Journal of the American Mosquito Control Association, 15(1):48–52, 1999

COMPARISON OF ISOZYME PATTERNS OF *AEDES AEGYPTI* POPULATIONS COLLECTED FROM PRE- AND POST-*BACILLUS THURINGIENSIS ISRAELENSIS* TREATMENT SITES IN THAILAND

KRIANGKRAI LERDTHUSNEE[1] AND THEERAPHAP CHAREONVIRIYAPHAP[2]

ABSTRACT. Isozyme patterns of 13 field-collected populations of *Aedes aegypti* from Thailand were compared using starch gel electrophoresis. Three populations were collected before the *Bacillus thuringiensis* var. *israelensis,* (*B.t.i.*) application was initiated. The other 10 populations were collected after the *B.t.i.* treatment. Results revealed that the number of polymorphic loci were lower in the *B.t.i.* treated populations as compared to controls. In addition, lower genetic variability was found in populations collected from *B.t.i.* treated sites (Mae Ka Sa [KS] and Mae Kud Luang [KL] village). These results are most likely due to a genetic bottleneck produced by the *B.t.i.* treatment. Heterozygosity increased in the months following *B.t.i.* treatment, probably because of immigration when the control program was withdrawn. However, the anticipated reduction in the expected heterozygosity was only observed in the KS site. This may be due to preexisting low heterozygosity in the KL population. No fixed differences in alleles were detected among the 13 populations.

KEY WORDS *Bacillus thuringiensis israelensis* control, *Aedes aegypti,* Thailand, heterozygosity

INTRODUCTION

Aedes aegypti (L.), a major vector of dengue virus, is commonly distributed throughout the tropics and subtropics. Increases in transportation have contributed to the movement of dengue virus-infected mosquitoes and viremic humans (Failloux et al. 1995). The taxonomic status and population genetics of *Ae. aegypti* are extremely important because of the wide involvement of this species in the transmission of human viral pathogens. The number of dengue cases has increased in the Asian region, including Thailand, which is considered to be a hyperendemic zone (Bhamarapravati 1990). Several control strategies have been employed to control *Ae. aegypti* larvae. Chemical control strategies, however, tend to cause environmental contamination and increase pressure for the evolution of insecticide resistance. Several efforts have been made to replace chemical insecticides with microbial larvicides such as bacteria (*Bacillus thuringiensis* var. *israelensis* [*B.t.i.*] and *Bacillus sphaericus*). These bacteria produce a toxic crystal protein during sporulation that has a specific activity against mosquito larvae (Aly et al. 1987). Several formulations are being developed and used in an operational control program (Mulla 1985).

In this study, we determined if *B.t.i.* treatment reduced genetic variabilty due to population bottlenecks. We analyzed and compared isozyme variability of *Ae. aegypti* populations collected before and after *B.t.i.* treatment. The study was conducted in 3 villages in northwestern Thailand.

MATERIALS AND METHODS

Mosquito populations: Specimens were collected from 13 populations of *Ae. aegypti* from 3 villages of Mae Ka Sa District in Mae Sot County, Tak Province, Thailand. Collections were made from July 1996 to April 1997, in the villages of Mae Ka Sa (KS) (20 km north of Mae Sot City), Mae Kud Luang (KL) (9 km southeast of Mae Ka Sa), and Mae Kud Sam Tha Mai (KT) (7 km southwest of Mae Ka Sa) (Fig. 1).

Specimens were collected from six sympatric populations of *Ae. aegypti* from Mae Ka Sa village (*B.t.i.* treated site). Of these, KS-C1 was collected before *B.t.i.* application started and 5 populations (KS-T1, KS-T2, KS-T3, KS-T4, and KS-T5) were obtained 1, 2, 3, 4, and 5 months, respectively after *B.t.i.* application (Table 1). Specimens were collected from five sympatric populations of *Ae. aegypti* from *B.t.i.* treated Mae Kud Luang village. Of these, KL-C1 was before treatment with *B.t.i.* and KL-T2, KL-T3, KL-t4, and KL-T5 were collected 2, 3, 4, and 5 months, respectively, after *B.t.i.* treatment. *Ae. aegypti* from Mae Kud Sam Tha Mai village (KT-C1 [July 1996], Kt-C2 [February 1997]) were untreated.

All *Ae. aegypti* samples were collected as larvae or pupae and reared individually to the adult stage. Fourth-instar larvae or pupal exuviae were preserved and each specimen was recorded and iden-

[1] National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 73/1 Rama VI Road, Phyathai, Bangkok 10400, Thailand.

[2] Department of Biology, Faculty of Liberal Arts and Science, Kasetsart University, Kamphaengsaen Campus, Nakorn Pathom 73140, Thailand.

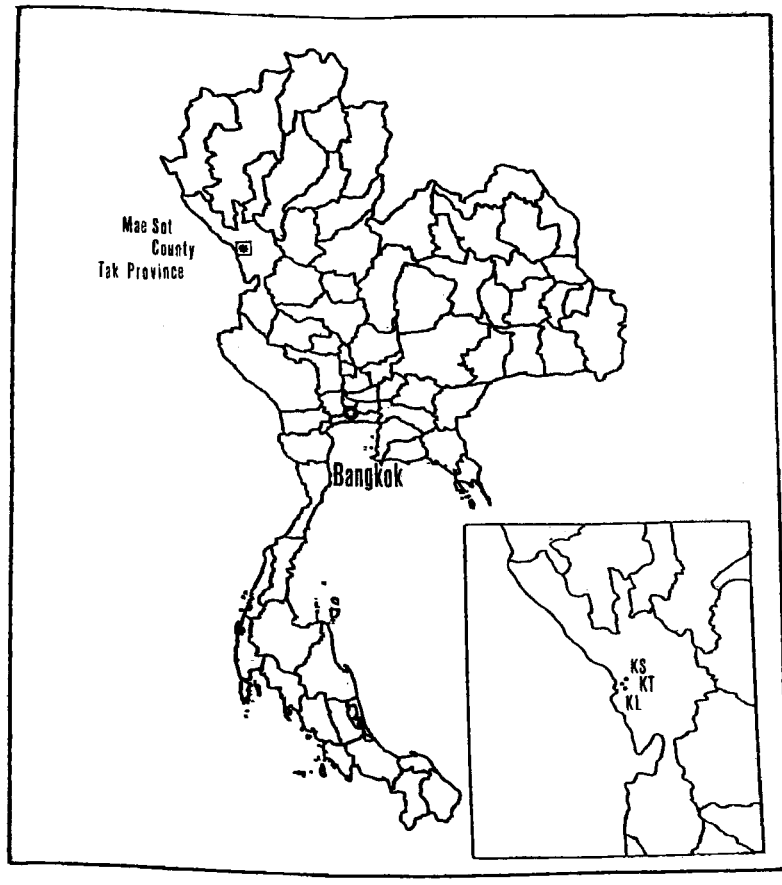

Fig. 1. Collection sites of 13 *Aedes aegypti* populations from Mae Ka Sa (KS), Mae Kud Luang (KL) and Mae Kud Sam Tha Mai (KT) Villages, Mae Sot County, Tak Province, Thailand.

tified. An average of 30 adults was stored at −70°C until used (tested) in electrophoretic analyses.

Starch gel electrophoresis: Starch gel electrophoresis was performed using 29 enzyme systems. Four buffer systems were used for the preliminary examination of each enzyme system to determine which buffer provided the best resolution. The 4 buffers were Tris-malate-ethylenediaminetetracetic acid (TMED) (Pasteur et al. 1988), Tris-citrate buffer system (TCss) (Shaw and Prasad 1970), lithium hydroxide (LiOH) (Pasteur et al. 1988), and the morpholine (Morph) (Clayton and Tretiak 1972). Horizontal starch gel electrophoresis followed Harris and Hopkinson (1976) as modified by Manguin et al. (1995). Electrophoresis was carried out on horizontal starch gels using 50 g of Sigma starch (Sigma Chemical Co., St. Louis, MO), 25 g of sucrose, and 450 ml of the appropriate gel buffer.

Each mosquito was ground in 25 μl of grinding buffer (25 μl/4 wicks) and the homogenate was absorbed onto 4 × 11-mm cellulose polyacetate wicks (Gelman Sciences Inc., Ann Arbor, MI). The TCss, LiOH, and Morph were run for 6 h at a constant voltage of 16 V/cm (Manguin et al. 1995). The

Table 1. Timetable of *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) treatment.

Village	Pre-*B.t.i.* July 1996	*B.t.i.* treatment Aug. 1996	Sept. 1996	Oct. 1996	Nov. 1996	Post-*B.t.i.* treatment Dec. 1996	Jan. 1997	Feb. 1997	March 1997	April 1997
Mae Ka Sa (KS)	KS-C1	—	—	—	—	KS-T1	KS-T2	KS-T3	KS-T4	KS-T5
Mae Kud Luang (KL)	KL-C1	—	—	—	—	—	KL-T2	KL-T3	KL-T4	KL-T5
Mae Kud Sam Tha Mai (KT)	KT-C1	—	—	—	—	—	—	KT-C2	—	—

Table 2. Electrophoretic enzyme systems screened on *Aedes aegypti* adults.

Enzyme system	E.C. number[1]	Symbol	No. loci[2]	Buffer[3]
Aconitase	4.2.1.3	*Acon*	2	TMED
Adenylate kinase	2.7.4.3	*Ak*	2	TCss
Aldehyde oxidase	1.2.3.1	*Ao*	1	LiOH
Arginine kinase	2.7.3.3	*Argk*	2	TCss
Esterase	3.1.1.1	*Est*	3	TMED
Fumarase	4.2.1.2	*Fum*	1	TCss
Glycerol dehydrogenase	1.1.1.72	*Gcd*	1	TMED
Glutamate oxaloacetate transaminase	2.6.1.1	*Got*	2	Morph
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	*G3pdh*	1	Morph
α-Glycerophosphate dehydrogenase	1.1.1.8	*Gpdh*	1	LiOH
Glucose-6-phosphate dehydrogenase	1.1.1.49	*G6pdh*	1	TMED
β-Hydroxyacid dehydrogenase	1.1.1.30	*Had*	1	TMED
Hexokinase	2.7.1.1	*Hk*	1	TMED
Isocitrate dehydrogenase	1.1.1.42	*Idh*	1	Morph
Leucine amino peptidase	3.4.11.1	*Lap*	2	LiOH
Malate dehydrogenase	1.1.1.37	*Mdh*	2	Morph
Malic enzyme	1.1.1.40	*Me*	1	LiOH
Mannose-6-phosphate isomerase	5.3.1.8	*Mpi*	1	TMED
Phosphoglucomutase	5.4.2.2	*Pgm*	1	Morph
Phosphoglucose isomerase	5.3.1.9	*Pgi*	1	LiOH
Pyruvate kinase	2.7.1.40	*Pk*	2	TCss
Triose phosphate isomerase	5.3.1.1	*Tpi*	1	Morph
Xanthine dehydrogenase	1.2.1.37	*Xdh*	1	LiOH
Total			32	

[1] Enzyme commission (E.C.) number.
[2] Number of scorable bands per phenotype.
[3] Refers to the electrophoresis buffer (see Materials and Methods). TMED, Tris-maleate-ethylenediaminetetraacetic acid; TCss, Tris-citrate buffer system; LiOH, lithium hydroxide; Morph, morpholine.

TMED system was run for 12 h at a constant voltage of 8 V/cm. Each gel was stained and incubated at 37°C for 15–60 min.

Data analysis: Genotype frequencies were analyzed the computer program BIOSYS-1 (Swofford and Selander 1981). The program calculated allele frequencies, heterozygosity per locus, and genetic variability measures, and performed a chi-square goodness-of-fit test for Hardy–Weinberg proportions.

RESULTS

Of 29 enzyme systems screened, 23 had good resolutions and 32 putative loci were scored (Table 2). Allele frequencies are available from the authors

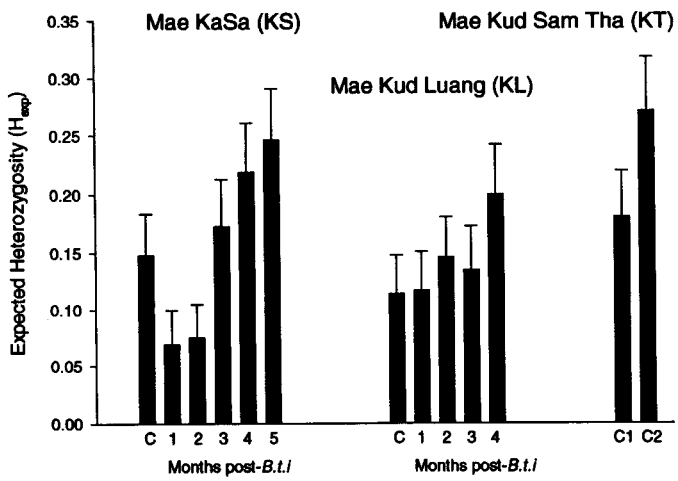

Fig. 2. Expected heterozygosity (H_{exp}) at 32 loci of 13 populations of *Aedes aegypti* before and after treatment with *Bacillus thuringiensis* var *israelensis*.

upon request. Six loci (*Argk*-1, *Est*-3, *Idh*-1, *Lap*-2, *Mdh*-2, and *Pgm*-1) were polymorphic in all populations, whereas *Est*-1, *Est*-2, *G3pdh*-1, *Pgi*-1, *Tpi*-1, and *Xdh*-1 were monomorphic in all populations. A total of 18–20 polymorphic loci were found in the pre-*B.t.i.* treatment KS-C1 population and in *T-C1* and *KT-C2* control populations. Fewer polymorphic loci (9–18) were detected in most populations collected from *B.t.i.* treatment sites (Fig. 2).

Mean heterozygosity

The mean observed heterozygosities of the pre-*B.t.i.* treatment populations KS-C1 and KL-C1 were 0.147 and 0.108, respectively (Fig. 2). Mean heterozygosities of sympatric untreated populations, KT-C1 and KT-C2, ranged from 0.172 to 0.259 (see Table 3).

Heterozygosity was lower in the 1 and 2 month post-*B.t.i.* treatment populations (KS-T1 and KS-T2), as compared to the 3, 4, and 5 month post-*B.t.i.* treatment populations (KS-T3, KS-T4, and KS-T5). Reasons for the relatively low mean heterozygosity in the pre-*B.t.i.* treatment KL remain unclear (Fig. 2). However, heterozygosity of *Ae. aegypti* populations collected from both KS and KL increased over time.

Departures from Hardy–Weinberg equilibrium

Of 416 tests, 8 significant deviations from the Hardy–Weinberg equilibrium were observed and these represent less than 5% type 1 error rate. The reasons for the apparent deviations seen in *Ak*-2 (KL-T5), *Argk*-1 (KT-C1 and KT-C2), *Hk*-1 (KT-C1), *Lap*-2 (KL-T2), *Mpi*-1 (KL-T5) and *Pgm*-1 (KT-C1 and KT-C2) are unknown. However, deviations may have been caused by small sample sizes of certain populations. Moreover, *Pgm*-1, which contained several alleles, was difficult to score and may have resulted in some minor scoring errors.

DISCUSSION

Lower genetic variability occurred in *Ae. aegypti* populations collected from *B.t.i.* treated sites in Mae Ka Sa (KS) and Mae Kud Luang (KL) villages. These results are most likely due to a genetic bottleneck produced by the *B.t.i.* treatment. Heterozygosity increased in the months following *B.t.i.* treatment, probably due to immigration when the control program was withdrawn. Significant loss of heterozygosity is probably due to an efficient control program, either from a regular Abate®-sand (American Cyanamid Co., Wayne, NJ) application or supplementary *B.t.i.* treatments, resulting in a population bottleneck followed by a founders effect when control was relaxed or withdrawn. Some alleles were found to temporarily disappear from the KS-T1 population. Some of these alleles reappeared in the specimens of KS-T2 or KS-T3. This could

Table 3. Measures of genetic variability at 32 loci of 13 populations of *Aedes aegypti*.

B.t.i.[1] treatment/village	Population	Number of polymorphic loci[2]	Mean sample size per locus[3]	Mean no. alleles per locus[3]	% loci polymorphic[2]	Mean heterozygosity	
						Direct count[3]	Hardy–Weinberg expected[3]
Mae Ka Sa (KS)	KS-C1 (pre-*B.t.i.*)	20	29.2 (1.5)	2.1 (0.2)	43.8	0.147 (0.035)	0.148 (0.036)
	KS-T1 (post-*B.t.i.*)	9	29.0 (1.4)	1.6 (0.2)	15.6	0.069 (0.031)	0.070 (0.031)
	KS-T2 (post-*B.t.i.*)	10	29.6 (1.4)	1.6 (0.2)	18.8	0.075 (0.030)	0.077 (0.031)
	KS-T3 (post-*B.t.i.*)	14	26.0 (0.0)	1.6 (0.2)	43.8	0.161 (0.037)	0.173 (0.040)
	KS-T4 (post-*B.t.i.*)	19	24.0 (0.3)	1.8 (0.2)	50.0	0.209 (0.040)	0.219 (0.042)
	KL-T5 (post-*B.t.i.*)	18	23.2 (0.8)	1.8 (0.2)	53.1	0.254 (0.045)	0.246 (0.044)
Mae Kud Luang (KL)	KL-C1 (pre-*B.t.i.*)	14	29.2 (1.5)	1.7 (0.2)	28.1	0.108 (0.032)	0.114 (0.034)
	KL-T2 (post-*B.t.i.*)	13	30.9 (0.9)	1.6 (0.1)	28.1	0.115 (0.034)	0.117 (0.034)
	KL-T3 (post-*B.t.i.*)	16	30.8 (0.9)	1.7 (0.1)	37.5	0.140 (0.035)	0.146 (0.036)
	KL-T4 (post-*B.t.i.*)	14	30.8 (0.9)	1.7 (0.2)	31.3	0.138 (0.038)	0.135 (0.038)
	KL-T5 (post-*B.t.i.*)	16	16.3 (0.8)	1.7 (0.1)	46.9	0.217 (0.042)	0.2 (0.042)
Mae Kud Sam Tha Mai (KT)	KT-C1 (control)	18	30.1 (1.4)	1.9 (0.2)	46.9	0.172 (0.039)	0.181 (0.039)
	KT-C2 (control)	18	18.2 (0.6)	1.9 (0.2)	56.3	0.259 (0.045)	0.271 (0.047)

[1] *Bacillus thuringiensis* var. *israelensis*.
[2] A locus is considered to be polymorphic if the frequency of the most common allele does not exceed 0.95.
[3] Values in parentheses are standard errors.

be explained by the disappearance of alleles in the population during treatment with *B.t.i.* tablets followed by reestablishment of alleles from immigrant mosquitoes from populations in untreated areas of the village. This result is in agreement with the study on genetic differentiation among *Ae. aegypti* populations from French Polynesia (Failloux et al. 1995).

The anticipated reduction in the expected heterozygosity was only observed in the KS site. This may be due to preexisting low heterozygosity in the KL population. The low level of genetic variablity found in KL-C1, a control pre-*B.t.i.*-treatment population, could be due to an efficient control program using Abate®-sand insecticide that was carried out yearly by the village health clinic during the beginning of the rainy season. A different control strategy using several chemical insecticides (i.e., fenitrothion, malathion, and temephos) was tested in Mae Kud Luang village but not in Mae Ka Sa and Mae Kud Sam Tha Mai villages.

ACKNOWLEDGMENTS

We thank Chiefs of Health Clinics at Mae Ka Sa, Mae Kud Luang, and Mae Kud Sam Tha Mai villages, Mae Sot County, Tak Province, Thailand. We thank Ken Linthicum of the United States Army Medical Component (USAMC), Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand, and Sylvie Manguin of Institut Français de Recherche Scientifique pour le Développement en Cooperation (IRD), Montpellier, France, for reviewing this manuscript and P. Juntarumporn for her technical assistance. This research study was funded by the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Bangkok, Thailand.

REFERENCES CITED

Aly, C., M. S. Mulla, W. Schnetter and B. Z. Xu. 1987. Floating bait formulations increase effectiveness of *Bacillus thuringiensis* var. *israelensis* against *Anopheles* larvae. J. Am. Mosq. Control Assoc. 3:583–588.

Bhamarapravati, N. 1990. Dengue overview. Southeast Asian J. Trop. Med. Public. Health. 21:634–635.

Clayton, J. W. and D. N. Tretiak. 1972. Amine-citrate buffers for pH control in starch gel electrophoresis. J. Fish Res. Board Can. 29:1169–1172.

Failloux, A. B., H. Darius and N. Pasteur. 1995. Genetic differentiation of *Aedes aegypti* the vector of dengue virus in French Polynesia. J. Am. Mosq. Control Assoc. 11:457–462.

Harris, H. and D. A. Hopkinson. 1976. Handbook of enzyme electrophoresis in human genetics. North-Holland Publ. Co., Amsterdam, The Netherlands.

Manguin, S., D. R. Roberts, E. L. Peyton, I. Fernandez-Salas, M. Barreto, R. Fernandez-Loayza, R. E. Spinola, R. M. Granaou and H. Mario. 1995. Biochemical systematics and population genetic structure of *Anopheles pseudopunctipennis,* vector of malaria in Central and South America. Am. J. Trop. Med. Hyg. 53:362–377.

Mulla, M. S. 1985. Field evaluation and efficacy of bacterial agents and their formulations against mosquito larvae, pp. 227–250. *In:* M. Laird and J. W. Miles (eds.). Integrated mosquito control methodologies, Volume 2. Academic Press, New York.

Pasteur, N., G. Pasteur, F. Bonhomme, J. Catalan and J. Britton-Davidian. 1988. Practical isozyme genetics. Ellis Horwood Limited, Chichester, England.

Shaw, C. R. and R. B. Prasad. 1970. Starch gel electrophoresis of enzyme. A compilation of recipes. Biochem. Genetics 4:297–320.

Swofford, D. L. and R. B. Selander. 1981. BIOSIS-1. A computer program for the analysis and allelic variation in population genetics and biochemical systematics. Illinois Natural History Survey, Champaign, IL.