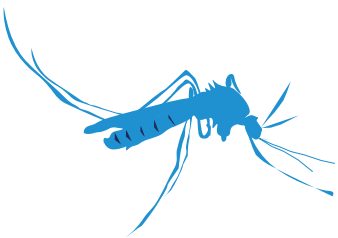
Discriminating concentrations of insecticides for monitoring resistance in *Culex quinquefasciatus* and *Culex tarsalis*

Report of a multi-centre laboratory study and WHO expert consultations



World Health Organization

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Contents

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Study investigators (organized alphabetically by country, principal investigator and co-investigator):
Fundação Oswaldo Cruz, Rio de Janeiro, Brazil: Dr Ademir J Martins, Dr Margarete Martins dos Santos Afonso, Dr José Bento Pereira Lima, Dr Elizabeth Ferreira Rangel, Dr Fernando Ariel Genta and Dr Samara Graciane da Costa-Latgé

Institut de Recherche pour le Développement, Montpellier, France: Dr Vincent Corbel, Dr Dominique Cerqueira and Mr Stephane Duchon

Centre for Biotechnology Research and Development, Kenya Medical Research Institute, Nairobi, Kenya: Dr Luna Kamau and Dr Damaris Matoke-Muhia

Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia: Mr Adanan Che Rus, Ms Nur Amirah Abdul Latif and Dr Zary Shariman Yahaya

Centers for Disease Control and Prevention, Atlanta (GA), United States of America (USA): Dr Audrey Lenhart, Ms R. Lucrecia Vizcaino Cabarrus, Ms Claudia J. Corredor-Medina, Dr Ephantus Muturi and Mr Christopher Sandi

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External contributors (experts who participated in WHO consultations, listed in alphabetical order by country):
Dr Luc Djogbenou, Université Abomey Calavi, Benin; Dr Josiane Etang, Malaria Research Laboratory, Organisation de coordination et de coopération pour la lutte contre les grandes endémies en Afrique Centrale, Yaoundé, Cameroon; Dr Kamaraju Raghavendra, Indian Council of Medical Research, National Institute of Malaria Research, New Delhi, India; Dr Ahmad Ali Enayati, School of Public Health, Mazandaran University of Medical Sciences, Sari, Islamic Republic of Iran; Dr Zahra Saeidi, School of Public Health, Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran; Dr Nazni Bt Hj Wasi Ahmad, Institute of Medical Research, Kuala Lumpur, Malaysia; Dr Adriana Flores, Universidad Autónoma

de Nuevo León, San Nicolás de los Garza, México; Dr Lalita Roy, Tropical and Infectious Disease Centre, B. P. Koirala Institute of Health Sciences, Dharan, Nepal; Dr João Pinto, Instituto de Higiene e Medicina Tropical, Universidade NOVA de Lisboa, Lisbon, Portugal; Dr Thomas Churcher, Imperial College London, London, United Kingdom; Dr Rosemary Lees, Liverpool School of Tropical Medicine, Liverpool, United Kingdom.

WHO headquarters (Department of Control of Neglected Tropical Diseases):
Dr Rajpal S. Yadav and Dr Raman Velayudhan conceptualized the study. Dr Yadav designed and coordinated the study with test sites for data collection, data curation and tracking progress; convened the WHO consultations; and reviewed and finalized the study report.

WHO regional and country office focal points:
Dr Emmanuel Chanda, WHO Regional Office for Africa, Brazzaville, Democratic Republic of the Congo; Dr Haroldo S da Silva Bezerra, WHO Regional Office for the Americas, Washington (DC), USA; Dr Samira Al-Eryani, WHO Regional Office for the Eastern Mediterranean, Cairo, Egypt.

Other stakeholders:[1]
Dr Susanne Stutz, BASF SE, Limbergerhof, Germany; Dr Sebastian Horstmann, Envu Mosquito Management, Monheim, Germany; Dr Barnabas Zogo, Sumitomo Chemicals Co. Ltd, Tokyo, Japan; Dr Anil Makkapati, Envu Mosquito Management, Singapore; Dr Mark Hoppe, Syngenta, Basel, Switzerland; Bill & Melinda Gates Foundation, Liverpool, United Kingdom; Dr Dave Malone, Bill & Melinda Gates Foundation, Liverpool, United Kingdom; Dr James Austin, BASF, Wake Forest (NC), USA; Dr William Jany, Dr Laetitia Leroy and Ms Joanna Tyszko, Clarke International LLC, St Charles (IL), USA.

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Declarations of interest and confidentiality undertakings

WHO received and reviewed declarations of interest and confidentiality undertakings from all the experts who participated in the WHO consultations on 27 March and 20 June 2023 and concluded that none could give rise to a potential or reasonably perceived conflict of interest related to the subjects discussed at the meetings.

[1] Representatives of donor agencies and commercial entities, who participated in the open sessions of the consultations with observer status and provided technical comments on the draft study report prepared by the WHO secretariat. Their technical comments and views were considered in the closed sessions, which were limited to the WHO experts and the secretariat. They did not participate in final approval of the report.

Abbreviations and acronyms

CDC	United States Centers for Disease Control and Prevention
CR	completion rate
DC	discriminating concentration
FIOCRUZ	Fundaçao Oswaldo Cruz, Brazil
h	hour/s
IRD	Institut de Recherche pour le Développement, France
KEMRI	Kenya Medical Research Institute
LC_{50}	lethal concentration required to kill 50% of mosquitoes tested
LC_{99}	lethal concentration required to kill 99% of mosquitoes tested
LC_{100}	lethal concentration required to kill 100% of mosquitoes tested
MERO	81% rapeseed oil methyl ester
min	minute/s
ppm	parts per million
SOP	standard operating procedure
TDC	tentative discriminating concentration
USA	United States of America
VCRU	Vector Control Research Unit (Malaysia)
WHO	World Health Organization

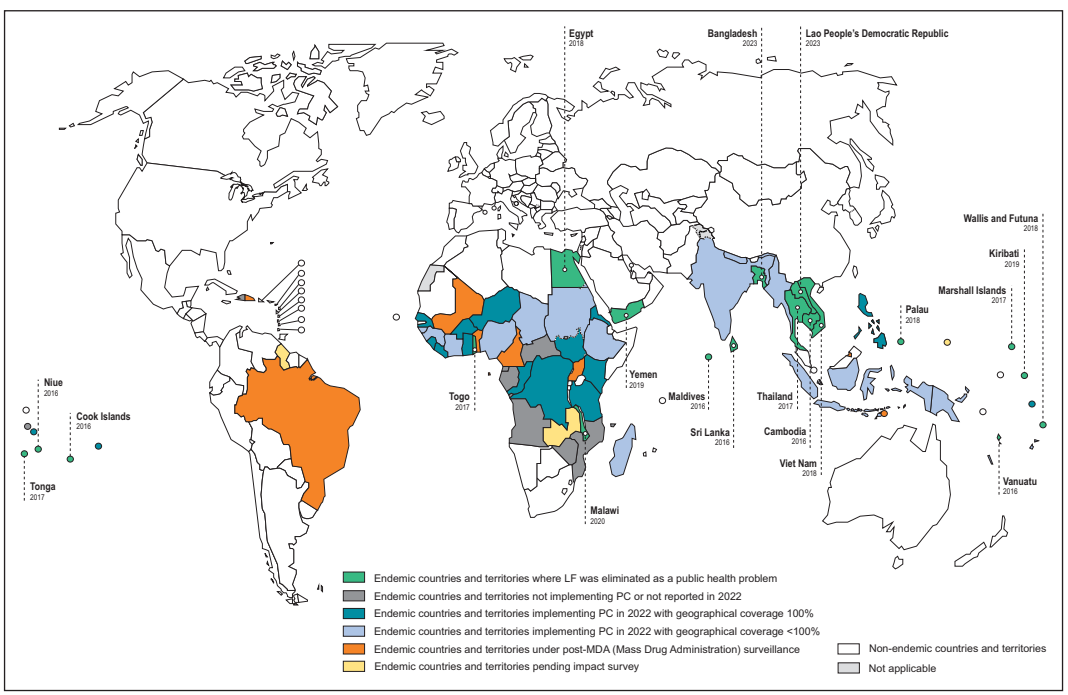
1. Introduction

Some species of *Culex* mosquitoes are important vectors of parasitic worms and arboviruses – namely, *Culex quinquefasciatus, Cx. pipiens, Cx. tarsalis, Cx. tritaeniorhynchus* and *Cx. annulirostris*. Among the major diseases transmitted by *Culex* species are Bancroftian filariasis, West Nile virus disease, Rift Valley fever, Japanese encephalitis, St Louis encephalitis, Murray Valley encephalitis and Ross River disease.

Lymphatic filariasis currently affects 863 million people in 47 countries worldwide (Fig. 1). A key component of the WHO Global Programme to Eliminate Lymphatic Filariasis is to stop the spread of the disease by administering an annual dose of medicines to the entire at-risk population (i.e. mass drug administration). A disease-specific target of the WHO road map for neglected tropical diseases 2021–2030 *(1)* is to eliminate lymphatic filariasis worldwide by 2030.

The options for control of *Culex* mosquitoes used in public health and environment programmes are environmental management, larviciding, space spraying to contain outbreaks of arboviral diseases such as West Nile virus, indoor residual spraying such as for control of Japanese encephalitis, and personal protection such as with insecticide-treated

Fig. 1. Global distribution of lymphatic filariasis in endemic countries, 2023



PC: preventive chemotherapy.

nets and skin-applied repellents. *Cx. quinquefasciatus* is considered to be a major nuisance mosquito in many urban and semi-urban tropical settings, and its distribution is likely to extend further globally *(2)*. Vector control is an important element in the prevention, control and elimination of major vector-borne diseases. The aim of the WHO Global Vector Control Response 2017–2030 *(3)* is to raise vector control high on the public health agenda in order to reduce the burden of vector-borne diseases. As large amounts of insecticide are used for control of Culex mosquitoes worldwide, it is essential to improve entomological surveillance, generate and share data on the susceptibility of major vector species, namely *Cx. quinquefasciatus* and *Cx. tarsalis*, and monitor insecticide resistance in adequate test protocols and diagnostic concentrations.

Although there is a global database on the resistance of malaria vectors to insecticides, none is available for *Culex* species; however, several scientific reports from all over the world indicate increasing resistance of *Culex* species to public health insecticides *(4–9)*.

In accordance with WHO guidance, insecticide resistance is monitored in field populations of mosquitoes in bioassays conducted with filter papers impregnated with a standard concentration of an insecticide (i.e. the diagnostic or discriminating concentration, DC).[1] The concept of an insecticide DC has clear advantages in terms of the cost and efficiency of testing, and it has been adopted widely for monitoring insecticide resistance in mosquitoes and other disease vectors *(10,11)*. WHO has recently updated insecticide DCs for testing the resistance of adult *Anopheles*, *Aedes* and sand fly species in WHO-supervised multi-centre studies *(12,13)*. Standard operating procedures (SOPs) have been developed for impregnating filter papers and conducting WHO tube tests *(14,15)*. In addition, a new WHO bottle bioassay method and its SOP have been developed to test the susceptibility of adult *Anopheles* and *Aedes* species to some existing and new insecticides that cannot be impregnated into filter papers for technical reasons *(16,17)*.

In the past, the DCs of only a few insecticides were established for testing the susceptibility of *Cx. quinquefasciatus* but were not addressed in WHO-supervised multi-centre studies (Table 1) *(11)*. Since then, many more vector control insecticides have been introduced, without determination of their DCs for monitoring resistance in field populations of *Culex* mosquitoes. After a needs assessment, therefore, WHO conducted a multi-centre laboratory study in 2022–2023 to determine the DCs of six insecticides against two vector species of major *Culex*-borne diseases, *Cx. quinquefasciatus* and *Cx. tarsalis*. These insecticides are currently used in insecticide formulations for the control of *Culex* species.

[1] WHO defines an insecticide DC as twice the lowest concentration that results systematically in 100% mortality after a 60-min exposure and a holding period of 24 h of a susceptible mosquito strain. A DC can also be defined as twice the 99% lethal concentration (LC_{99}) as determined in a relevant statistical model against a susceptible strain of an insect.

Table 1. Historical insecticide discriminating concentrations for WHO susceptibility tube tests with *Culex quinquefasciatus*

Insecticide class	Insecticide	DC (%)	Exposure period (h)	Carrier oil
Organochlorines	DDT	0.04	4	Risella
Pyrethroids	Deltamethrin	0.025	1	Silicone
	Lambda-cyhalothrin	0.025	1	Silicone
	Permethrin	0.25	3	Silicone
Carbamates	Propoxur	0.10	1	Olive
Organophospates	Fenitrothion	1.00	2	Olive
	Malathion	5.00	1	Olive

DC: discriminating concentration; h: hour/s.
Note: The holding period for recording was 24 h in all tests.
Source: WHO *(11)*.

WHO invited interested institutions to participate in the study in an open call published on the United Nations Global Marketplace portal and selected four suitable laboratories that have colonies of susceptible *Cx. quinquefasciatus* and *Cx. tarsalis* species. Additionally, the United States Centers for Disease Control and Prevention (CDC) volunteered to evaluate the susceptibility of *Cx. tarsalis*, because it transmits West Nile virus in the Region of the Americas.

The agendas and lists of participants in the WHO consultations are given in Annex 1. The interim results of the study were reviewed at a WHO consultation on 27 March 2023 and the final results in another session, on 20 June 2023 (Annex 2). A draft of the report, with the results of the study and the recommendations of experts to WHO, was then prepared and peer reviewed. A technical review of the report by stakeholders and by end users was solicited, to be organized by the WHO regional and country offices. The report was updated after the second WHO consultation, on 20 June 2023, with the recommendations of experts. The present report describes the study objectives and design and presents the results, conclusions and recommendations of the experts to WHO on new DCs for filter paper and bottle bioassays and on further studies.

2. Objectives of the study

The objectives of the study were to:

- determine concentration–response curves for four selected insecticide compounds for the WHO tube tests and two compounds for the WHO bottle bioassays against *Cx. quinquefasciatus* and *Cx. tarsalis*;
- establish and validate the DCs of the insecticides tested in the two bioassays; and
- identify gaps in and priorities for research for future work on insecticide DCs against *Culex* spp. vectors.

3. Participating laboratories

3.1 Lead coordinating institution

The Institut de Recherche pour le Développement (IRD), Infectious Diseases and Vectors, Ecology, Genetics, Evolution and Control Unit, Montpellier, France,[1] was selected by WHO to coordinate the study in participating laboratories, monitor progress, collect and analyse bioassay data and provide technical support if required. The IRD also communicated regularly with industry partners to discuss technical issues arising from the study, note any changes to test procedures (e.g. adjustment of the concentration of the surfactant in bottle bioassays) and obtain information on the insecticides (e.g. active ingredient composition, certificates of analysis, storage conditions, material safety data sheets).

3.2 Collaborating laboratories

As well as IRD, four other internationally recognized laboratories with extensive entomological capacity in four WHO regions participated in the study. All the laboratories had susceptible mosquito colonies and adequate facilities and capacity to conduct laboratory testing and are either formally designated WHO collaborating centres or have good capacity for testing insecticides (Table 2). They were contracted directly by WHO and were asked strictly to follow the standard study design (protocol) and the SOPs developed collaboratively.

[1] https://en.ird.fr and https://mivegec.fr/en

4. *Culex* species

As explained earlier, two main *Culex* vector species were selected for the study (Table 2). The criteria for selecting the species were that they are:

- known to be involved in transmission of lymphatic filariasis and/or West Nile virus;
- representative of a geographical region in which these diseases are either endemic or may pose a risk of disease transmission (in WHO's African, Americas, European and Western Pacific regions); and
- are colonized at the participating laboratories.

Only fully susceptible strains (defined as susceptible to insecticides in all major classes, with no detectable resistance mechanisms) were tested. A quality control check of the *Cx. quinquefasciatus* mosquito strains used in three centres (i.e. the SLAB strain at IRD and the Fundaçao Oswaldo Cruz (FIOCRUZ), and the Nairobi strain at the Kenya Medical Research Institute, Kenya, (KEMRI), was performed with either synergist bioassays or molecular tools (polymerase chain reaction or sequencing) to detect the presence of any resistance mechanisms. The results of these tests are summarized in Annex 2.

Table 2. Participating laboratories and susceptible *Cx. quinquefasciatus* and *Cx. tarsalis* species included in the study

WHO region	Country	Institution	Sand fly species and strain
African	Kenya	KEMRI, Nairobi	*Cx. quinquefasciatus* (Nairobi, Kenya; 2003)
Americas	Brazil	FIOCRUZ, Rio de Janeiro	*Cx. quinquefasciatus* (SLAB strain, California, USA, 1966)
	USA	CDC, Atlanta (GA)	*Cx. tarsalis* (YOLO strain, California, USA, 2003, NR-43026, MR4-CDC)
European	France	IRD, Montpellier	*Cx. quinquefasciatus* (SLAB strain, California, USA, 1966); *Cx. tarsalis* (YOLO strain, California, USA, 2003, NR-43026, MR4-CDC established at IRD, Montpellier in 2022)
Western Pacific	Malaysia	VCRU, Penang	*Cx. quinquefasciatus* (VCRU strain, Penang, 1978)

FIOCRUZ: Fundaçao Oswaldo Cruz; IRD: Institut de Recherche pour le Développement; KEMRI: Kenya Medical Research Institute; VCRU: Vector Control Research Unit.

5. Test compounds, carrier oils and surfactant

Six insecticide compounds were tested. Four were tested in WHO tube tests (alpha-cypermethrin, deltamethrin, malathion and bendiocarb) and two (transfluthrin and clothianidin) in WHO bottle bioassays (Table 3).

Insecticides, carrier oils and surfactant oil (81% rapeseed methyl ester oil, MERO, Envu, Mosquito Management [previously Bayer CropScience], Monheim, Germany) were supplied to the participating laboratories.

The insecticides tested belong to four different insecticide classes. They were categorized into two test groups (Table 3). Group 1 consisted of four compounds for which the WHO tube test method was suitable for establishing and validating DCs; and group 2 consisted of two compounds with distinct chemical properties and/or a mode of action that prevented their impregnation onto filter paper because of their instability; therefore, glass bottle bioassays were used to establish and validate DCs.

Preliminary studies showed that some of the new insecticides tend to crystallize on filter paper if they are impregnated with an inappropriate carrier oil, hence limiting the bio-efficacy and duration of the treated papers *(10)*. Silicone and olive oil were used as carrier oils for treatment of filter papers, while 81% MERO was used as a surfactant oil for coating glass bottles. The carrier oils and MERO were dissolved in acetone for impregnation of filter papers or coating bottles for bioassays, according to the WHO SOPs *(15,17)*.

All the test compounds and MERO were provided gratis to WHO by their respective manufacturers, with certificates of analysis and material safety data sheets. They were stored under appropriate conditions as per the manufacturers' instructions.

Table 3. Class, Test compound, Bioassay method, and Solvent, carrier oil or surfactant oil

Class	Test compound	Biossay method	Solvent, carrier oil or surfactant oil
Pyrethroids	Alpha-cypermethrin	Tube test	Acetone + silicone oil
	Deltamethrin	Tube test	Acetone + silicone oil
	Transfluthrin	WHO bottle bioassay	Acetone
Carbamates	Bendiocarb	Tube test	Acetone + olive oil
Organophosphates	Malathion	Tube test	Acetone + olive oil
Neonicotinoids	Clothianidin	WHO bottle bioassay	Acetone + MERO (1500 ppm)

h: hour/s; ppm: parts per million.

Note: In all tests, the time for drying the filter paper or bottle was 24 h, the exposure time was 1 h, and the susceptibility end-point was mortality 24 h after the 1-h exposure.

6. Study design

The activities conducted and their timelines were, in chronological order:

- deciding on the scope of the study and preparing a generic study outline (January 2022);
- posting a "request for proposal" on the United Nations Global Marketplace portal (March 2022);
- selecting suitable laboratories that met WHO requirements for a multi-centre study and signing technical service agreements with participating institutions (April–May 2022);
- harmonizing test protocols among laboratories, deciding susceptibility end-points, and delivering test compounds and other materials (June–July 2022);
- convening a virtual meeting with the participating investigators to orient them on correct use of test procedures (18 July 2022);
- performing tests in steps 1 and 2 with insecticide compounds to establish concentration–response curves in order to select the range of concentrations that kill 0–100% of mosquitoes (July 2022–February 2023);
- analysing data and drafting an interim report of the study (early March 2023);
- convening a WHO expert consultation to review the interim analysis of data from steps 1 and 2 to select tentative DCs (TDCs) for further testing in step 3 (27 March 2023);
- validating selected TDCs in step 3 and updating the study report (April–June 2023);
- convening a follow-up session of the WHO expert consultation to review the analysis of data from step-3 testing and to select the final DCs (20 June 2023); and
- finalizing, editing and publishing the study report (September 2023).

7. Standard operating procedures

Available SOPs for impregnation of filter papers for testing insecticide susceptibility of adult mosquitoes in WHO tube tests were used in this study *(14)*. The SOPs for testing the susceptibility of *Anopheles* and *Aedes* mosquitoes to insecticides in the WHO tube test and the WHO bottle bioassay were used for testing the susceptibility of *Cx. quinquefasciatus* and *Cx. tarsalis (15,17)*. The WHO bottle bioassays were conducted in 250-mL Wheaton glass bottles (with an actual volume of 310 mL) with either a holed netting piece fixed to the mouth for introduction of mosquitoes or a cap with a rubber flap (Fig. 2).

Fig. 2. Technique for introducing mosquitoes into a 250-mL glass bottle[a] with a holed netting piece (left) or a holed cap and a rubber flap (right)

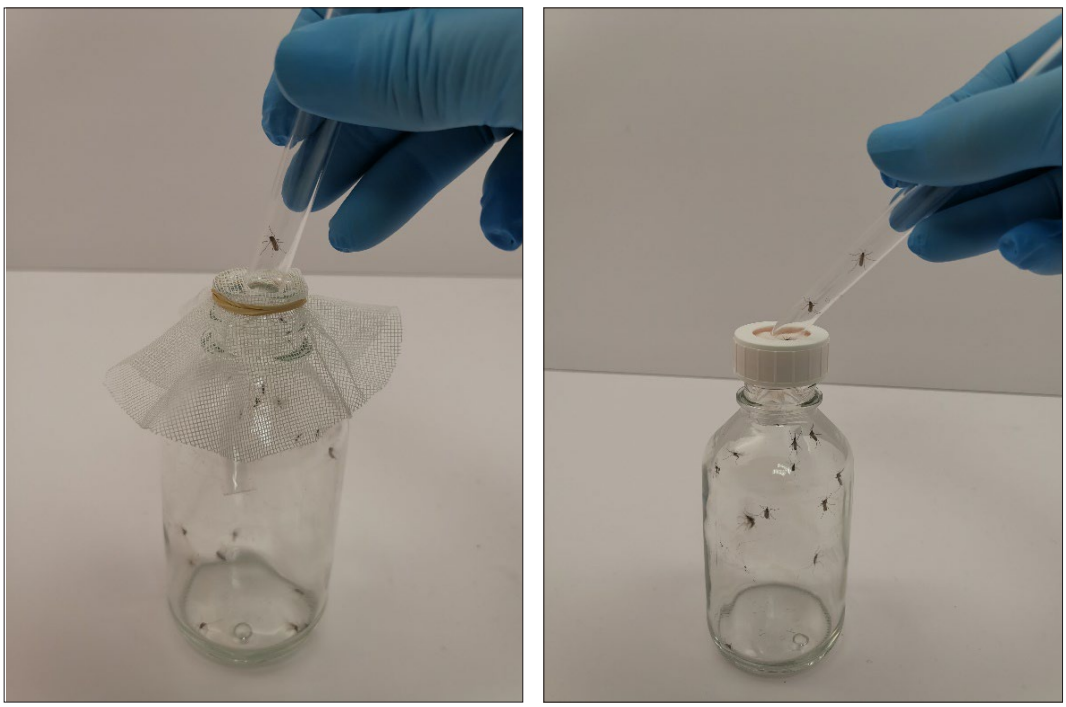


Photo credit: IRD/Mr Stéphane Duchon
[a] The total volume of a 250-mL Wheaton bottle is 310 mL.

Bottles were prepared with MERO as the surfactant. Briefly, to prepare one bottle with 1500 parts per million (ppm) of MERO, 1.66 µL MERO per bottle were required in view of the density of MERO of 0.9. The participating centres generally prepared larger stocks of MERO and acetone to coat bottles (e.g. for 100 mL of solution, 99.83 mL of acetone were added to 0.167 mL of MERO). An Excel spreadsheet is available online to calculate the amounts of insecticides, carrier oil or MERO and acetone, which can be downloaded from the WHO website.[1]

[1] https://cdn.who.int/media/docs/default-source/ntds/vector-ecology-mangement/calculation-tables-paper-impregnation-bottles-17jan2022-locked.xlsx

Uniform testing conditions were used by all the laboratories, comprising standard test protocols, bioassay methods, standard temperature and relative humidity, mosquito sample size per test, and the age and physiological status of the test mosquitoes *(12)*. The end-point of all the bioassays was mortality of mosquitoes recorded 24 h after exposure to an insecticide for a fixed exposure time of 1 h.

Preliminary testing of *Cx. quinquefasciatus* and *Cx. tarsalis* showed that bottles coated with MERO at 500–1500 ppm did not cause abnormal mortality (i.e. < 10%). Hence, the highest concentration of 1500 ppm MERO was adopted for testing clothianidin in WHO bottle bioassays as per the manufacturer's instructions. MERO was not used for testing transfluthrin, as previously recommended *(12,13)*.

The laboratories were asked to report to IRD and WHO any inconsistent results in test replicates for a given insecticide and test concentration, changes made to testing conditions or use of different concentrations of MERO to coat glass bottles.

8. Bioassays

The compounds were tested in three steps, described below. After completion of each step, the participating laboratories shared the results with IRD and WHO for analysis. IRD gave feedback to the laboratories on the validity of the tests and whether to proceed to the next step or to repeat or revalidate certain tests.

8.1 Preliminary screening bioassays to establish concentration–response curves for WHO tube tests and bottle bioassays (step 1)

The objective of the tests in step 1 was to perform preliminary bioassays to establish the range of concentrations of the test compounds that caused 0–100% mortality for each *Culex* species. The participating laboratories conducted initial exploratory bioassays to select a broad range of serial concentrations of each test compound to provide a range of responses (0–100% mortality of each *Culex* species and strain). Each laboratory impregnated Whatman no. 1 filter papers with 10–12 serial concentrations of the test compounds to test susceptibility in the WHO tube test according to the WHO SOP *(15)* or in glass bottles coated with serial concentrations of transfluthrin or clothianidin. Technical assistance was given on request by IRD and other collaborating laboratories in the preparation of stock solutions, impregnation of papers, coating of bottles and conducting bioassays. The impregnated papers and insecticide-coated bottles were dried for 24 h at room temperature. In the bioassays, insecticide-susceptible non-blood-fed female *Culex* aged 2–7 days were exposed to the serial concentrations of test compounds for 1 h. Susceptibility was recorded as percentage mortality of mosquitoes 24 h after a 1-h exposure (Table 4).

Table 4. General scheme for conducting WHO tube tests and bottle bioassays with *Cx. quinquefasciatus* and *Cx. tarsalis* in steps 1–3

Testing step	Numbers of test concentrations and controls	Minimum no. of mosquitoes per test concentration or control (tubes or bottles)	No. of replicates (test batches) per concentration or control	Total no. of mosquitoes tested	Expected outcome
1. Screening	Tests: 10–12 Control: 1	50 (25 × 2) Controls: 50 (25 × 2)	1	Tests: 500–600 Controls: 50	Range of concentrations that cause 0–100% mortality
2. Determination	Tests: 6 Control: 1	Tests: 100[a] (25 × 4) Controls: 50 (25 × 2)	3 (with 3 different batches of mosquitoes)	Tests: 1800 Controls: 150	LC_{50}, LC_{99} and LC_{100}, to select TDC
3. Validation	Tests: 1 Control: 1	Tests: 100 (25 × 4) Controls: 50 (25 × 2)	1	Tests: 100 Controls: 50	DC

DC: discriminating concentration; LC: lethal concentration; TDC: tentative discriminating concentration.

[a] Because of the difficulty of ensuring large numbers of *Culex* mosquitoes in colonies (especially for *Cx. tarsalis*), 50 mosquitoes were exposed per test concentration in some laboratories in step 2, and additional bioassays were conducted to achieve the required sample size for each test concentration.

8.2 Bioassays to establish concentration–response curves (step 2)

In step 2, the tube tests and WHO bottle bioassays were conducted in triplicate (when possible) to establish the LC_{50}, LC_{99} and LC_{100} (observed) and to select a TDC for each test compound. In this step, laboratories impregnated filter papers and coated glass bottles with the serial concentrations determined in step 1. At least six test concentrations and a control replicate were generally tested in step 2 to generate concentration–response curves. A step-2 test was considered valid if at least two concentrations killed < 50% of test mosquitoes, one concentration killed about 50%, two concentrations killed > 50% and one concentration killed about 100%. Each bioassay was performed three times with a given species (when possible), with 50–100 mosquitoes per concentration. Impregnated papers were usually used once but no more than three times and kept under suitable storage conditions between uses.

8.3 Validation of tentative discriminating concentrations against *Culex* species (step 3)

In this last step of testing, well-characterized, colonized, susceptible *Culex* strains were exposed to filter papers impregnated with each selected TDC or in bottles coated with the TDCs of each insecticide, as recommended by a WHO expert group, to record mortality. The

impregnated papers at "predefined" TDCs were prepared by the Universiti Sains Malaysia, tested for quality in coordination with WHO and supplied to all testing laboratories, while glass bottles were coated at the testing laboratories according to WHO SOPs *(17)*. The tube test or bottle bioassays were performed for a given insecticide TDC and *Culex* species with at least 100 mosquitoes per TDC and 50 mosquitoes for the control. All laboratories reported data to IRD and WHO for validation and analysis.

8.4 Reporting of data and monitoring of progress

The participating laboratories were responsible for collecting, checking quality, collating and reporting data on an Excel template developed by IRD and sending reports regularly to IRD and WHO, where the data were analysed and validated. Real-time feedback was given to the laboratories, and progress and achievement of milestones were monitored according to the completion rate (CR) of tests (Table 5). The raw data on all tests for a given insecticide and *Culex* species are archived at IRD.

CRs were calculated as follows:

$$\text{CR (\%)} = \frac{\text{Number of validated tests}}{\text{Total number of test to be performed}} \times 100$$

The CRs were estimated for each laboratory according to the testing steps completed and the compounds tested against their *Culex* species. Data were shared regularly with WHO, so that progress could be monitored, any necessary corrective measures taken, technical difficulties identified, and resolved and solutions proposed.

Table 5. Classification used to monitor progress and guide further action

Test completion rate (%)	Progress	Action	Communication frequency
0–35	Little	Strong follow-up, communication by e-mail, phone calls and video calls	Every 2–3 weeks or at WHO request
36–75	Moderate	Strengthened follow-up with regular exchange by e-mail, phone callsand video calls	Every 1–2 months or at WHO request
76–100	Good	Normal follow-up and exchange by e-mail and video calls	About every 3 months or at WHO request

Source: WHO *(12)*.

8.5 Data analysis, validation and interpretation of results

A Bayesian binomial model with a five-parameter logistics function developed at Imperial College London, United Kingdom, for a previous WHO study was used for analysis of the concentration–response data *(13)*. The modelling method used to analyse the multi-centre data has been published *(18)*. Briefly, the binomial sampling distribution was used to describe the outcome (i.e. mortality rates in each *Culex* spp. colony) after exposure to an insecticide or control. The model was fitted for all the bioassay results from each laboratory for each combination of insecticide and species tested to generate one concentration–response curve for each laboratory, insecticide and *Culex* spp. colony. The uncertainty of the estimate was determined by analysis of the range of concentration–response curves provided by the bioassays in each laboratory, for each species and insecticide. With this model, LC_{50} and LC_{99} and their ranges were estimated for each insecticide and species and for the country of each laboratory.

The LC_{99} and LC_{100} (from raw data) were both used to select TDCs for further testing in step 3, as they are more robust for determining mortality at high concentrations. When laboratories found different values for LC_{99} (or LC_{100}) for the same insecticide–species combination, the highest LC_{99} (or LC_{100}) was generally selected, although at the risk of overestimating a DC for a *Culex* species.

9. Results

9.1 Test completion rates

In all, 77 046 *Cx. quinquefasciatus* (71% of the total) and 30 691 *Cx. tarsalis* (29% of the total) were used to test six insecticides in either filter paper tests or WHO bottle bioassays (Fig. 3).

The test CRs were 94%, 90% and 100% in steps 1, 2 and 3, respectively (Table 6). CRs of 100% were achieved for transfluthrin, clothianidin, malathion and bendiocarb in step 1. In step 2, good CRs (> 75%) were achieved for all the insecticides. In step 3, the CRs were 100% for all the insecticides.

Fig. 3. Total numbers of *Culex quinquefascitus* and *Cx. tarsalis* tested in the WHO tube tests (with alpha-cypermethrin , deltamethrin, malathion and bendiocarb) and the WHO bottle bioassays (with clothianidin and transfluthrin)

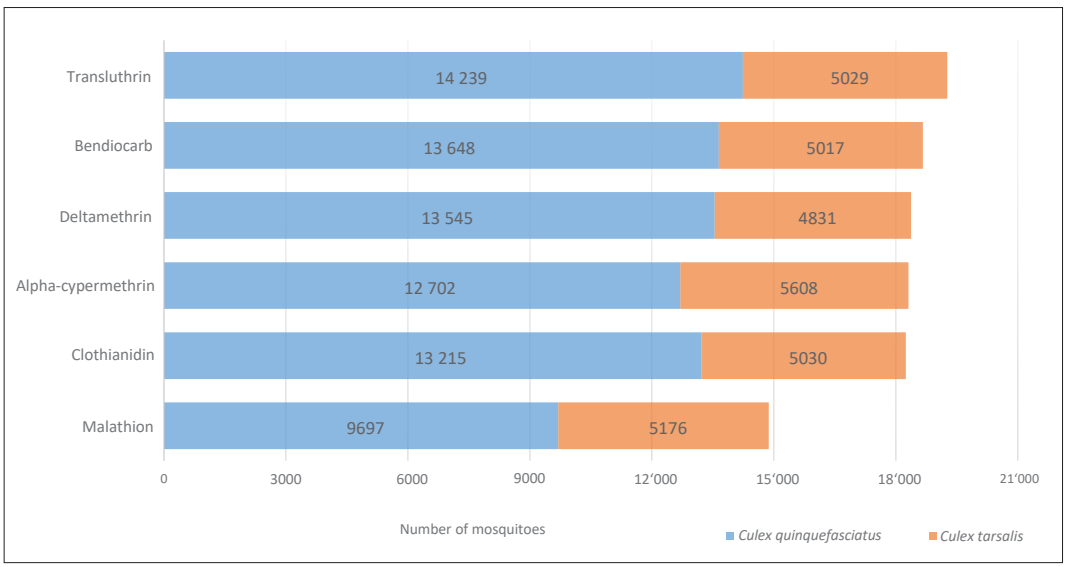


Table 6. Insecticides tested with *Cx. quinquefasciatus* and *Cx. tarsalis* and test completion rates in steps 1, 2 and 3

Step	No. of tests performed and validated	Pyrethroids			Carbamates	Organophosphates	Neonicotinoids	Total
	Insecticide	Alpha-cypermethrin	Deltamethrin	Transfluthrin	Bendiocarb	Malathion	Clothianidin	
	No. of tests performed	6	6	6	6	6	6	36
1	No. of validated tests	5	5	6	6	6	6	34
	CR (%)	83	83	100	100	100	100	94
2	No. of tests performed	18	18	18	18	18	18	108
	No. of validated tests	16	17	17	16	14	17	97
	CR (%)	89	94	94	89	78	94	90
3	No. of tests performed	11	10	14	10	6	12	63
	No. of validated tests	11	10	14	10	6	12	63
	CR (%)	100	100	100	100	100	100	100%

CR: completion rate.

CR: > 75% (good progress), 36–75% (moderate progress); < 35% (little progress)

9.2 Concentration–response curves, estimated LC_{99} and LC_{100} and selection of tentative discriminating concentrations

The concentration–response curves (steps 1–2) and the LC_{50}, LC_{99}, LC_{100} and TDCs for each insecticide and Culex species are presented below.

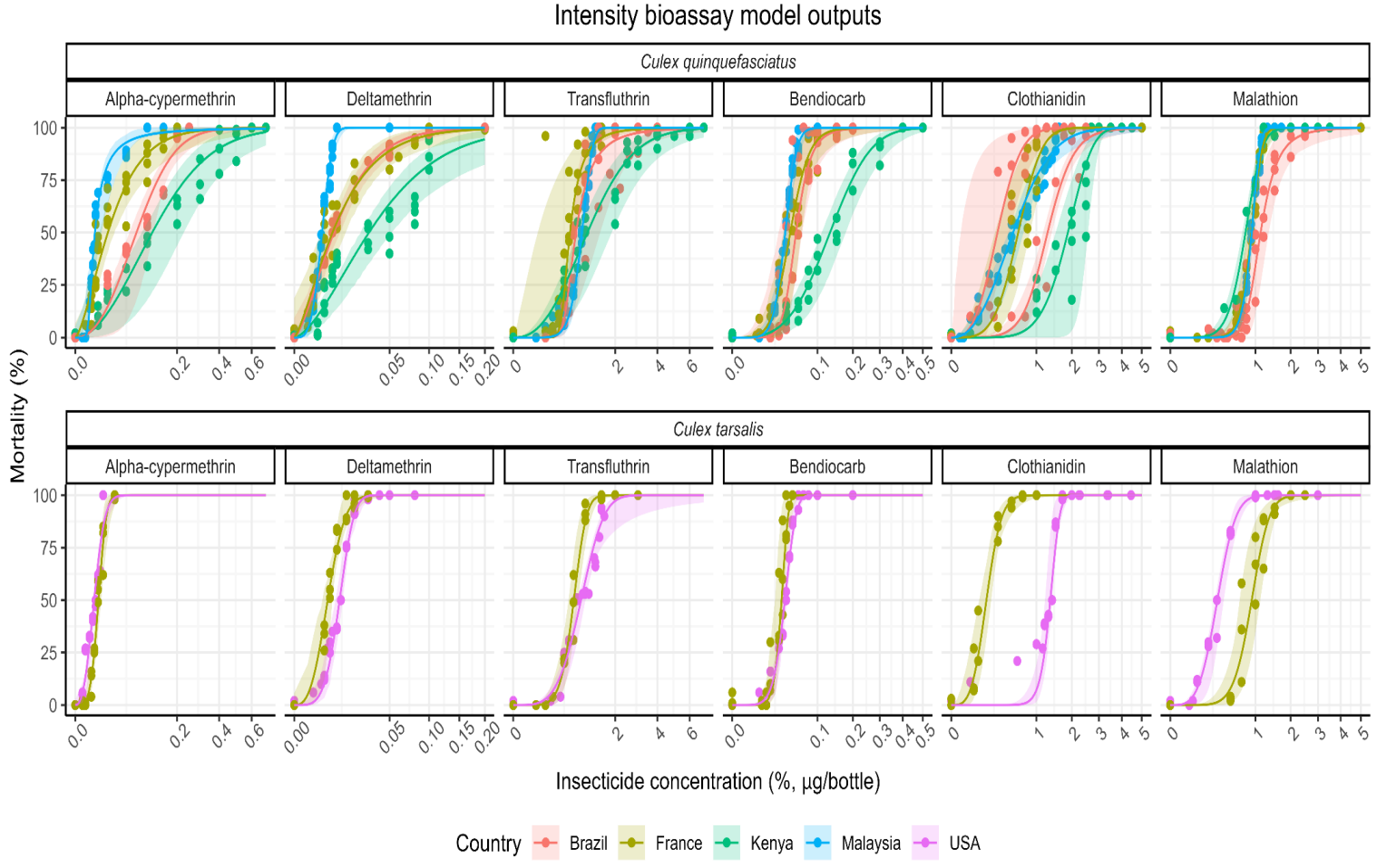
The data used for statistical analysis are summarized in Table 7. The total number of *Culex* tested per insecticide ranged from 14 103 with malathion to 17 459 with transfluthrin. The mean number of data points per insecticide ranged from 177 for malathion to 209 for bendiocarb. The mean number of mosquitoes per bioassay ranged from 705 for malathion to 797 for alpha-cypermethrin. For each insecticide–*Culex* species combination, insecticide concentrations that caused 0–100% mortality were used to establish the concentration–response curves and to estimate the LCs (Fig. 4).

Table 7. Data used for the statistical analyses

Insecticide	No. of bioassays	No. of data points	Total no. of mosquitoes tested	Mean no. of mosquitoes tested per bioassay	Insecticide concentration (%, or µg/bottle)		Mean mortality rate (%)		Mean mortality rate (%)	
					Mean	Max	Mean	Max	Mean	Max
Alpha-cypermethrin	21	193	16756	797.9	0.077	0.7	50.18	100	77.52	100
Deltamethrin	22	196	17108	777.6	0.030	0.2	54.16	100	74.81	100
Transfluthrin	23	204	17459	759.1	1.324	7	54.92	100	64.69	100
Bendiocarb	22	209	17417	791.7	0.074	0.5	53.06	100	66.91	100
Clothianidin	23	192	16602	721.8	1.173	5	57.31	100	60.50	100
Malathion	20	177	14103	705.2	0.943	5	50.32	100	59.88	100

Note: Both *Cx. quinquefasciatus* and *Cx. tarsalis* were tested with each insecticide. The minimum mortality and simulated mortality rate was 0%, and the maximum (max) mortality and simulated mortality rate was 100%.

Fig. 4. Concentration–response curves for each insecticide–species combination tested in either WHO tube tests (with alpha-cypermethrin, deltamethrin, bendiocarb and malathion) or the WHO bottle bioassays (with transfluthrin and clothianidin)



The results for each insecticide are described in detail below.

Alpha-cypermethrin

The LC_{99} and LC_{100} (estimated from the raw data) and the TDC selected for both *Culex* species are summarized in Table 8.

The LC_{99} for *Cx. quinquefasciatus* for each insecticide–species combination varied by participating laboratory (Table 8). For example, the LC_{99} with alpha-cypermethrin ranged from 0.34% (range, 0.071–0.37) against the VCRU strain to 0.86% (0.51–1.62) against the Nairobi strain at KEMRI, hence showing a 2.5 times difference in the susceptibility of the two strains to this insecticide. Similarly, the LC_{100} ranged from 0.1% for the VCRU strain to 0.6% for the Nairobi strain.

With *Cx. tarsalis*, the LC_{99} and LC_{100} were consistent in the two laboratories (CDC and IRD). The LC_{99} of alpha-cypermethrin against the YOLO strain ranged from 0.027 to 0.031, while the LC_{100} ranged from 0.015% to 0.03%.

On the basis of the evidence and in view of the uncertainty of the estimates, the following TDCs were selected for step-3 testing:

- *Cx. quinquefasciatus:* **1% and 2%** for testing at all participating institutions and an additional 2% for testing at KEMRI.
- *Cx. tarsalis:* **0.05%**.

Deltamethrin

The LC_{99} and LC_{100} values and the TDCs selected for both *Culex* species are summarized in Table 9.

As for alpha-cypermethrin, the estimated LC_{99} of deltamethrin against *Cx. quinquefasciatus* varied substantially among laboratories for different colonized strains.

The highest LC_{99} was found for Nairobi strain at KEMRI (0.39%; range, 0.22–1.41) and the lowest for the VCRU strain (0.012%; 0.010–0.015). The LC_{100} ranged from 0.01% for the VCRU strain to 0.2% for all other strains. Slight differences were seen between the estimated LC_{99} values and the LC_{100} observed values.

With *C. tarsalis*, the results were again more consistent. The LC_{99} against the YOLO strain from CDC ranged from 0.025% (0.014–0.04) at IRD to 0.027% (0.024–0.035) at CDC. The LC_{100} ranged from 0.026% to 0.028%.

On the basis of the evidence and in view of the uncertainty of the estimates, the following TDCs were selected for step-3 testing:

- *Cx. quinquefasciatus :* **0.4 and 0.8%**
- *Cx. tarsalis :* **0.05%.**

Transfluthrin

The LC_{99} and LC_{100} values and the TDCs selected for each species are summarized in Table 10. As for pyrethroids, some variation in test results was reported between testing centres and colonized strains. For example, the LC_{99} of *Cx. quinquefasciatus* was 1.43 µg per bottle (range, 1.30–1.62) for the VCRU strain and 6.01 µg/bottle (4.33–14.5) for the Nairobi strain, while the LC_{99} values were 2.74 (range, 1.47–4.31) for IRD and 4.56 (1.29–10.4) for the SLAB strain at FIOCRUZ. The LC_{100} ranged from 1.5 µg to 7 µg per bottle for all strains tested.

With *Cx. tarsalis*, the LC_{99} and LC_{100} values were consistent among the laboratories. The LC_{99} of alpha-cypermethrin for the YOLO strain was 1.35–2.28 µg per bottle, and the LC_{100} was 2 µg per bottle at both IRD and CDC.

On the basis of the evidence and in view of the uncertainty of the estimates, the following TDCs were selected for step-3 testing:

- *Cx. quinquefasciatus:* **4, 6 and 10 µg/bottle**
- *Cx. tarsalis:* **4 µg/bottle.**

Bendiocarb

The LC_{99} and LC_{100} and TDCs selected for each species are summarized in Table 11.

With *Cx. quinquefasciatus*, the LC_{99} varied from 0.079% (0.061–0.095) for the VCRU strain to 0.41% (0.347–0.57) for the Nairobi strain. Similar results were observed for the LC_{100} values estimated from the raw data, which ranged from 0.1% for the VCRU strain to 0.4% for the Nairobi strain.

The data obtained with *Cx. tarsalis* (YOLO strain from CDC) were more consistent. The LC_{99} and LC_{100} ranged from 0.05% to 0.07% for the two laboratories.

The following TDCs were selected for step-3 testing:

- *Cx. quinquefasciatus :* **0.4 and 0.8%**
- *Cx. tarsalis :* **0.15%.**

Malathion

The LC_{99} and LC_{100} values and the TDCs selected for both Culex species are summarized in Table 12.

As observed with other taxa, the estimated LC_{99} values were much higher for malathion than for the other insecticides. With *Cx. quinquefasciatus*, the LC_{99} was the lowest for the VCRU strain (1.27%, range 1.18–1.36) and highest for the FIOCRUZ strain (3.35%, range 1.79–9.99). The LC_{99} and LC_{100} data were consistent.

With *Cx. tarsalis,* the LC_{99} against the YOLO strain was 0.93–1.85 and the LC_{100} was 1.5–2%.

The following TDCs were selected for step-3 testing:

- *Cx. quinquefasciatus:* **5%**
- *Cx. tarsalis:* **5%.**

Clothianidin

The LC_{99} and LC_{100} values and the TDCs selected for each species are summarized in Table 13.

With clothianidin, the LC_{99} and LC_{100} values were relatively consistent among laboratories and strains. With Cx. quinquefasciatus, the LC_{99} ranged from 1.22 µg per bottle (range 0.66–3.06) for the SLAB strain at FIOCRUZ to 3.42 (2.97–3.83) for the Nairobi strain at KEMRI. The LC_{100} was 1.25–5 µg per bottle for all strains tested.

A similar trend was observed with *Cx. tarsalis*. The LC_{99} ranged from 0.60 µg per bottle (range 0.46–1.14) to 1.83 µg (1.56–1.98) against the YOLO strain hosted at IRD and CDC, respectively, and the LC_{100} values were 1–2 µg per bottle.

The following TDCs were selected for step-3 testing:

- *Cx. quinquefasciatus:* **5 and 10 µg per bottle**
- *Cx. tarsalis:* **3 and 5 µg per bottle.**

Table 8. LC_{50}, LC_{99} and LC_{100} (observed) values and selected TDC (%) of alpha-cypermethrin against *Culex* species in WHO tube tests

Species	Laboratory	No. of bioassays	Mean LC_{50} (%)	Range[a] (min–max)	Mean LC_{99} (%)	Range (min–max)	2 x LC_{99}	2 x LC_{100} (observed)	TDC (%) selected
Cx. quinquefasciatus	FIOCRUZ, Brazil	3	0.072	0.057–0.117	0.391	0.251–0.599	**0.78**	0.5	0.5 and 1% for all centres, and an additional TDC of 2% for KEMRI
	IRD, France	4	0.02	0.011–0.03	0.351	0.142–1.216	0.70	0.4	
	KEMRI, Kenya	3	0.114	0.085–0.227	0.857	0.516–1.623	1.7	**1.2**	
	VCRU, Malaysia	4	0.007	0.007–0.013	0.347	0.071–0.37	0.69	0.2	
Cx. tarsalis	IRD, France	3	0.01	0.009–0.013	0.027	0.019–0.039	0.054	0.06	0.05
	CDC, USA	4	0.007	0.006–0.008	0.031	0.024–0.044	**0.06**	0.03	

CDC: United States Centers for Disease Control and Prevention; FIOCRUZ: Fundaçao Oswaldo Cruz; IRD: Institut de Recherche pour le Développement; KEMRI: Kenya Medical Research Institute; LC: lethal concentration; max: maximum; min: minimum; TDC: tentative discriminating concentration max: maximum; VCRU: Vector Control Research Unit.

[a] Obtained from individual estimates for each insecticide and species (minimum and maximum).

In bold, LC_{99} or LC_{100} value selected for estimating the TDC.

Table 9. LC_{50}, LC_{99} and LC_{100} (observed) values and selected TDC (%) of deltamethrin against *Culex* species in WHO tube tests

Species	Laboratory	No. of bioassays	Mean LC_{50} (%)	Range[a] (min–max)	Mean LC_{99} (%)	Range (min–max)	2 x LC_{99}	2 x LC_{100} (observed)	TDC (%) selected
Cx. quinquefasciatus	FIOCRUZ, Brazil	3	0.009	0.007–0.011	0.139	0.094–0.224	**0.28**	0.4	0.4 and 0.8
	IRD, France	4	0.008	0.005–0.014	0.178	0.085–0.581	0.35	**0.4**	
	KEMRI, Kenya	4	0.031	0.024–0.052	0.386	0.221–1.41	**0.77**	**0.4**	
	VCRU, Malaysia	4	0.004	0.004–0.005	0.012	0.01–0.015	0.024	0.02	
Cx. tarsalis	IRD, France	3	0.006	0.004–0.008	0.025	0.014–0.04	**0.05**	0.03	0.05
	CDC, USA	4	0.011	0.009–0.013	0.027	0.024–0.035	**0.054**	0.06	

CDC: United States Centers for Disease Control and Prevention; FIOCRUZ: Fundaçao Oswaldo Cruz; IRD: Institut de Recherche pour le Développement; KEMRI: Kenya Medical Research Institute; LC: lethal concentration; max: maximum; min: minimum; TDC: tentative discriminating concentration; VCRU: Vector Control Research Unit.; max: maximum.

[a] Obtained from individual estimates for each insecticide and species (minimum and maximum).

In bold, LC_{99} or LC_{100} value selected for estimating the TDC.

Table 10. LC_{50}, LC_{99} and LC_{100} (observed) values and selected TDC (%) of transfluthrin against *Culex* species in WHO bottle bioassays

Species	Laboratory	No. of bioassays	Mean LC_{50} (µg/bottle)	Range[a] (min–max)	Mean LC_{99} (µg/bottle)	Range (min–max)	2 x LC_{99}	2 x LC_{100} (observed)	TDC (µg/bottle) selected
	FIOCRUZ, Brazil	4	0.76	0.657–1.453	4.56	1.292–10.4	**9.1**	**6**	4, 6 and 10
	IRD, France	4	0.62	0.116–0.766	2.738	1.472–4.312	5.5	**4**	
	KEMRI, Kenya	4	1.062	0.816–1.867	6.016	4.333–14.5	12[b]	14[b]	
	VCRU, Malaysia	4	0.911	0.845–0.965	1.435	1.309–1.626	2.9	3	
Cx. tarsalis	IRD, France	3	0.707	0.589–0.811	1.351	1.126–1.816	**2.7**	**4**	4
	CDC, USA	4	0.886	0.799–1.003	2.283	1.964–19.1	**4.6**	**4**	

CDC: United States Centers for Disease Control and Prevention; FIOCRUZ: Fundaçao Oswaldo Cruz; IRD: Institut de Recherche pour le Développement; KEMRI: Kenya Medical Research Institute; LC: lethal concentration; max: maximum; min: minimum; TDC: tentative discriminating concentration; VCRU: Vector Control Research Unit.

[a] Obtained from individual estimates for each insecticide and species (minimum and maximum).

[b] Unexpected high values compared to other centres. Data not taken into account in the selection of the TDC.

In bold, LC_{99} or LC_{100} value selected for estimating the TDC.

Table 11. LC_{50}, LC_{99} and LC_{100} (observed) values and selected TDC (%) of bendiocarb against *Culex* species in WHO tube tests

Species	Laboratory	No. of bioassays	Mean LC_{50} (%)	Range[a] (min–max)	Mean LC_{99} (%)	Range (min–max)	2 x LC_{99}	2 x LC_{100} (observed)	TDC (%) selected
Cx. quinquefasciatus	FIOCRUZ, Brazil	4	0.056	0.03–0.067	0.171	0.084–0.421	0.35	**0.4**	0.4 and 0.8
	IRD, France	4	0.046	0.037–0.066	0.15	0.079–0.432	0.30	0.3	
	KEMRI, Kenya	3	0.132	0.11–0.177	0.416	0.345–0.575	0.83	**0.8**	
	VCRU, Malaysia	4	0.039	0.037–0.043	0.079	0.061–0.095	0.16	0.2	
Cx. tarsalis	IRD, France	3	0.033	0.025–0.036	0.05	0.038–0.061	0.10	0.10	0.15
	CDC, USA	4	0.038	0.035–0.041	0.07	0.059–0.077	**0.14**	**0.14**	

CDC: United States Centers for Disease Control and Prevention; FIOCRUZ: Fundaçao Oswaldo Cruz; IRD: Institut de Recherche pour le Développement; KEMRI: Kenya Medical Research Institute; LC: lethal concentration; max: maximum; min: minimum; TDC: tentative discriminating concentration; VCRU: Vector Control Research Unit.

[a] Obtained from individual estimates for each insecticide and species (minimum and maximum).

In bold, LC_{99} or LC_{100} value selected for estimating the TDC.

Table 12. LC_{50}, LC_{99} and LC_{100} (observed) values and selected TDC (%) of malathion against *Culex* species in WHO tube tests

Species	Laboratory	No. of bioassays	Mean LC_{50} (%)	Range[a] (min–max)	Mean LC_{99} (%)	Range (min–max)	2 x LC_{99}	2 x LC_{100} (observed)	TDC (%) selected
Cx. quinquefasciatus	FIOCRUZ, Brazil	4	1.124	0.841–1.335	3.351	1.788–9.988	**6.7**	NA	5
	IRD, France	4	0.904	0.799–0.953	1.407	1.239–2.086	2.8	3	
	KEMRI, Kenya	1	0.778	0.714–0.841	1.554	1.372–1.843	3.1	**4**	
	VCRU, Malaysia	4	0.913	0.877–0.955	1.266	1.186–1.361	2.5	2.4	
Cx. tarsalis	IRD, France	3	0.914	0.664–1.115	1.85	1.561–3.191	3.7	**4**	5
	CDC, USA	4	0.306	0.275–0.393	0.936	0.698–1.201	1.9	3	

CDC: United States Centers for Disease Control and Prevention; FIOCRUZ: Fundaçao Oswaldo Cruz; IRD: Institut de Recherche pour le Développement; KEMRI: Kenya Medical Research Institute; LC: lethal concentration; max: maximum; min: minimum; NA: not available; TDC: tentative discriminating concentration; VCRU: Vector Control Research Unit.

[a] Obtained from individual estimates for each insecticide and species (minimum and maximum).

In bold, LC_{99} or LC_{100} value selected for estimating the TDC.

Table 13. LC_{50}, LC_{99} and LC_{100} (observed) values and selected TDC (µg/bottle) of clothianidin against *Culex* species in WHO bottle bioassays

Species	Laboratory	No. of bioassays	Mean LC_{50} (µg/bottle)	Range[a] (min–max)	Mean LC_{99} (µg/bottle)	Range (min–max)	2 x LC_{99}	2 x LC_{100} (observed)	TDC (µg/bottle) selected
Cx. quinquefasciatus	FIOCRUZ, Brazil	3	0.294	0.012–0.468	1.219	0.66–3.06	2.5	2.5	5 and 10
	IRD, France	4	0.617	0.264–0.852	1.712	1.244–2.579	3.4	**10**	
	KEMRI, Kenya	4	1.871	1.501–2.573	3.428	2.969–3.833	6.8	**5.4**	
	VCRU, Malaysia	4	0.526	0.453–0.72	2.865	1.891–6.944	5.7	4.0	
Cx. tarsalis	IRD, France	3	0.171	0.108–0.22	0.601	0.463–1.138	1.2	2.0	3 and 5
	CDC, USA	4	1.35	1.211–1.404	1.835	1.561–1.983	**3.7**	**4**	

CDC: United States Centers for Disease Control and Prevention; FIOCRUZ: Fundaçao Oswaldo Cruz; IRD: Institut de Recherche pour le Développement; KEMRI: Kenya Medical Research Institute; LC: lethal concentration; max: maximum; min: minimum; TDC: tentative discriminating concentration; VCRU: Vector Control Research Unit, Universiti Sains Malaysia.

[a] Obtained from individual estimates for each insecticide and species (minimum and maximum).

In bold, LC_{99} or LC_{100} value selected for estimating the TDC.

9.3 Validation of tentative discriminating concentrations (step 3)

A total of 20 TDCs were selected for further testing in step 3 against the two *Culex* species according to the results of step 2 (12 TDCs in filter paper test and 8 TDCs in bottle bioassays). In all, 63 bioassays (49 bioassays with *Cx. quinquefasciatus* and 14 with *Cx. tarsalis*) were conducted with a total of 6278 *Culex* mosquitoes.

In step 3, the mortality rate in *Culex* spp. was > 98% (i.e. the WHO susceptibility cut-off level) for most of the selected TDCs at the completion of step-2 testing (Tables 14, 15 and 16), except with alpha-cypermethrin (0.5%; 92–94% mortality; Tables 14) and transfluthrin (4 µg/bottle; 96% mortality; Table 16) against the Nairobi strain of *Cx. quinquefasciatus* at KEMRI. In quality control tests, kdr-resistant alleles (1014F) were detected in the Nairobi strain (see Annex 2 for details).

The results of step-3 testing were reviewed at the WHO consultation on 20 June 2023, which made recommendations for the final DCs.

Table 14. Mortality of *Cx. quinquefasciatus* and *Cx. tarsalis* with selected TDCs in WHO tube tests in step 3 (1-h exposure; 24-h recording time)

Insecticide	*Culex* species	Laboratory	TDC (%)	Mosquito mortality (%)	Number of mosquitoes tested
Alpha-cypermethrin	*Cx. quinquefasciatus*	IRD	0.5	100	101
		VCRU		100	100
		FIOCRUZ		100	101
		KEMRI		92[a,b]	100
		IRD	1	10	103
		VCRU		100	100
		FIOCRUZ		100	83
		KEMRI		100[a,b]	100
		KEMRI	2	100[a,b]	100
	Cx. tarsalis	IRD	0.05	100[a]	102
		CDC		100	113

Insecticide	*Culex* species	Laboratory	TDC (%)	Mosquito mortality (%)	Number of mosquitoes tested
Deltamethrin	*Cx. quinquefasciatus*	IRD	0.4	100	103
		VCRU		100	100
		FIOCRUZ		100	104
		KEMRI		100[a,b]	100
		IRD	0.8	100	101
		VCRU		100	100
		FIOCRUZ		100	91
		KEMRI		100[a,b]	100
	Cx. tarsalis	IRD	0.05	100[a]	100
		CDC		100	115
Bendiocarb	*Cx. quinquefasciatus*	IRD	0.4	100	102
		VCRU		100	100
		FIOCRUZ		100[a]	100
		KEMRI		100[a,b]	100
		IRD	0.8	100	104
		VCRU		100	100
		FIOCRUZ		100	81
		KEMRI		100[a,b]	100
	Cx. tarsalis	IRD	0.15	100[a]	99
		CDC		100	110
Malathion	*Cx. quinquefasciatus*	IRD	5	100[a]	101
		VCRU		100	100
		FIOCRUZ		100	100
		KEMRI		100[a,b]	100
	Cx. tarsalis	IRD	5	100[a]	97
		CDC		100	115

Green: 100% mortality; red: < 98% mortality.

CDC: United States Centers for Disease Control and Prevention; FIOCRUZ: Fundaçao Oswaldo Cruz; IRD: Institut de Recherche pour le Développement; KEMRI: Kenya Medical Research Institute; TDC: tentative discriminating concentration; VCRU: Vector Control Research Unit.

[a] Tests conducted with filter papers impregnated at the local institution due to delays in receiving the papers from VCRU, Malaysia.

[b] Tests conducted with a mosquito colony presenting *kdr* 1014F alleles.

After the WHO consultation on 20 June 2023, the remaining step-3 tests were also completed by IRD and KEMRI using papers impregnated at VCRU, Malaysia. The new results presented in Table 15 confirmed the previous results obtained with locally impregnated papers (Table 14).

Table 15. Mortality of *Cx. quinquefasciatus* and *Cx. tarsalis* with selected TDCs in WHO tube tests in step 3 by IRD and KEMRI using VCRU-supplied impregnated papers (1-h exposure; 24-h recording time, papers impregnated at VCRU, Malaysia)

Insecticide	*Culex* species	Laboratory	TDC (%)	Mosquito mortality (%)	Number of mosquitoes tested
Alpha-cypermethrin	*Cx. tarsalis*	IRD	0.05	100	99
	Cx. quinquefasciatus	KEMRI	0.5	94[a]	100
		KEMRI	1	100[a]	100
		KEMRI	2	100[a]	100
Deltamethrin	*Cx. tarsalis*	IRD	0.05	100	103
	Cx. quinquefasciatus	KEMRI	0.4	100[a]	100
		KEMRI	0.8	100[a]	100
Bendiocarb	*Cx. tarsalis*	IRD	0.15	100	101
	Cx. quinquefasciatus	KEMRI	0.4	100[a]	100
		KEMRI	0.8	100[a]	100
Malathion	*Cx. tarsalis*	IRD	5	100	103
	Cx. quinquefasciatus	IRD	5	100	103
		KEMRI	5	100[a]	100

Green: 100% mortality; red: < 98% mortality.
h: hour; IRD: Institut de Recherche pour le Développement; KEMRI: Kenya Medical Research Institute; TDC: tentative discriminating concentration.
[a] Tests conducted with a mosquito colony presenting *kdr* 1014F alleles.

Table 16. Mortality of *Cx. quinquefasciatus* and *Cx. tarsalis* with selected TDC of transfluthrin and clothianidin in WHO bottle bioassays in step 3 (1-h exposure; 24-h recording time)

Insecticide	*Culex* species	Laboratory	TDC (µg/bottle)	Mosquito mortality (%)	Number of mosquitoes tested
Transfluthrin	*Cx. quinquefasciatus*	IRD	4	100	98
		VCRU		100	102
		FIOCRUZ		100	81
		KEMRI		96[a]	100
		IRD	6	100	98
		VCRU		100	102
		FIOCRUZ		100	81
		KEMRI		100[a]	100
		IRD	10	100	103
		VCRU		100	102
		FIOCRUZ		100	86
	Cx. tarsalis	IRD	4	100	98
		CDC		100	109
Clothianidin	*Cx. quinquefasciatus*	IRD	5	100	100
		VCRU		100	101
		FIOCRUZ		100	88
		KEMRI		100[a]	100
		IRD	10	100	103
		VCRU		100	102
		FIOCRUZ		100	83
		KEMRI		100[a]	100
	Cx. tarsalis	IRD	3	100	101
		CDC		100	111
		IRD	5	100	100
		CDC		100	104

Green: 100% mortality; red: < 98% mortality.
CDC: United States Centers for Disease Control and Prevention; FIOCRUZ: Fundaçao Oswaldo Cruz; h: hour; IRD: Institut de Recherche pour le Développement; KEMRI: Kenya Medical Research Institute; LC: lethal concentration; max: maximum; min: minimum; TDC: tentative discriminating concentration; VCRU: Vector Control Research Unit.
[a] Tests conducted with a mosquito colony presenting *kdr* 1014F alleles.

10. Main problems encountered

While the laboratories reported no major problems in performing the required tests, the following issues were reported.

- Some difficulty was found in the regular production of sufficient numbers of mosquitoes for step-2 bioassays in triplicate, especially with *Cx. tarsalis*, which was found to be more difficult to breed in colonies. Consequently, some bioassays were conducted with 50 mosquitoes per test concentration and repeated at different intervals.
- The filter papers for step-3 testing were impregnated by VCRU, Malaysia, but quality control analysis at an independent laboratory and customs clearance of shipments of papers from VCRU to IRD and KEMRI took a long time. Therefore, as in step-1 and -2 testing, these two centres also used locally impregnated papers with selected TDCs and tested them in step 3. Additional bioassay data with VCRU-supplied papers were carried out by IRD and KEMRI after the WHO consultation on 20 June 2023 to confirm the results obtained with papers treated at those institutes (data were provided as additional information in Table 15).
- The results (LC_{99} or LC_{100}) of different laboratories when testing pyrethoids with *Cx. quinquefasciatus* in tube tests and the bottle bioassays showed varying tolerance of colonized strains to different insecticides. At KEMRI, high values for the LC were reported in tests with pyrethoids and *Cx. quinquefasciatus* in either tube tests or bottle bioassays. This laboratory reported survival of some mosquitoes at 24 h after exposure to the pyrethroid insecticides, even at high concentrations. A molecular analysis conducted by the Universidad Autónoma de Nuevo León, Mexico, with a few specimens of *Cx. quinquefasciatus* (Nairobi strain) showed the presence of the L1014F mutation in this strain (see results in Annex 2). Consequently, the expert group recommended exclusion of the data from KEMRI for pyrethroids and made final recommendations on the DCs for pyrethroids using the datasets from the other test centres. The KEMRI data were, however, considered to be valid for determination of the final DCs for malathion and bendiocarb. Quality control of the other mosquito colonies used for testing in synergist assays and polymerase chain reaction assays showed no evidence of any known mechanism of resistance to pyrethroids (i.e. 1014F *kdr* mutation and/or mixed function oxidase) for the SLAB strain tested at IRD and FIOCRUZ (Annex 2).
- Because of the limited number of participating laboratories, some data could not be cross-validated with those of other laboratories at the two sites selected for testing in *Cx. tarsalis*.

11. Conclusions and recommendations

The results of this study conducted in 2022–2023 in five laboratories to establish and validate the DCs of insecticides in order to monitor resistance in *Cx. quinquefasciatus* and *Cx. tarsalis*, the main vectors of lymphatic filariasis parasites and West Nile virus were assessed in a WHO consultation on 27 March 2023 to select TDCs for step-3 testing. The results of step-3 testing were assessed in a second consultation, on 20 June 2023, which made final recommendations to WHO on standard DCs and suggested that further studies be conducted to fill the identified knowledge gaps. The agendas and lists of participants in the WHO consultations are given in Annex 1.

The main outcomes and achievements of the multi-centre study were:

- conduct of tube tests and WHO bottle bioassays to test the susceptibility of *Cx. quinquefasciatus* and *Cx. tarsalis* to six insecticides used in public health (deltamethrin, alpha-cypermethrin, bendiocarb, malathion, transfluthrin, clothianidin);
- validated SOPs previously developed for *Anopheles* and *Aedes* spp. for testing insecticide resistance used in WHO tube tests and bottle bioassays with Culex mosquitoes;
- a central database of bioassay records for 107 737 *Culex* specimens created to provide additional data for studies of the reasons for different rates of mosquito mortality under different test conditions; and
- establishment and validation of 12 new DCs for six insecticides against two Culex species in either WHO tube tests or WHO bottle bioassays to monitor resistance in field populations of these species (Tables 17 and 18).

The results were reviewed at the WHO consultation on 20 June 2023, in which the experts made the following recommendations to WHO.

- The participating laboratories should characterize the test strains by various methods, such as synergist bioassays and molecular assays, to determine the quality of the mosquito colonies. In the absence of formal WHO guidance on test procedures for detecting molecular resistance markers, the experts recommended that WHO provide a standard method and test end-points for adequate characterization of insecticide resistance mechanisms in colonized mosquitoes for bioassays.
- Only those laboratories with mosquito colonies that are fully susceptible to insecticide DCs for different classes of insecticides should participate in future WHO concentration–response studies to avoid discrepancies in the determination of LCs and DCs.

- The Bayesian binomial model developed at Imperial College London, United Kingdom, to analyse intensity bioassay data *(18)* showed that LC_{99} values were more robust than LC99.9 values for determining mosquito mortality at high concentrations. Overall, it was estimated that the TDCs were likely to be 1.3–2.8 times higher when $LC_{99.9}$ values were used to calculate them rather than LC_{99} values. Better understanding of the variation in mortality in bioassays will indicate how best to select DCs in future studies.
- For the WHO bottle bioassay, MERO, which is produced by a single manufacturer, is currently the only WHO-recommended surfactant. The suitability of other surfactants or additives for coating bottles, such as SPAN 80, should be assessed in WHO-coordinated studies with different mosquito species and insecticide compounds.
- Investigations should be conducted to determine whether surfactants alter the effectiveness of some insecticides, which would make resistance monitoring much less sensitive. The capacity of surfactants to accelerate penetration of insecticides into insect cuticles or body parts should be further explored.
- More centres should be invited to participate in future WHO multi-centre studies, with a wider range of mosquito strains, to assess the consistency of bioassay results and provide better estimates of the DCs of insecticides.
- The DCs for other insecticides used in vector control products and of new insecticides likely to be used in the future should be determined with various strains of *Culex* spp.
- The purpose of WHO susceptibility tests is to detect the emergence of resistance to individual insecticides in a previously susceptible population. Consequently, current WHO guidance addresses tube test and bottle bioassay procedures to test the susceptibility of individual active ingredients and not their combination, or of formulated products. Although insecticide combinations and mixtures are required for vector control, it is recommended that mosquito resistance to each active ingredient be tested separately.
- Further monitoring of insecticide resistance in field populations of *Culex* mosquito vectors is recommended. Evidence should be collected by national disease control programmes and shared with WHO for monitoring resistance and developing insecticide resistance threat maps for *Culex* spp.

Table 17. Insecticide discriminating concentrations[a] for *Culex* species in WHO tube tests (24-h filter paper drying time; 1-h exposure; 24-h holding or recording time)

Insecticide	Species	Discriminating concentration (%)	Carrier oil (dissolved in acetone)
Alpha-cypermethrin	*Cx. quinquefasciatus*	0.5	Silicone
	Cx. tarsalis	0.05	
Deltamethrin	*Cx. quinquefasciatus*	0.4	Silicone
	Cx. tarsalis	0.05	
Bendiocarb	*Cx. quinquefasciatus*	0.4	Olive
	Cx. tarsalis	0.15	
Malathion	*Cx. quinquefasciatus*	5	Olive
	Cx. tarsalis	5	

[a] As mentioned in Table 1, the historical discriminating concentrations of DDT (0.04%), lambda-cyhalothrin (0.025%), permethrin (0.25%), propoxur (0.10%) and fenithrothin (1.00%), which were not validated in this study, remain unchanged.

Table 18. Insecticide discriminating concentrations for *Culex* species in WHO bottle bioassays (24-h bottle drying time; 1-h exposure; 24-h holding or recording time)

Insecticide	Species	Discriminating concentration (µg/bottle)	Solvent and surfactant oil
Transfluthrin	*Cx. quinquefasciatus*	4	Acetone
	Cx. tarsalis	4	
Clothianidin	*Cx. quinquefasciatus*	5	Acetone + MERO 1500 ppm
	Cx. tarsalis	5	

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Annex 1. Agendas and participants in WHO consultations

Agenda of consultation on 27 March 2023

WHO consultation to review results of the multi-centre study on determination of insecticide discriminating concentrations for monitoring resistance in *Culex* spp., 27 March 2023 (virtual meeting)

Open session		
14:00–14:05	- Opening remarks	Dr Raman Velayudhan, Unit Head, NTD/VVE, WHO
14:05–14:10	- Objectives of the consultation - Summary of interests declared by WHO experts - Meeting logistics - Overview of the process used for the study - Appointment of Chair, Co-chair and rapporteurs	Dr Rajpal Yadav, Scientist & study coordinator, NTD/VVE, WHO
Chair/Co-chair to take over the meeting		
14:10–15:00	- Presentation of the study design and method - Discussion	Dr Vincent Corbel, IRD
15:00–15:30	- Presentation of results of steps 1 and 2 and technical limitations	Dr Vincent Corbel, IRD
15:30–15:45	Coffee break	
15:45–16:15	- Statistical analysis of the multi-centre data	Ms Mara Kont, Imperial College London
16:15–17.00	- Discussion on TDCs of test compounds	All participants
Closed session (restricted to WHO experts, investigators and WHO secretariat)		
17:00–17:30	- Drafting of recommendations - Closure	Experts and investigators only

List of participants

Name	Institution
WHO experts	
Dr Luc Djogbenou	Universite Abomey Calavi, Benin
Dr Josiane Etang	Malaria Research Laboratory, Organisation de coordination et de coopération pour la lutte contre les grandes endémies en Afrique Centrale, Yaoundé, Cameroon
Dr Kamaraju Raghavendra	Indian Council for Medical Research, National Institute of Malaria Research, New Delhi, India
Dr Zahra Saeidi	School of Public Health, Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran
Dr Nazni Bt Hj Wasi Ahmad	Institute of Medical Research, Kuala Lumpur, Malaysia
Dr Adriana Flores	Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Mexico
Dr Lalita Roy	Tropical and Infectious Disease Centre, B. P. Koirala Institute of Health Sciences, Dharan, Nepal
Dr Thomas Churcher	Imperial College London, London, United Kingdom
Dr Rosemary Lees	Liverpool School of Tropical Medicine, Liverpool, United Kingdom
Collaborators and peer reviewers	
Dr Ademir Martins Jr	Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
Ms Luciana Diad	Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
Mr Thiago C. S. Ribeiro	Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
Dr Martha Ahumada	Instituto Nacional de Salud, Bogotá, Colombia
Dr Vincent Corbel	Institut de Recherche pour le Développement, Montpellier, France
Mr Stephane Duchon	Institut de Recherche pour le Développement, Montpellier, France
Ms Dominique Cerqueira	Institut de Recherche pour le Développement, Montpellier, France
Dr Luna Kamau	Kenya Medical Research Institute, Nairobi, Kenya
Dr Damaris-Matoke Muhia	Kenya Medical Research Institute, Nairobi, Kenya
Mr Adanan Che Rus	Vector Control Research Unit, Universiti Sains Malaysia, Penang, Malaysia
Ms Nur Amirah Abdul Latif	Vector Control Research Unit, Universiti Sains Malaysia, Penang, Malaysia
Dr Dominic P. Dee	Imperial College London, London, United Kingdom
Ms Mara K. Kont	Imperial College London, London, United Kingdom
Dr Audrey Lenhart	Centers for Disease Control and Prevention, Atlanta (GA), USA
Dr Lucrecia Vizcaíno Cabarrús	Entomology Branch, Centers for Disease Control and Prevention, Atlanta (GA), USA
Dr Christopher Sandy	Entomology Branch, Centers for Disease Control and Prevention, Atlanta (GA), USA

Stakeholders (observers)	
Dr James Austin	BASF, Wake Forest (NC), USA
Dr Mark Hoppe	Syngenta, Basel, Switzerland
Dr Sebastian Horstmann	Envu, Mosquito Management, Monheim, Germany
Dr William Jany	Clarke International LLC, St Charles (IL), USA
Dr Laetitia Leroy	Clarke International LLC, St Charles (IL), USA
Dr Anil Makkapati	Envu, Mosquito Management, Singapore
Dr Dave Malone	Bill & Melinda Gates Foundation, Liverpool, United Kingdom
Dr Susanne Stutz	BASF SE, Limbergerhof, Germany
Dr Barnabas Zogo	Sumitomo Chemicals Co. Ltd., Tokyo, Japan
WHO secretariat	
Dr Samira Al-Eryani	Regional Office for the Eastern Mediterranean, Cairo, Egypt
Dr Emmanuel Chanda	Regional Office for Africa, Brazzaville, Democratic Republic of the Congo
Dr Raman Velayudhan	Vector Control, Veterinary Public Health and Environment Unit, Department of Control of Neglected Tropical Diseases, WHO, Geneva, Switzerland
Dr Rajpal S. Yadav	Vector Control, Veterinary Public Health and Environment Unit, Department of Control of Neglected Tropical Diseases, WHO, Geneva, Switzerland
Dr Qingxia Zhong	Vector Control, Veterinary Public Health and Environment Unit, Department of Control of Neglected Tropical Diseases, WHO, Geneva, Switzerland

Agenda of consultation on 20 June 2023

WHO consultation to review results of the multi-centre study on determination of insecticide discriminating concentrations for monitoring resistance in *Culex* spp., 20 June 2023 (virtual meeting).

Open session		
14:00–14:10	- Opening remarks - Objectives of the consultation - Summary of interests declared by WHO experts - Appointment of Chair, Co-chair and rapporteur	Dr Rajpal Yadav, Scientist & study coordinator, NTD/VVE, WHO
Chair/Co-chair to take over the meeting		
14:10–15:00	- Presentation of results of step 3 and technical limitations	Dr Vincent Corbel, IRD
15:00–15:30	- General discussion (all participants)	Dr Vincent Corbel, IRD
15:30–15:45	- Coffee break	
Closed session (restricted to WHO experts, investigators and the WHO secretariat)		
15:45–17:30	- Conclusions and recommendations - Closure	Experts and investigators only

List of participants

Name	Institution
WHO experts (external contributors)	
Dr Luc Djogbenou	Universite Abomey Calavi, Benin
Dr Josiane Etang	Malaria Research Laboratory, Organisation de coordination et de coopération pour la lutte contre les grandes endémies en Afrique Centrale, Yaounde, Cameroon (Co-chair)
Dr Kamaraju Raghavendra	National Institute of Malaria Research, New Delhi, India (Rapporteur)
Professor Ahmad Ali Enayati	School of Public Health, Mazandaran University of Medical Sciences, Sari, Islamic Republic of Iran
Dr Zahra Saeidi	School of Public Health, Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran
Dr Nazni Bt Hj Wasi Ahmad	Institute of Medical Research, Kuala Lumpur, Malaysia
Dr Adriana Flores	Universidad Autónoma de Nuevo León, San Nicolás de los Garza, México (Chair)
Dr Lalita Roy	Tropical and Infectious Disease Centre, B. P. Koirala Institute of Health Sciences, Dharan, Nepal
Dr João Pinto	Instituto de Higiene e Medicina Tropical, Universidade NOVA de Lisboa, Lisboa, Portugal
Dr Thomas Churcher	Imperial College London, London, United Kingdom
Dr Rosemary Lees	Liverpool School of Tropical Medicine, Liverpool, United Kingdom

Collaborators and peer reviewers	
Dr Ademir Martins Jr	Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
Ms Luciana Dias	Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
Dr Martha Ahumada	Instituto Nacional de Salud, Bogotá, Colombia
Dr Vincent Corbel	Institut de Recherche pour le Développement, Montpellier, France
Mr Stephane Duchon	Institut de Recherche pour le Développement, Montpellier, France
Dr Luna Kamau	Kenya Medical Research Institute, Nairobi, Kenya
Ms Nur Amirah Abdul Latif	Vector Control Research Unit, Universiti Sains Malaysia, Penang, Malaysia
Mr Adanan Che Rus	Vector Control Research Unit, Universiti Sains Malaysia, Penang, Malaysia
Dr Dominic P. Dee	Imperial College London, London, United Kingdom
Ms Mara K. Kont	Imperial College London, London, United Kingdom
Stakeholders (observers)	
Dr Anil Makkapati	Envu, Mosquito Management, Singapore
Dr Mark Hoppe	Syngenta, Basel, Switzerland
Dr William Jany	Clarke International LLC, St Charles (IL), USA
Dr Laetitia Leroy	Clarke International LLC, St Charles (IL), USA
Ms Joanna Tyszko	Clarke International LLC, St Charles (IL), USA
WHO secretariat	
Dr Haroldo S da Silva Bezerra	Regional Office for the Americas, Washington (DC), USA
Dr Rajpal S. Yadav	Vector Control, Veterinary Public Health and Environment Unit, Department of Control of Neglected Tropical Diseases, WHO, Geneva, Switzerland

Annex 2. Quality control of the *Culex quinquefasciatus* SLAB and Nairobi strains used for testing

A2.1 Detection of *kdr* mutations and possible involvement of cytochrome P450 isozymes in *Culex quinquefasciatus* SLAB strain at IRD and FIOCRUZ

Synergist bioassays

Synergist bioassays were conducted with the *Cx. quinquefasciatus* SLAB strain against filter papers impregnated with deltamethin 0.01% and 0.05%, permethrin 0.1%, 0.3%, 0.75% and 1% and PBO 4% according to the WHO procedure *(1)*. Pre-exposure of mosquitoes for 1 h to PBO 4% did not increase mortality with deltamethrin (Fig. A2.1, no. of mosquitoes tested = 484) or permethrin (Fig. A2.2, no. of mosquitoes tested = 496), suggesting no involvement of cytochrome P450 isozymes in pyrethroid resistance.

Fig. A2.1 Synergist bioassays conducted with deltamethrin against SLAB strain of *Cx. quinquefasciatus*

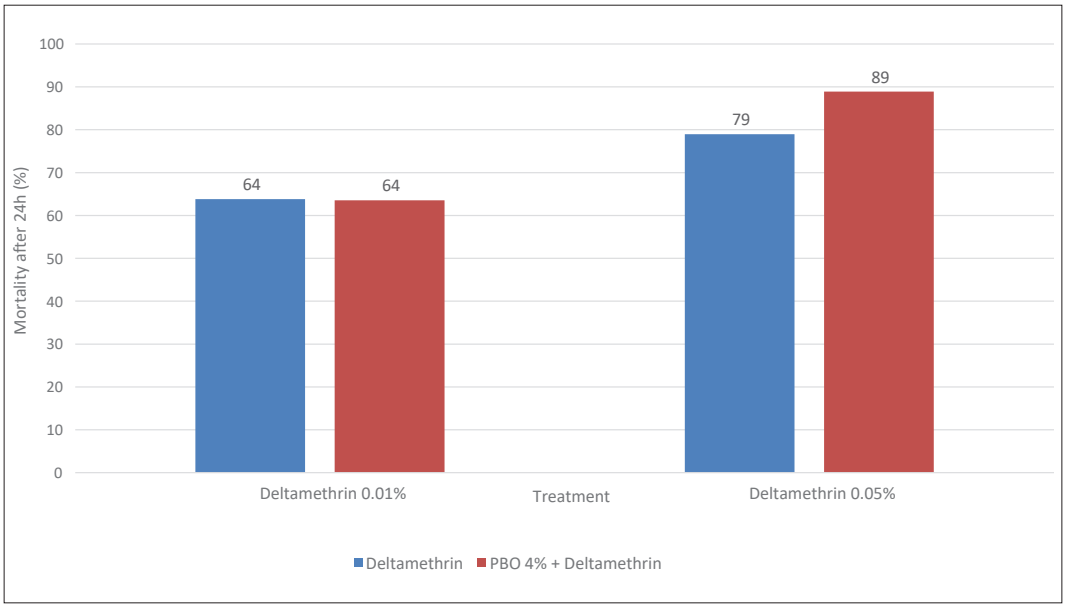
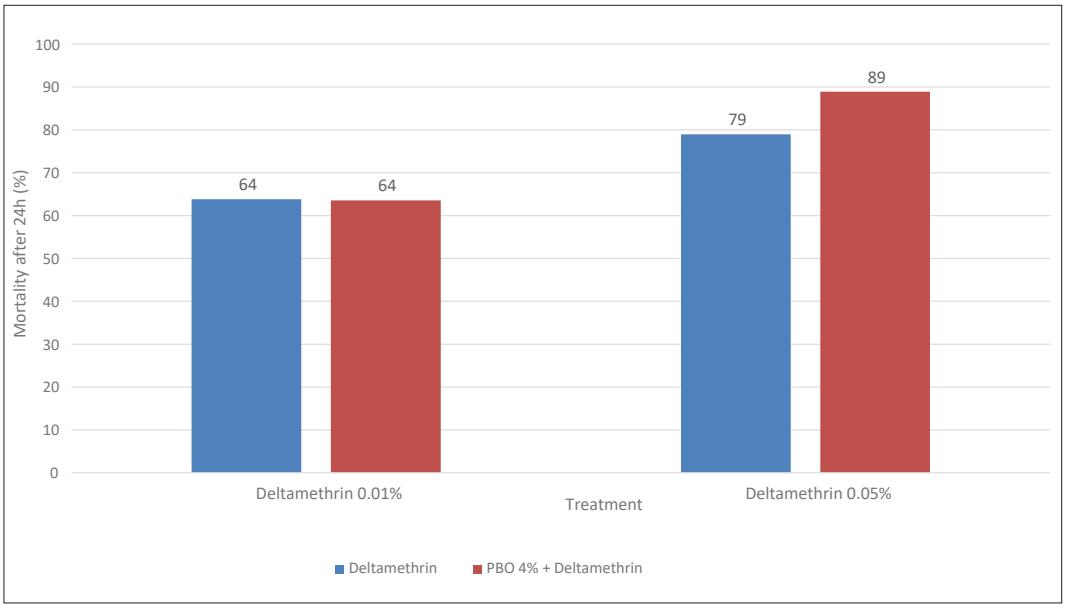


Fig. A2.2. Synergist bioassays conducted with permethrin against SLAB strain of *Cx. quinquefasciatus*



Molecular assays for *kdr* detection

The results of SANGER sequencing of the SLAB strain of *Cx. quinquefasciatus* did not demonstrate the presence of 1014F alleles in the 60 mosquito samples tested. Genetic polymorphism at the 1014 position was, however, reported in 47% of the samples tested (at four possible codons: TGT, TGA, TTA, TTT). Hence, there is little probability that *kdr*-resistant alleles are present in this strain (Table A2.1).

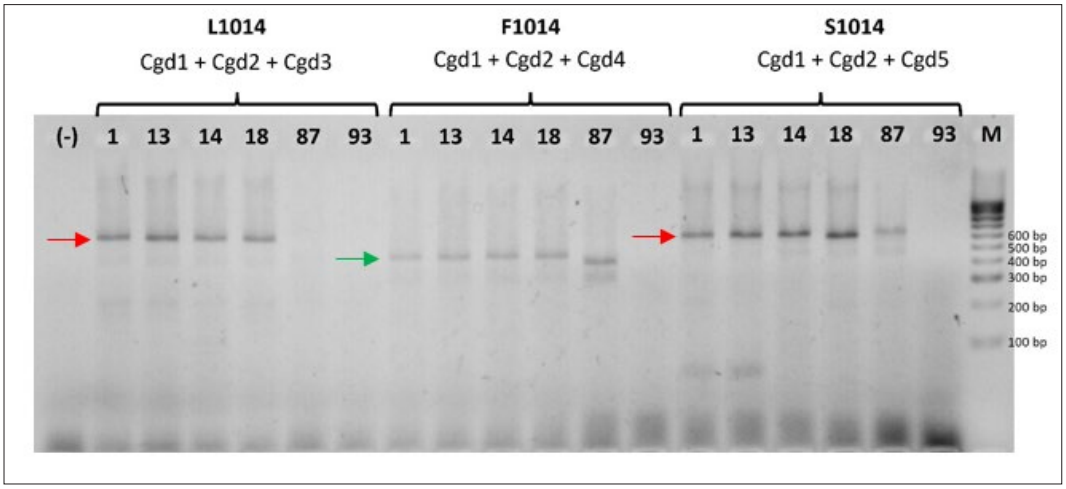
Table A2.1. Frequency of codons related to genetic polymorphism of kdr at position 1014

	TTA (1014S)	T, G/T, T/A (polymorphism)	TTT (1014F)
n	32	28	0
	53%	47%	0%

A2.2 Detection of the kdr mutations L1014F and L1014S in *Cx. quinquefasciatus* (Nairobi strain)

A molecular analysis was conducted at **Universidad Autónoma de Nuevo León**, Mexico, to detect possible *kdr* mutations (1014F and 1014S) in the *Cx. quinquefasciatus* Nairobi strain through melt curve and end-point polymerase chain reaction analysis with primers described elsewhere *(2)*. A DNA quality assessment with gel electrophoresis and spectrophotometric techniques was performed to ensure standardized, reproducible results. In the DNA quality assessment, only four of 100 samples showed DNA integrity, with an acceptable 260/280 ratio. These results indicated the presence of the 1014F mutant allele in the KEMRI strain, and mosquitoes were found to be homozygous for this mutation (Fig. A2.3).

Fig. A2.2. Polymerase chain reaction amplification analysis of the voltage-gated sodium channel containing mutations at the 1014 locus



Note: Numbers indicate the numbers of samples analysed. The red arrow highlights the ~540 bp fragment. The green arrow shows the ~380 bp fragment indicating the L1014F mutation.

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Global Neglected Tropical Diseases Programme
20 Avenue Appia
1211 Geneva 27
Switzerland

neglected.diseases@who.int

https://www.who.int/teams/control-of-neglected-tropical-diseases

9789240078970