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Research Article

Self-Limiting OX513A *Aedes aegypti* Demonstrate Full Susceptibility to Currently Used Insecticidal Chemistries as Compared to Indian Wild-Type *Aedes aegypti*

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OX513A *Aedes aegypti* is a genetically engineered strain carrying a self-limiting gene. Studies in several countries have shown the effectiveness of the strain at reducing pest *Aedes aegypti* populations. As a component of biosafety assessments relevant to Indian environments, OX513A and two Indian wild-type *Ae. aegypti* strains (from Aurangabad and Delhi) were tested for susceptibility to a range of commonly used insecticides in India, such as dichlorodiphenyltrichloroethane (DDT), malathion, deltamethrin, and permethrin using World Health Organization (WHO) testing kits and following WHO standard test procedures. Knockdown times (KDT) for all compounds were determined separately for male and female adults of the three mosquito strains. Results indicated that adults of OX513A, Aurangabad, and Delhi strains were resistant to DDT, yielding mortality rates of 90.9, 87.4, and 44.4% and 70.1, 3.0, and 6.0% for male and female adults, respectively. In contrast, adults of all three strains were found to be susceptible to malathion, deltamethrin, and permethrin, exhibiting mortalities between 98 and 100%. The magnitudes of susceptibility, based on the KDT_{50} values, were greater in the OX513A strain, as compared to wild-type strains of *Ae. aegypti* for all insecticides tested. The results confirm that, aside from historical resistance to DDT, OX513A has retained full sensitivity to these commonly used compounds and exhibits responses akin to those of susceptible Indian wild-type strains.

1. Introduction

Aedes aegypti (Linnaeus) is a cosmotropical mosquito that in recent decades has emerged as one of the most important vectors responsible for spreading several infectious viral diseases including dengue, chikungunya, and Zika [1, 2]. Dengue in particular has been of global concern due to its rapid expansion in both scope and scale. Presently, more than 2.5 billion people live in dengue-risk regions and an estimated 100 million new cases occur each year [3]. Up to a third of cases are suspected to occur in India, although pervasive underreporting within the region complicates epidemiological appraisals. The Indian "National Vector Borne Disease Control Programme" (NVBDCP) reported almost 100,000 confirmed dengue cases in 2015, resulting in 220 fatalities. In 2017, the national situation appears to equally severe with 129,329 cases and 200 deaths as of 12 November (http://nvbdcp.gov.in/den-cd.html). The World Health Organization (WHO) declared that, due to the lack of effective therapeutic treatments for dengue, the most effective means of disease mitigation are vector control. Strategies for vector management have so far proven less than fully effective, relying largely on insecticide applications in attempts to reduce local mosquito abundance to below disease transmission thresholds.

The intense selection pressures imposed by insecticides have resulted in the development of genes that encode for insecticide resistance. The lack of effective alternatives has driven the so-called pesticide treadmill syndrome, whereby the response to a loss of efficacy is to further increase

application rates and frequencies [4]. The net result, following such broad-scale and long-term insecticide use in the public health sector, is that in many areas resistance to commonly used products is almost ubiquitous, and vector control agencies have become largely ineffectual in terms of disease mitigation.

Recent advances in biotechnology have led to the development of alternative strategies for controlling *Ae. aegypti* populations, which for the first time could help curb the seemingly incessant rise in dengue prevalence. One such approach that is presently undergoing field-based evaluations in several countries involves the release of male adult OX513A, a genetically engineered strain of *Aedes aegyp*ti in which all individuals carry an inserted self-limiting gene and a marker gene. The self-limiting gene renders mating events between OX513A males and wild-type females as unsuccessful, with over 95% of the progeny dying before adulthood [5]. Initial studies from Grand Cayman, Brazil, and Panama have reported significant and sustained reductions in vector abundance, highlighting the potential of OX513A to reduce rates of disease transmission [6–8].

One aspect of biosafety relevant to the release of biological control agents, including OX513A, is whether the strain for release carries potentially deleterious genes such as those encoding for insecticide resistance. Even for cases where insects for release are unable or unlikely to persist, as for OX513A, their inherent susceptibility to insecticides is desirable for risk mitigation, if attempt to clear them from released environment is anticipated. Earlier studies on mating competitiveness and life history traits under Indian laboratory settings have shown the OX513A strain to be largely comparable to wild-type strains of *Ae. aegypti* [9].

This manuscript reports on a study that examined the insecticide resistance profile of the OX513A *Ae. aegypti* strain and compared responses to those of two Indian wild-type *Ae. aegypti* strains. The two wild-type *Ae. aegypti* strains tested were from urban locations, Aurangabad and New Delhi regions; Aurangabad region is located in the Western India and the New Delhi is located in the North region of India. Notably the only reliable method used for control of vector mosquitoes not only in India but also worldwide is using synthetic insecticides, imposing selection pressure over the course of time and eventually leading to insecticide resistance development. In the view, the two wild-type strains of urban locations were used in parallel to OX513A strain to test for insecticidal susceptible status.

2. Materials and Methods

2.1. OX513A Aedes aegypti L. The initial development of the OX513A *Ae. aegypti* strain was originally described by Phuc et al. [5]. OX513A contains a single insertion comprising two dominantly inherited genes. Firstly, a repressible self-limiting component confers a lethal phenotype only in immature life-stages. This consists of a tetO binding domain and a minimal promoter. Basal expression of the promoter produces a small amount of a cellular protein (tTAV) that binds tetO. TetO drives further expression of the promoter, which in turn produces more tTAV. This positive feedback produces increasing quantities of tTAV that become deleterious to cell function, ultimately resulting in death of the individual before adulthood. Contrastingly, in the presence of a repressor (tetracycline), tTAV is sequestered and is therefore unavailable to bind tetO. This precludes positive feedback and only the benign, basal level of tTAV, is produced. The second component is a gene that encodes for a fluorescent protein (DsRed2). DsRed2 is visible under a microscope equipped with specific filters (excitation of 510–550 nm and emission at 600 nm).

OX513A *Ae. aegypti* eggs (Oxitec Ltd., UK) were imported during September 2011 in accordance with the import permit (number BT/BS/17/328/2008-PID) issued by the Department of Biotechnology (DBT), Government of India, New Delhi. A cyclic colony was then maintained in Arthropod Containment Level II laboratory [10] under controlled environment conditions (27 ± 2°C, RH 70–80%, 12-hour light cycle). OX513A larvae were reared using tap water containing 30 μg/ml concentration of chlortetracycline with food provided daily to minimize excess. After emergence, adults were provided with 10% sucrose solution and damp germination papers for oviposition.

2.2. Wild-Type Aedes aegypti L. Eggs and adult stages of *Ae. aegypti* were collected from New Delhi (Nation Capital Territory, India) and Aurangabad (Maharashtra State, India) during 2011. Field samples were collected by placing ovitraps for egg collection and adults were collected by placing BG sentinel traps. The eggs collected were hatched in the laboratory and identified during adult stages following the identification keys described by Baraud [11]. The strains from the two regions were reared and maintained under controlled environment conditions (27 ± 2°C, RH 70–80%, 12-hour light cycle) and the cyclic colonies were named DEL and AWD for New Delhi and Aurangabad, respectively. Larvae were reared in tap water at a density of 1 larva per ml and were provided "LIQUIFRY" (Interpret, UK) fish food for the first day of development after hatching and were subsequently fed with ground Tetramin® fish food (Tetra, Germany) until pupation, with an increasing daily feeding regime. Pupae formed were sexed manually based on the size and introduced into the adult rearing cages (30 × 30 × 30 cm) at 1 : 2 ratio (male : female). After emergence, adults were provided with 10% sucrose solution and damp germination papers for oviposition.

2.3. Insecticides. Adults of all three strains were tested using WHO insecticide susceptibility testing kits, with insecticide-impregnated papers (Vector Control Research Centre, Universiti Sains, Malaysia). The discriminating concentrations, compounds, and chemical classes (in parentheses) were 4% dichlorodiphenyltrichloroethane (DDT) (organochlorine); 5% malathion (organophosphate); 0.75% permethrin (pyrethroid); and 0.05% deltamethrin (pyrethroid).

2.4. Bioassays. Insecticide tests were carried out during the period June 2015 to September 2015 as per the WHO bioassay procedure, using the susceptibility test kits against 4- to 5-day

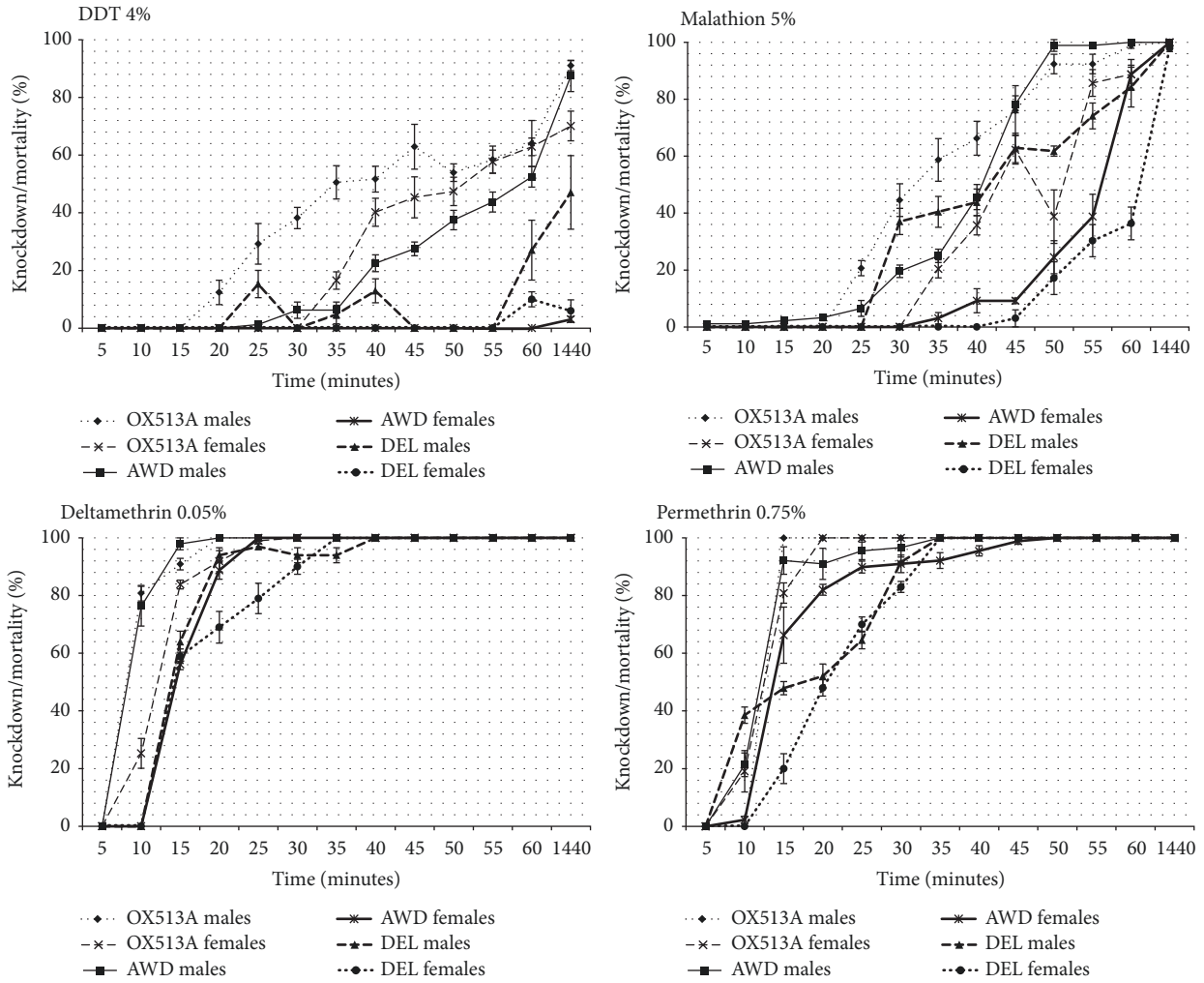

Figure 1: Knockdown/mortality percent of male and female adults of OX513A and wild-type *Aedes aegypti* from Aurangabad (AWD) and Delhi (DEL) against diagnostic concentrations of four synthetic insecticides. Error bars indicate standard errors.

old sugar-fed adults [12]. Tests for insecticide susceptibility against male and female mosquitoes of the three strains were performed on the same day for each insecticide. The strains used for bioassays were F_{11}, F_8, and F_{13} generations for OX513A, AWD, and DEL strains, respectively. Adult male and females were assessed separately and females were not blood-fed. Four replicates of 20–25 adults were used for treatment groups and three replicates of 20–25 adults for control groups. Preimpregnated papers for control groups used risella oil for DDT, olive oil for malathion, and silicone oil for permethrin and deltamethrin. Adult mosquitoes were exposed for a period of 60 minutes with cumulative knockdown recorded at 5-minute intervals. As per the WHO recommendations, a mosquito was considered "knocked down" if it was unable to stand or fly in a coordinated way or had fallen to the bottom of the exposure tube. After exposure, all mosquitoes were transferred to holding tubes, fed with 10% sucrose, and mortality recorded after 24 hours. As per the WHO recommendations, a mosquito was classified as dead if it was immobile or unable to stand or fly in a coordinated manner [12].

2.5. Statistical Analysis. The cumulative knockdown observations recorded during the insecticide exposure period were analysed by probit statistical software SPSS version 21 (IBM Corporation, New York, USA) to determine knockdown times (KT_{10}, KT_{50}, and KT_{99}) and 95% confidence intervals. For each experiment, mortality rates were first corrected for control mortality where control mortality was >5% [13]. Assessment criteria for corrected (where applicable) mortality rates were as follows: 98–100% indicated full susceptibility, <98% suggested resistance was likely, and <90% confirmed the presence of resistance [12]. The Pearson chi-square test (χ^2) was used to estimate the goodness-of-fit and linear regression (R^2) was used to evaluate the linearity of the response over time. Unless otherwise stated, results are presented to one decimal place and statistical analyses to at least two significant figures.

3. Results

Both knockdown and mortality data for all strain-compound combinations are presented in Table 1 (male adults), Table 2 (female adults), and Figure 1. Upon exposure to a 4%

Table 1: Mortalities and knockdown times (KDT) for male adults of OX513A (OX) and wild-type *Aedes aegypti* from Aurangabad (AWD) and Delhi (DEL) against diagnostic concentrations of four synthetic insecticides.

Insecticide (% conc)	Strain (n)	Mortality 24 h (%) Mean ± SE	KDT in minutes (95% CI)			Regression line	R^2	Pearson chi-square test χ^2 (df)	p value	Susceptibility status
			KDT_{10}	KDT_{50}	KDT_{99}					
DDT 4%	OX (89)	90.9 ± 1.8[a]	17.6 (13.3–21.1)	41.3 (37.2–46.8)	194.0 (136.0–351.3)	$y = 1.21x - 8.0$	0.90	25.6 (10)	0.004	Resistance likely
	AWD (80)	87.4 ± 5.4[a]	34.7 (32.0–36.9)	57.5 (54.4–61.9)	143.5 (119.1–188.2)	$y = 0.80x - 12.9$	0.86	3.7 (10)	0.961	Resistant
	DEL (85)	44.4 ± 12.8[b]	*55.6	*320.2	*7681.2	$y = 0.18x - 1.5$	0.18	102.1 (10)	<0.0001	Resistant
			#$F = 10.28$ (df = 2), $p = 0.005$							
Malathion 5%	OX (92)	100.0 ± 0	22.7 (20.5–24.5)	33.6 (32.0–35.3)	68.9 (62.4–78.6)	$y = 1.99x - 22.6$	0.95	16.1 (10)	0.098	Susceptible
	AWD (92)	100.0 ± 0	26.4 (18.7–30.8)	38.1 (34.3–42.2)	59.3 (52.7–72.7)	$y = 2.02x - 28.9$	0.88	109.0 (10)	<0.0001	Susceptible
	DEL (89)	100.0 ± 0	25.5 (20.9–28.8)	41.3 (38.1–44.9)	99.2 (80.9–140.0)	$y = 1.54x - 20.1$	0.92	34.5 (10)	<0.0001	Susceptible
Deltamethrin 0.05%	OX (99)	100.0 ± 0	5.9 (4.7–6.9)	8.7 (7.6–9.6)	17.2 (14.8–22.1)	$y = 0.93x + 58.4$	0.34	21.7 (10)	<0.017	Susceptible
	AWD (97)	100.0 ± 0	6.2 (5.4–6.8)	8.6 (8.0–9.1)	15.5 (14.1–17.7)	$y = 0.90x + 57.6$	0.33	2.6 (10)	0.989	Susceptible
	DEL (100)	100.0 ± 0	9.6 (5.6–12.2)	14.8 (11.4–17.5)	32.6 (25.7–56.1)	$y = 1.60x + 26.5$	0.58	95.7 (10)	<0.0001	Susceptible
Permethrin 0.75%	OX (100)	100.0 ± 0	9.9 (8.1–11.0)	12.5 (11.3–13.7)	19.1 (16.7–25.1)	$y = 1.40x + 37.9$	0.42	31.9 (10)	<0.0001	Susceptible
	AWD (89)	100.0 ± 0	7.6 (5.4–9.2)	12.0 (10.2–13.7)	27.9 (23.0–38.8)	$y = 1.20x + 35.0$	0.50	35.8 (10)	<0.0001	Susceptible
	DEL (96)	100.0 ± 0	7.9 (4.8–10.4)	15.4 (12.1–18.2)	51.5 (39.1–84.6)	$y = 1.59x + 19.8$	0.80	69.3 (10)	<0.0001	Susceptible

[#]Differences in mean values indicated by the same letters within rows are nonsignificant at the 0.05 level by one-way ANOVA using Tukey's b-test; *CI not calculable.

Table 2: Mortalities and knockdown times (KDT) for female adults of OX513A (OX) and wild-type *Aedes aegypti* from Aurangabad (AWD) and Delhi (DEL) against diagnostic concentrations of four synthetic insecticides.

Insecticide (% conc)	Strain (n)	Mortality 24 h (%) Mean ± SE	KDT in minutes (95% CI)			Regression line	R^2	Pearson chi-square test χ^2 (df)	p value	Susceptibility status
			KDT_{10}	KDT_{50}	KDT_{99}					
DDT 4%	OX (97)	70.1 ± 5.2[a]	32.5 (28.2–35.6)	50.0 (46.9–54.2)	109.3 (89.8–153.3)	$y = 1.29x - 20.2$	0.86	26.6 (10)	0.003	Resistant
	AWD (99)	3.0 ± 2.0[b]	--	--	--	--	--	--	--	Resistant
	DEL (100)	6.0 ± 7.7[b]	[*]82.0	[*]158.0	[*]518.7	$y = 0.08x - 1.7$	0.23	22.3 (10)	0.014	Resistant
			[#]$F = 102.04$; df = 2; $p < 0.0001$							
Malathion 5%	OX (98)	100.0 ± 0[a]	33.0 (27.9–36.4)	45.3 (42.2–48.7)	80.2 (68.7–107.6)	$y = 1.69x - 27.7$	0.80	50.8 (10)	<0.0001	Susceptible
	AWD (98)	100.0 ± 0[a]	36.2 (24.1–41.9)	58.5 (50.8–84.3)	139.3 (92.1–640.1)	$y = 1.08x - 20.9$	0.57	79.4 (10)	<0.0001	Susceptible
	DEL (99)	98.0 ± 1.2[a]	42.2 (30.5–51.0)	92.9 (68.0–362.8)	390.0 (165.6–22565.6)	$y = 0.56x - 11.0$	0.60	44.2 (10)	<0.0001	Susceptible
			[#]$F = 3.00$; df = 2; $p = 0.101$							
Deltamethrin 0.05%	OX (99)	100.0 ± 0	8.1 (7.3–8.9)	12.0 (11.3–12.6)	24.3 (22.2–27.5)	$y = 1.34x + 39.1$	0.52	6.0 (10)	0.813	Susceptible
	AWD (98)	100.0 ± 0	11.7 (10.8–12.4)	15.1 (14.5–15.7)	23.8 (22.2–26.2)	$y = 1.62x + 24.5$	0.59	6.3 (10)	0.792	Susceptible
	DEL (100)	100.0 ± 0	10.5 (8.0–12.4)	16.7 (14.6–18.5)	38.7 (32.6–51.3)	$y = 1.76x + 17.6$	0.71	39.7 (10)	<0.0001	Susceptible
Permethrin 0.75%	OX (89)	100.0 ± 0	9.3 (8.5–9.9)	12.2 (11.6–12.7)	20.0 (18.4–22.5)	$y = 1.22x + 34.5$	0.50	1.5 (10)	0.999	Susceptible
	AWD (89)	100.0 ± 0	8.7 (6.1–10.9)	15.3 (12.8–17.6)	42.7 (34.7–60.1)	$y = 1.44x + 21.2$	0.64	44.9 (10)	<0.0001	Susceptible
	DEL (100)	100.0 ± 0	14.0 (12.5–15.2)	20.5 (19.3–21.6)	41.0 (37.1–46.8)	$y = 2.05x + 1.9$	0.82	15.3 (10)	0.123	Susceptible

[#]Differences in mean values indicated by the same letters within rows are nonsignificant at the 0.05 level by one-way ANOVA using Tukey's b-test; [*]CI not calculable; [--]knockdown rates were insufficient to calculate.

concentration of the organochlorine, DDT, 50% knockdown of OX513A male adults was achieved in 41.3 minutes and 24-hour mortality was 90.9%. These values were not significantly different from those for AWD males. Although CI were not calculable for knockdown of DEL males with DDT, the 24-hour mortality rate (44.4%) was significantly lower than both the other strains. For male and female adults, knockdown times (KDT_{50}) for OX513A against DDT were 41.3 and 50 minutes, respectively, and the 24-hour mortality value (70.1%) observed for female adults was significantly lower than observed for males (90.9%). In the same manner, females of both wild types also demonstrated a greater tolerance than males of their respective strains. In addition, the 24-hour mortalities for AWD and DEL females against DDT (3.0 and 6.0%, resp.) were significantly lower from that of OX513A females. When assessed against the WHO interpretation criteria, data for DDT were sufficient to confirm the presence of resistance in all three strains.

For the organophosphate compound, malathion, despite significant variations observed in KDT values no differences emerged between either males or females of the three strains with respect to mortality observed after 24 hours of exposure to malathion. Only DEL females showed any survival after 24-hour exposure to the 5% discriminating concentration, yielding a mortality value of 98%. When assessed against the WHO interpretation criteria, data for malathion indicated that all three strains were susceptible.

At the WHO prescribed discriminating concentrations, data for permethrin and deltamethrin were consistent across all three strains. Knockdown times were similar for both these pyrethroids and consistently shorter than for either DDT or malathion. There were no consistent differences in KDT values between males and females and both sexes of all three strains gave 24-hour mortalities of 100% (Tables 1 and 2). When assessed against the WHO interpretation criteria, data for permethrin and deltamethrin indicated that all three strains were susceptible.

4. Discussion

The WHO classification scheme for resistant and susceptible responses conveniently categorizes results for strains in accordance with predetermined boundaries. This provides a consistent, standardized approach and a useful tool for determining the likely efficacy of insecticidal products at specific geographic localities. However, for more detailed examination of phenotypes or the subtleties of resistance-gene frequencies, it is important to examine the full breadth of responses. For example, the WHO categorizations are unable to determine the extent of resistance within surviving individuals, which could be fundamental to predicting how resistance levels may change in response to applied doses.

Although final classifications for all strains were the same for each of the individual insecticides, for two of the compounds (DDT and malathion) significant differences between responses were apparent. When comparing 24-hour mortality rates for DDT across males and females, OX513A showed significantly less resistance than either Indian strain. Indeed, OX513A males yielded a value of >90% categorizing them as "resistance likely," contrasting with both wild-type males that scored as "resistance confirmed." There are numerous reports documenting the establishment and increase of resistance to DDT in *Ae. aegypti* as a worldwide phenomenon, likely due to prolonged usage of DDT in vector control programs [14–17] during the latter half of the last century. These data were consistent with those previously reported for OX513A in relation to Malaysian *Ae. aegypti* [18].

Presence of resistance to DDT in mosquito strains has been reported to correlate with moderate resistance to pyrethroids [19]. Our results demonstrated that the strains tested were resistant to DDT and yet susceptible to pyrethroids. Although specific resistance mechanisms against DDT in insects have not always been fully characterized, increased metabolism of DDT by the glutathione S-transferase (GST) enzyme family and mutations in *kdr* genes of voltage-gated sodium channels have both been associated with DDT resistance development. Both DDT and pyrethroids affect the nervous system by modifying the gating kinetics of voltage-sensitive sodium channels, and mutations in sodium channels can cause binding failures with DDT and pyrethroids, underlying reports of cross-resistance between DDT and pyrethroids [20–25]. In this study, the lack of cross-resistance to pyrethroids has implicated metabolic resistance as opposed to target-site alterations [16]. For malathion, DEL adult females (98%) were the only strain/sex combination to show survival at this application rate. Although technically scoring as susceptible in terms of the WHO classification system, the presence of a low frequency of organophosphate resistance genes in this population should not be overlooked. Malathion is routinely used in fogging operations for mosquito control during the peak epidemic periods of Japanese encephalitis and dengue in northeastern states, due to which the dengue vectors in the environment are at continuous selection. It has been suggested that this poses a threat to efficacy of malathion in the long-term [17].

The widespread use of synthetic insecticides against pests of medical importance has been ongoing for over 70 years. Despite our familiarity with such practices, our understanding of the risk-benefit relationships is still far from complete. Regardless of the situation to hand, it is clear that when alternatives exist, due to the fact that human and environmental risks that they can pose insecticidal treatments are most appropriate as a final line of defense. Deploying them in such a manner also minimizes any selection for resistance, helping to retain efficacy for as long as possible. It is only when insecticidal treatments are fully effective that they are capable of delivering reductions in vector prevalence significant enough to counterbalance the risks associated with their use. Our present tests clearly indicate that OX513A strain is equally susceptible to insecticides as the wild-type susceptible strains. Notably, OX513A strain is a self-limiting strain and would not persist in the environment when released in the environment as vector control tool, and the susceptibility of the strain towards the commonly used insecticides is an added benefit to impose risk mitigation plan and to clear them from the specific released environment if desired.

5. Conclusion

The problem is not the use of insecticides per se; it is the lack of credible alternatives capable of delivering life-saving performance without additional inherent risk. Contemporary genetic approaches, such as OX513A, may soon fill that gap. It is important to emphasize that OX513A strain is self-limiting strain and does not persist in the environment when released, thus the present susceptibility tests suggest the strain poses few long-term risks when implemented in the vector control program. In the meantime, regardless of the scientific rigor and ground-breaking potential, transparent evaluation and implementation of genetic technologies are persistently thwarted by a cultural and political aversion to the new, a fear of that which is not understood. A willingness to objectively comprehend and evaluate perceived risk is the key to overcoming such uncertainties; without which, antipathy towards genetic technologies is nothing short of an irrational phobia.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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