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Quantitative Sequencing for the Determination of Kdr-type Resistance Allele (V419L,L925I, I936F) Frequencies in Common Bed Bug, Cimex lectularius L., (Hemiptera: Cimicidae) Populations Collected from Israel

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12   **Quantitative Sequencing for the Determination of Kdr-type Resistance Allele (V419L,**

13   **L925I, I936F) Frequencies in Common Bed Bug, *Cimex lectularius* L., (Hemiptera:**

14   **Cimicidae) Populations Collected from Israel**

15

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30   **Running head:** Palenchar et al: Knockdown Resistance in Bed Bugs from Israel

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34   **ABSTRACT**

35   Human bed bug infestations have dramatically increased worldwide since the mid-1990s. A

36   similar phenomenon was also observed in Israel since 2005, when infestations were reported

37   from all over the country. Two single nucleotide polymorphisms (V419L and L925I) in the bed

38   bug voltage sensitive sodium channel confer *kdr*-type resistance to pyrethroids. Using

39   quantitative sequencing (QS), the resistance allele frequencies of Israeli bed bug populations

40   from across the country were determined. Genomic DNA was extracted from samples of 12

41   populations of bed bugs collected from Israel and DNA fragments containing the V419L or

42   L925I and I936F mutations sites were PCR amplified. The PCR products were analyzed by QS

43   and the nucleotide signal ratios calculated and used to predict the resistance allele frequencies of

44   the unknown populations. Results of the genetic analysis show that resistant nucleotide signals

45   are highly correlated to resistance allele frequencies for both mutations. Ten of the 12 tested

46   populations had 100% of the L925I mutation and 0% of the V419L mutation. One population

47   was heterogeneous for the L925I mutation and had 0% of the V419L mutation and another

48   population was heterozygous for the V419L mutation and had 100% of the L925I mutation.

49   I936F occurred only at low levels. These results indicate that bed bugs in Israel are genetically

50   resistant to pyrethroids. Thus, pyrethroids should only be used for bed bug management with

51   caution using effective application and careful monitoring procedures. Additionally, new and

52   novel-acting insecticides and non-chemical means of controlling bed bugs should be explored.

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56   **Key Words:** *Cimex lectularius,* common bed bug, Israel, knockdown resistance (*kdr*),

57   pyrethroids, quantitative sequencing.

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75   **INTRODUCTION**

76   *Cimex lectularius* L., the common bed bug, is an obligatorily hematophagous

77   ectoparasitic insect that feeds on a variety of warm blooded mammals including humans

78   (Usinger 1966, Boase 2004, Thomas et al. 2004, Reinhardt and Siva-Jothy 2007, Tawatsin et al.

79   2011, Zhu et al. 2010). *C. lectularius* is widely distributed throughout temperate parts of the

80   globe, and poses serious economic, psychological, and physiological problems to humans.

81   Effective monitoring and managing of bed bug populations and preventing or delaying

82   development of insecticide resistance are keys to reducing and eliminating the ramifications that

83   accompany their presence.

84   Human reactions to *C. lectularius* bites are highly variable (Boase 2004, Thomas et al.

85   2004, Wang et al. 2011). The initial bite of a bug is usually not felt. However, later the bite site

86   itches, swells, burns, becomes inflamed or forms a weal and is very disturbing for the victim.

87   There may be a central punctum at the site of the bite. Recurrent bites are often concentrated on

88   the arms, legs and back, as well as on the face around the eyes. Lesions are frequently present in

89   linear or clustered arrangements (Mumcuoglu, 2008). It is generally believed that bed bugs are

90   not likely a vector for human disease (Blow et al. 2001, Boase 2004, Moore and Miller 2006,

91   Reinhardt and Siva-Jothy 2007, Romero et al. 2007, Weeks et al. 2011, Doggett et al. 2012).

92   Recently, Salazar et al. (2014) have shown that the common bed bug may be vector of

93   *Trypanosoma cruzi* and could pose a risk for vector-borne transmission of Chagas disease.

94   However, there is no evidence that the common bed bud actually transmits this pathogen in the

95   field.

96   *C. lectularius* was a well-known parasite in human dwellings throughout the world until

97   the end of WWII. The prevalence of common bed bug infestations decreased dramatically in

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98   developed countries until the 1980s, largely due to the introduction and widespread use of

99   insecticides, such as DDT and malathion, and probably the improvement in housing conditions

100 and hygiene (Usinger 1966, Reinhardt and Siva-Jothy 2007). Bed bug infestations increased

101 dramatically after the mid-1990s in the United States, the United Kingdom, various other

102 European countries (Koganemaru and Miller 2013), Australia, Brazil, Chile (Faundez and

103 Carvajal 2014) and China (Wang et al. 2013).

104   Infestations by the common bed bug were very common in what today is Israel until

105 WWII (Kaufmann 1938, Dolev 2007). With the intensive use of DDT and lindane, the parasite

106 became very rare and the few remaining pests were most probably those that became resistant to

107 DDT (Levinson 1953, Cwilich et al. 1957, Gratz 1958, 1959, Busvine 1977, Mumcuoglu, 2008).

108 During the years of 2006-2008, a 50-150% increase in the reported cases of infestations occurred

109 in comparison to the period of 2001-2005. Bed bugs were mainly reported in hotels, residences

110 and prisons, as well as in industrial buildings (Mumcuoglu and Shalom 2010). The most

111 commonly used insecticides during this time were pyrethroids, followed by organophosphorous

112 and carbamate insecticides. Banning the use of organophosphates inside houses and work places,

113 as well as increasing numbers of foreign workers and new immigrants from developing

114 countries, were considered additional factors for the increased number of bed bug infestations.

115   Bed bugs have proven to be very difficult pests to control, and insecticides have been

116 used widely to control existing infestations (Thomas et al. 2004) and prevent reinfestations

117 (Weeks et al. 2011). The selection of insecticide-resistant bed bugs is a significant contributing

118 factor to their recent resurgence. Various strains of bed bugs from different geographical

119 locations can have different resistance mechanisms (Yoon et al. 2008, Kilpinen et al. 2008, Zhu

120 et al. 2013, Romero et al. 2009, Bai et al. 2011).

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121   Bed bugs have demonstrated strong resistance to the pyrethroids (Seong et al. 2010), a

122   class of insecticide that has been favored for bed bug control due to their high efficacy and low

123   mammalian toxicity (Yoon et al. 2008). To date, bed bugs have developed multiple resistance

124   mechanisms against pyrethroids, such as target site insensitivity due to mutations in the alpha-

125   subunit of the voltage-sensitive sodium channel (VSSC), increased xenobiotic metabolism, and

126   decreased cuticular penetration (Yoon et al. 2008, Zhu et al. 2013, Adelman et al. 2011,

127   Mamidala et al. 2011, Koganemaru et al. 2013, Dang et al., 2013).

128   Pyrethroids target neuronal VSSCs of insects, which functions to generate and propagate

129   action potentials in neurons (Zhu et al. 2010, Castèle and Catterall 2000). Pyrethroids selectively

130   modify VSSC causing "repetitive discharge in motor and sensory axons and convulsive activity",

131   which leads to paralysis and death (Morin et al. 2002). Widespread use of DDT following World

132   War II may have inadvertently selected bed bugs for pyrethrins/pyrethroid resistance as DDT

133   and pyrethrins/pyrethroids share the same target site (Busvine 1958, Zhu et al. 2010, Moore and

134   Miller 2006, Boase 2004, Yoon et al. 2008). Point mutations in the alpha subunit of the VSSC in

135   insects confer resistance to pyrethroids through target site insensitivity, resulting in knockdown

136   resistance (*kdr)* (Yoon et al. 2008). *Kdr* and *kdr*-type mutations have been identified in many

137   insects and result in DDT resistance and cross-resistance to the pyrethrins/pyrethroids (Clark et

138   al. 2013).

139   Two mutations, a valine to leucine (V419L) and a leucine to isoleucine (L925I), have

140   been identified by Yoon et al. (2008) in the highly deltamethrin-resistant common bed bug

141   population (NY-BB), which conferred *kdr*-type resistance to pyrethroids. Both of these

142   mutations result from single nucleotide polymorphisms (SNPs: **G**TC→**C**TC and **C**TT→**A**TT for

143   V419L and L925I, respectively) in the alpha subunit gene of the common bed bug VSSC

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144　(*Clvssc).* A novel mutation I936F has also been identified in a single *d*-allethrin-resistant

145　common bed bug population from Adelaide, Australia, but it appears to give only low levels of

146　resistance and has not been validated as a *kdr*-type mutation functionally (Dang et al. 2014).

147　　　　Using this information, a quantitative sequencing (QS) protocol was developed to

148　establish a population-based genotyping method as a molecular monitoring tool to predict the *kdr*

149　allele frequencies in common bed bug populations (Seong et al. 2010). The *kdr* allele frequencies

150　in different bed bug populations calculated by QS correlated well with filter contact vial bioassay

151　data, confirming the role of the two mutations in pyrethroid resistance (Seong et al. 2010).

152　Similar approaches based on these initial findings have been used widely to establish the role of

153　these two mutation in pyrethroid resistance in many common bed bug populations worldwide

154　(Zhu et al. 2010, Tawatsin et al. 2011, Durand et al. 2012, Tawatsin et al. 2013, Dang et al.

155　2014).

156　　　　The purpose of this study was to predict the resistance allele frequencies of *kdr*-type

157　mutations in common bed bug populations collected from 12 locations across Israel. This

158　information is critical for those attempting to manage bed bug infestations in Israel, including

159　homeowners and pest management professionals, at a time when current approaches are failing

160　and infestations are increasing. Prior testing in other countries has shown that the frequencies of

161　*kdr*-type alleles are highly correlated to pyrethroid resistance (Yoon et al. 2008, Seong et al.

162　2010). Therefore, we hypothesize that the same two *kdr*-like mutations found in pyrethroid-

163　resistant bed bugs elsewhere (V419L and L925I) will also be found at high frequencies in the

164　Israeli populations. Additionally, previous information has identified that the L925I mutation is

165　particularly important in conferring neuronal insensitivity and pyrethroid resistance in bed bugs

166   (Yoon et al. 2008, Seong et al. 2010, Zhu et al. 2010). This study aims to contribute additional

167   information towards validating or rejecting that hypothesis.

168

169   **MATERIALS AND METHODS**

170   **Collection of common bed bug samples**

171   Bed bugs were collected by pest management professionals in Israel prior to treating the

172   infested area with insecticide. The 12 collection sites were five apartments, three one-family

173   houses, two hotels and two industrial buildings. Each population came from a different location

174   in Israel (Table 1). Bed bugs (8-12 per location) were individually removed from their normal

175   habitats, including but not limited to beds, wall carpets and frames and stored in 70% ethanol

176   prior to processing. Bed bugs were collected between February 2011 and April 2012 and sent to

177   the Pesticide Toxicology Laboratory, University of Massachusetts-Amherst, for genotyping

178   analysis by QS.

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181   **Sample selection for DNA extraction**

182   All stored bed bugs from a common collection site/population were washed by removing

183   the 70% ethanol and placing them into a 1.5 mL tube with 1 ml DNase free, double-distilled,

184   H_2O (ddH_2O) and gently mixing the tubes by hand shaking. After 1-2 min, the wash solution was

185   removed using a pipette. For each population, a single test sample, labeled BBM1 to BBM12,

186   was prepared by first separating the different developmental stages. Life stages were determined

187   by visual inspection according to Boase (2004) and Thomas et al. (2004). A single life stage (the

188   largest and most abundant in the population) was selected as the test sample while all other bed

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189   bugs were recombined and stored in 95% ethanol at -20°C. All test samples had multiple bed

190   bugs of the same life stage (Table 2).

191   **Genomic DNA extraction**

192   Genomic DNA (gDNA) was extracted from the 12 test samples (BBM1-12) using the

193   DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's instructions.

194   Briefly, all bed bugs in a test sample were placed into 2 mL micro-centrifuge tubes containing

195   180 µL Buffer ATL, 0.9 µL reagent DX and five stainless steel grinding balls (0.25 g, SPEX

196   Sample Prep, Metuchen, NJ) and homogenized in an automated homogenizer (Geno Grinder

197   2010, SPEX Sample Prep, Metuchen, NJ) at 1250 strokes per min for 1 min. Proteinase K (20

198   µL) was added to the tube and incubated for 3-24 h at 56°C. Following incubation, the lysate was

199   transferred to a 1.5 mL tube with 100% ethanol (200 µL) and Buffer AL (200 µL) and vortexed.

200   The sample was transferred to a DNeasy Mini spin column (Qiagen), and centrifuged for 30 s at

201   6000g. The flow through was discarded and the column placed into a new collection tube before

202   washing with 500 µL buffer AW1, followed by 500 µL buffer AW2 with centrifuging for 30 s at

203   20,000g at each wash. The column was dried by centrifugation for 3 min at 20,000g before DNA

204   elution. The column was placed into a new collection tube and incubated for 1 min with 50 µL

205   buffer AE and centrifuged for 1 min at 6,000g to elute the DNA. This step was repeated to

206   maximize total DNA yield.

207   **Quantification of gDNA**

208   1 µl of extracted gDNA from a single test sample was applied to a NanoDrop ND 8000

209   UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE), absorbance at 260 nm

210   determined and used to calculate the DNA concentration in ng of DNA μL^{-1} using a modified

211   Beer-Lambert equation. DNA purity was also determined by the ratio between absorbencies at

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212   260 and 280 nm, with ratios between 1.5 and 1.8 indicative of pure samples. Samples were

213   diluted to a concentration of 10 ng/µL and stored at -20°C.

214   **PCR amplification of gDNA**

215   15 µL of a master mix (12 µL ddH$_2$O, 5 µL 5X Advantage HD Buffer, 1.75 µL 2.857

216   mM dNTP mixture, 0.25 µL Advantage HD Polymerase and 1 µL of a 10 mM solution

217   containing the forward and reverse primers for either V419L or L925I and I936F, Table 3) were

218   mixed with 5 µL of template DNA (10 ng/µL) and placed onto an Eppendorf Mastercycler Pro

219   (Hamburg, Germany) thermal cycler operated using the following parameters: 1 cycle at 95.0°C

220   for 1 min; by 35 cycles of: 30 s at 95.0°C, 30 s at 61.0°C; and 1 min at 72.0°C. Gel

221   electrophoresis (0.9% agarose) was used to verify quality and quantity of PCR DNA fragment

222   amplification using ethidium bromide (1 µL of 10 mg/mL stock) staining to visualize PCR

223   products under UV light. Where present, positive controls used an individual bed bug sample

224   (BBS5) obtained from a laboratory-reared bed bug colony (FL-BB, Yoon et al, 2008). Negative

225   controls for the PCR reaction used ddH$_2$0 as template, and in all cases no band was present after

226   PCR amplification. This confirms that all reagents were free from gDNA contamination.

227   **PCR product purification***:*

228   The QIAquick PCR Purification Kit (Qiagen) was used for PCR product purification

229   following the manufacturer's instructions. Briefly, 5 volumes of buffer PB were added to 1

230   volume PCR product, mixed with 10 µL of 3M sodium acetate buffer, transferred to a QIAquick

231   spin column placed in a 2 mL collection tube and centrifuged for 30 s at 20,000g. The column

232   was first washed with 750 µL guanidine HCl (30 s) and then with 750 µL buffer PE (30 s). After

233   drying by centrifugation, DNA was eluted into a 1.5 mL tube by applying 50 µL and then 30 µL

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234　　of Buffer EB and centrifuging at 20,000g for 1 min. Gel electrophoresis (0.9% agarose) was used

235　　to verify quantity and quality of products as above.

236　　**Standard curve for QS analysis**

237　　　　The QS protocol developed by Seong et al. (2010) was followed and standard curves

238　　generated using the deltamethrin-resistant NYS-BB (Seong et al. 2010) and the insecticide-

239　　susceptible FL-BB (Yoon et al. 2008) strains of bed bugs for both mutations. Resistant and

240　　susceptible nucleotide signal intensities for each mixture were determined from sequence

241　　chromatograms and used to calculate nucleotide signal ratios (NSR) ([resistant nucleotide signal]

242　　/ [resistant nucleotide signal + susceptible nucleotide signal]). Resulting nucleotide signal ratios

243　　were plotted against known resistant allele frequencies to generate standard curve equations as

244　　well as lower and upper prediction band equations (Fig. 1) using Sigma Plot 10.0 (Systat

245　　Software Inc., San Jose, CA). The standard curves were used to predict resistance allele

246　　frequencies for the 12 unknown Israeli BBM populations at the 95% confidence level (Seong et

247　　al. 2010). A standard curve for the I936F mutation was not generated as the NSR values were

248　　consistently low.

249

250　　**Determination of unknown resistant allele frequencies**

251　　　　Triplicates for each test sample (BBM1-12), containing 5 µL of a 5 µM sequencing

252　　primer solution (3VLQS for V419L and 5LIQS for L925I and I936F, Table 3), were mixed with

253　　10 µL of purified PCR product (Fig. 2) at a concentration of (1 ng/µL), loaded into a 96 well

254　　plate and sent to GeneWiz (South Plainfield, NJ) for sequencing. Sequence chromatograms were

255　　analyzed using Chromas lite software (Technelysium Pty Ltd., Tewantin, Australia) to determine

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256   the nucleotide signal intensity at the respective mutation site (Fig. 3). NSR were calculated and

257   used to predict resistance allele frequency from the Standard curves (Seong et al. 2010).

258

259   **RESULTS**

260   Standard curves for the V419L and L925I mutations were generated from known

261   populations of deltamethrin-resistant and susceptible bed bugs (Fig. 1). The equation for the

262   V419L and L925I standard curves were: $Y = 3.6754(1-[0.7271]^x)$ and $Y = 2.0146(1-[0.4914]^x)$,

263   respectively, where Y is the resistance allele frequency. The curves show that the resistant and

264   susceptible nucleotide signal intensities, which were used to determine the NRSs, were highly

265   correlated to resistance allele frequencies with an R^2 value of 0.998 and 0.996 for the V419L and

266   L925I curves, respectively. The V419L curve had smaller 95% prediction bands than the L925I

267   curve, which may be due to different efficiencies in the dideoxy nucleotide terminator

268   incorporation between the resistant and susceptible nucleotides in the sequencing reaction

269   (Korch and Drabkin 1999).

270   After extracting gDNA from the 12 Israeli bed bug populations, absorbencies of the

271   eluents were determined spectrophotometrically to determine the concentration and purity. For

272   each test sample, the DNA concentrations (ng/μL) and purity (A260/A280 values) were as

273   follows: BBM1 (123, 1.8), BBM2 (53, 1.7), BBM3 (43, 1.9), BBM4 (13, 2.9), BBM5 (76, 2.1),

274   BBM6 (41, 1.9), BBM7 (94, 2.0), BBM8 (72, 1.9), BBM9 (120, 1.9), BBM10 (78, 2.1), BBM11

275   (120, 2.2), BBM12 (5.5, 2.2) All test samples yielded sufficient DNA (~10 ng/μL) of required

276   purity for PCR amplification.

277   Two separate PCR reactions were performed on the 12 test sample eluents (BBM1-12) to

278   amplify DNA fragments encompassing the V419L or L925I and I936F mutation sites within the

12

279   alpha subunit gene of *Clvssc*. The size and quality of the PCR products were determined by

280   electrophoresis on a 0.9% agarose gel with ethidium bromide staining. The expected sizes of the

281   V419L or L925I and I936F PCR products were 354 and 360 base pairs (bp), respectively.

282   The PCR products were purified and subjected to electrophoresis (Fig. 2). Bands at the

283   expected sizes for the V419L or the L925I and I936F mutations confirmed the presence of the

284   alleles of interest, and a band was present in the positive control, while no band was present in

285   the negative control. Purified PCR products were sent to GeneWiz for sequencing. These

286   sequences were analyzed with the QS protocol to determine the presence or absence of the three

287   point mutations in the 12 test samples.

288   The resistance allele frequencies at the V419L mutation for all populations were 0.0 with

289   the exception of BBM9, which was heterogeneous with a resistance allele frequency of 0.39

290   (Table 4). The resistance allele frequency of 0.0 corresponds to a 0% incidence of the V419L

291   resistant allele in 11 of the 12 test samples. The 0.39 resistance allele frequency associated with

292   one of the 12 populations samples (BBM9) corresponds to a 39% presence of the resistant allele

293   at this site.

294   The resistance allele frequency for the L925I mutation was 1.0 (100% presence of the

295   L925I-resistant allele, all bed bugs within a sample have the mutation) for 11 of the 12

296   populations tested (Table 4). The exception was the BBM10 sample, which had a resistance

297   allele frequency of 0.61 (61%), indicating that this population was heterogeneous for the L925I

298   mutation. NSRs were likewise determined for the I936F mutation for all samples but only ranged

299   from 0.06 to 0.22 with an average NRS of 0.09.

300

301   **DISCUSSION**

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302   Previously published research has shown that genetic resistance to DDT and the

303   pyrethroids in common bed bugs is conferred, in part, by the presence of one or both of the

304   V419l and L925I mutations. The goal of this project was to build upon these initial findings and

305   determine the *kdr*-type resistance allele frequencies using QS in 12 bed bug populations

306   collected from Israel in order to characterize the level and extent of these mutations, which lead

307   to nerve insensitivity, so that monitoring and management strategies can be put into place.

308   Results of the DNA extraction show that the modified methods used are generally

309   successful. For BBM1-12, the average DNA concentration was 69.8 ng/µL and only one sample

310   (BBM12) was below the 10 ng/µL concentration prescribed for PCR. The low concentration of

311   the BBM12 sample may partly be due to the low sample size and life stage used (n =2, third

312   instars). BBM12 is the only sample of the original twelve that used third instars and additionally

313   has a sample size less than or equal to all other samples. Nevertheless, the gDNA extractions,

314   PCR amplification and purification protocols provided sufficient and suitable products for the

315   QS analysis.

316   Results of the QS analysis provided useful information as to the state of *kdr*-based

317   DDT/pyrethroid resistance in Israel bed bug populations. Eleven out of the 12 samples

318   populations lacked the V419L mutation but all 12 populations had the L925I mutation. The only

319   populations with differing genotypes were BBM9 and BBM10. BBM9 was heterogeneous in

320   terms of the presence of the V419L mutation (RAF = 0.39) but was homogeneous L925I

321   mutation (RAF = 1.0). BBM10 lacked the V419L mutation (RAF = 0.0) but was heterogeneous

322   for the L925I mutation (RAF = 0.61). Overall, only the BBM10 population did not have a RAF

323   of 1.0 for the L925I mutation. None of the 12 sampled populations had the I936F mutation at

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324 either high or intermediate levels and this mutation appears to either play no role in the genetic

325 resistance to the pyrethroids or only a minor role.

326   A 2010 study by Zhu et al. provides relevant information for comparison to our current

327 results. These authors investigated *kdr*-type mutations in 93 bed bug populations from across the

328 USA and separated the populations into the following four haplotypes based on their genotypes:

329 A (neither mutation), B (L9251 but not V419L), C (both mutations), and D (V419L but not

330 L9251). Of the 93 populations, haplotype B was the most common (44%), followed by C (39%),

331 A (12%) and D (3%). These results are comparable with the current results from Israeli bed bug

332 populations. Eleven out of the 12 populations tested in this study were haplotype B (BBM1-8

333 and BBM10-12) (92%), the most common haplotype. BBM9 was haplotype C (8%), the next

334 most common haplotype. None of the samples tested in this study were haplotype A (0%) or D

335 (0%). The most compelling piece of information comparing the current study to the Zhu et al.

336 (2010) study is the frequencies of the L925I versus V419L mutations. Haplotype B, while the

337 most common haplotype in both of these studies, is overwhelming the most common in the

338 Israeli populations (Fig. 4). Interestingly, while 36 of the 93 populations tested by Zhu et al.

339 (2010) were haplotype C (39%), only one of the bed bug populations tested in this study showed

340 this haplotype (8%). Most recently, Dang et al. (2014) have shown that that there are high

341 frequencies of *kdr*-type mutations associated with Australian bed bug populations, indicating the

342 *kdr*-based pyrethroid resistance is also widespread across Australia. As found in Israel and

343 elsewhere, haplotype B was most prevalent, haplotypes A and C were rare and haplotype D was

344 not detected in Australia. Interestingly, two strains maintained in the laboratory, 'Moonee Ponds'

345 and 'Darlinghurst II', appear to be losing the V419L mutation and becoming predominately

346 haplotype B over time.

15

347   In 2008, Yoon et al. suggested that the L9251 "may play a critical role in deltamethrin

348   resistance", after finding a cloned VSSC plasmid containing only this mutation, while all others

349   contained both the L926I and V419L mutations. Seong et al. (2010) tested common bed bugs

350   collected from Yongsan, Korea in 1993, 2007, 2008, and 2009 for *kdr*-type resistance allele

351   frequencies using similar methods as the ones used in this study. Their findings showed no

352   mutation for bed bugs collected in 1993, full saturation at both mutation sites in 2007, partial

353   resistance for L925I (86%) and V419L (8%) in 2008, and 100% resistance for L925I with 0%

354   resistance for V419L in 2009. The authors asserted that the L925I mutation appears to have been

355   selected more intensively then the V419L mutation. The results from the Israeli bed bug

356   populations presented here support his notion and further suggest that a fitness disadvantage may

357   be associated with the V419L mutation.

358   Based on the trend previously shown in genetic analysis of *kdr*-based resistance in bed

359   bugs in the USA, selective pressure appears to cause bed bugs exposed to pyrethroids to select

360   both mutations but over time the V419L mutation is lost, leaving only the L925I mutation in

361   heavily selected populations. If the use of pyrethroids for controlling bed bugs continues in

362   Israel, selective pressure will likely favor the L925I haplotype. The results of this current study

363   show that this may have already begun in Israel as 11 out of the 12 populations are homozygous

364   for the L925I mutation with very few bed bugs maintaining the V419L mutation.

365

366   **Conclusion**

367   This paper aimed to elucidate the status of genetic resistance to pyrethroids in bed bug

368   populations collected from Israel. Based on QS results, genetic testing showed that the majority

369   of Israeli bed bugs examined in this study are resistant to pyrethroids by the *kdr* mechanism.

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370   Continued use of pyrethroids on Israeli bed bugs will further select for a highly resistant

371   populations. To effectively control these pests, alternative methods of treatments should be

372   explored.

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556   **Table 1. Location and habitat of the 12 bed bug populations collected from Israel**

Population #	Location	Habitat
1	Jerusalem	Apartment
2	Tel Aviv	Apartment
3	Kfar Sava	Industrial Building
4	Kfar Sava	House
5	Haifa	Industrial Building
6	Hadera	Apartment
7	Eilat	Hotel
8	Tel Aviv	Hotel
9	Netanya	Apartment
10	Nazareth	House
11	Rehovot	Apartment
12	Jerusalem	House

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563 **Table 2. Sample sizes and life stages of the 12 tested QS samples from bed bug**
564 **populations collected from Israel**

Population	Sample Size	Life Stage Used
BBM1	4	Adult
BBM2	2	Adult
BBM3	3	Fourth instar
BBM4	4	Fourth instar
BBM5	6	Adult
BBM6	10	Fourth instar
BBM7	2	Adult
BBM8	4	Adult
BBM9	2	Adult
BBM10	5	Fourth instar
BBM11	3	Fourth instar
BBM12	2	Third instar

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576   **Table 3. PCR and QS primers used for the amplification of DNA fragments containing**
577   **the V419L or L929I and I936F mutations and for sequencing reactions**

Primer name	Sequence	Purpose
3'PCRVL	5'-CTGATGGAGATTTTGCCACTGATG-3'	VL sense PCR Primer
5'PCRVL	5'-GTCCGTGGCACATGTTGTTCTTCA-3'	VL antisense PCR Primer
3'PCRLI	5'-GGAGTTCGCCATCAGGGAATCTAT-3'	LI sense PCR Primer
5'PCRLI	5'-GGTCTATCAGTTTTGAGGTCATTCAG-3'	LI antisense PCR Primer
3'QSVL	5'-CCTCTTCAGCTTCTTCTTCTT-3'	VL sequencing primer
5'QSLI	5'- GTGTTTAAGCTGGCTAAGTCATGGCC-3'	LI sequencing primer

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Table 4. Nucleotide Signal Ratios (NSR) and Resistant Allele Frequencies (RAF) for bed bug samples.

Population	V419L				L925I				I936F		
	Nucleotide Signals		NSR	RAF (SD[1])	Nucleotide Signals		NSR	RAF (SD[1])	Nucleotide Signals		NSR
	Sus.	*Res.*			*Sus.*	*Res.*			*Sus.*	*Res.*	
BBM1	599	0	0.0	0.0	0	548	1.0	1.0	1049	93	0.08
BBM2	743	0	0.0	0.0	0	552	1.0	1.0	989	155	0.14
BBM3	683	0	0.0	0.0	0	582	1.0	1.0	1064	109	0.09
BBM4	516	0	0.0	0.0	0	513	1.0	1.0	979	271	0.22
BBM5	664	0	0.0	0.0	0	549	1.0	1.0	1039	81	0.07
BBM6	678	0	0.0	0.0	0	574	1.0	1.0	1082	156	0.13
BBM7	577	0	0.0	0.0	0	564	1.0	1.0	1042	106	0.09
BBM8	667	0	0.0	0.0	0	577	1.0	1.0	1046	62	0.06
BBM9	402	226	0.36	0.399 (0.032)	0	549	1.0	1.0	1093	95	0.08
BBM10	811	0	0.0	0.0	340	354	0.51	0.613 (0.002)	974	222	0.19
BBM11	757	0	0.0	0.0	0	561	1.0	1.0	1057	114	0.10
BBM12	749	0	0.0	0.0	0	555	1.0	1.0	1044	121	0.10

[1]SD = Standard Deviation.

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609   **Figure Captions**

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611   **Fig. 1.** Standard curves for V419L and L925I mutations. Resistant nucleotide signals from QS

612   were plotted against corresponding resistant allele frequencies at the V419L (A) and L925I (B)

613   mutations sites. Equations of the lines are listed above each graph. Black linear regression lines

614   are shown with 95% confidence bands in red. Nucleotide signal ratios were calculated as

615   follows: resistant nucleotide signal / [resistant nucleotide signal + susceptible nucleotide signal].

616   **Fig. 2.** Agarose gel (0.9%) electrophoresis of BBM V419L (A) and L925I purified PCR

617   products. Lane numbers correspond to BBM test samples (i.e., 1, 2, 3 for BBM1, BBM2, BBM3

618   etc.), a 100 basepair (bp) ladder (L, 100 – 1,200 bp, select marker sizes are indicated on left),

619   positive control (+, BBS5 from prior experimentation), and a negative control (-, ddH2O). An

620   asterisk (*) indicates the expected size of the respective PCR product and corresponds to the

621   experimental bands indicating successful PCR of the target DNA fragment. PCR products were

622   visualized under ultraviolet light using ethidium bromide staining.

623   **Fig. 3.** Sequencing chromatograms showing differences between susceptible, mixed, and

624   resistant nucleotide signals. On the left hand side, the VSSC amino acid substitution that results

625   in a *kdr* mutation is indicated, and the corresponding base pair change is bolded and underlined.

626   These same base pairs are indicated by an arrow in the chromatogram sequence, and its identity

627   is boxed on the chromatogram base pair readout. "N" indicates that there is no sequence

628   consensus, i.e. the population is mixed and contains both resistant and susceptible alleles.

629   **Fig. 4.** Geographic distribution of *kdr*-type mutations in common bed bug populations collected

630   from Israel. Pie charts show haplotype frequencies for the twelve bed bug populations tested.

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631   Haplotypes: A (neither mutation, black), B (L925I only, white), C (both mutations, grey).

632   Haplotype D (V419L only) is not represented in any population and is omitted.

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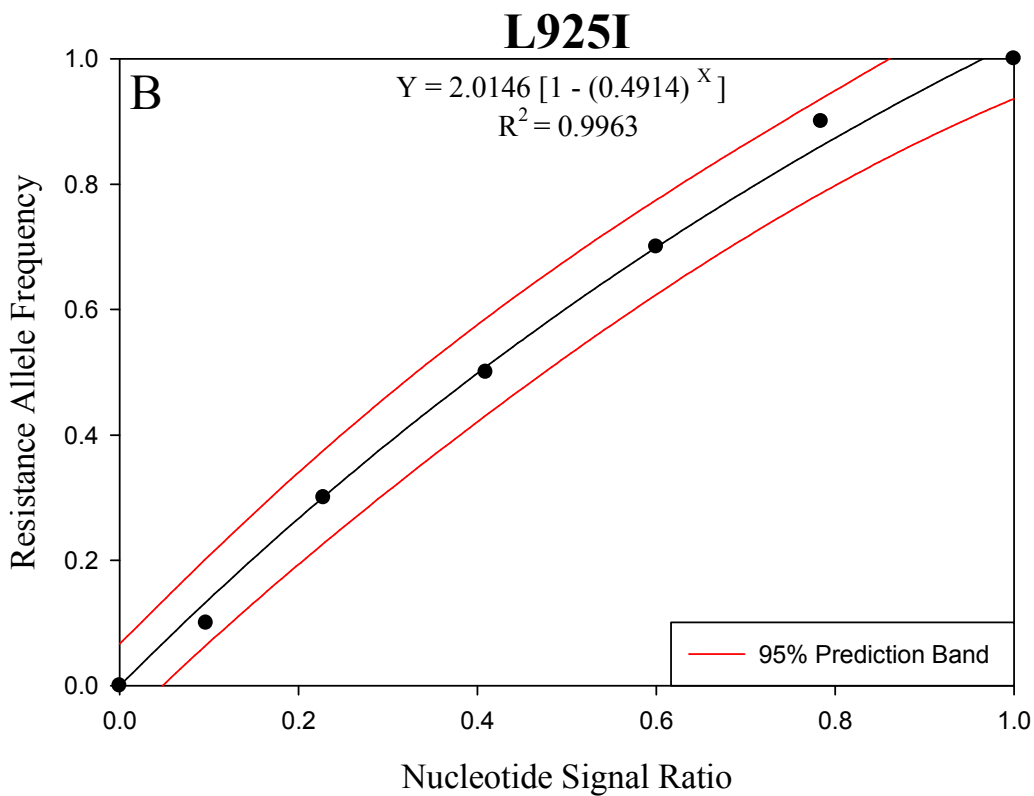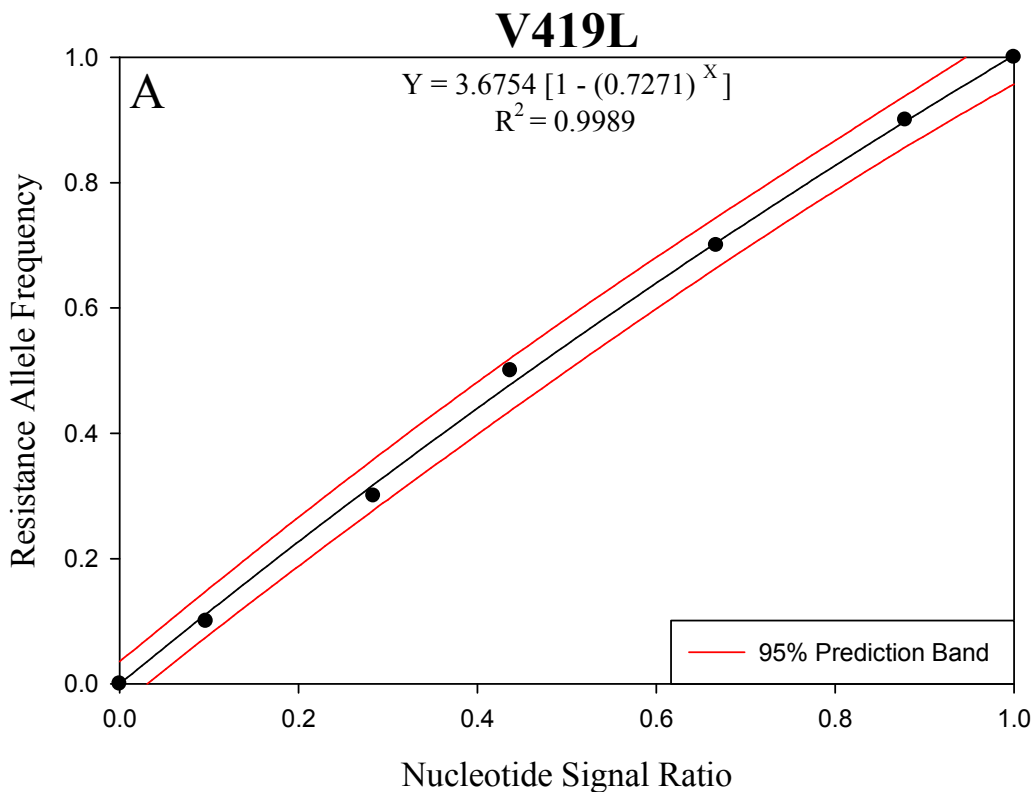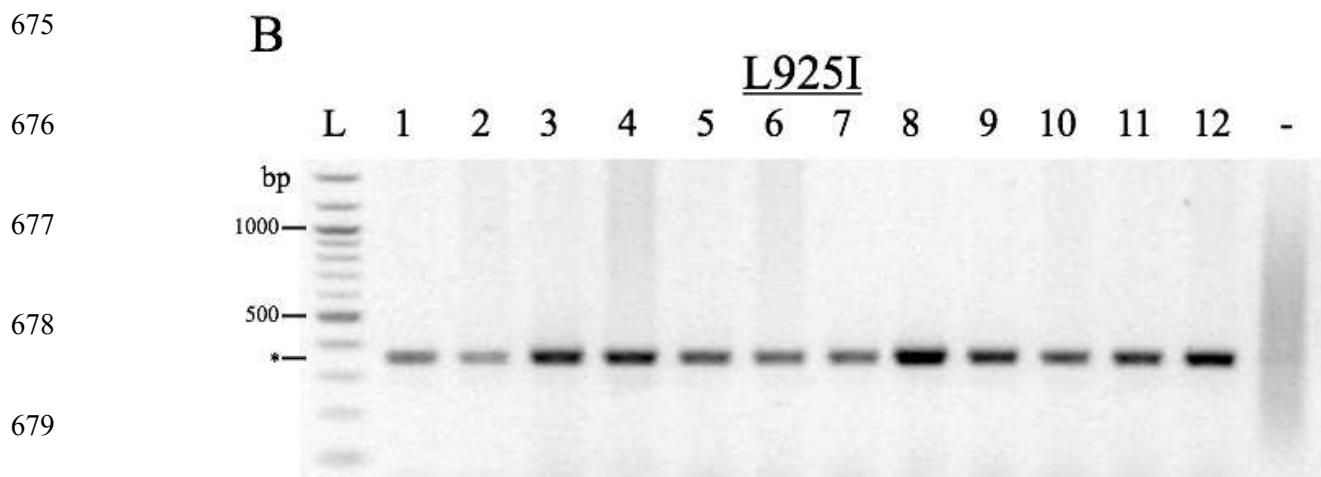636

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648   Fig 1.

V419L

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$$Y = 3.6754 \left[1 - (0.7271)^X \right]$$
$$R^2 = 0.9989$$

Resistance Allele Frequency

Nucleotide Signal Ratio

— 95% Prediction Band

L925I

B

$$Y = 2.0146 \left[1 - (0.4914)^X \right]$$
$$R^2 = 0.9963$$

Resistance Allele Frequency

Nucleotide Signal Ratio

— 95% Prediction Band

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670   Fig 2.
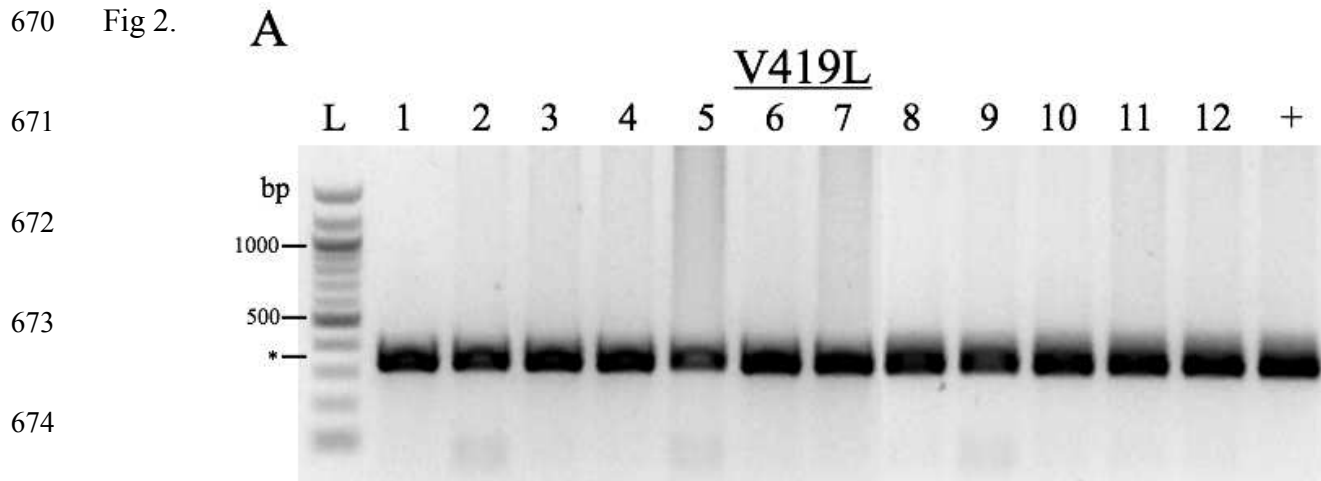
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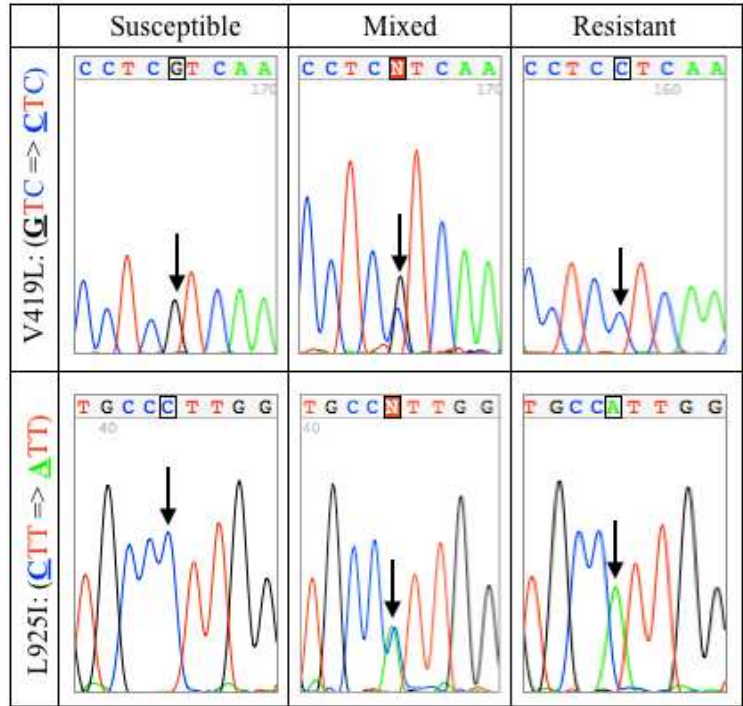
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687　Fig 3.

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703   Fig 4.

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Table 3. PCR and QS primers used for the amplification of DNA fragments containing the V419L or L929I and I936F mutations and for sequencing reactions

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5'PCRVL	5'-GTCCGTGGCACATGTTGTTCTTCA-3'	VL antisense PCR Primer
3'PCRLI	5'-GGAGTTCGCCATCAGGGAATCTAT-3'	LI sense PCR Primer
5'PCRLI	5'-GGTCTATCAGTTTTGAGGTCATTCAG-3'	LI antisense PCR Primer
3'QSVL	5'-CCTCTTCAGCTTCTTCTTCTT-3'	VL sequencing primer
5'QSLI	5'- GTGTTTAAGCTGGCTAAGTCATGGCC-3'	LI sequencing primer

Table 4. Nucleotide Signal Ratios (NSR) and Resistant Allele Frequencies (RAF) for bed bug samples.

Population	V419L				L925I				I936F		
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BBM5	664	0	0.0	0.0	0	549	1.0	1.0	1039	81	0.07
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BBM7	577	0	0.0	0.0	0	564	1.0	1.0	1042	106	0.09
BBM8	667	0	0.0	0.0	0	577	1.0	1.0	1046	62	0.06
BBM9	402	226	0.36	0.399 (0.032)	0	549	1.0	1.0	1093	95	0.08
BBM10	811	0	0.0	0.0	340	354	0.51	0.613 (0.002)	974	222	0.19
BBM11	757	0	0.0	0.0	0	561	1.0	1.0	1057	114	0.10
BBM12	749	0	0.0	0.0	0	555	1.0	1.0	1044	121	0.10

[1]SD = Standard Deviation.