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METHODS OF TESTING AND ANALYZING EXCITO-REPELLENCY RESPONSES OF MALARIA VECTORS TO INSECTICIDES[1]

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ABSTRACT. A new test system that includes an excito-repellency test box, test procedures, and statistical treatment of data is described. The method consists of enclosing 25 mosquitoes in an exposure chamber lined with insecticide-treated or untreated (control) test papers. Each chamber has a single portal for mosquitoes to escape to a receiving cage, and numbers escaping are manually recorded at 1-min intervals. The exposure chamber accommodates a screened, 2nd chamber that, when placed in the exposure chamber, prevents the mosquitoes from making physical contact with test papers. A full assay utilized one exposure chamber that permits physical contact with insecticide-treated papers, one chamber that permits physical contact with control papers, one chamber that prevents physical contact with insecticide-treated papers, and a 4th chamber that prevents contact with control papers. After insecticide exposure, test populations are held for observations on 24-h mortalities. A survival analysis approach is described for estimating mosquito escape rates and for comparing differences in mosquito escape rates, with or without physical contact with insecticide, among populations, insecticides, and doses of insecticide.

INTRODUCTION

Assays for evaluating behavioral responses of malaria vectors to insecticide residues have been reviewed by Muirhead-Thomson (1960), Coluzzi (1963), Busvine (1964), and Elliott (1972). The test of greatest value for studies of insecticide avoidance was described by Coluzzi (1963) as a box with slits for escaping. Such a box was described by Rachou et al. (1973) and is referred to as the excito-repellency test box. Similar excito-repellency test boxes are described by Rachou et al. (1973), Charlwood and Paraluppi (1978), Roberts et al. (1984), Rozendaal et al. (1989), and Evans (1993). In excito-repellency tests, mosquitoes are released inside a box lined with sprayed paper. Outlets in the form of out-projecting baffles permit the mosquitoes to escape into 2 separate cages. The baffles prevent the mosquitoes from reentering the box and the numbers escaping are counted by time postrelease. The difficulties of working with test boxes were described by Roberts et al. (1984). Major problems relate to the difficulties in introducing specimens into the boxes, removing live specimens at the end of test periods, and providing a standardized insecticide dose. The lack of an appropriate method of data analysis has been another shortcoming of the test method. Earlier methods did not test for behavioral responses without physical contact with insecticide-treated papers.

Described herein are improved boxes for testing behavioral responses of adult *Anopheles* mosquitoes with or without physical contact with insecticide residues. Survival analysis methods are described for the statistical treatment of test data.

MATERIALS AND METHODS

The test method consists of enclosing 25 mosquitoes in an exposure chamber lined with insecticide-treated or untreated (control) papers. Each exposure chamber has a single portal for mosquitoes to escape to a receiving cage. The exposure chamber accommodates a screened, 2nd chamber (inner chamber) that, when placed in the first chamber, prevents the mosquitoes from making physical contact with test papers. Under test conditions, mosquitoes are enclosed within the exposure chamber and the only source of light comes from the exit portal. A full assay consists of 4 exposure chambers of 2 treatment chambers and 2 control chambers, as shown in Table 1. Treatment chambers are lined with test papers impregnated with insecticide and an oil-based carrier. Control chambers are lined with papers impregnated with carrier alone. One treatment chamber permits tarsal contact with insecticide. The second treatment chamber includes the inner chamber, so mosquitoes cannot make tarsal contact with insecticide. For brevity, tests with or without the inner chambers, for either treatment or control papers, are referred to as contact trials (no inner chamber) or noncontact trials (with an inner chamber).

Components of the excito-repellency chambers are illustrated and numbered in Fig. 1. Except for a inner panel (No. 1), the exposure chamber is constructed of metal and can be chemically cleaned. The exposure chamber (No. 4) is constructed of stainless steel and each chamber is 34 × 32 × 32

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Table 1. Test conditions for evaluating behavioral responses of malaria vectors to insecticide residues.

Papers lining the exposure chamber	With or without contact with test papers	
	With	Without
With insecticide (treatment chambers)	X	X
Without insecticide (control chambers)	X	X

cm. The front panel is 32 × 32 cm and is equipped with an escape portal (No. 6). The escape portal is an outward projecting funnel (exit funnel), 14.75 cm at its base. The top and bottom of the exit funnel are 14 cm long and converge, leaving a 1.50-cm-wide opening (a horizontal slit) through which the mosquitoes can escape from the exposure chamber. The back of the exposure chamber is a hinged metal door (No. 5) that closes tightly. The exposure chamber is also equipped with an inner removable rear panel (No. 1). This panel fits inside the back of the exposure chamber, abuts 4 small flanges inside the chamber, and serves to imprison

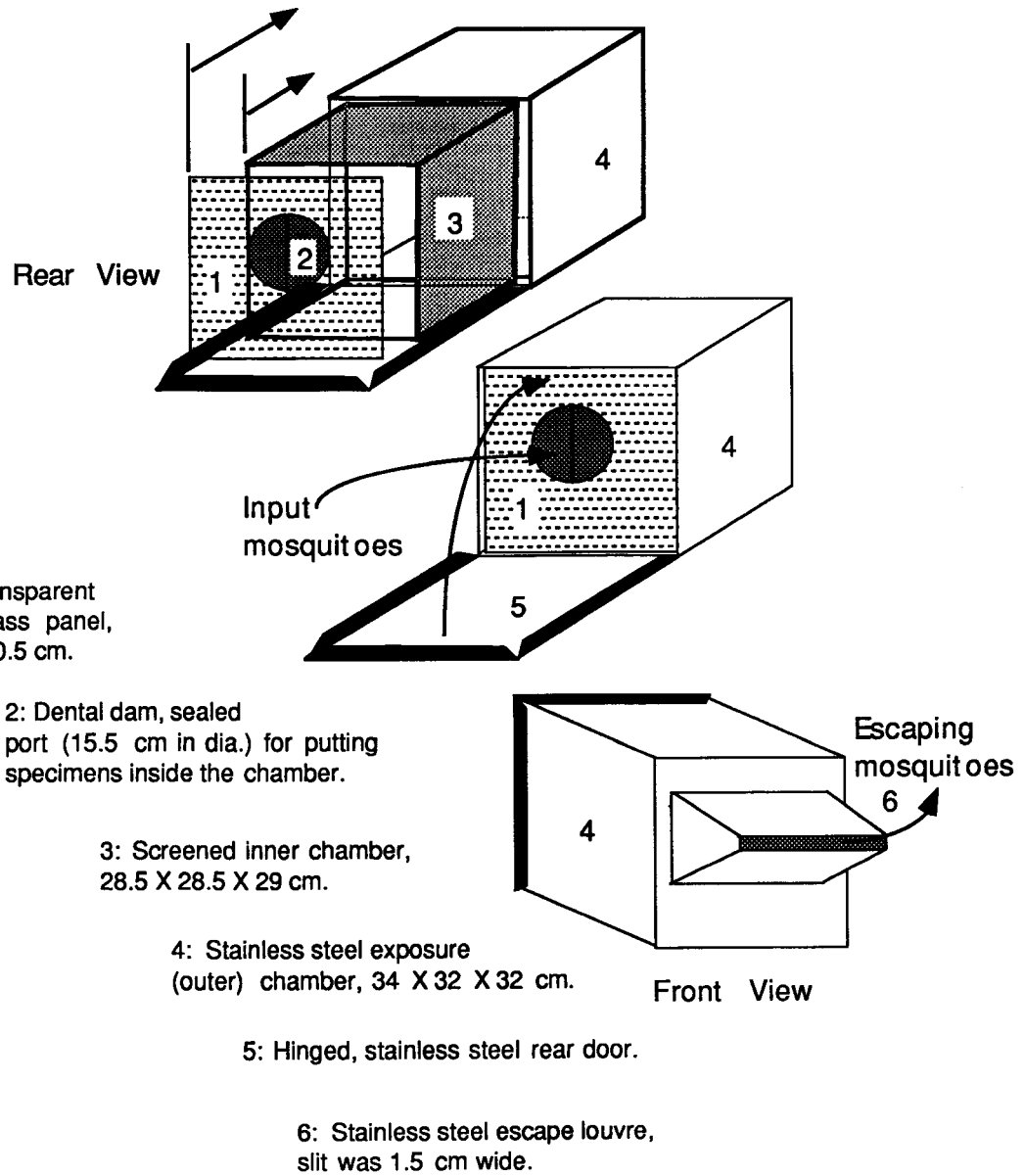

Fig. 1. An excito-repellency test box for the study of behavioral responses of mosquitoes to insecticides.

the test population inside the exposure chamber. Plexiglas™ is used for the inner rear panel so mosquitoes can be observed inside the chamber. The Plexiglas panel is equipped with a large round hole (15.5 cm in diameter) that is sealed with a split piece of dental dam (No. 2). This sealed opening is used for placing mosquitoes inside and for removing mosquitoes from the chamber. The 2 rear panels fulfill several requirements. First, the test population must be in darkness so imprisoned specimens can orient on light filtering through the escape funnel; thus the chamber needs to be solid and nontransparent. This requirement is fulfilled by closing the rear metal door at the start of each test. Second, the investigator needs to see inside the chamber to check for dead versus live specimens, both pre- and posttesting, and to remove live specimens at the end of the test. The ability to see inside the chamber is fulfilled by using transparent Plexiglas for the inner rear panel. Third, a self-sealing portal is needed for placing a test population inside the chamber and for removing specimens from the chamber at the end of each test. This requirement is fulfilled by using a split dental dam seal on the 15.5-cm-diameter opening in the Plexiglas rear panel.

The frame of the inner chamber (No. 3) is constructed of 0.62 × 0.62-cm aluminum beams. The structure of each chamber is 28.5 × 28.5 × 29 cm and the inner surface of each is covered with metal screening. A fine mesh metal screen, 52 cells per inch, covers the top, bottom, and 2 side walls of the inner chamber. The inner chamber is open ended, with 0.62-cm rubber gaskets on the front and back beams. When placed in the outer chamber, the front gasket seals small gaps between the front stainless steel panel and the inner chamber. Likewise, the rear gasket seals gaps between the Plexiglas panel and the inner chamber. The inner screen surface is no closer than 0.62 cm from the surface of test papers, and it prevents mosquitoes from making tarsal contact with the surface of test papers.

The receiving cage is a one-gal (1.6-liter) ice cream carton with a screened top. The cage fits over the outward projecting exit funnel. A section of orthopedic stocking is attached to an opening in the side of the carton. The funnel of the exposure chamber is inserted through the stocking and through the opening of the receiving cage.

Test papers lining the exposure chambers are first clipped, with metal paper clips, to large sheets of clean white typing paper. The large papers are taped together in a ribbon effect. Then, with test papers attached, the ribbon of paper is placed against the sides, top and bottom of the exposure chamber. Papers are secured to the walls by pairing a small magnet on outside of the wall with a paper clip on the inside wall, the paper is secured by attraction between the magnets and paper clips. Test papers are not positioned on the front or back of the exposure chamber.

A full test requires 4 groups of 25 mosquitoes (test population) each. With 2 investigators, test populations can be introduced into each of the 4 exposure chambers in approximately 1 min. Before mosquitoes are introduced into the exposure chambers, exit funnels are sealed with Styrofoam™ inserts. A 3-min rest period has been used to permit mosquitoes to adjust to test chamber conditions in other test procedures (Busvine 1964); therefore, a 3-min interval is used in the present procedure. After 3 min, the Styrofoam insert is removed from each of the escape funnels to initiate the observation period. Numbers escaping from exposure chambers to receiving cages are recorded manually at 1-min intervals; after 5 min of observation, receiving cages are replaced with clean cages. The exchange of receiving cages facilitates the accurate counting of numbers escaping for each time interval.

A survival analysis approach is used to estimate the rates of mosquitoes escaping from chambers. In the excito-repellency test, there are only 2 possible outcomes for a specimen: it will either escape or not escape from the exposure chamber. Binary test data are optimized for survival analysis techniques by only working with counts of specimens that do not escape. However, an estimate of escape rate or probability of escape is obtained by subtracting from one the estimated rate or estimated probability of remaining in the exposure chamber. These statistically defined estimates for 1-min observation periods can be used to compare differences in mosquito escape rates among populations, insecticides, and concentrations (doses) of insecticides, in either contact or noncontact trials. The analytical results can be presented either as proportions escaping or proportions remaining in exposure chambers.

In this analysis, mosquitoes that escape are treated as "deaths" and those remaining in the exposure chamber from one minute to the next as "survivals." Specimens in the exposure chamber at the end of the test are treated as "censored." In survival analysis terminology, the survival time of a specimen is thought to be censored when the end point of interest (in our case, escape from the exposure chamber) has not been observed for that specimen (Lee 1992, Collett 1994). Time (min) for 50% and 90% of the test population to escape is estimated with the life table method, and these estimates are used as "escape time" summary statistics (ET_{50} and ET_{90}).

The log-rank method is used to compare patterns of escape behavior (analogous to survival curves). This test is designed to detect differences between survival curves that result when the death (or escape) rate in one group is consistently higher than the corresponding rate in a 2nd group and the ratio of these 2 rates is consistent over time (in survival analysis, this is also called the proportional hazard rate). With excito-repellency data, the basic idea underlying the log-rank test involves examining es-

cape observations by 1-min intervals. To test the null hypothesis, we calculate the observed escape and expected escape in each 1-min interval. The data are analyzed by use of tabular data presenting columns for time, number observed to escape, number expected to escape, and difference between observed and expected. We then combine the tabular data for each test to give an overall measure of the deviation of the observed escape values from their expected values by each 1-min test interval. The log-rank method was proposed by Mantel and Haenzel (1959); it is also called the Mantel–Cox and Peto–Haenzel methods (Mantel and Haenzel 1959).

The log-rank test has a chi-square distribution with k degrees of freedom, where k is the number of groups-1. A statistical software package, STATA®[5], can be used for this analysis to test for differences among or between populations, dose levels, and insecticides.

RESULTS AND DISCUSSION

The World Health Organization's (WHO) recommended tests of malaria vectors for behavioral responses to insecticides do not discriminate between contact versus noncontact stimulation (WHO 1975). The tests are based on the concept that malaria vectors respond to insecticides only after physical contact with the chemical and this concept is unrealistic.

As measured in the excito-repellency test (Chareonviriyaphap et al. 1997), noncontact repellency is not as quick or pronounced a behavioral response as contact irritancy. In the field, most mosquitoes stimulated to prematurely exit houses will probably do so only after physical contact with insecticide residues. However, the mosquito must first enter the house before it can make contact with insecticide. Except for a specimen that enters and then exits the house in pursuit of a host, the stimuli for the 3 behavioral acts of entering, resting indoors, and eventually exiting the house are different.

Noncontact repellency probably exerts its most powerful influence by preventing mosquitoes from entering houses. This repellency action has been documented in several field studies against several vector species (Roberts and Andre 1994). As examples, Roberts and Alecrim (1991) showed with experimental houses that *Anopheles darlingi* Root females practically stopped entering a house after it was sprayed with DDT, approaching a 100% reduction in indoor biting. Smith and Webley (1968) showed a 60–70% reduction in house entering by *Anopheles gambiae* Giles females after an experimental house was sprayed with DDT. Shalaby (1966) showed that, in comparison with a control house, 75% fewer *Anopheles culicifacies* Giles specimens were collected inside a DDT-sprayed house, even with all surfaces screened to preclude physical contact with DDT. With this background, it is clear that the excito-repellency test may provide a direct measure of vector responsiveness to the irritant affects of insecticides, and the results might be predictive of actions inside of houses under field conditions. However, the excito-repellency test is perhaps less informative of insecticidal impact on the act of entering a sprayed house.

Behavioral responses of vectors to insecticides are important, but generally neglected areas of study. Progress in understanding the importance of insecticide avoidance behaviors has been impeded by the lack of acceptable test systems. To date, no WHO-recommended test methods discriminate between contact versus noncontact insecticide-induced behaviors. No tests are easily conducted or, excluding the excito-repellency test box, provide a powerful and reproducible result. Additionally, no test data are amenable to sophisticated statistical analyses. The excito-repellency test box, the test, and data analysis methods (test system) described in this report were designed to resolve some of the problems identified by Roberts et al. (1984). The test system has already been used in an extensive study of behavioral responses of different *Anopheles albimanus* Wiedemann populations to DDT, permethrin, and deltamethrin (Chareonviriyaphap et al. 1997). Using this test system in combination with susceptibility, isozyme, and esterase tests, Chareonviriyaphap et al. (1997) obtained no evidence of relationships between physiological resistance and behavioral responses of *An. albimanus* females to insecticides.

The excito-repellency test system described in this report offers the following desirable attributes:

1. Exposure chambers are constructed of metal and can be chemically cleaned for use with different doses and types of insecticides.
2. Screened inserts provide a capability to test behavioral responses without physical contact with insecticide.
3. Mosquitoes are easily transferred to the exposure chambers and are easily removed from the chambers after the test is complete.
4. Counts by 1-min intervals are sensitive to rapid behavioral responses to insecticides.
5. Highly reproducible test results are obtained (Chareonviriyaphap et al. 1997).
6. The survival analysis method is a robust treatment of data that minimizes the loss of information.
7. Comparative summary statistics in the form of escape times for 50 and 90% (ET_{50} and ET_{90}) of test specimens to escape exposure chambers can be estimated.
8. Specimens that escape at different time intervals and specimens that remain in the exposure

[5] STATA® statistical software was provided by Stata Corporation, 702 University Drive East, College Station, TX 77840.

chamber throughout the test can be held and scored for 24-h mortalities.

In our studies (Chareonviriyaphap et al. 1997), we employed test papers that were prepared according to WHO specifications by the United States Army Center for Health Promotion and Preventive Medicine, Aberdeen Proving Ground, MD. Test papers are also available from the WHO.

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