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REPORT OF A MULTI-CENTRE LABORATORY STUDY AND WHO EXPERT CONSULTATIONS

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Determining discriminating concentrations of insecticides for monitoring resistance in sand flies: report of a multi-centre laboratory study and WHO expert consultations

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[1] The other stakeholders were representatives of the 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 limited to the WHO experts and the secretariat. The stakeholders did not participate in final approval of the report.

Declaration of interest and confidentiality undertaking

WHO reported that it had received and reviewed declarations of interest and confidentiality undertaking from all the external contributors i.e., experts who participated in the WHO consultations and had concluded that none could give rise to a potential or reasonably perceived conflict of interest related to the subjects discussed at the meetings.

Abbreviations and acronyms

ALIP	Aklilu Lemma Institute of Pathobiology
CR	completion rate
DC	discriminating concentration
FIOCRUZ	Fundaçao Oswaldo Cruz
ICDDRb	International Centre for Diarrhoeal Disease Research
ICMR	Indian Council of Medical Research
IPT	Institut Pasteur de Tunis
IRD	Institut de Recherche pour le Développement
KEMRI	Kenya Medical Research Institute
LC	lethal concentration
MERO®	81% rapeseed oil methyl ester
NIH	National Institute of Health (Colombia)
NIMR	National Institute of Malaria Research
PBO	piperonyl butoxide
ppm	parts per million
RMRIMS	Rajendra Memorial Research Institute of Medical Sciences
SOP	standard operating procedure
TDC	tentative discriminating concentration
WHO	World Health Organization

1. Introduction

Of the 200 countries and territories for which data on leishmaniases were reported to WHO in 2020, 98 were considered endemic for the disease, including 71 that were endemic for both visceral and cutaneous leishmaniasis transmitted by sand fly vectors *(1)*. The target of the World Health Organization (WHO) road map for neglected tropical diseases 2021–2030 *(2)* is elimination of visceral leishmaniasis as a public health problem worldwide. Vector control is an important element in the prevention, control and elimination of major vector-borne diseases *(3)*, mainly with insecticides. The aim of the WHO Global Vector Control Response 2017–2030 *(4)* is to ensure that vector control is high on the public health agenda in order to reduce the burden of vector-borne diseases, including leishmaniases. Improved entomological surveillance and monitoring of insecticide resistance are the main pillars of the response. Effective vector control with insecticidal products is essential for control of leishmaniases, as is generation of data on the susceptibility of sand fly vectors to insecticides in order to manage insecticide resistance.

Although there is a global database on the resistance of malaria vectors to insecticides, none is available for sand flies; however, scattered reports from leishmaniases-endemic countries indicate emerging resistance of sand fly vectors to insecticides. In the South-East Asia Region, Phlebotomus argentipes sand flies are fully resistant to DDT but are still mainly susceptible to pyrethroid, organophosphate and carbamate insecticides applied indoors for control of malaria or visceral leishmaniasis *(5–7)*. Detection of a non-synonymous mutation, L1014F, in a voltage-gated sodium channel gene in the wild population of *Ph. argentipes* indicates the presence of pyrethroid tolerance and possible emergence of resistance to pyrethroids *(8)*. In Sri Lanka, insecticide resistance due to altered esterases and insensitive acetylcholinesterase was reported in sand flies *(9)*. Emerging insecticide resistance in sand flies has also been reported in other countries *(10, 11)*; e.g., in Sudan, where various degrees of susceptibility to a number of insecticides was reported in wild populations of the cutaneous leishmaniasis vector, *Ph. papatasi (12)*.

A major limitation to monitoring the frequency of phenotypic resistance of wild populations of sand flies is the lack of WHO standard test procedures and of filter papers impregnated with insecticide-discriminating concentrations for major sand fly vector species *(7)*. Insecticide-impregnated filter papers produced by the Universiti Sains Malaysia in coordination with WHO for testing susceptibility of mosquitoes are used in several countries to monitor resistance in sand fly populations. Furthermore, no WHO-validated bottle bioassay was available to test the susceptibility of sand flies to insecticides that cannot be impregnated onto filter papers, and a standard method had to be developed.

Insecticide resistance is monitored in field populations of mosquitoes by conducting bioassays with filter papers impregnated with a standard concentration of an insecticide (known as 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 has been adopted widely for the purposes of monitoring insecticide resistance in mosquitoes and other disease vectors *(13)*.

After a needs assessment, WHO conducted a multi-centre laboratory study in 2020–2022 to determine the DCs of selected insecticides in major class groups that are used for leishmaniasis vector control. WHO invited interested institutions to participate in the study through an open call, which was published on the United Nations Global Marketplace portal, and selected suitable laboratories in countries that had colonies of susceptible sand fly species.

Standard operating procedures (SOPs) for testing the resistance of sand flies to insecticides in the WHO tube test and the WHO bottle bioassay were developed collaboratively. Insecticides, carrier oils, surfactant oil (81% rapeseed methyl ester oil, MERO®, Envu, Mosquito Management (previously Bayer CropScience), Monheim, Germany), test kits and glass bottles were supplied to the participating laboratories. The study was performed in three steps.

The interim results of the study were reviewed at a WHO consultation on 31 January 2022 and the final results in another session on 29 June 2022 *(***Annex***)*. Thereafter, a draft of the report incorporating the results of the study and recommendations of experts to WHO was prepared and peer reviewed. Besides this, a technical review of the report by key end-users was solicited via the WHO regional and the country offices in countries endemic with leishmaniases. The peer reviewed report was finalized in consultation with the experts who had previously participated in the WHO consultations.

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.

[1] WHO has defined 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. The DC can also be defined as twice the 99% lethal concentration (LC99) as determined in a relevant statistical model against a susceptible strain of an insect.

2. Objectives of the study

The objectives of the study were to:

- develop an SOP for the WHO tube test (filter paper bioassay) for determining insecticide DCs;
- develop and validate a WHO bottle bioassay for determining DCs of insecticides that are unstable or cannot be impregnated onto filter paper;
- determine concentration–response curves for seven insecticide compounds and a synergist, piperonyl butoxide (PBO), for the WHO tube tests and one compound for the bottle bioassays;
- determine an optimum percentage concentration of PBO to be used in synergist bioassays against sand flies;
- establish and validate the DCs of the insecticides tested in the study against selected sand fly species; and
- identify gaps in and priorities for research for future work on insecticide DCs against sand flies.

3. Participating laboratories

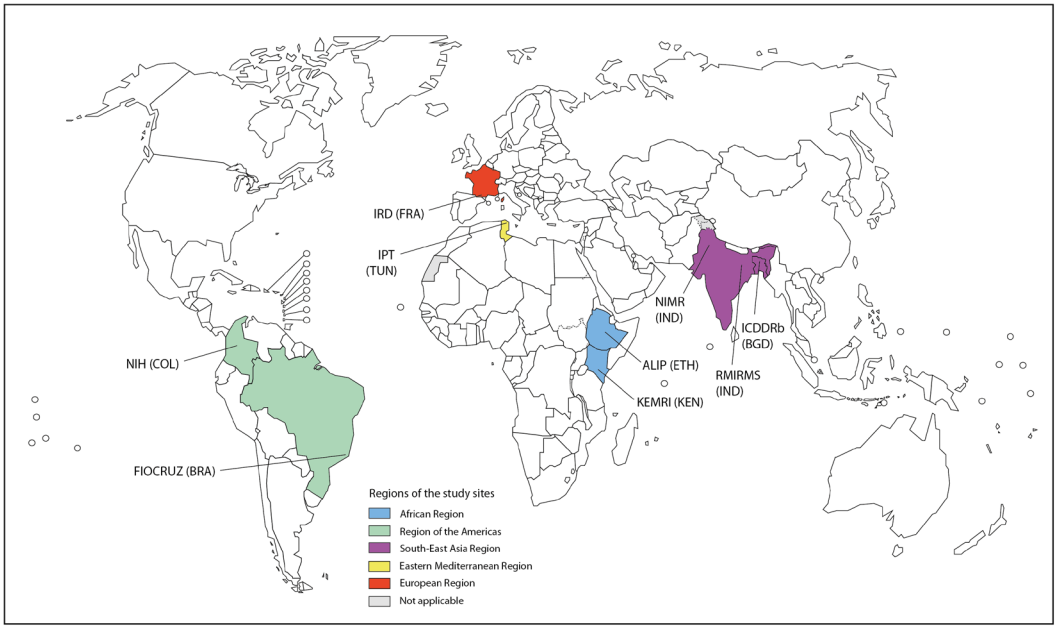
3.1 Lead coordinating institution

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. 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 receive information on the insecticides (e.g., active ingredient composition, certificates of analysis, storage conditions, material safety data sheets).

3.2 Collaborating laboratories

Nine internationally recognized laboratories with extensive entomological capacity in all WHO regions initially agreed to participate to the study. All the laboratories had susceptible sand fly colonies and adequate facilities and capacity to conduct laboratory testing and are

Fig 1. Locations of the eight participating laboratories



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Data Source: World Health Organization
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The abbreviations of names of laboratories are defined in **Table 1**

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

either formally designated WHO collaborating centres or have good capacity for testing insecticides. They were contracted directly by WHO and were asked to strictly follow the standard study design (protocol) and the SOPs developed collaboratively. Only eight (**Fig. 1, Table 1**) completed the study, as one laboratory was excluded due to loss of its colony of *Ph. chinensis* sand flies.

Table 1. Participating laboratories and susceptible sand fly species included in the study

WHO region	Country	Institution	Sand fly species and strain
African	Kenya	Kenya Medical Research Institute (KEMRI), Nairobi	*Phlebotomus dubosqui* (Baringo, Kenya, 1980)
	Ethiopia	Aklilu Lemma Institute of Pathobiology (ALIP), Addis Ababa University, Addis Ababa	*Phlebotomus longipes* (Selale, Ethiopia, 2021)
Americas	Brazil	Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro	*Lutzomyia longipalpis* (LABFISI/Jacobina, Brazil, 1970)
	Colombia	National Institute of Health (NIH), Bogotá	*Lutzomyia longipalpis* (El Callejón, Ricaurte, Colombia, 2002)
Eastern Mediterranean	Tunisia	Institut Pasteur de Tunis (IPT), Tunis	*Phlebotomus papatasi* (Felta, Tunisia, 2017)
European	France	Institut de Recherche pour le Développement (IRD), Montpellier	*Phlebotomus papatasi* (Zaragoza, Spain, 2013)
European	India	Indian Council of Medical Research (ICMR) – Rajendra Memorial Research Institute of Medical Sciences, Patna, and National Institute of Malaria Research, New Delhi	*Phlebotomus argentipes* (Bihar, India, 2020)
	Bangladesh	International Centre for Diarrhoeal Disease Research (ICDDRb), Dhaka	*Phlebotomus argentipes* (Trishal, Bangladesh, 2016)

4. Test compounds

Seven insecticide compounds were tested in sand fly species in WHO tube tests and one (clothianidin) in WHO bottle bioassays (**Table 2**).

The insecticides included in the study belong to four different insecticide classes. They were categorized into two test groups:

- group 1: seven compounds (six insecticides and the synergist PBO) for which the WHO tube test method was suitable for establishing and validating DCs; and
- group 2: one compound, clothianidin, which has distinct chemical properties and/or a mode of action that means it cannot be impregnated onto filter papers because of its instability; therefore, glass bottle bioassays were used to establish and validate DCs.

Preliminary studies showed that some 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 *(14)*. The WHO bottle bioassay developed for testing the susceptibility of mosquitoes, which was modified from the bottle bioassay of the United States Centers for Disease Control and Prevention for mosquitoes, was adapted to test the susceptibility of sand flies and to record mortality 24 h or later after exposure to an insecticide for a fixed time (i.e., 1 h), similarly to the method with impregnated papers.

Table 2. Test compounds, solvent, carrier oil or surfactant-oil used in WHO tube tests and bottle bioassays

Class	Test compound	Bioassay method	Solvent, carrier oil or surfactant-oil
Pyrethroids	Deltamethrin	Tube test	Acetone + silicone oil
	Permethrin (40:60 cis:trans isomer ratio)	Tube test	Acetone + silicone oil
	Alpha-cypermethrin	Tube test	Acetone + silicone oil
Carbamates	Bendiocarb	Tube test	Acetone + olive oil
Organophosphates	Malathion	Tube test	Acetone + olive oil
	Pirimiphos-methyl	Tube test	Acetone only[a]
Synergists	PBO	Tube test	Acetone + silicone oil
Neonicotinoids	Clothianidin	WHO bottle bioassay	Acetone + MERO® (800 ppm)

[a] No oil is used in impregnation of filter papers with pirimiphos-methyl, according to the manufacturer's instructions.
In all tests, the time for drying of filter paper or bottle was 24 h, the exposure time was 1 h, and the susceptibility endpoint was mortality 24 h after the 1 h exposure.

MERO®: 81% rapeseed methyl ester oil.

The endpoint of all the bioassays was mortality of sand flies. The aim of bioassays with PBO was to determine the optimum percentage concentration to be used in synergist bioassays against sand flies. The solvent (acetone) and carrier oils used to impregnate filter papers for bioassays complied with the WHO guidelines for tube tests. For the WHO bottle bioassays with clothianidin, a surfactant oil (i.e., MERO®) diluted in acetone was used to coat the bottles. 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.

5. Sand fly species

For logistical reasons and to ensure a manageable study size, five main sand fly vector species were selected for the study (**Table 1**). The criteria for selecting the species were that they are:

- known to be involved in the transmission of visceral or cutaneous leishmaniasis;
- representative of a geographical region in which visceral or cutaneous leishmaniasis is endemic (Africa, Asia, Europe, Central and South America or the Middle East); and
- are colonized at the participating laboratories.

Only fully susceptible sand fly strains (defined as susceptible to insecticides in all major classes with no detectable resistance mechanism) were tested.

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;
- advertising a "request for proposal' on the United Nations Global Marketplace portal (January 2020);
- selecting suitable laboratories that meet WHO requirements for a multi-centre study and signing technical service agreements with participating institutions (February–September 2020);
- harmonizing test protocols among laboratories, deciding susceptibility endpoints and delivering test compounds and other materials (March–October 2020);
- meeting with participating investigators (8 October 2020);
- orienting the investigators on correct use of test procedures through video tutorials developed by the IRD Worldwide insecticide resistance network (October 2020);
- modifying and adapting the WHO bottle bioassay for mosquitoes to test the susceptibility of sand flies to clothianidin (October–December 2020);
- performing preliminary tests of serial concentrations of test compounds to establish concentration–response curves to assess the range of concentrations that kill 0–100% of sand flies (January–May 2021);
- performing complete sets of bioassays (in triplicate) to estimate goodness-of-fit (slope and P value), LC_{99} and LC_{100}[1] and their ranges and variation in the mortality rates for each insecticide–sand fly species combination by computing the absolute difference in mortality rates from each data point on the best-fit line (June–December 2021);
- analysing data, drafting an interim report and peer review (December 2021–January 2022);
- 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 (31 January 2022);
- validating selected TDCs in step 3 and updating the study report (February–June 2022);
- convening a follow-up session of the WHO expert consultation to review the analysis of data from step 3 and select final DCs (29 June 2022);
- further validating certain identified TDCs (July–September 2022);
- updating the study report and peer review (September–October 2022); and
- finalizing and editing the SOPs and the study report for publication (November 2022).

[1] This is the lowest concentration killing 100% of mosquitoes in bioassays.

The compounds were tested in the sequential steps described in sections 6.2, 6.3 and 6.4. 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 tests and whether to proceed to the next step or to repeat or revalidate certain tests.

6.1 Developing test procedures

The initial procedures for testing the susceptibility of adult sand flies to insecticides in WHO tube tests, for coating bottles with insecticides and for conducting bottle bioassays were developed by adapting recently published WHO SOPs for testing the susceptibility of mosquitoes *(15, 16)*. Similarly, the initial procedure for determining the optimum percentage concentration of PBO to be used in synergist bioassays against sand flies and the ability of PBO to restore susceptibility of sand flies to pyrethroid insecticides in tube tests was developed by adapting a recently pubished WHO SOP for mosquitoes *(17)*.

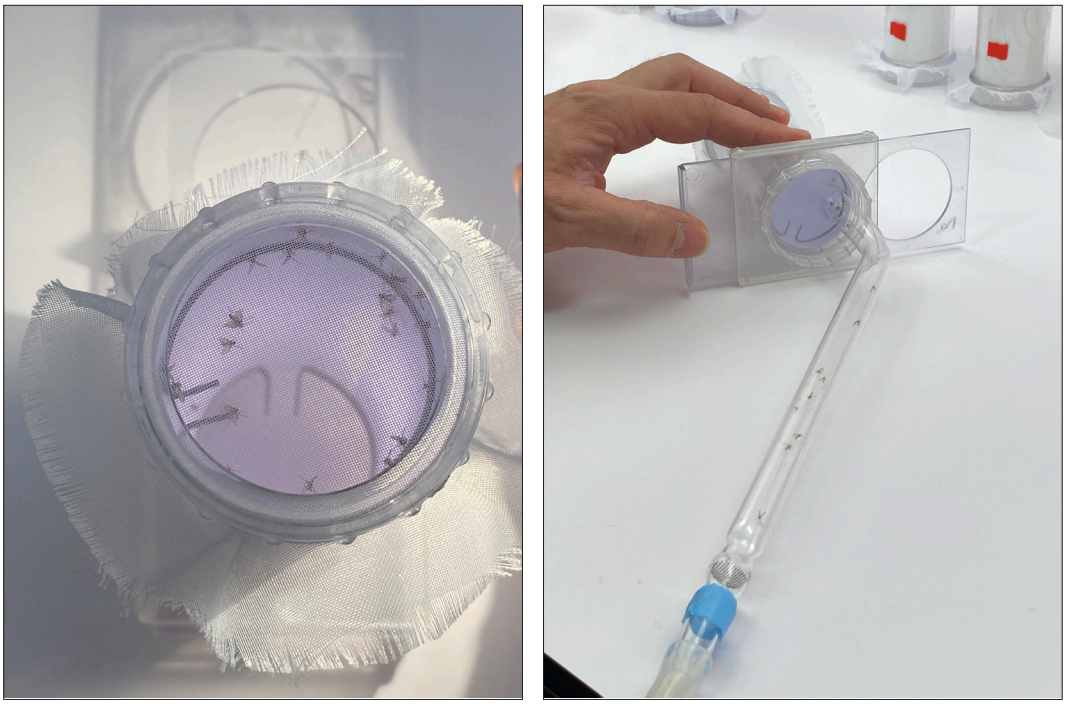
The test procedures for sand flies were then expressed as SOPs after peer review by technical experts. The SOPs will be published separately.

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

Uniform testing conditions were adopted by all the laboratories, comprising standard test protocols, bioassay methods, temperature and relative humidity, sample size per test, the age and physiological status of sand flies and use of a screen of suitable mesh size for the bioassay tubes (**Fig. 2**) and holed netting peace or holed caps fitted with a rubber flap to facilitate introduction of sand flies into bottles (**Fig. 3**). The laboratories were asked to report to IRD and WHO inconsistent results in test replicates for a given insecticide and test concentration, changes made to testing conditions or use of different concentrations of the surfactant to coat glass bottles.

The objective of the tests in step 1 was to perform preliminary bioassays to establish the range of concentrations with mortality between 0 and 100% for the test compounds for each sand fly 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, i.e., 0–100% mortality of each sand fly species. 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 (18) or in bottles coated with serial concentrations of 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 clothianidin-coated bottles were dried for 24 h at room temperature. In the bioassays, susceptible non-

Fig 2. Netting screen on bioassay tubes (hole size, < 500 µm) that prevents escape of sand flies during testing (left); introducing sand flies into the tube (right) (photo credit: IRD/Dr Stéphane Duchon)



The plastic tubes in the mosquito susceptibility test kits currently supplied by Universiti Sains Malaysia have a wide mesh screen, which cannot prevent escape of sand flies that are smaller than mosquitoes.

blood-fed female sand flies aged 3–7 days were exposed to the serial concentrations of test compounds for 1 h. Susceptibility was recorded as percentage mortality of sand flies 24 h after a 1-h exposure. During step 1, the susceptibility of 50 sand flies was tested in only one replicate at each concentration (**Table 4**).

For the bioassays with PBO, laboratories determined the highest percentage concentration that had no lethal effect on susceptible sand flies (i.e., the sub-lethal concentration) taken from the colonies 24 h after a 1-h exposure to filter papers impregnated with serial concentrations of PBO.

Fig 3. Showing technique for introduction of sand flies into a 250-mL glass bottle[a] with a holed netting piece (left) (photo credit: WHO/Dr Rajpal Yadav); a glass bottle with a holed cap and a rubber flap for introduction of sand flies as an alternative technique (right) (photo credit: IRD/Dr Stéphane Duchon)

[a]The total volume of a 250-mL graduated bottle is 310 mL.

Table 4. Scheme for conducting WHO tube tests and bottle bioassays in steps 1–3

Testing step	Numbers of test concentrations and controls	Minimum no. of sand flies per test concentration or control (tubes or bottles)	No. of replicates (test batches) per concentration	Total no. of sand flies tested	Expected outcome
1. Screening	Tests: 10–12 Control: 1	50 (25 x 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 x 4) Controls: 50 (25 x 2)	3	Tests: 1800 Controls: 150	LC_{50}, LC_{99} and LC_{100} and to select TDC
3. Validation	Tests: 1 Control: 1	Tests: 100 (25 x 4) Controls: 50 (25 x 2)	1	Tests: 100 Controls: 50	DC

DC: discriminating concentration; TDC: tentative discriminating concentration

[a] Because of the difficulty of ensuring large numbers of adult sand flies in colonies, 50 sand flies were usually exposed per test concentration in step 2, and additional bioassays were conducted to achieve the required sample size for each test concentration.

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

In step 2, the tube tests and bottle bioassays were conducted in triplicate to establish LC_{50}, LC_{99} and LC_{100} (observed) and to select the TDC for each test compound. In this step, laboratories impregnated filter papers and coated glass bottles with serial concentrations determined in step 1. The results were analysed in a Bayesian binomial model (see section 6.5.1) to determine the concentration:LC ratios and to assess differences in mortality rates with test conditions.

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 sand flies, one concentration killed about 50%, two concentrations killed > 50% of sand flies and one concentration killed about 100% of sand flies. Each bioassay was performed three times with a given species (when possible) with 50–100 sand flies per concentration. Impregnated papers were usually used once but no more than three times and kept under suitable storage condition between uses.

In tests of the capacity of the synergist PBO to increase the lethal action of a pyrethroid insecticide (e.g., alpha-cypermethrin), susceptible sand flies were first exposed to

incremental concentrations of PBO for 1 h and then exposed for an additional 1 h to filter paper impregnated with the LC50 of alpha-cypermethrin to determine the extent of susceptibility restored by pre-exposure of sand flies to PBO.

6.4 Validation of tentative discriminating concentrations against various sand fly species (step 3)

In the final step, TDCs were selected during a WHO expert consultation on 31 January 2022 on the basis of the results of step-2 testing. The participants recommended that, when possible, a single TDC be adopted for each compound and sand fly species tested in order to minimize the number of unique DCs for production of impregnated papers for routine monitoring of resistance in national disease control programmes. The participants decided by consensus to choose a single DC for each compound for all sand fly species, acknowledging the risk of either under- or over-estimation of the resistance of single species. After the consultation, WHO compiled a list of the TDCs for each insecticide to be tested in step 3 against sand fly species (or group of species if relevant). Filter papers were then impregnated at the selected TDC of each insecticide at the Universiti Sains Malaysia and sent to the relevant participating laboratories for testing. No PBO-impregnated filter papers were sent, as no TDC was to be tested.

For bottle bioassays, bottles were coated with the TDC of clothianidin or with a control. The bioassays comprised one test with four replicates of clothianidin for a given sand fly species, with at least 100 sand flies tested per TDC and 50 per control replicate. The data were reported to IRD and WHO for final analysis.

6.5 Piperonyl butoxide synergist bioassays

The aim of assays with the synergist PBO was to determine not a DC (as for other compounds) but the optimum percentage concentration for synergist–insecticide bioassays against sand flies. The commonly accepted definition of the optimum concentration of a synergist is the highest concentration that does not kill the targeted species, known as the "sub-lethal" concentration. The tests involved exposing sand fly species to increasing concentrations of PBO, from 0.1% to 20%, for 1 h in WHO tube tests and recording mortality after 24 h.

The bioassays were carried out by the ICMR-Rajendra Memorial Research Institute of Medical Sciences, India, to determine the percentage concentration of PBO for a synergistic effect on pyrethroids against susceptible sand flies (step 2). Briefly, susceptible female Ph. argentipes were exposed to PBO within the range of 0.1–25% for 1 h and then exposed for 1 h to filter papers impregnated with alpha-cypermethrin at the LC50 to determine the first concentration at which the mortality rate reached a plateau. The test conditions were the same as those for testing insecticides (i.e., 27 ± 2 °C temperature and 75 ± 10% relative humidity).

6.6 Data analysis and reporting

6.6.1 Analysis, validation and interpretation of data

The participating laboratories were responsible for collecting, checking the quality, collating and reporting data on an Excel® template developed by IRD and sending reports regularly to IRD, where the data were analysed and validated, and real-time feedback was given to the laboratories. The raw data on all the tests, with identity cards with all information for a given insecticide and sand fly species, are archived at IRD.

A Bayesian binomial model with a five-parameter logistics function was developed at Imperial College London, United Kingdom, for analysis of the concentration–response data from the study. The binomial sampling distribution was used to describe the outcome (mortality rates in sand flies) 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 sand fly species. 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} for each insecticide and species combination were used to select TDCs for step-3 testing.

6.6.2 Reporting of data and monitoring of progress

All the participating laboratories, including IRD, sent their bioassays results to WHO, where progress and achievement of milestones were monitored in system based on the completion rate (CR) of tests (**Table 5**). CRs were calculated as follows:

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

The CRs were estimated for each laboratory according to the testing steps completed and the compounds tested against their sand fly 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

7. Results

7.1 Test completion rates

Excluding synergist tests with PBO, 109 861 sand flies were used to test seven insecticides in either tube tests or bottle bioassays, of which 66% (n = 72 966) were tested against *Phlebotomus* spp. and the others against *Lutzomyia longipalpis* (**Fig. 4**). The test CRs were 95% for step 1, 79% for step 2 and 100% for step 3 (**Table 6**). CRs of 100% were achieved for all the insecticides except clothianidin (63%) in step 1. In step 2, good CRs (i.e., > 75%) were achieved for all the insecticides except clothianidin (58%) and bendiocarb (71%). In step 3, the CR was 100% for all insecticides tested in both tube tests and bottle bioassays.

Fig 4. Total numbers of sand flies (Phlebotomus and Lutzomyia spp.) tested in WHO tube tests (alpha-cypermethrin, deltamethrin, malathion, pirimiphos-methyl, bendiocarb) and WHO bottle bioassays (clothianidin)

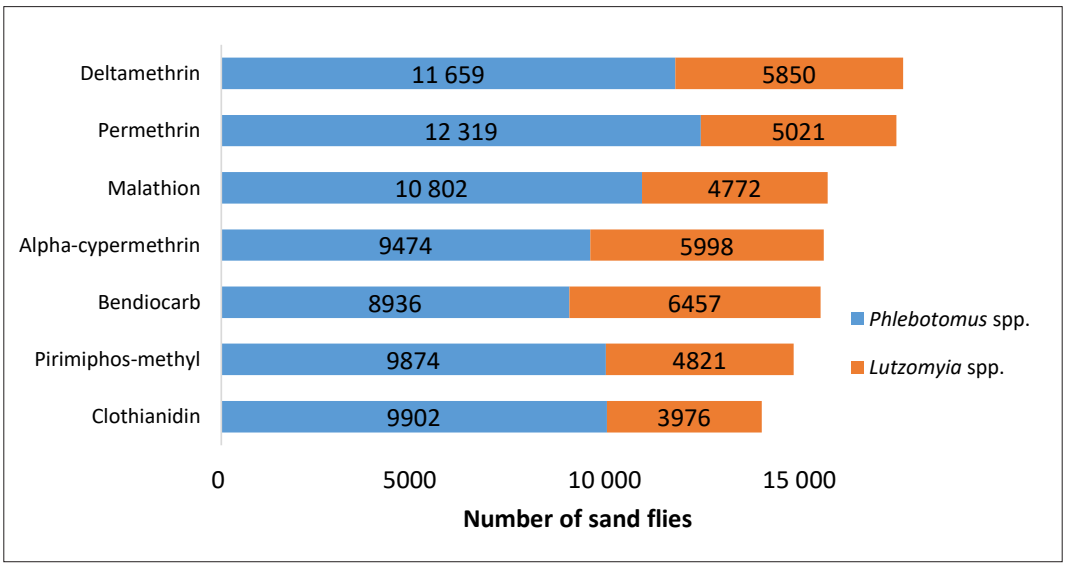


Table 6. Insecticides and sand fly species tested and test completion rates (CRs) in steps 1, 2 and 3

Step	Insecticide class	Pyrethroids			Carbamates	Organophosphates		Neonicotinoids	Total
	Insecticide	Alpha-cypermethrin	Deltamethrin	Permethrin	Bendiocarb	Malathion	Pirimi-phos-methyl	Clothianidin	
	Sand fly species	*Ph. argentipes* *Ph. papatasi* *Ph. longipes* *Ph. dubosqui* *Lu. longipalpis*	*Ph. argentipes* *Ph. papatasi* *Ph. longipes* *Ph. dubosqui* *Lu. longipalpis*	*Ph. argentipes* *Ph. papatasi* *Ph. longipes* *Ph. dubosqui* *Lu. longipalpis*	*Ph. argentipes* *Ph. papatasi* *Ph. longipes* *Ph. dubosqui* *Lu. longipalpis*	*Ph. argentipes* *Ph. papatasi* *Ph. longipes* *Ph. dubosqui* *Lu. longipalpis*	*Ph. argentipes* *Ph. papatasi* *Ph. longipes* *Ph. dubosqui* *Lu. longipalpis*	*Ph. argentipes* *Ph. papatasi* *Ph. longipes* *Ph. dubosqui* *Lu. longipalpis*	
	No. of species tested	5	5	5	5	5	5	5	5
1	No. of tests performed	8	8	8	8	8	8	8	64
	No. of validated tests	8	8	8	8	8	8	5	59
	CR (%)	100	100	100	100	100	100	63	95%
2	No. of tests performed	24	24	24	24	24	24	24	169
	No. of validated tests	20	22	23	17	18	19	14	132
	CR (%)	79	92	96	71	75	79	58	79%
3	No. of tests performed	8	12	16	12	14	9	14	85
	No. of validated tests	8	12	16	12	14	9	14	85
	CR (%)	100	100	100	100	100	100	100	100%

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

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

The concentration–response curves (step 2) and the LC_{99}, LC_{100} and TDCs for each insecticide and sand fly species are presented below.

The current definition of the DC of an insecticide is twice the LC_{99} or LC_{100} of the compound for a given test species. In our statistical framework, we observed that the simulated mortality of sand flies never reached 100% (i.e., the infinite values were close to 100%), resulting in a "flat" concentration–response curve at high concentrations. This may have affected the estimated LC values, especially at the top of the curve, resulting in high uncertainty around $LC_{99.9}$. To overcome this limitation, LC_{99} (and not $LC_{99.9}$) and LC_{100} (when available) were both used to select TDCs for 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, the highest LC_{99} (or LC_{100}) was selected, at the risk of over-estimating a DC for the sand fly species. A comparison of data across species and taxa (e.g., mosquitoes), however, gave us confidence in the results and in the selection of TDCs for validation tests in step 3.

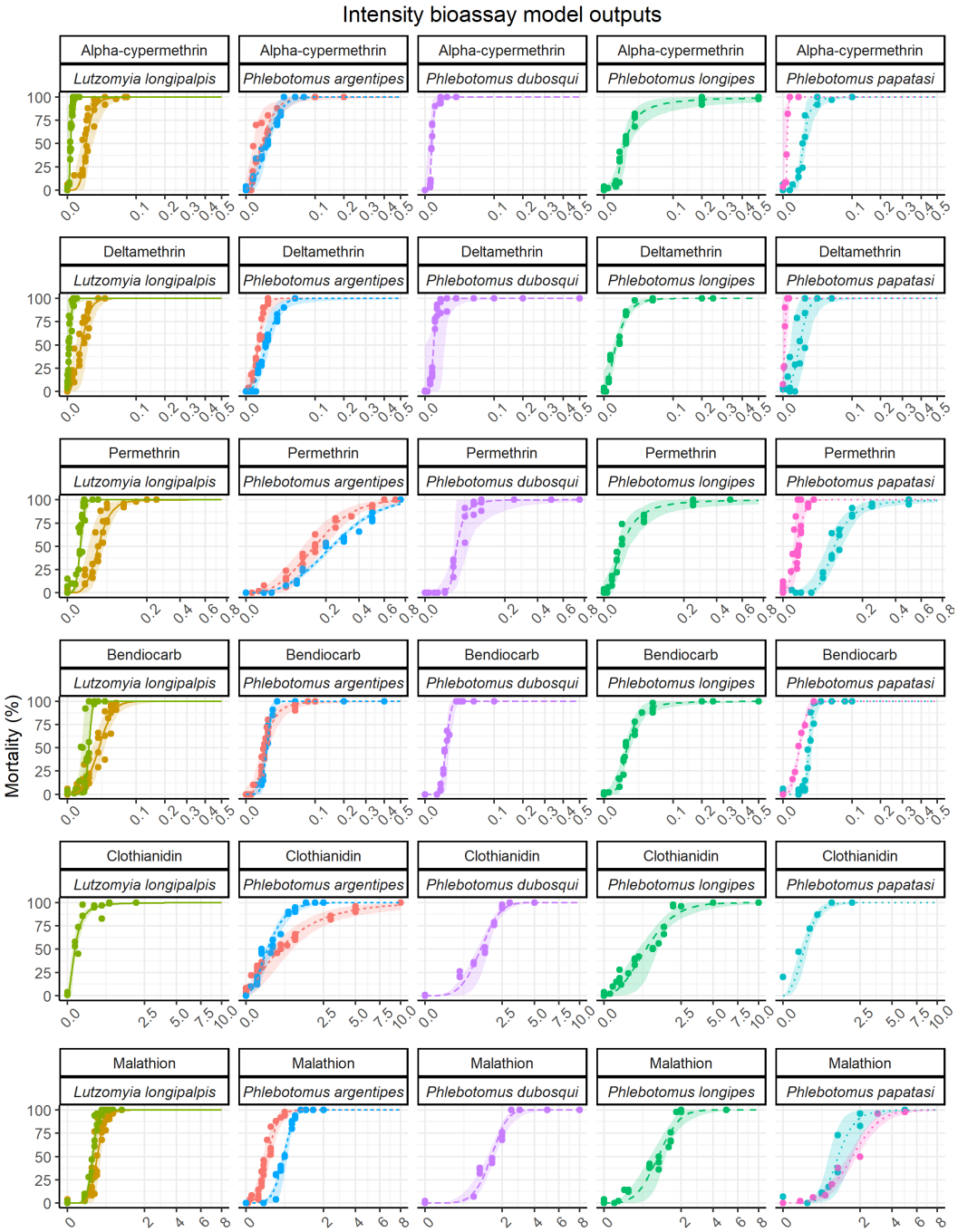
The data used for statistical analysis are summarized in **Table 7**. The mean number of sand flies tested per insecticide ranged from 10 639 for clothianidin to 15 159 for permethrin. For each insecticide–sand fly-species combination, insecticide concentrations that caused 0–100% mortality were used to establish the concentration–response curves and to estimate the LCs (**Fig. 5**).

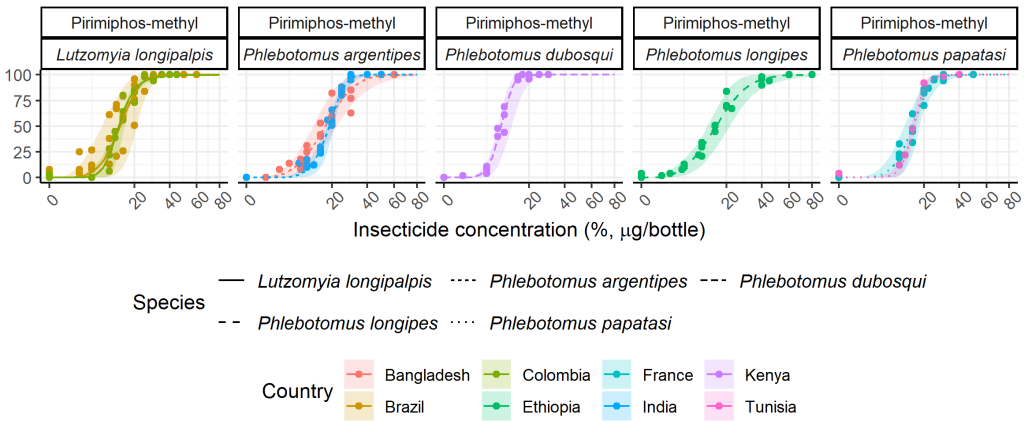
Table 7. Data used for the statistical analyses

Insecticide	Test method	No. of data points	Mean no. of sand flies per bioassay	Total no. of sand flies for which data analysed	Insecticide concentration (% for filter papers and µg per bottle)			Mean mortality rate (%)
					Min	Max	Mean	
Alpha-cypermethrin	Tube test	204	560	14 006	0	0.50	0.02	89.5
Deltamethrin	Tube test	220	582	15 144	0	0.50	0.01	84.6
Permethrin	Tube test	240	561	15 159	0	0.75	0.11	78.1
Bendiocarb	Tube test	205	581	13 355	0	0.50	0.03	92.0
Malathion	Tube test	198	555	12 759	0	8	0.83	70.6
Pirimiphos-methyl	Tube test	205	530	13 256	0	80	18	73.0
Clothianidin	Bottle bioassay	146	626	10 639	0	10	1.4	57.3

Five species of sand fly were tested with each insecticide, and the maximum mortality rate was 100%.

Fig 5. Concentration–response curves for each insecticide–species combination tested in either WHO tube tests (pyrethroids, carbamate and organophosphate) or bottle bioassays (clothianidin)





Alpha-cypermethrin

The LC_{99} and LC_{100} (estimated from the raw data) and the TDC selected for each species are summarized in **Table 8**. The estimates of LC_{99} vary substantially by sand fly species, for example, the LC_{99} being 0.7% (range, 0.39–3.0) for *Ph. longipes* and 0.007% (0.002–0.012) for *Ph. dubosqui*. The LC_{100} ranged from 0.01% for *Ph. dubosqui* to 0.5% for *Ph. longipes*.

The LC_{99} for each insecticide–species combination varied widely among laboratories (**Table 8**). For example, the LC_{99} of alpha-cypermethrin against *Ph. papatasi* ranged from 0.046% (0.018–0.055) at IRD to 0.001% (0.000661–0.00131) at IPT, indicating ultra-susceptibility of the IPT's sand fly colony to this insecticide. For *Lu. longipalpis*, also, a 30 times difference in LC_{99} was observed between FIOCRUZ (0.043%) and NIH (0.001%).

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

- *Ph. dubosqui*: 0.02%
- *Ph. papatasi, Ph. argentipes* and *Lu. longipalpis*: 0.1%
- *Ph. longipes*: 1%

Deltamethrin

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

As for alpha-cypermethrin, the estimated LC_{99} of deltamethrin varied substantially among laboratories for different test species. The highest LC_{99} was found for *Ph. longipes* (0.05%; range, 0.049–0.057) and the lowest for *Ph. dubosqui* (0.009%; range, 0.005–1.8 x 10^{25}).

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

- *Ph. dubosqui*: 0.02% and 0.05%
- *Ph. papatasi* and *Lu. longipalpis*: 0.05%
- *Ph. argentipes* and *Ph. longipes*: 0.05% and 0.1%

Permethrin

The LC_{99} and LC_{100} values and the TDCs selected for each species are summarized in **Table 10**.

The estimated LC_{99} of permethrin also varied substantially by species and among laboratories. *Ph. longipes, Ph. argentipes* and *Ph. papatasi* were most tolerant to permethrin (LC_{99}, 0.6–1%) and *Ph. longipalpis* and *Ph. papatasi* the least tolerant (LC_{99}, 0.15%).

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

- *Lu. longipalpis* and *Ph. dubosqui*: 0.5% and 1%
- *Ph. papatasi, Ph. argentipes* and *Ph. longipes*: 1% and 1.5%

Bendiocarb

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

Much less variation among laboratories and among species was observed with bendiocarb than with the pyrethroids. The LC_{99} varied from 0.016% (0.009–0.016) for *Lu. longipalpis* to 0.18% (0.074–0.30) for *Ph. longipes*. Similar results were observed for the LC_{100} values estimated from the raw data, which ranged from 0.02% for *Ph. dubosqui* to 0.2% for *Ph. longipes*.

The following TDCs were selected for step-3 testing:

- *Ph. dubosqui* and *Ph. papatasi*: 0.05%
- *Lu. longipalpis* and *Ph. argentipes*: 0.1%
- *Ph. longipes*: 0.4%

Malathion

The LC_{99} and LC_{100} values and tentative DCs selected for each species are summarized in **Table 12**.

The estimated LC_{99} values were much higher for malathion than for the other insecticides. The LC_{99} was the lowest for *Lu. longipalpis* (0.7%) and the highest for *Ph. papatasi* (5%). The LC_{99} and LC_{100} data were consistent.

The following TDCs were selected for step-3 testing:

- *Lu. longipalpis* and *Ph. argentipes*: 2%
- *Ph. dubosqui* and *Ph. longipes*: 5%
- *Ph. papatasi*: 5% and 10%

Pirimiphos-methyl

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

The estimated LC_{99} values for pirimiphos-methyl were similar for different species, except for *Ph. dubosqui*, which showed the greatest susceptibility, with an LC_{99} of 17 mg/m^2. The LC_{99} for all the other species ranged from 28 (28.1–28.1[1]) mg/m^2 for *Ph. papatasi* to 53 (41–64) mg/m^2 for *Ph. longipes*. A similar trend was found for the LC100 values.

The following TDCs were selected for step-3 testing:

- *Ph. dubosqui*: 50 and 100 mg/m^2
- *Lu. longipalpis*, *Ph. papatasi*, *Ph. argentipes* and *Ph. longipes*: 100 mg/m^2

Clothianidin

The LC_{99} and LC_{100} values and the TDCs selected for each species are summarized in **Table 14**.

As with pirimiphos-methyl, the LC_{99} and LC_{100} values were relatively consistent among laboratories and sand fly species. For example, the LC_{99} ranged from 1.24 (0.68–2.19) for *Lu. longipalpis* to 5.16 (4.9–6.13) for *Ph. longipes*. A similar trend was observed for the LC_{100}, which ranged from 1–5 µg per bottle for all species. Much higher LC_{99} and LC_{100} values were reported for Ph. argentipes by ICMR than by ICDDRb; hence, these data were not considered in selecting the TDC for clothianidin.

The following TDCs were selected for step-3 testing:

- *Ph. papatasi* and *Lu. longipalpis*: 3 µg/bottle
- *Ph. argentipes*: 3 µg and 4 µg/bottle
- *Ph. dubosqui*: 6 µg/bottle
- *Ph. longipes*: 10 µg/bottle

[1] The value is from one bioassay in one laboratory; the result is uninformative as the range is estimated from a single curve.

Table 8. LC_{50}, LC_{99} and LC_{100} (observed) values and selected TDC of alpha-cypermethrin against sand flies 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
Ph. papatasi	IRD, France	2	0.009	0.008–0.011	0.046	0.018–0.055	**0.091**	NA	0.1
	IPT, Tunisia	1	0.000	3.7×10^{-4} –3.7×10^{-4}	0.001	0.000661–0.00131	0.002	NA	
Lu. longipalpis	FIOCRUZ, Brazil	4	0.007	0.005–0.011	0.043	0.018–0.052	0.086	**0.14**	0.1
	NIH, Colombia	4	0.000	2.0×10^{-4} –3.2×10^{-4}	0.001	9.6×10^{-4}–0.001	0.003	0.004	
Ph. argentipes	ICDDRb, Bangladesh	4	0.005	0.002–0.007	0.060	0.059–0.301	0.12	0.06	0.1
	ICMR-RMRIMS, India	3	0.009	0.008–0.009	0.061	0.044–0.07	**0.12**	0.10	
Ph. dubosqui	KEMRI, Kenya	3	0.001	8.5×10^{-4} –9.3×10^{-4}	0.007	0.002–0.012	0.013	**0.02**	0.02
Ph. longipes	ALIP, Ethiopia	4	0.009	0.008–0.011	0.702	0.394–3.004	1.40	**1%**	1

NA: not available; min: minimum; 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 9. LC_{50}, LC_{99} and LC_{100} (observed) values and selected TDC of deltamethrin against sand flies 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
Ph. papatasi	IRD, France	3	0.006	0.002–0.01	0.026	0.011–0.039	**0.05**	0.05	0.05
	IPT, Tunisia	1	0.000	5.0×10^{-5}–5.0×10^{-5}	0.000	2.0×10^{4}–2.0×10^{4}	0.000	NA	
Lu. longipalpis	FIOCRUZ, Brazil	4	0.004	0.003–0.008	0.025	0.018–0.026	**0.05**	0.04	0.05
	NIH, Colombia	4	0.000	3.7×10^{5}–2.2×10^{-4}	0.002	7.2×10^{-4}–0.003	0.003	0.002	
Ph. argentipes	ICDDRb, Bangladesh	2	0.003	0.003–0.003	0.016	0.015–0.024	0.03	**0.1**	0.05 and 0.1
	ICMR-RMRIMS, India	4	0.009	0.008–0.009	0.051	0.051–0.109	**0.10**	0.1	
Ph. dubosqui	KEMRI, Kenya	6	0.001	0.001–0.014	0.009	0.005–1.8×10^{25}	**0.02**	0.02	0.02 and 0.05
Ph. longipes	ALIP, Ethiopia	2	0.003	0.003–0.003	0.052	0.049–0.057	**0.10**	0.1	0.05 and 0.1

NA: not available; min: minimum; 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 permethrin against sand flies in WHO tube tests

Species	Institution	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 (5)
Ph. papatasi	IRD, France	3	0.082	0.069–0.099	0.618	0.324–0.809	**1.2**	**1%**	1 and 1.5
	IPT, Tunisia	2	0.005	0.003–0.008	0.033	0.007–0.027	0.065	0.06	
Lu. longipalpis	FIOCRUZ, Brazil	4	0.030	0.017–0.038	0.149	0.122–0.16	0.29	**0.4**	0.5 and 1
	NIH, Colombia	3	0.005	0.004–0.006	0.014	0.011–0.018	0.027	0.04	
Ph. argentipes	ICDDRb, Bangladesh	4	0.147	0.126–0.168	0.739	0.704–0.943	1.5	**1.5**	1 and 1.5
	ICMR-RMRIMS, India	2	0.231	0.219–0.232	1.040	1.026–20.6	**2.1**	1.2	
Ph. dubosqui	KEMRI, Kenya	5	0.032	0.015–0.051	0.148	0.075–1.8 x 10^8	0.29	**0.5**	0.5 and 1
Ph. longipes	ALIP, Ethiopia	4	0.010	0.007–0.015	0.632	0.183–2.142	**1.3**	**1**	1 and 1.5

NA: not available; min: minimum; 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 11. LC_{50}, LC_{99} and LC_{100} (observed) values and selected TDC of bendiocarb against sand flies in WHO tube tests

Species	Institution	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
Ph. papatasi	IRD, France	3	0.014	0.013–0.016	0.028	0.019–0.028	**0.056**	0.06	0.05
	IPT, Tunisia	1	0.006	0.006–0.006	0.023	0.023–0.023	0.045	0.04%	
Lu. longipalpis	FIOCRUZ, Brazil	3	0.021	0.015–0.026	0.078	0.069–0.092	**0.15**	NA	0.1
	NIH, Colombia	3	0.010	0.005–0.01	0.016	0.009–0.016	0.031	0.04	
Ph. argentipes	ICMR-RMRIMS, India	3	0.009	0.008–0.009	0.022	0.019–0.021	0.043	0.04	0.1
	ICDDRb, Bangladesh	4	0.007	0.006–0.008	0.096	0.073–0.138	**0.19**	0.16	
Ph. dubosqui	KEMRI, Kenya	3	0.009	0.009–0.011	0.019	0.015–0.022	0.038	**0.04**	0.05
Ph. longipes	ALIP, Ethiopia	3	0.011	0.011–0.012	0.180	0.074–0.301	0.36	**0.4**	0.4

NA: not available; min: minimum; 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 12. LC_{50}, LC_{99} and LC_{100} (observed) values and selected TDC of malathion against sand flies in WHO tube tests

Species	Institution	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
Ph. papatasi	IRD, France	2	1.025	0.80–1.39	3.58	2.70–3.34	**7.2**	10	5 and 10%
	IPT, Tunisia	1	1.607	1.60–1.60	5.23	5.23–5.23	**10**	NA	
Lu. longipalpis	FIOCRUZ, Brazil	3	0.296	0.21–0.34	0.86	0.35–0.89	**1.7**	NA	2%
	NIH, Colombia	3	0.211	0.19–0.24	0.44	0.32–0.49	0.9	1.2	
Ph. argentipes	ICDDRb, Bangladesh	4	0.148	0.11–0.17	0.66	0.58–0.76	1.3	2	2%
	ICMR-RMRIMS, India	3	0.466	0.46–0.52	1.02	0.92–1.07	**2.1**	2	
Ph. dubosqui	KEMRI, Kenya	3	1.462	1.43–1.50	2.95	2.87–3.40	**5.9**	5	5%
Ph. longipes	ALIP, Ethiopia	4	0.978	0.88–1.10	2.95	2.45–3.43	**5.9**	5	5 %

NA: not available; min: minimum; 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 13. LC_{50}, LC_{99} and LC_{100} (observed) values and selected TDC of pirimiphos-methyl against sand flies in WHO tube tests

Species	Institution	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 (mg/m^2)
Ph. papatasi	IRD, France	3	14.93	13.3–16.6	30.9	28–35	62	**100**	100
	IPT, Tunisia	1	15.30	15.3–15.3	28.0	28.1–28.1	56	80	
Lu. longipalpis	FIOCRUZ, Brazil	4	13.74	9.474–19.5	31.3	26.7–32.5	62	60	100
	NIH, Colombia	2	13.30	13.2–14.8	38.4	31.2–38.6	77	**80**	
Ph. argentipes	ICDDRb, Bangladesh	4	16.93	13.6–19.9	49.3	38.6–59.8	**99**	80	100
	ICMR-RMRIMS, India	4	18.73	18.5–19.6	33.6	31.2–35.3	67	80	
Ph. dubosqui	KEMRI, Kenya	3	8.90	8.411–11.1	17.2	15.6–22.5	35	**50**	50 and 100
Ph. longipes	ALIP, Ethiopia	4	15.21	13.7–18.0	53.4	41.2–64.6	**107**	120	100

NA: not available; min: minimum; 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 14. LC_{50}, LC_{99} and LC_{100} (observed) values and selected TDC of clothianidin against sand flies in WHO bottle assays

Species	Institution	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 (µg/bottle)
Ph. papatasi	IRD, France	1	0.1	0.18–0.18	1.24	1.23–1.23	**2.5**	2	3
Lu. longipalpis	NIH, Colombia	2	0.024	0.019–0.032	1.24	0.68–2.19	**2.5**	2	3
Ph. argentipes	ICDDRb, Bangladesh	4	0.46	0.41–0.60	16.18	15.8–27.1	32[b]	20[b]	3 and 4
	ICMR-RMRIMS, India	3	0.18	0.17–0.22	1.99	1.82–2.18	**3.9**	3	
Ph. dubosqui	KEMRI, Kenya	3	1.28	1.30–1.36	3.42	3.23–3.54	**6.8**	6	6
Ph. longipes	ALIP, Ethiopia	4	0.72	0.69–0.82	5.16	4.91–6.13	**10.3**	10	10

NA: not available; min: minimum; max: maximum

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

[b] Due to unexpected high estimates, these LC values were not considered for selecting the TDC

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

7.3 Optimum concentration of piperonyl butoxide for synergist bioassays

In all, 2553 sand flies were tested with PBO. The susceptibility of *Phlebotomus* and of *Lutzomyia* spp. to PBO differed (**Fig. 6**). When the *Phlebotomus* spp. were exposed to PBO at a concentration of 1–4%, no mortality was observed in *Ph. papatasi* (IRD, France), *Ph. dubosqui* (KEMRI, Kenya) or Ph. argentipes (ICDDRb, Bangladesh), but 10–16% mortality was observed in *Ph. papatasi* at IPT, Tunisia.

At low concentrations of PBO (0.5–4%), the susceptibility of *Lu. longipalpis* was higher (5–29 %) than that of *Phlebotomus* spp. (10–16%).

In the additional tests at ICMR-Rajendra Memorial Research Institute of Medical Sciences, in which susceptible female *Ph. argentipes* were pre-exposed for 1 h to a given PBO concentration (range, 0.1–25%) followed by exposure for 1 h to filter papers impregnated with the LC_{50} of alpha-cypermethrin (0.01%), PBO increased the toxicity of alpha-cypermethrin by about 30% when the concentration exceeded 0.5% (**Fig. 7**), then rapidly reached a plateau of mortality. The mortality rate did not increase markedly at concentrations of PBO above 25%; however, the rate never reached 100% with any PBO concentration.

Fig 6. Concentration–response of PBO against *Phlebotomus* spp. and *Lutzomyia longipalpis*

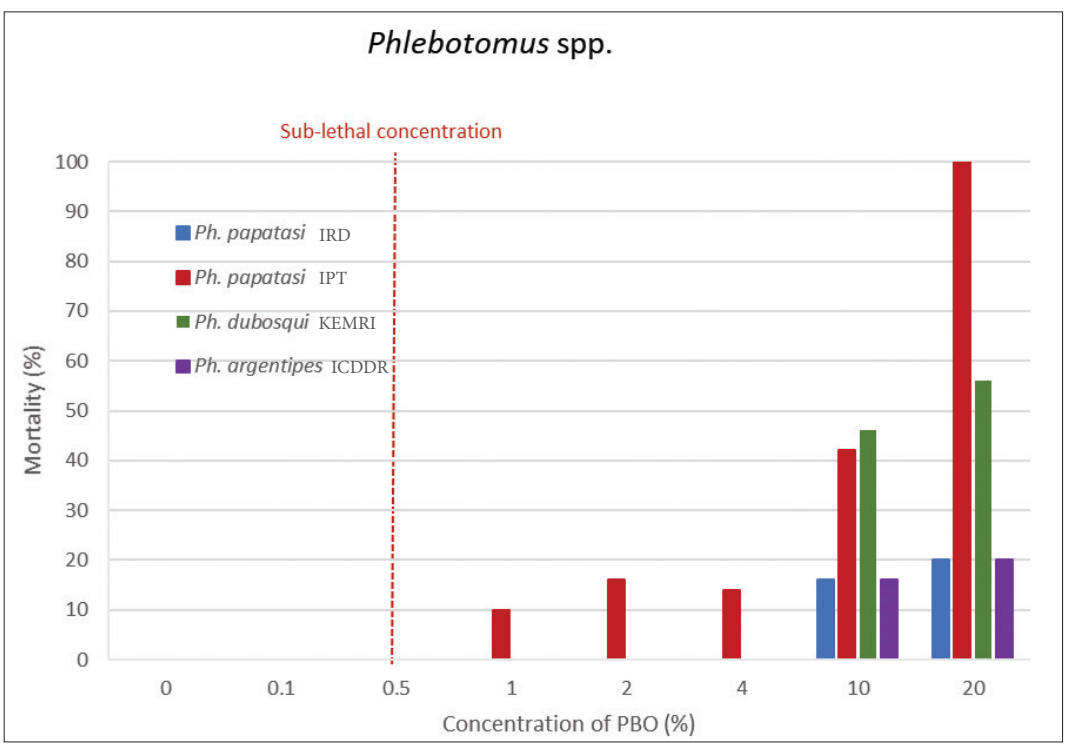


Fig. 6 Cont'd. Concentration–response of PBO against *Phlebotomus* spp. and *Lutzomyia longipalpis*

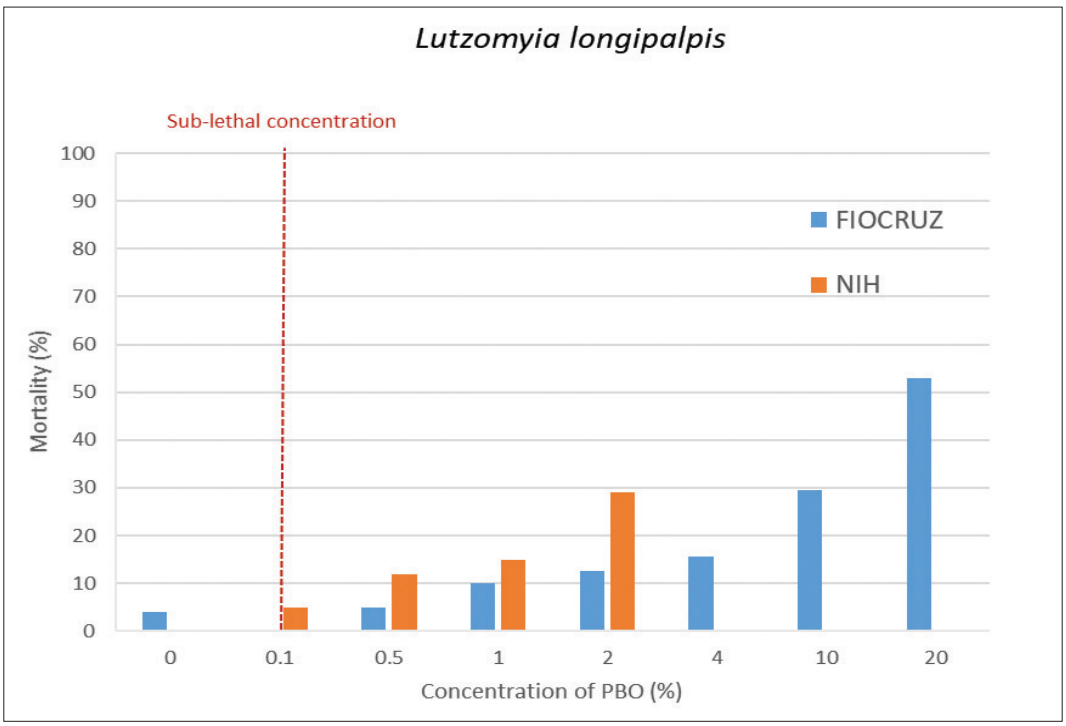
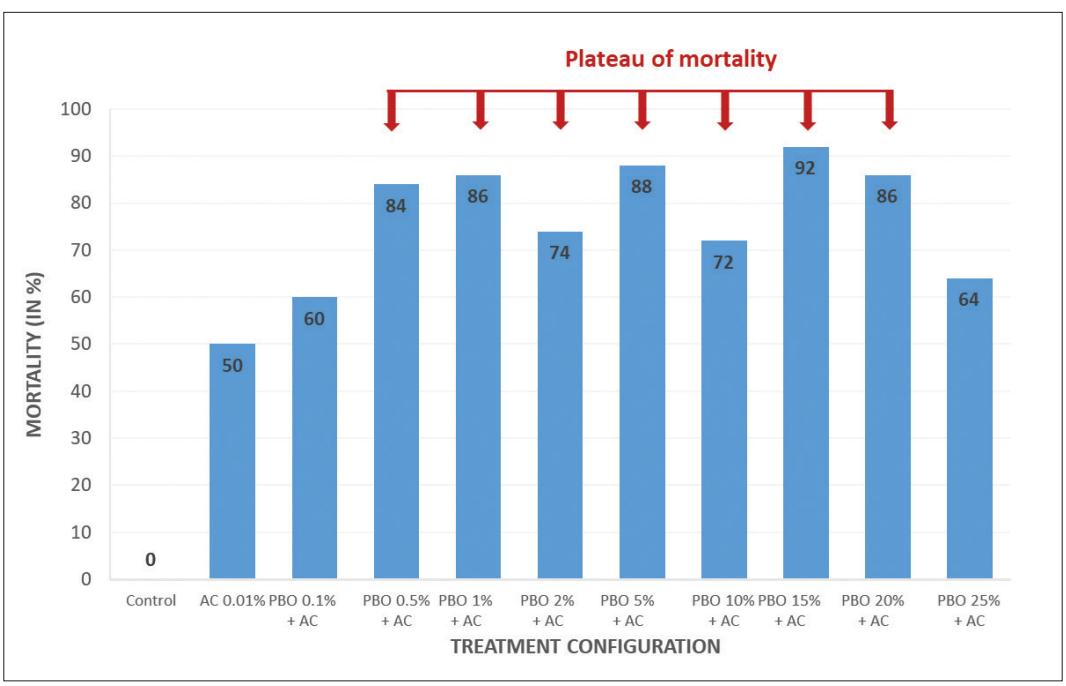


Fig 7. Potentiation induced by PBO (at 0.1–25%) on alpha-cypermethrin 0.01% (at LC_{50}) against susceptible strains of *Ph. argentipes* (ICMR-Rajendra Memorial Research Institute of Medical Sciences, India)



[a] **On the X axis, tests with control, alpha-cypermethrin 0.01% alone (AC) and pre-exposure to serial concentrations of PBO (0.1–25%) followed by 0.01% of AC.**

7.4 Validation of tentative discriminating concentrations in WHO tube tests (step 3)

A total of 21 TDCs were selected for further testing in step 3 against five sand fly species according to the results of step 2. In all, 85 bioassays (65 bioassays with *Phlebotomus* spp. and 20 with *Lutzomyia* spp.) with 9046 sand flies were conducted.

The mortality rate in sand flies was > 98% (i.e., the WHO susceptibility cut-off level) at most of the selected TDCs (**Tables 15** and **16**), except with 0.1% bendiocarb against *Lu. longipalpis*, 2% malathion against *Ph. argentipes* and 3–4 µg/bottle clothianidin against *Ph. argentipes* in some laboratories. With malathion and clothianidin, 100% mortality was achieved in repeated tests; however, repetition of the tests with bendiocarb at 0.1% did not change the outcome; 100% mortality was reached only by increasing the concentration to 0.15%.

The results in step 3 were then reviewed in a WHO consultation to recommend final DCs.

Table 15. Mortality of *Phlebotomus* and *Lutzomyia* spp. exposed to TDC in WHO tube tests in step 3 (1-h exposure; 24-h recording time)

Insecticide	Species of sand fly	Laboratory	TDC tested (%)	Mortality rate (%)	No. of sand flies tested
Alpha-cypermethrin	*Ph. papatasi*	IRD	0.1	100	111
		IPT	0.1	100	104
	Lu. longipalpis	FIOCRUZ	0.1	100	104
		NIH	0.1	100	135
	Ph. argentipes	ICDDRb	0.1	100	100
		ICMR-RMRIMS	0.1	100	100
	Ph. dubosqui	KEMRI	0.02	100	100
	Ph. longipes	ALIP	1	100	100
Deltamethrin	*Ph. papatasi*	IRD	0.05	100	106
		IPT	0.05	100	100
	Lu. longipalpis	FIOCRUZ	0.05	100	125
		NIH	0.05	100	131
	Ph. argentipes	ICDDRb	0.05	100	102
		ICMR-RMRIMS	0.05	99	100
		ICDDRb	0.1	100	102
		ICMR-RMRIMS	0.1	100	100
	Ph. dubosqui	KEMRI	0.02	100	100
		KEMRI	0.05	100	100
	Ph. longipes	ALIP	0.05	100	100
		ALIP	0.1	100	101

Insecticide	Species of sand fly	Laboratory	TDC tested (%)	Mortality rate (%)	No. of sand flies tested
Permethrin (40:60 cis:trans isomer ratio)	*Ph. papatasi*	IRD	1.0	100	107
		IPT	1.0	100	100
		IRD	1.5	100	107
		IPT	1.5	100	95
	Lu. longipalpis	FIOCRUZ	0.5	100	100
		NIH	0.5	100	127
		FIOCRUZ	1.0	100	102
		NIH	1.0	100	116
	Ph. argentipes	ICDDRb	1.0	100	103
		ICMR-RMRIMS	1.0	99	100
		ICDDRb	1.5	100	100
		ICMR-RMRIMS	1.5	100	100
	Ph. dubosqui	KEMRI	0.5	100	100
		KEMRI	1.0	100	100
	Ph. longipes	ALIP	1.0	100	100
		ALIP	1.5	100	99
Bendiocarb	*Ph. papatasi*	IRD	0.05	100	102
		IPT	0.05	100	100
	Lu. longipalpis	FIOCRUZ - R1	0.1	94	102
		FIOCRUZ - R2	0.1	95	101
		FIOCRUZ - R3	0.1	93	102
		FIOCRUZ	0.15	100	102
		FIOCRUZ	0.2	100	102
		NIH	0.1	100	135
	Ph. argentipes	ICDDRb	0.1	100	101
		ICMR-RMRIMS	0.1	100	100
	Ph. dubosqui	KEMRI	0.05	100	100
	Ph. longipes	ALIP	0.4	100	100

Insecticide	Species of sand fly	Laboratory	TDC tested (%)	Mortality rate (%)	No. of sand flies tested
Malathion	*Ph. papatasi*	IRD	5	100	104
		IPT	5	100	100
		IRD	10	100	111
		IPT	10	100	99
	Lu. longipalpis	FIOCRUZ	2	100	102
		NIH	2	100	134
	Ph. argentipes	ICDDRb	2	100	102
		ICMR-RMRIMS - R1	2	79	200
		ICMR-RMRIMS - R2	2	100	100
		ICMR-RMRIMS	3	100	100
		ICMR-RMRIMS	4	100	100
		ICMR-RMRIMS	5	100	100
	Ph. dubosqui	KEMRI	5	100	100
	Ph. longipes	ALIP	5	100	98
Pirimiphos-methyl	*Ph. papatasi*	IRD	100 mg/m^2	100	101
		IPT	100 mg/m^2	100	101
	Lu. longipalpis	FIOCRUZ	100 mg/m^2	100	99
		NIH	100 mg/m^2	100	153
	Ph. argentipes	ICDDRb	100 mg/m^2	100	103
		ICMR-RMRIMS	100 mg/m^2	99	100
	Ph. dubosqui	KEMRI	50 mg/m^2	100	100
		KEMRI	100 mg/m^2	100	100
	Ph. longipes	ALIP	100 mg/m^2	100	101

R1, R2 and R3: test replicates
Green: 100% mortality; orange: 98–99% mortality: red: < 98% mortality.

Table 16. Mortality of *Phlebotomus* and *Lutzomyia* spp. exposed to TDC of clothianidin in WHO bottle bioassays in step 3 (1-h exposure; 24-h recording time)

Species of sand fly	Laboratory	TDC tested (µg/bottle)	Mortality rate (%)	No. of sand flies tested
Ph. papatasi	IRD	3	100	105
	IPT	3	99	103
Lu. longipalpis	FIOCRUZ	3	100	123
	NIH	3	100	186
Ph. argentipes	ICDDRb	3	97	101
	ICMR-RMRIMS	3	93	98
	ICDDRb	4	100	101
	ICMR-RMRIMS - R1	4	94	99
	ICMR-RMRIMS - R2	4	100	100
	ICMR-RMRIMS	6	100	100
	ICMR-RMRIMS	8	100	100
	ICMR-RMRIMS	10	100	97
Ph. dubosqui	KEMRI	6	100	100
Ph. longipes	ALIP	10	100	101

R1, and R2: test replicates
Green: 100% mortality; orange: 98–99% mortality: red: < 98% mortality.

8. Main problems encountered

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

- Some delay was experienced in supplying insecticides, carrier oils and other materials to the test sites at the beginning of the study in late 2020 because of logistical problems due to the coronavirus disease (COVID-19) pandemic.
- Difficulty was experienced in regular production of enough sand flies in the colonies. The test laboratory in Shanghai, China, lost its sand fly colony and had to be excluded from the study. The laboratory in Ethiopia replaced *Ph. orientalis* with *Ph. longipes* because of technical difficulty in increasing the colony of *Ph. orientalis* during the COVID-19 pandemic. In other laboratories, due to the difficulty of producing sand flies, the number per concentration was reduced to 50 instead of 100, but the number of replicates was increased to achieve the sample size.
- Because of the limited number of participating laboratories, some data could not be cross-validated with those of other laboratories at two sites (*Ph. longipes* in Ethiopia and *Ph. dubosqui* in Kenya). When cross-validation of data was possible at two laboratories (for *Ph. papatasi*, *Ph. argentipes* and *Lu. longipalpis*), the mortality rates differed markedly among laboratories for the same insecticide–species combination. The *Ph. papatasi* strain at IPT, Tunisia, and *Lu. longipalpis* strain at NIH, Colombia, were suspected of being "hyper-susceptible" to pyrethroids, as the LC_{99} values obtained from tests at IRD, France, and FIOCRUZ, Brazil, with the same species (but with different strains) were consistent with those reported for other sand fly species as well as for mosquitoes *(14)*. Although there is no clear explanation for this difference, it was presumably due to differences in the genetic structure of the sand fly strains tested (e.g., presence of different sibling species) and/or differences in the extent of exposure of the field populations to insecticides before being colonized. It was also observed that some of the TDCs selected in step 2 did not cause > 98% mortality of sand flies in step 3 in different laboratories. Therefore, additional replicates and/or higher concentrations of insecticides were tested in order to achieve 100% mortality. After discussion at the WHO consultation, the lowest concentrations of test compounds that resulted systematically in 100% mortality of the test species were selected.
- One laboratory found difficulty in performing bottle bioassays with clothianidin mixed with 800 ppm of MERO®, and the tests had to be repeated several times before the desired outcomes were achieved. At other test sites, however, 800 ppm of MERO® was found to be adequate for testing clothianidin in bottle bioassays, and this concentration was therefore adopted.

- Difficulty was found in establishing a concentration of PBO that optimally synergized pyrethoids against susceptible sand flies. It should be noted that the sand fly colonies used in this study did not show resistance to pyrethroids, which may explain why 100% mortality was not reached, regardless of the concentration of PBO tested. The study should be followed up with oxidase- resistant sand flies to assess potentiation by PBO of various pyrethroids more accurately.
- Local restrictions due to the COVID-19 pandemic prevented access to the laboratories at almost all sites, which significantly extended the study timeline.

9. Conclusions and achievements

The results of the study in 2020–2022 involving eight laboratories to establish and validate DCs of insecticides to monitor the resistance of the main sand fly vectors of *Leishmania* parasites to insecticides were assessed in a WHO consultation on 31 January 2022 to select TDCs for step-3 testing, and the results from step 3 were assessed in a consultation on 29 June 2022, which made final recommendations to WHO on the DCs and subsequent studies required to fill the identified gaps. The agendas and participants in the two consultations are listed in the **Annex.**

The main achievements of the study are:

- conducted tube tests and WHO bottle bioassays for testing the susceptibility of the four main *Phlebotomus* spp. (*Ph. papatasi, Ph. argentipes, Ph. longipes, Ph. dubosqui*) and *Lu. longipalpis* to seven insecticides used in public health (deltamethrin, alpha-cypermethrin, bendiocarb, malathion, pirimiphos-methyl, clothianidin);
- developed a network of eight laboratories in different regions with the capacity for testing the susceptibility of sand flies to insecticides and further built their capacity for studies with standard designs and SOPs;
- validated a statistical framework to analyse concentration–response data and generate relevant metrics, minimizing measurement errors and background mortality of sand flies;
- created a central database of bioassay records for > 100 000 sand flies, which will provide data for studies of the source of differences in sand fly mortality under different test conditions;
- established and validated 18 new DCs of seven insecticides with five sand fly species in either WHO tube tests or WHO bottle bioassays to monitor resistance in sand flies (**Tables 17 and 18**); and
- conducted a WHO tube test with PBO, a synergist, against susceptible strains of four sand fly species (*Ph. papatasi, Ph. argentipes, Ph. dubosqui* and *Lu. longipalpis*) to determine the concentration to be used in synergist–pyrethroid bioassays. Although the studies were limited, an interim concentration of 0.5% PBO was adopted for further synergist bioassays against sand flies.

Table 17. Insecticide discriminating concentrations (DCs) for sand fly species in WHO tube tests (24-h filter paper drying time; 1-h exposure; 24-h holding or recording time)

Insecticide	Species	DC (%)	Carrier oil or solvent
Alpha-cypermethrin	*Ph. papatasi*	0.1	Silicone oil
	Ph. argentipes	0.1	
	Lu. longipalpis	0.1	
	Ph. dubosqui	0.02	
	Ph. longipes	1	
Deltamethrin	*Ph. papatasi*	0.05	Silicone oil
	Ph. argentipes	0.05	
	Ph. dubosqui	0.02	
	Ph. longipes	0.05	
	Lu. longipalpis	0.05	
Permethrin (40:60 cis:trans isomer ratio)	*Ph. papatasi*	1	Silicone oil
	Ph. argentipes	1	
	Ph. dubosqui	0.5	
	Ph. longipes	1	
	Lu. longipalpis	0.5	
Bendiocarb	*Ph. papatasi*	0.05	Olive oil
	Ph. argentipes	0.1	
	Ph. dubosqui	0.05	
	Ph. longipes	0.4	
	Lu. longipalpis	0.15	
Malathion	*Ph. papatasi*	5	Olive oil
	Ph. argentipes	2	
	Ph. dubosqui	5	
	Ph. longipes	5	
	Lu. longipalpis	2	
Pirimiphos-methyl[a]	*Ph. papatasi*	100 mg/m^2	Acetone alone
	Ph. argentipes	100 mg/m^2	
	Ph. dubosqui	50 mg/m^2	
	Ph. longipes	100 mg/m^2	
	Lu. longipalpis	100 mg/m^2	

[a] DC expressed as mg/m^2, as no carrier oil is used to treat filter papers with pirimiphos-methyl.

Table 18. Clothianidin DCs for sand fly 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
Clothianidin	*Ph. papatasi*	4	Acetone + MERO® 800 ppm
	Ph. argentipes	4	
	Ph. dubosqui	6	
	Ph. longipes	10	
	Lu. longipalpis	4	

MERO®: 81% rapeseed oil methyl ester

10. Recommendations to WHO

After a review of the results of the studies and the discussions at the WHO consultations, the following recommendations were made to WHO.

- **Investigate the causes of the between-laboratory differences in mortality of *Ph. papatasi* and *Lu. longipalpis***. The bioassays at different participating laboratories showed significant (30–50 times) differences in the LC_{99} values for pyrethroids in *Lu. longipalpis* and *Ph. papatasi* strains. These differences are a cause of concern as they may result in over- or under-estimates of the insecticide DCs for a given species. *Lu. longipalpis* is a biological complex of cryptic species, and its sibling species differ widely in several behavioural traits *(19)*. Molecular studies should be conducted to determine the genetic structures of the different strains of *Lu. longipalpis*, any genetic differences and how they affect the tolerance of different strains to insecticides. Although no cryptic species of *Ph. papatasi* have been reported or suspected, the high insecticide susceptibility of the Tunisian strain could be due to very low exposure to insecticides before establishment of the colony at IPT in 2017. Further studies at more testing laboratories would allow cross-validation of the bioassay results and more reliable test outcomes.
- **Optimum concentration of PBO for synergist bioassays**. In the PBO–pyrethroid synergy studies, 0.5% PBO did not cause abnormal mortality (i.e., < 20%) in control tests with both *Ph. papatasi* and *Lu. longipalpis* and was sufficient to potentiate the toxicity of alpha-cypermethrin against susceptible *Ph. argentipes* (an increase in the mortality rate over that with alpha-cypermethrin alone of about 20%). The mortality rate was not much higher at concentrations > 0.5%, and 100% mortality of sand flies was not achieved, regardless of the PBO concentration tested up to 25%. The WHO expert consultation acknowledged that the absence of mixed-function oxidases in the *Ph. argentipes* strain tested in India might explain the moderate elevation in mortality rate after exposure to increasing PBO concentrations. Consequently, the consultation recommended:
  - adoption of an interim concentration of 0.5% PBO for synergist–insecticide bioassays against sand flies in order to obtain more evidence in various susceptible colonies and sand fly species; and
  - further potentiation studies with well-characterized sand fly strains resistant to pyrethroid mixed-function oxidases in order to better understand the action of PBO and provide necessary evidence to eventually revise the WHO recommendation.
- **Insecticide tolerance of *Ph. longipes***. Both the LC_{99} and LC_{100} values indicate that the *Ph. longipes* strain colonized at ALIP, Ethiopia, to insecticides is higher than that of other sand fly species. It is not clear whether this is a general trait of this species or whether this strain, which was colonized recently (2021) has a resistance

mechanism. Additional tests in at least one other laboratory with a different strain of *Ph. longipes* would be necessary to confirm this possibility. If this cannot be achieved rapidly, well-known genetic markers (e.g., *kdr*) could be investigated in collaboration with research institutions with the appropriate capacity. In addition, concentration–response bioassays with representative insecticides could be repeated with the same strain after five or 10 generations of colonization of the species, as, in principle, the level and frequency of resistance decrease after several generations of inbreeding.

- **Monitoring of insecticide resistance in field populations of sand flies**. This study is a first attempt to establish and validate the DCs of new and existing public health insecticides against five sand fly species that transmit leishmaniases. In view of the high inter-laboratory variation in estimates for some insecticide–species combination, the new DCs for sand flies should be tested in other settings and conditions. Evidence should be collected by national disease control programmes and shared with WHO for further assessment, which might revise the DCs in due course.

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Annex. Agendas and participants in WHO consultations

Agenda of consultation on 31 January 2022

WHO consultation to review interim results of the multi-centre laboratory study for determining insecticide discriminating concentrations for monitoring of resistance in sand fly vectors of leishmaniases, 31 January 2022 [Virtual meeting, 14:00–17:30 CET]

Objectives:

1. To assess interim results from steps 1 and 2 of the concentration-response studies with selected insecticide compounds at 8 different laboratories against sand fly vectors;
2. To determine and recommend tentative discriminating concentrations (TDCs) of test compounds either impregnated on filter papers or tested in WHO bottle bioassays;
3. To discuss technical challenges and limitations, if any, and suggest measures to resolve them; and
4. To update progress in developing standard operating procedures (SOPs) for testing the susceptibility of sand flies.

Open session		
14:00–14:10	- Opening remarks	Dr Raman Velayudhan, Unit Head, NTD/VVE
14:10–14:20	- 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, NTD/VVE
Chair/Co-chair to take over the meeting		
14:20–14:40	- Presentation of the study design and method - Discussion	Dr Vincent Corbel, IRD
14:40–15:40	- Presentation of results of steps 1 and 2 and technical limitations	Dr Vincent Corbel, IRD
15:40–16:00	- Statistical analysis of the multi-centre data	Ms Mara Kont, Imperial College London
16:00–16:10	- Coffee break	
16:10–17:00	- Discussion on TDCs of test compounds	All participants
Closed session (restricted to WHO experts, investigators and the WHO secretariat)		
17:00–17:30	- Drafting of recommendations - Closure	Experts and investigators only

List of participants

Name	Institution
WHO experts	
Dr João Pinto	Instituto de Higiene e Medicina Tropical, Universidade NOVA de Lisboa, Lisbon, Portugal (Chairperson)
Dr Audrey Lenhart	Entomology Branch, Centers for Disease Control and Prevention, Atlanta (GA), United States of America (Rapporteur)
Dr Rosemary Susan Lees	Liverpool School of Tropical Medicine, Liverpool, United Kingdom
Dr Thomas Churcher	Imperial College, London, United Kingdom
Professor Ahmad Ali Enayati	School of Public Health, Mazandaran University of Medical Sciences, Sari, Islamic Republic of Iran
Dr Adanan Che Rus	Vector Control Research Unit, School of Biological Sciences,Universiti Sains Malaysia, Penang, Malaysia
Dr Pie Müller	Swiss Tropical and Public Health Institute, Basel, Switzerland
Professor Arti Prasad	Public Health Entomology Laboratory, MLS University, Udaipur, India
Collaborators and peer reviewers	
Dr Ademir J Martins	Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
Ms Margarate Afonso	Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
Dr Martha Liliana Ahumada	Instituto Nacional de Salud, Bogotá, Colombia
Dr Martha Quinones	University of Colombia, Bogotá, Colombia
Dr Vincent Corbel	Institut de Recherche pour le Développement, Montpellier, France
Ms Dominique Cerqueira	Institut de Recherche pour le Développement, Montpellier, France
Dr Stephane Duchon	Institut de Recherche pour le Développement, Montpellier, France
Dr Manju Rahi	Indian Council of Medical Research, New Delhi, India
Dr Diwakar Singh Dinesh	Indian Council of Medical Research-Rajendra Memorial Research Institute of Medical Sciences, Patna, India
Dr Krishna Pandey	Indian Council of Medical Research-Rajendra Memorial Research Institute of Medical Sciences, Patna, India
Dr Kamaraju Raghavendra	Indian Council of Medical Research-National Institute of Malaria Research, New Delhi, India
Dr Vaishali Verma	Indian Council of Medical Research-National Institute of Malaria Research, New Delhi, India
Dr Elyes Zhioua	Unit of Vector Ecology, Institut Pasteur de Tunis, Tunis, Tunisia
Dr Ifhem Chelbi	Unit of Vector Ecology, Institut Pasteur de Tunis, Tunis, Tunisia
Mr Debashsi Ghosh	International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh
Dr Rajib Chowdhury	National Institute of Preventive and Social Medicine, Dhaka, Bangladesh
Dr Luna Kamau	Kenya Medical Research Institute, Nairobi, Kenya
Dr Damaris-Matoke Muhia	Kenya Medical Research Institute, Nairobi, Kenya
Dr Sisay Dugassa	Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Addis Aba-ba, Ethiopia
Ms Mara Kont	Imperial College, London, United Kingdom

Stakeholders (observers)	
Dr Dave Malone	Bill & Melinda Gates Foundation, Liverpool, United Kingdom
Dr Angus Spiers	Innovation-2-Impact Project, Liverpool, United Kingdom
Dr Susanne Stutz	BASF, Limburgerhof, Germany
Dr James Austin	BASF, Wake Forest (NC), United States of America
Dr Mark Hoppe	Syngenta, Stein, Switzerland
Ms Lisa Eppler	Syngenta, Basel, Switzerland
Mr Raj Saran	Syngenta, Vero Beach (FL), United States of America
Dr Kunizo Mori	Mitsui Chemicals Agro, Inc. (consultant), Tokyo, Japan
Dr John Lucas	Sumitomo Chemicals Co. Ltd (consultant), London, United Kingdom
Dr Barnabas Zogo	Sumitomo Chemicals (UK) plc, United Kingdom
WHO secretariat	
Dr Rajpal S. Yadav	Vector Control, Veterinary Public Health and Environment Unit, Department of Control of Neglected Tropical Diseases, WHO, Geneva, Switzerland
Dr Raman Velayudhan	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
Ms Lucía Fernández Montoya	Global Malaria Programme, Geneva, Switzerland
Dr Haroldo S. da Silva Bezerra	Regional Office for the Americas, Washington (DC), USA
Dr Emmanuel Chanda	Regional Office for Africa, Brazaville, Democratic Republic of the Congo (unable to attend)
Dr Samira Al-Eryani	Regional Office for the Eastern Mediterranean, Cairo, Egypt

Agenda of WHO consultation (virtual) on 29 June 2022

WHO consultation to review results of the multi-centre study on determination of insecticide discriminating concentrations for monitoring resistance in sand flies, 29 June 2022 (final meeting). [Virtual meeting, 14:00–17:30 CEST]

Scope of the meeting

After reviewing data at the WHO consultation (virtual) on 31 January 2022,[1] the WHO expert group advised establishment of 21 tentative discriminating concentrations of insecticides against *Phlebotomus* and *Lutzomyia* spp. of sand flies and specific instructions for further testing in step 3 for confirmation of the tentative discriminating concentrations.

The aim of the final WHO consultation is to validate test procedures (SOPs) and review the data from step-3 bioassays. The meeting will also review the scientific evidence on PBO testing and decide whether a concentration for synergist bioassays against sand flies can be recommended.

According to the outcome of step 3 testing and recommendations of the consultation, the final study report along with SOPs will be published by WHO to provide guidance on insecticide discriminating concentrations for monitoring resistance in sand flies.

Agenda (open session)		
14:00–14:05	- Welcoming remarks	Dr Raman Velayudhan, Unit Head, NTD/VVE
14:05–14:10	- Scope and objectives of the meeting; declarations of interest; appointment of Chair, Co-chair and rapporteurs	Dr Rajpal Yadav, Scientist, NTD/VVE
14:10–15:00	- Review of step-3 bioassay data from studies completed in 2022 - Discussion and recommendations to WHO on discriminating concentrations	Dr Vincent Corbel, IRD
15:00–15:30	- Review of SOPs (WHO tube test and WHO bottle bioassay) for final validation and dissemination - Discussion	Dr Rajpal Yadav, Scientist, NTD/VVE
15:30–15:45	- Coffee break	
15:45–16:15	- Review of evidence on the potentiation effect of PBO on pyrethroids against susceptible sand flies - Discussion and recommendations for synergist bioassays	
16:15–16:30	- Any other relevant technical matters	Dr Vincent Corbel, IRD
Closed session (restricted to experts, investigators and the WHO secretariat)		
16:30–17:25	- Conclusions and final recommendations	Experts and investigators
17:25–17:30	- Closure	Experts and investigators

[1] Determining discriminating concentrations of insecticides for monitoring resistance in sand flies: Interim report of a multi-centre laboratory study. February 2022 (unpublished).

List of participants

Name	Institution
Invited experts	
Dr João Pinto	Instituto de Higiene e Medicina Tropical, Universidade NOVA de Lisboa, Lisbon, Portugal (Chairperson)
Dr Audrey Lenhart	Entomology Branch, Centers for Disease Control and Prevention, Atlanta, United States of America (unable to attend)
Dr Rosemary Susan Lees	Liverpool School of Tropical Medicine, Liverpool, United Kingdom (Rapporteur)
Dr Thomas Churcher	Imperial College, London, United Kingdom
Professor Ahmad Ali Enayati	School of Public Health, Mazandaran University of Medical Sciences, Sari, Islamic Republic of Iran
Dr Adanan Che Rus	Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia
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Ms Margeret Afonso	Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
Dr Martha Liliana Ahumada	Instituto Nacional de Salud, Bogotá, Colombia
Dr Vincent Corbel	Institut de Recherche pour le Développement, Montpellier, France
Dr Stephane Duchon	Institut de Recherche pour le Développement, Montpellier, France
Ms Dominique Cerqueira	Institut de Recherche pour le Développement, Montpellier, France
Dr Manju Rahi	Indian Council of Medical Research (ICMR), New Delhi, India (unable to attend)
Dr Kamaraju Raghavendra	Indian Council of Medical Research-National Institute of Malaria Research, New Delhi, India
Dr Vaishali Verma	Indian Council of Medical Research-National Institute of Malaria Research, New Delhi, India
Dr Krishna Pandey	Indian Council of Medical Research-Rajendra Memorial Research Institute of Medical Sciences, Patna, India
Dr Elyes Zhioua	Unit of Vector Ecology, Institut Pasteur de Tunis, Tunisia
Dr Ifhem Chelbi	Unit of Vector Ecology, Institut Pasteur de Tunis, Tunisia
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Dr Damaris-Matoke Muhia	Kenya Medical Research Institute, Nairobi, Kenya
Dr Sisay Dugassa	Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Addis Ababa, Ethiopia

Stakeholders (observers)	
Dr Susanne Stutz	BASF, Limbergerhof, Germany
Dr James Austin	BASF, Wake Forest (NC), United States of America
Dr Mark Hoppe	Syngenta, Stein, Switzerland
Dr Sebastian Horstmann	Envu, Mosquito Management, Monheim, Germany
Dr Kunizo Mori	Mitsui Chemicals Agro, Inc. (Consultant), Tokyo, Japan
Dr John Lucas	Sumitomo Chemicals Co.Ltd. (Consultant), London, United Kingdom
Mr John Invest	Sumitomo Chemicals Co. Ltd. (Consultant), London, United Kingdom
Dr Barnabas Zogo	Sumitomo Chemicals Co. Ltd., Tokyo, Japan
WHO secretariat	
Dr Rajpal S. Yadav	Vector Control, Veterinary Public Health and Environment Unit, Department of Control of Neglected Tropical Diseases, WHO, Geneva, Switzerland
Dr Raman Velayudhan	Vector Control, Veterinary Public Health and Environment Unit, Department of Control of Neglected Tropical Diseases, WHO, Geneva, Switzerland
Ms Lucía Fernández Montoya	Global Malaria Programme, WHO, Geneva, Switzerland
Dr Haroldo S. da Silva Bezerra	Regional Office for the Americas, Washington (DC), United States of America
Dr Emmanuel Chanda	Regional Office for Africa, Brazaville, Democratic Republic of the Congo
Dr Samira Al-Eryani	Regional Office for the Eastern Mediterranean, Cairo, Egypt

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