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Original Article

PYRETHROID RESISTANCE STATUS OF *AEDES ALBOPICTUS* (SKUSE) COLLECTED IN NAGASAKI CITY, JAPAN

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Abstract

Insecticide susceptibility tests were conducted on *Aedes albopictus* adults and larvae of F1 colonies collected from Nagasaki City, Japan. The results were compared with those of several such colonies collected from other locations in Japan. The larvae collected from Nagasaki City, as well as those from several other locations in Japan showed high resistance to *d*-T_{80}-allethrin. Adult susceptibility tests showed that the adults of almost all tested colonies were highly resistant to DDT, except for those of the Yonaguni colony, while more than half of the adults of the Nagasaki and Fukuoka colonies were resistant to permethrin. No single point mutation in the voltage-gated sodium channel was detected in any of the tested colonies. Bioassay by using synergists (DEM, DEF, PBO, and DMC) indicated cytochrome P450 activity, which might be related to pyrethroid detoxification. A clear relationship between the metabolic factors that might explain the cross resistance between DDT and pyrethroids was not observed in both the adults and larvae.

Key words : *Aedes albopictus*, pyrethroid, DDT, resistance, *kdr*, synergist

Introduction

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are the most common vector-borne diseases. They affect at least 2.5 billion people in tropical and subtropical countries, the areas at risk of transmission (Bonet *et al.*, 2007 ; Gubler, 1998a ; 1998b ; Guzman *et al.*, 2010). Japan, which is located in a temperate area, has experienced epidemic dengue outbreaks in several coastal cities in 1942 – 1945 (Hotta, 1998). The primary vectors of DF and DHF are *Aedes aegypti* (L) and *Aedes albopictus* (Skuse) (Gubler, 1998a ; 1998b). *Ae. albopictus* is assumed to be the vector responsible for dengue outbreaks in Japan (Hotta, 1998). *Ae. albopictus* inhabits tropical regions but is also found in temperate regions, including Japan, Europe, America, and Australia (Bonilauri *et al.*, 2008 ; Powers and Logue, 2007). In addition to being a vector of dengue virus, *Ae. albopictus* is the main vector of chikungunya (CHIK) virus (Bonilauri *et al.*, 2008; Powers and Logue, 2007). Although DHF has not been prevalent in Japan for the past 50 years (Hotta, 1998) and CHIK outbreaks have never been reported in Japan, the possibility of viral disease outbreak exists because of the common distribution of the vector *Ae. albopictus*. Vector control is an essential measure for controlling the outbreak of viral diseases, and the use of insecticides might be the most common and effective method of vector control.

For proper vector control, it is crucial to understand the insecticide resistance status of *Ae. albopictus*. This allows for precise selection of an appropriate insecticide. Only few studies on insecticide resistance in *Ae. albopictus* have been conducted in Japan. Toma *et al.* (1992) reported the susceptibility of seven colonies of *Ae. albopictus* from Ryukyus Island to 11 types of insecticides, including DDT, organophosphates,

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carbamates, and pyrethroids, and concluded that all strains were susceptible to all insecticides, except DDT. Dipping method was used by Suzuki and Mizutani (1962) to test the susceptibility of *Ae. albopictus* larvae collected from Kawasaki (Tokyo) and Kawashima (Nagasaki) to organochlorines and organophosphates. The larvae showed tolerance to the above insecticides. Moreover, Kawada *et al.* (2010) found possible pyrethroid resistance in *Ae. albopictus* larvae collected from some locations in Nagasaki City.

Here, we report the pyrethroid resistance status of *Ae. albopictus* collected from Nagasaki City in comparison with that in specimens collected from several other locations in Japan. The possible mechanism of resistance is also discussed.

Materials and Methods

Collection of mosquito larvae

Field collection of *Ae. albopictus* larvae was performed in 19 city parks in Nagasaki City and an additional place in the campus of Nagasaki University (ITM) (Nagasaki Prefecture, **Fig. 1**), Amami Island (Kagoshima Prefecture), and Yonaguni Island (Okinawa Prefecture). Other colonies from the National Institute of Infectious Diseases (Tokyo, Japan) were used as references in this study. The laboratory colonies were obtained from Kurume City (Fukuoka Prefecture), Fukuoka City (Fukuoka Prefecture), Hatsukaichi City (Hiroshima Prefecture), Takarazuka City (Hyogo Prefecture) Higashikurume (Tokyo), and Ikaken (Tokyo) **(Fig. 1)**. Collection sites in Nagasaki City were selected according to Kawada *et al.* (2010) who reported some locations where the mosquitoes showed low susceptibility to pyrethroid. Collection of larvae was performed from May 9 to September 1, 2011. Mosquito larvae were directly collected, mainly from catch basins, using a metal dipper (diameter, 13 cm). If mosquito larvae were not available, eggs were collected using an ovitrap placed among the shrubbery in the park. The collected larvae were brought to the laboratory and reared under laboratory conditions (27℃, 70 % relative humidity). Identification was carried out when female adults emerged, in accordance with Tanaka *et al.* (1979).

Insecticides and synergists used in the study

An emulsifiable concentrate of 90% *d*-T_{80}-allethrin (Sumitomo Chemical Co., Ltd., Tokyo, Japan) was used for the simplified knockdown bioassay for larvae (Kawada *et al.*, 2009). For the World Health Organization (WHO) bioassay of adult mosquitoes, insecticide-impregnated paper containing a diagnostic dose of permethrin (0.75%) and DDT (4%) (USM, Malaysia) was used. Inhibitor of DDT dehydrogenase activity, 4-chloro-α-(chlorophenyl)-methyl-benzenemethanol (DMC, chlorphenetol ; 98%, CAS number 80-06-8 ; Sigma-Aldrich, Germany), inhibitor of cytochrome P450 monooxygenase activity, 5-[2-(2-butoxyethoxy)ethoxymethyl]-6-propyl-1,3-

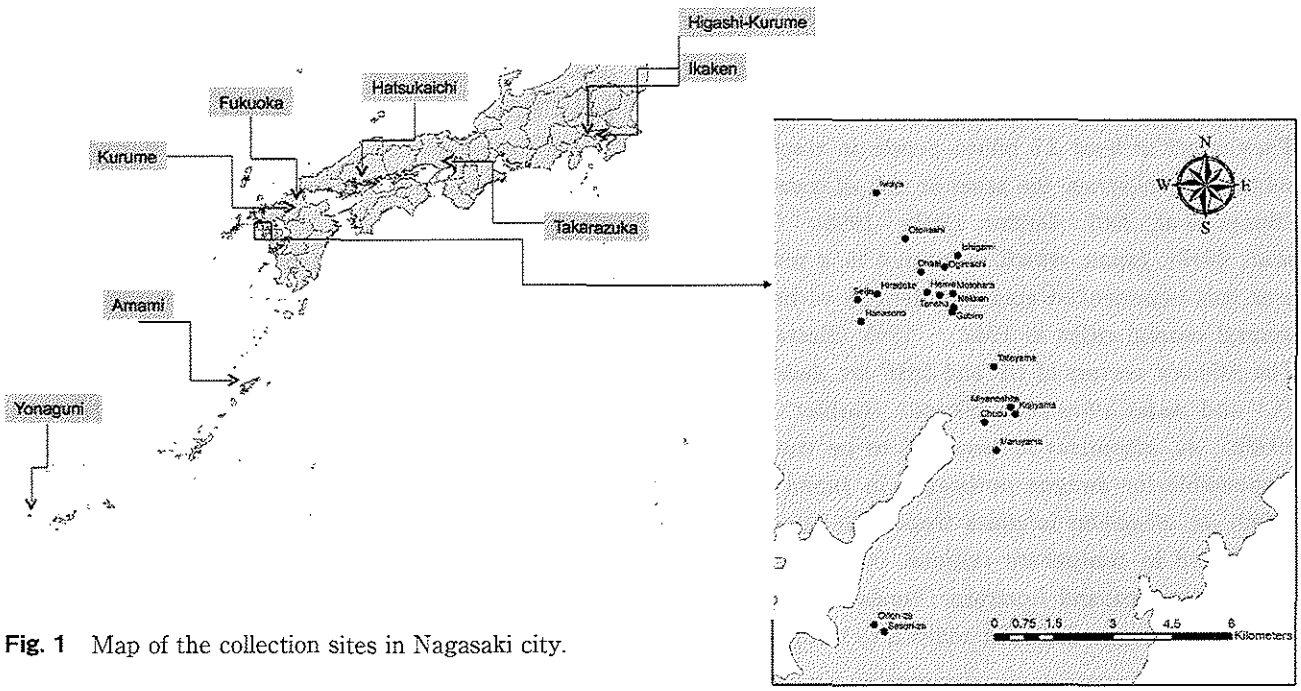

Fig. 1 Map of the collection sites in Nagasaki city.

benzodoxole (PBO, piperonyl butoxide ; 98%, CAS number 51-03-6, WAKO Pure Chemical Industries, Osaka, Japan), inhibitor of glutathione S-transferase activity, diethyl (2z-2-butenedioate) (DEM, diethyl maleate ; ≥96%, CAS number 141-05-9, Sigma-Aldrich, Saint Louis, MO, USA), and inhibitor of esterase activity, S,S,S-tributyl phosphorotrithionate (DEF, Tribuphos ; 97%, CAS number 78-48-8, WAKO Pure Chemical Industries, Ltd, Japan) were used as synergists for permethrin and DDT.

Simplified knockdown bioassay using larvae

The simplified bioassay for the detection of knockdown susceptibility of larvae was carried out according to Kawada *et al.* (2009). The bioassay was carried out using F1 progeny of each collected colony. A larva was placed in a 20-ml glass vial with water. An emulsifiable concentrate of 90% *d*-T_{80}-allethrin was diluted using deionized water to obtain 250 ppm concentration. After releasing the larva, 32 μl or 8 μl of the solution was added to the glass vial to obtain a concentration of 0.4 and 0.1 ppm, respectively. Twenty larvae were used for each concentration. Knockdown of the larvae was observed for 30 min. The larvae that sank to the bottom of the glass vial and could not swim, float, or were paralyzed, were considered as the knockdown larvae and time to knockdown was recorded for each larva. The knockdown data were summarized for each colony. The time required for 50% knockdown (KT_{50}) was scored according to the following six categories : 1, <5 min ; 2, 5－<10 min ; 3, 10－<15 min ; 4, 15－<20 min ; 5, 20－<30 min ; and 6, >30 min. The susceptibility index was calculated as the product of the score at 0.1 ppm and 0.4 ppm. Thus, mosquitoes with a susceptibility index 1 were considered the most susceptible and those with susceptibility index of 36 were considered the least susceptible to *d*-T_{80}-allethrin.

Insecticide susceptibility test for adults

Adult bioassay was conducted according to the standard WHO susceptibility or resistance test protocol (WHO, 1998). Insecticide-impregnated paper containing 0.75% permethrin and 4% DDT was used for the test. Twenty unfed female mosquitoes (2- to 5-day-old F1 progeny of field-collected or laboratory colonies) were released in the WHO test tube kit for 1 h and the time for knockdown was recorded. After exposure, the mosquitoes were transferred to a holding tube lined with untreated paper and provided with cotton soaked with 5% sucrose solution as the meal. Mortality was recorded after 1 day. KT_{50} and average mortality were calculated for the mosquito colony.

Susceptibility tests for adults by using synergists

Further susceptibility tests by using synergists were performed. Synergist mixed in a 0.3-μl acetone solution was topically applied to female mosquitoes with maximal non-lethal dose of PBO and DEF (1 μg/female), DEM (1.5 μg/female), and DMC (2 μg/female). Female adults were first anesthetized using carbon dioxide for 3 min, and then transferred to a container containing dry ice to maintain the effect anesthesia. A 0.3-μl aliquot of acetone solution containing the synergist at the required concentration was applied on the dorsal prothorax of the female by using an automatic applicator (Burkard Manufacturing Co. Ltd, Rickmansworth, England). The topical treatment was performed 1－2 h prior to the WHO test to maximize the effect of the synergists (Bonnet *et al.*, 2009). The bioassay was repeated to obtain three replicates, with 10 females per replicate.

Susceptibility test for larvae by using synergists

The test was conducted according to the standard WHO (1981) larval susceptibility test. A series of 100-ml aliquots of the designated concentration of *d*-T_{80}-allethrin from 0.4 ppm to 0.006 ppm was prepared in a plastic cup. For the bioassay by using synergists, a 0.5-ml aliquot of ethanol solution containing PBO, DEF, and DEM was added to the above solution to obtain constant PBO (0.6 ppm), DEF (1 ppm), and DEM (1 ppm) concentrations. Twenty-five late third or early fourth instar larvae were released into the above solutions by using a metal strainer. Control experiments were carried out by adding the same concentration of synergists without *d*-T_{80}-allethrin. Dead and moribund larvae were recorded after 24 h of exposure. The larvae that sank and could not swim when stimulated were considered as moribund. The percent mortality was calculated for each concentration and corrected using Abbott's formula in cases where the mortality of the control was 20% or less (Abbott, 1925). The mortality data were subjected to regression analysis on log dosage, and their LC_{50}, slope, and heterogeneity (χ^2) were calculated according to Finney (1971). The percent suppression of synergists was calculated according to formula described bellow (Fakoorziba *et al.*, 2009).

% suppression = [1 -(LC_{50} of *d*-T_{80} allethrin with synergist)/(LC_{50} of *d*-T_{80} allethrin)] x100

Detection of *kdr* mutation

Direct sequencing was performed to investigate the presence of point mutations in voltage-gated sodium channels (I1011, L1014, V1016, and F1534) previously shown in *Ae. aegypti* (Brengues *et al.*, 2003 ; Chang *et al.*, 2009 ; Saavedra-Rodriguez *et al.*, 2007) and *Ae. albopictus* (Kasai *et al.*, 2011). After larval bioassay, the whole body of a larva was placed in a 1.5-ml PCR reaction tube and homogenized with a mixture of extraction solution (20 μl) + tissue preparation solution (5 μl) (REDExtract-N-AmpTM Tissue PCR Kit, Sigma, USA). All larvae used for the bioassay irrespective of dead or alive were extracted. Initial fragment amplification was performed using primers AaSCF20 (5′-GACAATGTGGATCGCTTCCC-3′) and AsCR21 (5′-GCAATTCTGGGCTTGTTAACTTG-3′) for the detection of I1011, L1014, and V1016 (Domain II) ; and AaSCF7 (5′-GAGAACTCGCCGATGAACTT-3′) and AaSCR7 (5′-GACGACGAAATCGAACAGGT-3′) for F1534 (Domain III), under the following conditions: 94℃ for 3 min, followed by 35 cycles of 94℃ for 15 s, 55℃ for 30 s, and 72℃ for 30 s, and then 72℃ for 10 min. Amplified fragments of the expected size were purified using ExoSAP-IT (USB Corporation, Cleveland, OH, USA). DNA sequencing was carried out using primers AaSCF3 (5′-GTGGAACTT CACCGACTTCA3′) for Domain II analysis and AaSCR8 (5′-TAGCTTTCAGCGGCTTCTTC-3′) for Domain III analysis. A BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Japan) was used for DNA sequencing. PCR was performed under the following conditions : 96℃ for 1 min, followed by 25 cycles of 96℃ for 10 s, 50℃ for 5 s, and 50℃ for 2 min. Direct DNA sequencing was performed using a 3730 DNA Analyzer (Applied Biosystems, Japan). The electropherogram of the targeted amino acid was analyzed using MEGA 4.0 public domain software (http://www.megasoftware.net/). The unique DNA haplotype sequences were deposited in DNA Data Bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp/index-j.html).

Statistical Analysis

The median KT_{50} and LC_{50} were calculated using SPSS 19.0 Software (IBM Corp., Armonk, NY, USA). Correlations between larval susceptibility indices and adult mortality when treated using 0.75% permethrin and 4% DDT-impregnated paper were determined using the non-parametric Spearman correlation test in SPSS 19.0.

Results

Simplified knockdown bioassay using larvae

Susceptibility indices of *Ae. albopictus* collected from Nagasaki City and other places in Japan ranged from 12 to 36 **(Table 1)**. Among the 20 colonies from Nagasaki City, 10 showed the highest susceptibility index (36), six colonies showed an index of 24－30, and four colonies showed an index of 12－18, indicating that more than half of the Nagasaki colonies were highly tolerant to *d*-T_{80}-allethrin. Among eight colonies collected from other places in Japan, two colonies showed a susceptibility index of 36, two colonies showed an index of 24－30, and the other four colonies showed an index of 12－18. Otonashi, Seijo, Hanasono, and Kojiyama colonies from Nagasaki City and Ikaken, Fukuoka, Amami, and Yonaguni colonies showed high susceptibility to *d*-T_{80}-allethrin.

Insecticide susceptibility test for adults

Adult susceptibilities to permethrin and DDT by WHO tube test of F1 colonies collected from Nagasaki City are shown in **Table 2**. When 90% mortality was adopted as the threshold for the detection of resistance, 12 among 20 colonies collected from Nagasaki City were categorized as permethrin-resistant colonies. On the other hand, only one colony (Fukuoka) was categorized as permethrin-resistant among the eight colonies collected from other places in Japan. By using the same criteria as above, all colonies, except the Yonaguni colony, were categorized as DDT-resistant. Nineteen Nagasaki colonies showed very low mortality (< 40%), indicating that these colonies were highly resistant to DDT. No correlation was observed between larval susceptibility indices and adult susceptibility to permethrin ($N = 28$, $P = 0.273$) and DDT ($N = 28$, $P = 0.964$).

Susceptibility tests for adults by using synergists

Synergistic activities of DEM, DEF, DMC, and PBO to DDT and permethrin in *Ae. albopictus* adults are shown in **Table 3**. Two Nagasaki colonies (Chuou and Heiwa) showed high resistance to both pyrethroids and DDT. The Yonaguni colony was susceptible to both insecticides, and the Higashikurume colony was susceptible to pyrethroids and resistant to DDT. Synergistic activity with DDT was the highest for DEM in both the Chuou and Heiwa colonies, while DMC and PBO were not effective. On the other hand,

Table 1 Susceptibility index and frequency of *kdr* mutation (L1011, L1014, V1016, and F1534) of larvae collected in Nagasaki city and other places in Japan.

Collection site	Code	Susceptibility Index[1)]	Frequency of *kdr* Mutation		DDBJ Accesion Number	
			Number Examined	Allelic Frequency	D II	D III
Gubiro	Gubiro	36	20	0	AB827803	AB827818
Iwaya	U039	36	20	0	AB827808	AB827823
Chuou	E010	36	20	0	AB827801	AB827816
Heiwa	N0102	24	20	0	AB827804	AB827819
Otonashi	U005	18	20	0	AB827813	AB827827
Ishigami	N020	36	20	0	AB829520	AB828346
Ogimachi	N019	36	20	0	AB828339	AB828350
Seijo	N051	12	20	0	AB828346	AB828353
Hiradoko	N014	30	20	0	AB827805	AB827820
Maruyama	S001	30	20	0	AB829521	AB828347
Tateyama	E017	36	20	0	-	AB828354
Nekken	ITM	36	20	0	AB828338	AB828349
Ohashi	N007	36	20	0	AB827811	AB827826
Motohara	N016	36	20	0	AB828337	AB828348
Orion-za	O008	30	20	0	AB828340	AB828351
Hanasono	N017	18	20	0	AB828336	AB828345
Tenshu	N009	24	20	0	AB828343	AB828355
Sasori-za	O012	24	20	0	AB828341	AB828352
Kojiyama	E022	18	20	0	AB827809	AB827824
Miyanoshita	E007	36	20	0	AB827810	AB827825
Ikaken[2)]		18	20	0	AB827807	AB827822
Takarazuka[3)]		36	20	0	AB827814	AB827828
Kurume[4)]		36	20	0	AB827812	AB829522
Fukuoka[5)]		18	20	0	AB827802	AB827817
Hatsukaichi[6)]		30	20	0	AB827806	AB827821
Amami[7)]		12	20	0	AB827800	AB827816
Yonaguni[8)]		18	20	0	AB828344	AB828356
Higashikurume[9)]		30	20	0	-	-

1) [Score at 0.1 ppm (1-6)] X [Score at 0.4 ppm (1-6)] of *d*-T_{80}-allethrin. Each 10 larvae were tested for 0.1 and 0.4 ppm.
2) Transferred from The National Institue of Infectious Diseases 3) Hyogo Perfecture
4), 5) Fukuoka Perfecture 6) Hiroshima Perfecture 7) Kagoshima Perfecture
8) Okinawa Perfecture 9) City of Tokyo

DEM, DMC, and PBO were synergistic with DDT in the Higashikurume colony. A comparison could not be made among synergists with permethrin, because all synergists showed 100% mortality, although the decrease in KT_{50} in the Chuou and Heiwa colonies when treated using synergists might indicate the effect of the synergists.

Table 2 Knockdown time and mortality of *Ae. albopictus* adults by WHO tube test with 0.75% permethrin and 4% DDT.

Collection site	Code	0.75% permethrin[1]		% Mortality	4% DDT[1]		% Mortality
		KT_{50}[2,3]	KT_{90}[2,3]		KT_{50}[2,3]	KT_{90}[2,3]	
Gubiro	Gubiro	29.2 (25.3 - 33.9)	54.0 (44.4 - 73.9)	90.0	>60	>60	0
Iwaya	U039	23.8 (14.8 - 35.7)	42.2 (29.8 - 141.7)	75.0	>60	>60	0
Chuou	E010	41.5 (36.8 - 46.6)	56.3 (49.5 - 72.8)	65.0	>60	>60	0
Heiwa	N0102	32.8 (29.2 - 37.0)	49.4 (42.9-62.3)	75.0	>60	>60	0
Otonashi	U005	36.0 (31.4 - 41.4)	61.2 (51.3 - 82.6)	81.0	>60	>60	10.0
Ishigami	N020	28.3 (25.1 - 31.9)	44.0 (38.1 - 54.9)	92.2	>60	>60	30.0
Ogimachi	N019	26.0 (23.0 - 29.6)	42.9 (36.8 - 54.2)	90.0	>60	>60	10.0
Seijo	N051	33.5 (30.0 - 37.4)	47.4 (41.7 - 58.5)	95.0	>60	>60	5.0
Hiradoko	N014	44.3 (40.7 - 48.0)	60.0 (54.4 - 70.8)	80.0	>60	>60	15.0
Maruyama	S001	24.0 (22.01- 26.3)	28.7 (26.2 - 33.9)	100	>60	>60	10.0
Tateyama	E017	14.8 (8.2 - 20.4)	20.7 (16.4 - 94.6)	100	>60	>60	10.0
Nekken	ITM	21.6 (19.1 - 24.5)	34.4 (29.4 - 44.8)	85.0	>60	>60	20.0
Ohashi	N007	28.2 (24.9 - 31.7)	43.0 (37.3 - 53.6)	100	>60	>60	15.0
Motohara	N016	31.0 (27.8 - 34.5)	40.8 (36.3 - 51.5)	100	>60	>60	25.0
Orion-za	O008	32.5 (28.4 - 37.3)	55.2 (46.5 - 73.0)	100	>60	>60	40.0
Hanasono	N017	27.7 (24.9 - 30.9)	36.2 (32.4 - 45.5)	85.0	>60	>60	15.0
Tenshu	N009	23.7 (21.4 - 26.6)	33.2 (29.0 - 41.8)	85.0	>60	>60	5.0
Sasori-za	O012	19.1 (17.4 - 21.2)	25.5 (22.6 - 32.8)	100	>60	>60	0
Kojiyama	E022	38.2 (29.7 - 37.0)	44.0 (39.1- 55.1)	85.0	>60	>60	10.0
Miyanoshita	E007	19.7 (17.7 - 21.9)	27.6 (24.3 - 34.7)	90.0	>60	>60	85.0
Ikaken		18.5 (7.3 - 47.2)	37.2 (22.3 - 2833.1)	100	>60	>60	30.0
Takarazuka		20.3 (17.9 - 23.1)	33.1 (28.3 - 42.9)	95.0	>60	>60	65.0
Kurume		32.0 (29.3 -34.9)	44.1 (39.8 - 51.5)	96.7	>60	>60	6.7
Fukuoka		27.4 (24.6 - 30.8)	36.6 (32.3 - 46.6)	61.1	>60	>60	65.0
Hatsukaichi		47.6 (39.8 - 62.3)	>60 -	100	>60	>60	10.0
Amami		20.5 (18.5 - 22.9)	28.3 (24.9 - 36.1)	100	>60	>60	30.0
Yonaguni		19.3 (17.0 - 21.9)	31.3 (26.7 - 40.9)	100	52.8 (44.4-70.7)	>60	100
Higashikurume		15.1 (13.6 9 -16.6)	23.2 (20.5 - 28.4)	100	>60	>60	33.3

1) Number of adults tested was 10 with 3 replicates for each test.
2) KT_{50}, time (min) required for 50% knockdown; KT_{90}, time (min) required for 90% knockdown
3) Figures in parentheses indicate 95% confidence interval

Susceptibility tests for larvae by using synergists

The Chuou and Heiwa colonies, which showed high resistance to pyrethroids, were compared with the Higashikurume and Yonaguni colonies. In all colonies, PBO was more effective than DEF in reducing the LC_{50} of *d*-T_{80}-allethrin **(Table 4)**. Synergism of DEM was not as high as that of PBO in the Chuou colony and the Heiwa colony.

Detection of *kdr* mutation

Mutations in the voltage-gated sodium channel (I1011, L1014, V1016, and F1534) were detected in 20 larvae used in the simplified larval knockdown test **(Table 1)**. Not a single mutation was found in 20 colonies collected in Nagasaki City and 8 colonies collected in the other places in Japan.

Discussion

The present study confirms that most *Ae. albopictus* colonies collected in Nagasaki City, and one colony collected in Fukuoka City, showed resistance to pyrethroids. Moreover, it was surprising that almost all colonies collected in Japan, except for the Yonaguni colony, were found to be resistant to DDT. The present study might be the first report for widespread

Table 3 Susceptibility of adult *Ae. albopictus* to DDT and Permethrin by WHO tube test with synergists. [1)]

Colony	Treatment[2)]	KT_{50}	KT_{90}	% Mortality
Chuou	DDT [3)]	>60	>60	6.7
	DDT+DEM	>60	>60	60.0
	DDT+DEF	>60	>60	50.0
	DDT+DMC	>60	>60	26.7
	DDT+PBO	>60	>60	23.3
	PER [4)]	26.3 (24.3 - 26.6)	34.2 (31.1 - 40.3)	76.7
	PER+DEM	20.5 (19.1 - 23.4)	27.1 (24,4 - 32.4)	100
	PER+DEF	18.8 (17.8 - 19.9)	30.3 (32.2 - 35.6)	100
	PER+DMC	20.9 (19.0 - 23.2)	32.8 (28.9 - 39.8)	100
	PER+PBO	10.3 (6.7 - 13.9)	27.7 (1.2 - 44.6)	100
Heiwa	DDT	>60	>60	16.7
	DDT+DEM	>60	>60	73.3
	DDT+DEF	>60	>60	53.3
	DDT+DMC	>60	>60	26.7
	DDT+PBO	>60	>60	23.3
	PER	32.1 (28.2 - 37.1)	68.8 (55.9 - 94.9)	86.7
	PER+DEM	12.1 (9.2 - 15.2)	21.2 (16.7 - 36.0)	100
	PER+DEF	19.1 (17.6 - 20.8)	30.3 (28.6 - 32.1)	100
	PER+DMC	16.8 (15.2 -18.1)	21.5 (19.7 - 25.6)	100
	PER+PBO	16.8 (6.7 - 13.9)	21.5 (19.7 - 25.6)	100
Higashikurume	DDT	>60	>60	6.7
	DDT+DEM	>60	>60	93.3
	DDT+DEF	>60	>60	20.0
	DDT+DMC	>60	>60	73.3
	DDT+PBO	>60	>60	53.3
	PER	23.8 (21.9 - 26.0)	33.0 (29.6 - 39.2)	100
	PER+DEM	20.6 (16.7 - 22.8)	32.6 (28.7 - 39.6)	100
	PER+DEF	22.4 (20.3 - 24.7)	34.9 (30.7 - 42.4)	100
	PER+DMC	23.5 (21.6 - 26.7)	33.4 (29.8 - 39.9)	100
	PER+PBO	20.6 (19.2 - 22.4)	26.6 (24.1 - 31.7)	100
Yonaguni	DDT	>60	>60	93.3
	DDT+DEM	>60	>60	100
	DDT+DEF	>60	>60	100
	DDT+DMC	>60	>60	100
	DDT+PBO	>60	>60	100
	PER	26.3 (24.3 - 28.6)	34.2 (31.1 - 40.3)	100
	PER+DEM	20.1 (17.2 - 24.4)	28.1 (23.6 - 43.6)	100
	PER+DEF	20.1 (17.2 - 24.1)	27.3 (24.1 - 33.2)	100
	PER+DMC	20.6 (19.2 - 22.4)	26.7 (24.1 - 31.8)	100
	PER+PBO	21.3 (19.6 - 23.4)	25.5 (22.1 - 26.7)	100

1) Number of adults tested was 10 with 3 replicates for each test.
2) Synergists of of non-lethal dose (DEM, 1.5 μg/insect ; DEF and PBO, 1 μg/insect ; DMC, 2 μg/insect) was tropically treated 1-2 hours prior to the WHO test.
3) DDT 4% 4) Permethrin 0.75%

DDT resistance in this species in Japan, although some studies have also reported DDT resistance in some local Japanese populations (Miyagi *et al.*, 1989 ; Suzuki, 1962 ; 1963 ; Suzuki and Mizutani, 1962 ; Toma *et al.*, 1992). Widespread DDT resistance in mosquitoes and other insects in Japan is probably attributable to the widespread use of the chemical in the nationwide control programs of 1945－1962 (Kasai *et al.*, 2007 ; Toma *et al.*, 1992). It is interesting that only the Yonaguni colony was susceptible to DDT out of all the colonies collected in Japan. The same result was reported by Toma *et al.* (1992), in which the Yonaguni and Minami Daito colonies were susceptible to DDT from among several colonies collected from Okinawa Prefecture. The above facts confirm that extensive DDT treatment was not performed in those islands during the vector control program (Miyagi *et al.*, 1996).

Kawada *et al.* (2010) suggested the possibility of cross-resistance between pyrethroids and DDT in *Ae. albopictus* collected from Nagasaki City, because they showed resistance to pyrethroids. The organized and massive larvicidal treatment of graveyard containers with DDT formulations in the 1950s is thought to be one of the main causes for the development of pyrethroid resistance in this species, given that no other massive treatments with pyrethroids were conducted in this area. Kasai *et al.* (2007) suggested the same hypothesis concerning pyrethroid resistance in *Culex pipiens pallens* Coquillet in Japan. Possible mechanisms that might cause cross-resistance between pyrethroids and DDT include mutations in voltage-gated sodium channels (*kdr*) and enhanced cytochrome P450 monooxygenase activity. In the present study, however, no *kdr* mutation was found in the *Ae. albopictus* colonies. The contribution of PBO was not notable in enhancing DDT efficacy, indicating that neither *kdr* mutation nor cytochrome P450 is a major cause of detoxification of DDT. Enhancement of esterase activity, which is thought to be blocked by DEF and glutathion *S*-transferase, which is blocked by DEM, were suggested in DDT-resistant colonies. However, the toxicological roles of both the above enzymes have not been recognized as DDT-resistance factors yet, although a few studies have suggested correlations between the above enzymes and DDT resistance (Lumjuan *et al.*, 2011; Ranson *et al.*, 2011; Sarkar *et al.*, 2009). Enhancement of DDT dehydrochlorinase, which is blocked by DMC, might

Table 4 Susceptibility of *Ae. albopictus* larvae to *d*-T_{80}-allethrin with synergists. [1]

Colony	Treatment[2]	LC_{50}	LC_{95}	Slope ± SE	χ^2 (df)	% Suppression
Chuou	ALT[3]	0.200 (0.172 - 0.238)	0.949 (0.684 -1.511)	2.434 ± 0.236	5.017 (3)	-
	ALT+PBO	0.061 (0.054 - 0.069)	0.200 (0.164 - 0.262)	3.184 ± 0.284	2.718 (3)	69.5
	ALT+DEF	0.137 (0.120 - 0.158)	0.578 (0.447 - 0.818)	2.63 3± 0.228	2.838 (3)	31.5
	ALT[4]	0.222 (0.166 - 0.309)	0.422 (0.305 - 1.083)	5.906 ± 0.619	9.146 (3)	-
	ALT+DEM[4]	0.103 (0.094 - 0.113)	0.202 (0.175 - 0.247)	5.595 ± 0.572	4.078 (3)	53.6
Heiwa	ALT	0.103 (0.092 - 0.115)	0.266 (0.219 - 0.349	3.978 ± 0.390	2.938 (3)	-
	ALT+PBO	0.037 (0.023 - 0.063)	0.049 (0.029 - 0.205)	3.547 ± 0.310	14.371 (3)	64.1
	ALT+DEF	0.060 (0.040 - 0.091)	0.188 (0.155 - 0.696)	3.290 ± 0.283	10.212 (3)	41.7
	ALT[4]	0.111 (0.063 - 0.019)	0.525 (0.274 - 2.292)	2.239 ± 0.215	10.342 (3)	-
	ALT+DEM[4]	0.088 (0.039 - 0.183)	0.447 (0.206 - 7.721)	2.462 ± 0.214	16.111 (3)	20.7
Higashikurume	ALT	0.021 (0.013 - 0.035)	0.075 (0.043 - 0.339)	3.043 ± 0.254	11.784 (3)	-
	ALT+PBO	0.012 (0.009 - 0.017)	0.044 (0.028 - 0.107)	3.000 ± 0.259	16.174 (3)	42.9
	ALT+DEF	0.018 (0.010 - 0.033)	0.064 (0.034 - 0.644)	2.091 ± 0.240	6.146 (3)	14.3
Yonaguni	ALT	0.029 (0.021 -0.020)	0.088 (0.032 - 0.410)	3.394 ± 0.291	7.858 (3)	-
	ALT+PBO	0.014 (0.009 - 0.022)	0.022 (0.035 - 0.180)	4.084 ± 0.381	13.251(3)	51.7
	ALT+DEF	0.019 (0.018 - 0.021)	0.040 (0.035 - 0.050)	5.189 ± 0.519	3.952 (3)	34.5

1) Number of larvae tested was 30 with 3 replicates for each test.
2) Synergists of of non-lethal concentration (PBO, 0.6 ppm; DEF, 1.0 ppm; DEM, 1.0 ppm) was treated additionally.
3) *d*-T_{80}-allethrin
4) Tested in the different day using the different egg batches from the same colony.

be one of the main resistance factors in the Higashi-Kurume colony but the contribution of this enzyme was low in the Chuou and Heiwa colonies. The contribution of synergists in reducing pyrethroid resistance in adult *Ae. albopictus* is not clear, because all synergists caused 100% mortality in the adult susceptibility tests. However, the decrease in KT_{50} in the treatment by using DEF, DEM, and PBO might explain the existence of synergism with pyrethroid. On the other hand, PBO was the most effective in reducing the LC_{50} of *d*-T_{80}-allethrin in the larval stage, indicating that cytochrome P450 might be one of the main resistance factors.

This study highlights the wide distribution of pyrethroid tolerance or resistance in *Ae. albopictus* in Nagasaki City and the widespread DDT resistance in this species in Japan. In the first half of the 1950s, DDT and BHC were sprayed at the peripheries of graveyards or into flower vases on gravestones in Nagasaki City. Following the ban on DDT use in the 1970s, organophosphates such as diazinon, temephos, fenthion, and fenitrothion were used instead of DDT, and since then, no pyrethroid insecticide has been used for mosquito control. Moreover, an organized system for mosquito control in Nagasaki City has been canceled, in accordance with the revision of the Infectious Disease Prevention Law in 2000. Therefore, pyrethroid resistance in *Ae. albopictus* populations in Nagasaki City is considered attributable to the massive and organized treatment by using DDT in the 1950s as a mosquito larvae control measure (Kawada *et al.*, 2010). To conclude, however, we could not elucidate a clear relationship among resistance factors that might explain the cross-resistance between DDT and pyrethroids in pyrethroid-resistant *Ae. albopictus* in Japan. It is clear that kdr mutation has not occurred in *Ae. albopictus* during the course of the past, widespread DDT treatment. It can be hypothesized that multiple metabolic factors might possibly play roles in detoxification of pyrethroids, and some of them might be common to both DDT and pyrethroid detoxification mechanisms. Further biochemical study is necessary for clarifying the above hypothesis.

Pyrethroid resistance in *Ae. albopictus* is not a serious worldwide problem at present. Pyrethroids provide one of the most promising countermeasures for controlling mosquitoes. Development of pyrethroid resistance, therefore, will be a major hindrance to mosquito control programs. At present, there are no suitable chemical substitutes for pyrethroids. A regular monitoring system for insecticide susceptibility, including a simple biochemical evaluation system that can elucidate the modes of resistance, should be given priority in mosquito resistance management.

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長崎市内で採集されたヒトスジシマカ *Aedes albopictus*（Skuse）のピレスロイド抵抗性について

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長崎市内および日本国内の他地域で採集されたヒトスジシマカ *Aedes albopictus*（Skuse）について、幼虫および成虫の殺虫剤感受性調査を実施した。幼虫に対する簡易的な感受性試験において、長崎市内で採集されたコロニーの多く、および他地域で採集されたヒトスジシマカの幾つかが *d*-T_{80}-アレスリンに対し抵抗性を示した。また、与那国島で採集されたコロニーを除く全てのコロニーの成虫はDDTに対して高い抵抗性を示し、長崎市内採集コロニーの半数以上および他地域採集コロニーのうち福岡で採集されたコロニーがペルメトリンに対して抵抗性であった。いずれのコロニーからも電位依存性ナトリウムチャンネルのミューテーション（*kdr*）は検出されなかった。協力剤（DEM, DEF, PBO, DMC）を用いた成虫および幼虫の感受性試験の結果、長崎市内採集のヒトスジシマカにおけるピレスロイド抵抗性にはcytochrome P450に関連した酸化代謝が関与していることが示唆されたが、DDTとピレスロイド間の交差抵抗性を裏付ける結果は得られなかった。