Original Article

Serologic Evidence of Exposure to West Nile Virus among Humans in Ibadan, Southwestern Nigeria

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ABSTRACT

Background: Cases of fever without diagnoses of malaria and/or typhoid are usually regarded as undifferentiated febrile illness (UFI) in Nigeria. We studied the contribution of WNV (an arbovirus) to UFI in humans in southwestern Nigeria.

Materials and methods: Sera from 188 consenting humans visiting two health care facilities in Ibadan, and 25 horse grooms from Lagos and Ibadan were screened for WNV antibodies by cELISA and a subset by PRNT. Pertinent demographic/clinical data were collected and blood samples screened for *Plasmodium spp, Salmonella typhi* and *S. paratyphi* antibodies. Student's t-test and binary logistic regression were used for data analysis.

Results: Overall, 156 participants (73.2%, 95% CI: 67.3-79.2 [n=213]) were positive for WNV antibodies. Being clinically ill was associated (p=0.001) with WNV seropositivity while "active" and "non-active" participants had comparable (p=0.21) seroprevalence of 74.6% and 62.5%, respectively. Forty-five percent (18/40) of febrile participants had WNV antibodies only, thereby accounting for UFI. The 18-66 year olds had higher (75.8%) seroprevalence than those ≤ 17 years (47.4%) while seropositivity obtained for the horse grooms (56.0%) was significantly lower than for the remaining 188 individuals (75.5%). Participants were mostly exposed to WNV (75.5%) than *Plasmodium* (33.5%) and *S. typhi* (39.9%) while PRNT showed that 10.5% of tested humans had protective WNV antibodies.

Conclusions: This study revealed serologic evidence of exposure of the participants to WNV and contribution of the virus (or related flaviviruses) to UFI in the study area. High prevalence of the antibodies indicates endemicity of southwestern Nigeria for WNV.

Key words: Humans, anti-WNV antibody, prevalence rate, undifferentiated febrile illness, southwestern Nigeria.

West Nile virus (WNV) is a neurovirulent, zoonotic mosquito-borne virus belonging to the genus *Flavivirus* in the family Flaviviridae; it is responsible for outbreaks of meningitis and encephalitis[1]. The virus is maintained in an enzootic cycle between ornithophilic mosquitoes and birds, but can also infect and cause disease in horses and humans[2]. In humans, infection begins following a bite from a WNV-infected female mosquito (e.g. *Culex* mosquito)[3]. WNV was regarded as a mild pathogen causing self-limiting outbreaks but this notion changed when the virus caused large epidemics with high impact on human and animal health, hence it has been termed a re-emerging zoonotic pathogen[4]. Recently, the geographic range of WNV has been extended to all continents of the world with significant increase in incidence in humans, horses and birds[5]. The virus is found in Africa, Asia and Europe, the Mediterranean region, the Middle East, Australia and the

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Americas[6, 7]. It was first isolated from a 37 year-old febrile woman in the West Nile District of northern Uganda in 1937[8]. The first reported human epidemic occurred in Israel in 1951, and the first isolation of WNV from *Culex* mosquito was made in 1952[9]. Epidemics of WNV infection in humans have occurred in Algeria, Romania, Russia and South Africa[10, 11, 12, 13]. Occurrence of WNV in mosquitoes and detection of specific antibodies in febrile patients have been documented in northern Nigeria[14, 15].

Surveillance for WNV activity in any population can be achieved through seroprevalence study. Hence, animals such as camels, goats, cattle, sheep and horses in Nigeria and Romania have been reported as having hemagglutination-inhibiting antibodies to WNV[10, 11, 16, 17]. Plaque reduction neutralization test (PRNT) has also been used for sero-surveillance in the USA and Nigeria[14, 15, 18] and for determination of correlate of sero-protection[19].

Nigeria is endemic for malaria and typhoid fevers. Hence, many cases of febrile illness are usually regarded by physicians as either of these diseases[15, 21]. Also, when malaria and typhoid tests are negative for a given patient, such cases are commonly regarded as "undifferentiated febrile illness (UFI)" especially when the patients fail to respond to anti-malarial drugs[17]. Since most health facilities in Nigeria lack the capacity to conduct arboviral investigations on such "UFI" patients, physicians treat them symptomatically.

In an earlier study of horses in southwestern Nigeria, high WNV antibody prevalence was reported with establishment of enzootic status among the horses[22]. It is therefore necessary to test the humans and grooms in daily contact with horses for evidence of exposure to WNV. Association of WNV with cases of UFI has only been reported in two studies from northern Nigeria[14, 15] and, to our knowledge, evidence of WNV infection in humans has not been reported in southwestern Nigeria in recent years. We therefore hypothesized that WNV infection has no association with febrile illness in humans in southwestern Nigeria and that humans in this region have no evidence of exposure to the virus.

MATERIALS AND METHODS:

Description of human participants and locations:

This study was conducted between June, 2011 and October, 2012. The human participants in this study were resident in Ibadan, Oyo state, and Onosa and Ajah, Lagos state, southwestern Nigeria. They were mostly Yorubas of male and female genders. The participants largely comprised two groups: a group that visited healthcare facilities for medical test or medical care while the other group consisted of horse grooms who handled and took care of horses in their stables or polo clubs. The participants visited two different healthcare facilities, namely the Central Medical Diagnostic Center (CMDC), Total Garden, Ibadan (N07.398°, E003.910°) and Department of Family Medicine, University College Hospital (UCH), Ibadan (N07.4015°, E003.90345°). Those from CMDC were apparently healthy referral patients from Adeoyo State Hospital, Ibadan which provides medical care to mostly residents of Ibadan who visit the laboratory for tests ranging from pregnancy to malaria parasite (MP) and Widal (for typhoid fever), among others. The humans from UCH, which is a tertiary healthcare facility that provides medical services majorly to patients from southwestern Nigeria, other parts of the country and neighboring countries, comprised apparently healthy and clinically ill participants. The horse grooms were all males, apparently healthy

and mostly of Hausa extraction with few Yorubas. They belonged to the Eleyele Polo club (N06.4500°, E003.4333°), Ibadan, and the Onosa (N06.47001°, E003.80226°) and Ajah (N06.46700°, E003.57255°) horse ranches. They groom and exercise the horses daily, and accompany them to various locations in Nigeria for annual polo tournaments. Oyo and Lagos states are located in the tropical rainforest in southwestern Nigeria and enjoy wet season from April/May to October/November and dry season from November to March/April.

Study design and sample size:

This is a cross-sectional serosurvey of individuals from CMDC and UCH, and horse grooms from Eleyele polo club, Ibadan and horse ranches in Lagos. Only humans who completed consent forms were consecutively included in this study. With the assistance of physicians, pertinent demographic and clinical data were obtained from participants in UCH; these include fever, headache, muscular/joint pains and abdominal pains/discomfort. Being basically a diagnostic laboratory, there was no physician at CMDC, and it was not logistically possible to employ the services of one at the Centre. Hence, some clinical data were not obtained for the CMDC cohort.

In order to establish contribution of WNV infection to UFI among participants, the blood and serum samples of participants from UCH and CMDC were subjected to malaria parasite and Widal tests as well as WNV antibody detection ELISAs. However, the horse grooms were only tested for WNV antibodies.

Using the formula for estimating sample size[23, 24], and 80.16% prevalence rate of WNV antibody in humans of northern Nigeria[14], a sample size of 246 humans was arrived at. However, for logistic reasons, we could only study 213 human participants.

Blood sample collection, serum preparation and storage:

About 7ml blood sample was aseptically collected by a phlebotomist from each participant via venepuncture. At UCH, 1ml of the collected blood was dispensed into clean EDTA-treated vial and sent to the Medical Microbiology and Parasitology laboratory for MP test while 2ml blood was dispensed into labeled anti-coagulant-free Universal bottle and screened for the presence of anti-O and anti-H antibodies against *Salmonella typhi*, and *S. paratyphi* A, B and C by the Widal agglutination test the same day. The MP and Widal tests were also done at CMDC. Sera prepared from each of the remaining blood samples were appropriately labeled and stored at -80°C until assayed for WNV antibodies. The remaining blood samples left to clot at room temperature were spun at 3,000 rpm for 10 minutes. The supernatant (serum) from each sample was collected with a new Pasteur pipette into appropriately labeled Eppendorf tubes and stored at -80°C until tested for WNV antibodies in the Virology laboratory, Department of Veterinary Microbiology and Parasitology, University of Ibadan.

Inclusion criteria for the study were male and female patients of all ages with/without fever, headache and muscular/joint pains with or without neurologic signs, and willingness to provide samples for MP and Widal tests while exclusion criterion was unwillingness of patients to participate in the study.

Malaria parasite test for detection of *Plasmodium species*:

Within one hour of blood sample collection and using established protocol[21, 25], separate thick and thin blood films were made from each blood sample on clean glass slides, labeled accordingly and examined by microscopy. The blood

samples were handled carefully as potentially infectious.

Widal slide agglutination assay:

Each serum sample was subjected to Widal slide agglutination test for detection of anti-O and -H antibodies against *S. typhi*, and *S. paratyphi* A, B and C using the rapid Cromatest® Febrile Antigen Kit (LINEAR CHEMICALS S.L., Barcelona, Spain) according to manufacturer's instruction. When the rapid slide agglutination test was positive for any sample, it was further confirmed by tube agglutination test as described by Opara *et al.*[21]. A serum sample was considered positive when it produced visible agglutination at ≥ 1:80 dilution for O antigen and ≥ 1:160 for H antigen. When a sample gave agglutination at 1:40 dilution for only one of the eight antigens, the test was repeated.

Enzyme-linked immunosorbent assay (ELISA) for detection of WNV antibodies:

Prior to performing the ELISA on human sera, individual serum sample was spotted in two replicates on Whatman FTA® card and appropriately labeled. Punched-out discs were soaked in elution buffer and the RNA eluted from the cards was subjected to real-time RT-PCR using WNV-specific primers and probes to detect WNV RNA (as evidence of viraemia). Since none of the tested sera had detectable WNV RNA, they were therefore tested for only WNV IgG antibodies using West Nile Virus Antibodies Enzyme Immunoassay (DIA. PRO, Diagnostic Bioprobes Srl Via G, Milano, Italy) for qualitative determination of antibodies to WNV in human serum. The assay was done according to manufacturer's instructions.

Plaque reduction neutralization test (PRNT):

Two-fold serial dilutions of test, and positive and negative control sera were incubated for about 60 minutes with equal volume of virus at 37°C in 5% CO_2. The respective serum-virus mixtures were incubated with about 2-day-old confluent Vero C1008 cells in twelve-well tissue culture plates for 60 minutes at 37°C and 5% CO_2 along with wells containing virus-only and medium-only controls. Following overlay with 3% carboxymethylcellulose, plates were incubated at 37°C and 5% CO_2 for 3-5 days. After fixation and staining, plaques in each well were counted and titers calculated using percentage reduction in plaques compared to the WNV control wells (50% reduction in number of plaques [$PRNT_{50}$] was used as neutralizing end-point)[19, 26]. Human serum with titer of, at least, 1:10 was regarded as positive with protective anti-WNV antibodies.

Data analysis:

Results obtained were presented with descriptive statistics at 95% confidence interval (CI). In order to establish associations between study variables, Student's t-test, Chi-square and binary logistic regression (BLR) analysis were used with statistical significance set at $p \leq 0.05$. The analyses were done using Excel spreadsheet and SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS:

Study participants:

A total of two hundred and thirteen (male and female) participants were studied. The overall age of the participants ranged from 0.5-66.0 years (yrs), mean age was 31.8 yrs [95% CI: 30.0-33.6]. Table 1 shows the distribution of the human participants involved in the study. Forty of the 82 patients recruited in UCH had fever (≥ 38.3°C) as observed by the attending physician and they presented with various clinical signs/symptoms such as fever, headache, muscular/joint and abdominal pains. The remaining 42 patients from UCH were afebrile but had other clinical

signs/symptoms that simulated malaria and or typhoid fever as stated above. A total of 136 study participants (5 from UCH, 106 from CMDC and 25 horse grooms) were apparently healthy. The remaining 77 participants, all from UCH, had pertinent clinical signs/symptoms.

Malaria parasite test for detection of *Plasmodium species*:

Out of the 188 blood samples (82 from UCH and 106 from CMDC) tested for malaria parasite, 63 tested positive for *Plasmodium falciparum*, giving an overall prevalence rate of 33.5% (95% CI: 26.8-40.3). By location, 19 (23.2%, 95% CI: 14.0-32.3) of the 82 participants from UCH were MP-positive with five of them having headache, fever and body pains to give 26.3% concordance between clinical signs/symptoms and laboratory diagnosis of malaria. In comparison, 44 of the 106 humans screened at CMDC tested positive for MP to give a location-specific prevalence rate of 41.5% (95% CI: 32.1-50.9).

Widal slide agglutination assay

A total of 188 participants were tested by the Widal slide agglutination test. The titer of positive sera for O antigens of *S. typhi* and *paratyphi* ranged from 1:80 to 1:320; for the H antigens however, it ranged from 1:160 to 1:640. In all, 75 sera (39.9%, 95% CI: 32.4-46.9) had antibodies (agglutinins) indicative of typhoid fever. For location-specific antibody prevalence, 30 of the 82 participants from UCH were positive to give 36.6% [95% CI: 26.2-47.0]; 10 (33.3%) of these had fever and abdominal discomfort/pains that simulated clinical typhoid, thereby showing concordance between clinical and laboratory diagnosis of the disease. Of the 106 participants from CMDC, 44 were positive for Widal test to give a prevalence rate of 41.5% [95% CI: 32.1-50.9].

Table 1: Distribution of human participants in the study.

Group	Number of participants	Mean age (yrs)	p-value	Gender Male	Female
UCH	82	38.9	0.001 [a]	24	58
CMDC	106	26.5		37	69
UCH	82	38.9	0.005 [a]	24	58
GROOMS	25	31.1		25	0
CMDC	106	26.5	0.09	37	69
GROOMS	25	31.1		25	0

[a]=significant difference in mean age

WNV serology:

Serologic assays were done for the 213 individuals (82 from UCH, 106 from CMDC and 25 horse grooms from Lagos and Ibadan). A total of 156 humans had detectable WNV antibodies by ELISA, giving overall WNV IgG antibody prevalence of 73.2% [95% CI: 67.3-79.2]. The prevalence of WNV antibodies among the 188 subset (those from UCH and CMDC) was 75.5% [95% CI: 69.4-81.7]. The participants from UCH that were seropositive were 72, giving a location-specific prevalence rate of 87.8% [95% CI: 80.7-94.9] while in CMDC, 70 of the 106 participants had WNV antibodies giving a prevalence rate of 66.0% [95% CI: 57.0-75.1%]. Therefore, compared to the CMDC cohort, those from UCH, had significantly higher WNV antibody prevalence with *p*-value of 0.001 and odds ratio (OR) of 3.7 (95% CI: 1.7-8.0).

Regarding clinical picture, group-specific prevalence rates showed that 36 of the 40 febrile patients from UCH had WNV antibodies, to give prevalence rate of 90.0% [95% CI: 80.7-99.3]. Of the remaining 42 non-febrile patients in UCH, 35 (83.3%, 95% CI: 72.1-94.6) had WNV

Table 2: Variables analyzed for association with WNV antibody positivity in humans

Variables	Number tested	Number positive (%)	Odds ratio (95% CI)	p-value
Participant group				
CMDC	106	70 (66.0)	1.5 (0.6-3.7)	0.35
UCH	82	72 (87.8)	5.7 (2.0-15.8)	0.001
Groom	25	14 (56.0)		
Gender				
Male	86	59 (68.6)		
Female	127	97 (76.4)	1.5 (0.8-2.7)	0.21
Age (years)				
≤ 17	19	9 (47.4)		
18-66	194	147 (75.8)	3.5 (1.3-9.1)	0.008
Clinical condition				
Ill	77	71 (92.2)	7.1 (2.9-17.5)	0.001
Apparently healthy	136	85 (62.5)		
Exposure tendency				
Active	189	141 (74.6)	1.8 (0.7-4.4)	
Non-active	24	15 (62.5)		0.21
MP test				
Positive	63	48 (76.2)	1.1 (0.5-2.1)	
Negative	125	94 (75.2)		0.88
WIDAL test				
Positive	74	59 (79.7)	1.5 (0.7-3.0)	0.28
Negative	114	83 (72.8)		

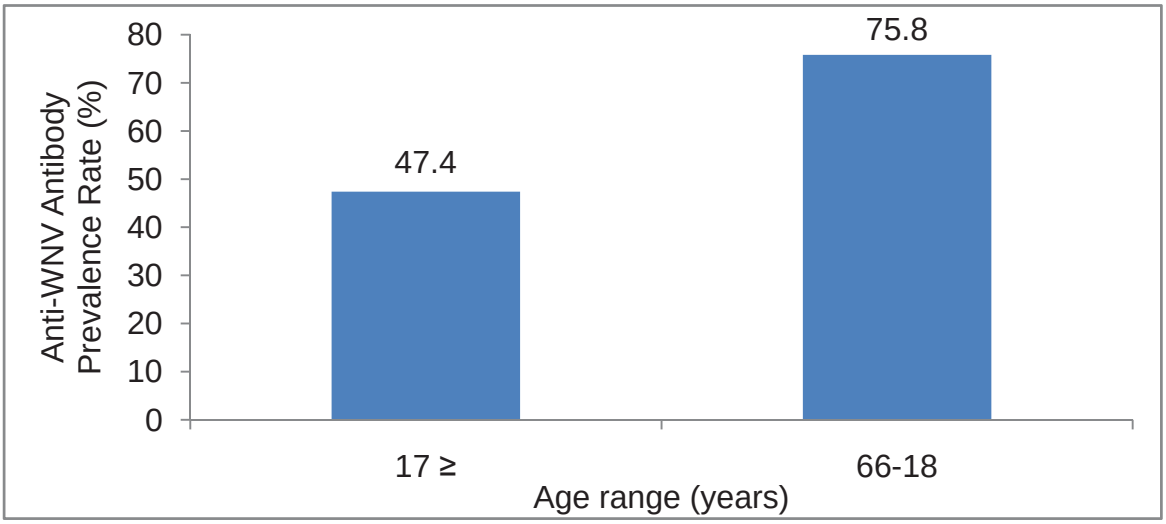

Figure 1**:** WNV antibody prevalence in relation to age of participants.

antibodies. Comparison of the prevalence rates of the febrile and non-febrile patients showed they were statistically comparable (p = 0.38).

Out of the 136 apparently healthy participants (106 from CMDC, 5 from UCH and 25 horse grooms) screened by MP and typhoid tests, 85 were positive for WNV antibodies giving a prevalence rate of 62.5% [95% CI: 54.4-70.6].

The remaining 77 participants, all from UCH, had relevant clinical

signs/symptoms; of these, 71 (92.2% [95% CI: 82.2-98.2]) were positive for WNV antibodies. Comparison of the prevalence rates of these two different clinical groups indicated association between clinical illness and WNV seropositivity (OR: 7.1 [95% CI: 2.9-17.5], p = 0.001).

In order to investigate differences in exposure to WNV by age group, the participants were divided into two: those ≤ 17 yrs and 18-66 yrs. The former recorded 47.4% seroprevalence (9/19, 95% CI: 24.9-69.8%) while the latter gave 75.8% (147/194, 95% CI: 69.7-81.8%). Statistical analysis showed that the older age group had significantly higher (p = 0.008) WNV seroprevalence with OR of 3.5 compared to the ≤ 17 yrs age group.

The participants were also divided into two groups to reflect propensity for outdoor activities which possibly exposed them to WNV mosquito vectors. The first group was the "non-active" participants in the age range 0.5-14 yrs and 56-66 yrs (n=24) who were more likely to be indoors. Fifteen of these (62.5%) had WNV antibodies. In the second group designated as "active" with participants aged 16-55 yrs, WNV seropositivity was 74.6% (141/189); comparison of the two group-specific prevalence rates showed they were statistically comparable (p = 0.21). It was observed, however, that participants in the age range 0.5-14 yrs had 50.0% (6/12) WNV antibody prevalence while those aged 56-66 yrs had seroprevalence of 75.0% (9/12).

Among the 25 horse grooms who were all males, 14 (56.0%, 95% CI: 36.5-75.5) had WNV antibodies. Of the 12 from Eleyele Polo club, Ibadan, eight (66.7%) had WNV antibodies while six (46.2%) of the 13 grooms in Lagos were seropositive. Comparison of WNV seroprevalence between the 25 grooms and the remaining 188 participants showed that the latter had significantly higher seroprevalence with a p-value of 0.04 and OR of 2.4 (95% CI: 1.0-5.7).

Binary logistic regression and Chi-square analyses to establish associations between other participants' variables and WNV antibody positivity are as shown in Table 2. It was observed that WNV antibody prevalence rate significantly increased with increasing age of participants (Figure 1). The results of PRNT on the human sera are shown in Table 3.

Identification of participants with undifferentiated febrile illness:

The analysis to establish contribution of WNV infection to undifferentiated febrile illness involved 188 participants (the patients in UCH and CMDC) that were tested for MP, typhoid and WNV antibodies. Only the participants from UCH, however, had clinical data. Overall, 167 participants (88.8% [95% CI: 84.3-93.3]) were positive for, at least, one of the three tests (Figure 2).

It was observed that 24 participants (12.8%, [95% CI: 8.0-17.5%]) were positive while 21 (11.2%, [95% CI: 6.7-15.7%]) were negative for all the three tests. Of the latter, only one participant (4.7%) was free of all the pertinent clinical signs/symptoms.

A total of 58 persons (30.9%, [95% CI: 24.2-37.5%]) were positive for only WNV antibody (Figure 2) with 40 of them being among the UCH cohort and having febrile illness (≥ 38.3°C). Notably, 18 (45.0%, [95% CI: 29.6-60.4%]) of the 40 febrile patients tested positive for only WNV antibody (i.e. they were negative for both MP and Widal tests). These probably represented participants with condition described as "undifferentiated febrile illness". Other various outcomes of the three tests are as shown in Figure 2.

Table 3: Protection status of tested human sera

Number Tested	Positive by cELISA	Positive by PRNT	cELISA/PRNT concordance	Protection status
1	+	+ (1:10)	Yes	10.5%
1	_	+ (1:10)	No	(protective)
2	_	_	Yes	89.5% (not
15	+	_	No	protective)

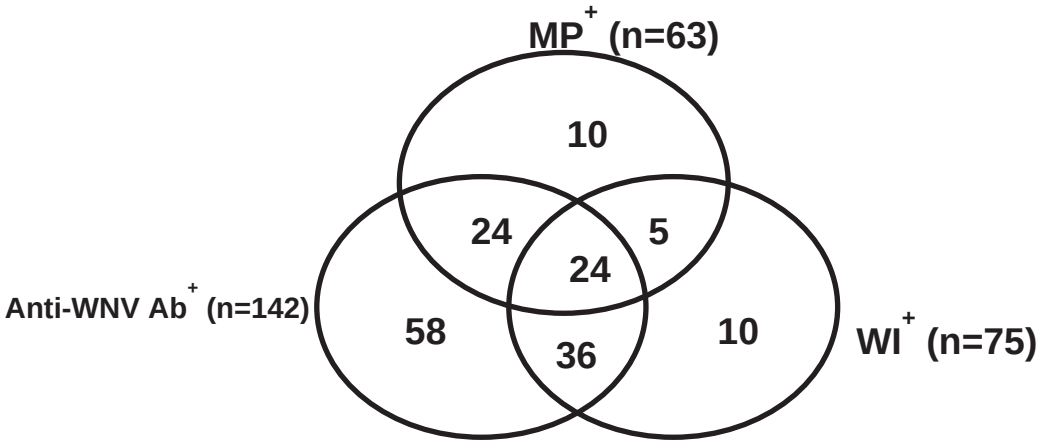

Figure 2: Venn diagram showing inter-relationship between positive results for WNV antibody, MP and Widal tests.

DISCUSSION:

In order to establish exposure of humans (including horse grooms) in southwestern Nigeria to WNV and provide information on possible contribution of WNV to cases usually referred to as "undifferentiated febrile illness", we studied sera of 213 human participants. The observation that humans from UCH were significantly older in average age than either of the other two cohorts suggests that older participants were more likely to seek health care services in UCH (tertiary health institution) than younger ones.

The overall anti-WNV antibody prevalence rate of 73.2%, though less than 80.16% observed by Baba *et al.*[14] in northern Nigeria, was still considerably high; the various group-specific prevalence rates were also high (Table 2). Control or elimination of infectious disease agents rests on protective immunity in a sufficient proportion of a given population of humans (or animals)[27]. Hence, in agreement with previous observation[28], the high prevalence of WNV antibody in this study partly explains the absence of West Nile disease (WND) outbreaks in humans in southwestern Nigeria. Also, as earlier reported in humans and horses[14, 22, 29], the high seroprevalence obtained is an indication of widespread or high WNV activity and endemicity in southwestern Nigeria. The high WNV seropositivity in this study could be partly explained by the location of Oyo, Lagos and surrounding states in the humid tropical rainforest region of Nigeria characterized by heavy rainfall and collection of water in peri-domestic environments that favor

mosquito breeding[30]. In a similar study in Gabon[42], a relatively high location-specific anti-WNV antibody prevalence rate (23.7%) was reported from the forested regions of the country. On the contrary, Baba *et al.*[14] in the semi-arid zone of Nigeria reported high WNV IgG prevalence rate of 80.16% among febrile patients, which is slightly higher than the 73.2% obtained in this study; they concluded that their study area was endemic for flavivirus.

Though the group-specific WNV prevalence rates were generally high, the UCH participants had the highest prevalence rate with about 4 and 5 times higher likelihood of being WNV-seropositive respectively compared to those from CMDC and the horse grooms (who were apparently healthy at the time of sampling). This could be due to the fact that majority of the UCH cohort (77 out of 82) presented with one clinical sign/symptom or the other during sampling. Moreover, the analysis in this study has shown that being clinically ill was significantly associated with WNV seropositivity. Another possible reason could be that the study participants visiting UCH, a tertiary health care facility that receives patients from all parts of southwestern Nigeria as well as other states in Nigeria and beyond, had been exposed to WNV (or related flavivirus)-infected mosquitoes in their various homes or neighbourhoods.

Although the 106 participants from CMDC, which were mostly referral cases from Adeoyo State Hospital and other health care facilities in Oyo State, did not have clinical data and were all apparently healthy at the time of sampling, they also had high WNV seroprevalence rate. Thus, the WNV seroprevalence rates of the UCH and CMDC cohorts were statistically comparable, implying similar exposure of the participants to WNV. These high group-specific and overall WNV seroprevalence rates indicate endemicity of southwestern Nigeria for WNV.

Apart from participants visiting health care facilities, 25 horse grooms that daily cared for horses were tested for presence of WNV antibody. Although more than half (14/25, 56.0%) of the grooms were positive for WNV antibodies, their seroprevalence was significantly lower compared to that of participants from UCH and CMDC (75.5%). This suggests that horse grooming may not constitute a significant risk factor for WNV infection in southwestern Nigeria although the observation could also be attributed to the smaller sample size of the grooms. In a previous report[22], high (90.3%) WNV seroprevalence was detected in the horses cared for by grooms. Comparison of WNV seroprevalence rates, which is an indicator of exposure to WNV, in the two species revealed that the horses[43] had significantly higher seroprevalence with 7 times likelihood ($p = 0.001$, OR = 7.0 [95% CI: 2.7-18.0]) of being seropositive than their handlers. This shows that the horses were more exposed to WNV which could be an indication that the mosquito vectors had a biting/feeding preference for horses than their grooms, even though they live in the same environment. Additionally, the open stables of the horses could have exposed them more to mosquito vectors compared to the grooms who slept indoors at night. In a similar study of 1,280 sera from 80 jockeys and grooms, 100 fowlers, 500 blood donors, 600 healthcare workers, 100 veterinary surgeons and 100 hunters in Italy, zero WNV seroprevalence was reported[20]. While this report from Italy might suggest that being around horses does not pose a significant risk of WNV infection, it is contrary to the findings of the current study in which WNV antibodies were detected in horse grooms. The WNV seropositivity obtained for

horse grooms in the present study is probably due to endemicity of southwestern Nigeria for the virus and widespread activity of *Culex* mosquito vectors which have been reported to be abundant in the region[43].

It was observed that the 18-66 years old participants had significantly higher WNV seroprevalence and were about 4 times more likely to be positive than the ≤ 17 year age-group. This suggests that the propensity for WNV infection increases with advancing human age and could be due to the tendency for the older individuals ("active" age group) to stay outdoors more than the ≤ 17 year old participants ("non-active" age group), which predisposes the former more to mosquito bites. These observations are similar to that of El Rhaffouli *et al*. [35] that young individuals who were more likely to stay outdoors for work or leisure (socio-economic activities) had greater exposure to WNV vectors. In a previous study, Baba *et al*.[14] attributed high rate of exposure to mosquito bites and subsequent WNV infection to large-scale tree planting in Borno State, Nigeria that provided shelter for mosquitoes and shade for human outdoor activities. Another plausible reason for the observation might be the endemicity of southwestern Nigeria for WNV as observed in this study and previous studies involving horses in the region[22, 29]. Moreover, the high seropositivity obtained for the older age-group could partly explain the absence of West Nile disease in southwestern Nigeria as already reported[28]. The observation, however, that 78.9% of the seropositive individuals detected by ELISA were negative by PRNT may account for the absence of WND in the study area because these individuals might actually possess CF or HI antibodies or antibodies against related flaviviruses which, to a great extent, are also protective against WNV infection[33].

The fact that no significant difference in WNV antibody prevalence exists between the MP-positive and MP-negative groups (Table 2) suggests that although *Plasmodium spp.* and WNV are transmitted by mosquitoes, the vector species of these two infectious agents are different and they both cause disease independent of each other in their human hosts. This is corroborated by previous reports showing that malaria is transmitted majorly by *Anopheles* mosquitoes and WNV by *Culex* species[44,45]. Similarly, the comparable WNV antibody prevalence rates obtained for the Widal test-positive and -negative individuals in this study shows that presence of typhoid fever is not associated with WNV seropositivity.

With respect to protective WNV neutralizing antibody, the fact that majority (89.5%) of the sera tested by PRNT were negative even when 78.9% were ELISA-positive suggests that the participants were likely exposed to other antigenically related flaviviruses. It could also be that the antibodies were actually specific for WNV but non-neutralizing (probably complement fixing or haemagglutination-inhibiting).

Interestingly, one serum sample that was negative for WNV antibodies by cELISA was not only positive by PRNT, it had a protective titer of 1:10[19] (Table 3). Since PRNT is a highly specific assay, it can be inferred that the individual was certainly exposed to WNV and that PRNT also has considerable sensitivity for detection of WNV antibodies. This study has shown that none of the sera tested by real-time RT-PCR contained WNV RNA (evidence of viraemia), indicating that none of the participants' had ongoing WNV infection at the time of sampling. Though herd immunity against WNV has not been

established in any animal species, the high WNV antibody prevalence rate among the participants in this study was probably responsible for clearance of the viraemia prior to sampling[36, 37]. Wang *et al*.[38] made similar observation of no WNV RNA in human sera. The only recent study similar to this in southwestern Nigeria is the study by Opaleye *et al*.[39] in which none of the 185 blood donors tested in Osun state had detectable WNV-IgM. In northern Mexico, Rodrıguez *et al*.[33] also reported that out of 588 febrile individuals, 44 encephalitic patients and 800 asymptomatic blood donors, none had detectable WNV-IgM. Since IgM is known to persist in human blood for about 2-3 months to more than a year[40], its non-detection in this study despite the presence of WNV-IgG indicates that exposure of the WNV antibody-positive humans had occurred, at least, more than three months prior to sampling.

The observation that 88.8% of the 188 participants (excluding the horse grooms) analysed were positive for, at least, one of the three pathogens (*Plasmodiun falciparum*, *Salmonella typhi or paratyphi* A, B and C, and WNV antibody) was not unexpected as Nigeria is known to be endemic for malaria and typhoid fevers[15, 21]. Likewise, the observed high WNV seropositivity has established southwestern Nigeria as being endemic for WNV and this is further buttressed by previous observation that WNV was enzootic among horses in the same area[22].

Also, the finding that clinically ill participants were about 7 times (OR: 7.1, p = 0.001) more likely to be WNV seropositive than the apparently healthy ones shows that, although southwestern Nigeria is endemic for malaria and typhoid fever, clinical illness presenting with fever and other relevant signs/symptoms in health care facilities could likely be due to WNV or antigenically related flaviviruses such as yellow fever or dengue that are rarely considered during diagnosis of febrile conditions in the country; most health care centres lack the facilities and trained personnel to investigate arboviral causes of fever. Additionally, the fact that participants had greater odds of being positive for WNV antibodies compared to MP and Widal tests showed that they were mostly exposed to WNV or a related flavivirus. The results presented in Figure 2 further corroborate greater exposure of the participants to WNV as those positive for WNV antibody alone were about six times more than those positive for only MP or typhoid test. These findings underscore the need to consider WNV when patients present with fever/headache/body pains at health care facilities in southwestern Nigeria. Some limitations of this study are that information on the yellow fever vaccination status of the participants was not collected, while the sera were also not tested for antibodies to endemic yellow fever and dengue viruses. These would have, revealed possible cross-reactivity of the tested sera with WNV.

It is noteworthy that of the 40 participants from UCH with fever, 18 (45.0%) were seropositive for only WNV. This sub-population could therefore be described as individuals with undifferentiated febrile illness as previously reported[15, 41]. This observation is buttressed by the fact that significant association (p = 0.001) existed between being clinically ill and WNV antibody positivity in the present study.

In conclusion, this study revealed that humans in southwestern Nigeria had serologic evidence of exposure to WNV, even though none of the tested sera had evidence of viraemia. The high seroprevalence of WNV antibodies obtained is an indication of endemicity of southwestern Nigeria for WNV. Additionally, the observation that many of

the tested humans had no protective/neutralizing antibodies against WNV, even when they were positive for WNV antibodies by cELISA, suggests serologic cross-reactivity with antigenically related flaviviruses or that the WNV antibodies detected were non-neutralizing. The study participants were mostly exposed to WNV than either *Plasmodium spp* or *S. typhi/paratyphi*. Moreover, the high prevalence of antibodies against WNV or related flaviviruses could account for the absence of West Nile neurologic disease among humans in the study area. Finally, WNV infection accounted for about half (45.0%) of the cases of undifferentiated febrile illness among the participants involved in this study while humans of "active" and "non-active" age groups had similar exposure to WNV.

ETHICAL CONSIDERATIONS:

The study design was approved by the Oyo State Research Ethical Review Committee (AD13/479/147) and the University of Ibadan/UCH Ethics Committee (UI/EC/11/0173).

CONFLICT OF INTEREST:

We declare that this research was conducted without any commercial or financial relationships that could imply conflict of interest.

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AVAILABILITY OF DATA UPON REQUEST:

Data obtained from this study would be made available upon request.

AUTHORS' CONTRIBUTIONS:

1. WFS and DOO: Conceived and designed the study, and also analysed and interpreted the data.
2. WFS: Drafted the article; WFS and DOO revised it critically for important intellectual content.
3. WFS and DOO: Approved the final version to be published.
4. WFS and DOO: Agree to be accountable for accuracy and integrity of all aspects of the work.

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