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SILENT CIRCULATION OF ARBOVIRUSES IN CAMEROON
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# SILENT CIRCULATION OF ARBOVIRUSES IN CAMEROON

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## ABSTRACT

***Objectives:*** **To investigate the silent circulation and transmission of arthropod-borne viruses (arboviruses) in the Fako Division of Cameroon.**
***Design:*** **This survey was conducted based on clinical observations and laboratory diagnosis; field collections of mosquitoes.**
***Setting:*** **This study was conducted in the Fako Division of South West Cameroon.**
***Subjects*****: One hundred and two sera were obtained from febrile patients (with negative laboratory findings for malaria and typhoid fever) at clinics in the Fako Division, and diurnal anthropophilic mosquitoes (4,764) collected.**
***Interventions:*** **Virus isolation was attempted from these, and sera were screened for antibodies against 18 African arboviruses by haemagglutination inhibition (HI) and complement fixation (CF) tests.**
***Results*****: No virus was isolated. Fifty three of 79 (67.1%) sera reacted with one or more viral antigens. Twenty nine sera (36.7%) reacted with members of the genus *Alphavirus*, with Chikungunya (CHIKV) and O'nyong-nyong (ONNV) viruses as the most frequent (34.2%). Forty six sera (58.2%) reacted with members of the genus *Flavivirus*: 24 (30.4%) were cross-reactive, but 11.4% reacted monotypically with Zika, 5.1% with yellow fever virus (YFV), 5.1% with dengue virus-2 (DENV-2), 2.5% with DENV-1 and 1.3% with Wesselsbron virus, respectively. The plaque reduction neutralisation test used to specify the agent that elicited the response could not resolve 33.3% of the cross reactions between CHIKV and ONNV. Neutralising antibody titres against ONNV and CHIKV were very high indicating probable re-infection.**
***Conclusion:*** **Our results indicate previously undetected circulation of arboviruses in Cameroon, and suggest that they are important, overlooked public health problems.**

## INTRODUCTION

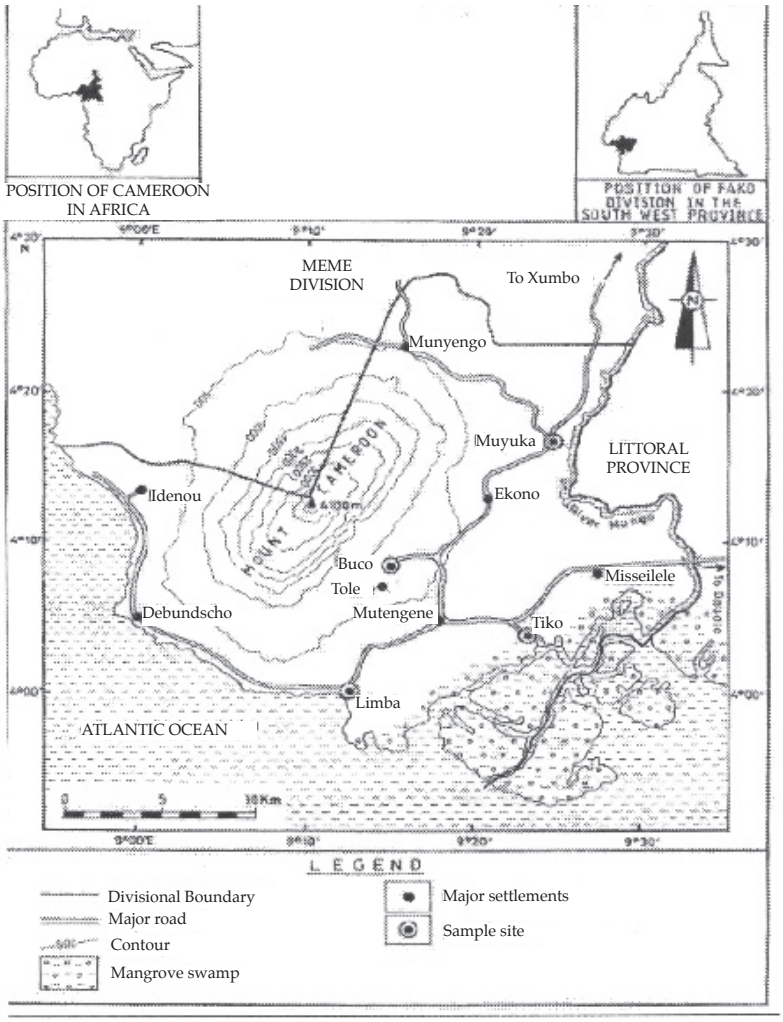
Arthropod-borne viruses (arboviruses) are responsible for numerous human infections worldwide, and cause both epidemics of serious disease as well as endemic disease in some areas. Despite documented evidence for their presence in virtually all African countries including Cameroon (1), they are almost never part of routine laboratory diagnoses and very often go unnoticed, probably accounting for morbidity and frequent debilitation. Arboviral infections indiscriminately manifest in humans with symptoms similar to those of malaria and typhoid fever, the most common causes of febrile illness in sub-Saharan Africa. The most common arboviral diseases of humans include dengue fever (DEN), yellow fever (YF) and chikungunya (CHIK). Dengue is characterised by fever, severe myalgia and arthralgia, and rash. Except for the painful symptoms, which led to the name "breakbrone fever," classic DEN is a relatively mild disease and is usually not fatal (2). A more serious form of DEN, dengue haemorrhagic fever (DHF), is characterised by bleeding from the skin, gums, and gastrointestinal tract and sometimes by circulatory

failure and shock (3,4). DEN is the fastest growing disease of the world with more than 2 billion people at risk, and the World Health Organization (WHO) estimates that "there may be 50 million cases of dengue infection worldwide every year" (4). Chikungunya is a dengue-like disease that can occur as a massive epidemic, as was the case in early 2006 on the French island of La Réunion (5). Yellow fever is caused by the yellow fever virus (YFV) and begins with fever, chills, headache, and backache, followed by nausea and vomiting, followed by a yellowing of the skin termed jaundice that gives the disease its name. In severe cases, the virus produces lesions in the infected organs, and haemorrhage occurs.

These three arboviruses and many others are transmitted by diurnally active mosquitoes, mainly from the genus *Aedes*, even though some other groups may be important vectors. Numerous mosquitoes have been suspected and some incriminated as vectors of various arboviruses in Cameroon during the colonial and early postcolonial eras by surveys conducted by scientists from the Centre Pasteur and Office de la Recherche Scientifique et Technique Outre-Mer (ORSTOM). Unfortunately, after the last (1976) publication of these surveys, there was a 25-year gap before Fontenille and Toto reported the introduction of the *Aedes albopictus*, a potential new vector of DF and YF into southern Cameroon (6-8). Later, Ndip *et al.* (9) Kuniholm *et al.* (10) and Demanou *et al.* (11), reported prevalence of antibodies (Abs) to some arboviruses in the serum of febrile patients in Cameroon, and Peyrefitte et *al.*, isolated the CHIKV from a patient. In this survey, we sought to further investigate the latent circulation of arboviruses, characterise the agents and assess the risk of infection in Cameroon, with reference to the Fako Division (Figure 1).

**Figure 1**

*Map of the Fako division showing the locations of collection*



## MATERIALS AND METHODS

*Study population:* One hundred and two sera were anonymously collected from consenting febrile patients (59 males and 43 females) consulting at the Provincial Hospital Annex and Mount Mary Health Centre in Buea, and the Cameroon Development Corporation Central Clinic, Tiko. These are the major settlements in the Fako division and comprise almost 80% of the population. The hospitals in Buea and Tiko receive a very large number of patients from the region, with patients admitted from throughout the division. Laboratory diagnoses had excluded malaria and typhoid fever, the most common causes of fevers in Cameroon. Blood was collected in clean collection tubes, the serum separated and stored at -80°C.

Diurnally active anthropophilic mosquitoes (4,764) were obtained from human landing catches in Buea, Limbe and Tiko, by experienced collectors who were vaccinated against YF, received malaria medication, and had taken ivermectin to prevent onchocerciasis/river blindness. All collections (serum and mosquitoes) strictly followed our protocol approved by the Ministry of Public Health and the administrations of the participating hospitals/clinics. Mosquitoes were equally stored at -80°C. All samples were transported on dry ice to the University of Texas Medical Branch (UTMB), Galveston, Texas, USA.

*Virus isolation attempts:* Although there was a conspicuous breach in the chain of cold during the transportation of the samples to the USA, virus isolation was nevertheless attempted (3). Mosquitoes were sorted on a chill table and pooled by species and by sex, homogenised in cell culture medium, centrifuged, filtered and 300 µL of each pool inoculated in a monolayer of mosquito (C6/36) and African green monkey kidney (Vero) cells. Human sera were inoculated directly into monolayers of Vero cells (300 µL) or diluted (1:10) and inoculated into C6/36 cells. Cell cultures were incubated at appropriate temperature for 7 and 14 days respectively for $C^{6/36}$ and Vero, and monitored daily for cytopathic effect (CPE). At the end of the incubation period, cells were harvested and screened by indirect immunofluorescence assay using mouse Abs against three members of the genus *Flavivirus* (YFV, DENV and ZIKAV). All virus isolation attempts were negative, possibly because of the rupture in the chain of cold, and are not further discussed in this paper. However, these failures and increasing difficulties in international transportation of diagnostic material stressed the need for the transfer of equipment (for appropriate containment) and diagnostic reagents to Cameroon. This is being properly addressed, and the development of high level biosafety containment facilities is underway.

*Preparation of antigens:* Arbovirus antigens (Ags) were obtained from the World Reference Collection for Emerging Viruses and Arboviruses (WRCEVA) at UTMB (Table 1). Prior to use in haemagglutination inhibition test (HI), Ags were titrated to determine optimal concentration (4 U used in test) and pH to use.

*Haemagglutination-inhibition tests:* The sera were screened for the presence of Abs to African arboviruses. They were treated with acetone to remove lipids and non-specific inhibitors, and absorbed with goose cells to remove natural agglutinins. Titres were recorded as the highest dilution causing total or almost total inhibition of agglutination.

*Complement fixation:* Complement fixation test (CF) was performed using 2 units of complement and some of the Ags previously prepared. The sera were incubated at 60°C for 20 minutes to inactivate naturally occurring human complement before testing (13). Titres were recorded as the highest dilution giving 3+ or 4+ fixation of complement on a scale of 0 to 4+.

*Plaque reduction neutralisation tests*: Because some sera cross-reacted with multiple Ags (especially CHIK and ONNV), they were tested by the plaque reduction neutralisation test (PRNT) to discriminate between these infections. The Ross strain of the CHIKV and the MP730 strain of the ONNV obtained from the WRCEVA were amplified and titrated. Each virus stock was diluted to a titre of 800 PFU/ml (plaque forming units per ml) and used in all assays. Serial 2-fold dilutions of the 27 sera cross-reactive with CHIKV and ONNV were prepared, mixed with equal volumes of the virus stock, incubated at 4°C overnight and inoculated into monolayers of Vero cells. The cultures were then incubated until plaques formed. The titre of the neutralising Abs was considered as the highest dilution that would neutralise 80% or more of the virus in the stock used. Cross-reaction was resolved if there was a 4-fold or greater difference in the titre of the Abs to one of the viruses over the other.

## RESULTS

*Haemagglutination-inhibition:* A total of 79 human sera was tested by HI for antibodies against 18 arthropod-borne viruses. The results (Table 1) indicate that 53 (67.1 %) sera reacted with one or more antigens. No evidence was found of immunity to viruses of the *Orthobunyavirus*, *Phlebovirus* or *Thogotovirus* genera. Twenty nine sera (36.7%) reacted with *Alphavirus* antigens, with CHIKV and ONNV as the most frequent (34.2%). Forty six sera (58.2%) reacted with *Flavivirus* antigens. Of this total, 24 (30.4%) were cross-reactions, but four (5.1%) reacted monotypically with YF, nine (11.4%) with ZIKA, one (1.3%) with Wesselsbron (WSL) and two (2.5%) with a titre against YF and ZIKA, respectively, greater than with other *Flavivirus* antigens. In addition, four (5.1%) sera reacted with DEN-2 and two (2.5%) with DEN-1.

*Complement fixation:* Eighteen sera were studied using CF test and the presence of antibodies was confirmed in six sera reacting with CHIK and ONN antigens, six sera reacting with ZIKA among 10 HI positive, and one with SF (Table 2). These results indicate that most responses to ONNV were recent responses (titre ≥1:32).

**Table 1**
*List of antigens used and prevalence of haemagglutination inhibition antibodies in sera from Cameroonian patients*

| Genus | Antigen | Number positive (%) | Geometric mean titre |
|---|---|---|---|
| *Alphavirus* | *Chikungunya* (CHIKV) | 27 (34.2) | 173 |
| | *Middleburg* (MIDV) | 21 (26.6) | 35 |
| | *O'nyong nyong* (ONNV) | 27 (34.2) | 179 |
| | *Semliki Forest* (SFV) | 21 (26.6) | 41 |
| | *Sindbis* (SINDV) | 11 (13.9) | 40 |
| *Flavivirus* | *Dengue-1* (DENV-1) | 30 (38.0) | 76 |
| | *Dengue-2* (DENV-2) | 31 (39.2) | 100 |
| | *Dengue-3* (DENV-3) | 28 (35.4) | 49 |
| | *Dengue-4* (DENV-4) | 28 (35.4) | 66 |
| | *Spondweni* (SPOV) | 19 (24.1) | 58 |
| | *Wesselbron* (WSLV) | 33 (41.8) | 74 |
| | *West Nile* (WNV) | 27 (34.17) | 49 |
| | *Yellow fever* (YFV) | 34 (43.03) | 65 |
| | *Zika* (ZIKAV) | 30 (37.97) | 101 |
| *Orthobunyavirus* | *Ingwavuma* (INGV) | 0 | 0 |
| | *Tahyna* (TAHV) | 0 | 0 |
| *Phlebovirus* | *Rift Valley* (RVFV) | 0 | 0 |
| *Thogotovirus* | *Thogotovirus* (THOV) | 0 | 0 |

**Table 2**
*Complement fixation test results*

| Sample ID | *Alphavirus* | | | | *Flavivirus* | | |
|---|---|---|---|---|---|---|---|
| | ONN | CHIK | SF | MID | YF | WSL | ZIKA |
| 2 | | | | | 16/≥32 | 16/≥32 | |
| 18 | | | | | | | 8/8(1:80) |
| 22 | | | | | | | 0 (1:40) |
| 46 | | | | | | | 0 (1:80) |
| 47 | | | | | | | 16/8 (1:160) |
| 50 | | | | | | | 32/8 (1:320) |
| 51 | | | | | | | 32/8 (1:320) |
| 59 | | | | | | | 0 (1:40) |
| 65 | | | | | | | 0 (1:80) |
| 77 | | | | | | | 16/8 (1:80) |
| 78 | | | | | | | 16/8 (1:160) |
| 5 | 32/≥32 | 32/8 | | | | | |
| 15 | 32/≥32 | 0 | | | | | |
| 37 | 64/≥32 | 8/8 | | | | | |
| 45 | 32/≥32 | 0 | | | | | |
| 52 | | | 16/≥32 | 0 | | | |
| 58 | ≥256/≥32 | 0 | | | | | |
| 69 | 64/≥32 | 0 | | | | | |

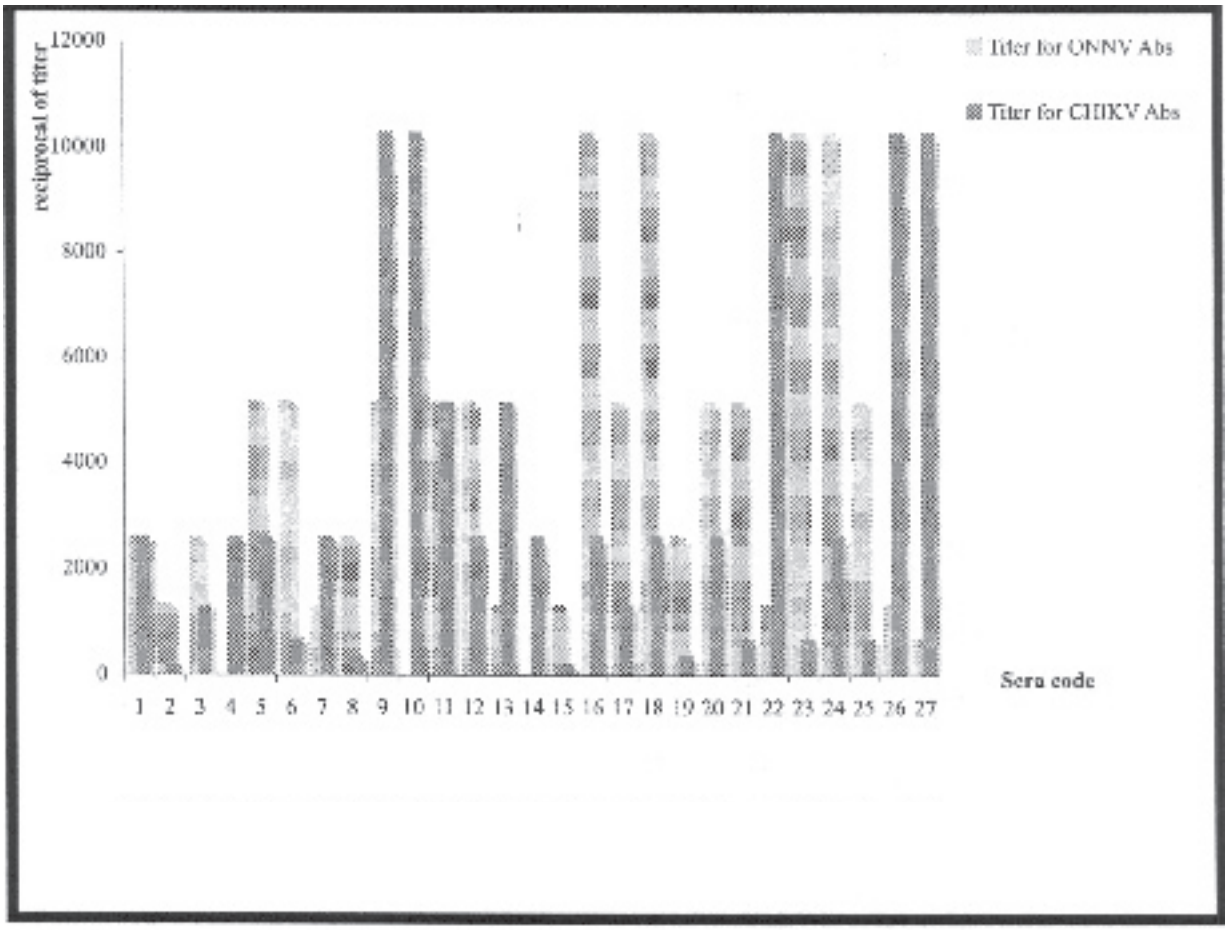
Numerator=titre of Ag used; denominator=titre of Ab used. Ab titre of 1:32 or more represents primary response; brackets indicate HI titre.

*Plaque reduction neutralisation test.* The PRNT used to specify the agent that elicited the response could not resolve 33.3% of the cross reactions between CHIKV and ONNV (antibody titres were within 2-fold), probably because both agents circulate in the study area. However, they clearly indicated that neutralising Ab titres against ONNV and CHIKV were generally very high, with 70% above 1:2560, indicating probable recent infections. In 48.1% of cases, neutralising Abs to ONNV had a titre at least 4-fold higher than those for CHIKV, indicating that the response was mounted to an infection with the ONNV, while in 14.8% of sera, Abs to CHIKV were at least 4-fold higher (Figure 2).

## DISCUSSION

This research adds to the recent resumption of activities to monitor arbovirus circulation in Cameroon. The study of arboviruses in Cameroon was undertaken during the colonial era in the British part of the country then attached to the territory of Nigeria, and more extensively in the early years of independence of the country, mainly focusing around Yaoundé, the capital city. MacNamara reported the isolation the *Kumba virus*, which was ultimately recognised as a strain of the SFV (15), from *Eretmapodites grahami* mosquitoes. Joint teams from the Centre Pasteur du Cameroun and the ORSTOM continuously investigated the epidemiology, ecology and dynamics of arboviral transmission from 1963 to the mid-1970s, including identification of vectors and potential reservoirs (16-19). Their investigations yielded among other major achievements the

**Figure 2**
*Comparative chart of neutralising antibodies for CHIKV and ONNV*



discovery of three new viruses, *Nkolbisson virus* of the *Rhabdoviridae, Okola virus* of the family *Bunyaviridae,* and *Yaoundé virus* of the genus *Flavivirus*, as well as indicated that at least 23 arboviruses circulated in the forests of Cameroon (20). However, they found no evidence for the presence of any of the major human arboviruses known from other parts of Africa (16,19-24).

Our findings indicate that several arboviral infections of humans are probably endemic in Cameroon and that two or more arboviruses probably infect many people. This is especially true for infections with ZIKAV, ONNV and CHIKV, for which there is a relatively high prevalence of Abs and very high neutralisation titers (Figure 2). Although these agents have never been, or are rarely isolated in Cameroon, the prevalence of the ONNV antibodies appears to be higher than that of the CHIK as had been observed in natural reservoirs (17). This may be accounted for by the fact that ONNV is transmitted by the malaria vectors (*Anopheles* spp.), which are certainly the most abundant African haematophagous insects. Posey and his collaborators (25) recently recorded an outbreak of ONNV in a refugee camp in Cote d'Ivoire and recommended laboratory diagnosis for arboviruses when massive outbreak of febrile rash illness occurs. CHIKV and ONNV are two closely related alphaviruses that are difficult to distinguish clinically or using serology, and for many years ONNV was considered by some investigators as a variant of CHIKV. Chikungunya, although not usually fatal, is a very painful and seriously debilitating disease. It recently caused an important epidemic on islands in the Indian Ocean (La Reunion, Comoros and Madagascar) with more than 300,000 cases reported, a major epidemic in India with up to 6.5 million cases, and also caused a small epidemic in Italy. Reports of fatalities and transmission from mother to child accompanied these epidemics (4). ZIKAV, which had not been previously reported in Cameroon, appears as the most important *Flavivirus* of humans in the Fako Division accounting for more than 11% of the fevers of unknown origin. It was found infecting patients in neighbouring Nigeria and causing an epidemic with symptoms including jaundice (26). Three of the patients having Abs to ZIKA in our study displayed jaundice, which was also seen in two patients with DENV-2 abs and one patient with YFV and WSLV. Yellow fever had not been reported from Cameroon since the last epidemics that affected the Adamawa highlands of the country and was poorly documented. The abs to YFV found in our study surely resulted from exposure to infectious mosquito bites because none of the positive patients had been vaccinated for YF. The data gathered in this survey enabled us determine that there was a high risk for an outbreak of YF in Fako, which was confirmed last September 2009 with two confirmed cases of infection. There is now a national vaccination scheme for YF in the country and data suggest that between 72-87% of the population is now covered (27). The serotypes of DENV (-1 and -2) known to occur in Africa are also present in Cameroon. DENV-2 is on of the fastest spreading infectious agents in the world. Its importance in Cameroon is probably overlooked just like that of the other arboviruses because the area is endemic for malaria and typhoid fever for which diagnosis is readily available. Our findings generally corroborate those of recent studies in Cameroon (9,10). However, we have surprisingly not diagnosed any evidence of exposure to TAHV, which is relatively high in the study of Kuniholm and collaborators (10). This suggests more in-depth investigation to map the relative distribution of the various Arboviruses that circulate in the area.

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