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Two New Alternatives to the Conventional Arm-in-Cage Test for Assessing Topical Repellents

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Abstract

European guidelines for testing attractant and repellent efficacy (i.e., Product type 19 [PT19]) have been in revision since 2017. A key topic of discussion is the current approach to evaluating topical repellents. The European Chemical Agency has stated field testing should be avoided because of mosquito-borne disease risks. However, the most common laboratory method, the arm-in-cage (AIC) test, may limit the reliable extrapolation of lab results to field conditions. This study's main goal was to assess alternative laboratory methods for evaluating topical mosquito repellents that use mosquito landing rates more representative of those in the field. The study took place at three European testing labs using 30 study participants per test and the mosquito, *Aedes albopictus* (Skuse, 1894, Diptera: Culicidae). In phase 1, a conventional AIC test and a sleeved AIC test were performed. Respectively, the arm area exposed was 600 and 100 cm^2, and cage volume was 0.040 and 0.064 m^3. Mosquito density was the same for both: 1 female/840 cm^3. In phase 2, room-based testing (40 ± 5 mosquitoes in 25–30 m^3) was used as a proxy for field testing. The mosquito repellent employed was 15% N,N-diethyl-m-toluamide in ethanol at two doses: 1 and 0.5 g/600 cm^2. The protection times measured at each laboratory were analyzed both separately and together using nonparametric (Kruskal–Wallis) test. The two alternatives methods showed to be potential alternatives to the current AIC method recreated field mosquito landing rates and achieved reproducible protection times across laboratories.

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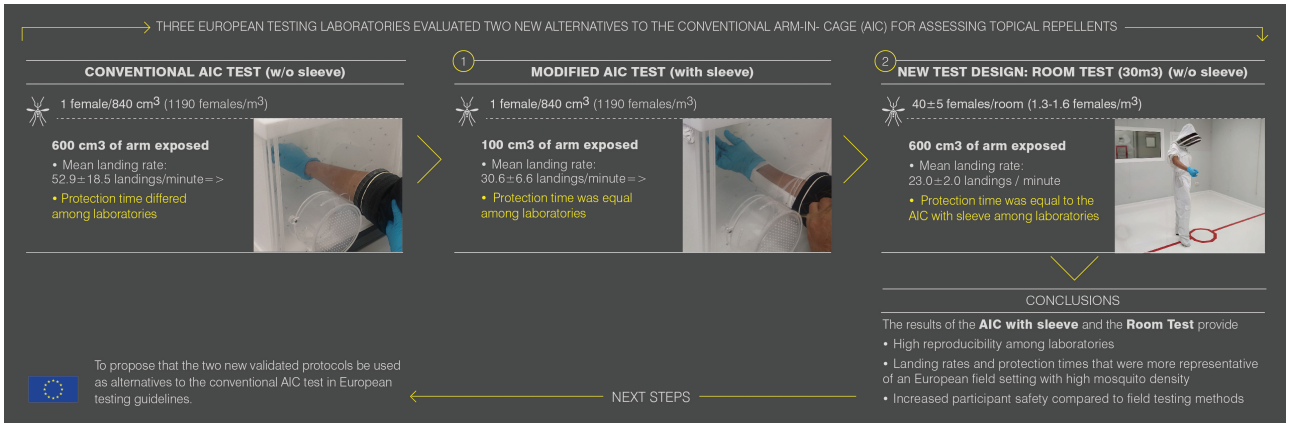
Graphical abstract



Key words: *Aedes albopictus*, bioassay, room test, landing rate, testing protocols

The incidence of vector-borne diseases (VBDs) has increased worldwide in recent decades (Weaver and Reisen 2010, Gould et al. 2017, Gossner et al. 2018), underscoring the importance of studying them more extensively. This trend also highlights the need to build more efficient, integrated vector management programs that employ physical, biological, environmental, and chemical measures to prevent and control VBD transmission.

Among the products approved for use by consumers are personal protection products (PPPs), which include topical and spatial repellents. PPPs play an important role in reducing interactions between humans and insects thereby minimizing human exposure to insect bites during outdoor activities and thus also reducing the risk of disease transmission. Such products are therefore increasingly important tools in the fight against mosquito-borne diseases (Verhulst et al. 2007).

No vaccines are currently available for many of the most frequent VBDs (Gossner et al. 2018), which presents a threat to human health. For example, local outbreaks of imported arboviruses have occurred on different continents, including Europe (Grandadam et al. 2011, Lourenço and Recker 2014, Schaffner and Mathis 2014) and the Americas (Moore and Mitchell 1997, Ruiz-Moreno et al. 2012, Hennessey et al. 2016). Consequently, people are increasingly relying on preventive measures, including the use of PPPs. Over recent years, the demand for repellents and household insecticides has soared during periods of heightened mosquito activity (Chouhan and Deshmukh 2020).

Depending on the country or administrative region, insecticides and repellents may be regulated as pesticides, quasi-drugs, or cosmetics. In Europe, the marketing and use of such products is regulated by European Biocidal Products (BPR) Regulation 528/2012 (The European Parliament and the Council of the European Union 2012). Before insecticides and repellents can be sold on the European market, certain technical requirements must be met: their chemical formulation and properties must be described in detail, their toxicological risks for humans and the environment must be assessed, and their efficacy during intended use must be quantified. Furthermore, these data are used to gain final approval for product labels, which include efficacy claims (European Chemical Agency [ECHA] 2019c).

In 2012, the ECHA updated its guidelines for evaluating the efficacy of insecticides and repellents (ECHA 2011). Since 2017, revision has been underway of the guidelines for Product type 19 (PT19), a category that includes attractants and repellents (ECHA 2018b, c, d; U.S. Environmental Protection Agency [EPA] 2019a, b).

At present, the efficacy of topical mosquito repellents is most commonly evaluated in the laboratory using the arm-in-cage (AIC) test, described by the World Health Organization (WHO 2009) and the EPA (2010). However, an important question has recently arisen: do the protection times estimated by the AIC test accurately reflect the duration of protection a consumer would experience under outdoor conditions? The AIC test is used to estimate the complete protection time (CPT) of topical repellents (i.e., formulated as lotions, creams, wipes, or sprays) under laboratory conditions. To evaluate a topical repellent, WHO guidelines state that 200–250 host-seeking female mosquitoes are to be placed in a cage with sides measuring between 35 and 40 cm, such that mosquito density is equivalent to 3,125–3,900 females/m^3 (1 female/320 cm^3). By comparison, EPA guidelines state that 200 host-seeking female mosquitoes are to be placed in cages measuring approximately 61 × 61 × 61 cm, such that mosquito density is equivalent to 881 females/m^3 (1 female/1,160 cm^3). The product to be tested is then applied to the forearms of human volunteers (of mixed sexes), who introduce their arms into the cage every 30 or 60 min. To characterize landing and/or probing activity, the arm is left in the cage for 3 min during each exposure period. The test typically runs for either up to 8 h following product application or until the product no longer provides complete protection. In WHO guidelines, CPT is the time elapsed between product application and the first mosquito landing and/or instance of probing. In the EPA guidelines, CPT is the time elapsed between product application and efficacy failure, where the latter is defined on a study-specific basis. For example, it can be the time between product application and the first efficacy failure event that has been confirmed within 30 min by a second similar event.

Although the AIC test is a well-accepted and internationally recognized method, its CPT estimates for topical repellents may be lower than the CPT estimates obtained under field conditions. Past research has examined the correlation between CPTs measured in the laboratory using the conventional AIC test and CPTs measured under field conditions. In general, landing rate increased proportionally with mosquito density, which led in turn to shorter CPTs, as seen in the conventional AIC test (Obermayr et al. 2010, Colucci

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and Müller 2018). In another study, Moreno-Gómez et al. (2020) showed that the mosquito landing rate obtained in the conventional AIC test (229 landings/min) significantly exceeded the landing rate obtained in at afield site in Europe (26.8 landings/min).

In this vein, when the ECHA Efficacy Working Group (EFF WG) met in Helsinki on 3–4 December 2018, European authorities and industry representatives discussed whether they should further develop one of the sections of the European testing guidelines for mosquito repellents (i.e., the chapter entitled "Simulated-use test for topical repellents against mosquitoes applied on human skin"), in which the AIC test is described (ECHA 2018b). During the discussions, ECHA advised that field-based evaluation methods should be avoided because, although such methods provide results that are more representative of outdoor conditions, there are increasing concerns about VBDs in field settings (Seyler et al. 2009, Rocklöv et al. 2016). This position is contrary to that of the EPA, which expressly requires field testing at two different sites that is overseen by the Human Studies Review Board (EPA 2010). Since field testing can present health risks because the infection status of mosquitoes is unknown, it was decided that European AIC testing should be adjusted to allow repellents to be evaluated under controlled laboratory conditions that better simulate field conditions. Two further aims were stated in this project. First, the new method should yield results leading to meaningful label claims, namely more accurate protection times for European consumers using the product under outdoor conditions. Second, the new method should generate reproducible results at different testing laboratories. It was therefore agreed that European authorities, industry representatives, and researchers at European testing laboratories would work together to revise current testing parameters and requirements with a view to developing European laboratory methods for topical repellent evaluation. This collaborative study assessed two alternative testing methods to the conventional AIC test with the objective of obtaining more accurate estimates of repellent protection time by better simulating the actual conditions faced by consumers engaging in outdoor activities in Europe.

Materials and Methods

The test designs and evaluation parameters were agreed upon when the EFF WG met on 27–28 March 2019 (ECHA 2019b). The study was performed from July 2019 to February 2020. It was divided into two phases. The first phase took place from July to October 2019, and AIC testing was performed (cage volumes: 0.040 and 0.064 m^3). The second phase took place from November 2019 to February 2020, and room testing was performed (room volumes: 25–30 m^3).

During the first phase of the study, the objective was to determine whether the European AIC methodologies being evaluated could recreate the landing rate observed in a natural area of Europe highly infested with mosquitoes. Three European testing laboratories participated in the research: Henkel (Spain), Tecnalia (Spain), and i2L Research (UK). This phase of the study examined the effect of three variables—mosquito number, repellent dose, and sleeve use (which reduced the area of skin exposed from 600 to 100 cm^2)—on protection time and landing rate.

The results of the first phase were used to answer four key questions. First, were the results of AIC testing reproducible among laboratories? Second, how was landing rate affected by mosquito number and sleeve use? Third, how did sleeve use impact protection time? Fourth, how did dose (0.5 vs 1.0 g/600 cm^2) affect protection time?

During the second phase of the study, the goal was to develop a laboratory-based method for evaluating topical repellents under controlled conditions that could serve as an effective alternative to both the conventional AIC test, as described in WHO (2009) and EPA (2010) AIC guidelines and field-based testing. This alternative method, called the room test (RT), was implemented in a laboratory setting but used a significantly larger test enclosure (a room of 25–30 m^3) than the AIC method. The increased space allowed the mosquitoes to fly around more freely. Furthermore, study participants could fit their entire bodies within the testing rooms even if only one of their forearms was exposed to the mosquitoes. This experimental set-up was understood to allow more natural mosquito behavior while simultaneously limiting the disease risks faced by study participants (since mosquitoes in the laboratory are guaranteed to be pathogen free). Additionally, it could control for other environmental factors that commonly vary in the field. The results of the RT were then compared with the results of AIC testing, obtained during phase 1, to assess their consistency in estimating protection times.

During the first phase, i2LResearch did not achieve the target landing rate during the sleeveless AIC test, meaning its results could not be validated. For reasons of availability, a different group of testing laboratories took part in the second phase of the study (i.e., the RT): Henkel (Spain), Tecnalia (Spain), and BioGenius (Germany).

The results of the second study phase were used to answer three key questions. First, were the results of the RT reproducible among laboratories? Second, how did dose (0.5 g/600 vs 1.0 g/600 cm^2) affect protection time? Third, how did landing rate and protection time compare for room-based testing versus AIC testing?

During both study phases, the following experimental conditions were the same:

Insect Species

ECHA (2011) guidelines for PT19 efficacy testing state that repellents should be tested using *Aedes* species since they are the most aggressive group of mosquitoes. In this study, all tests were performed with *Aedes albopictus* (Skuse 1895), although each laboratory used its own specific strain, reared in-house over many years. Mosquito origin differed: the Tecnalia strain comes from mosquitoes collected in the field (Spain) in 2015; the i2LResearch strain comes from mosquitoes collected in Mauritius in 2013; Henkel obtained its strain from the Entostudio Test Institute (Italy) in 2013; and Biogenius obtained its strain from BEI Resources (United States) in 2015.

Mosquito rearing conditions were as follows: temperature of 25 ± 2°C, relative humidity of 60 ± 5%, and photoperiod of 12:12 (L:D). The mosquitoes were nonblood-fed females 5–10 d in age. They were given sugar water prior to and during the testing period to promote their good health.

Study Participants

The work conducted herein was approved by the ethics committee of Henkel AG & Co. KGaA. It met the company's corporate standards, which ensure health, safety, and respect for the environment as well as the protection and ethical treatment of all study participants. Participants (aged 18–55 yr) were recruited and signed a written informed consent form, which explained the purpose and procedures of the study as well as their role and responsibilities and the voluntary right to refuse or withdraw from the study at any point. As per EU guidelines, participants were asked to avoid the use of nicotine, alcohol, fragrances (perfumes, body lotions, soap, etc.), and repellents for 12 h prior to and during testing (ECHA 2018a).

Thirty participants, 10 per laboratory, took part in each phase. During AIC testing, 16 of the participants were male and 14 were female; in the RT, 14 of the participants were male and 16 were

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female. Prior to testing, the skin to be exposed was washed with unscented soap, rinsed with water, rinsed with 70% ethanol or isopropyl alcohol, and then dried with an uncontaminated towel (ECHA 2018a). Between exposure periods, study participants remained in air-conditioned rooms and kept their activity levels low.

Climatic Conditions

Temperature and relative humidity were kept at 25.0 ± 2°C and 60 ± 5%, respectively, because preliminary trials using WHO (2009) recommendations (27 ± 2°C and ≥80 ± 10%), showed that, over long periods of time (8 h), mosquito activity was disrupted at higher temperatures (27°C) in the RT. Such was not the case at lower temperatures (25°C; unpublished data).

Repellent Formula

The repellent formula was provided by Endura S.p.A. (Italy). It did not contain any fragrances, and the co-formulants were alcohol based. The active substance was 15% N,N-diethyl-m-toluamide (DEET; CAS number 134-62-3), which was chosen because it is one of the most widely marketed chemical-based insect repellents. It has been in use worldwide since the 1950s (Fradin and Day 2002), and the WHO (2009) recommends that it be employed as the positive control when evaluating topical repellents. Because of its long history of use, DEET has become the gold standard against which other repellents are compared, and it has been employed in tests with many types of arthropods (Debboun et al. 2014). The percentage of DEET was chosen to ensure that the repellent would result in more than 8 h of CPT at the highest dose, which facilitated comparisons among laboratories.

Doses

Two doses of repellent were evaluated: 1 g of product/600 cm^2 of skin surface and 0.5 g of product/600 cm^2 of skin surface. The first dose was chosen based on WHO (2009) and EPA (2010) recommendations. The second dose was chosen because, in the future, it is likely that only lower doses will be considered acceptable when conducting Human Health Risk Assessments (HHRAs) of BPR-approved active compounds, especially those with higher concentrations of active substances (≥15%).

The amount of repellent to be applied was calculated based on the arm measurements (circumference and length) of each participant, taking into account the area to be exposed. This quantity of repellent was spread on the forearm using a pipette and distributed using a glove-covered finger.

End Points

Mosquito activity was measured on the untreated arm by counting the number of landings that took place during each exposure period. For the treated arm, the endpoint was the first confirmed probe. A landing occurred when a flying mosquito alighted on the skin without probing or biting. When mosquitoes land, they assume a typical posture and try to taste the skin with the proboscis. In this study, the mosquitoes were shooed away at this point by shaking the arm, so as to prevent biting. Probing occurred when a mosquito penetrated the skin with her mouthparts without ingesting any blood. Each study participant was paired with an experienced researcher who could accurately count the number of landings.

Evaluation Parameters

Protection time was quantified by measuring CPT and 99% protection because both metrics provide useful information when it comes to methodological validation.

Complete Protection Time

CPT is the duration of time over which protection is equal to 100%. There are different ways to define CPT. In WHO (2009) guidelines, CPT is the time between repellent application and the first mosquito landing and/or instance of probing. In the EPA guidelines, CPT is the time between repellent application and repellent efficacy failure, which is defined on a study-specific basis; for example, repellent efficacy failure can be said to have occurred when a first failure event is followed by a second failure event within 30 min. However, in the revised European guidelines, a novel and more conservative definition of CPT has been adopted. It is calculated as follows: A first instance of probing is noted that must be confirmed by a second instance of probing during the same or the subsequent 3-min exposure period. Then, the exposure period preceding the first probing event is identified; CPT is thus the time between repellent application and this preceding exposure period. In this study, CPT was measured according to European guidelines.

Percentage Protection

Percentage protection expressed the duration of repellent protection in terms of the percent reduction in landings/instances of probing attributable to the repellent for each participant. This metric was calculated as follows:

$$\%\ \text{protection} = (C - T) \times 100/C,$$

where C = number of landings on the untreated control arm; T = number of probing instances on the treated arm; and one figure was calculated: 99% protection (P99).

Frequency of Exposure Intervals

Participants were exposed to the mosquitoes every hour for up to 8 h or until percentage protection fell below 99%, whichever came first.

Study phase 1: Experimental Conditions Exclusive to AIC Testing

Mosquito Density and Testing Enclosures

The European authorities and industry representatives agreed that, even from a conservative perspective, the mosquito density specified in WHO (2009) guidelines (minimum of 1 female/320 cm^3) was not representative of even the worst infestation levels found in Europe. In contrast, EPA guidelines indicate that a density of 1 female/1,160 cm^3 should be used. Based on unpublished observations, Ulla Gordon and Sergej Sperling (Biogents AG) have recommended that an intermediate value of 1 female/840 cm^3 be employed, a figure arising from the results of a previous study (Obermayr et al. 2010). This intermediate value was discussed during the EFF WG workshop dedicated to the revision of the PT19 efficacy guidelines, which took place on 19–20 October 2017, in Berlin, and it was agreed that this density would be used during the AIC testing performed here. For reasons of availability, two of the laboratories used cages with a volume of 0.040 m^3, and the other laboratory used cages with a volume of 0.064 m^3. Although mosquito density was kept constant, cage volume differed among laboratories, which meant that the absolute numbers of mosquitoes also differed. Thus, 60 mosquitoes

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were used in the 0.040-m^3 cages, whereas 90 mosquitoes were used in the 0.064-m^3 cages.

Landing Rate

It was agreed beforehand that, for a test to be validated, the minimum landing rate on the untreated arm would need to be 10 landings/30 s or 20 landings/min (WHO 2009, ECHA 2018a). During each exposure period, the number of landings on the untreated arm was measured for a total of 1 min. The results were then extrapolated from 1 to 3 min to calculate percentage protection and landing rate. Setting the measurement period to 1 min for the untreated arm reduced the probability that study participants would be bitten. For the arm treated with repellent, the exposure period lasted 3 min. The mosquitoes in the cage were replaced with new mosquitoes if the target landing rate was not achieved (WHO 2009, ECHA 2018a).

Exposure Area

One of the variables evaluated was test design. During the test, the participants' hands were protected by gloves, but their forearms were exposed. Two different surface areas of exposure were used: 600 and 100 cm^2 (Fig. 1):

Sleeveless AIC Test

The complete forearm, representing a surface area of approximately 600 cm^2, was exposed. It was treated with the doses described above. This is the standard approach used in the conventional AIC test described in WHO (2009) and EPA (2010) guidelines, with the exceptions cited above.

Sleeved AIC Test

During the EFF WG meeting (3–4 December 2018), it was agreed that the area of exposure should be reduced from 600 to 100 cm^2 (5 cm × 20 cm) to avoid exposing the complete forearm and to limit the occurrence of bites (Obermayr et al 2010). Only the underside of the lower arm (covered by fewer hairs) was exposed. Identical sleeves were used at all the testing laboratories. They were made of 2-mm-thick unscented white elastic polyester that was washable; it was fit snugly around the arm using Velcro. This material could not be penetrated by mosquitoes. The sleeve was not removed between exposure periods. The study participants each had their own set of sleeves, one for the untreated arm and the other for the treated arm. During the test, if a mosquito was perched on the edge of the sleeve rather than on the participant's skin, the landing/probing instance was not counted. During repellent application, the product was applied to an area slightly larger than the area to be exposed (i.e., there was overlap between the area treated with repellent and the area covered by the sleeve). The dose was calculated accordingly.

Study phase 2: Experimental Conditions Exclusive to the Room Test

Testing Cabins

For reasons of availability, two of the testing laboratories used a 30-m^3 room and the third used a 25-m^3 room. The rooms' walls were white and made of a washable material (Fig. 2).

The rooms were not ventilated so as to minimize variability among laboratories.

Every day before a testing trial was started, the rooms were checked for insecticide contamination. At least 10 sugar-fed females (5–10 d old) were released into the room and left there for 30 min. They were given 10% sugar solution on cotton wool. The room was declared contaminated or in unsatisfactory condition if the knockdown effect on the mosquitoes was higher than 10% during this period. If no contamination was detected, the first set of mosquitoes

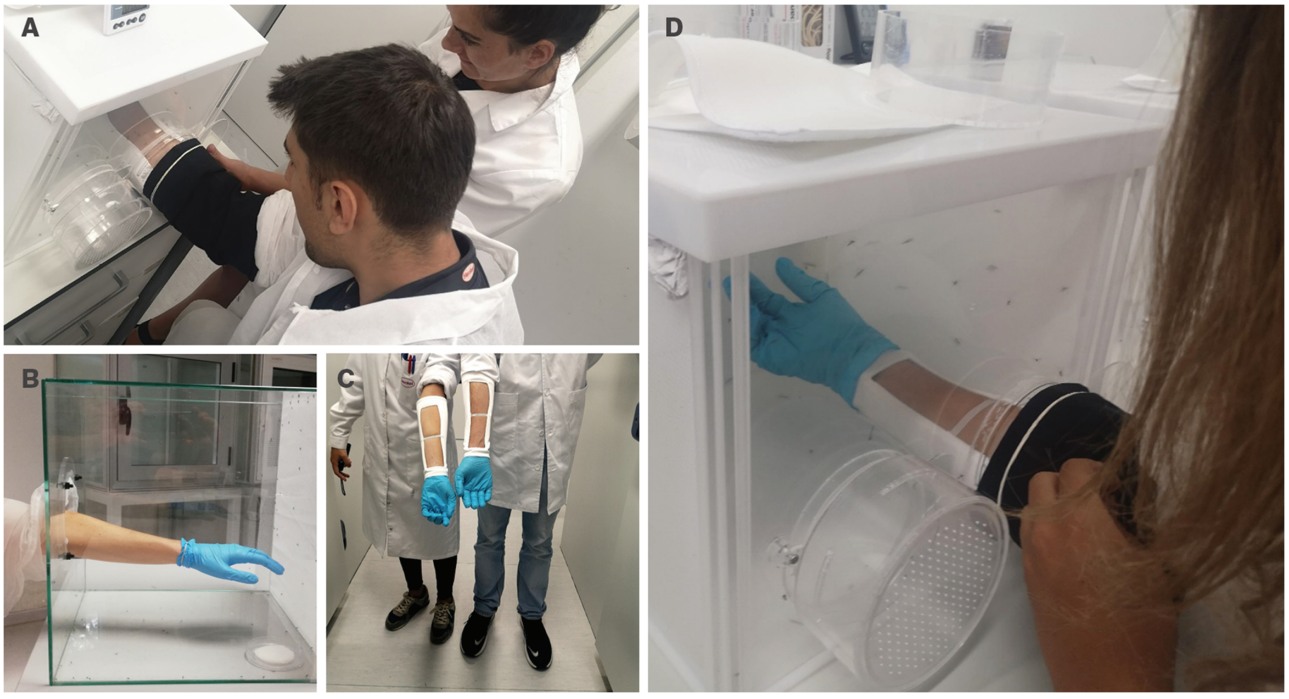

Fig. 1. Arm-in-cage (AIC) testing. (a) Study participant with his arm in the cage and the study director who is counting/confirming landing number, (b) cage (0.064 m^3) in which a study participant's full forearm (600 cm^2) is exposed, (c) study participants displaying their sleeved forearms, and (d) cage (0.040 m^3) in which a study participant's sleeved forearm is exposed (100 cm^2).

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was removed, and a second set of mosquitoes was released into the room for use during testing.

Density

The mosquito density used in the RT was intended to replicate a range of landing rates (20–30 landings/min) that encompassed the maximum landing rate observed during field testing (26.8 landings/min; Moreno-Gómez et al. 2020). This target range also fits with the WHO (2009) criterion that must be met for AIC results to be validated: the minimum landing rate on the untreated area must be 10 landings/30 s or 20 landings/min. The number of mosquitoes necessary to attain this rate was then determined over the course of preliminary trials, and the testing laboratories took into account the aggressiveness of their mosquito strains. This preliminary research revealed that the target landing rate could be attained by using 40–50 mosquitoes per room, with the precise number depending on the laboratory and strain aggressiveness.

Fig. 2. One of the 30-m^3 rooms used in the RT at the Henkel R&D International Laboratory.

Landing Rate

The standard reference method for measuring human exposure to mosquito bites in the field is the human landing catch (HLC) method, where mosquitoes are captured when they land and attempt to feed on human subjects (WHO 1975). In this study, however, the HLC method was not used because it was important that procedures remain consistent between the AIC tests and the RT. Consequently, the number of landings that occurred during the 3-min exposure period was recorded without capturing the mosquitoes. Although *Ae. albopictus* prefers to bite the ankles (Shirai et al. 2002), the participants' forearms were exposed in the RT. Again, the aim was to remain consistent with AIC testing.

Exposure Period

The mosquitoes were released into the room and allowed to acclimatize for 30 min. Then, a study participant wearing full body protection (i.e., light beekeeper suit, gloves, and hospital booties; Fig. 3) entered the room and walked around inside for 2 min; during this time, the forearms were completely covered. Next, the person stopped walking and exposed her or his untreated arm for 3 min, during which time the number of landings was recorded. During the exposure period, the study participants shook off the mosquitoes before they started biting. The person then covered her or his untreated arm and exposed the treated arm for 3 min.

Statistical Analysis

Treatment medians and their associated confidence intervals were calculated using the Kaplan–Meier survival analysis method described in WHO (2009), using MedCalc (v. 19.6.4; MedCalc Software, Ostend, Belgium). All statistical comparisons were performed using nonparametric (Kruskal–Wallis) tests. For comparisons involving more than two treatments (e.g., comparison of three labs), the Conover–Iman multiple comparison procedure with the Bonferroni correction for multiplicity was applied to determine which treatments were significantly different from each other. All the statistical comparisons employed an α level of 0.05.

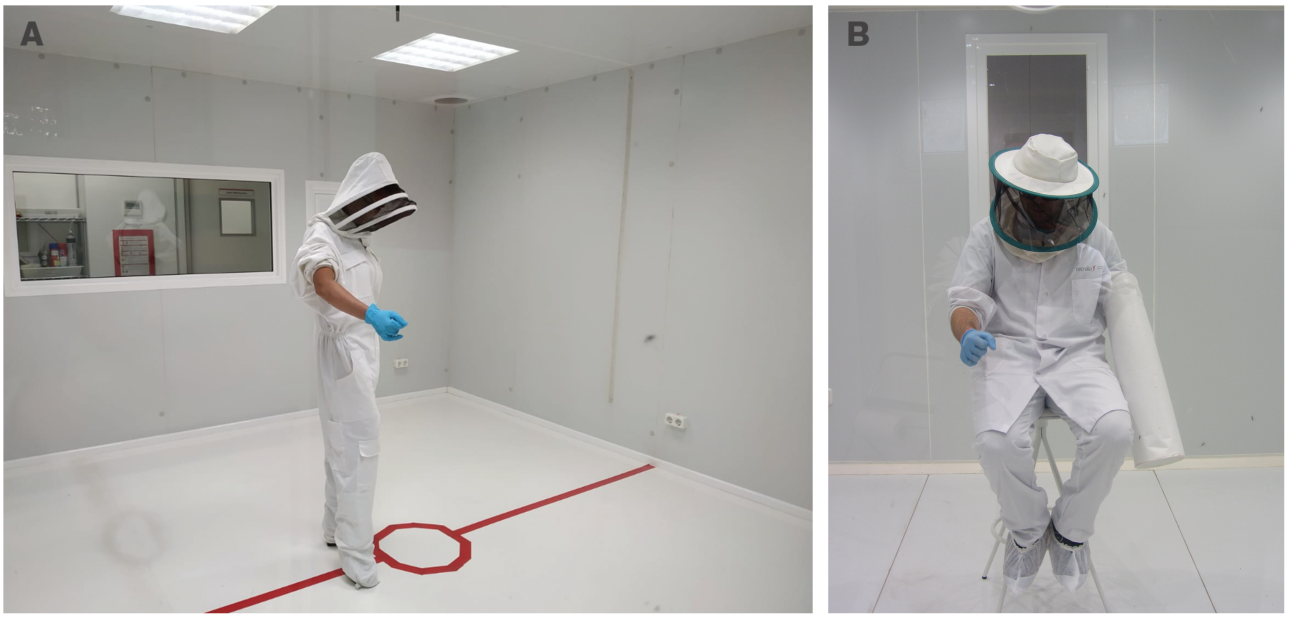

Fig. 3. The room test. (a) Study participant dressed in the protective suit exposing a forearm and (b) study participant whose treated forearm is covered with a plastic tube that is not in contact with the skin so as to avoid repellent removal during the test.

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Table 1. Effects of dose and arm-in-cage (AIC) test design (sleeveless and sleeved) on protection time among laboratories (median and 95% confidence intervals)

	Protection time (h)	Dose							
		1.0 g				0.5 g			
		Laboratory			*P*	Laboratory			*P*
		i2L Research	Henkel	Tecnalia		i2L Research	Henkel	Tecnalia	
Sleeveless AIC test	CPT	2.0[b]	5.0[a]	4.0[b]	<0.001	1.0[b]	4.0[a]	0.0[c]	<0.001
	(95% CI)	(1.2–2.8)	(4.0–6.0)	(3.1–4.9)		(0.3–1.7)	(3.4–4.6)	(0.0–0.0)	
	P99	3.0[b]	5.0[a]	5.0[a]	<0.001	1.0[b]	4.0[a]	1.0[b]	<0.001
	(95% CI)	(2.6–3.4)	(4.0–6.0)	(4.4–5.6)		(0.0–2.0)	(3.0–5.0)	(0.6–1.4)	
Sleeved AIC test	CPT	NA	6.0[a]	6.0[a]	0.481	NA	3.0[a]	2.0[b]	0.015
	(95% CI)		(4.5–7.5)	(5.5–6.5)			(1.6–4.4)	(1.3–2.7)	
	P99		6.0[a]	6.0[a]	0.481		3.0[a]	2.0[a]	0.279
	(95% CI)		(4.5–7.5)	(5.6–6.4)			(1.6–4.4)	(1.0–3.0)	

The two doses were 1.0 and 0.5 g, and the three laboratories were i2L Research, Henkel, and Tecnalia. The tests were performed using a formulation containing 15% DEET. Mosquito density was one female/840 cm^2. An α level of 0.05 was used to determine statistical significance. Within a given row and dose, any two medians that do not share letters are significantly different. Not applicable (NA): the results of the sleeved AIC test performed at i2L Research were excluded from the analysis because the target minimum landing rate was not attained. CPT, complete protection time.

Results

AIC Testing

The following four key questions were addressed:

(1) Were the results of AIC testing reproducible among laboratories?

The effects of laboratory, dose, and sleeve use on protection time were evaluated. Although there were significant differences in protection time among laboratories in the combined analysis, pairwise analysis revealed certain similarities. Henkel obtained significantly longer protection times (CPT, PT99) than did i2L Research and Tecnalia at both doses when the sleeve was not used. However, when the sleeve was used, Henkel and Tecnalia did not differ in their protection times at either dose (except for CPT at 0.5 g/600 cm^2; Table 1).

(2) How was landing rate affected by mosquito number and sleeve use?

Although mosquito density was constant (as described in Materials and methods), mosquito number differed depending on cage volume, and the presence of more mosquitoes led to a higher landing rate in the sleeveless AIC test. This relationship should be noted and explored further. However, sleeve use appeared to address this concern, eliminating the significant effect of mosquito number on landing rate (Table 2). The use of the sleeve had two additional key effects: 1) it significatively reduced the landing rate, by 40% when 60 mosquitoes were present and by 48% when 90 mosquitoes were present and 2) it led to landing rates that more greatly resembled those in the field (26.8 landings/min; Moreno-Gómez et al. 2020; Table 2).

(3) How did sleeve use influence protection time?

The effects of sleeve use on protection time were examined for each laboratory. For the results of the comparisons among laboratories, see question 1 and Table 1. At Henkel, sleeve use did not significantly affect protection time. In contrast, at Tecnalia, sleeve use resulted in significantly longer CPTs for both doses and significantly higher P99 values for the 0.5-g dose (Table 3).

Sleeve use had additional benefits. By limiting the area of skin exposed, it increased the accuracy of data collection and reduced the study participants' stress during testing (Supp Mater [online only]: video footage of the sleeved and sleeveless AIC tests).

(4) How did dose affect protection time?

Dose consistently affected protection time. The 1-g dose resulted in significantly longer protection times than did the 0.5-g dose at all the laboratories regardless of sleeve use (Table 4).

Room Test

The following three key questions were addressed:

(1) Were the results of the room test reproducible among laboratories?

The effects of laboratory and dose on protection time were evaluated. Variation among testing laboratories was partially controlled by using the landing rate as an evaluation parameter. At all three laboratories, the mean landing rate remained above 20 landings/min throughout the 8-h test period (BioGenius: 24.41 ± 2.58, Henkel: 24.67 ± 3.18, and Tecnalia: 23.07 ± 3.37).

Protection times were significantly longer at Henkel than at Tecnalia. They did not differ between Henkel and BioGenius, nor did they differ between BioGenius and Tecnalia (Table 5).

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Table 2. Effect of mosquito number and sleeve use on landing rate (mean ± SD)

	Mosquito number/cage volume/landing rate		*P*
	60 mosquitoes (0.040-m³ cages)	90 mosquitoes (0.064-m³ cages)	
Sleeved AIC test	26.41 ± 6.59	35.42 ± 6.52	0.1335
Sleeveless AIC test	44.23 ± 20.72	73.17 ± 23.57	<0.001

The tests were performed using a formulation containing 15% DEET. Mosquito density was one female/840 cm³. An α level of 0.05 was used to determine statistical significance.

Table 3. Effects of arm-in-cage (AIC) test design (sleeveless and sleeved) and dose on protection time (median and 95% confidence intervals)

	Protection time (h)	Laboratory								
		i2L Research			Henkel			Tecnalia		
		Sleeveless AIC test	Sleeved AIC test	*P*	Sleeveless AIC test	Sleeved AIC test	*P*	Sleeveless AIC test	Sleeved AIC test	*P*
Dose: 0.5 g	CPT (95% CI)	1.0 (0.3–1.7)	NA	NA	4.0[a] (3.4–4.6)	3.0[a] (1.6–4.4)	0.510	0.0[b] (0.0–0.0)	2.0[a] (1.3–2.7)	<0.001
	P99 (95% CI)	1.0 (1.0–2.0)			4.0[a] (3.0–5.0)	3.0[a] (1.6–4.4)	0.756	1.0[b] (0.6–1.4)	2.0[a] (1.0–3.0)	0.009
Dose: 1.0 g	CPT (95% CI)	2.0 (1.2–2.8)	NA	NA	5.0[a] (4.0–6.0)	6.0[a] (4.5–7.5)	0.905	4.0[b] (3.1–4.9)	6.0[a] (5.5–6.5)	0.035
	P99 (95% CI)	3.0 (2.6–3.4)			5.0[a] (4.0–6.0)	6.0[a] (4.5–7.5)	0.844	5.0[a] (4.4–5.6)	6.0 [a] (5.6–6.4)	0.229

The two doses were 1.0 g and 0.5 g, and the three laboratories were i2LResearch, Henkel, and Tecnalia. The tests were performed using a formulation containing 15% DEET. Mosquito density was one female per 840 cm³. An α level of 0.05 was used to determine statistical significance. Within a given row and dose, any two medians that do not share letters are significantly different. Not applicable (NA): the results of the sleeved AIC test performed at i2LResearch were excluded from the analysis because the target minimum landing rate was not attained. CPT, complete protection time.

(2) How did dose affect protection time?

In the RT, the lower dose consistently resulted in lower protection times at all three laboratories (Table 6).

(3) How did landing rate and protection time compare for room-based testing versus AIC testing?

Landing Rate: Landing rates in the sleeveless AIC test were higher than landing rates in the sleeved AIC test and in the RT. Because landing rate was fixed during the RT (i.e., maintained at 20–30 landings/min), there was less variability among study participants and laboratories in the RT than in the AIC tests, where mosquito density was the fixed variable. In addition, the results of the sleeved AIC test showed reduced variability among study participants when compared with the results of the conventional AIC (Fig. 4).

Protection Time: At Tecnalia, there was no difference in CPT between the RT and the sleeved AIC test at either dose. At Henkel, the results were dose dependent. There was an effect of test method on CPT for the 1.0-g dose, but not for the 0.5-g dose. Finally, BioGenius did not perform AIC testing, so only its RT results can be compared with those of the other laboratories. The RT results obtained at BioGenius were not significantly different from the RT results obtained at Henkel (Tables 5 and 7).

Discussion

The two overarching objectives of this study were as follows: 1) to ascertain whether the results from the alternative laboratory methods (sleeved AIC and room test) indicated that reproducible findings could be obtained across different laboratories and 2) to determine whether the results could better simulate field landing rates and thus more accurately estimate CPT. Results for the two methods—the sleeved AIC test and the RT—suggest that, if progressively standardized among laboratories, these approaches could, serve as alternatives to the conventional AIC test described in WHO (2009) and EPA (2010) guidelines for evaluating topical repellents under laboratory conditions. Furthermore, the study's findings suggest that CPTs from sleeveless AIC tests may not be reproducible among the three laboratories, probably because of the high landing rate associated. Both alternative methods were able to recreate a landing rate similar to the one observed in recent field research in an area of Europe highly infested with *Ae. albopictus* (26.8 landings/min; Moreno-Gómez et al. 2020). Moreover, this landing rate fits with the WHO (2009) criterion for validating AIC results (i.e., the landing rate on the untreated area must equal 10 landings/30 s or 20 landings/min. Consequently, these two methods could potentially be used to simulate the conditions that a European consumer might encounter outdoors, while also limiting the health risks to study participants that are involved in field testing given that the infection status of wild mosquitoes cannot be guaranteed. Furthermore, the sleeved AIC test and the RT yielded seemingly reproducible estimates of CPT across the testing laboratories and significatively lower levels of variability among study participants.

In the first phase of the study, a conventional AIC test was employed. Mosquito density was fixed at 1 female/840 cm³, as decided the European EFF WG, but because testing cages varied somewhat in volume across laboratories, the number of mosquitoes needed to

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Table 4. Effects of dose and arm-in-cage (AIC) test design (sleeveless and sleeved) on protection time (median and 95% confidence intervals)

	Protection time (h)	Laboratory								
		i2LResearch			Henkel			Tecnalia		
		Dose		*P*	Dose		*P*	Dose		*P*
		0.5 g	1.0 g		0.5 g	1.0 g		0.5 g	1.0 g	
Sleeveless AIC test	CPT	1.0[b]	2.0[a]	0.002	4.0[b]	5.0[a]	0.013	0.0[b]	4.0[a]	<0.001
	(95% CI)	(0.3–1.7)	(1.2–2.8)		(3.4–4.6)	(4.0–6.0)		(0.0–0.0)	(3.1–4.9)	
	P99	1.0[b]	3.0[a]	<0.001	4.0[b]	5.0[a]	0.016	1.0[b]	5.0[a]	<0.001
	(95% CI)	(0.0–2.0)	(2.6–3.4)		(3.0–5.0)	(4.0–6.0)		(0.6–1.4)	(4.4–5.6)	
Sleeved AIC test	CPT	NA	NA	NA	3.0[b]	6.0[a]	0.018	2.0[b]	6.0[a]	0.001
	(95% CI)				(1.6–4.4)	(4.5–7.5)		(1.3–2.7)	(5.5–6.5)	
	P99				3.0[b]	6.0[a]	0.018	2.0[b]	6.0[a]	0.003
	(95% CI)				(1.6–4.4)	(4.5–7.5)		(1.0–3.0)	(5.6–6.4)	

The two doses were 1.0 g and 0.5 g, and the three laboratories were i2LResearch, Henkel, and Tecnalia. The tests were performed using a formulation containing 15% DEET. Mosquito density was one female per 840 cm^3. An α level of 0.05 was used to determine statistical significance. Within a given row and dose, any two medians that do not share letters are significantly different. Not applicable (NA): the results of the sleeved AIC test performed at i2LResearch were excluded from the analysis because the target minimum landing rate was not attained. See Table 1 for the results of the comparisons among laboratories. CPT, complete protection time.

achieve that density was not always the same (i.e., 60 mosquitoes in 0.040 m^3 cages and 90 mosquitoes in 0.064 cm^3 cages). As a result, it was found that in general the absolute number of mosquitoes appeared to matter more than density in shaping outcomes (i.e., landing rate and protection time). Indeed, as mosquito number increased, landing rate increased, and CPT decreased. This relationship was extremely pronounced at Tecnalia, which used the higher number of mosquitoes (i.e., 90). Its landing rates in the sleeveless AIC test were twice as high (71.45 ± 29.14 landings/min) as those in the sleeved AIC test (34.83 ± 9.46 landings/min). These results appear to explain why CPT was much shorter for the sleeveless AIC test than for the sleeved AIC test also why CPTs differed between Tecnalia and Henkel, the latter having that employed a smaller number of mosquitoes (i.e., 60). The CPTs for the sleeveless AIC test performed at i2LResearch were an anomaly insofar as there was no correlation between mosquito numbers, landing rate, and CPT. Although no explanation for this apparent incongruity has been discovered, it is thought that mosquito strain sensitivity might be involved.

Other studies using the WHO AIC test in the laboratory and the HLC method in the field have observed the same relationship in a variety of mosquito species (Colucci and Müller 2018): *Ae. aegypti* (Linnaeus 1762) (Diptera: Culicidae), *Anopheles stephensi* (Liston 1901), and *Culex quinquefasciatus* (Say 1823) (Diptera: Culicidae) in the laboratory and *Ae. cinereus/geminus*, *Ae. Vexans* (Meigen 1830) (Diptera: Culicidae), and *An. plumbeus* (Stephens 1828) (Diptera: Culicidae) in the field; (Obermayr et al. 2010): *Ae. aegypti* in laboratory and *Ae. vexans* (Meigen 1830) and *Ochlerotatus sticticus* (Meigen 1838) (Diptera: Culicidae) in the field; Barnard et al. (1998): *Ae. aegypti* and *An. quadriannulatus* in the laboratory). However, it is worth noting that landing rates may be affected by multiple factors, including mosquito strain, the presence of alternate blood meals, and environmental conditions (Petrić et al. 2014, Brugman et al. 2017). In this study, the latter two factors were taken into consideration, and the research was carried out under controlled laboratory conditions using a single mosquito species (albeit different strains).

Because the landing rate was extremely high in the conventional AIC test, resulting in low CPT values, an alternative AIC methodology was explored. In the sleeved AIC test, participants only partially expose their forearms (i.e., a surface area of 100 cm^2), which differ from the approach described in WHO (2009) and EPA (2010) guidelines, where participants expose their entire forearms (i.e., a surface area of 600 cm^2). The underlying objective of this alternative AIC test was to simulate the landing rates observed during field testing. It was found that sleeve use had several clear benefits. First, it reduced the landing rate by 40–50% overall and subsequently increased CPT at both repellent doses. Second, it increased the reproducibility of the results among laboratories compared with the conventional AIC. Third, it made it possible to recreate the maximum landing rate found in the field that was mentioned above (26.8 landings/min; Moreno-Gómez et al. 2020). Fourth, by limiting skin exposure, it reduced the stress experienced by study participants and facilitated mosquito counts.

Because sleeve use led to such a marked increase in CPT, the decision was made to try an alternative testing approach that employed a larger space (i.e., rooms of 25–30 m^3 in volume), so as to better simulate natural mosquito behavior and conditions of exposure in the outdoors. This new method centered on mosquito landing rate rather than on mosquito density, allowing mosquito numbers to be modulated to attain the target landing rate and thus accounting for possible differences in strain aggressiveness. The objective was to

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Table 5. Effects of dose on protection time among laboratories during the room test (median and 95% confidence intervals)

Protection time ()	Dose							
	1.0 g				0.5 g			
	Laboratory			*P*	Laboratory			*P*
	BioGenius	Henkel	Tecnalia		BioGenius	Henkel	Tecnalia	
CPT	8.0[a]	8.0[a]	6.0[b]	0.001	3.0[a]	4.0[a]	2.0[b]	0.015
(95% CI)	(6.6–7.4)	(7.8–8.0)	(5.0–7.0)		(2.2–3.8)	(3.0–5.0)	(1.6–2.4)	
P99	7.0[ab]	8.0[a]	6.0[b]	0.025	3.0[ab]	4.0[a]	2.0[b]	0.036
(95% CI)	(6.2–7.8)	(6.6–8.0)	(5.0–7.0)		(2.0–4.0)	(3.0–5.0)	(1.6–2.4)	

The two doses were 1.0 g and 0.5 g, and the three laboratories were BioGenius, Henkel, and Tecnalia. The tests were performed using a formulation containing 15% DEET. The minimum mosquito landing rate was 20 landings/min, which was achieved using 45 ± 5 mosquitoes per room. An α level of 0.05 was used to determine statistical significance. Within a given row and dose, any two medians that do not share letters are significantly different. CPT, complete protection time.

Table 6. Effects of dose on protection for each laboratory during the room test (median and 95% confidence intervals)

Protection time (h)	Laboratory								
	BioGenius			Henkel			Tecnalia		
	Dose		*P*	Dose		*P*	Dose		*P*
	0.5 g	1.0 g		0.5 g	1.0 g		0.5 g	1.0 g	
CPT	3.0[b]	7.0[a]	<0.001	4.0[b]	8.0[a]	<0.001	2.0[b]	6.0[a]	<0.001
(95% CI)	(2.2–3.8)	(6.2–7.8)		(3.0–5.0)	(7.8–8.0)		(1.6–2.4)	(5.0–7.0)	
P99	3.0[b]	8.0[a]	<0.001	4.0[b]	8.0[a]	<0.001	2.0[b]	6.0[a]	<0.001
(95% CI)	(2.0–4.0)	(7.2–8.0)		(3.0–5.0)	(6.6–8.0)		(1.6–2.4)	(5.0–7.0)	

The two doses were 1.0 g and 0.5 g, and the three laboratories were BioGenius, Henkel, and Tecnalia. The tests were performed using a formulation containing 15% DEET. The minimum mosquito landing rate was 20 landings/min, which was achieved using 45 ± 5 mosquitoes per room. See Table 5 for the results of the comparisons among laboratories. CPT, complete protection time.

compare the protection time obtained in the RT with the two AIC tests (sleeveless or sleeved). In the RT, study participants had their entire bodies within the room, but only their forearms were exposed, like in the AIC tests. Overall, 45 (±5) mosquitoes were needed to obtain the minimum target landing rate of 20 landings/min. On average, all the laboratories maintained the mean landing rate at or above this value for the full 8 h of the test.

As noted above, one of the main objectives of the study was to assess whether test results could potentially be reproducible across laboratories. Although the CPTs estimated in the RT were not entirely similar among the laboratories (see Table 7), the estimates of 99% protection were statistically equivalent for both doses at Henkel, BioGenius, and Tecnalia. This finding suggests that the results were reproducible.

When the results of the RT and the AIC tests were compared, it was found that the RT's CPT estimates were statistically equivalent to those of the sleeved AIC test. In contrast, they differed significantly from those of the sleeveless AIC test that yielded shorter CPTs.

Two practical conclusions arise from these results. First, testing should focus on establishing a constant mosquito landing rate, as was done in the RT, rather than on establishing a constant mosquito density, as is stipulated in WHO (2009) and EPA (2010) guidelines for AIC testing. Second, the sleeve could serve as a useful tool for attaining the target landing rate during AIC testing. In particular, sleeve use made it possible to obtain landing rates that simulated those observed during field testing.

Focusing on mosquito landing rate rather than on mosquito density makes sense if the goal is to furnish label information that is accurate for product use outdoors. As opposed to mosquito density, mosquito landing rate is easily measured in the field, facilitating comparisons with laboratory studies. Earlier, it was mentioned that, field research observed a landing rate of 20–30 landings/min in an outdoor area in Europe that was highly infested with *Ae. albopictus*. However, such research can and should be expanded beyond *Ae. albopictus* to other mosquito species found in Europe. Indeed, landing rate measured in the field for other mosquitoes (using the HLC method) are lower than the minimum target landing rate measured in this study (i.e., 20 landings/min). For example, a study in Switzerland found that, for *Aedes cinereus* (Meigen 1818) (Diptera: Culicidae), *Aedes geminus* (Peus 1970) (Diptera: Culicidae), *Aedes vexans* (Meigen 1830) (Diptera: Culicidae), and *Anopheles plumbeus* (Stephens 1828) (Diptera: Culicidae), landing rates in the field were around 0.276 landings/min (range: 0.0–0.432) in the Langholz Forest and 0.0342 landings/min (range: 0.0–0.336) in the Thurauen Nature Reserve (Colucci and Müller 2018). Similarly, a study conducted in 2017 in southern England found that *Coquillettidia richiardii* (Ficalbi 1889) (Diptera: Culicidae), *Anopheles maculipennis* (Shute 1936) (Diptera: Culicidae), and *Culex modestus* (Ficalbi 1889) (Diptera: Culicidae) had landing rates of 0.0084–0.168 landings/min (Brugman et al. 2017). Outside of Europe, in California, biting rates in the field were 1.5 bites/min on the arm and 3 bites/min on the leg for *Ochlerotatus melanimom* (Dyar 1928) (Diptera: Culicidae), *Ae. vexans*, and *Ochlerotatus*

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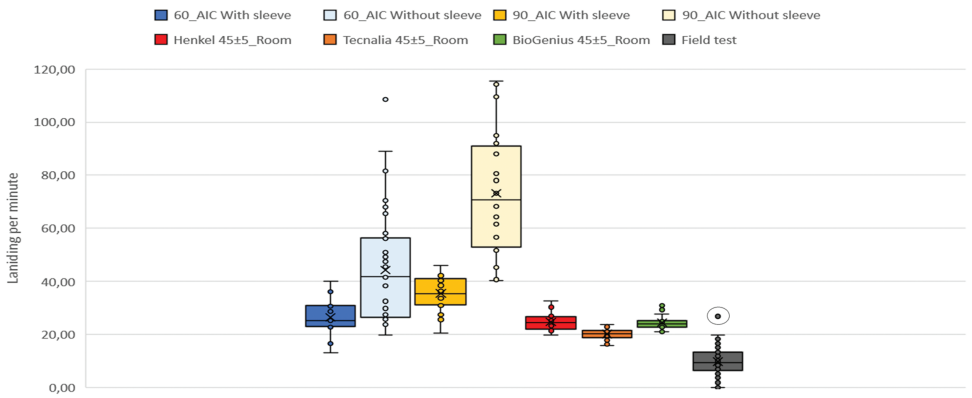

Fig. 4. Effect of test method on landing rate. The number of mosquitoes used in the sleeveless arm-in-cage (AIC) test, the sleeved AIC test, and the RT was 60, 90, and 45 ± 5, respectively. The results from a field (Moreno-Gómez et al. 2020) test are also provided, where the maximum landing rate obtained (26.8 landings/min) is circled.

Table 7. Effect of test method and dose on protection time for each laboratory (median and 95% confidence intervals)

Laboratory	Dose	Protection time (hours)	Test method			*P*
			Sleeved AIC test	Sleeveless AIC test	Room test	
Tecnalia	0.5 g	CPT	2.0[a]	0.0[b]	2.0[a]	<0.001
		(95% CI)	(1.3–2.7)	(0.0–0.0)	(1.6–2.4)	
		P99	2.0[a]	1.0[b]	2.0[a]	0.004
		(95% CI)	(1.0–3.0)	(0.6–1.4)	(1.6–2.4)	
	1.0 g	CPT	6.0[a]	4.0[b]	6.0[a]	0.012
		(95% CI)	(5.5–6.5)	(3.1–4.9)	(5.0–7.0)	
		P99	6.0	5.0	6.0	0.108
		(95% CI)	(5.6–6.4)	(5.6–6.4)	(5.0–7.0)	
Henkel	0.5 g	CPT	3.0	4.0	4.0	0.770
		(95% CI)	(1.6–4.4)	(3.4–4.6)	(3.0–5.0)	
		P99	3.0	4.0	4.0	0.929
		(95% CI)	(1.6–4.4)	(3.0–5.0)	(3.0–5.0)	
	1.0 g	CPT	6.0[b]	5.0[b]	8.0[a]	0.023
		(95% CI)	(4.5–7.5)	(4.0–6.0)	(7.8–8.0)	
		P99	6.0	5.0	8.0	0.196
		(95% CI)	(4.5–7.5)	(4.0–6.0)	(6.6–8.0)	
BioGenius	0.5 g	CPT	ND	ND	3.0	ND
		(95% CI)			(2.2–3.8)	
		P99	ND	ND	3.0	ND
		(95% CI)			(2.0–4.0)	
	1.0 g	CPT	ND	ND	7.0	ND
		(95% CI)			(6.2–7.8)	
		P99	ND	ND	8.0	ND
		(95% CI)			(7.6–8.0)	

The test methods were the sleeved arm-in-cage test, the sleeveless AIC test, and the room test. The two doses were 1.0 and 0.5 g, and the three laboratories were BioGenius, Henkel, and Tecnalia. Test were performed using a formulation containing 15% DEET. The AIC tests (sleeved and sleeveless) were structured around mosquito landing rate: a minimum of 20 landings/min achieved using 45 ± 5 mosquitoes. An α level of 0.05 was used to determine statistical significance. Within a given row and dose, any two medians that do not share letters are significantly different. ND, no data; this test configuration not performed by this laboratory. See Table 5 for the results of the comparisons among laboratories. CI, confidence interval; CPT, complete protection time.

increpitus (Dyar 1916) (Diptera: Culicidae) (Carroll and Loye 2006). Consequently, it should be possible to extend this approach to less aggressive mosquito species [e.g., *Culex pipiens* (L. 1758)] when testing repellents subject to European guidelines. The key will be to adjust mosquito number to achieve a minimum landing rate of 20 landings/min, as agreed upon at the EFF WG meetings, or to lower the rate even further for with other species based on species-specific of worst case scenarios in the field.

To ensure that repellents are tested under conditions that better reflect consumer use, it is also essential to examine dosage. In both phases of this study, two doses were tested. The first, 1 g/600 cm^2, is the dose recommended by WHO (2009) guidelines. However, efficacy testing is undergoing a mandated shift to align with HHRA guidelines for biocidal products (ECHA 2019c), which apply stricter dosage standards. As a result, the second dose was lower—0.5 g/600 cm^2. In general, the lower dose resulted in significantly lower CPTs. For example, CPT was as low as 0 h during the sleeveless AIC test in one laboratory, likely because of the higher landing rates associated with this design type. Indeed, the sleeveless AIC test appeared to strongly underestimate potential CPT at the low dose,

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given that longer CPTs were obtained with different test configurations, notably those in which landing rates were more aligned with the higher landing rate obtained in field (i.e., the sleeved AIC test and the RT). Given the above objective, it is also important to compare these sleeveless AIC results obtained with the results of field studies during which *Aedes* and *Anopheles* species were present. Previous field research has evaluated the CPTs associated with the use of topical repellents containing the same percentage of the same active substance (15% DEET) at one of the same doses (1 g/600 cm^2) as in this study (Moore et al. 2007, Colucci and Müller 2018). It was found that CPTs were definitely longer than 0 h, which could at least partially be explained by the lower mosquito landing rate under field conditions (Moore et al. 2007, Colucci and Müller 2018). Taken in tandem, these findings highlight the importance of carefully choosing laboratory methodologies because this choice can influence estimates of protection time, which must be accurately measured to prevent consumers from overapplying topical repellents.

This study confirmed that the two alternative methods described here—the sleeved AIC test and the RT—successfully simulated mosquito landing rates observed in the field in Europe. Its results also provide indications that these methods could assess how repellents will perform under outdoor conditions of use without exposing study participants to the health risks associated with field testing. In contrast, the traditional approach, the sleeveless AIC test, resulted in higher mosquito landing rates, which strongly suggests that it may underestimate actual consumer protection times. Furthermore, there was seemingly greater reproducibility among laboratories and less variability among study participants for the sleeved AIC test and the RT than for the sleeveless AIC test. It is essential that efficacy tests produce consistent results because they will be performed at a broad range of testing laboratories. Given these findings, these two new tests could represent reliable alternatives to the conventional AIC test when it comes to evaluating topical repellents based on European regulatory standard.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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Data Availability

The data sets generated during and/or analyzed during the study are available from the corresponding author upon reasonable request.

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