Identification of TCT, a novel knockdown resistance allele mutation and analysis of resistance detection methods in the voltage-gated Na^+ channel of *Culex pipiens pallens* from Shandong Province, China

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Received July 30, 2012; Accepted November 9, 2012

DOI: 10.3892/mmr.2012.1184

Abstract. The present study aimed to investigate deltamethrin resistance in *Culex pipiens pallens* (*C. pipiens pallens*) mosquitoes and its correlation with knockdown resistance (*kdr*) mutations. In addition, mosquito-resistance testing methods were analyzed. Using specific primers in polymerase chain reaction (PCR) and allele-specific (AS)-PCR, *kdr* gene sequences isolated from wild *C. pipiens pallens* mosquitoes were sequenced. Linear regression analysis was used to determine the correlation between the mutations and deltamethrin resistance. A *kdr* allelic gene was cloned and sequenced. Analysis of the DNA sequences revealed the presence of two point mutations at the L1014 residue in the IIS6 transmembrane segment of the voltage-gated sodium channel (VGSC): L1014F, TTA→TTT, replacing a leucine (L) with a phenylalanine (F); L1014S, TTA→TCA, replacing leucine (L) with serine (S). Two alternative kdr-like mutations, L1014F and L1014S, were identified to be positively correlated with the deltamethrin-resistant phenotype. In addition a novel mutation, TCT, was identified in the VGSC of *C. pipiens pallens*. PCR and AS-PCR yielded consistent results with respect to mosquito resistance. However, the detection rate of PCR was higher than that of AS-PCR. Further studies are required to determine the specific resistance mechanism. PCR and AS-PCR demonstrated suitability for mosquito resistance field tests, however, the former method may be superior to the latter.

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Key words: *Culex pipiens pallens*, resistance mechanism, knockdown resistance alleles, allele-specific-polymerase chain reaction technique

Introduction

Mosquito-borne diseases, including malaria (1), dengue fever (2), Japanese encephalitis (3) and yellow fever (4), are a serious threat to human health. In China, *Culex pipiens pallens* (*C. pipiens pallens*) is important for transmission of Japanese encephalitis and filariasis (5). Chemical agents, including pyrethroid pesticides, are primarily used as prevention and control measures for mosquitoes due to their high efficacy and low toxicity. However, the frequent utilization of pyrethroid pesticides has led to insecticide resistance. Therefore, studies on effective prevention and control methods against mosquitoes have become a top priority.

The main mechanisms of insecticide resistance include target and metabolic resistance and reduction of cuticle penetration in the mosquito (6). A reduction in insecticide target sensitivity leads to target resistance (knockdown resistance, *kdr*). The resistance of the insect nervous system to pyrethroid insecticides is largely caused by point mutations that result in single nucleotide polymorphisms (SNPs). Previous studies demonstrated that pyrethroid insecticide resistance is caused by point mutations in the S6 transmembrane segment of domain II of para-sodium channels in the mosquito (7,8). In the majority of cases, an A→T transition at position 1014 is observed, resulting in a leucine (L)-to-phenylalanine (F; L1014F) mutation (9-11). In *Anopheles arabiensis*, a T→C transition at position 1014 results in an L-to-serine (S; L1014S) mutation (12).

In *Anopheles gambiae* from West Africa, it was previously shown that frequency of the L1014F mutation was correlated with survival of pyrethroid exposure (10,13). Ranson *et al* demonstrated that the *kdr* substitution L1014S is correlated with resistance to dichlorodiphenyltrichloroethane (DDT) and pyrethroids in these species (8). Harris *et al* (14) identified two V1016I mutations in domain II, segment 6 (IIS6) and F1534C in IIIS6 in the voltage-gated sodium channel (VGSC) of *Aedes aegypti* from the Grand Cayman. The F1534C mutation was reported to be closely correlated with resistance to DDT and permethrin (14). Previously, L1014F and L1014C substitutions

have been associated with β-cypermethrin resistance in *Anopheles sinensis* (15,16). Two alternative *kdr* mutations, L1014F and L1014S, have been reported in *C. pipiens pallens*. The present study aimed to investigate deltamethrin resistance levels in wild and sensitive *C. pipiens pallens* populations. The correlation between *kdr* allelic mutation and resistance levels of *C. pipiens pallens* was determined through gene sequencing and allele-specific polymerase chain reaction (AS-PCR).

Materials and methods

Mosquito collection. *C. pipiens pallens* were collected in larvae form from five separate districts in Jinan (36˚38' N, 116˚56' E), Zibo (36˚41' N, 117˚59' E), Weifang (36˚38' N, 118˚30' E), Qingdao (35˚41' N, 119˚44' E) and Jining (35˚18' N, 116˚29' E) and in Shandong Province, China between June 2011 and October 2011 (Fig. 1). Specimen collection was performed in rural sewage rivers. However, mosquitoes collected from Qingdao were gathered from an uninhabited island. Sensitive strains (protected from contact with insecticides for 20 years), routinely reared in our laboratory were used as the reference. All the larvae were raised in pork liver and yeast powders to adulthood in insectaria with 12-h indoor illumination and temperature maintained at 23-26˚C

Insecticide bioassays. Adult bioassays were performed on 1- to 3-day-old female mosquitoes using World Health Organization (WHO) (17) filter paper impregnated with paraffin liquid and deltamethrin (final concentration, 0.05%). Each population contained 25 female mosquitoes and experiments on populations were repeated four times. Knockdown quantity following 10, 20, 30, 40, 50 and 60 min of exposure was recorded. Following exposure, mosquitoes were transferred to a holding tube and 10% sugar solution was supplied on a cotton pad. Mortality was scored following a 24-h recovery period and the mosquitoes were refrigerated at -70˚C for DNA extraction.

Kdr allele amplification and sequencing. Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) from individual mosquito specimens. The *kdr* alleles were amplified using PCR and AS-PCR. Primers (Table I) were designed based on the cDNA sequence of *C. quinquefasciatus* para-sodium channel gene α subunit (Genbank accession number, BN001092). Primers C1 and C2 were used for PCR and C1, C2, C3, C4 and C5 for AS-PCR. *Taq* hot start polymerase (1.25 units; Takara Bio, Inc., Shiga, Japan), 2.5 mM each dNTP, 15 mM $MgCl_2$, 20 μM each of the primers and 50 ng genomic DNA comprised a 50-μl total volume PCR mixture. PCR conditions were as follows: initial denaturation at 94˚C for 5 min; 32 cycles of 94˚C for 40 sec, 55˚C for 50 sec and 72˚C for 1 min, with a final extension at 72˚C for 8 min. AS-PCR conditions were as follows: initial denaturation at 94˚C for 5 min; 40 cycles of 94˚C for 1 min, 55˚C for 2 min and 72˚C for 2 min, with a final extension at 72˚C for 10 min.

Statistical analysis. Linear regression analysis was used to determine the correlation between the mutations and deltamethrin resistance. The positive rates of PCR and AS-PCR were compared using the χ^2 test. $P<0.05$ was used to indicate a statistically significant difference.

Table I. PCR primer sequences.

Name	Sequence (5'-3')
C1	CCT GCC ACG GTG GAA CTT
C2	GGA CAA AAG CAA GGC TAA GAA
C3	CCA CCG TAG TGA TAG GAA ATT TA
C4	ACG CTG GAA TAC TCA CGA CTG
C5	ACG CTG GAA TAC TCA CGA CA

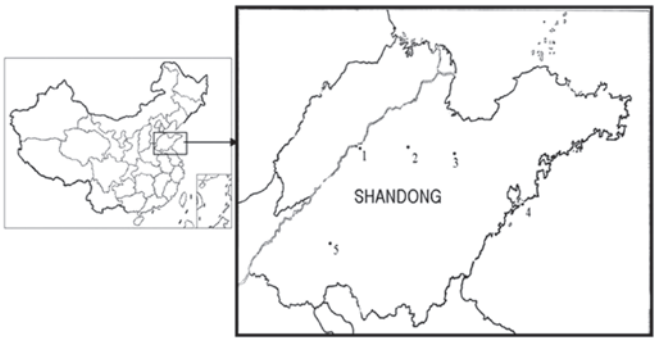

Figure 1. Map of China demonstrating the distribution of mosquito sampling sites. 1, Jinan (36˚38' N, 116˚56' E); 2, Zibo (36˚41' N, 117˚59' E); 3, Weifang (36˚38' N, 118˚30' E); 4, Qingdao (35˚41' N, 119˚44' E); and 5, Jining (35˚18' N, 116˚29' E).

Results

Deltamethrin resistance bioassay. Knockdown rates of *C. pipiens pallens* female mosquitoes following 60 min exposure to paper impregnated with 0.05% deltamethrin and the mortality rates following the 24-h recovery period are listed in Table II. According to WHO resistance evaluation indicators, mortality rates for the sensitive (Sn), initially resistant (M; resistance may exist, but further validation is required) and resistant (R) groups were identified as between 98 and 100%, 80 and 97% and <80%, respectively. Specimens from Jining, Jinan, Zibo and Weifang were included in the R group, those from Qingdao in the M group and those from sensitive strains in the Sn group.

Sequencing results of kdr alleles and a new substitution. A 521-bp DNA sequence from individual mosquitoes was separated and amplified from 622 specimens of *C. pipiens pallens* using PCR (Fig. 2A). TTA is the wild-type codon sequence in the 1014 site of the sodium channel gene II S6 (L1014). DNA sequence analysis revealed the presence of two point mutations at the site: L1014F, TTA→TTT, replacing L with F; L1014S, TTA→TCA, replacing L with S. The *kdr* genotype and allele frequencies of the phenotypes, determined by the deltamethrin resistance bioassay in *C. pipiens pallens* populations of various regions, are shown in Table III.

Alignment of the VGSC amino acid sequence of C. *pipiens pallens* populations is evident in Fig. 2B. In addition, a new

Table II. Bioassay results of deltamethrin resistance in *Culex pipiens pallens*.

Sampling point	No. of specimens	Knockdown rate in 1 h (%)	Mortality rate (%)	Level of resistance
Jining	102	32.35	37.25	R
Jinan	106	54.72	39.62	R
Zibo	96	70.83	64.58	R
Weifang	108	51.85	72.22	R
Qingdao	105	41.90	83.81	M
Sensitivity	105	98.10	98.10	S

R, resistant; M, initially resistant (resistance may exist, but further validation is required); S, sensitive.

Table III. The *kdr* genotype and allele frequency of phenotypes determined by the deltamethrin resistance bioassay in various regions of *Culex pipiens pallens* populations.

Region (Gr.)	TTA/TTA	TTT/TTT	TCA/TCA	TTA/TTT	TTA/TCA	TTT/TCA	TTA (%)	TCA (%)	TTT (%)
Jining (R)	5	34	13	2	2	8	17.16	21.57	61.27
Jining (Sn)	7	19	1	5	2	4			
Jinan (R)	7	27	10	6	3	10	37.27	19.81	42.92
Jinan (Sn)	26	9	3	2	2	1			
Zibo (R)	15	9	3	4	2	1	70.31	6.25	23.44
Zibo (Sn)	46	8	1	6	1	0			
Weifanf (R)	9	10	2	1	5	0	67.14	4.29	28.57
Weifang (Sn)	55	16	0	7	0	0			
Qingdao (R)	6	5	1	2	1	0	65.54	5.34	29.12
Qingdao (Sn)	57	21	1	3	3	3			
Sensitivity (R)	1	1	0	0	0	0	81.91	0.00	18.09
Sensitivity (Sn)	82	15	0	6	0	0			

kdr, knockdown resistance; Gr., group; R, resistant; Sn, sensitive.

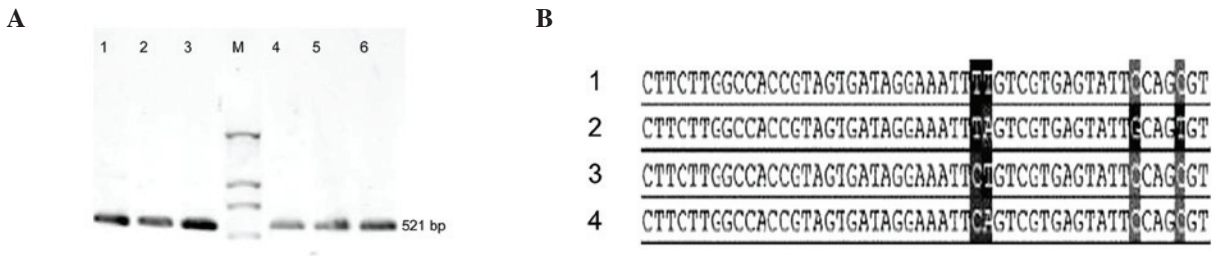

Figure 2. (A) Agarose gel electrophoresis of PCR production of sodium channel gene II S6 fragment in *Culex pipiens pallens (C. pipiens pallens)*. Bands: M, marker 2,000 bp; 1, Jining; 2, Jinan; 3, Zibo; 4, Weifang; 5, Qingdao; 6, sensitivity *C. pipiens pallens*. (B) Alignment of voltage-gated sodium channel amino acid sequence of *C. pipiens pallens* populations. Rows: 1, L1014F: TTA→TTT; 2, L1014: TTA no mutation; 3, L1014S: TTA→TCT; 4, L1014S: TTA→TCA.

substitution in codon 1014 within IIS6 from TTA to TCT (n=2), replacing an L with an S, was identified using PCR. Substitutions were homozygous for the S allele in deltamethrin survivors from Qingdao *C. pipiens pallens*.

Frequencies of the L1014F mutation from Jining, Jinan, Zibo, Weifang, Qingdao and sensitive C. *pipiens pallens* were 61.27, 42.92, 23.44, 28.57, 29.12 and 18.09%, respectively. Survival rates following the 24-h test period in the mosquito contact tube were 62.75, 60.38, 35.42, 27.78, 16.19 and 1.90%, respectively. Regression analysis demonstrated a correlation between the survival rate of the mosquito contact tube and the frequency of *kdr* L1014F alleles (y=1.317995x-10.6122, R^2=0.74068, P<0.05; Fig. 3A).

Frequencies of the L1014S mutation from Jining, Jinan, Zibo, Weifang and Qingdao *C. pipiens pallens* were 21.57, 19.81, 6.25, 4.29 and 5.34%, respectively. Survival rates

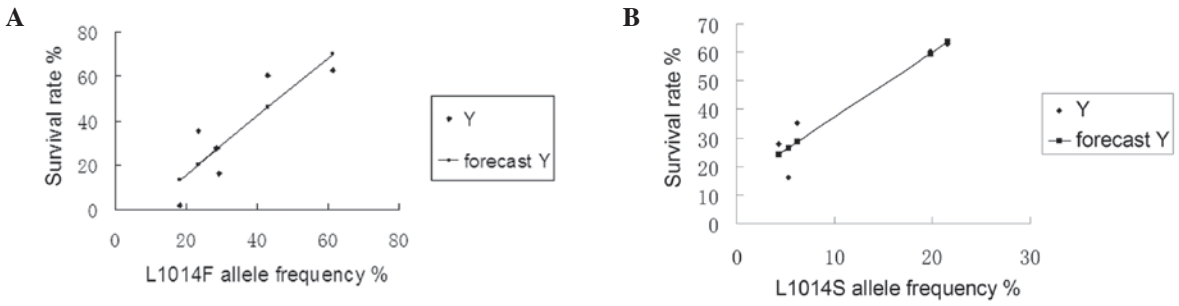

Figure 3. (A) Correlation between the mosquito survival rate and L1014F allele in *C. pipiens pallens*. (B) Correlation between the mosquito survival rate and L1014S allele in *C. pipiens pallens*.

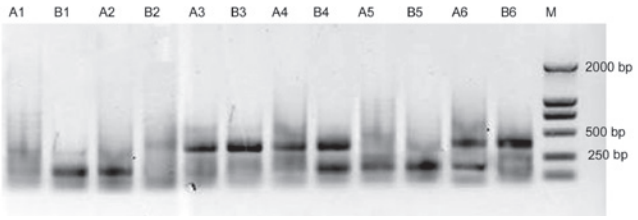

Figure 4. Results of AS-PCR test of *kdr* allele of *C. pipiens pallens*. 'A' samples were amplified with the primers C1, C2, C3 and C4 to detect the 1014L (TTA) and 1014S (TCA) alleles; 'B' samples were amplified with primers C1, C2, C3 and C5 to detect the 1014L and 1014F (TTT) alleles. M, marker 2,000 bp; A1B1, L1014F/L1014F (TTT/TTT) homozygous mutant (RR); A2B2, L1014S/L1014S (TTT/TTT) homozygous mutant (RR); A3B3, L1014/L1014 (TTA/TTA) wild homozygous (SS); A4B4, L1014/ L1014F (TTA/TTT) mutation heterozygotes (RS); A5B5, L1014S/L1014F (TCA/TTT) mutation heterozygotes (RS) A6B6, L1014/L1014S (TTA/TCA) mutation heterozygotes (RS). R, resistant; S, sensitive; AS-PCR, allele-specific-polymerase chain reaction.

following the 24 h test period in the mosquito contact tube were 62.75, 60.38, 35.42, 27.78 and 16.19%, respectively. Regression analysis revealed a substantial correlation between the survival rate in the mosquito contact tube and the frequency of *kdr* L1014S alleles (y=2.283074x+14.35824, R^2=0.899342, P<0.05; Fig. 3B).

Optimization of kdr mutation diagnostic assays. Based on the sequence data, AS-PCR was performed in *C. pipiens pallens* from Jining. In this test, C1 and C2 were used as outer primers to expand the fragment of the *kdr* allele (521 bp). Moreover, C3, C4 and C5 were used as specific inter-primers to amplify a 389- and 176-bp fragment for wild-type (codon TTA) and mutant (codons TCA and TTT) alleles, respectively. As a result, the sample for the wild homozygous gene TTA/TTA (SS) produced a band corresponding to 389 bp. The sample for the mutant (TCA/TTT) genes produced a band at 176 bp (Fig. 4). We obtained 64 (62.75%, a total of 102) specimens. As determined by the χ^2 test, the positive rate of RT-PCR was identified as significantly higher than that of AS-PCR (χ^2=44.273304, P=0.000000, P<0.05). Notably, 89 (87.25%) specimens were successfully amplified by this method following three repetitions. The positive rates of PCR and AS-PCR were compared using the χ^2 test, demonstrating that the former method is more effective than the latter (χ^2=11.830850, P=0.000583, P<0.05).

Discussion

The use of chemical agents as prevention and control measures for mosquitoes is common practice due to increased efficacy and lower mammalian toxicity (18,19). However, the intensive use of pyrethroids has triggered severe insecticide resistance in this vector and resulted in a reduced effect in vector control (20,21). Therefore, determination of insecticide resistance and elucidation of the mechanism by which it is developed is important. In this investigation, *C. pipiens pallens* from human-inhabited areas developed high resistance (mortality rate, 37.3-72.2%) to pyrethroid insecticides. The sample from the uninhabited island of Qingdao (mortality rate, 83.8%) demonstrated low resistance to pyrethroid insecticides. Previously, *C. pipiens pallens* exhibited low resistance and sensitivity levels (mortality rate: 82.8-91.5%) to cypermethrin, permethrin and deltamethrin between 2004 and 2006 (22). This resistance level has substantially increased in the last five years.

In the present study, the L1014F mutation in the IIS6 transmembrane segment of VGSC was positively correlated with the deltamethrin-resistant phenotype (R^2=0.74068). Studies performed in *C. pipiens pallens* (23) and *An. sinensis* (15) reported similar conclusions. However, Abdalla *et al* (24) did not identify a correlation between the *kdr* L1014F gene mutation and pyrethroid insecticide-resistant phenotype in an analysis of 14 natural populations of *Arab Anopheles* in the Sultan region. However, the sample size (<50) of that study is considered insufficient to analyze this correlation by a number of research groups (25). The present study analyzed 622 *C. pipiens pallens* samples to ensure that the experiment was accurate.

A number of studies have identified a response in L1014S mutants to pyrethroid insecticide selection (26-28). Kawada *et al* (12) identified the L1014S mutation in the larvae of *A. gambiae* and *A. arabiensis*. Singh *et al* (29) also studied *Anopheles* from the Alba area and the mutation was first verified in *Anopheles stephensi*. A study on the RSP-ST-resistant strains of *A. gambiae* identified a significantly increased proportion of the *kdr* gene mutant L1014S/L1014S (RR) homozygotes in cypermethrin-resistant groups that were correlated with the cypermethrin-resistant phenotype (8). These observations are consistent with results of the present study, in which the *kdr* gene L1014S mutation was identified to correlate with the deltamethrin-resistant phenotype

(R^2=0.899342). By contrast, Chen *et al* (23) detected no correlation between *C. pipiens pallens* in China and L1014S mutations of pyrethroid insecticide resistance. However, only one L1014S/L1014S (RR) homozygote was reported in this study and therefore the resistance caused by L1014S mutations may be ignored. The present study identified 35 L1014S/L1014S (RR) homozygous mosquitoes (L1014S allele frequency=118). Therefore, the results of the present study have a higher degree of credibility.

Considering the importance of the L1014S (TCA) mutation in the development of deltamethrin resistance, we hypothesized that the TCT mutation from Qingdao *C. pipiens pallens* is correlated with insecticide resistance. This hypothesis was previously demonstrated in *An. sinensis* (15). TTG (L), in codon 1014 of sodium channel gene II S6, was mutated to TTT (F) or TGT (cysteine, a new mutation) in *An. sinensis*. However, TCA and TCT code for the amino acid S. The *kdr* gene L1014S mutation is closely correlated with the deltamethrin-resistant phenotype (R^2=0.899342) and was demonstrated by bioassay to cause increased resistance in the insect (15,23). We hypothesize that the TCT (S) mutation identified in Qingdao *C. pipiens pallens* has a similar function. Additional studies are required to determine this conjecture.

Two parallel PCR systems are suitable for identification of one idiotype in AS-PCR. Observation of a 389-bp, 176-bp band or both bands represents wild homozygous TTA/TTA (SS), homozygote (RR) or heterozygote genotype (RS) samples, respectively. Considering the simplicity and cost-effectiveness of the technique, AS-PCR is widely used in studies on resistance of mosquitoes, including *An. sinensis* (30) and *A. gambiae* (31). In addition, this method eliminates steps, including purification and sequencing. However, Bass *et al* (32) previously reported cases of non-specific extension and primer dimers that affect the accuracy of the results. In the present study, a statistically significant difference was observed in the detection rate of AS-PCR and gene sequencing methods (χ^2=11.830850, P=0.000583, P<0.05). Therefore, we conclude that AS-PCR is suitable for large-scale field inspections, due to its simplicity and cost-effectiveness. However, PCR methods may prove superior to AS-PCR in mosquito resistance field tests for mutations and deltamethrin resistance.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (no. 81271877).

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