O’nyong-nyong (ONN) is an alphavirus in the family Togaviridae in the Semliki Forest antigenic complex. Fever caused by this virus first appeared in the Acholi people of northwestern Uganda in February 1959. Locally, the disease was called “o’nyong-nyong”, meaning very painful weakening of joints. Workers at the East African Virus Research Institute in Entebbe recovered the virus from the blood of infected patients and from the mosquitoes Anopheles (Cellia) funestus Giles was presumed to be the principal vector because it was the most abundant mosquito species from which a strain of ONN virus was isolated. This virus was isolated for the first time from a culicine species, Mansonia (Mansonioideis) uniformis Theobald. Bwamba virus and Nyando virus were also isolated from An. funestus.

O’nyong-nyong fever (ONN) virus. This documented the first cases of ONN fever in Uganda since the end of the last outbreak in 1962. Thus, epidemic ONN fever had re-emerged after an absence of 35 years. In December 1996, authorities of the Ministry of Health invited the Centers for Disease Control and Prevention (CDC) to join the investigations of the epidemic in southcentral Uganda. By this time, the epidemic had spread into the districts of Ssembabule, Mbarara, and Masaka. With the onset of the main dry season, the epidemic started to decrease, although virus transmission as shown by human cases continued throughout the study reported here (Sanders EJ and others, unpublished data). The entomologic studies reported here represent the work conducted between January 27 and February 2, 1997. The objectives of these studies were 1) to confirm the role of anophelines in ONN fever epidemics, and 2) to examine other mosquito species as epidemic vectors of ONN virus.

MATERIALS AND METHODS

Study area. All investigations were carried out at Bbaale village (31°17’E, 0°40’S), Kooki County, in the Rakai District of southcentral Uganda. Bbaale is one of the many villages on the banks of Lake Kijanebalola, a small lake of about 45 km², with many swampy lagoons spread out in the valleys. The country consists of low lying, gently rolling hills, with an altitude between 1,200 and 1,500 meters above mean sea level. The hills are covered with slightly wooded grassland. The valleys have scattered thick woodlands, but generally no forests. The valleys are predominantly cultivated with banana and coffee plantations. Other crops included cassava, sweet potatoes, potatoes, sorghum, groundnuts, beans, and maize. Many areas near the banks of the lake are also cultivated. Livestock include goats and sheep.

Homesteads, comprising 2 or 3 buildings per compound, are scattered along a village road winding through banana plantations. Each homestead consisted of a single family. Most houses in the village are made of mud and reeds with grass-thatched roofs or iron-sheet roofing. The entomologic collections were made in and around randomly selected homesteads along the village road. Collections were made in the main sleeping house of selected homesteads.
Mosquito collection and handling. Two methods of mosquito collection were used. The first was knockdown pyrethrum spray catches using hand-held spray pumps or aerosol spray cans. Pyrethrum was diluted 0.2% (2 mL/L) in kerosene. This was used as the spray mixture. Houses were sprayed during the daytime. Occupants of the houses to be sprayed were alerted the evening before and requested to keep their houses closed until the spray team arrived. The second method was CDC light traps with incandescent light that were placed inside and/or outside of randomly selected houses overnight. The trapped mosquitoes were aspirated into collection tubes and were immobilized with pyrethrum spray. The collection from each house was separated into anophelines and culicines, counted, and placed separately in labeled vials. The vials were placed in a cool box with ice packs (4°C) for later transfer into liquid nitrogen at the field station (usually 5–7 hr later).

Virus isolation. Mosquitoes from individual collections at each homestead were identified to species by using the appropriate keys.18–20 Species were then grouped into pools of 50 or less mosquitoes. The pooled mosquitoes were placed into chilled mortars and triturated in 1.6 ml of BA-1 diluent (1× Medium 199 with Hanks’ salts, 0.05 M Tris, pH 7.6, 1% bovine albumin, 0.35 g/L of sodium bicarbonate, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 1 μl/ml of fungizone). Blood-engorged mosquitoes were decapsulated, and the heads placed into chilled mortars and triturated in 1.6 ml of BA-1 diluent. The mosquito suspensions were clarified by centrifugation.

One hundred microliters of each of the suspensions were inoculated on Vero cells in 6-well plastic plates. The plates were incubated for 1 hr at 37°C to allow attachment of the virus to cells; the inoculated cell cultures were then overlaid with a nutrient medium containing 1% agarose (SeaKem LE agarose: FMC BioProducts, Rockland, ME). After 5 days of incubation at 37°C in a 5% carbon dioxide incubator, a second overlay of nutrient agarose medium containing 1% neutral red dye was added over the first overlay. The plates were observed daily for 14 days for plaque formation.

Virus identification. The infective agents were initially identified by indirect immunofluorescence assay tests. Diluted virus suspensions were inoculated on Vero cells in 25-mL flasks. After 4–7 days, when the cytopathic effect involved at least 25% of the Vero cell monolayer, the cells were harvested and diluted. Infected cells were detached from the flask by trypsinization and were resuspended in phosphate-buffered saline, pH 7.4, containing 5% fetal bovine serum. Suspensions of the infected Vero cells were added to wells of teflon-coated 12-spot slides (10 μl/well). Slides were air-dried and fixed in cold acetone. The infected cells were then tested by adding a series of polyclonal mouse hyperimmune ascitic fluids or National Institutes of Health hyperimmune ascitic fluids or National Institutes of Health reagents. Anti-mouse antibody conjugated to fluorescent isothiocyanate was then added to visualize antibodies to the virus. Further definitive identification of the isolates was made by carrying out plaque reduction neutralization tests following the method of Lindsey and others.21

RESULTS

Mosquito collections. A total of 10,050 mosquito specimens were collected from Bbaale between January 27 and February 2, 1997 from within and around 34 houses (Table 1). The collection contained 4 anopheline and 11 culicine species. More mosquito specimens were collected by knockdown pyrethrum spray catches (5,285) than by using indoor (3,746) or outdoor light traps (1,019). Most of the anophelines (94%) were collected by knockdown pyrethrum spray catches inside houses, while more culicines (70%) were collected by night indoor light trapping. The mean ± SE number of mosquitoes collected per house by pyrethrum spray catches was 166 ± 22 (n = 34). The mean ± SE number of collections per light trap was 67 ± 8.5 (n = 56) and 40 ± 6 (n = 26), respectively, for traps set inside and outside of houses.

All 34 houses that were sprayed were positive for anophelines. Many houses (65%) had between 100 and 300 anopheline specimens at a single collection, with a mean ± SE of 204 ± 22 (n = 22) anopheline mosquitoes per house. One house had 533 anopheline specimens at a single knockdown spray collection. The mean ± SE number of anopheline and culicine mosquitoes collected from each of the sprayed houses were 128 ± 21 and 15 ± 3 (n = 34), respectively. Less than 2.5% of the mosquitoes collected in outside traps were anophelines, with a mean ± SE of 1.6 ± 0.4 (n = 26) mosquitoes per light trap. The mean ± SE number of culicines collected in outside light traps was 36 ± 7.7 (n = 26). For the 56 trap nights, 7.1% of the mosquitoes collected in indoor light traps were anophelines (mean ± SE = 4.4 ± 1.1, n = 56). A few traps did not contain any anophelines. The mean ± SE number of culicines collected in the same traps was 62.8 ± 8.6 (n = 56).

The numbers of males and females of the different species that were randomly chosen and processed for ONN virus isolation are shown in Table 2. This was about 60% of the collection. The sample consisted mainly of female mosquitoes (86%). The most common species were An. funestus (46%) and Culex (Culex) univittatus Theobald (28%). Few An. gambiae (1 male and 1 female) were collected. Almost 90% of An. funestus specimens were collected by knockdown pyrethrum spray catches, while more than half of the Mansonia (Mansoniaoides) uniformis (Theobald), Ma. (Mansoniaoides) africana (Theobald), and Cx. univittatus were collected by indoor light trapping.

Virus isolations. Of the 10,050 mosquito specimens collected, 5,741 (including 760 male An. funestus) were tested for presence of viruses at CDC in Fort Collins, Colorado (the rest remained at UVRI). A total of 571 pools of mosquitoes were tested. Four isolates of three different viruses were obtained: two isolates of ONN virus, and one isolate each of Bwamba (BWA) and Nyando (NDO) viruses, both members of the genus Bunyavirus of the family Bunyaviridae.
One of the ONN virus isolates was obtained from a pool of 50 female *An. funestus* mosquitoes; the other ONN isolate was obtained from a pool of 11 female *Ma. uniformis* mosquitoes. The BWA virus (family Bunyaviridae) was isolated from another pool of 50 female *An. funestus* mosquitoes and NDO virus (family Bunyaviridae) was isolated from a pool of 37 *An. funestus* mosquitoes.

Table 3 shows results of cross-serum plaque-reduction neutralization tests for BWA virus isolate Ug Ar 1888 and NDO virus isolate Ug Ar 1712 compared with the prototype BWA virus and prototype NDO virus. Prototype Eretmapodites virus (Eth Ar 147) was included to clearly determine the NDO virus isolate, to which it is closely related antigenically. The NDO virus isolate was obtained from a pool of 50 female *An. funestus* mosquitoes. This isolate was next in abundance, accounting for 11% of the collection. *Anopheles gambiae* composed 3% of the collection from this area.

**Anopheles funestus** was the most abundant mosquito species collected (about 45%), and a strain of ONN virus was isolated from a pool of 50 females of these mosquitoes. This species of mosquito was already known to be a vector of ONN virus and was presumed to be the principal vector in the 1959–1962 epidemic. The species was the principal epidemic vector at the time we sampled was *An. funestus*. It is therefore apparent that the principal epidemic vector at the time we sampled was *An. funestus*.

**Table 3**

<table>
<thead>
<tr>
<th>Virus</th>
<th>BWA</th>
<th>NDO</th>
<th>Eth Ar 147</th>
<th>Ug Ar 1712</th>
<th>Ug Ar 1888</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2,560</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>1,280</td>
<td>&lt;20</td>
</tr>
<tr>
<td>NDO</td>
<td>&lt;20</td>
<td>640</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Eth Ar 147</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>40</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Ug Ar 1712</td>
<td>&lt;20</td>
<td>640</td>
<td>&lt;20</td>
<td>2,560</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Ug Ar 1888</td>
<td>640</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>1,280</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

* Titers of neutralizing antibody to virus isolated from Bbaale village, Rakai District, Uganda conducted in and around human habitations. The species of mosquitoes collected were generally similar to what was collected in the first epidemic, particularly the collections from Kakuto (31°29′E, 0°53′S) and Buyamba (31°25′E, 2°42′S), which are near Bbaale village. Buyamba and Kakuto are about 10 km and 40 km, respectively, from Bbaale village. Over the 35-year period between collections, there has apparently been only a limited change in the endophilic mosquito species of this area. In the 1959–1962 collections, *An. funestus* was the most abundant, constituting more than 80% of the collection. *Mansonia uniformis* was next in abundance, accounting for 11% of the collection. *Anopheles gambiae* composed 3% of the collection from this area.

**DISCUSSION**

During the 1959–1962 ONN fever epidemic, the epidemic vector species of ONN virus were endophilic mosquitoes. Thus, the entomologic investigations reported here were conducted in and around human habitations. The species of mosquitoes collected were generally similar to what was collected in the first epidemic, particularly the collections from Kakuto (31°29′E, 0°53′S) and Buyamba (31°25′E, 2°42′S), which are near Bbaale village. Buyamba and Kakuto are about 10 km and 40 km, respectively, from Bbaale village. Over the 35-year period between collections, there has apparently been only a limited change in the endophilic mosquito species of this area. In the 1959–1962 collections, *An. funestus* was the most abundant, constituting more than 80% of the collection. *Mansonia uniformis* was next in abundance, accounting for 11% of the collection. *Anopheles gambiae* composed 3% of the collection from this area.

**Table 2**

Mosquito species collected from Bbaale village, Rakai District, Uganda

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>No. of females</th>
<th>No. of males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Pools</td>
</tr>
<tr>
<td><em>Anopheles</em> (Anopheles) costani</td>
<td>Laveran</td>
<td></td>
</tr>
<tr>
<td><em>Anopheles</em> (Cellia) funestus</td>
<td>Giles</td>
<td></td>
</tr>
<tr>
<td><em>Anopheles</em> (Cellia) gambiae</td>
<td>Giles</td>
<td></td>
</tr>
<tr>
<td><em>Anopheles</em> (Cellia) pharoensis</td>
<td>Theobald</td>
<td></td>
</tr>
<tr>
<td>Aedes (Stegomyia) simpsoni s.l.</td>
<td>(Theobald)</td>
<td></td>
</tr>
<tr>
<td>Culex (Culex) picipes</td>
<td>(Theobald)</td>
<td></td>
</tr>
<tr>
<td>Culex (Culex) univittatus</td>
<td>(Theobald)</td>
<td></td>
</tr>
<tr>
<td>Culex (Culex) pipiens quinquefasciatus Say</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culex (Eumelanomaya) rima</td>
<td>Theobald</td>
<td></td>
</tr>
<tr>
<td>Coquillettia (Coquillettia) cristata</td>
<td>(Theobald)</td>
<td></td>
</tr>
<tr>
<td>Coquillettia (Coq.) microannulata</td>
<td>(Theobald)</td>
<td></td>
</tr>
<tr>
<td>Mansonia (Mansoniodes) africana</td>
<td>(Theobald)</td>
<td></td>
</tr>
<tr>
<td>Mansonia (Mansoniodes) formosus</td>
<td>(Theobald)</td>
<td></td>
</tr>
<tr>
<td>Uranotaenia (Pseudoficalbia) ornata</td>
<td>Theobald</td>
<td></td>
</tr>
<tr>
<td>Uranotaenia (Uranotaenia) [Edwards gp. A] sp. 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Total</em></td>
<td>4,939</td>
<td>526</td>
</tr>
</tbody>
</table>

* Positive pools = pools from which O’nyong-nyong virus strains were isolated. – = 0.
Corbet and others, while discussing earlier studies on the An. funestus series, indicated that the vector species of the 1959–1963 ONN fever epidemic was An. funestus since other species of the series do not enter human habitations and are assumed to be non-anthropophilic. Based on this assumption, all the collected specimens were considered to be An. funestus.

Haddow and others had expressed the opinion that mosquitoes of the genus Mansonia Blachard might be vectors of ONN virus, even though no ONN virus isolate was obtained from the Mansonia spp. collected during the 1959–1962 ONN fever epidemic. Our isolation of ONN virus from a pool of female Ma. uniformis mosquitoes is the first reported isolation of this virus from a culicine species. Until now, ONN virus was the only arthropod-borne virus thought to be transmitted exclusively by anophelines.

This ONN virus isolate suggests that Ma. uniformis mosquitoes may transmit ONN virus. Mansonia uniformis is a medium-sized, very scaly culicine that is very common and widely spread in most of Africa south of the Sahara. It also occurs in Asia, the Pacific, and northern Australia. This species commonly feeds on humans both inside and outside of houses, primarily at night. Like An. funestus, it commonly rests inside dwellings. Mansonia uniformis accounted for 18% of the collected mosquitoes. Therefore, it may have played a limited role as a vector in the 1996–1997 ONN fever epidemic. However, the vector status and competence of this species for ONN virus are unknown. Isolates of several other viruses have previously been obtained from this species (BWA, Chikungunya, Ndumu, Rift Valley fever, Spondweni, and Wesselsbron viruses). It therefore appears to be an important vector of arboviruses. Its geographic distribution has implications for the spread of ONN virus to the continents of Asia and Australia.

The BWA and NDO viruses, both members of the genus Bunyavirus of the family Bunyaviridae, were also isolated from An. funestus. The BWA virus together with Pongola virus form the Bwamba serogroup, while NDO virus together with Eretammapodites virus form the Nyando serogroup of viruses. These viruses are known to infect vertebrates, and are widely spread in east Africa and many other countries south of the Sahara.

Anopheles funestus is a vector of several viral diseases including BWA, NDO, ONN, Akabane, Orongo, Pongola, Semliki Forest, Tatagouine, and Tanga viruses. It is apparent that not only was An. funestus important in the transmission of ONN virus during the epidemic, but it may also have been concomitantly transmitting NDO and BWA viruses. Mansonia uniformis mosquitoes are also known to naturally infect with BWA virus. The NDO virus was first isolated during the investigations of the 1959–1960 ONN fever epidemic from An. funestus mosquitoes collected in Kisumu in western Kenya. Theiler and Downs also reported the isolation of two strains of BWA virus during the 1960s, one from a man and the other from An. funestus mosquitoes from the Masaka District, which at that time was inclusive of the present Rakai District. These isolates were obtained during the investigations of the 1959–1962 ONN fever epidemic. More recently, a strain of BWA virus was isolated from one of the field staff of the Rakai HIV project working in this same area just before the 1996–1997 ONN fever epidemic (Sempala SDK, and others, unpublished data). Thus, the three viruses (ONN, BWA, and NDO) appear to co-exist with transmission by the same vectors.

Acknowledgments: We express our gratitude to Duane J. Gubler, John Roehrig, and Rebecca Devours (CDC, Fort Collins, CO) and S. D. K. Sempala (UVRI, Entebbe, Uganda) for facilitating the CDC team travel to Uganda. Thanks are due to Lee Dunster and Manuela Kranz (Kenya Medical Research Institute, Nairobi, Kenya) and Fred Senfuka (UVRI, Entebbe, Uganda) for assistance in the field. The technical assistance of Marvin Godsey, Christine Hopp, Nick Karabatos, Bruce Cropp, and Denise Martin (CDC, Fort Collins, CO) and Anthony Mukuye and Peter Nawanga-Lule (UVRI, Entebbe, Uganda) in the laboratory investigations is highly appreciated. The advice of Robert Shope (University of Texas Medical Branch, Galveston, TX) and Elizabeth Marum (CDC, Kampala, Uganda) is gratefully acknowledged.

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