Case Report

Evidence of arbovirus co-infection in suspected febrile malaria and typhoid patients in Nigeria

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Abstract

Introduction: Clinical symptoms of malaria and typhoid infections are virtually indistinguishable from those initially seen in many arbovirus infections. Here we describe arbovirus co-infection detected in 310 sera samples collected from febrile, clinically suspected malaria/typhoid patients in Borno State, Nigeria.

Methodology: Tested initially for *Plasmodium falciparum* by microscopy and for *Salmonella* Typhi by Widal test, samples were subsequently tested for chikungunya (CHIKV), yellow fever (YFV), dengue (DENV) and West Nile viruses (WNV) by plaque reduction neutralization test.

Results: While 92% of patients tested positive for malaria, typhoid, an arbovirus infection, or a combination of one or more of these types of infections, less than 1% of the patients tested positive for malaria alone and only 3.9% tested positive for typhoid alone. Approximately half of the patients tested positive for infection with a single arbovirus (48%) regardless of the presence or absence of malaria or typhoid. Of those who showed 90% to 95% virus neutralization, 67.7% had neutralizing antibodies against DENV, 50% against CHIKV, 25% against WNV and 8.7% against YFV. Eight per cent tested negative against all six pathogens, suggesting that other arboviruses not tested for in this study may also be circulating in Nigeria.

Conclusions: The results suggest that misdiagnosis of arbovirus co-infections as malaria infections, combined with a lack of virus surveillance and underreporting of arbovirus infections, increases the potential for undetected and uncontrolled spread of important vector-borne arboviruses becoming serious underlying public health concerns in Nigeria.

Introduction

In Nigeria, malaria is generally ascribed to all febrile illnesses unless confirmed through laboratory testing. Febrile patients regularly exhibit symptoms thought to be caused by malaria or typhoid but which are also commonly observed with arbovirus infections. It is common practice for patients with fever to visit a health-care facility only if fever persists after self-medication with two or three different anti-malaria treatments.

Arboviruses cause widespread morbidity in Nigeria but there is little documented research on their burden and distribution. The mosquito species responsible for the transmission of dengue (DENV), yellow fever (YFV), chikungunya (CHIKV), and West Nile viruses (WNV) (*Aedes* spp*., Culex* spp*.*) and those responsible for transmitting *Plasmodium falciparum (Anopheles* spp*.*) are well established in Nigeria and prevalent in Borno and surrounding states [1].

The prevalence of these arboviruses in north-eastern Nigeria has not been comprehensively analysed, with many of the previous sero-prevalence surveys conducted in the mid to late 1970s having been set in southern Nigeria [2,3,4,5]. In this study we investigate the presence of neutralization antibodies against DENV, CHIKV, WNV, and YFV using a well-established PRNT assay [6,7] in a cohort of 310 febrile

patients suspected of being positive for malaria or typhoid, and tested for both.

Methodology

Study areas

Between July and December 2008, a total of 310 blood samples were collected from patients exhibiting febrile illness who visited University of Maiduguri Teaching Hospital (UMTH), a tertiary health institution which serves as a referral health centre for six states in northeastern Nigeria (specifically, Borno, Taraba, Adamawa, Yobe, Gombe, and Bauchi), and neighbouring African countries (Chad to the northeast, Niger to the north and Cameroon to the East) (Figure 1). Individual patient hospital records showed that the common clinical manifestations in these cases at the time of sample collection were fever, headache, nausea, vomiting, myalgia and arthralgia, while abdominal discomfort, chest pain, dizziness and diarrhoea were less common. Demographic data

Figure 1. Geographical location of Maiduguri, Borno State, Nigeria

including age and sex of the patients; the date of onset of symptoms were obtained through interview at the time of sample collection. The samples tested here were collected during both the rainy season (July to September) and the cool, dry *Harmattan* season (October to March).

Blood samples

Approval for the study and informed consent was obtained from the Ethical Committee of the UMTH and the patients. The patients tested in this study had no record of yellow fever vaccination and verbally attested to this. Only patients with fever (> 102.2˚F / 39˚C) who were suspected of malaria/typhoid infection were included in the study. Both male and female patients were tested (140 male: 170 female), with patient age ranging from younger than one year to older than 80 years and a mean age of 32 (Table 1). Blood (5 ml) was collected by venipuncture and allowed to clot at room temperature. Serum was carefully collected after centrifugation at 8,000 rpm for 5 minutes and stored at –20˚C until tested. All 310 samples were tested initially for *Plasmodium falciparum* by microscopy and *Salmonella* Typhi by Widal test at the Immunology Laboratory of UMTH. Subsequent plaque reduction neutralization tests (PRNT) for CHIKV, YFV, DENV and WNV were carried out at IRCCS Burlo, Istituto di Igiene e Medicina Preventiva, Virologia, and the Laboratory of Molecular Virology, ICGEB in Trieste, Italy. Confirmatory testing and data analysis were performed at the Health Protection Agency, Porton Down, United Kingdom.

Microscopy

Blood was collected by finger-prick and venipucture. The skin area to be sampled was swabbed with 70% alcohol and allowed to dry before collection. Thick and thin blood films were made on clean slides and labelled accordingly as recommended by the World Health Organization (WHO) [8]. Thin films were fixed with methanol and stained with 3% Giemsa stain of pH 7.0 for 30 minutes. Taking the number of leucocytes per microliter of blood as 6,000, parasite density of blood using the thick film was expressed as follows: parasite count x 6,000, divided by the number of white blood cells (WBCs) counted [8]. Thick films were used to determine the parasite densities while thin films were used to identify the parasite species and infective stages. Stained slides were examined under the light microscope using a x100 objective lens with immersion oil.
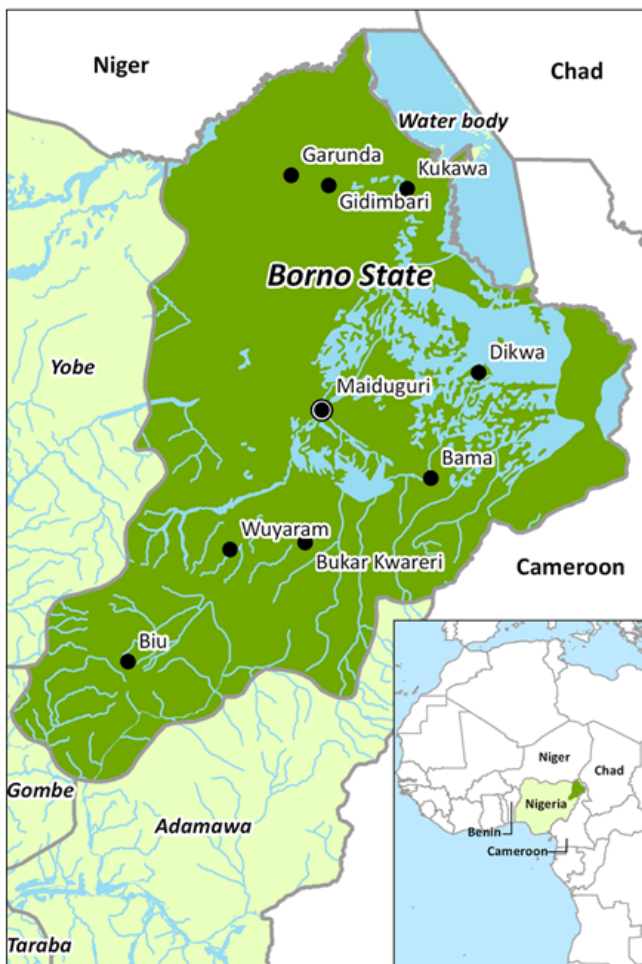
Table 1. Absolute numbers of neutralization antibodies detected to circulating arboviruses in febrile patient sera stratified by age and sex. *Male (M) to female (F) ratio. Infection prevalence expressed as a percentage of sera positive for infection (n = 285). †8.1% (25/310) of sera were negative for all pathogens tested here with a male to female ratio of 11:14.

Age (years)	Patients* (M : F)	DENV 1-4	WNV	YFV	CHIKV
<1	1 (1:0)	1	0	0	1
1-9	8 (5:3)	5	1	0	3
10-19	40 (14:26)	25	9	5	20
20-29	110 (44:66)	72	24	10	52
30-39	77 (36:41)	52	19	4	39
40-49	41 (23:18)	22	10	2	21
50-59	20 (8:12)	11	5	2	0
60-69	10 (7:3)	5	2	2	6
70-79	1 (1:0)	1	0	0	0
≥ 80	2 (1:1)	2	1	0	1
All ages (M : F)	310 (140:170)	193 (93:100)	71 (23:48)	25 (12:13)	143 (63:80)
Prevalence (%) †	**91.90**	**67.71**	**24.91**	**8.77**	**50.17**

Widal agglutination reaction

Serodiagnosis of *Salmonella* Typhi infection was conducted by the Widal test, which detects agglutinating antibodies against the O and H antigens of *Salmonella* Typhi and "H" antigens of *S.* Paratyphi A and *S.* Paratyphi B [9]. The O antigen is the somatic antigen of *Salmonella* Typhi and is shared by *Salmonella* Paratyphi A*, S.* Paratyphi B*,* other *Salmonella* species, and other members of the Enterobacteriaceae family. Antibodies against the O antigen, which are predominantly IgM, rise and disappear early in the illness [10]. The H antigens are flagellar antigens of *Salmonella* Typhi*, S.* Paratyphi A and *S.* Paratyphi B. Antibodies to H antigens are both IgM and IgG, rising late in the illness and persisting longer [9,10]. The protocol was carried out as previously described by Ayse *et al.* [11]. Titers of antibodies against the antigens were deemed significant where single acute phase samples resulted in O > 1 : 160 and H > 1 : 320.

Neutralization tests

Sera were tested for CHIK, YF, DEN 1-4 and WNV viruses by plaque reduction neutralization test (PRNT). PRNT was performed on baby hamster kidney (BHK-21) cells (ATCC, Manassas, VA, USA) in 24-well plates. Briefly, 100 µl of 2-fold serum dilution in serum free Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) was incubated for one hour with 100 µl of the virus working solution (10-20 plaque forming units / 50µl of the final volume of virus-serum mixture). The mixture was incubated 37°C with 5% CO_2 for one hour, then 50µl of the virus-serum mixture was added to the cell suspension in triplicate. The infected plates were incubated at 37°C with 5% CO_2 for one hour. Each well received 1 ml of 3% low viscosity carboxyl methylcellulose (CMC) made up in DMEM with 2% heat-inactivated fetal bovine serum, 1% glutamine, 2 mM and 100 U of penicillin with 100 µg/ ml streptomycin (Sigma, St. Louis, MO, USA). The infected cells were incubated for three days under the same conditions as described above. The plaques were fixed with 10% formaldehyde-phosphate buffered saline (PBS) for 30 minutes and stained with 0.5% crystal violet in 80%/20% PBS solution for 30 minutes. Plates were washed with a copious amount of water, air dried and plaques counted. The PRNT was calculated so that the end-point titres were considered reciprocal of the last solution showing the desired percentage reduction in plaque counts. Sera that showed complete or 90% to 95% neutralization were considered positive ($PRNT_{90}$). The following criteria were required to validate the test: (i) the integrity of the uninfected cell monolayer (negative control) was maintained; (ii) the appropriate plaque counts per well were determined by back-titration of input virus; (iii) there was no increase in plaque counts with negative control serum; (iv) the

PRNT titre of the positive control serum was appropriate; and (v) no serum toxicity was noticed in low serum dilutions. Each test plate contained a negative control comprising the serum of an individual who tested negative for each of the six pathogens described here. The plaque reduction by the patient's antibody to the virus was expressed as the proportion of plaques formed in the serum-virus samples divided by the average number of plaques in the positive controls. The proportion was then expressed as a percentage reduction. Owing to the high degree of cross-reactivity of flaviviruses, the specificity of the test was increased to determine the end point titre of the infecting virus by cross-neutralization of the $PRNT_{90}$ positive samples. Positive controls comprised of serum known to be positive for each of the antigens tested were used and included: CHIKV S27 (AF

369024), WNV NY99 (DQ211652), DENV-1 Hawaii A, DENV-2 New Guinea, DENV-3 TC3, DENV-4 TC25, and YFV French neurotropic virus (U21055), which were kindly donated by Berhnard Notch Research Institute for Tropical Medicine (BNITP, Hamburg, Germany). On receipt, viruses were titrated on BHK cells.

Results

Of the 310 febrile sera samples tested here, 285 (92%) tested positive for malaria, typhoid, an arbovirus infection, or a combination of one or more of these types of infections. Twenty-five (8%) of the 310 patient sera tested here were negative for all six pathogens. As shown in Figure 2, only 2 patients tested positive for malaria alone (0.65%), whereas 47 were co-infected with malaria and at least one

Figure 2. Distribution of co-infection of DENV, CHIKV, WNV, YFV, Malaria (*P. falciparum*) and Typhoid (*S. typhimurium)* in febrile patient sera by sex (n = 310)
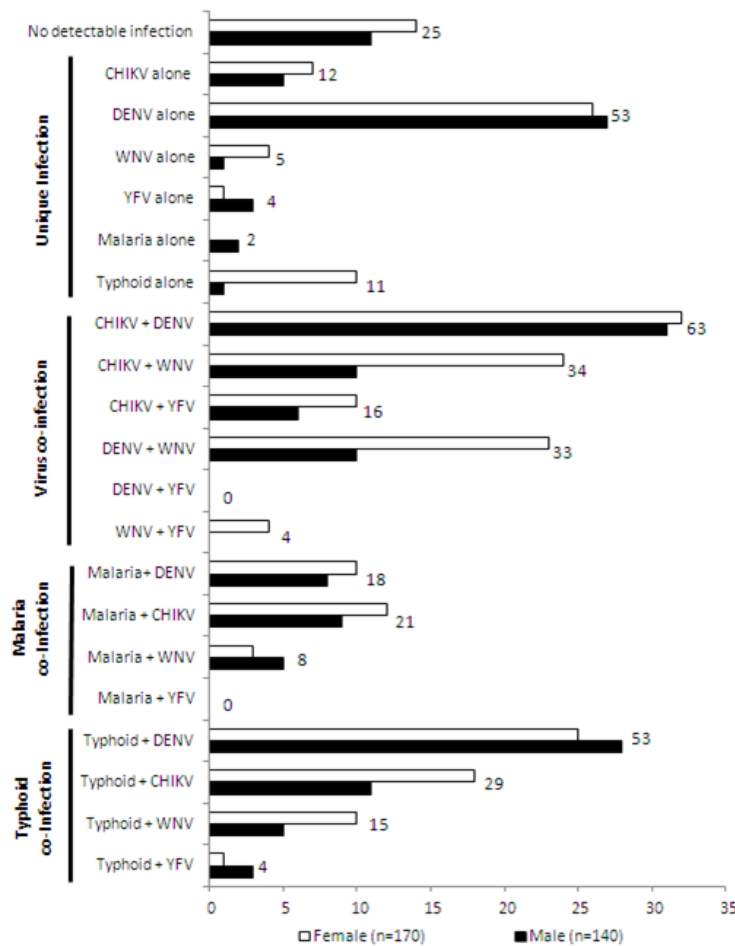
Numbers on right of graph represent total samples testing positive for pathogen and include 25 samples testing negative for all 6 pathogens.

arbovirus (16.5%). Eleven patients tested positive for typhoid alone (3.9%), while 101 tested positive for infection with one or more arboviruses and typhoid (35.4%). No patients tested positive for both malaria and typhoid. Approximately half of the patients tested positive for infection with a single arbovirus (48%) regardless of presence or absence of malaria or typhoid. Of 285 samples who showed ≥ 90-95% virus neutralization, 193 (67.71%) had neutralizing antibodies against DENV, 143 (50.17%) against CHIKV, 71 (24.91%) against WNV, and 25 (8.77%)

against YFV (Table 1). Within each of these groups, subsets had neutralizing antibodies to only one of each of DENV (95/193, 49.2%), CHIKV (25/143, 17.4%), WNV (11/71, 15.4%), and YFV (6/25, 24%) respectively, regardless of malaria/typhoid co-infection status (Table 2).

The prevalence of multi-virus co-infections was widespread, with 219 sera samples (76.8%) testing positive for more than one arbovirus in the absence of malaria or typhoid, compared to 85 (29.8%) in the presence of malaria (n = 14) or typhoid (n = 23). Of

Table 2. Absolute numbers and total prevalence (%) of arbovirus circulation / co-circulation in a cohort of 310 febrile patient sera testing positive for 4 distinct arboviruses, typhoid (T) and malaria (M)

Infection type*	DENV	WNV	YFV	CHIKV
Unique arbovirus	53	5	4	12
Unique arbovirus (+ typhoid)	37	5	2	6
Unique arbovirus (+ malaria)	5	1	0	7
Total unique arbovirus infections (regardless of T/M)†	*95*	*11*	*6*	*25*
Co-infection without T/M (+1 virus)	48	17	14	59
Co-infection without T/M (+2 viruses)	24	27	3	27
Total virus co-infections without T/M	*72*	*44*	*17*	*86*
Co-infection with typhoid (+1 virus)	13	7	2	20
Co-infection with typhoid (+2 viruses)	3	3	0	3
Total >1 virus co-infected with typhoid	*16*	*10*	*2*	*23*
Co-infection with malaria (+1 virus)	9	3	0	10
Co-infection with malaria (+2 viruses)	4	4	0	4
Total >1 virus co-infected with malaria	*13*	*7*	*0*	*14*
Total arbovirus co-infections (regardless of T/M)	*98*	*60*	*19*	*118*
Total numbers of infections (unique + co-infections)	**193**	**71**	**25**	**143**
Total infection prevalence (% unique + co-infections)	**67.71**	**24.91**	**8.77**	**50.17**

*25/310 sera tested negative for all 6 pathogens here and are excluded from calculations here, (n = 285).
†No samples were positive for both typhoid and malaria.
N/A indicates that the total number of infections detected is higher than the number of patient sera samples due to multiple infections of the same samples, this prevalence is therefore be expressed as a percentage.

these, dual infection with CHIKV/DENV was most commonly observed (39/219, 17.8%), with CHIK/YFV and CHIKV/WNV dual infections showing (13/219, 5.9%) and (7/219, 3.2%) prevalence, respectively. DENV/WNV dual infection was observed in 9 (4.1%) of the 219 co-infected sera. WNV/YFV dual infections were only observed in (1/219) 0.45% of all virus co-infections. No samples tested positive for DENV/YFV dual infection.

Of all 310 samples tested, none were positive for both typhoid and malaria, indicating independency of these diagnoses (Fisher's exact test P < 0.001). Assuming that the likelihood for each diagnosis is independent from each other diagnosis, a smaller than expected number of patients with positive diagnoses for typhoid / CHIKV (P = 0.005) and DENV/YFV (P < 0.001), was observed, whereas a higher than expected number had neutralization antibodies for malaria/CHIKV (P = 0.007), WNV/CHIKV (P < 0.001) and YFV/ CHIKV (P = 0.011). All other tested pathogens showed no significant difference in their likelihood of double positive diagnoses (P > 0.1 in all cases). We also investigated the profile of patient age, comparing patients with each diagnosis to the rest of the cohort, as shown in Table 1. A significant difference in the distribution of age in CHIKV positive patients was observed compared to their counterparts (P = 0.004, by Fisher's exact test). No significant overall age difference was observed among others in the total cohort (data not shown, P > 0.1 in all cases). We observed differences between the frequencies of positive diagnoses in males and females for WNV (P = 0.022, by Fisher's exact test) where a higher than expected number of females (48 females and 23 males) compared to males positive for diagnosis was noted; however, this was not the case for any other diagnosis. A different age distribution between males and females (P = 0.022, by Fisher's exact test) was observed in individuals with a positive diagnosis of DENV, where a significantly higher frequency of younger females was observed (Table 1). This trend did not seem to occur with any of the other diagnoses (P > 0.1).

Discussion

Arboviral diseases are among the most important of the emerging infectious diseases. The prominent resurgence and geographic spread of these diseases in locations where susceptible arthropod vectors and hosts provide permissive conditions for arboviruses to cause major epidemics are well established [12,13,14,15].

There are many possible factors involved; these include demographic changes (global population increase and increased urbanization), changes in sociological behaviour (modern transportation), agricultural development (new irrigation techniques and deforestation), possible global climate changes, changes in government public health strategies, and evolutionary changes in the pathogen genomes in response to the factors mentioned above. The need for continued international surveillance and improvement of public health infrastructures to meet existing and future emerging disease threats is therefore vital.

Several of the pathogens that produced neutralizing antibodies in the patient sera in this study have previously been detected in Nigeria [6,7]. However, approximately one in ten of the febrile patients tested negative for all six pathogens, suggesting that other pathogens not tested for in this study but causing febrile illness may also be circulating [2,3,4,5]. These are also unlikely to be attributable to lack of sensitivity of the malaria microscopy assay, as it remains the most reliable and sensitive technique for malaria diagnosis in an endemic country such as Nigeria. However, it could be speculated that other arboviruses (O'nyong nyong, Bwamba, Tataguine, Thogoto, Wesselsbron, CCHFV, Zika, Lassa virus and Tick-borne encephalitis viruses such as Dugbe) that are capable of causing febrile illness but were not tested for as part of this study, may be circulating in the environment, as proposed by earlier investigations [16,17,18,19]. All of the patients involved in this study attested to not having received the YF vaccine, suggesting that only a low level of YFV circulation exists in the areas investigated. Additionally, no historic YFV outbreak data were available in the study area, even though the arthropod species (*Aedes* spp.) that transmit the virus are prevalent [1]. Due to its geographical location, unique topography and diverse fauna, Borno state has a considerable range of mosquito vectors circulating throughout the year. The samples tested here were taken from patients who reside in Borno state, five neighbouring states (Yobe, Taraba, Adamawa, Bauchi and Gombe) and three neighbouring countries (Chad, Niger, and Cameroon; Figure 1). The hospital serves as a referral centre to these states, and residents of the neighbouring countries can take advantage of cross-border socioeconomic activities for their health care, highlighting possible cross-border transmission.

The samples tested in this study were collected between July and December, which falls during both the rainy season (July to September) and *Harmattan*

(cool, dry) season (October to January) in north-eastern Nigeria. Highest antibody levels to DENV and WNV were reported during the rainy season with peaked activities in July [6]. The seasonal distribution of other pathogens such as CHIKV, YFV, *P. faliciparum* and *S.* Typhi was not previously reported in this region of Nigeria, so YFV and CHIKV could be assumed to share similar seasonal variation as DENV, as all three viruses are transmitted by *Aedes* mosquitoes.

In this study, patients infected with DENV or CHIKV were the highest in numbers, with neutralization antibodies against either virus or against both (co-infection). A disproportionate number of patients had positive diagnoses for WNV/CHIKV and YFV/CHIKV infections involving two or more pathogens. Given that cross-reactivity between *alphavirus* (CHIKV) and *flaviviruses* (WNV, YFV, DENV) of the established plaque reduction neutralization tests used here was ruled out, we suggest that individuals infected with one of these viruses may have been infected by mosquitoes carrying more than one virus, or were bitten by more than one infected mosquito [20.21]. Since *Aedes aegypti* and *Aedes albopictus* are the principal vectors of DENV, YFV, and CHIKV, co-infection of these viruses is consistent with several other reports from La Reunion Island, Gabon, India and Thailand, where many different arboviruses are endemic [22,23,24].

These co-infections may provide an opportunity for genetic exchange between virus strains, allowing the viruses to mutate, potentially resulting in change in clinical severity or vector specificity, as was seen with chikungunya virus in the 2007 Reunion epidemic [25]. Genetic mutations among these organisms and a lack of arbovirus diagnostic test capabilities have important medical and veterinary implications.

Due to the nature of the route of infection (*i.e.*, mosquito), those at greatest risk of infection are those at highest exposure to circulating mosquitoes. In this study the febrile patients ranged from a six-month-old boy to an 82-year-old woman (both infected with DENV). *A. aegypti* is a day biting mosquito and commonly found inside homes, where young children and older women spend greater amounts of time than other age groups, potentially placing them at greater risk of infection than others [26].

The limitation of this study lies in the fact that patients without symptoms were not tested. Since many arbovirus infections are asymptomatic, high levels of antibodies could potentially be detected in apparently healthy populations. Although the

neutralization test antibodies from acute phase sera may not necessarily denote recent infections, it does reveal the endemicity and ongoing transmission of these viruses, and how they co-exist within the same host. We have discussed single and dual arbovirus infections, both in the presence and absence of malaria and typhoid to present a focussed and clear data set. However, several of the samples tested in this study tested positive for three arboviruses in addition to either malaria or typhoid. Another study that includes a representative healthy cohort is underway to further assess the prevalence of these multiple virus infections among healthy populations within the same study area.

Conclusion

Four important arboviruses causing disease in humans are co-circulating in malaria and typhoid endemic areas in Nigeria. A significant proportion of suspected malaria/typhoid patients tested negative for both but positive for one or more arbovirus infections. Misdiagnosis of arbovirus co-infections as malaria infections and under-reporting of arbovirus infections through lack of effective arbovirus surveillance programmes may be a cause for serious underlying public health concerns in the study area, in particular through the potential of undetected and uncontrolled spread of severe vector-borne human diseases, such as chikungunya or dengue hemorrhagic fevers.

Through the implementation of improved arbovirus surveillance programmes and diagnostic assays, quality of care for the patients can be improved, and the costs and risks associated with current drugs becoming ineffective against ubiquitous pathogens in Nigeria can be reduced.

Borno state has a considerable range of mosquito vectors circulating throughout the year. Since WNV is transmitted by *Culex* mosquitoes whereas DENV, CHIKV, YFV, and WNV are transmitted by *Aedes* mosquitoes, the seroprevalence pattern according to the vector prevalence in the area should be a focus of future research.

In further studies, sera will be tested for additional viral pathogens, circulating viruses will be isolated and characterised, and novel assays implemented. Additionally, a systematic vector prevalence study on the distribution of *Aedes* and *Culex* mosquito species in Borno state would be valuable to determine which of these assays are most suitable to use in local populations. Strains of viruses currently circulating in Borno state have been isolated from human sera and will be sequenced and fully characterized for the development of up-to-date, low cost, accurate

diagnostic assays so that local health-care facilities can perform differential diagnosis of febrile cases for arboviruses.

Acknowledgements

References

1. Okogun GR, Nwoke B, Okere A, Anosike J, Esekhegbe A (2003) Epidemiological implications of preferences of breeding sites of mosquito species in Midwestern Nigeria. Ann Agric Environ Med 10: 217-222.
2. Fagbami A (1977) Epidemiological investigations on arbovirus infections at Igbo-Ora, Nigeria. Trop Geogr Med 29: 187-191.
3. Moore DL, Causey OR, Carey DE, Reddy S, Cooke AR, Akinkugbe FM, David-West TS, Kemp GE (1975) Arthropod-borne viral infections of man in Nigeria, 1964-1970. Ann Trop Med Parasitol 69: 49-64.
4. Tomori O, Monath TP, O'Connor EH, Lee VH, Cropp CB (1981) Arbovirus infections among laboratory personnel in Ibadan, Nigeria. Am J Trop Med Hyg 30: 855-861.
5. Olaleye OD, Omilabu SA, Faseru O, Fagbami AH (1988) 1987 yellow fever epidemics in Oyo State, Nigeria: a survey for yellow fever virus haemagglutination inhibiting antibody in residents of two communities before and after the epidemics. Virologie 39: 261-266.
6. Baba MM, Saron MF, Diop O, Mathiot C, and Adeniji JA (2006) West Nile virus in mosquitoes and febrile patients in a semi-arid zone in Nigeria. The journal of American Science 2: 28-34.
7. Baba MM, Saron MF, Vorndam AV, Adeniji JA, Diop O, Olaleye D (2009) Dengue Virus Infections in Patients Suspected of Malaria/Typhoid in Nigeria. Journal of American Science 5: 129-134.
8. Agomo PU, Okonkwo CA, Asianya OO, Okoh HI, Nebe OJ (2001) Comparative evaluation of Immuno-chromatographic test (ICT) and Parasight-F for the rapid diagnosis of falciparum malaria in Nigeria. Afr J Clin Exp Microbiol 2: 45.
9. Olopoenia LA and King AL (2000) Widal agglutination test - 100 years later: still plagued by controversy. Postgrad Med J 76: 80-84.
10. Rodrigues C (2003) The Widal test - more than 100 years old: abused but still used. J Assoc Physicians India 51: 7-8.
11. Ayse W, Onder E, Banu B (2002) Widal test in diagnosis of typhoid fever in Turkey. Clin Vaccine Immunol 4: 938-941.
12. Powers AM, Logue CH (2007) Changing patterns of Chikungunya virus: re-emergence of a zoonotic arbovirus. Gen Virol 88: 2363-2377.
13. Gubler DJ (2001) Human arbovirus infections worldwide. Ann N Y Acad Sci 951: 13-24.
14. Mackenzie JS, Gubler DJ, Petersen LR (2004) Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med 10 Suppl 12: 98-109.
15. Ozer N (2006) West Nile virus and its vectors. Mikrobiyol Bul 40: 121.
16. Guyer B (1972) Serological survey for arboviruses in Igbo-Ora, western Nigeria. Ann Trop Med Parasitol 66: 243-250.
17. Causey OR, Kemp GE, Madbouly MH, Lee VH (1969) Arbovirus surveillance in Nigeria, 1964-1967. Bull Soc Pathol Exot Filiales 62: 249-253.
18. Fagbami A (1978) Human arthropod-borne virus infections in Nigeria. Serological and virological investigations and Shaki, Oyo State. J Hyg Epidemiol Microbiol Immunol 22: 184-189.
19. Boorman JP, Draper CC (1968) Isolations of arboviruses in the Lagos area of Nigeria, and a survey of antibodies to them in man and animals. Trans R Soc Trop Med Hyg 62: 269-277.
20. Adesina OA and Odelola HA (1991) Ecological distribution of Chikungunya haemagglutination inhibition antibodies in human and domestic animals in Nigeria. Trop Geogr Med 43: 271-275.
21. Olaleye OD, Omilabu SA, Ilomechina EN, Fagbami AH (1990) A survey for haemagglutination-inhibiting antibody to West Nile virus in human and animal sera in Nigeria. Comp Immunol Microbiol Infect Dis 13: 35-39.
22. Vazeille M, Mousson L, Martin W, Failloux AB (2010) Orally co-infected *Aedes albopictus* from La Reunion Island, Indian Ocean, can seliver both sengue and chikungunya infectious viral particles in their saliva. PLoS Neglected Tropical Disease 6: 706.
23. Dieudoné Nkoghe EML, Ollomo B, Nze-Nkogue C, Becquart P, Grard G, Pourrut X, Charrel R, Moureau G, Ndjoyi-Mbiguino A, and De Lamballerie X (2009) Concurrent Chikungunya and Dengue Virus Infections during Simultaneous Outbreaks, Gabon, 2007. Emerg Infect Dis 15: 591-593.
24. Chahar HS, Bharaj P, Dar L, Guleria R, Kabra SK, S. (2009) Co-infections with Chikungunya Virus and Dengue Virus in Delhi. India Emerg Infect Dis 15: 1077-1080.
25. Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S (2007) A single mutation in chikungunya virus affects vector specificity and epidemic potential. PLoS Pathog 3: 201.
26. Garcia-Rejon J, Loroño-Pino MA, Farfan-Ale JA, Flores-Flores L, Del Pilar Rosado-Paredes E, Rivero-Cardenas N, Najera-Vazquez R, Gomez-Carro S, Lira-Zumbardo V, Gonzalez-Martinez P, Lozano-Fuentes S, Elizondo-Quiroga D, Beaty BJ, Eisen L (2008) Dengue virus-infected Aedes aegypti in the home environment. Am J Trop Med Hyg 79: 940-950.
27. Baba MM, Logue CH, Oderinde B, Abdulmaleek H, Williams J, Marcello A, D'Agaro P and Hewson R (2012) Arbovirus co-infections in suspected febrile malaria and typhoid patients: A worrisome situation in Nigeria (2012). J Antivir Antiretrovir Suppl 1: 1948-5964.

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