Studying DDT Susceptibility at Discriminating Time Intervals Focusing on Maximum Limit of Exposure Time Survived by DDT Resistant Phlebotomus argentipes (Diptera: Psychodidae): an Investigative Report

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SUMMARY: Extensive application of routine insecticide i.e., dichlorodiphenyltrichloroethane (DDT) to control Phlebotomus argentipes (Diptera: Psychodidae), the proven vector of visceral leishmaniasis in India, had evoked the problem of resistance/tolerance against DDT, eventually nullifying the DDT dependent strategies to control this vector. Because tolerating an hour-long exposure to DDT is not challenging enough for the resistant P. argentipes, estimating susceptibility by exposing sand flies to insecticide for just an hour becomes a trivial and futile task. Therefore, this bioassay study was carried out to investigate the maximum limit of exposure time to which DDT resistant P. argentipes can endure the effect of DDT for their survival. The mortality rate of laboratory-reared DDT resistant strain P. argentipes exposed to DDT was studied at discriminating time intervals of 60 min and it was concluded that highly resistant sand flies could withstand up to 420 min of exposure to this insecticide. Additionally, the lethal time for female P. argentipes was observed to be higher than for males suggesting that they are highly resistant to DDT’s toxicity. Our results support the monitoring of tolerance limit with respect to time and hence points towards an urgent need to change the World Health Organization’s protocol for susceptibility identification in resistant P.argentipes.

INTRODUCTION

Controlling vector populations in order to manage vector-borne epidemics has been one of the preferred options for a long time. Presently, insecticide resistance, a pervasive natural phenomenon that occurs after an extensive, indiscriminate, and prolonged application of a particular insecticide, is high; it became a lingering issue, obstructing every strategy for controlling the vector populations in Integrated Vector Management (IVM) and Integrated Disease Management (IDM). The development and severity of resistance to insecticides in vectors are primarily controlled by human actions while lack of concern in dealing with resistance can set the stage for upsurge in vector populations leading to the reversals in public health programs.

In the context of visceral leishmaniasis (VL), it should be noted that adult female Phlebotomus argentipes Annandale and Brunetti (Diptera: Psychodidae), inoculated with mature parasite Leishmania donovani (Kinetoplastida: Trypanosomatidae) serve as a secondary, invertebrate host as well as vector for the lethal disease VL or Kala-azar which primarily affects the human hosts in Indian subcontinent (1). Therefore, in Indian subcontinent VL transmission is of anthroponotic nature i.e., transmission of parasite is from human to sand fly and vice-versa. Additionally, dichlorodiphenyltrichloroethane (DDT) is undoubtedly an insecticide of choice to control the nuisance caused by P. argentipes due to lower procurement cost and easier application as compared to carbamates and other insecticides available (2).

Historically, phlebotomine sand flies in India were susceptible to all insecticides prior to 1976. However, continued spraying of 1 g /m² DDT since 1976 to control Kala-azar in Bihar (3) evoked the problem of resistance among them. During 1979, the case with the highest degree of DDT resistance to P. papatasi was reported from North Bihar (4-5), while resistance to DDT in P. argentipes was reported first from the village of Samastipur district (6). Since then, many studies have been performed for portraying the development and dominance of insecticide resistance against DDT in P. argentipes from different pockets of Bihar (7-15) after its first emergence and testimonial reporting in the case of house flies (16).

Prior to screening insecticide resistance/susceptibility in the field, performing reliable laboratory tests for observing insects’ susceptibility towards any insecticide following an hour-long exposure to that insecticide impregnated filter paper at discriminating concentrations are supposed to be very relevant and noteworthy. This method is in accordance with the recommendations of the World Health Organization (WHO), (17).

However, an increase in the number of survivors in
each replicate of the DDT susceptibility test indicates increasing insecticide resistance among \( P. \ argentipes \) (15). In that situation, susceptibility estimation following insecticide exposure to sand flies for just an hour seems to be trivial and futile. To resolve this issue, the WHO strongly recommends for an increment in time of exposure to the discriminating concentration of insecticide (18). But till now, none of the reports explicates the maximum limit of insecticide exposure time to which highly resistant \( P. \ argentipes \) can combat its effect. Therefore, the present study was conducted as a preliminary assessment to investigate the maximum limit of DDT (4%) exposure time to which highly resistant \( P. \ argentipes \) could resist the effect of DDT and survive. This study objective adds to an attribute for an urgent need of change in rhetoric form of the WHO protocol for susceptibility estimation among highly resistant \( P. \ argentipes \).

**MATERIALS AND METHODS**

**Sampling of DDT resistant sand flies:** DDT resistant sand flies were derived from the DDT resistant \( P. \ argentipes \) colony (12th generation at the time of the writing of this manuscript) that was housed at the insectary of Rajendra Memorial Research Institute of Medical Sciences (Indian Council of Medical Research), Agamkuan, Patna-07, Bihar, India. The original colony of DDT resistant \( P. \ argentipes \) was established during 2014-2015 by collecting sand flies, testing their susceptibility towards DDT at a diagnostic dose of 4% (15) and rearing under the controlled conditions of the insectarium with stable temperature and relative humidity of 28 ± 2°C and 80 ± 5% respectively with 12 h of light/dark cycles (19-23). Additionally, the non-resistant sand flies serving as control samples for the bioassay test were procured from the general colony of \( P. \ argentipes \) being reared under the controlled environment of insectarium of this research institute. All the susceptibility bioassay tests were conducted at the controlled laboratory environment of Vector Biology and Control Department, RMRIMS (ICMR), Agamkuan, Patna-07, Bihar, India.

As the maintenance procedure for the \( P. \ argentipes \) colony (of resistant and non-resistant towards 4% DDT), rabbits were used to provide blood meals to the freshly emerged adult sand flies (15); these rabbits were obtained from the Animal House Division of RMRIMS (ICMR), Agamkuan, Patna-07, Bihar, India. After completion of the blood-feeding process for 2-3 h, the rabbits were returned back to the animal house where they were kept in separate animal cages (12 sq. ft. with grids of 2.5 inches) and were provided with food and water according to the diet chart of the animal house of the research institute. In this study, "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed when conducting experiments that involve animals. Protocols established by an animal ethics committee of Indian Council of Medical Research (ICMR), Government of India, were also followed while conducting the experiments that used animals during this research study. All experiments were conducted under the guidance of the Institutional Ethical Committee of RMRIMS (ICMR), Agamkuan, Patna-07, Bihar, India.

**Insecticide susceptibility test:** Investigatory experiments for exploring the maximum limit of insecticide exposure time to which DDT resistant \( P. \ argentipes \) can resist the effect of DDT were carried out with one-day old, sucrose-fed, adult sand flies obtained from the 1st, 3rd and 5th generations of the colony that had been reared during the months of September 2014, November 2014, and February 2015, respectively. The insecticide susceptibility tests were carried out in accordance with the protocol established by the WHO (17) and the followed in the previous reporting (15).

Insecticide susceptibility testing apparatus used in our study contained green-dot marked ‘holding tube’ for lining with filter paper, red-dot marked ‘exposure tube’ for lining with 4% DDT impregnated paper and a sliding unit, purposely for screwing and joining both the plastic tubes (125 mm length and 44 mm in diameter) of the apparatus. Papers were fastened along the walls of tubes with the help of metal clips affixed at both ends of the holding and exposure tubes (17). Overall 16 test replicates were performed by the gentle release

**Table 1. Susceptibility test result for estimating LT\(_{50}\), LT\(_{90}\) and LT\(_{95}\) for resistant \( P. \ argentipes \) responded towards the prolonged exposure of DDT at discriminating time intervals of 60 min**

| Observed parameter | Insecticide exposure time (in min) |
|--------------------|------------------------------------|
|                    | 60 | 120 | 180 | 240 | 300 | 360 | 420 | 480 |
| **CONTROL**        |    |     |     |     |     |     |     |     |
| No. of sand flies tested (NT) |   |     |     |     |     |     |     |     |
| Alive               | 40 | 40  | 40  | 40  | 40  | 40  | 40  | 40  |
| mortality %         | 0  | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| **EXPERIMENT**      |    |     |     |     |     |     |     |     |
| No. of sand flies tested (NT) |   | 60  |      | 50  | Male); Total = 110 |
| No. of Alive (NA) ± % | 109 ± 99.09% | 106 ± 96.36% | 82 ± 74.54% | 56 ± 50.90% | 32 ± 29.09% | 3 ± 2.72% | 0 ± 0% | 0 ± 0% |
| No. of Senseless (NS) ± % | 1 ± 0.90% | 3 ± 2.72% | 15 ± 13.63% | 22 ± 20% | 13 ± 11.81% | 10 ± 9.09% | 1 ± 0.90% | 0 ± 0% |
| No. of Dead (ND) ± % | 0 ± 0% | 1 ± 0.90% | 13 ± 11.81% | 32 ± 29.09% | 65 ± 59.09% | 97 ± 88.18% | 109 ± 99.09% | 110 ± 100% |
| Observed Mortality=ND/NT*100 | 0 | 0.90 | 11.81 | 29.09 | 59.09 | 88.18 | 99.09 | 100 |
| Lethal Time for 50% tested \( P. \ argentipes \) (LT\(_{50}\)) against 4% DDT | 280 min; at CI of 95% |
| Lethal Time for 90% tested \( P. \ argentipes \) (LT\(_{90}\)) against 4% DDT | 370 min; at CI of 95% |
| Lethal Time for 95% tested \( P. \ argentipes \) (LT\(_{95}\)) against 4% DDT | 400 min; at CI of 95% |
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Table 2. Observed Lethal Time (LT) values for the experimented male and female *P. argentipes*

| Observed Lethal Time (LT) against 4% DDT | Experimented *P. argentipes* |
|------------------------------------------|------------------------------|
|                                          | Male sand flies | Female sand flies |
| LT50                                     | 60.33 min; at 282.36 min; at Cl of 95% | Cl of 95% |
| LT90                                     | 128.04 min; at 389.00 min; at Cl of 95% | Cl of 95% |
| LT95                                     | 133.00 min; at 472.07 min; at Cl of 95% | Cl of 95% |

RESULTS

Out of 16 experimental replicates, only 11 test replicates were included in the evaluation of results due to successful completion of the test; the results of the remaining 5 experimental sets were discarded due to unsatisfactory results because of test samples (i.e., sand flies) escaping or being trapped crushed inside the screw of experimental setup during the experimental session. Therefore, from the study of overall 11 experimental replicates, it was observed that out of 110 tested *P. argentipes* (comprising 60 females and 50 males), 99.09\% had competitively tolerated the threshold exposure period of an hour towards DDT, suggesting an urgent need for the estimation of their susceptibility status towards the insecticide, as recommended by the WHO (17-18). For that purpose, experimental setups were upheld for prolonged exposure of sand flies towards the insecticide. Approximately 50\% test mortality along with record numbers of senseless sand flies (i.e., 20\%) were observed in 300 min and 240 min respectively of prolonged exposure to DDT. Moreover, highly resistant sand flies struggled to survive for 420 min for their survival and ultimately achieved absolute mortality in 480 min of insecticide exposure. The LT50, LT90, and LT95 values for overall experimented *P. argentipes* were observed to be at 280 min, 370 min, and 400 min, respectively with 95\% confidence interval (CI; Table 1). However, the LT values against 4\% DDT for killing 50\%, 90\% and 95\% experimented male sand flies (i.e., 60.33 min, 128.04 min, and 133 min) were comparatively lower than those recorded for the experimented female *P. argentipes* (i.e., 282.36 min, 389 min, and 472.07 min) respectively with 95\% CI (Table 2). The statistical analysis of data revealed the significance of the result ($\chi^2 = 9.7; d.f = 4; p = 0.04$). The data on resistant *P. argentipes*’ response towards the prolonged exposure of DDT for estimating LT50, LT90, and LT95 at the discriminating time intervals of 60 min have been illustrated in Table 1, Fig. 1, and Table 2.

DISCUSSION

For many years, DDT has been used worldwide for controlling sand flies through direct intervention or inadvertently as a collateral benefit of anti-malaria campaigns (24-30). Continuous and rigorous use of DDT for controlling insects had definitely invited unavoidable circumstances of lowest susceptibility among the *P. argentipes* in Vaishali district at Bihar (24, 9-11). In terms of insecticide effect, susceptibility and resistance factors are antagonistic to each other. The lowest susceptibility towards a particular insecticide is supposed to be the other face of the same coin indicating the highest resistance against that insecticide and vice-versa among the insect population (15).

In a recent report, dealing with the susceptibility profile of *P. argentipes* derived from different pockets of Bihar, sand flies of the Daulatpur Chandi village of Vaishali district (25.683°N, 85.216°E) had exhibited the highest resistance towards DDT (14). Over here, the percentage mortality range (41%-52.73\%) and corrected mortality rate (44.83\%) for *P. argentipes* observed with...
insecticide tube assay recommended by the WHO, were estimated to be the lowest as compared to the other districts of Bihar, indicating the presence of sand flies that were highly resistant towards DDT (15).

Performing laboratory tests for observing insects’ susceptibility following an hour exposure to insecticide impregnated paper, at discriminating concentrations is relevant and noteworthy prior to screening insecticide resistance/susceptibility for insects in the field (17). However, the technique itself possess various limitations, such as environmental factors, quality control and shelf life of insecticide impregnated papers, insufficient number, and physiological conditions of testing samples participating the experimental session (17-18, 31), that even a minute fluctuation in any of the factor(s) may cause deviations from the expected outcome with respect to the insects’ susceptibility to an insecticide. In that situation, accessing susceptibility at different exposure time as well as increasing exposure time corresponding to the large number of survivors or resistant insects is also recommended by the WHO (18). However, to date, there have been no reports explaining the maximum limit of insecticide exposure time to which resistant P. argentipes can combat its effect. Therefore, an urgent demand of this study had paved the way for elucidating an approach for quantifying the maximum limit of DDT (4%) exposure time to which resistant P. argentipes struggle to resist the effect of DDT for their survival.

On the basis of bioassay experiment performed in accordance with the insecticide susceptibility test recommended by the WHO at varying time interval of 60 min, it was observed that the maximum number of resistant P. argentipes survived the threshold period of 60 min of DDT exposure and tolerated the insecticidal pressure up to 420 min of this insecticide exposure and achieved absolute mortality in 480 min. In our experiments, we focused on the mortality of test specimens rather than the knockdown time (KT), because under the effect of continuous exposure of DDT, the sand flies were expected to die faster and in higher numbers (17-18); hence, estimating KT becomes futile and can be neglected. However, Lethal time (LT) has been calculated as 280 min, 370 min and 400 min for killing 50%, 90% and 95% test sand flies respectively, under the effect of DDT. These figures are much higher than the previously observed results documented from the different pockets of Bihar state (3-6, 8-11). From the earlier studies, LT50 for P. argentipes against 4% DDT was ranged between 52-69 min (11) whereas later this value i.e., LT against DDT for killing 50% and 90% population of P. argentipes was reported to have increased up to 1.28 h and 3.57 h respectively (6). Additionally, the present study reveals high LT values for killing 50%, 90% and 95% experimented female P. argentipes (i.e., 282.36 min, 389 min and 472.07 min) as compared to that of male sand flies (i.e., 60.33 min, 128.04 min and 133 min). This suggests that female sand flies are highly resistant to the toxic effects of an insecticide (10-14, 24, 27-30), making it physically potent to draw blood meal from the vertebrate host and offer favorable environment to pathogenic parasite for harboring them into its gut (1). Therefore, the result from this study, depicts an increased level of LT among P. argentipes, providing fresh, baseline information to an extent with respect to the exposure time to which highly resistant sand flies can combat the pressure of DDT for their survival. The result strongly corroborates with the developed status of insecticide resistance at peak (15, 32) and adds an attribute towards an urgent need of change in rhetoric form of the WHO protocol for susceptibility identification in resistant P. argentipes, ultimately controlling the menace caused by them.

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Conflict of interest None to declare.

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