Arboviral Disease Vectors Surveillance and Control Guideline

October 2021
Addis Ababa
Ethiopia

Arboviral Disease Vectors Surveillance and Control Guideline

Public Health Entomology Research Team

October 2021

Addis Ababa
Ethiopia

Foreword

This technical guideline was written to consolidate information and procedures for surveillance and control of mosquitoes that transmit Arthropod-borne viruses (arboviruses) diseases such as Yellow fever, Dengue, Chikungunya and Zika viruses. It is guiding those entomologists, vector control technicians, and other individuals responsible for conducting entomological vector surveillance and vector control during the arboviral investigation. This new guideline will receive a periodic review and will be updated to ensure that the information presented reflects current technology and guidance. Individuals using this guideline are encouraged to submit comments and suggestions for improvement.

Table of Contents

Page

List of Abbreviations

CDC……Center for Disease Control & prevention

CFR……. Case Fatality Rate

CHIKV……...Chikungunya virus

CI……. Container Index

DHF……. Dengue Hemorrhagic fever

EPHI……Ethiopian Public Health Institute

EYE……. Eliminate Yellow Fever Epidemic

HI……House Index

IEC……. Information Education & Communication

KDT……Knockdown time

PCR……. Polymerase Chain Reaction

RNA……Ribonucleic Acid

SNNPR……...South Nation Nationality People Region

WHO……. World health organization

YF……. Yellow fever

1. Introduction

Arbovirus (arthropod-borne virus) applies to any virus that is transmitted to humans and/or other vertebrates by certain species of blood-feeding arthropods, chiefly insects (flies and mosquitoes) and arachnids (ticks). Three main virus families for arboviruses that cause infections in humans; flaviviridae, togaviridae and bunyaviridae, thus found in tropical and subtropical regions.

The *Aedes* mosquito is responsible for the transmission of many arthropod-borne viruses (arboviruses), including dengue virus, yellow fever virus, Zika virus, chikungunya virus and West Nile virus. These arboviruses pose increasing global public health concerns because of their rapid geographical spread and increasing disease burden.

Those viruses are transmitted by infected female mosquitoes in the genus *Aedes*. Mosquitoes are flying insects that develop and emerge from small pocket water bodies, from which the winged adults emerge. Only the adult female mosquitoes bite humans to feed on blood for the development of eggs. While an infected female mosquito biting a healthy person to take blood, it transmits the virus or becomes infected while biting an infected person vise versa. *Aedes* mosquitoes are common in most of the urban areas on account of deficient water management, presence of non-degradable unused tyres and long-lasting plastic containers as well as increasing urban agglomerations, and where the inability of the public health community to mobilize the population to respond to the need to eliminate mosquito breeding sites. Natural larval habitats are rare but include tree holes, leaf axles, and rock. Under the optimal conditions, the life cycle of the aquatic stage of the *Aedes* Aegypti can be as short as seven days. At low temperatures, however, it may take several weeks. During the rainy season, the risk of virus transmission is greater.

Entomological surveillance is essential to guide the program on designing and implementing appropriate intervention as well as to generate research-based decision-making evidence. Its additional benefit is to assess the susceptibility status, behavior of the mosquito, and decay rate of the sprayed chemicals used for interventions. In addition, the surveillance can tell the relevance and urgency of undertaking a mass vaccination and/or vector control campaign. Such a study requires some expertise and methodology that guide the experts to do effective research in entomology. Therefore, this guideline describes how entomological interventions are used to confirm arbovirus vector distribution and disease burden in specific areas. The intervention technique also describes how to respond to an outbreak of arbovirus disease and seizes not to occur additional cases.

2. Objectives

The primary objective of this guideline is to guide *Aedes* mosquitoes surveillance and control in response to risk reduction of dengue, chikungunya, Zika, and yellow fever viruses across Ethiopia. Information generated from the surveillance can be used to determine changes in the geographical distribution and density of *Aedes* vectors as well as to design, implement and evaluate vector control programs. All this information is facilitating appropriate and timely decisions regarding *Aedes* mosquitos' vector interventions. Therefore, this document is intended for the use of research institutions, universities and local public health officials, and vector control specialists.

3. Vector biology

Aedes mosquito, a vector of Zika, Dengue, Yellow fever and Chikungunya, is a dark mosquito with a single silver stripe on the top of the thorax and white-banded legs. Their abdomen is dark brown to black and they have white scales on their thorax that form a violin-like pattern. Each tarsal segment of the hind legs has white bands. These white bands make them look like they are stripped. Adult *Aedes* mosquitoes grow to a small size of about 4-7 millimeters. Females are larger than males. Mostly found outdoors and associated with thickets and dense vegetation. Only the adult female mosquitoes bite humans to feed on blood and aggressive daytime biter.

Figure 1: shows *Aedes* aegypti.

Life stages of *Aedes* mosquito

Eggs

Eggs look like black dirt.

- Adult, female mosquitoes lay eggs on the inner walls of containers with water, above the waterline.

- Eggs stick to container walls like glue. They can survive drying out for up to 8 months and more. Mosquito eggs can even survive a winter.
- Mosquitoes only need a small amount of water to lay eggs. Bowls, cups, fountains, tires, barrels, vases, and any other container storing water make a great "nursery."

Larvae

- Larvae live in the water. They hatch from mosquito eggs. This happens when water (from rain or a sprinkler) covers the eggs.
- Larvae can be seen in the water. They are very active and are often called "wigglers."

Pupae

- Pupae live in the water. An adult mosquito emerges from the pupa and flies away.

Adult

- Adult female mosquitoes bite people and animals. Mosquitoes need blood to produce eggs.
- After feeding, female mosquitoes look for water sources to lay eggs.
- *Aedes mosquitoes* don't fly long distances (not more than 300m). In its lifetime, these mosquitoes will only fly within a few blocks.
- *Aedes mosquitoes* prefer to live near breeding sites and bite people.
- Because *Aedes* mosquitoes bite people and animals, they can live in or near homes or in neighboring woods.
- *Ae. aegypti* mosquitoes live indoors and outdoors, while *Ae. albopictus* lives outdoors.

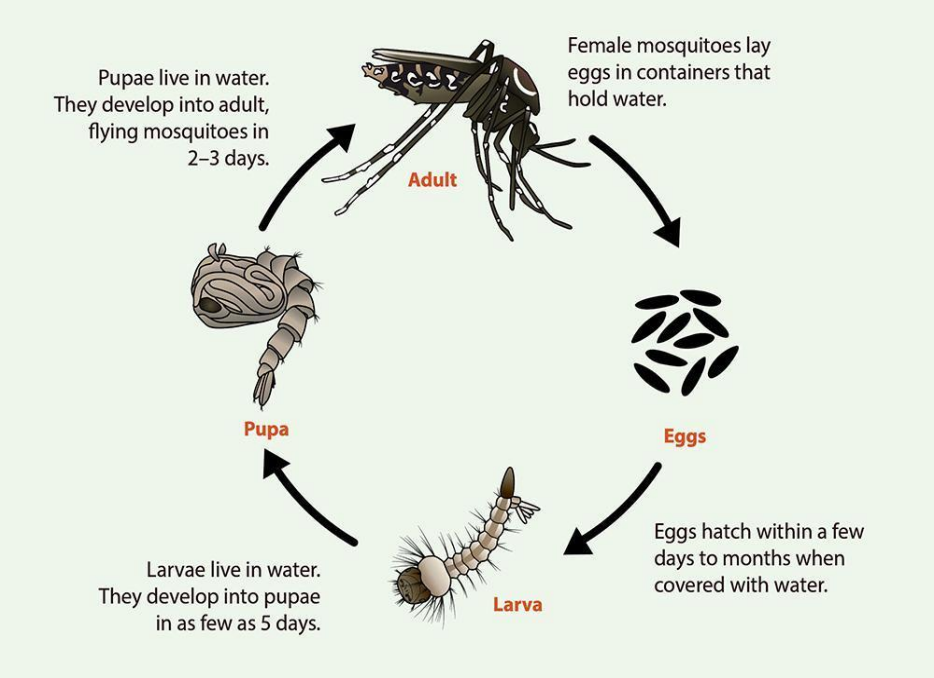


Figure 2: Life cycle of Aedes aegypti.

4. Bio ecology and geographical distribution of *Aedes* vectors in Africa

Aedes aegypti

Aedes aegypti has two subspecies that are distinguished by ecological and behavioral characteristics. *Ae*. aegypti aegypti, the domestic and peri-domestic forms found in tropical urban areas, is believed to have evolved from *Ae.* aegypti formosus, the ancestral African tree-hole form. Previous studies have shown that Ae. aegypti aegypti exists in Asia and the New World, while in Africa both subspecies exist, but with a limited distribution of *Ae.* aegypti aegypti on Africa's east coast.

The presence of *Ae.* aegypti aegypti in west and central Africa is still debated, mainly because of the lack of reliable methods to distinguish the different subspecies. Available morphological keys are not sufficiently accurate to distinguish *Ae.* aegypti aegypti from *Ae.* aegypti formosus or intermediary forms.

Despite the taxonomic debate, we can assume that, in all of Africa, there are at least two different *Ae.* aegypti populations based on bionomics:

A. A domestic, anthropophilic subpopulation that bites during the daytime, especially in the late afternoon and uses artificial containers (water storage containers, cans, old tyres, abandoned

containers, etc.) as breeding sites. Due to the artificial nature of these breeding sites, this *Ae.* aegypti subpopulation is present throughout the year.

B. A wild, zoophilic subpopulation breeding in natural habitat (rock holes, tree holes, fruit husks, etc.).

Aedes africanus group

Aedes africanus group, which is a complex of four sibling species: *Ae.* Africanus, *Ae.* neo africanus, *Ae.* opok *and Ae.* pseudo africanus, which were previously confounded. *Aedes* africanus is found in forest areas, but can also be found in forest galleries of savannah regions. The species is abundant in the east and central Africa, but it has a more localized distribution in west Africa. It is primatephilic and very aggressive to humans. It bites preferentially in the forest canopy, but can also bite at the ground level. It has crepuscular activity, yet also becomes active during the day if a human enters its habitat an "intrusion effect". The other species of the group have similar bioecological patterns to *Ae.* africanus.

Aedes luteocephalus

Aedes luteocephalus is another species of the subgenus Stegomyia morphologically similar to *Ae.* africanus group species. It is simio anthropophilic, has a crepuscular behavior and takes blood meals at the canopy, but occasionally bites on the ground. The species are abundant in forest habitats and may be present in very rare situations in the villages surrounding the forest.

Aedes simpsoni group

For many years, it was assumed that the *Ae.* simpsoni group had a wide geographical distribution in Africa. However, recent studies have shown behavioral divergences in diverse geographical contexts, indicating the existence of a morphologically similar group. These studies split the simpsoni group into three sibling species: *Ae.* simpsoni, *Ae.* bromeliae and *Ae.* lilii. These species are abundant in the forest, and in transition areas of forest and savannah (ecotone) with heavy rainfalls. In forest areas, they are present throughout the year, while in pre-forestry areas their activity is seasonal.

Aedes bromeliae

Aedes bromeliae is present in several African countries but has important behavioral variations that might affect its vector status depending on geographical context. It is the major YF vector in East Africa; there is evidence of association with YFV in nature and the vector is abundant with an anthropophilic feeding pattern. The mosquito is especially aggressive during the afternoon, but it may also bite at dusk and early in the morning. In west Africa, although its presence is

reported, it does not seem to play a role in arbovirus disease transmission to humans because of its zoophilic tendencies. The species shares the same natural breeding sites as *Ae.* simpsoni (plant leaves, tree holes, fruit husks, etc.), but also uses containers in East Africa in the domestic and per domestic environment.

Aedes lilii

Aedes lilii appears to have a geographical distribution limited to east Africa. The species does not seem to be involved in the arbovirus disease transmission in this part of the continent because it does not bite humans. Its breeding sites are sylvatic.

Aedes (Diceromyia) *furcifer/ taylori* group

The *Aedes* (Diceromyia) farcie/ taylori group was initially considered to be composed of two species: *Ae.* taylori and *Ae.* furcifer. *Ae.* furcifer has been split into *Ae.* furcifer ss. and *Ae.* cordellieri recently. The three species are found in west, central and south Africa, but only *Ae.* furcifer and *Ae.* taylori seems to be well established in West Africa and central Africa. *Ae.* cordellieri is the only species of the group present in east Africa.

These species share several bio-ecological characteristics. They are absent in forest areas and well represented in the savannah areas in open spaces. Their breeding sites are natural and include mainly tree holes, bamboo, fruit and so on. They are simio anthropophilic with a crepuscular peak in biting activity.

Aedes furcifer is the only species of the group that has activity in villages bordering the forest environment. Such behavior confers to this species a very important role in domestic transmission cycles. *Ae.* taylori and *Ae.* cordellieri remains confined to the wild habitat.

Aedes (Aedimorphus) *vittatus*

Aedes (Aedimorphus) vittatus is the only species of the subgenus Aedimorphus involved in arbovirus disease transmission. *Ae.* vittatus has been found associated with YFV in nature and its ability to transmit was proven experimentally. Nevertheless, it is still considered to be an accessory vector, since there is very little information. It was only incriminated in the epidemic that occurred in Sudan in 1940 and was suspected to have played an accessory role during outbreaks that occurred in the Jos Plateau, Nigeria, in 1959 and 1969. The species has pronounced seasonal dynamics, with an early peak of density at the beginning of the rainy season, which suggests a limited role in sylvatic transmission because the virus tends to emerge towards the end of the rainy season. *Ae.* vittatus is a savannah species that is abundant in rocky areas. It is especially prevalent in West African forest galleries, but it is also common in villages

near the forest. Females have daily and nocturnal activity with a significant crepuscular peak. They bite a wide range of vertebrate hosts, with a strong anthropophilic trend in specific locations.

5. Arbovirus Outbreaks Overview in Ethiopia

5.1. Yellow Fever

Yellow fever is an acute arbovirus disease transmitted primarily by *Aedes* species. The distribution of yellow fever in Africa and the population at risk of the disease is little known. However, Geographically Ethiopia lies in the so-called yellow fever belt which ranges from 15^0N to 10^0S of the equator and includes 34 countries in Africa with a combined population of about 468 million at risk. Based on the EYE Strategy risk classification, Ethiopia is ladled as at high risk.

Yellow fever outbreaks have been reported during the period 1950- 2020, particularly in the southwestern part of the country. During the period 1960-1962: Gamo Gofa, Kaffa, Shoah districts were affected by the YF outbreak. The largest YF outbreak occurred in 1966, in Arbaminch District (Gamo Gofa Zone, in which 2,200 cases and 450 deaths (CFR = 20.5%) were reported. Yellow Fever outbreak occurred in 2013 in the South Omo Zone of Southern Nations and Nationalities Regional state, in which 141 cases and 43 deaths (CFR = 30.5%) were reported. In 2018, a YF outbreak occurred in the Wolaita zone, Offa woreda, a total of 35 suspected yellow fever cases were reported, of which 10 were reported dead. The last yellow fever outbreak occurred in the SNNP region, Gurage Zone Inemoreena Enor District, in 2020 and reported 86 cases.

5.2. Chikungunya

Chikungunya refers to an infection by the Chikungunya virus (CHIKV). CHIKV is known to be transmitted by the Aedes mosquito. The name Chikungunya means “bends up” in the native language of southeastern Tanzania, and refers to the symptoms of Chikungunya fever.

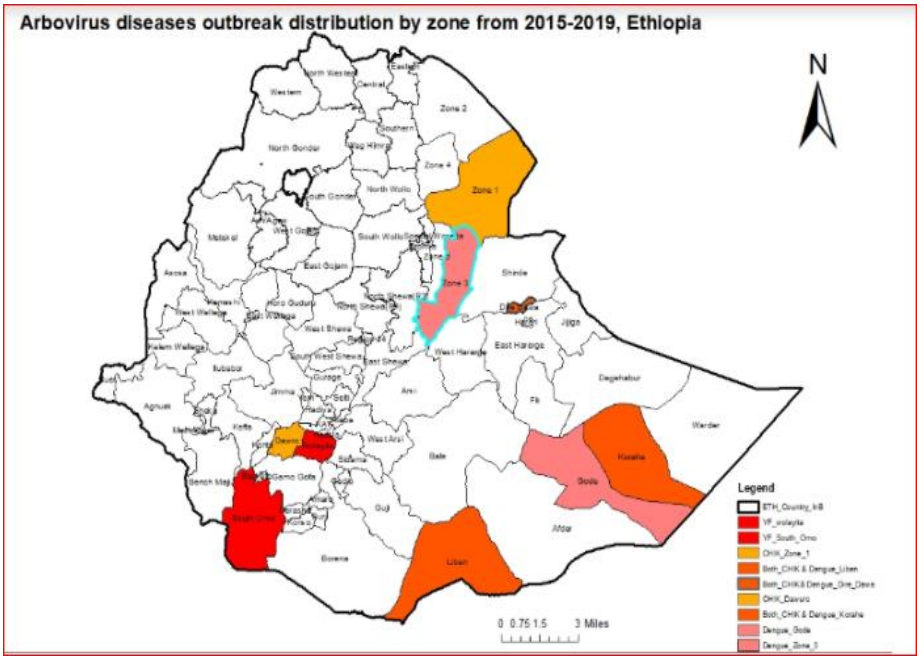
In Ethiopia, more than 53,200 people have been infected by the Chikungunya virus. The outbreak affected mostly the eastern parts of Ethiopia. The first-ever confirmed case of a Chikungunya infection in Ethiopia was identified in June 2016 from the Suuf sub-district, Somalia region. In Afar (Adaar woreda), 1,167 people were affected with no deaths from March-May, 2019. In May 2019 in Kebridahar town, Somali region, 123 people were affected. Chikungunya outbreak also occurred in Dawro zone, SNNP region on May 25, 2019 and affected

around 380 people with no death. In Dire Dawa the largest and mixed occurrence of Chikungunya and Dengue fevers. It was reported at the end of July 2019 and persisted for about 3 months and during this time affected 51,530 people.

5.3. Dengue fever

Dengue refers to an infection by any of the four related virus serotypes, DEN-1, DEN-2, DEN-3, and DEN-4. They are transmitted to humans by mosquito species: *Aedes* aegypti, *Ae.* albopictus, and *Ae.* polynesiensis. Human infection by the virus leads to Dengue fever and occasionally Dengue hemorrhagic fever. Dengue hemorrhagic fever (DHF) results from reinfection by a different Dengue serotype.

In Ethiopia, Dengue fever outbreak started on September 16, 2019 in the Afar region, Gewane and Amibara woredas. It affected around 588 and more people. However, in Amibara woreda it was mixed with chikungunya like Dire Dawa.



6. Entomological sample Collection

6.1. Vector surveillance

There are two types of Vector surveillance methods: larval and adult mosquito vector surveillance. It is critical for effective arboviral diseases control as it provides information on changes in vector populations, Vector species present, vector-borne diseases and control measures that need to be conducted. Moreover, it is essential for early detection of exotic species with public health importance and its prevention and control methods. The larval and adult

mosquito surveillance provides relevant information for the control program. It is used to determine the appropriate type of intervention and identify the sites and time the innervation impacted most on the disease.

Objectives of surveillance indicated

1.Detect presence/absence
2.Assess population density
3.Assess seasonality
4.Determine habitat preference
5. Determine biting behavior
6. Determine resting behavior
7. Determine host preference
8. Detect pathogen infection

6.2. Methods of Arboviral Disease Vectors Survey

Household Sampling Methods

Vector Surveys should be done in a randomly selected household. The households included in the surveys are selected by systematic random sampling technique. This requires an initial estimate of the number of households to visit in each locality based on demographics data to be statistically significant (confidence interval of 95%). Therefore, the number of households will be determined by dividing the population size of the community by the estimated number of people living in a house. Once a sample size is determined for each affected locality, the number will be divided and equally allocated to the number of households where arbovirus cases have been recorded (household index). The minimum sample size that one needs to visit in one locality is 100 houses. Each index household will be a starting point. After visiting this index household, the next household to be visited will be selected using a systematic sampling procedure. The sampling step will be calculated using the ratio of the number of households in the community to the number of households to be sampled.

$K = \frac{N}{n}$, Where, K = the gap between every household; N = total number of households in the surveyed village; n = the sample size.

6.3. Larval mosquito surveillance

Larval surveillance and control is a critical component of an effective integrated vector management program because it has an impact on the adult population, and hence reduces arboviral parasite transmission. Vector larval collection is a critical activity in vector surveillance**.** The basic sampling unit is the house or premise, which is systematically searched for water holding containers. All artificial containers containing water, inside households and

outside in the courtyard, will be inspected using a flashlight. All natural breeding sites containing water in the domestic and per domestic environment will also be inspected. When a container is found harboring at least one larvae or pupae, it is considered positive. In this case, a sample of larvae and pupae is taken and kept alive in a bottle containing water from the breeding site. The bottle is carefully labeled with information concerning the date of collection, the house number, the type of breeding site and so on (Annex II). Then they will be reared in mosquito cages or the collections containers covered with a net.

Figure 4. (A) *Discarded tyres (B) Different plastic water tank (C) False banana plant "Inset" (D) Clay pot & others.*

The information obtained from larval collections includes determination of the vector species, identification of preferred breeding sites for each species, determination of the geographical distribution of vectors, evaluation of anti-larval measures, and collecting samples for rearing adults in the insectary**.**

Basic equipment

larvae trays, Pipette, Plastic cup with net cover, Dipper, Cage, Rubber band, Cotton, Marker, Registration form, Flashlights and accessories (e.g. batteries and light), waterproof notepad, Eppendorf tube, Forceps, Identification Key, Petri dishes, Pencil, Larvae food, Sugar solution (10%), Hand lenses, a Bag to put all equipment, Compound microscope.

6.4. Indices used for larval surveys

Three indices are commonly used to measure *Ae*. aegypti density levels:

A. The House (premises) Index (HI): Presence of houses or premises positive for *Aedes* larvae.

The house index has been used most widely for measuring population levels.

The HI is calculated as follows

$$HI = \frac{No.of\ Houses\ Positive\ for\ Mosquito\ Larvae}{No.of\ Houses\ inspected} \times 100$$

B. Container Index (CI): Presence of water holding containers positive for *Aedes* vector larvae. The container index provides information on the proportion of water holding containers that are positive.

$$CI = \frac{No.of\ Positive\ Containers}{No.of\ water\ holding\ containers\ inspected} \times 100$$

C. Breteau Index (BI): Number of *Aedes* positive containers per 100 houses in a specific locality. The breteau index establishes a relationship between positive containers and houses and is considered to be the most informative. In the course of gathering the basic information for calculating the breteau index, it is possible and highly desirable to obtain a profile of the larval habitat characteristics by simultaneously recording the relative abundance of the various container types either as potential or actual sites of mosquito production (e.g., number of positive drums per 100 houses, number of positive tires per 100 houses, etc.).

$$BI = \frac{No.of\ Mosquito\ Positive\ Containers}{No.of\ Houses\ inspected} x\ 100$$

Note that: According to the World Health Organization (WHO, 1986), there is an epidemic risk when these indices are more than the thresholds of ≥ 20 for the Breteau and ≥5% for the house index.

6.5. Pupal/demographic survey

If the types of containers with the highest rates of adult mosquito emergence are known in a community, they can be selectively targeted for source reduction (e.g. elimination) or other vector control interventions to optimize the use of limited resources. A pupal/demographic survey is an operational research tool to identify these most epidemiologically important types of containers. Unlike the traditional *Stegomyia* (*Aedes*) indices described above, pupal/demographic surveys measure the total number of pupae in different classes of containers in a given community. Such surveys are far more labor intensive than the larval surveys previously described, and are not envisaged for routine monitoring of *Aedes* populations. The collection of demographic data enables the calculation of the ratio between the numbers of pupae (a proxy for adult mosquitoes) and persons in the community. There is growing evidence that, together with other epidemiological parameters such as dengue serotype-specific seroconversion rates and temperature, it is possible to determine the level of vector control needed in a specific location to inhibit virus transmission. This remains an important area for research, with potential for public health application.

Pupal Index (PI): No. of pupae per 100 houses. It is used to estimate the relatively newly emerged adult population.

$$PI = \frac{No.\ of\ pupae}{/No.of\ Houses\ inspected} x\ 100\ ^{1}$$

6.6. Adult mosquito collection

The objective of prospecting the adult stage is to identify those mosquitoes that prefer to bite humans (anthropophilic) in the study area, to estimate their density, to study their biological and ecological behavior (e.g. aggressiveness and biting behavior indoor and outdoor, and resting behavior indoor and outdoor) and their association with arbovirus disease in nature. In the domestic environment, mosquito capture will be conducted indoors and outdoors to measure the exophagic or endophagic trends of potential vectors.

Adult Surveys:

A. **Landing collection:** Landing collection of humans is a sensitive means of detecting low level infestations of *Ae* aegypti, but are very labor intensive. Because adult males have low dispersal

1

rates, their presence can be a reliable indicator of clear proximity to hidden larvae habitats. It is usually expressed in terms of landing counts per man hour.

B. **Resting collection:** During periods of inactivity, adult mosquitoes typically rest indoors, especially in bedrooms and mostly in dark places, such as cloth closets and other sheltered sites. Resting collection requires systematic searching of these sites for adult mosquitoes with the aid of flashlights. Following a standard timed collection routine in selected rooms of each house, densities are recorded as the number of adults per house or number of adults per man hour of human efforts.

There are several methods of capturing adult mosquitoes. However, Human landing collection is the method that provides the best data as it is specific to human-vector contact. However, it is important to mention the ethical considerations of this method of collection, which could expose the workers to mosquito bites. We thus recommend the human landing collection method, after providing training for field workers, immunizing against arbovirus (if available) and prescribing malaria prophylaxis. Alternative methods to collect *Aedes* mosquitos are numerous, such as CDC Light Trap, Indoor residual spraying with pyrethroid, Aspiration of resting adult mosquitoes in rooms and surrounding vegetation, Backpack aspirator, sticky trap and other odor-baited traps that use different chemical or natural lures, but none of them competes with the human landing collection.

Basic equipment

Aspirator, CDC light trap & batteries, Paper cup with net, Petri dish, Cotton, Liquid nitrogen and container, forceps, cages, White sheet of cloth, Pyrethrum solution, Hand sprayer, Forceps, backpack aspirator and accessories.

Adult Biting Index (ABI) or Human Landing Rate (HBR)

$$ABI = \frac{No.of\ Mosquito\ Sp.Collected}{No.of\ Human\ Bait}\ per\ hour$$

Interpretation of adult density:

- >2/human/hr = high risk
- <2/human/hr = low risk

C. **Oviposition traps:** Ovitraps are devices used to detect the presence of *Ae* aegypti where the population density is low and larval surveys are largely unproductive (when the Breteau index is less than 5) as well as normal conditions. The ovitrap is used for *Ae* aegypti surveillance in

urban areas to evaluate the impact of adulticidal space spraying on the adult female population.

Table 1. The overview of the main mosquito collection methods

Collection method	Life stage	Advantage	Disadvantage	Objective
Ovitrap	Eggs	Simple	Variable efficiency	1,2,3
Dipper sampling	Larvae, Pupae	Direct result	Some habitats missed	2,4
Gravid Trap	Oviposition female	Relatively simple	Not very efficient	1,2,3,7,8
Human Landing collection	Host-seeking female	Direct result	Laborious, ethical issues	2,5
BG-Sentinel	Adults	Efficient for specific species	Cost	1,2,3,8
Mosquito Magnet: CDC trap	Adults	Moderately efficient	Cost	1,2,3,8
Aspirator	Adults	Direct result	Laborious, inefficient	2,6,8

6.7. Analysis of findings

A. **Vector:** Presence or absence of the primary and secondary vectors, geographic distribution.

B. **Larval indices:** high or low indices based on WHO figure, mosquito density vs. cases, etc.

C. **Breeding sites:** kind of container (i.e. drum, tyre, etc.), type (artificial/natural), location (indoor/outdoor), size (large/small), most common container, species density vs type (artificial/natural) etc.

D. **Abundance curves:** This risk assessment must be done in each affected village or sub-village to localize and delimitate precisely the area at risk. The abundance curves are used to determine the typology of breeding sites and to draw a curve of their abundance in each locality. This analysis will be performed to estimate the abundance of the breeding sites containing water and then determine the number found positive. In each case, first classify breeding sites according to

their localization in the domestic or per domestic environment. Next, look to see which specific types of containers are mostly infested in a given area.

E. Community: Socio-economic factors & behavioral practices, lifestyle, beliefs, presence or absence of legislations on arbovirus disease prevention & control, etc.

7. Species identification and transport of samples to the laboratory

7.1. Morphological species identification

Although several techniques including molecular and DNA-based techniques have been developed in mosquito taxonomy, adult and immature morpho-taxonomy are preferable for their simplicity and easy application in the field. Morpho-taxonomy involves the use of morphological characteristics to identify species based on the phenotype (qualitative) and/or use of dimensions (quantitative) of their structures. Features such as markings on thoracic and abdominal segments, leg coloration, markings on the lateral edge of the scutum, as well as the color of the scutum are all used in taxonomy.

7.1.1. Adult stage Identification

Mosquitoes possess only one pair of functional wings- the fore wings and the hind wings are represented by a pair of small knob-like halteres. They belong to the suborder Nematocera of the order. They can be distinguished from other flies of a somewhat similar shape and size (for example Dixidae, Chaoboridae and Chironomidae) by (1) the possession of a conspicuous forwardly projecting proboscis, (2) the presence of numerous appressed scales on the thorax, legs, abdomen and wing veins, and (3) a fringe of scales along the posterior margin of the wings. Frequently, but not always, the thorax, legs and wing veins of the adult culicines are covered with dull-coloured, often brown, scales. The abdomen is often covered with brown or blackish scales, but some whitish scales may occur on most segments. Adults are recognized more by their lack of ornamentation than by any striking diagnostic characters. The tip of the female abdomen is not pointed but blunt. Claws on all tarsi are simple and those on the hind tarsi are very small. Examination under a microscope shows that all tarsi have a pair of small fleshy pulvilli. Many, but not all, *Aedes* adults have conspicuous patterns on the thorax formed by black, white or silvery scales, and in some species yellow and/or brownish scales are present. The legs often have dark and white rings. *Aede*s aegypti, often called the yellow fever mosquito, is readily recognized by the lyre-shaped silver markings on the lateral edges of the scutum.

Scales on the wing veins of *Aedes* mosquitoes are narrow and usually mainly black. In *Aedes*, the abdomen is often covered with black and white scales forming distinctive patterns, and in the female, the abdomen is pointed at its tip.

Although several techniques including molecular and DNA-based techniques have been developed in *Aedes* taxonomy, adult morpho-taxonomy is preferable for its simplicity and easy application in the field Morpho-taxonomy involves the use of morphological characteristics to identify species based on the phenotype (qualitative) and/or use of dimensions (quantitative) of their structures. Features such as markings on thoracic and abdominal segments, leg coloration, markings on the lateral edge of the scutum, as well as the color of the scutum are all used in taxonomy.

Most *Aedes* adults have conspicuous black, white or silvery scales patterns on the thorax and the abdomen. The legs often have black and white rings. Scales on the wing veins of *Aedes* mosquitoes are narrow and are usually more or less all black except maybe at the base of the wing. Spiracular areas are also without scales or bristles. The abdomens of adult females are pointed at the tip.

All larvae and pupae collected should be placed in labeled paper cups and reared into adults. Adult mosquitoes collected from villages and emerging from aquatic stages are killed by freezing and identified using a chill table microscope and morphological keys. Care should be taken to identify mosquitoes separately according to their geographical and ecological origin. Males are separated from females, and engorged, separated from non-fed females, and pooled with a maximum of 10 individuals per species, by gender, and geographical and ecological origins. The samples should be classified according to their geographical origin. Each sample should be documented by filling out a form and providing information on at least important aspects (see Annex II for a sample form). Killed mosquitos are frozen in nitrogen liquid and transported to the laboratory, then stored at –70 °C or –180 °C until tested for virus by real-time PCR and/or viral isolation.

Morphological features used in the identification keys

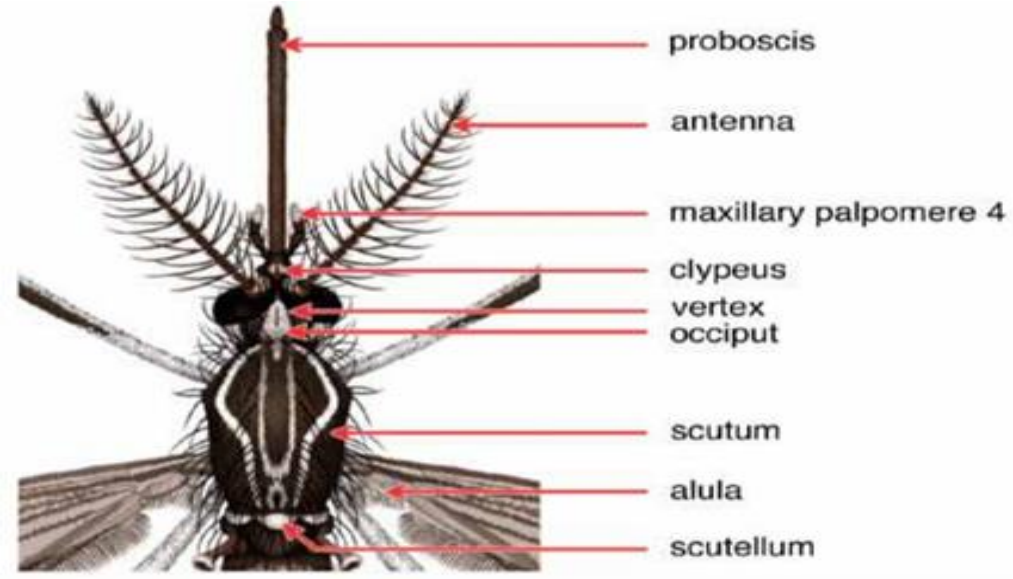


Figure 4A. Dorsal view of adult head and thorax - *Aedes (Stegomyia) aegypti*.

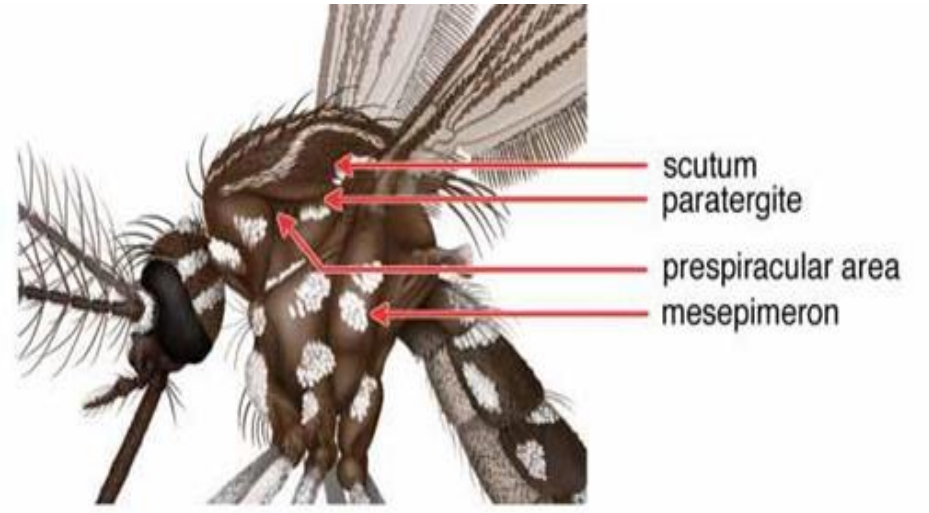


Figure 4B. Lateral view of adult head, thorax and abdomen (part) – Aedes (Stegomyia) aegypti.

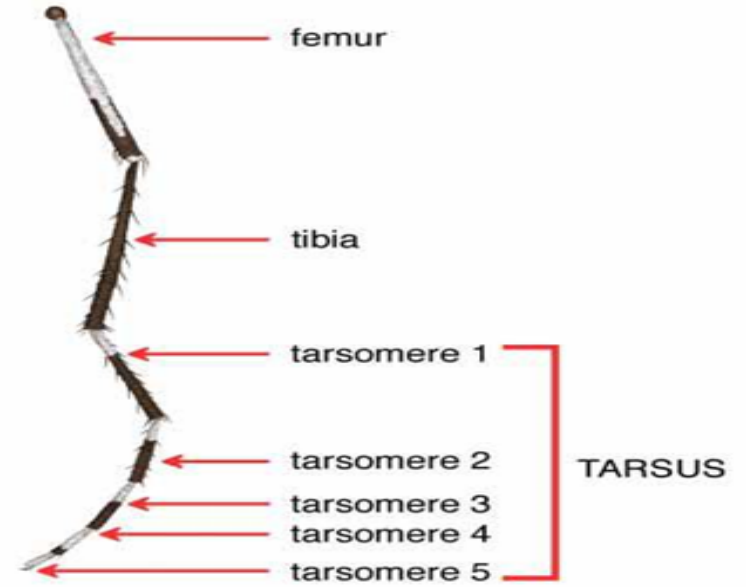


Figure 5. Anterior view of hind leg – *Aedes (Stegomyia) aegypti*.

7.1.2. Immature stage Identification

Immature stage identification of *Aedes* mosquitoes is dependent on identification of mosquito breeding sites, examination of potential breeding locations and selection of water samples. Since mosquitoes breed in almost any kind of water body it is important to check all water bodies during a larval or pupal survey.

Larvae Identification

Mosquito larvae morphologically differ greatly from that of an adult mosquito. Some common features of a mosquito larva, which separates them from other true flies, all mosquito larvae are dorso ventrally flattened and the body divided into three parts; head, thorax and abdomen. Head bears several structures like antenna, eye, mental plate, mouth brush, and upper and lower head hair, pre antennal and antennal hair. The larval abdomen is segmented; usually bears 8 to 10 segments. Lateral hair may or may not be present. Arising from the eighth segment, in all species except the *Anopheles*, is a breathing tube, or siphon, which is thrust up through the surface of the water, and through which the larva breathes atmospheric air. The siphon has a double row of spines near the base, where it joins the eighth segment and one or more paired hair tufts along its length. Air plugs are present in siphon tubes that control Comb and anal gills are present in the anal segment. When coming to the surface of the water to breath, non *anopheline* wiggle tails hang head downward, seemingly suspended from the tip of the breathing tube. The larval head is

large, usually globular, and is provided with two prominent dark colored eyes. Just in front of the eyes lie the antennae, or feelers, which bear a tuft of hairs at or near the middle. The upper surface of the head is provided with a number of paired hairs, which are of importance in classification. The thorax is clothed with numerous lateral tufts of hair. The abdomen is composed of nine segments, each provided with tufts of hair, and bearing on the eighth segment a number of spines or scales, arranged in a triangular patch, in a row, or a band the larvae can be identified by a simple method and by preparation of permanent slides using a series of dichotomous pictorial identification keys.

Anopheline larvae seldom go to the bottom, but float at the top of the water, in the surface film, parallel with the surface. An excellent mode of illustrating the difference between the two types of larvae is to view them in a glass of water which is held about 4 inches above eye level, about 6 inches from the face. Anopheles larvae are variously colored, sometimes being grass green, gray, reddish-brown, or black. Often there is a longitudinal stripe on the back of the abdomen, which may be broken by spots of lighter or darker pigment. The young wiggle tails are dark in color, mottled on the backs with flecks of white. The head is much smaller than the thorax and is somewhat pear shaped. The antennae do not hang down like those of non anopheline, but arise from the sides of the head, and project straight forward. On the dorsal surface of the third to seventh segments of the abdomen are short rosettes of hair, the so-called palmate hairs, which serve as aids to keep the larva afloat. All species considered here have more than one pair of tufts on the breathing tube, which differentiates them immediately from the larvae of the other divisions. In all species of *Culex,* the antennal tuft projects from an offset about two-thirds the distance from base to tip, the antenna being thick at the base, the part beyond the tuft slender.

The larvae of the genus *Aedes* are separated on the basis of the character and arrangement of the scale patch and the siphonal spine. In *Aedes*, the scale patch consists of few to many pointed scales, not finely fringed as in *Culex*. There is only one pair of tufts beyond the spines on the breathing tube. The antenna is usually uniformly tapering, with the tuft at or near the middle. *Aedes aegypti* is long and grayish, usually with a darker head and breathing tube when fully grown. It moves with a characteristic larva when disturbed, jerking from side to side in a wide arc as it descends to the bottom. When breathing, it hangs almost straight down from the water surface. The dorsal head hairs have a single hair in the middle instead of the usual tuft. The scale patch of the eighth abdominal segment is composed of 10 black spines in a single row. Each

spine has a pointed base and long terminal spine with several curved spines at each side. The breathing tube is short, stout, somewhat barrel shaped, a little more than twice as long as broad, with a double row of spines at its base, followed by a single pair of tufts. The spines are long, with two large and a few small teeth near the base.

7.1.3. Egg Identification

An *Aedes* species egg is about 0.8mm in length and under magnification looks like mouse droppings and could be found alone or in groups up to several hundred. These eggs can be seen by the naked eye however it would be hard to count or remove without either a magnifying glass or a microscope. Aedes species eggs can remain out of the water for extended periods and remain fertile. With the addition of water, these eggs will be able to hatch, unless they've gone into diapause (a resting period) for the winter. Mosquitoes lay eggs individually (*Anopheles*), in rafts (Culex) on the water and individually pasted above the waterline (Figure 5).

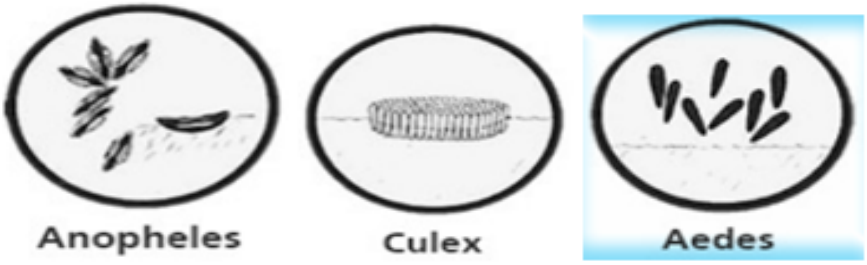


Figure 8. Anopheline and culicilin eggs

Traditionally, egg identification can be conducted in water containers and other diversified locations. This can be through placing a paddle (stick) in a partially-filled water container for Aedes mosquitoes to lay eggs on and Paper lining can be a better option, as it provides more surface area**.**

To identify *Aedes* mosquito eggs:

Set and collect eggs from natural and artificial ovitraps. Remove oviposition materials or papers and transport to the laboratory in a plastic bag containing a moist sponge or paper towel to maintain humidity. Affix the portion of the oviposition paper containing eggs to a microscope slide using double-sided tape. If the number of eggs on the oviposition paper is low, all eggs can be examined. If the number of eggs on the oviposition paper is high, a sample of eggs can be tested. Examine the microscope slide under a compound microscope using a light source that illuminates from above. It is helpful to toggle between 10x and 40x when viewing the eggs.

7.2. Sample handling and transportation

Adult mosquitoes are always preserved dry to allow these features to be observed. The traditional method of preparing adult mosquitoes is by pinning: the mosquito is killed by exposure to Ethanol or alcohol. All collected mosquitoes would be identified to species or genera level and have to be preserved with Liquid nitrogen gas or 100% RNA later in an ice pack containing a box and kept at -70 to -80 ºC for both transportation and storage in the laboratory. To determine if a mosquito has any arboviruses, it is necessary to extract this RNA from the mosquito. RNA can degrade rapidly at room temperature, so mosquitoes collected from the field must be stored appropriately as soon as possible. This can be done by using cold temperatures (-80°C freezers, liquid nitrogen and dry ice) or commercially available reagents that preserve RNA. Stored mosquitoes can then be processed in the laboratory.

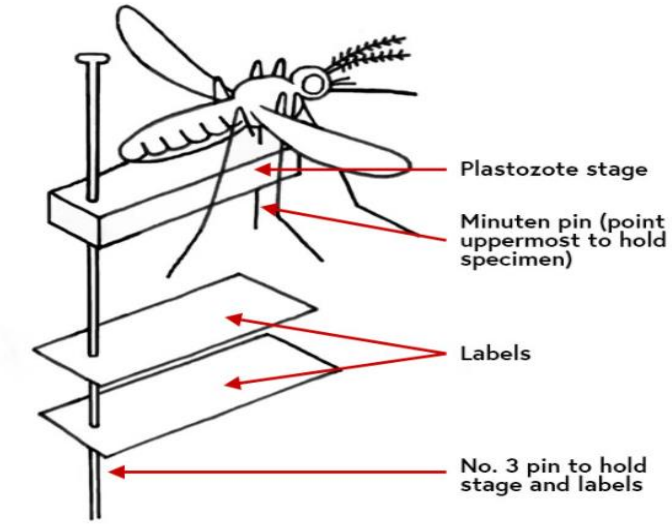


Figure 6. Shows preparing adult mosquitoes by pinning for identification.

Mosquito larvae should not be presented dry but in water soluble media, such as Berlese's fluid, Gater's fluid or Hoyer's fluid. To prepare the specimen, sufficient fluid should be placed on a microscope slide and the larva lowered into its dorsal side up. A coverslip can then be applied over the specimen, and this can be sealed with a waterproof substance such as nail varnish. Finally, the slide should be labeled with details of the specimen.

7.3. Molecular Identification

Continuous evaluation of possible arboviral transmission risk and timely application of correct mosquito control is must be based on surveillance of mosquito species in their corresponding immature stages. Adult specimens from various sites that have been preserved for a long time may have lost somebody parts or features (e.g. Scales, legs, etc.) which are important for accurate morphological identification. This could make species identification very boring because taxonomic experts, therefore, have to be consulted to verify every identified species.

However, polymerase chain reaction (PCR)-based genetic assays are relatively easy and less expensive to develop and use in both laboratory and field situations. Eventually, they may offer an intense device for carrying out meaningful surveillance of vital vector species without the necessity of basing identification only on adult stages. Mosquito researchers and public health organizations that need to rapidly identify numerous numbers of samples or different samples that may contain various vector species can use PCR as opposed to utilizing conventional tedious classifying and identification techniques.

DNA extraction

DNA molecules are extracted from the whole body of mosquitoes using a DNA extraction kit. Then, the extracted DNA molecules are treated with RNaseeI according to the manufacturer's instructions and stored at -20°C

DNA isolation from frozen live or ethanol preserved mosquito

1. For grinding specimens, use a razor blade to cut off the cap and rim of a 0.67 mL micro centrifuge tube. Make a pestle by inserting a 1.5mL blue pipette tip into the smaller 0.67mL micro centrifuge tube (this is a cheaper and time saving alternative to using and re-sterilizing ground glass homogenizers).

2. Rinse alcohol-preserved specimens in sterile distilled water briefly to remove excess alcohol. Frozen or live animals can be ground directly.

3. Grind animal on the inside wall of the larger tube (with pestle) in 20-30 µL of Lysis buffer. Once sufficiently ground, add the Lysis buffer to a total volume of 1ml. Add 100 µl of 10% SDS and 4 µL of Proteinase K (20 mg/ml) [keep enzyme mixture on ice] and mix.

4. Incubate at 55°C with rotation/shaking for a minimum of 4 hours to overnight. It can be used to extract DNA from small portions of larger crustaceans or insects. All steps are performed at room temperature unless specified.

Steps:

5. Add 500 µL of equilibrated phenol (pH 8.0) and shake the tube well for 5-10 min.

6. Centrifuge for 10 min at 12,000 rpm in a micro centrifuge.

7. Remove the aqueous phase (usually the top layer) and place it into a fresh tube. Try not to collect any portion of the bottom layer when the sample is transferred. Repeat steps 3-5 on the aqueous sample once (or until the interface between the lower organic phase and the upper aqueous phase is clean).

8. Add 500 µL of chloroform, place it on the rotator for 5-10 min, and centrifuge for 10 min. Remove the aqueous phase (top layer) and place it in a new tube.

9. At this point you can stop and continue with the DNA precipitation steps at a later time, or amplify directly from the sample (if concerned about losing DNA at precipitation steps, storage is not recommended for long term).

10. Add cold 100% ethanol to the sample tube from step 6 above (fill to the top).

11. Place at -80°C for 15 min.

12. Centrifuge for 25 min at 12,000 rpm in a micro centrifuge.

13. Discard alcohol carefully by pipetting, without disturbing the pellet (if the pellet is visible). Check pipette tip before ejecting to ensure that pellet was not drawn into pipette tip during alcohol removal. If the pellet is inside the pipette tip, return the pellet to the tube and spin again.

14. Add 1000 µL of 70% ethanol and vortex for a few sec.

15. Centrifuge at 12,000 rpm in a micro centrifuge for 10 min. Check for pellets as above.

16. Air dry at room temperature or place in a vacuum centrifuge without heat.

17. Re suspend DNA pellet in 100 µL sterile water.

18. Place the DNA sample in a 50-55°C water bath for 5-10 min to dissolve DNA pellet before amplification. Thoroughly mix DNA samples before any further procedures. Use 1-2 µL samples for PCR.

19. Keep a working DNA sample in the refrigerator (i.e., 30 µL aliquot) to avoid freeze/thaw cycles and place the remainder of the DNA sample at -20°C for future use.

Insect preparation: This preparation can be used on small insects and whole small insects (or part of a larger insect, such as a leg) is ground with a pipette tip in 100 µL of "quick and dirty" extraction buffer (1X PCR Buffer) in a 1.5mL tube.

- ❖ The ground insect in solution is placed in a boiling water bath for 15 min.
- ❖ The tube is centrifuged at 14,000 rpm to get rid of debris.
- ❖ The supernatant containing crude DNA preparation is ready for PCR and can be transferred to a new tube.
- ❖ The crude DNA solution is stored at -20°C. Different concentrations of the crude preparation need to be tested for PCR amplification.

Polymerase chain reaction

All Polymerase Chain Reactions (PCRs) are performed in a total volume of 20 µl for 35 cycles, using 100–200 ng of genomic DNA in each reaction as templates. The reaction mixture contained 400 nM of each primer, 1.5 mM of MgCl2, 1 unit of Taq DNA polymerase, 0.2 mM of dNTPs, and 2 µl of 10 X reaction buffer, with the final volume being adjusted to 20 µl with Double Distilled Water (DDW). The amplification program was set as follows: 5 min at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 80 s, and an additional final extension at 72 °C for 10 min. The amplified amplicons are purified using the DNA gel purification kit (GF-1 Vivantis, Malaysia). To confirm the expected size band, PCR assay is sequenced using the appropriate primers. The expected size bands after gel purification are sequenced and subsequently analyzed and assembled via Chromas (version 2.31, 2005), DNA star (version 7.10, 2006), and BLAST by NCBI online. The

amplicons with sizes close to the predicted range were sequenced using forward and reverse Gene Specific Primers (GSPs) in two sequencing readings.

Aedes species-specific diagnostic PCR assay

The ability to discriminate between *Aedes* species using morphological characteristics may be compromised if distinctive coloration is worn away or if specimen damage occurs. At the molecular level, the multi-copy variable second internal transcribed region (ITS2) of ribosomal DNA can be used to discriminate between different species. Because this assay is based on sequence variations within the region, it is possible for PCR amplification of specimens other than *Ae. aegypti* and *Ae. albopictus* may yield similar results.

PCR cycle conditions 94°C/5min x 1 cycle (95°C/1min, 54°C/30sec, 72°C/1min) x 35 cycles 72°C/5min x 1 cycle 4°C hold.

Prepare PCR Master Mix for 1.25 µL PCR reactions. Add reagents in the order presented

96	48	1	Reagent
1590 µL	795 µL	15.9 µL	Distilled H_2O
500 µL	250 µL	5.0 µL	5X PCR buffer
100 µL	50 µL	1.0 µL	DNTP(2Mm concentration)
50 µL	25 µL	0.5 µL	CP-P1A (10pmol/ µL)-F[GTGGATCCTGTGAACTGCAGGACACATG]
50 µL	25 µL	0.5 µL	CP-P1B(10pmol/ µL)-R[GTGTCGACATGCTTAAATTTAGGGGGTA]
100 µL	50 µL	1.0 µL	Mgcl2(25Mm)
10 µL	5 µL	0.1 µL	Promega Go Taq DNA polymerase (5U/ µL)
2.4ml	1.2 ml	24 µL	Total (To each 24 µReaction add 1 µL template DNA)

Run samples on a 1.5% agarose gel stained with EtBr or another intercalating agent such as SYBR Green or Gel Red

Mitochondrial DNA PCR Assays for *Aedes* Mosquitoes

Mitochondrial DNA (mtDNA) is one of the most commonly studied regions in insect systematics due to its high rate of homoplasmy. Within the mtDNA, there are several segments of which only a few are routinely examined in aedines: cytochrome c oxidase I (COI), cytochrome c oxidase II (COII), cytochrome b, and NADH ubiquinone oxidoreductase

Cytochrome oxidase I (COI)

PCR cycle conditions 95°C/5min x 1 cycle (97°C/30s, 40°C/45s, 72°C/60s) x 35 cycles 72°C/5min x 1 cycle 10°C hold.

Prepare PCR Master Mix for 1, 48, or 96 25µl PCR reactions. Add reagents in the order presented

96	48	1	Reagent
1587.5 µl	793.75 µl	15.875 µl	Distilled H_2O
500 µl	250 µl	5 µl	5X PCR buffer
100 µl	50 µl	1 µl	dNTP (2mM concentration)
100 µl	50 µl	1 µl	$MgCl_2$ (25 mM concentration)
100 µl	50 µl	1 µl	CI-J-1632 (10pmol/µl)
100 µl	50 µl	1 µl	CI-N-2191 (10pmol/µl)
12.5 µl	6.25 µl	.125 µl	GoTaq DNA polymerase (5U/ µl)
2.5 ml	**1.25 ml**	**25 µl**	**Total (to each 25 µl reaction add 1 µl template DNA)**

Run samples on a 1.5% agarose gel stained with an intercalating agent such as EtBr, load 10µl of the sample. You will expect an approximately 597 bp product.

NADH ubiquinone oxidoreductase

PCR cycle conditions 98°C/2min x 1 cycle (95°C/30s, 45°C/30s, 72°C/45s) x 5 cycles (95°C/30s, 46°C/45s, 72°C/45s) x 28 cycles 72°C/5min x 1 cycle 10°C hold

Prepare PCR Master Mix for 1, 48, or 96 25µl PCR reactions. Add reagents in the order presented

96	48	1	Reagent
1587.5 µl	793.75 µl	15.875 µl	Distilled H_2O
500 µl	250 µl	5 µl	5X PCR buffer
100 µl	50 µl	1 µl	dNTP (2mM concentration)
100 µl	50 µl	1 µl	$MgCl_2$ (25 mM concentration)
100 µl	50 µl	1 µl	ND5F (10pmol/µl)
100 µl	50 µl	1 µl	ND5R (10pmol/µl)
12.5 µl	6.25 µl	.125 µl	GoTaq DNA polymerase (5U/ µl)
2.5 ml	**1.25 ml**	**25 µl**	**Total (to each 25 µl reaction add 1 µl template DNA)**

Run samples on a 1.5% agarose gel stained with an intercalating agent such as EtBr, load 10µl of the sample.

Note that: Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 reactions to compensate for imprecise measurements.

8. Detection of arbovirus in mosquito vectors by real-time PCR

The real-time PCR is an advanced version of the classical PCR. It is based on the binding of a fluorescent signal to the accumulation of amplification products in the reaction. The accumulation of the signal is measured and displayed as a graph over time.

Identified mosquitoes should be identified to species morphologically or through molecular techniques. Blood-fed individuals have to be separated from gravid and unfed individuals and pooled according to environmental/habitats, collection geography and time. Non-blood-fed female mosquitoes have to be pooled into groups of 50 or less, by species, location of collection and tested for Arboviral RNA using reverse transcription, real-time polymerase chain reaction (PCR). The Mosquitoes have to be homogenized by adding 1mL of a 50:50 mixture of phosphate-buffered saline (PBS) and 2X lysis buffer and three steel shots using a high-speed mechanical homogenizer (Retsch MM 300) for 4 minutes at 20 cycles/second. Each homogenized pool will be centrifuged for 2 minutes at 13,000 rpm. RNA will be extracted from mosquito pools using an ABI Prism 6100 Nucleic Acid and eluted in a final volume of 60 µL of elution solution. A region of the Viral RNA envelope gene will be detected using real-time, reverse transcription-polymerase chain reaction (RT-PCR). The thermocycling will be performed on an ABI Prism 9700HT sequence detector following the TaqMan One-Step RT-PCR Master Mix. The amplification threshold has to be determined by serial dilutions of controls and a maximum cycle threshold for samples to be considered positive will be estimated. Mosquitoes can be processed during the RNA extraction and RT-PCR on 96-well plates and each plate contains at least two positive extraction controls and an additional RT-PCR positive control. All plates had at least 10 negative controls (blanks) distributed evenly on the plate for quality control. Maximum likelihood estimates for infection rates have to be calculated using the Pooled Infection Rate.

Viral RNA extraction and DNA synthesis

RNA has to be extracted from pooled mosquito samples using the QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. 140 uL of serum was added to 560 uL AVL buffer containing carrier RNA into a 1.5 uL microcentrifuge tube and mixed by pulse-vortexing for 15 sec followed by incubation at room temperature for 10 min. Protein precipitation was followed by adding 560 uL of absolute alcohol mixed by pulse-vortexing for 15 sec. The lysate is then passed through the silica-gel column, and the column has

to be washed twice with 500 uL of washing buffers AW1 and AW2, respectively. Finally, RNAs are carefully eluted by 60 uL of buffer AVE equilibrated temperature and the extracted mosquito RNA should be stored at -20˚C before amplification. To convert extracted RNA into cDNA, 4 uL of RNA template is combined into 16 uL of a master mix containing 4uL of 5XVILO Reaction Mix, 2uL of 10xSuperscript Enzyme Mix and 10uL nuclease free water; the whole volume was brought up to 20uL. The tube contents are mixed by vortexing for 15 seconds and then incubated in the thermocycler at the following conditions: 25°C for 10 min, 42°C for 60 min; the reaction is terminated at 85°C for 5 min and 4°C as the hold temperature.

Arbovirus detection by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The cDNA amplicons are used for RT-PCR amplification (AgPath-ID™ One-Step RT-PCR Kit, Applied Biosystems, USA) using primers targeting virus genera or specific arboviruses. A total of 20 uL of master mix need to be prepared containing 10uL of 2xDream DreamTaq green PCR Master Mix (Thermo Scientific, Calsband, USA), 1uL of both forward and reverse primer, 1uL of cDNA and 7 uL of nuclease-free water up to 20uL. Primers targeting Bunyavirus, Alphavirus and Flavivirus (Table I) were used to detect the presence of arbovirus specific genera. For samples that tested positive with genus primers, were tested further with primers that target conserved genes in the specific viruses belonging to the genus in question (Table II). The PCR cycling performed was the following: an initial denaturation step at 94°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing temperatures at 57°C for 60 seconds each, extension at 72°C for 30sec. The reaction mixture in each PCR tube is then subjected to a final extension step at 72°C for 10 min. the PCR amplification of the targeted gene for a specific virus in the cDNA is performed in a 25 uL reaction containing: 12.5 uL of 2xDream Taq Green PCR master mix (Thermo Scientific), 0.5 uL each of forward and reverse primer, 2 uL of the cDNA and 9.5 uL of water to top up to 25 uL. thermal profiles are performed on a Gene Amp PCR system 9700 (Applied Biosystems, USA). PCR products are then separated by electrophoresis and visualized under UV light.

Table 2: DNA sequences of the primers used for detection of arbovirus genera

Virus	Target gene or protein	Primer	Sequence (5'→3'	Position	PCR product size (bp)
Alphavirus	NSP4	VIR2052F	TGG CGC TAT GAT GAA ATC TGG AAT GTT	6971-6997	150
		VIR2052R	TAC GAT GTT GTC GTC GCC GAT GAA	7086-7109	
Bunyavirus	N Protein	BCS82C	ATC ACT GAG TTG GAG TTT CAT GAT GTC	86-114	251
		BCS332V	GCTGT TCC TGT TGC CAG GAA AAT	309-329	
Flavivirus	NS5	FU1	TAC AAC ATG ATG GGA AAG AGA GAG AA	9007-9032	220
		CFD2	GTG TCC CAG CCG GCG GTG TCA TCA GC	9308-9283	

Table 3: DNA sequence of the primers used for screening of specific viruses

Virus	Primers	Sequence (5'-3')	Region, position
CHIKV	CHIK3F	CAC ACG TAG CCT ACC AGT TTC	5'NTR, 14-112
	CHIK3R	GCT GTC AGC GTC TAT GTC CAC	
DENV	D1 38-65	TCA ATA TGC TGA AAC GCG CGA GAA ACC G	3'UTR, 10520-10541
	D2 455-483	TTG CAC CAA CAG TCA ATG TCT TCA GGT TC	3'UTR, 10674-10694
RVFV	RVF3	CAG ATG ACA GGT GCT AGC	Gn, 876
	RVF4	CTA CCA TGT CCT CAA T	GlyM, 2817-2840
YFV	CAG	CGA GTT GCT AGC AAT AAA CAC ATT TGG A	Polypro. 43 - 71
	YF7	AAT GCT CCC TTT CCC AAA TA	Polyprot. 1293-1312

9. Testing insecticide susceptibility of the vectors

Mosquito sampling and rearing

Representative sites need to be identified in arboviral diseases affected areas for the assessment of the insecticide resistance status of Aedes populations. These should include neighborhoods with the highest insecticide application in public health. Larval stages are easier to collect from the most productive breeding sites and should be kept alive and taken to a local or centralized insectary facility for rearing. Usually, the second or F2 generation is used, as enough larvae or adult mosquitoes are needed for the necessary tests. Only female mosquitoes should be used in

the tests. It is recommended that susceptibility tests be performed on non-blood fed females of 3–5 days old.

Testing Conditions

The optimum conditions for phenotypic susceptibility tests are 27 ± 2°C temperature, 75 ± 10 % relative humidity and low illumination that are usually maintained in an insectary. Where such infrastructure is not available, the tests should be done indoors in a building free from insecticidal contamination while maintaining optimum humidity and temperature using local procedures and avoiding extreme illumination and wind. Where possible, subsequent comparison tests should be made under similar conditions of temperature and humidity.

Materials

- 1 mosquito cage
- Live female mosquitoes (140 healthy specimens needed for testing)
- 14 plastic tubes (125mm L x 44mm D)
- 7 steel spring-wire clips
- 7 copper spring-wire clips
- Red marker, or 5 red dot stickers
- Yellow marker, or 2 yellow dot stickers
- 14 sheets clean white paper (12 x 15 cm)
- 5 sheets insecticide-impregnated paper
- 2 sheets oil-impregnated control paper
- 7 pads of cotton wool
- 10% sugar water solution
- 140 Eppendorf tubes 4

Procedures for susceptibility testing

1. Prepare seven holding tubes by rolling seven sheets of clean white paper (12 x 15 cm) into a cylinder shape. Individually insert each cylinder into a separate holding tube, and fasten it into position with a steel spring-wire clip. Attached the tubes to slides.
2. In each of the holding tubes, aspirate 20 active female mosquitoes (in batches) from a mosquito cage through the filling hole in the slide. After the mosquitoes have been transferred, close the slide unit and set the holding tubes in an upright position for one hour at optimum test conditions. After one hour, replace any knocked-down, dead or damaged mosquitoes with healthy ones.
3. Prepare seven additional tubes in the same manner as the holding tubes. Mark five with a red dot (exposure tubes) and two with a yellow dot (control tubes). Line each exposure tube with a sheet of insecticide-impregnated paper, and the two control tubes with a sheet of oil impregnated control paper. Fasten the papers to each tube with a copper spring-wire clip.

4. Attach the five exposure tubes to the vacant position on the slides, and open the slide unit. Blow the mosquitoes gently into the exposure tubes. Once all the mosquitoes are in the exposure tubes, close the slide unit, detach the exposure tubes and set them upright. Fill the two control tubes with mosquitoes in the same way.
5. Keep the mosquitoes in the exposure and control tubes for one hour. Make sure that the tubes are set in an upright vertical position with the mesh-screen on top.

6. After one hour, transfer the mosquitoes back to the holding tubes by reversing the procedure outlined in Step 4. Set all the holding tubes upright, with the mesh-screen on top. Soak a pad of cotton wool in 10% sugar water solution and place it on the mesh-screen.

7. Maintain the mosquitoes in the holding tubes for a 24-hour recovery period, under the optimum conditions previously described. It is important to keep the holding tubes in a place free from extreme temperature and humidity, ideally in an insectary facility. Temperature and humidity should be recorded during the recovery period.

8. At the end of the 24-hour recovery period, count and record the number of dead mosquitoes (refer to Annex 1 for definitions of knockdown and mortality). If mosquito mortality in the control tubes exceeds 10%, correct the mortalities of all treated groups using Abbott's formula (below). Discard the test and repeat if the corrected mortality in the control tubes exceeds 10%.

$$Corrected\ mortality = \frac{\%\ mortality\ with\ treated\ paper\ -\ \%mortality\ in\ control}{100\ -\ \%mortality\ in\ control} x\ 100$$

9. If supplementary tests (biochemical or molecular) are necessary after completing the susceptibility test, transfer each mosquito (dead or alive) to an individual, clearly labeled Eppendorf tube. Refrigerate and store the tubes until they can be processed for supplementary testing.

10. Corrected mortality (%) = % mortality with treated paper — % mortality with control 100 — % mortality with control X 100

Interpretation of results

In light of new knowledge and the need for prompt action to counter the spread of resistance among vector populations, guidance on interpreting the results of the WHO bioassay test has been revised. The current recommendations are as follows:

- Mortality between 98–100%: Susceptibility is indicated
- Mortality less than 98%: Resistance suggested. Further tests are needed to verify.
- Mortality between 90%–97% (corrected if necessary): Presence of resistant genes in the vector population must be confirmed. The confirmation of resistance may be obtained by performing additional bioassay tests with the same insecticide on the same population or on the progeny of any surviving mosquitoes (reared under insectary conditions) and/ or by conducting molecular assays for known resistance mechanisms. If at least two additional tests consistently show mortality below 98%, then resistance is confirmed.
- Mortality less than 90%: Confirmation of the existence of resistant genes in the test population with additional bioassays may not be necessary, as long as a minimum of 100 mosquitoes were tested. However, further investigation of the mechanisms and distribution of resistance should be undertaken.

Procedures to test larvae

Larvae collection

Aedes larvae can be collected from the most productive breeding containers by using dippers or by the use of ovitraps. The larvae must be sorted into different instars in the insectary.

Test Conditions

The optimum conditions for larvae susceptibility tests are similar as for adult mosquitoes: a proper insectary facility in which enough larvae are reared for the tests is required. This should include a bench in which the testing cups can be maintained without disturbance during the tests. Note that for some insecticides such as insect growth regulators, the tests could last around 2 weeks. 27 ± 2oC temperature, 75 ± 10 % relative humidity and low illumination that are usually maintained in an insectary. Where such infrastructure is not available, the tests should be done indoors in a building free from insecticidal contamination while maintaining optimum humidity and temperature using local procedures and avoiding extreme illumination and wind. Where possible, subsequent comparison tests should be made under similar conditions of temperature and humidity.

Materials

- 140 x 3th –4th instar larvae
- 1 pipette capable of delivering 100–1000 µl
- 5 x 1 ml pipettes (insecticides) ·
- 1 x 1 ml pipette (control)
- 100 x 100 µl disposable tips
- 100 x 500 µl disposable tips
- 3 droppers with rubber suction bulbs
- 1 small strainer or a loop of plastic screen
- 7 disposable cups (if not available, use 120ml and 250ml glass bowls/beakers)
- 1 graduated measuring cylinder
- Data recording forms
- Log–probit software or paper
- Alcohol (or organic solvent)

Insecticide and bacterial larvicide susceptibility tests

Mosquito larvae can be exposed to a wide range of insecticide concentrations to test the activity range of the insecticide. After determining the mortality of larvae in this range of concentrations, a narrower range (of 4–5 concentrations, yielding between 10% and 95% mortality in 24 or 48 hours) is used to determine LC50 and LC90 values. The same procedure is carried out for a susceptible strain and a field test population. Procedures for insecticide and bacterial larvicide testing.

Procedures for larvicide testing

1. Dilute the appropriate volume of insecticide with 100ml or 200ml of water in disposable test cups or vessels to obtain the desired target dosage, starting with the lowest concentration.
2. Transfer 20 late 3rd/early 4th instar larvae to each cup. Small, unhealthy or damaged larvae should be removed and replaced.
3. After 24 hours of exposure, record larval mortality. For slow acting insecticides, 48hour exposure may be required. Count moribund larvae and add to numbers of dead larvae to calculate mortality percentage. Dead larvae are those that cannot be induced to move when they are probed with a needle in the siphon or the cervical region. Moribund larvae are those incapable of rising to the surface or not showing the characteristic diving reaction when the water is disturbed.
4. Four or more replicates are set up for each concentration and an equal number of controls are set up simultaneously with tap water, to which 1 ml alcohol (or the organic solvent used) is added. Each test should be run three times on different days.

5. If more than 10% of the control larvae pupate in the course of the experiment, the test should be discarded and repeated. If the control mortality is between 5% and 20%, the mortalities of treated groups should be corrected according to Abbott's formula (below).

$$Corrected\ mortality = \frac{\%mortality\ in\ test\ -\ \%mortality\ in\ control}{100 - \%mortality\ in\ control} x\ 100$$

6. Lethal concentrations (LC) which result in 50%, 90% and 99.9% mortality (LC50, LC90 and LC99.9) values are calculated from a log dosage–probit mortality regression line using computer software programs, or estimated using log probit paper.

7. When using a susceptible strain, twice the calculated LC99.9 is used as a discriminating or diagnostic concentration to test resistance in field populations. For example, the discriminating concentration for the insecticide temephos has been established as 0.012 mg/L. If a rapid assessment of the status of temephos resistance of a field population is required, this discriminating dose could be used.

8. Resistance ratios (RR) are often calculated and useful to monitor the evolution of insecticide resistance in a field population. The way to calculate a RR is to divide the LC50 of the field population by the LC50 of a susceptible strain. When RR is 10 the mosquitoes are highly resistant.

Insect growth regulators (IGRs) tests

IGRs have a delayed action on treated larvae. These juvenile hormone analogues interfere with the transformation of late instar larvae to pupae and then to adults, whereas chitin synthesis inhibitors inhibit cuticle formation and affect all instars and immature stages of the mosquito. In IGRs tests, mortality is assessed every two or every three days until adult emergence, or mortality in immature stages. An accurate initial count of larvae is essential because of the cannibalistic or scavenging behavior of larvae during the long exposure period. Larvae should also be provided with a small amount of food at two day intervals until the test ends. The effect of IGRs on mosquito larvae is expressed in terms of the percentage of larvae that do not develop into successfully emerging adults, or adult emergence inhibition (IE%). The same procedure is carried out for a susceptible mosquito strain and a wild/field population.

Procedures for IGR testing

1. Expose third instar larvae to a range of IGR concentrations, in the same way as chemical larvicide test procedures.

2. Count mortality or survival every two or every three days until the complete emergence of adults, or mortality in immatures.

3. At the end of the observation period, calculate the impact of the IGR (expressed as IE%) based on the number of larvae that do not develop successfully into viable adults. In recording IE% for each concentration, moribund and dead larvae and pupae, as well as adult mosquitoes not completely separated from the pupal case, are considered as "affected". The number of successfully emerged adults may also be counted from the empty pupal skins. The test is terminated when all the larvae or pupae in the controls have died or emerged into adults. Any deformities or morphogenetic effects that occur in moulting immature mosquitoes or emerging adults are also recorded.

4. Combine the data from all replicates of each concentration. Total or mean emergence inhibition can be calculated on the basis of the number of third stage larvae exposed. The overall emergence of adults reflects activity. IE% is calculated using the following formula:

$$\%IE = 100 - \frac{\%adult\ emerged\ in\ treated\ batches\ x100}{\%adult\ emerged\ in\ control\ batches}$$

5. If adult emergence in the control is less than 90%, the test should be discarded and repeated. Where the percentage is between 91% and 99%, correct the data using Abbott's formula.

6. Perform probit regression analysis on IE values obtained at each concentration to determine IE50 and IE90 values (using computer software programs or estimated from a log–probit paper).

7. Resistance ratios (RR) can be used to monitor the evolution of insecticide resistance in a field population. Calculate a RR by dividing the IE50 of the field population by the IE50 of a susceptible strain. RR <5 indicates a susceptible field population; RR from 5—10 indicates moderate resistance, and RR >10 indicates high resistance.

10. Managing Insecticide Resistance

While non-insecticide-based tools for controlling *Aedes* are available and should be prioritized, the use of insecticides is necessary as a rapid intervention to interrupt disease transmission, particularly during outbreaks of mosquito-borne disease. Few insecticide active ingredients are available for public health use, and to minimize the impact of insecticide resistance in a control

program, appropriate decisions need to be made. Usually, the first option to be selected by a control program is the least expensive insecticide with the highest effectiveness against the vector populations, which also has a low risk to applicators and bystanders. Development of insecticide resistance drives a change to more expensive options, compromising coverage. In the absence of insecticide pressure, insecticide resistance may be reduced or reverted. It is this reversion to susceptibility which is the underlying assumption behind any effective resistance management strategy. However, reversion rates are variable and may be very slow, particularly when a chemical insecticide has been used for many years. Insecticide resistance management can be undertaken using insecticides in conjunction with other nonchemical insecticidal vector control methods. This approach is suitable for Aedes mosquitoes since different tools are available for adult and larval control, including chemical insecticides with unrelated classes/modes of action such as IGRs or biologically derived toxins (i.e. Bti, spinosad) for larval control. Options to prevent or limit the development of insecticide resistance include

Insecticide rotation: To preclude the emergence of resistance, insecticides of unrelated classes with different modes of action should be sprayed in rotation, ideally in a bi-annual cycle. This is good practice and should be implemented wherever possible, even before resistance has been reported.

Combining interventions: As adult and larval control must be done simultaneously to have an impact on vector populations, different classes of insecticides with different modes of action should be used for each stage (e.g. using an organophosphate as an adulticide, combined with a Bti or Bti+Bs, or IGR as larvicides).

Mosaics: One compound is used in one geographic area, and a compound of different insecticide classes and modes of action (i.e. organophosphate and pyrethroid) are used in neighboring areas. This strategy, while theoretically robust, is logistically difficult to implement – especially in an epidemic setting where rapid response is key to contain a disease outbreak. Resistance management and mode of action in order to successfully develop and implement rotation, combination or mosaic resistance management strategies, knowledge of the mode of action and/or chemical class of the available insecticide products is essential. Knowledge of the resistance mechanism developed by the local population of mosquitoes is also important, since cross-resistance may occur between different classes of insecticides that share the same resistance mechanism. Synergist assays, biochemical and molecular tests are available for this

purpose. Collaborative work with academic or research institutes will help control programs generate this useful information.

11. Vector control

The Aedes mosquito uses a wide range of confined larval habitats, both man-made and natural. However, it may not be feasible or cost-effective to attempt to control the immature stages in all such habitats in a community. Some man made container habitats produce large numbers of adult mosquitoes, whereas others are less productive. Consequently, control efforts should target the habitats that are most productive and hence epidemiologically more important rather than all types of containers, especially when there are major resource constraints. Such targeted strategies require a thorough understanding of the local vector ecology and the attitudes and habits of residents pertaining to the containers.

Different containers require specific control measures that depend on the nature of the container and how it is used. Five general types of containers produce *Aedes* mosquitoes: -

- Phytotelmata (tree holes, leaf axils, etc.)
- Non-essential or disposable containers (food and drink containers, tires, broken appliances)
- Useful containers (water storage vessels, potted plants and trivets, animal drinking pans, paint trays, toys, pails, septic tanks)
- Cavities in structures (fence poles, bricks, uneven floors and roofs, roof gutters, air-conditioner trays)
- Outdoor underground structures (storms drains, water meters, public wells, septic tanks)

11.1. Prioritization of areas for intervention

- **Priority 1:** localities where an outbreak of Arbovirus disease had occurred.
- **Priority 2:** localities high larval indices **HI ≥5%** and/or **BI ≥20.**
- **Priority 3:** localities relatively low larval Indices **HI <5%** and/or **BI <20.**
- **Priority 4:** localities where there are no arbovirus cases and low *Aedes* densities.

11.2. Environmental or Larval Source Management

Larval source management (LSM)) is the management of aquatic habitats that are potential larval habitats for mosquitoes to prevent the completion of development of the immature stages. Environmental management seeks to change the environment to prevent or minimize vector propagation and human contact with the vector pathogen by destroying, altering, removing or recycling non-essential containers that provide larval habitats.

Habitat modification: a permanent alteration to the environment, including landscaping, surface water drainage, filling and land reclamation, coverage of water storage containers with mosquito-proof lids or permanent slabs and coverage of the water surface with impenetrable to vectors,

Habitat manipulation: a recurrent activity including water level manipulation, e g. flushing streams, drains,

Changes to human habitation or behavior: actions to reduce human–vector contact, such as installing mosquito screening on windows, doors and other entry points, and using mosquito nets while sleeping during daytime.

Mosquito-proofing of water-storage containers: Water storage containers can be designed to prevent access by mosquitoes for oviposition. Containers can be fitted with tight lids or if rain-filled, tightly-fitted mesh screens can allow for rainwater to be harvested from roofs while keeping mosquitoes out.

Repellents: repellents have also been proposed for use in high-risk groups, such as pregnant mothers. Despite the potential to provide individual protection against bites from arboviral vectors, the deployment of the above personal protective methods in large scale public health campaigns has been limited, at least partially due to the scarcity of evidence of their public health value. Daily compliance and appropriate use of the repellents seem to be major obstacles to achieving such potential impact. Repellents are a common means of personal protection against mosquitoes and other biting insects. The formulations (lotions or sprays) based on the following active ingredients are recommended by WHO: - DEET (N, N-diethyl-3-toluamide)- IR3535- icaridin (KBR 3023 or Picaridin)- dimethyl phthalate, benzyl benzoate, dimethyl carbamate and ethyl hexanediol.

Treated clothing: treated clothing has not been evaluated by WHO. They aim to reduce the risk of mosquito biting. School children should adhere to these practices whenever possible.

Impregnating clothing with chemicals such as permethrin can be especially effective in preventing mosquito bites.

Untreated clothing: Wearing long sleeves and trousers with stockings may protect the arms and legs, the preferred sites for mosquito bites.

Mats, coils and aerosols: Household insecticidal products, namely mosquito coils, pyrethrum space spray and aerosols have been used extensively for personal protection against mosquitoes. Electric vaporizer mats and liquid vaporizers are more recent additions that are marketed in practically all urban areas.

Solid waste management

The benefits of reducing the amount of solid waste in urban environments extend beyond those of vector control. Applying many of the basic principles can contribute substantially to reducing *Ae. aegypti* larval habitats. Proper storage, collection and disposal of waste are essential for protecting public health. The basic rule of "reduce, reuse, recycle" is highly applicable. Efforts to reduce solid waste should be directed against discarded or non-essential containers, particularly if they have been identified in the community as important mosquito-producing containers.

Solid waste should be collected in plastic sacks and disposed of regularly. The frequency of collection is important: twice per week is recommended for housefly and rodent control in warm climates. Integration of *Ae. aegypti* control with waste management services is possible and should be encouraged.

Street cleansing: A reliable and regular street cleansing system that removes discarded water bearing containers and cleans drains to ensure they do not become stagnant and breed mosquitoes will both help to reduce larval habitats of *Ae. aegypti* and remove the origin of other urban pests.

11.3. Chemical control

Adulticidals

Methods of chemical control that target adult vectors are intended to impact on mosquito densities, longevity and other transmission parameters. Adulticides are applied either as residual surface treatments or as space treatments.

Residual treatment: Residual surface treatment has both adulticiding and larviciding effects. Suitable insecticides can be applied with hand-operated compression sprayers. Power sprayers

can be used to treat large accumulations of discarded containers (e.g. tyre dumps) rapidly. Care must be taken not to treat containers used to store potable water.

Space spray: space spray, technically a fog, is a liquid insecticide dispersed into the air in the form of hundreds of millions of tiny droplets less than 30μm in diameter. The objective of space spraying is the massive, rapid destruction of the adult vector population. Space spraying is recommended for control only in emergency situations to suppress an ongoing epidemic or to prevent an incipient one. Continuous entomological and epidemiological surveillance should be conducted to determine the appropriate application schedule and the effectiveness of the control strategy.

The efficiency of space spraying is dependent on: - method of release (aircraft, vehicle, hand-held equipment), - fog types (cold or thermal), - droplet size, application rate, climatic conditions, - building structures, configuration and penetration of space sprays, - target area size, - terrain and accessibility, Peak flight times. Space spraying can be applied as (i) thermal fogging or (ii) cold fogging. Formulations such as wettable powders (WP), suspension concentrates (SC) and water-dispersible granules (WG) are unsuitable for space spraying. An appropriate formulation must be chosen and the label instructions carefully followed for all applications.

Formulations for space spraying include:

Hot fogging concentrates (HN) are a formulation suitable for application by thermal fogging equipment, either directly or after dilution.

Ultra-low-volume liquid (ULV) is a homogenous liquid ready for use through ulv equipment which is specially formulated for low volatility.

Emulsion, oil in water (EW) is a heterogeneous fluid formulation consisting of a solution of insecticide in an organic liquid dispersed as fine globules in a continuous water phase.

Emulsifiable concentrate (EC) is a homogenous liquid formulation to be applied as an emulsion after dilution in water or oil.

Larviciding

Larviciding is the regular application of biological or chemical insecticides to water bodies. Although chemicals are widely used to treat *Aedes* larval habitats, larviciding should be considered as complementary to environmental management and except in emergencies should be restricted to containers that cannot otherwise be eliminated or managed. Larvicides may be impractical to apply in hard-to-reach natural sites such as leaf axils and tree holes. For treatment

of drinking water, temephos and methoprene can be applied at dosages of up to 1 mg of active ingredient (a.i.) per liter (1 ppm); pyriproxyfen can be applied at dosages up to 0.01 mg a.i. per liter (0.01 ppm).

Types of Larvicides:

Oils and surface films: these agents include petroleum distillates and monomolecular surface films (mmf) such as isostearyl alcohol made from renewable plant oils. They act by suffocating larvae or disrupting surface tension, inhibiting the ability of larvae to rest and breathe at the surface of the water causing them to drown and interfering with adult emergence. They are considered effective in the control of the larval stage but may be impacted by wind or absorbed by vegetation. These agents will affect any aquatic invertebrate requiring use of the air-water interface for breathing, resting or egg-laying. Re-treatment is needed weekly.

Synthetic organic chemicals: organophosphates are synthetic organic chemicals that can kill mosquito larvae by interfering with the enzyme acetylcholinesterase, which is required to regulate nerve transmission in all organisms. the organophosphate temephos has been used extensively as a larvicide against blackfly larvae in the West Africa onchocerciasis control Program, against copepods in the guinea worm eradication program, and against *Aedes* larvae in domestic water storage containers in dengue control programs. Pyrethroids are toxic to fish and may select for resistance, and therefore must not be used for control of mosquito larvae.

Bacterial larvicides: bacterial larvicides (BL) include products based on the insecticidal crystal proteins produced by bacillus thuringiensis var. israelensis (BTI), and bacillus sphaericus (BS). Upon ingestion by mosquito larvae, these proteins are modified by enzymes in the larval midgut and then bind with specific receptors on the midgut epithelium, resulting in pore formation and interruption of feeding and homeostasis. Frequency of re-treatment with bacterial larvicides can range from 1 to 4 weeks depending on formulation, habitat, temperature, and species.

Spinosyns: spinosyns are metabolites extracted from fermentation using the bacterium saccharopolyspora spinosa. spinosyns acts as a nicotinic acetylcholine receptor (nachr) allosteric activator. It is available as an emulsifiable concentrate, dispersible tablets, granules and suspension concentrate, and has very low acute toxicity to mammals.

Insect growth regulators (IGR): (IGR) have two groups:

1. Juvenile hormone mimics such as methoprene and pyriproxyfen, which prevent the development of larvae and pupae into adults,

2.Chitin synthesis inhibitors such as diflubenzuron and triflumuron, which kill larvae when they moult

3.**Biological control**: the introduction of natural predators into water bodies. Larviciding should be implemented during the population bottleneck of the target vector. For example, when arbovirus transmission is at its lowest, to reduce the density of adult mosquitoes before the rainy season begins. The number of water bodies requiring treatment during winter is reduced as such larviciding becomes more manageable and therefore likely to be cost effective.

12. Bibliography

Alexander N, Lenhart AE, Romero-Vivas CME, Barbazan P, Morrison AC, Barrera R, Arredondo- Jimenez JI, Focks DA. (2006). Sample sizes for identifying the key types of container occupied by dengue-vector pupae: the use of entropy in analyses of compositional data. Annals of Tropical Medicine and Parasitology 100: S5-S16.

Barrera R, Mackay AJ, Amador M (2013). A novel autocidal ovitrap for the surveillance and control of Aedes aegypti. Journal of the American Mosquito Control Association. 29:293-296.

Bangs MJ, Tan R, Listiyaningsih E, Kay BH, Porter KR (2001). Detection of dengue viral RNA in Aedes aegypti (Diptera: Culicidae) exposed to sticky lures using reverse-transcriptase polymerase chain reaction. Journal of Medical Entomology 38:720-724.

Beebe NW, Saul A (1995). Discrimination of all members of the Anopheles Punctulatus complex by polymerase chain reaction-restriction fragment length polymorphism analysis. American Journal of Tropical Medicine and Hygiene 53(5):478–481 DOI 10.4269/ajtmh.1995.53.478.

Burkhalter KL, Savage HM (2017). Detection of Zika virus in desiccated mosquitoes by real-time reverse transcription PCR and plaque assay. Emerging Infectious Diseases 23(4):680-81.

CDC (2016). Zika Virus. http://www.cdc.gov/zika/index.html, Chikungunya Virus, http://www.cdc.gov/Chikungunya/index.html, and Dengue, http://www.cdc.gov/Dengue/, all updated periodically.

Edwards FW (1941). Mosquitoes of the Ethiopian region: III culicine adults and pupae. London, British Museum (Natural History).

Ferrara L, Germain M, Hervy JP (1984). Aedes (Diceromyia) furcifer (Edwards, 1913) et Aedes (Diceromyia) taylori (Edwards, 1936): le point sur la différentiation des adultes. Cahier ORSTOM Série Entomologie Médicale et Parasitologie, 22:95–98.

Gould LH, Osman MS, Farnon EC, Griffith KS, Godsey MS, Karch S, Mulenda B, El Kholy A, Grandesso F, de Radiguès X, Brair ME, Briand S, El Tayeb el SM, Hayes EB, Zeller H, Perea W (2008). An outbreak of YF with concurrent Chikungunya virus transmission in South Kordofan, Sudan, 2005. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 102(12):1247–1254.

Huang YM (1990). The subgenus Stegomyia of Aedes in the Afrotropical region, I. The Africanus group of species (Diptera: Culicidae). Contributions of the American Entomological Institute, 26:1–90.

Huang YM (1986a). Aedes (Stegomyia) bromeliae (Diptera: Culicidae), the yellow fever virus vector in East Africa. Journal of Medical Entomology, 23:196–200.

Huang YM (1986b). Notes on the Aedes (Diceromyia) furcifer group, with a description of a new species (Diptera: Culicidae). Proceedings of the Entomological Society of Washington, 88(4):634–649.

Jupp PG (1997). Mosquitoes of southern Africa: Culicinae and Toxorhynchitinae. Hartbeespoort, South Africa, Ecoglide cc Publishers.

Jupp PG (1998). Aedes (Diceromyia) furcifer (Edwards) and Aedes (Diceromyia) cordellieri Huang in southern Africa: distribution and morphological differentiation. Journal of the American Mosquito Control Association, 14(3):273–276.

Kirk R (1941). An epidemic of yellow fever in the Nuba Mountains, Anglo–Egyptian Sudan. Annals of Tropical Medicine and Parasitology, 35:67–108.

Kimberlin DW, Brady MT, Jackson MA, Long SS (2015). Arboviruses report of the committee on infectious disease. 30th ed. Elk Grove, IL: American Academy of Pediatrics; 2015. p. 240-246.

Lee VH, Moore DL (1972). Vectors of the 1969 yellow fever epidemic on the Jos Plateau, Nigeria. Bulletin of the World Health Organization, 46(5):669–673.

Lewis DJ, Hughes TP, Mahaffy AF (1942). Experimental transmission of yellow fever by three common species of mosquitoes from the Anglo–Egyptian Sudan. Annals of Tropical Medicine and Parasitology, 36:34–38.

Leopoldo M.2004. Pictorial keys for the identification of mosquitoes (Diptera: Culicidae) associated with Dengue Virus Transmission Zootaxa 589: 1–60.

Powell JR, Tabachnick WJ, Arnold J (1980). Genetics and the origin of a vector population: Aedes aegypti, a case study. Science, 208:1385–1387.

Powell JR, Tabachnick WJ (2013). History of domestication and spread of Aedes aegypti—a review. Memórias do Instituto Oswaldo Cruz Suppl 1:11-17.

Tabachnick WJ, Powell JR (1979). A world-wide survey of genetic variation in the yellow fever mosquito, Ae. aegypti. Genetics Research, 34:215–219.

Tamura K, Nei M (1993). Estimation of the number of nucleotide substitutions in the control region of Mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10(3):512–526 DOI 10.1093/oxfordjournals.molbev.a040023.

WHO (1986). Prevention and control of yellow fever in Africa. Geneva, WHO.

WHO (2016) Eliminate Yellow Fever Epidemics (EYE) 2017 – 2026).

WHO (2014). Rapid field entomological assessment during yellow fever outbreaks in Africa; Handbook for Methodological field approaches for scientists with a basic background in entomology.

WHO (2009). Dengue guidelines for diagnosis, treatment, prevention and control: new edition. Geneva: World Health Organization.

WHO (1998). Tests procedures for insecticide resistance monitoring in malaria vectors, bio efficacy and persistence of insecticides on treated surfaces. Report of the WHO Informal Consultation (WHO/MAL/98), Geneva, WHO.

WHO (2009). Dengue guidelines for diagnosis, treatment, prevention and control: new edition. Geneva: World Health Organization.

13. Annexs

Annex I

Form for recording pooled mosquitoes

Woreda:----------

Kebele:----------

Geographical origin	Localization	Date	Method of collection	ID number	Species	Sex		Feeding status	Number of specimens	RT-PCR Result
						M	F			

Annex II

Prototype of Aquatic Stages reporting form

Date----------

Village----------

House No.	No of hab.U.	Type of breeding sites																				Total number of +ve container		GPS
		Plastic tank				Bucket/bowls				Clay jar				Tires		Flowerpot		Well		Inset leave axil				
		Indoor		Outdoor		Indoor		Outdoor		Indoor		Outdoor												
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	

S No.	Guideline prepared by	Institute
1	Solomon Kinde	EPHI
2	Fekadu Gemechu	EPHI
3	Yonas Wuletaw	EPHI
4	Aleminesh Hilemariyam	EPHI
5	Wondimeneh Mekuriyaw	EPHI
6	Abate W/tinsaye	EPHI
7	Araya Enkubay	EPHI
8	Fitsum Tesfaye	EPHI
9	Dr Esayas Kinfe	EPHI
10	Dr Wondatir Nigatu	EPHI
11	Tilahun Kebede	MoH
12	Bederi Abduletif	MoH