need for active surveillance and intensive education on arbovirus activities in the environment cannot be emphasized.

The detection of DEN RNA in *Aedes species* has demonstrated the important role of the vector in the epidemiology of dengue infections in the environment.The presence of DEN RNA in male *Aedes aegypti* and *Aedes Species* is evident of vertical transmission in Nigeria and this compared favorably with previous report in Mexico (Gunther et. al. 2007). In agreement with Miagostovich et. al.(1988), one of the DEN IgM negative serum was found positive (DEN-3) by RT-PCR, suggesting that most of the IgM negative samples in this study could have been false. This probably contributed to the low prevalence rate of DEN infections obtained in this study. The consideration of the time of onset of symptoms during sample collection would have given more precise information on the status of these patients with regards to recent DEN infections in Nigeria. This is because specimens taken earlier than six days after onset would have a variable percentage of false negatives due to insufficient time for antibody development. Also, a small percentage of patients have detectable IgM antibodies on the day that symptoms

begin and most patients become positive by the sixth day after onset (Vorndam and Kuno 1997)). Therefore the few IgM positive sera in this study yielded no viral RNA probably because the time of sample collection did not favor RT-PCR result. It is therefore imperative to employ the two techniques (MAC-ELISA and RT-PCR) in proper diagnosis of DEN infections.

As the clinical symptoms associated with DEN infections are indistinguishable from those of many other viral, bacterial and parasitic infections, specific diagnostic tests assume critical importance in the unequivocal identification of DEN infections (Hapugoda et. al. 2007).This implies that, the prodromal phase of DEN infections could be mistaken for malaria/ typhoid fevers, which are hyper endemic in this environment. Therefore, it is important to include this virus (and possibly other endemic arboviruses) in the differential diagnosis of febrile illnesses in Nigeria. Surveillance with good laboratory services serves as an "early warning system" against impending outbreak of arbovirus infections

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FIGURE 1: DEN IgM AND THE CORRESPONDING IgG ANTIBODY

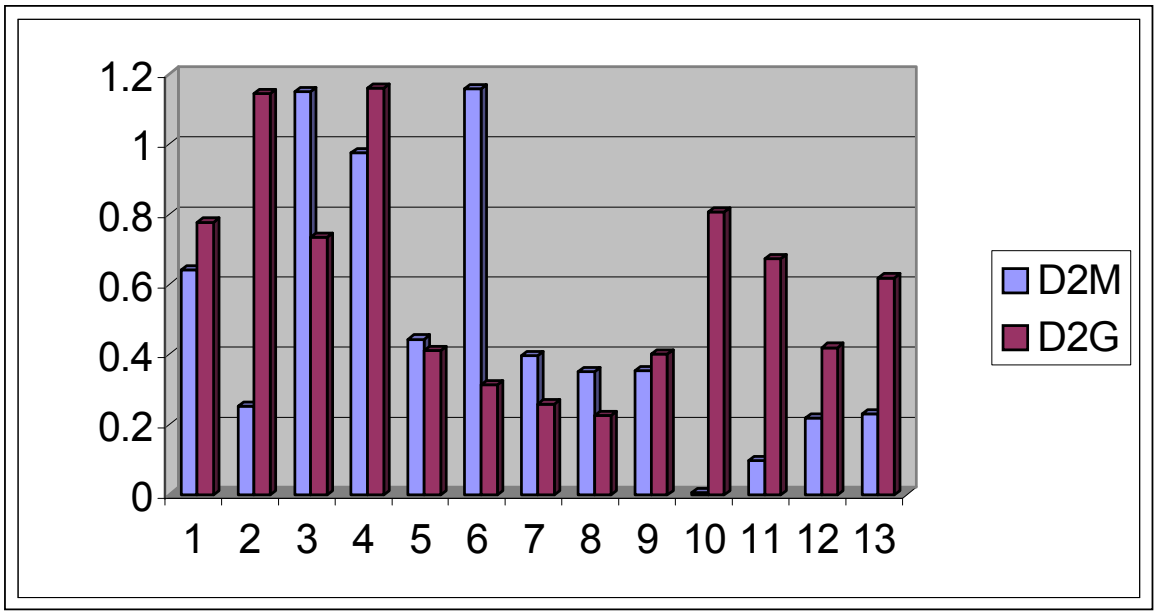

TABLE 1: DENGUE VIRUS INFECTIONS IN DIFFERENT ECOLOGICAL ZONES IN NIGERIA

	ECOLOGICAL ZONE	TOWN/ CITY	TOTAL NO TESTED	NO POSITIVE (%)	DENGUE SEROTYPE	REACTION WITH NORMAL Ag
1	RAIN FOREST	IBADAN	442	4(0.9)	D2	6(1.4%)
2	SUDAN SAVANNA	KANO	267	0(0)	NONE	6(2.2%)
3	WOODED/GRASS SAVANNA	GOMBE	341	0(0)	NONE	14 (4.1%)
4	GRASS SAVANNA	ABUJA	281	1(0.36)	D2	7 (2.5%)
5	DELTAIC SAVANNA	CALABAR	317	3(0.1)	D2	1(0.1%)
6	SAHEL SAVANNA	MAIDUGURI	300	5 (1.67)	D1 AND D2	5 (1.67%)
	TOTAL		1948	13 (0.67)		39 (2.0%)
	* NO REACTION WITH NORMAL ANTIGEN					
	** REACTED WITH NORMAL ANTIGEN					

FIGURE 2: ZONAL DISTRIBUTION OF DEN IgG ANTIBODIES

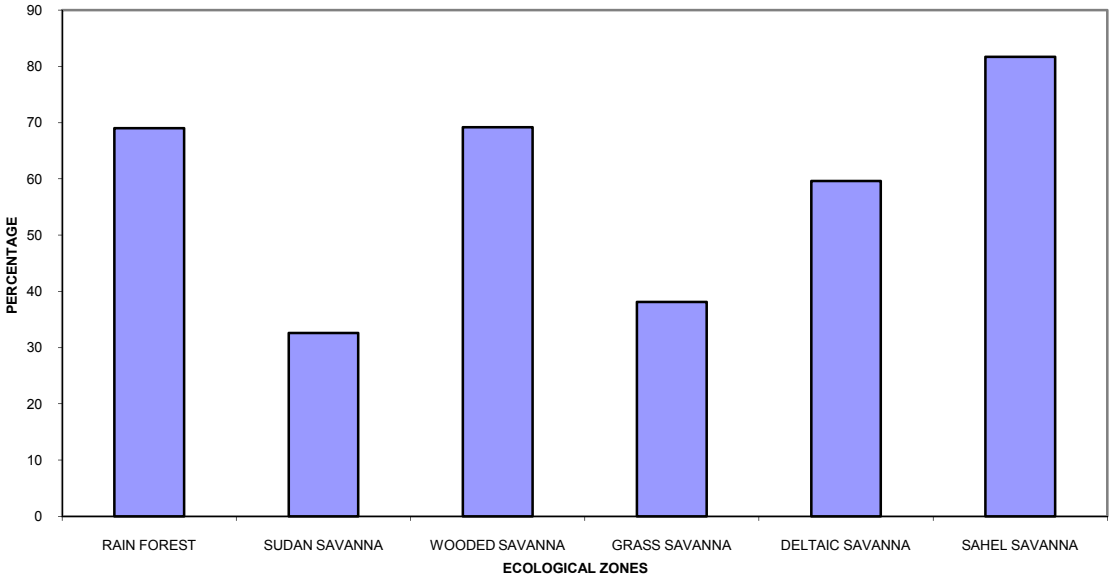

TABLE 2:THE SUMMARY (RESULT) OF RT-PCR ON *AEDES SPECIES*

				RTPCR		
MOSQUITO SPECIES	**SEX**	**NO OF POOL TESTED**	**D1**	**D2**	**D3**	**D4**
Aedes aegypti	female	27	1	2	0	4
Aedes aegypti	males	7	0	1	2	1
Aedes species	females	12	0	0	1	1
Aedes species	males	9	0	1	1	0
unidentified	not known	4	0	0	2	2
Total		59	1	4	5	4

FIGURE 2: ZONAL DISTRIBUTION OF DEN IgG ANTIBODIES