Studying DDT susceptibility at discriminating time interval focusing maximum limit of exposure time survived by DDT resistant *Phlebotomus argentipes* (Diptera: Psychodidae) - an investigatory report

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Received: November 28, 2015. Accepted: January 16, 2017
Published online: February 28, 2017
DOI: 10.7883/yoken.JJID.2015.604
Studying DDT susceptibility at discriminating time interval focusing maximum limit of exposure time survived by DDT resistant *Phlebotomus argentipes* (Diptera: Psychodidae) - an investigatory report

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**Key words:** Dichlorodiphenyltrichloroethane (DDT), *Phlebotomus argentipes*, insecticide resistance, insecticide exposure, Lethal time (LT).

**Running Title:** Maximum exposure time for resistant sand flies.
Abstract

Extensive application of routine insecticide i.e., Dichlorodiphenyltrichloroethane (DDT) to control Phlebotomus argentipes (Diptera: Psychodidae), the proven vector of Visceral leishmaniasis (VL) in India, had evoked the problem of resistance/ tolerance against DDT, eventually nullifying the DDT dependent strategies to handle the nuisance caused by them. Because tolerating an hour exposure of DDT is not so challenging for resistant P. argentipes, estimating susceptibility by exposing sand flies to insecticide for just an hour becomes trivial and futile task.

Therefore, present bioassay study was carried out to investigate the maximum limit of exposure time to which DDT resistant P. argentipes combat its effect 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 minutes and concluded that highly resistant sand flies can withstand up to 420 minutes of this insecticide exposure. Also, the Lethal time (LT) for female P. argentipes were observed to be higher than that of male suggesting its nature of being highly resistant to its toxicity. The result is supportive for monitoring the tolerance limit with respect to time and hence attribute towards an urgent need of change in rhetoric form of WHO protocol for susceptibility identification in resistant P. argentipes.

Introduction

For controlling vector borne epidemics, controlling vector populations remained highlighted as a preferred option since long. Presently, insecticide resistance being powerful, pervasive natural phenomenon, occurs after an extensive, indiscriminate and prolonged application of particular insecticide, and hence serve as a smoldering issue, obstructing every strategy for controlling vector population under Integrated Vector Management (IVM) as well as Integrated Disease Management (IDM). The development and severity of resistance to insecticides in vectors are primarily controlled by the human action while ignorance of concern in dealing with resistant can set the stage for explosions in vector populations leading to the reversals in the public health protection programs.
In context to the Visceral leishmaniasis (VL), it is to 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 lethal disease Visceral leishmaniasis (VL) or Kala-azar that is being transmitted by them affecting primarily 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. Also, Dichlorodiphenyltrichloroethane (DDT) is undoubtedly an insecticide of choice to control the nuisance caused by *P. argentipes* due to lower procurement cost and application as compared to carbamates and other available insecticides (2).

Historically, in India, prior to 1976, phlebotomine sand flies were supposed to be susceptible against all insecticides. Continued spraying of 1g /m² DDT since 1976, to control Kala-azar in Bihar (3) had evoked the problem of resistance among them. During 1979, the highest degree of DDT resistance cases were reported in *P. papatasi* from North Bihar (4-5) while resistance to DDT in *P. argentipes*, was firstly reported from the village of Samastipur district (6). Since then, lots of work had been conducted 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 case of house flies (16).

As per the recommendations of World Health Organization (WHO), performing reliable laboratory tests for observing insects’ susceptibility towards an insecticide following an hour exposure to insecticide impregnated filter paper, at discriminating concentrations is relevant and noteworthy prior screening insecticide resistance/ susceptibility for insects in the field (17). But, an increase in the number of survivors in each replicate of the DDT susceptibility test indicates increasing instances of insecticide resistance among *P. argentipes* (15). And in that situation, susceptibility estimation following insecticide exposure to sand flies for just an hour seems to be trivial and futile. To sort out the problem, WHO strongly recommends for an increment in time of exposure to the discriminating concentration of insecticide (18). But, till now, none of the report explicates the maximum limit of
insecticide exposure time to which highly resistant *P. argentipes* combat its effect. Therefore, the present study was conducted as a preliminary check-up to investigate the maximum limit of DDT (4%) exposure time to which highly resistant *P. argentipes* struggle to uphold the effect of DDT for their survival. Study objective adds an attribute towards an urgent need of change in rhetoric form of WHO protocol for susceptibility estimation among highly resistant *P. argentipes*.

**Materials and Methods**

1. **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 manuscript documentation) that is being maintained at the insectarium 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 its susceptibility towards DDT at a diagnostic dose of 4% (15) and rearing under the controlled conditions of insectarium being maintained at the constant temperature and relative humidity of 28 ± 2°C and 80 ± 5% respectively with a 12 h: 12 h light/dark cycle (19-23). Along with that, non-resistant sand flies for serving as a control sample for bioassay were procured from the general colony of *P. argentipes* being reared under the controlled environment of insectarium of the 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.

Under maintenance procedure of the *P. argentipes* colony (of resistant and non-resistant towards 4% DDT), rabbits deployed for providing a blood meal to the freshly emerged adult sand flies (15), were obtained from the Animal House Division of RMRIMS (ICMR), Agamkuan, Patna-07, Bihar, India. After completion of blood-feeding process for 2-3 hours, the rabbits were then returned back to the animal house where they were separately kept in animal cages (12 sq ft with grids of 2.5 inches) and supplemented with food and water according to the diet chart of animal house of the research institute.
For the present study "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed for conducting research experiments involving animal. Protocols led by an animal ethics committee of Indian Council of Medical Research (ICMR), Government of India, were also followed in conducting the present research work involving experiments using animal subject. All experiments had been conducted under the guidance of the Institutional ethical committee of RMRIMS (ICMR), Agamkuan, Patna-07, Bihar, India.

2. Insecticide Susceptibility Test

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

Insecticide susceptibility testing apparatus exploited in the present 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 44mm in diameter) of an apparatus. Papers were fastened along the wall of tubes with the help of a metal clips affixed at the both ends of holding and exposure tubes (17). Overall 16 test replicates were performed by the gentle release of batches of 10 sand flies, comprising both sexes into each 16 test replicates with the help of an oral aspirator. Along with the 16 test replicates, 2 replicates of 20 sand flies for control were also held parallel to the test. The sand flies were held in the holding tubes for about an hour, purposely for acclimatization to the environment of experimental setup. After an hour, all the *P. argentipes* were transferred to an exposure tube through the hole present at the sliding unit of testing apparatus. The insecticide susceptibility testing apparatus and 4% DDT impregnated papers (developed by WHO by the Universiti Sains Malaysia, which is based in Penang,
Malaysia, with manufacturing year 2013) were procured from National Vector Borne Disease Control Programme (NVBDCP), Government of India, New Delhi, India in the year 2013.

Physical status of experimental sand flies in each exposure tubes were minutely observed and recorded as actively alive, senseless and dead, at each interval of 60 minutes until cent percent mortality was achieved at the end of each test replicates. In this regard, ‘alive’ and ‘dead’ status of the testing insect has been clearly elaborated by the WHO (17) but for the present study ‘senseless’ is a new terminology denoting the physical condition of sand flies in which they remains inactive under the insecticidal effect but possess some movements in their wings and legs. On completion of test, the data were subjected to probit statistical analysis using the SPSS (IBM Statistics) Version 21 software program for determining LT\textsubscript{50}, LT\textsubscript{90} and LT\textsubscript{95} of DDT against \textit{P. argentipes}. The results are represented in tabular (\textbf{Table 1}) as well as in graphical form (\textbf{Figure 1}). Also, the LT values for killing 50%, 90% and 95% population of experimented male and female \textit{P. argentipes} was observed and illustrated in \textbf{Table 2}.

\textbf{Results}

Out of 16 experimental replicates, only 11 test replicates were considered for the result evaluation due to successful achievement of satisfactory result while the results of remaining 5 experimental sets were discarded off due to unsatisfactory results produced either by escaping of test samples (i.e., sand flies) or by their trapping/ crushing inside the screw of experimental set-up during the experimental session. Therefore, from the study of overall 11 experimental replicates, it was observed that out of 110 tested \textit{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 estimation of their susceptibility status towards the insecticide, as recommended by 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 minutes and 240 minutes respectively of prolonged exposure to DDT. Moreover,
highly resistant sand flies struggled for 420 minutes for their survival and ultimately achieved absolute mortality in 480 minutes of insecticide exposure. The LT$_{50}$, LT$_{90}$ and LT$_{95}$ values for overall experimented $P. \text{argentipes}$ were observed to be at 280 minutes, 370 minutes and 400 minutes respectively at a confidence interval (CI) of 95% (Table 1). However, the LT values against 4% DDT for killing 50%, 90% and 95% experimented male sand flies (i.e., 60.33 minutes, 128.04 minutes and 133 minutes) were observed to be comparatively lowered as recorded for that of experimented female $P. \text{argentipes}$ (i.e., 282.36 minutes, 389 minutes and 472.07 minutes) respectively at a confidence interval (CI) of 95% (Table 2). The statistical analysis of data revealed the significance of the result ($\chi^2 =9.7; \text{d.f=4}; \text{p=0.04}$). The data of resistant $P. \text{argentipes}$ responded towards the prolonged exposure of DDT for estimating LT$_{50}$, LT$_{90}$ and LT$_{95}$ at the discriminating time intervals of 60 minutes has been illustrated in Table 1, Figure 1 and Table 2.

Discussion

For many years, DDT has been exploited worldwide for controlling sand flies through direct intervention or inadvertently as a collateral benefit of anti-malaria campaigns (24-30). Continuous and rigorous perusal of DDT for controlling insects’ nuisance had definitely invited unavoidable circumstances of lowest susceptibility among the $P. \text{argentipes}$ of Vaishali district at Bihar (24, 9-11). In terms of insecticide effect, susceptibility and resistance factors are antagonistic to each other, lowest susceptibility towards particular insecticide is suppose to be the other face of 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 $P. \text{argentipes}$ derived from the different pockets of Bihar, sand flies’ of the Daulatpur Chandi village of Vaishali district (25.6833 °N, 85.2167° E), lying at the northern side of Ganges course, that is about 62 kilometers away from Patna, the capital of Bihar, India, had exhibited the highest resistance towards DDT (14). Over here, the percentage mortality range (41.00-52.73) and corrected mortality rate (44.83%) for $P. \text{argentipes}$
under insecticide tube assay recommended by the World Health Organization (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 viz., 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) can deviate from the expected outcome with respect to the susceptibility of an insect towards any insecticide. In that situation accessing susceptibility at varying exposure time interval as well as an increment in exposure time following to the large number of survivors or resistant insects, in test replicates is also recommended by WHO (18). But, till date, no report explicates the maximum limit of insecticide exposure time to which resistant *P. argentipes* combat its effect. Therefore, an urgent demand of this study had paved way for elucidating an approach for quantifying the maximum limit of DDT (4%) exposure time to which resistant *P. argentipes* struggle to uphold the effect of DDT for their survival.

On the basis of bioassay experiment performed in accordance with insecticide susceptibility test recommended by WHO at varying time interval of 60 minutes, it was observed that the maximum number of resistant *P. argentipes* survived the threshold period of 60 minutes of DDT exposure and tolerated the insecticidal pressure up to 420 minutes of this insecticide exposure and achieved absolute mortality in 480 minutes. For present experiment we were very much focused upon the mortality of test specimen rather than the Knockdown Time (KT) because under the effect of continuous exposure of DDT, mortality of sand flies were expected to be faster and higher (17-18) and in that situation estimation of KT become futile and can be neglected. However, Lethal time (LT) has been calculated as 280 minutes, 370 minutes and 400 minutes for killing 50%, 90% and 95% test sand flies.
respectively, under the effect of DDT that are very much higher as compared to the previously observed results documented from the different pockets of Bihar state (3-6, 8-11). From the earlier studies, \( LT_{50} \) for \( P. \) argentipes against 4% DDT was ranged between 52-69 minutes (11) whereas this value i.e., LT against DDT for killing 50% and 90% population \( P. \) argentipes was reported to be increased up to 1.28 hours and 3.57 hours respectively (6). Also, present study reveals the high LT values for killing 50%, 90% and 95% experimented female \( P. \) argentipes (i.e., 282.36 minutes, 389 minutes and 472.07 minutes) as compared to that of male sand flies (i.e., 60.33 minutes, 128.04 minutes and 133 minutes) suggesting and supporting its nature of being highly resistant to the toxic effects of an insecticide (10-14, 24, 27-30) and 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, in this regard, present study result, depicting an increased level of LT provides fresh, baseline information on 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 hence adds an attribute towards an urgent need of change in rhetoric form of WHO protocol for susceptibility identification in resistant \( P. \) argentipes.

In conclusion, insecticide resistance or susceptibility has serious connotations upon the vector and disease control programs and hence present result possesses implications upon monitoring the tolerance limit with respect to time. Therefore, present study offers an opportunity for introducing new and effective approaches of vector control viz., combined application of different class of insecticide, insecticide rotation, etc., that can effectively overtake the side-effects of an increased insecticide tolerance among the population of \( P. \) argentipes, ultimately controlling the menace caused by them.

Acknowledgements

Present work is a part of study under PhD programme [Registration No. 3700/PhD. (Sc.) proceed 2014] entitling “Study of behavioral responses in the offspring emerged out from single parent insect
of 4% DDT resistant strain *Phlebotomus argentipes* (Diptera: Psychodidae) the vector of kala-azar (Visceral leishmaniasis) in Indian subcontinent” progressing under the supervision of University of Calcutta, Kolkata, India and financially sponsored by University Grant Commission, India with vide grant number [RGNF 2012-13-ST-BIH-20305] and RMRIMS (ICMR), Agamkuan, Patna-07, Bihar, with study title “Understanding behavior profile of DDT resistant sand flies for exploring scope of IGR as an alternate technique for containing the population of *P. argentipes*” [Id.No. INT-119-VBC/2015] approved by the Scientific Advisory Committee (SAC) of RMRIMS (ICMR) in year 2015.

The authors cordially express their gratitude towards NK Sinha, SA Khan, AK Mandal, BB Prasad and all members of Vector Biology and Control Department for their excellent technical support and help during the experiment and manuscript documentation session. Thanks are also due to the SAC of RMRIMS (ICMR), Institutional Ethical Committee (IEC) as well as to the Animal Ethics Committee (AEC) for their mandatory approval to conduct the research study. Last but not least, we are also thankful to the research publication committee of RMRIMS for considering the manuscript worth publication.

**Conflict of Interests**

None to declare

**References**


Figure Legend

**Figure 1**: Graphical representation for results of resistant *P. argentipes* responded towards the prolonged exposure of DDT at discriminating time intervals of 60 minutes.

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**Additional files**

None
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 minutes.

<table>
<thead>
<tr>
<th>Observed parameters</th>
<th>Insecticide Exposure time (in Minutes)</th>
<th>CONTROL</th>
<th>EXPERIMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(15 Female, 25 Male); Total=40</td>
<td>(60 Female, 50 Male); Total=110</td>
</tr>
<tr>
<td>No. of sand flies tested (NT)</td>
<td></td>
<td>Alive</td>
<td>No. of Alive (NA)± %</td>
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<tr>
<td></td>
<td></td>
<td>40</td>
<td>109± 99.09 %</td>
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<td></td>
<td></td>
<td>40</td>
<td>106± 96.36 %</td>
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<td>40</td>
<td>82± 74.54 %</td>
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<td>56± 50.90 %</td>
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<td>40</td>
<td>32± 29.09 %</td>
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<td>40</td>
<td>3± 2.72 %</td>
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<td>alive</td>
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<td>0.90</td>
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</table>

Lethal time for 50% tested $P. argentipes$ ($LT_{50}$) against 4% DDT: 280 Minutes; at CI of 95%.

Lethal time for 90% tested $P. argentipes$ ($LT_{90}$) against 4% DDT: 370 Minutes; at CI of 95%.

Lethal time for 95% tested $P. argentipes$ ($LT_{95}$) against 4% DDT: 400 Minutes; at CI of 95%.
Table 2: Observed Lethal Time (LT) values for the experimented male and female *P. argentinipes*.

<table>
<thead>
<tr>
<th>Observed Lethal Time (LT) against 4%DDT</th>
<th>Experimenterd <em>P. argentinipes</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male sand flies</td>
</tr>
<tr>
<td>LT&lt;sub&gt;50&lt;/sub&gt;</td>
<td>60.33 Minutes; at CI of 95%</td>
</tr>
<tr>
<td>LT&lt;sub&gt;90&lt;/sub&gt;</td>
<td>128.04 Minutes; at CI of 95%</td>
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<tr>
<td>LT&lt;sub&gt;95&lt;/sub&gt;</td>
<td>133.00 Minutes; at CI of 95%</td>
</tr>
</tbody>
</table>
Figure 1-updated

P. argentipes responded towards DDT [in %]

Prolonged Exposure time towards DDT [in minutes]