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The Entomological Society of Korea



Susceptibility status of larval Aedes aegypti mosquitoes in the Western Region of Saudi Arabia

Journal:	Entomological Research
Manuscript ID	ENR-21-012.R1
Manuscript Type:	Research Paper
Date Submitted by the Author:	08-Mar-2021
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Keywords:	1.000 Biology, Ecology and Behaviour, 3.003 Insecticide resistance < 3.000 Medical and Veterinary Entomology, 10.000 Molecular entomology and ecology
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SCHOLARONE[™] Manuscripts

Susceptibility status of larval Aedes aegypti mosquitoes in Saudi Arabia 56 7 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 temphos 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 temphos 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 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 temphos, but the documented LC_{50} of 61.8 mg/L, appears unfeasibly high being far beyond the LC_{50} 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.

43 Materials and Methods

44 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).

53 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 (LC_{50}) and the resistance ratio relative to New Orleans (RR_{50}).

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3	56	<table 1=""></table>
4	57	
5	58	Bioassays were performed using temephos (Sigma-Aldrich, Dorset, UK), or <i>Bti</i> (Vectobac®12AS 1.2%, 1200
6	59	ITU/mg. A total of eight different concentrations of <i>Bti</i> and nine of temephos were used for each strain (Table
7	60	2&3). The concentrations were selected as they have been reported to result in larval mortality between 10%
8	61	and 95% (15). The data was used to calculate the lethal dose that kills 50% (LC ₅₀) in each population.
9	62	Dilutions of temephos (stock dissolved in absolute ethanol) with distilled water up to a total volume of 200mL
10	63	are detailed in Table 2. For each concentration of each insecticide, three or four replicates of a pool of
11	64	approximately 25 late third or early fourth instar larvae were tested along with a negative control pool;1mL
12	65	absolute ethanol mixed into 199 mL of distilled water for temephos or into 100ml of distilled water for Bti
13	66	assays.
14	67	<table 2=""></table>
15	68	<table 3=""></table>
16	69	
17	70	Vectobac stock (1.2%) was diluted by adding 1ml of the stock (1.2%) to 99 ml distilled water to obtain
12	71	0.012% (120pmm) which was used in the experiment.
10	72	All larval bioassays were performed in 6 cm in diameter plastic bowls; Mortality was recorded after 24h of
20	73	exposure. Any larvae failing or unable to swim up to the surface independently were counted as dead. Any
20	74	larvae that had pupated during exposure were omitted from the total count.
21	75	
22	76	Statistical analysis
23	77	The mortality (%) was calculated for the number of mosquitoes or larvae that were dead after 24h exposure.
24	78	The LC ₅₀ value for the larval bioassays was calculated using probit regression analysis (SPSS version 24). If
25	79	chisq >0.05, confidence limits were adjusted accordingly (SPSS does this unless the fit is terrible, if it is it
26	80	will not calculated Cis). The resistance ratio (RR) was calculated by comparison of the resistant Makkah and
27	81	Jeddah strains against the susceptible New Orleans strain using the formula below to monitor the level of
28	82	insecticide resistance in a field population.
29	83	Resistance ratio (RR) = LC_{50} of resistant strain
30	84	LC ₅₀ of susceptible strain
31	85	
32	86	
33	87	Results
34	88	Larval bioassays
35	89	Mortality was not observed in any strain in the control assays. Based on the mortality rate across different
36	90	concentrations of temephos and <i>Bti</i> , resistance to the larvicides was higher in field strains when compared to
37	91	the New Orleans strain (Table 4 and Table 5).
38	92	<1 able 4>
39	93	<1 able >>
40	94	Indeed in both the temphos bloassays, the LU_{50} confidence intervals were not overlapping in comparisons
41	93	of any strain, indicating a significant difference in mortality between the strains (1 able 6). However, whilst
42	90 07	indicates limited/no registences 5 10 moderate registence and 10 is substantial registence. Therefore, head
43	97	an this closefficient no definitive resistance, to temperhap and <i>Pti</i> was identified in any of the strains tested
44	90	on this classification, no definitive resistance to temphos and <i>Bit</i> was identified in any of the strains tested.
45	100	
46	100	Table 62
47	101	
48	102	Diceussion
49	103	The current study was conducted to assess the suscentibility of larval Ae geownti to commonly used
50	104	insecticides in the cities of leddah and Makkah Larval bioassays did not detect resistance in either Makkah
51	105	or leddah to temephos or Bti (all resistance ratios <5 compared to a standard suscentible strain (17). In
52	107	contrast extreme temphos of Dir (an resistance in A_{ρ} account larvae from Jazan (I C_{co} -61.8 mg/L) was reported in
53	108	2016 (4) When compared to the average I C_{50} of multiple separate studies of the susceptible reference strains
54	109	Rockefeller New Orleans and Bora Bora (18) this equates to a resistance ratio above 10 000 far exceeding
55	110	the highest ratio of 224 previously recorded (in Brazil: (18)) This estimate from Iazan thus appears unlikely
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Saudi Arabia seems appropriate.

higher LC_{50} values than the susceptible New Orleans strain.

variation exists which might be selected to higher levels in future.

other options such as insecticide growth regulators.

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to be correct, and in the absence of additional data, a provisional assessment of temephos susceptibility in

Temephos resistance in Ae. aegypti larvae has been recorded globally including British Virgin Islands (19),

Thailand (20), Brazil (21), Cuba (22), Colombia (23), Martinique (24) and Santiago island (25). Whilst the

current data suggest susceptibility, it is important to note that both Saudi Arabian strains showed significantly

Bacillus thuringiensis israelensis (Bti) is a bacterial derived toxin that has been widely used for vector

control. The populations from Jeddah and Makkah were susceptible to this compound in comparison (based

on a resistance ratio <5) with the New Orleans strain. Almost all other studies have reported similar findings,

including Martinique populations (highly resistant to most insecticides) that were susceptible to Bti compared

to the Bora-Bora strain, Santiago island, Cameroon and Malaysia (18). Although *Bti* resistance is apparently

absent in Ae. aegypti populations to date, resistance has detected in Culex pipiens, from Syracuse, New York

which had a resistance ratio of 33-fold when compared to the S-Lab susceptible strain (26). Resistance to Bti

has also been demonstrated in Aedes rusticus Rossi mosquitoes, selected for resistance through annual Bti

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

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

were still relatively low compared to when mosquitoes are selected for 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 LC_{50} value than the New Orleans strain, suggesting that, as with temphos,

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

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139 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.

and consideration of add 143 Authors' contributions

AMA-N collected the field samples, performed the larval bioassays, analysed data and drafted the manuscript. SA conducted the larval bioassays and analysed data. DW conceived and designed the experiments, drafted the manuscript and analysed data. All authors read and approved the final manuscript.

- 40 147 Acknowledgment
- 148 The authors are indebted all the municipal staff (administrators and laboratory technicians in the vector 149 control programme) of Makkah and Jeddah in Saudi Arabia. We are also thankful to Dr Craig Wilding in 150 Liverpool John Moores University for his co-operation during this work.

43 150 Elverpool John Moore

- 44 152 The authors declare no conflicts of interest.
 - 153 Funding
- 46 154 This work was supported by a PhD Studentship from the Saudi Cultural Bureau to Ashwaq M Al Nazawi 47 155

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

Temephos a	assay	Bacillus thuringiensis israelensis 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	

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

Final concentration	Volume of stock solution	Volume of distilled water
(mg/L)	(mL)	(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	Desired concentration	Volume 2
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

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.

	Temephos Concentration (mg/L)								
Strain	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*.

	Bacillus thuringiensis israelensis (Bti) Concentration (ppm)							
Strain	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
Inclu Inclu	0	0	16.4	19 6	50.2	04.0	07.5	100
Jeddan neid	0	0	10.4	48.0	39.2	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

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

53			-		
		Temeph	nos assay	Bti as	say
	Strain	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	0.00041ª (0.000276-0.000537)	1
	Makkah lab	0.017 ^b (0.014-0.019)	1.7	n/a	n/a
	Jeddah lab	0.029° (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 LC_{50} 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.

$ \begin{array}{c} 1\\ 2\\ 3\\ 4\\ 5\\ 6\\ 7\\ 8\\ 9\\ 10\\ 11\\ 12\\ 13\\ 14\\ 15\\ 16\\ 17\\ 18\\ 19\\ 20\\ 21\\ 22\\ 23\\ 24\\ 25\\ 26\\ 27\\ 28\\ 29\\ 30\\ 31\\ 32\\ 33\\ 34\\ 35\\ 36\\ 37\\ 38\\ 39\\ 40\\ 41\\ 42\\ 43\\ 44\\ 45\\ 46\\ 47\\ 48\\ 49\\ 50\\ 51\\ 52\\ 53\\ 54\\ 55\\ 55\\ 55\\ 55\\ 55\\ 55\\ 55\\ 55\\ 55$	261 262				
51 52 53 54 55 56 57 58			7	7	
59 60					

Temephos	s assay	Bacillus thuringiensis israelensis 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	

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

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

Final concentration	Volume of stock solution	Volume of distilled water
(mg/L)	(mL)	(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	Volume of stock solution	Desired concentration	Volume 2
(stock) ppm	(mL)	(100mL)-ppm	(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

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.

	Temephos Concentration (mg/L)								
Strain	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*.

		Bacillus thuringiensis israelensis (Bti) Concentration (ppm)						
Strain	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

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

	Temephos assay		Bti assay	
Strain	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° (0.025-0.034)	2.9	0.001483 ^b (0.001341-0.001629)	3.6
Makkah field	n/a		0.000834° (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 LC_{50} 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.

Susceptibility status of larval *Aedes aegypti* mosquitoes in the Western **Region of Saudi Arabia**

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 temphos 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. Larval bioassays did not detect resistance in Makkah and Jeddah to temephos or *Bti* where a resistance ratio <5 compared to the New Orleans susceptible strain. 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.

Larval bioassays did not detect resistance in either Makkah or Jeddah to temephos or Bti (all resistance

- ratios <5 compared to a standard susceptible strain
- Key words: Mosquito larvae, Larval bioassay, Bti, temephos 22

Introduction

In Saudi Arabia, insecticides are extensively used to combat mosquito-borne diseases and other household pests, as well as in agriculture (Aziz et al., 2014). 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 (World Health Organization, 1997). 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 (Aziz et al., 2014, Mahyoub et al., 2013, Alsheikh et al., 2016). However, the extensive use of chemical insecticides has led to the development of insecticide resistance in Ae. aegypti worldwide including Saudi Arabia (Yaicharoen et al., 2005, Ranson et al., 2010, Marcombe et al., 2009, Jirakanjanakit et al., 2014, Rodríguez et al., 2005, Al Nazawi et al., 2017, Aziz et al., 2011, Alsheikh et al., 2016). In 2011, Ae. aegypti strains from Makkah were found to be resistant to lambda-cyhalothrin, deltamethrin, permethrin, bendiocarb and cyfluthrin (Aziz et al., 2011, Al Nazawi et al., 2017) but still susceptible to pirimiphos-methyl (actellic) and Bacillus thuringiensis israelensis Bti (Bacilod) (Aziz et al., 2011). In addition, Jeddah strains showed high prevalence of resistance to the pyrethroid deltamethrin and permethrin and the carbamate bendiocarb (Al Nazawi et al., 2017) 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 (vet remained susceptible to cyfluthrin) (Alsheikh et al., 2016). The larvae were reported as highly resistant to temphos, but the documented LC_{50} of 61.8 mg/L, appears unfeasibly high being far beyond the LC₅₀ reported for other temephos resistant populations in the world (Biber et al., 2006, dos Santos Dias et al., 2017) 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.

Materials and Methods

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,

2		
3	56	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
4	57	which was collected in 03-04/2016(Al Nazawi et al., 2017). The field strain was collected in 01-02/2018.
5	58	The larvae were reared to adults under insectary conditions of 27 ± 2 °C, 75% $\pm 10\%$ R.H and L12:D12
6	59	photoperiod as described by (Al Nazawi et al., 2017). Two reference strains, Cayman, a multiply resistant
7	60	lab strain, though reported as lacking temephos resistance (Harris et al., 2010), and the standard
8	61	(ubiquitously-susceptible) strain New Orleans were used. All strains were raised under the same standard
9	62	insectary conditions at the Liverpool School of Tropical Medicine (Al Nazawi et al., 2017).
10	63	Larval Bioassays
11	64	Larval bioassays were carried out on <i>Aedes</i> strains shown in Table 1 according to the WHO protocol (World
12	65	Health Organization, 2005) to determine the lethal concentrations (LC ₅₀) and the resistance ratio relative to
13	66 67	New Orleans (RR ₅₀).
14	68	
15	60	Bioassaus were performed using temephos (Sigma Aldrich Dorset UK) or Rti (Vestabos®12AS 1.2% 1200
16	70	ITU/mg. A total of eight different concentrations of <i>Bti</i> and nine of temenhos were used for each strain (Table
17	70	2&3) The concentrations were selected as they have been reported to result in larval mortality between 10%
18	72	and 95% (World Health Organization 2005). The data were used to calculate the lethal dose that kills 50%
19	$7\bar{3}$	(LC_{50}) in each population. Dilutions of temephos (stock dissolved in absolute ethanol) with distilled water
20	74	up to a total volume of 200mL are detailed in Table 2. For each concentration of each insecticide, three or
21	75	four replicates of a pool of approximately 25 late third or early fourth instar larvae were tested along with a
22	76	negative control pool;1mL absolute ethanol mixed into 199 mL of distilled water for temephos or into 100ml
23	77	of distilled water for <i>Bti</i> assays.
24	78	<table 2=""></table>
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20	80	
27	81	Vectobac stock (1.2%) was diluted by adding 1ml of the stock (1.2%) to 99 ml distilled water to obtain
28	82	0.012% (120pmm) which was used in the experiment.
29	83	All larval bioassays were performed in 6 cm in diameter plastic bowls; Mortality was recorded after 24h of
30 21	04 85	exposure. Any larvae failing or unable to swim up to the surface independently were counted as dead. Any
ו כ כי	85 86	larvae that had pupated during exposure were officied from the total count.
22	87	Statistical analysis
34	88	The mortality (%) was calculated for the number of mosquitoes or larvae that were dead after 24h exposure
35	89	The LC ₅₀ value for the larval bioassays was calculated using probit regression analysis (SPSS version 24)
36	90	(Finney, 1971). If chisq >0.05, confidence limits were adjusted accordingly (SPSS does this unless the fit is
37	91	terrible, if it is it will not calculated Cis) (Finney, 1971). The resistance ratio (RR) was calculated by
38	92	comparison of the resistant Makkah and Jeddah strains against the susceptible New Orleans strain using the
39	93	formula below to monitor the level of insecticide resistance in a field population.
40	94	Resistance ratio (RR) = LC_{50} of resistant strain
41	95	$L\overline{C_{50}}$ of susceptible strain
42	96	
43	9/	
44	98	Results
45	99 100	Larval bloassays Mortality was not observed in any strain in the control assays. Decad on the mortality rate agrees different
46	100	concentrations of temenhos and <i>Rti</i> resistance to the larvicides was higher in field strains when compared to
47	102	the New Orleans strain (Table 4 and Table 5)
48	102	<pre>Table 4></pre>
49	104	<table 5=""></table>
50	105	Indeed in both the temphos bioassays, the LC_{50} confidence intervals were not overlapping in comparisons
51	106	of any strain, indicating a significant difference in mortality between the strains (Table 6). However, whilst
52	107	there is significant variation in susceptibility, current guidelines (Mazzarri and Georghiou, 1995), suggest
53	108	that a resistance ratio <5 indicates limited/no resistance; 5-10 moderate resistance, and >10 is substantial
54	109	resistance. Therefore, based on this classification, no definitive resistance to temephos and Bti was identified
55	110	in any of the strains tested.
56	111	
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3	112	
4	113	<table 6=""></table>
5	114	
6	115	Discussion
7	116	The current study was conducted to assess the suscentibility of larval Ae approxitient to commonly used
8	117	insecticides in the cities of leddah and Makkah. Larval bioassays did not detect resistance in either Makkah
0 0	118	or leddah to temenhos or <i>Bti</i> (all resistance ratios <5 compared to a standard suscentible strain (World Health
5 10	110	Organization 2016). In contrast, extreme temphos resistance in A_{ext} account larvae from Jaran (I $C_{ex}=61.8$
10	120	mg/L) was reported in 2016 (Alsheikh et al. 2016). When compared to the average L C_{cc} of multiple separate
11	120	studies of the suscentible reference strains Rockefaller New Orleans and Rora Rora (Moves et al. 2017).
12	121	this equates to a resistance ratio above 10,000 for exceeding the highest ratio of 224 previously recorded (in
13	122	Brazil: (Moves et al. 2017)) This estimate from Jazan thus appears unlikely to be correct and in the absence
14	123	of additional data, a provisional assassment of temphos suspentibility in Saudi Arabia same appropriate
15	124	Tamenhos resistance in <i>Ac. accunti</i> larvae has been recorded globally including British Virgin Islands (Wirth
16	125	and Georghiou (1000). Theiland (Donlowet et al. 2005). Brogil (Melo Santos et al. 2010). Cuba (Bisset et
17	120	alu Georginou, 1999), Thananu (Foliawat et al., 2003), Biazii (Meio-Sainos et al., 2010), Cuba (Bisset et al., 2011), Calambia (Crisalas et al., 2012) Martinigua (Mercamba et al., 2012) and Santiago island (Pasha
18	127	at., 2011), Coloniola (Orisales et al., 2013), Martinique (Marconioe et al., 2012) and Santiago Island (Nocia at al. 2015). Whilet the current date suggest susceptibility, it is important to note that both Soudi Archien
19	120	et al., 2015). Whilst the current data suggest susceptionity, it is important to note that both saudi Arabian strains showed significantly higher I C values then the susceptible New Orleans strain
20	129	Sually showed significantly higher LC ₅₀ values that the susceptible new Orleans sually used for yester
21	121	buculus inuringensis isruelensis (bii) is a bacterial derived toxin that has been widery used for vector
22	131	on a registence ratio <5) with the New Orleans strain. Almost all other studies have reported similar findings
23	132	including Martinique nonvilations (highly registent to most insectioides) that were suscentible to <i>Bti</i> compared
24	133	to the Borg Borg strain. Sontiago island Comproon and Malaysia (Moyes et al. 2017). Although <i>Bti</i>
25	134	to the Dola-Dola strain, Santiago Island, Cameroon and Malaysia (Moyes et al., 2017). Although Dil resistance is apparently absent in <i>Aa. gaginti</i> populations to date, resistance has detected in <i>Cular ninians</i> .
26	136	from Surgeuse New York which had a registence ratio of 22 fold when compared to the S I ab suscentible
27	130	strain (Paul et al. 2005). Resistance to <i>Rti</i> has also been demonstrated in <i>Adas rusticus Rossi</i> mosquitoes
28	138	selected for resistance through annual <i>Rti</i> treatment in Jarval sites in the Rhône-Alpes region. The mosquitoes,
 29	130	collected in the treatment area had a moderate resistance ratio up to 7.9 fold compared to the untreated area
30	140	(Bover et al. 2012) The attained resistance levels were still relatively low compared to when mosquitoes are
30 31	140	selected for resistance to other insecticides (Boyer et al. 2012). The multiple active to vince free days of the contract of t
27	141 142	Cry11A and Cyt1A produced by <i>Bti</i> might act at different recentors, making evolution of resistance to <i>Bti</i>
52 22	142	very difficult (Wirth 2013) Nevertheless, both Saudi nonulations showed a significantly higher I C., value
22 24	143 144	than the New Orleans strain suggesting that as with temphos variation exists which might be selected to
54 25	144	higher levels in future
30	146	Therefore although the <i>Ae accurati</i> nonulation in Jeddah and Makkah are still suscentible to temenhos
30	147	rotational application of <i>Rti</i> and temenhos, or another larvicide to which there is full susceptibility would be
3/	148	advisable to slow down evolution of resistance to either of them thus retaining their efficacy over extended
38	149	periods of use in vector control. Our findings suggest the notential to develop resistance to both insecticides
39	150	may exist and thus mixture or rotation is advisable along with continued monitoring and consideration of
40	151	other options such as insecticide growth regulators
41	152	other options such as insociolae growth regulators.
42	102	
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44	153	Conclusion
45	153	Aedes account from Makkah and Jeddah remain suscentible to the larvicides assessed in this study and thus
46	155	larval source management and larviciding could remain an effective tool in control, but regular monitoring
47	156	and consideration of additional alternatives is advised
48	157	Authors' contributions
49	158	AMA-N collected the field samples performed the larval bioassays analysed data and drafted the
50	159	manuscript SA conducted the larval bioassays and analysed data DW conceived and designed the
51	160	experiments drafted the manuscript and analysed data All authors read and approved the final manuscript
52	161	Acknowledgment
53	162	The authors are indebted all the municipal staff (administrators and laboratory technicians in the vector
54	163	control programme) of Makkah and Jeddah in Saudi Arabia. We are also thankful to Dr Craig Wilding in
55	164	Livernool John Moores University for his co-operation during this work
56	165	Competing interests
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3	166	The authors declare no conflicts of interest.
4	167	Funding
5	168	This work was supported by a PhD Studentship from the Saudi Cultural Bureau to Ashwaq M Al Nazawi
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ble 1. Number of Ae. aegypti larvae from Jeddah and Makkah used in each bioassay at different times.

Temephos assay		Bacillus thuringiensis israelensis 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	

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

Final concentration	Volume of stock solution	Volume of distilled water
(mg/L)	(mL)	(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	Volume of stock solution	Desired concentration	Volume 2
(stock) ppm	(mL)	(TOUML)-ppm	(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

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*.

	Bacillus thuringiensis israelensis (Bti) Concentration (ppm)							
Strain	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

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

	Temeph	nos assay	Bti assay		
Strain	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	0.00041ª (0.000276-0.000537)	1	
Makkah lab	0.017 ^b (0.014-0.019)	1.7	n/a	n/a	
Jeddah lab	0.029° (0.025-0.034)	2.9	0.001483 ^b (0.001341-0.001629)	3.6	

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Makkah field	n/a	0.000834°	2.1
		(0.000688-0.000982)	
Jeddah field	n/a	0.00098 ^{b,c}	2.4
		(0.000882-0.001076)	
Cayman	n/a	0.001018 ^{b,c}	2.5
		(0.000882-0.001157)	

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 LC_{50} 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.