Susceptibility status of larval *Aedes aegypti* mosquitoes in Saudi Arabia

Ashwaq M Al Nazawi¹, Simon Ashall², David Weetman³

¹Preventive Medicine Department, Public Health Directorate, Ministry of Health, Jeddah 22246, Saudi Arabia

²School of Life Sciences, Keele University, Staffordshire, ST5 5BG, UK

³Department of Vector Biology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK

Abstract

Vector control programs worldwide are facing the challenge of mosquitoes becoming resistant to available insecticides. Larviciding is a crucial preventative measure for dengue control but data on insecticide resistance of larval *Ae. aegypti* in the Middle Eastern Region are limited. This study assesses the susceptibility status of *Ae. aegypti* collected from the two most important dengue foci in Saudi Arabia, Jeddah and Makkah, to important chemical and biological larvicides; the organophosphate temephos and *Bacillus thuringiensis israelensis* (*Bti*). Whilst worldwide, and particularly in Latin America, high-level resistance to temephos is common, Jeddah and Makkah populations exhibited full susceptibility to both temephos and *Bti*. These data suggest each can be considered by vector control programs for preventative dengue control in the region, as part of temporal rotations or spatial mosaics to manage insecticide resistance.

Key words: Mosquito larvae, Larval bioassay, *Bti*, temephos

***Corresponding author**

E-Mail: amalnazawi@moh.gov.sa (Dr. Ashwaq Alnazawi)

1.Introduction

In Saudi Arabia, insecticides are extensively used to combat mosquito-borne diseases and other household pests, as well as in agriculture (1). *Aedes aegypti* is primarily controlled by larvicides such as Spinosad (Natular®), *Bacillus thuringiensis israelensis* (*Bti*) toxin (VectoBac®), pyriproxyfen and diflubenzuron. Adulticides such as deltamethrin, permethrin, cyfluthrin and fenitrothion are also used for fogging and indoor residual spraying to reduce the density of adult mosquitoes during outbreak situations (2). Temephos, *Bti*, Spinosad and insect growth regulatory hormones such as pyriproxyfen are used as larvicides in breeding sites, but *Bti* and Spinosad are more common in Jeddah and Makkah (1, 3, 4). However, the extensive use of chemical insecticides has led to the development of insecticide resistance in *Ae. aegypti* worldwide including Saudi Arabia (4-11). In 2011, *Ae. aegypti* strains from Makkah were found to be resistant to lambda-cyhalothrin, deltamethrin, permethrin, bendiocarb and cyfluthrin (10, 11) but still susceptible to pirimiphos-methyl (actellic) and *Bacillus thuringiensis israelensis* *Bti* (Bacilod) (11). In addition, Jeddah strains showed high prevalence of resistance to the pyrethroid deltamethrin and permethrin and the carbamate bendiocarb (10) but no studies to date have been considered larvicides. In Jazan, the population was resistant to lambda-cyhalothrin, DDT, bendiocarb and showed moderate resistance to permethrin, deltamethrin and fenitrothion (yet remained susceptible to cyfluthrin) (4). The larvae were reported as highly resistant to temephos, but the documented LC₅₀ of 61.8 mg/L, appears unfeasibly high being far beyond the LC₅₀ reported for other temephos resistant populations in the world (12, 13) suggesting further investigation is essential.

A major limitation of the control program in the region is the limited surveillance to monitor the effectiveness of control intervention, or changes in the resistance of populations that may undermine the control efforts. We therefore assess the susceptibility status of the sole local dengue vector *Ae. aegypti* collected from Makkah of Saudi Arabia to larvicides (temephos and *Bti*). The outcome of this study will provide reliable, updated data on the resistance profile of larval *Ae. aegypti* populations from Saudi Arabia and may provide indication of which insecticides may be more effective.

2. Materials and Methods

2.1 Mosquito strain

Aedes aegypti larvae were collected from multiple breeding sites in two dengue endemic areas in Makkah (Lab= 21°45'2.13 N, 39°92'1.96 E; field=21°40'7.70 N, 39°86'3.19 E) and Jeddah (Lab=21°35'2.13 N, 39°13'9.42 E; field=21°60'3.97 N, 39°27'2.49 E). The lab strains were fifth generation from the original field which was collected in 03-04/2016. The field strain was collected in 01-02/2018. The larvae were reared as described by (10). Two reference strains, Cayman, a multiply resistant lab strain, though reported as lacking temephos resistance (14), and the standard (ubiquitously-susceptible) strain New Orleans were used. All strains were raised under the same standard insectary conditions at the Liverpool School of Tropical Medicine (10).

2.2 Larval Bioassays

Larval bioassays were carried out on *Aedes* strains shown in **Table 1** according to the WHO protocol (15) to determine the lethal concentrations (LC₅₀) and the resistance ratio relative to New Orleans (RR₅₀).

Table 1 Number of *Ae. aegypti* larvae from Jeddah and Makkah used in each bioassay at different times.

Temephos assay		<i>Bacillus thuringiensis israelensis</i> assay	
Strain	Sample size	Strain	Sample size
New Orleans	900	New Orleans	650
Makkah lab strain	900	Makkah lab strain	590
Jeddah lab strain	900	Jeddah lab strain	650
		Makkah field strain	546
		Jeddah field strain	567
		Cayman	605

Bioassays were performed using temephos (Sigma-Aldrich, Dorset, UK), or *Bti* (Vectobac®12AS 1.2%, 1200 ITU/mg. A total of eight different concentrations of *Bti* and nine of temephos were used for each strain (**Table 2&3**). The concentrations were selected as they have been reported to result in larval mortality between 10% and 95% (15). The

data was used to calculate the lethal dose that kills 50% (LC₅₀) in each population. Dilutions of temephos (stock dissolved in absolute ethanol) with distilled water up to a total volume of 200mL are detailed in **Table 2**. For each concentration of each insecticide, three or four replicates of a pool of approximately 25 late third or early fourth instar larvae were tested along with a negative control pool; 1mL absolute ethanol mixed into 199 mL of distilled water for temephos or into 100ml of distilled water for *Bti* assays.

Table 2 Temephos stock dilution with distilled water up to 200 ml to obtain the appropriate final concentrations.

Final concentration (mg/L)	Volume of stock solution (mL)	Volume of distilled water (mL)
0.08	1	199
0.07	0.875	199.125
0.06	0.75	199.25
0.04	0.5	199.5
0.03	0.375	199.625
0.02	0.25	199.75
0.01	0.125	199.875
0.005	0.0625	199.9375
0.0025	0.03125	199.96875

Table 3 *Bacillus thuringiensis israelensis* (*Bti*) stock dilution with distilled water from the stock solution at 1.2%.

C1 (stock) ppm	Volume of stock solution (mL)	Desired concentration (100mL)-ppm	Volume 2 (mL)
120	0.005	0.0059997	100.005
120	0.003	0.003599892	100.003
120	0.002	0.002399952	100.002
120	0.001	0.001199988	100.001
120	0.00075	0.000899993	100.00075
120	0.0005	0.000599997	100.0005
120	0.0002	0.00024	100.0002
120	0.0001	0.00012	100.0001

Vectobac stock (1.2%) was diluted by adding 1ml of the stock (1.2%) to 99 ml distilled water to obtain 0.012% (120ppm) which was used in the experiment.

All larval bioassays were performed in 6 cm in diameter plastic bowls; Mortality was recorded after 24h of exposure. Any larvae failing or unable to swim up to the surface

independently were counted as dead. Any larvae that had pupated during exposure were omitted from the total count.

2.3 Statistical analysis

The mortality (%) was calculated for the number of mosquitoes or larvae that were dead after 24h exposure. The LC₅₀ value for the larval bioassays was calculated using probit regression analysis (SPSS version 24). If chisq >0.05, confidence limits were adjusted accordingly (SPSS does this unless the fit is terrible, if it is it will not calculate CIs). The resistance ratio (RR) was calculated by comparison of the resistant Makkah and Jeddah strains against the susceptible New Orleans strain using the formula below to monitor the level of insecticide resistance in a field population.

$$\text{Resistance ratio (RR)} = \frac{\text{LC}_{50} \text{ of resistant strain}}{\text{LC}_{50} \text{ of susceptible strain}}$$

3. Results & Discussion

3.1 Larval bioassays

Mortality was not observed in any strain in the control assays. Based on the mortality rate across different concentrations of temephos and *Bti*, resistance to the larvicides was higher in field strains when compared to the New Orleans strain (**Table 4 and Table 5**).

Table 4 Average percentage mortality of *Ae. aegypti* larvae from Makkah and Jeddah and the susceptible strain, New Orleans exposed to nine concentrations of temephos.

Strain	Temephos Concentration (mg/L)								
	0.0025 (%)	0.005 (%)	0.01 (%)	0.02 (%)	0.03 (%)	0.04 (%)	0.06 (%)	0.07 (%)	0.08 (%)
Makkah	0	0	9.18	70	89.6	100	99	99	100
Jeddah	1	0	1.02	32	79	73	94.8	98	100
New Orleans	5.2	9.9	52.6	81	96	99	100	100	100

Table 5 Average percentage mortality of *Ae. aegypti* larvae from Makkah, Jeddah (field and lab strains), New Orleans and Cayman strains exposed to eight concentrations of *Bti*.

Strain	<i>Bacillus thuringiensis israelensis</i> (<i>Bti</i>) Concentration (ppm)							
	0.00012 (%)	0.00024 (%)	0.0006 (%)	0.00089 (%)	0.0012 (%)	0.002 (%)	0.0036 (%)	0.006 (%)
Makkah field	10	19.3	26.2	58.1	63.2	98.8	98.4	100
Jeddah field	0	0	16.4	48.6	59.2	94.9	97.5	100
Jeddah lab	0	5	12.7	21.9	32.2	72.9	93.6	100
New Orleans	20.5	22.9	59.5	70	98.9	100	100	100
Cayman	5.3	12.5	21.6	43.1	52.2	95	100	100

Indeed in both the temephos bioassays, the LC₅₀ confidence intervals were not overlapping in comparisons of any strain, indicating a significant difference in mortality between the strains (**Table 6**). However, whilst there is significant variation in susceptibility, current guidelines (16), suggest that a resistance ratio <5 indicates limited/no resistance; 5-10 moderate resistance, and >10 is substantial resistance. Therefore, based on this classification, no definitive resistance to temephos and *Bti* was identified in any of the strains tested.

Table 6 Lethal concentrations of temephos and *Bti* that kills 50% of *Ae. aegypti* strains.

Strain	Temephos assay		<i>Bti</i> assay	
	LC ₅₀ , mg/L (95% C.I.)	RR	LC ₅₀ , ppm (95% C.I.)	RR
New Orleans	0.010 ^a (0.009-0.011)	1	0.00041 ^a (0.000276-0.000537)	1
Makkah lab	0.017 ^b (0.014-0.019)	1.7	n/a	n/a
Jeddah lab	0.029 ^c (0.025-0.034)	2.9	0.001483 ^b (0.001341-0.001629)	3.6
Makkah field	n/a		0.000834 ^c (0.000688-0.000982)	2.1
Jeddah field	n/a		0.00098 ^{b,c} (0.000882-0.001076)	2.4
Cayman	n/a		0.001018 ^{b,c} (0.000882-0.001157)	2.5

Shared letters within a column indicate no significant difference based on overlapping confidence limits. The Makkah lab *Bti* assay was not calculated (n/c) because a very poor fit of the probit model meant that LC₅₀ confidence intervals could not be reliably estimated even with a correction factor. Makkah and Jeddah field strains were not assessed (n/a) with temephos because the strains were not available at LSTM. The RR shown in the table indicates the resistance ratio.

200

201 The current study was conducted to assess the susceptibility of larval *Ae. aegypti* to

202 commonly used insecticides in the cities of Jeddah and Makkah. Larval bioassays did not

203 detect resistance in either Makkah or Jeddah to temephos or *Bti* (all resistance ratios <5

204 compared to a standard susceptible strain (17). In contrast, extreme temephos resistance in

205 *Ae. aegypti* larvae from Jazan (LC₅₀=61.8 mg/L) was reported in 2016 (4). When compared

206 to the average LC₅₀ of multiple separate studies of the susceptible reference strains

207 Rockefeller, New Orleans and Bora Bora (18), this equates to a resistance ratio above

208 10,000, far exceeding the highest ratio of 224 previously recorded (in Brazil; (18)). This

209 estimate from Jazan thus appears unlikely to be correct, and in the absence of additional

210 data, a provisional assessment of temephos susceptibility in Saudi Arabia seems

211 appropriate.

212 Temephos resistance in *Ae. aegypti* larvae has been recorded globally including British

213 Virgin Islands (19), Thailand (20), Brazil (21), Cuba (22), Colombia (23), Martinique (24)

214 and Santiago island (25). Whilst the current data suggest susceptibility, it is important to

215 note that both Saudi Arabian strains showed significantly higher LC₅₀ values than the

216 susceptible New Orleans strain.

217 *Bacillus thuringiensis israelensis* (*Bti*) is a bacterial derived toxin that has been widely

218 used for vector control. The populations from Jeddah and Makkah were susceptible to this

219 compound in comparison (based on a resistance ratio <5) with the New Orleans strain.

220 Almost all other studies have reported similar findings, including Martinique populations

221 (highly resistant to most insecticides) that were susceptible to *Bti* compared to the Bora-

222 Bora strain, Santiago island, Cameroon and Malaysia (18). Although *Bti* resistance is

223 apparently absent in *Ae. aegypti* populations to date, resistance has detected in *Culex*

224 *pipiens*, from Syracuse, New York which had a resistance ratio of 33-fold when compared

225 to the S-Lab susceptible strain (26). Resistance to *Bti* has also been demonstrated in *Aedes*

226 *rusticus* Rossi mosquitoes, selected for resistance through annual *Bti* treatment in larval

227 sites in the Rhône-Alpes region. The mosquitoes collected in the treatment area had a

228 moderate resistance ratio up to 7.9 fold compared to the untreated area (27). The attained

229 resistance levels were still relatively low compared to when mosquitoes are selected for

230 resistance to other insecticides (27). The multiple active toxins-Cry4A, Cry4B, Cry11A

and Cyt1A- produced by *Bti* might act at different receptors, making evolution of resistance to *Bti* very difficult (28). Nevertheless, both Saudi populations showed a significantly higher LC₅₀ value than the New Orleans strain, suggesting that, as with temephos, variation exists which might be selected to higher levels in future.

Therefore, although the *Ae. aegypti* population in Jeddah and Makkah are still susceptible to temephos, rotational application of *Bti* and temephos, or another larvicide to which there is full susceptibility, would be advisable to slow down evolution of resistance to either of them thus retaining their efficacy over extended periods of use in vector control. Our findings suggest the potential to develop resistance to both insecticides may exist and thus mixture or rotation is advisable, along with continued monitoring, and consideration of other options such as insecticide growth regulators.

4. Conclusion

Aedes aegypti from Makkah and Jeddah remain susceptible to the larvicides assessed in this study and thus larval source management and larviciding could remain an effective tool in control, but regular monitoring and consideration of additional alternatives is advised.

Acknowledgment

The authors are indebted all the municipal staff (administrators and laboratory technicians in the vector control programme) of Makkah and Jeddah in Saudi Arabia. We are also thankful to Dr Craig Wilding in Liverpool John Moores University for his co-operation during this work.

References

1. Al-Shami SA, Mahyoub JA, Hatabbi M, Ahmad AH, Rawi CSMJP, vectors. An update on the incidence of dengue gaining strength in Saudi Arabia and current control approaches for its vector mosquito. 2014;7(1):258.
2. World Health O. Dengue haemorrhagic fever : diagnosis, treatment, and control. Geneva: World Health Organization; 1997.
3. Mahyoub JA, Ghramh HA, AL-Ghamdi K, Farooqi N. The Potency of Aedes species in transmitting dengue fever virus with evaluating the susceptibility of vector larval stages to some insecticides. Egyptian Academic Journal of Biological Sciences C, Physiology and Molecular Biology. 2013;5(2):109-15.
4. Alsheikh A, Mohammed W, Noureldin E, Daffalla O, Shrwani K, Hobani Y, et al. Resistance status of Aedes aegypti to insecticides in the Jazan Region of Saudi Arabia. Biosci, Biotech Res Asia. 2016;13(1):155-62.
5. Yaicharoen R, Kiatfuengfoo R, Chareonviriyaphap T, Rongnoparut P. Characterization of deltamethrin resistance in field populations of Aedes aegypti in Thailand. Journal of Vector Ecology. 2005;30(1):144.
6. Ranson H, Burhani J, Lumjuan N, Black IV WC. Insecticide resistance in dengue vectors. TropIKA net [online]. 2010;1(1).
7. Marcombe S, Carron A, Darriet F, Etienne M, Agnew P, Tolosa M, et al. Reduced efficacy of pyrethroid space sprays for dengue control in an area of Martinique with pyrethroid resistance. The American journal of tropical medicine and hygiene. 2009;80(5):745-51.
8. Jirakanjanakit N, Saengtharatip S, Rongnoparut P, Duchon S, Bellec C, Yoksan S. Trend of temephos resistance in Aedes (Stegomyia) mosquitoes in Thailand during 2003–2005. Environmental entomology. 2014;36(3):506-11.
9. Rodríguez MM, Bisset JA, De Armas Y, Ramos F. Pyrethroid insecticide-resistant strain of Aedes aegypti from Cuba induced by deltamethrin selection. Journal of the American Mosquito Control Association. 2005;21(4):437-45.
10. Al Nazawi AM, Aqili J, Alzahrani M, McCall PJ, Weetman D. Combined target site (kdr) mutations play a primary role in highly pyrethroid resistant phenotypes of Aedes aegypti from Saudi Arabia. Parasites & vectors. 2017;10(1):161.
11. Aziz AT, Dieng H, Hassan AA, Satho T, Miake F, Salmah MRC, et al. Insecticide susceptibility of the dengue vector Aedes aegypti (Diptera: culicidae) in Makkah City, Saudi Arabia. Asian Pacific Journal of Tropical Disease. 2011;1(2):94-9.
12. Biber PA, Dueñas JR, Almeida FL, Gardenal CN, Almirón WR. Laboratory evaluation of susceptibility of natural subpopulations of Aedes aegypti larvae to temephos. Journal of the American Mosquito Control Association. 2006;22(3):408-11.
13. dos Santos Dias L, da Graca Macoris MdL, Andrighetti MTM, Otrera VCG, dos Santos Dias A, da Rocha Bauzer LGS, et al. Toxicity of spinosad to temephos-resistant Aedes aegypti populations in Brazil. PLoS One. 2017;12(3).
14. Harris AF, Rajatileka S, Ranson H. Pyrethroid resistance in Aedes aegypti from Grand Cayman. The American journal of tropical medicine and hygiene. 2010;83(2):277-84.

15. World Health Organization. Guidelines for laboratory and field testing of mosquito larvicides. 2005.
16. Mazzarri MB, Georghiou GP. Characterization of resistance to organophosphate, carbamate, and pyrethroid insecticides in field populations of *Aedes aegypti* from Venezuela. Journal of the American Mosquito Control Association. 1995;11(3):315-22.
17. World Health Organization. Monitoring and managing insecticide resistance in *Aedes* mosquito populations. 2016.
18. Moyes CL, Vontas J, Martins AJ, Ng LC, Koou SY, Dusfour I, et al. Contemporary status of insecticide resistance in the major *Aedes* vectors of arboviruses infecting humans. PLoS neglected tropical diseases. 2017;11(7).
19. Wirth MC, Georghiou G. Selection and characterization of temephos resistance in a population of *Aedes aegypti* from Tortola, British Virgin Islands. Journal of the American Mosquito Control Association. 1999;15(3):315-20.
20. Ponlawat A, Scott JG, Harrington LC. Insecticide susceptibility of *Aedes aegypti* and *Aedes albopictus* across Thailand. Journal of Medical Entomology. 2005;42(5):821-5.
21. Melo-Santos M, Varjal-Melo J, Araújo A, Gomes T, Paiva M, Regis L, et al. Resistance to the organophosphate temephos: mechanisms, evolution and reversion in an *Aedes aegypti* laboratory strain from Brazil. Acta Tropica. 2010;113(2):180-9.
22. Bisset J, Rodríguez M, Ricardo Y, Ranson H, Perez O, Moya M, et al. Temephos resistance and esterase activity in the mosquito *Aedes aegypti* in Havana, Cuba increased dramatically between 2006 and 2008. Medical and Veterinary Entomology. 2011;25(3):233-9.
23. Grisales N, Poupardin R, Gomez S, Fonseca-Gonzalez I, Ranson H, Lenhart A. Temephos resistance in *Aedes aegypti* in Colombia compromises dengue vector control. PLoS neglected tropical diseases. 2013;7(9):e2438.
24. Marcombe S, Mathieu RB, Pocquet N, Riaz M-A, Poupardin R, Sélior S, et al. Insecticide resistance in the dengue vector *Aedes aegypti* from Martinique: distribution, mechanisms and relations with environmental factors. PLoS ONE. 2012;7(2):e30989.
25. Rocha HDR, Paiva MHS, Silva NM, De Araujo AP, De Azevedo Camacho DRR, Da Moura AJF, et al. Susceptibility profile of *Aedes aegypti* from Santiago Island, Cabo Verde, to insecticides. Acta Tropica. 2015;152:66-73.
26. Paul A, Harrington LC, Zhang L, Scott JG. Insecticide resistance in *Culex pipiens* from New York. Journal of the American Mosquito Control Association. 2005;21(3):305-9.
27. Boyer S, Paris M, Jegou S, Lempérière G, Ravanel P. Influence of insecticide *Bacillus thuringiensis subsp. israelensis* treatments on resistance and enzyme activities in *Aedes rusticus* larvae (Diptera: Culicidae). Biological Control. 2012;62(2):75-81.
28. Wirth MC. Mosquito resistance to bacterial larvicidal toxins. The open Toxinology Journal. 2013;3(1):126-40.