A NEW HIGH-PERFORMANCE PCR DIAGNOSTIC FOR THE DETECTION OF PYRETHROID KNOCKDOWN RESISTANCE KDR IN ANOPHELES GAMBIAE

FREDERIC TRIPET,* JENNIFER WRIGHT, AND GREG LANZARO
Department of Entomology, University of California Davis, Davis, California

Abstract. Monitoring the spread of the knockdown resistance allele kdr in areas of extensive pyrethroid use is critical to vector-control projects. Currently available methods for detecting kdr from DNA samples are characterized by poor amplification, time-consuming steps, and primers that exhibit frequent null alleles. We describe a new PCR diagnostic that uses fluorescent primers based on conserved priming sites and enables simple detection of the kdr allele on a sequencer. Using samples from a West African Anopheles gambiae population, we show that the new PCR yielded significantly higher rates of amplification and more accurate estimates of kdr frequency. The method works equally well for the leucine to phenylalanine substitution found in West Africa and the East African leucine to serine substitution.

INTRODUCTION

Malaria still accounts for 300 million to 500 million clinical cases and 1.5 million to 3 million deaths per year in Africa.1,2 The main vectors of malaria in this region are the members of the Anopheles gambiae complex that comprises at least seven sibling species, among which Anopheles arabiensis and Anopheles gambiae sensu stricto are the most important species in terms of spatial distribution and abundance.3–5 An. gambiae s.s. has been further subdivided into five chromosomal forms based on extensive polytene chromosome studies6–8 and the M and S molecular forms—based studies of the ribosomal DNA region on the x-chromosome.9–11 In some parts of Africa, the distribution of M and S molecular types is linked to the chromosomal forms.10 In others, the relationship breaks down but populations differing in their molecular forms are still thought to characterize partially or completely isolated populations.11–13

Current vector-control projects in Africa rely heavily on the use of pesticides for treating bed nets or for residual spraying of domiciles. Different studies have demonstrated the efficacy of insecticide-treated net (ITN) programs for locally curbing malaria incidence.14 Pyrethroid compounds are the only insecticides approved for such use due to their lower toxicity to mammals, faster kill speed, and comparatively better excito-repellent effects than other compounds.15 Unfortunately, resistance to pyrethroids has recently emerged in West and East Africa, and its fast spread suggests that it will become a major hindrance to malaria control programs targeting adult mosquitoes.16 A common form of resistance to pyrethroids is the knockdown resistance (kdr), which is linked to a single nucleotide substitutions in the voltage-gate sodium channel gene that results in target-site resistance to pyrethroids and cross-resistance to DDT.17 In West Africa, the substitution results in a change from leucine to phenylalanine (TTA to TTT)18 and was first detected in populations of the Savanna chromosomal form in coastal Ivory Coast.19 Since then, the kdr allele has also been detected in Savanna populations from Benin, Burkina Faso, Guana, and Mali.20–25 In East Africa, kdr resistance is due to a substitution from leucine to serine (TTA to TCA) at the same codon as the substitution observed in West Africa.17 This kdr allele has been detected in Kenya where its frequency has significantly increased as a result of recent ITN programs.26 Because of the rapid spread of kdr resistance in West Africa and its emergence in East Africa, large-scale surveys of the current distribution of resistance throughout the geographical range of major vectors are critical to successful spraying and ITN projects. The distribution of the kdr allele can also be used to detect barriers to gene flow among populations of vectors and in this regard is relevant to the study of reproductive isolation between the chromosomal and molecular forms of An. gambiae s.s.27–28

Two methods have been commonly used to detect the presence of the kdr allele and estimate its frequency from large numbers of individuals.18,29 The first method is a multiplex PCR that uses a cocktail of 4 primers to generate diagnostic band patterns on agarose gels.18 The very low amplification efficiency of this PCR precludes the analyses of DNA samples of low concentration or of poor quality. Because of high rates of null-alleles in the 4 priming sequences, estimates of the frequency of the kdr allele in wild populations are likely to be inaccurate and some samples cannot be amplified at all. The second approach is a simple PCR amplification followed by sequence-specific oligonucleotide probing (PCR-SSOP).29 The additional hybridization steps required by the latter method may explain why it has not been used as extensively as the multiplex PCR. Moreover, one of the primers is identical to that used in the multiplex PCR, hence null-alleles are to be expected using this procedure, too. Finally, a third method based on a hot ligation oligonucleotide assay (HOLA) has recently been developed that allows for convenient scoring by visualization of a fluorescein-labeled reporter after allele-specific ligation to a detector.30 This approach is more complex and time consuming than the multiplex PCR but has the advantage of requiring no electrophoresis equipment.30 As an initial PCR amplification is never-sufficient to amplify the kdr allele using a gene fragment analyzer. We demonstrate the advantages of the method by comparing the allelic frequencies obtained with the new PCR with those we obtained using the multiplex PCR in a previous study of the kdr distribution in an An. gambiae population on Bioko Island.
Equatorial Guinea. The method can easily be adjusted to detect the kdr substitution characterizing resistant East African populations. This was shown using DNA from crosses between susceptible and resistant An. gambiae strains in the laboratory. The new PCR maximizes amplification efficiency and alleviates most of the problems due to poor DNA quality and the presence of null-alleles. Consequently, it should generally produce estimates that are based on larger sample sizes and are comparatively more accurate than in past studies.

MATERIALS AND METHODS

Field-collected mosquitoes. Field-collected mosquitoes came from the city of Malabo (3°21′N, 8°40′E), Bioko Island, Equatorial Guinea, in West Africa. For comparing the two PCR assays for detecting the West African kdr allele (Leu-Phe substitution), we used only those DNA extracts that were successfully characterized as An. gambiae s.s. and the M and S form of Anopheles gambiae s.s. PCR assays31,32 (see previous study28 for details).

Laboratory strains and crosses. We obtained the RSP strain of An. gambiae that is an established kdr-resistant S-form strain colonized from mosquitoes collected in Kenya and maintained at the Malaria Research & Reference Reagent Resource Center (MR4; Manassas, VA) repository at the Centers for Disease Control (strain reference number MRA-762). We also used the Kisumu1 strain, a kdr-susceptible S-form strain colonized from the area of Kisumu in Kenya (strain reference number MRA-334). To optimize our PCR assay for the East African kdr allele (Leu-Ser substitution) we produced mosquito families that were homozygote and heterozygote for resistance. Newly emerged adults from the RSP and Kisumu strains were combined in two cages of 30 RSP females with either 30 RSP or Kisumu males as well as a cage with 30 Kisumu males and females. After 3 days, females were presented daily with a bloodmeal. Two days later, gravid females were placed in single tubes for single laying oviposition. Egg-batches that hatched successfully were reared to 2nd instar larval stage at which point DNA was extracted from the larvae using the DNAzol extraction buffer and accompanying protocol (Invitrogen, Carlsbad, CA).

kdr detection by multiplex PCR. Detection of the Leu-Phe kdr mutation in the population from Bioko was first made using the PCR diagnostic test developed by Martinez-Torres and colleagues18 with modifications aimed at improving amplification efficiency. We used 2 mL of extracted genomic DNA and AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) in a 25 μL reaction. The primers Agd1, Agd2, Agd3, and Agd4 and other reactions were identical to those used in Martinez-Torres et al.18 PCR amplifications were done on an MJ Research PTC-200 thermal cycler (MJ Research, Watertown, MA) and included an initial 13 minutes at 95°C, 1 minute at 95°C, 30 seconds at 48°C, 30 seconds at 72°C for 60 cycles and a final extension step at 72°C for 10 minutes. Amplified fragments were run on 1.5% agarose gels.

kdr detection by PCR elongation with fluorescence. A set of two forward primers and one reverse primer were designed using the software Oligo version 6. The forward primers were designed bearing in mind that they had to have high priming efficiency but nevertheless show high annealing specificity to the kdr susceptible and resistant alleles. We opted for primers with similar sequences except for the kdr substitution site located at their 3′ end (Figure 1 and Table 1). A single reverse primer was designed just outside of intron 2 and was selected on the basis of its high priming efficiency and compatibility with the forward primers (Figure 1 and Table 1). The optimized PCR reaction mix for detecting the Leu-Phe substitution included 0.25 μL of AGSWA primer labeled with green fluorescence (5′ Hex), 0.12 μL of AGRWA primer labeled with blue fluorescence (5′ Fam), 0.25 μL of the AGREV primer, 0.45 μL of DNTPs (10 mM), and 0.125 μL of Taq (Eppendorf, Hamburg, Germany) and 1 μL of DNA template in a 25 μL reaction with 10X buffer, 5X PCR enhancer (Eppendorf, Hamburg, Germany), and 2.5 μL magnesium chloride (25 mM). PCR amplifications were done on MJ Research PTC-200 thermal cycler (MJ Research, Watertown, MA) and included an initial 2 minutes at 95°C, 1 minute at 95°C, 30 seconds at 63°C, 30 seconds at 72°C for 25 cycles and a final extension step at 72°C for 5 minutes. For the Leu-Ser substitution conditions only 0.2 μL of AGSWA primer labeled with green fluorescence (5′ Hex) was used. PCR products were diluted 40 times in H2O before being mixed with Genescan 400HD size standard (Applied Biosystems) and run on an ABI 3100 capillary sequencer (Applied Biosystems). The gels were analyzed using the ABI PRISM Genescan Analysis Software (Applied Biosystems).

RESULTS

Estimation of kdr allele (Leu-Phe) frequency in Bioko. Multiplex PCR assay. PCR amplification was generally poor with the multiplex PCR hence nonamplified and ambiguous samples were run a second time. After these two attempts, 53 of 75 (70.6%) individuals were successfully amplified (Table 2). However, scoring the genotypes of individuals was generally difficult because of low amplification (Figure 2). In some cases, only one of the two expected bands was amplified indicating mismatches in one of the priming sites. Such null-alleles potentially affected 15 of the 53 amplified samples (28.3%). Poor amplification was also responsible for the erroneous scoring of an S-form individual as heterozygote for kdr resistance. As reported by Reimer and others28 kdr resistance is only present in the M-form An. gambiae population from the city of Malabo on Bioko Island, Equatorial Guinea. Based on all genotypes (individuals with one band missing included), the estimated frequency of the kdr allele in

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Figure 1. Partial sequence of the voltage-gate sodium-channel gene (bps 921 to 1190) showing the location of two introns and of the coding region that bears the substitutions responsible for kdr resistance in West and East Africa (position 1104 and 1105 in bold). The priming regions of the allele-specific fluorescent forward primers for the detection of the West African (bps 1081 to 1105) and East African (bps 1080 to 1104) alleles as well as the priming site of universal reverse primer are indicated (arrows).
the M form was 35.2%. When only M-form individuals that showed amplification of two bands were considered, the frequency of the \textit{kdr} allele was 44.0%.

\textit{PCR elongation with fluorescence.} To match the amount of effort used for the multiplex PCR, we re-ran nonamplified and ambiguous samples once. After those two rounds of PCR, 70 of 74 samples (93.3%) were amplified. All 70 samples were genotyped by visual inspection of the green (susceptible) and blue (resistant) fluorescent peaks present at size 110 bps on the chromatographs generated by Genescan (Figure 3 and Table 2). There were no ambiguities in the scoring of genotypes. The frequency of the \textit{kdr} allele calculated from the frequency of genotypes was, in this case, equal to 50.0%.

\textbf{Detection of \textit{kdr} allele (Leu-Ser) in laboratory crosses.} The PCR elongation assay with fluorescent primers gave equally good results for the Leu-Ser substitution characteristic of \textit{kdr} resistance in East Africa. As expected, all progeny of the 2 Kisumu strain families exhibited chromatographs typical of susceptible individuals (large green peaks) (Table 3, families 1 and 2). Similarly, families involving the resistant RSP strain produced progeny that were entirely identified as resistant (families 3 and 4). As expected, crosses between the susceptible and resistant strains produced hybrid susceptible-resistant progeny (Table 3, families 5 and 6).

**DISCUSSION**

Here we show that the new PCR elongation assay with fluorescent primers provides a reliable method for detecting

\begin{table}[h]
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\begin{tabular}{l|c|c|c|c|c|c|c}
| Form | \textit{s/s} & \textit{r/r} & \textit{r/s} & \textit{s/r} & \textit{s/r} or \textit{r/r} & \textit{r/r} or \textit{s/s} & \textit{r/s} or \textit{s/r} |
|-------|-----------|-----------|-----------|-----------|----------------|----------------|----------------|
| M     | 15        | 0         | 104       | 0         | 5              | 27             | 54             |
| \textit{PCR} | 7        | 5         | 27        | 0.36      | 14             | 48             | 62             |
| \textit{Fluorescence} | 27        | 0         | 0         | 0         | 0              | 0              | 27             |
\end{tabular}
\caption{Comparison of the frequency of homozygote susceptible (\textit{s/s}), homozygote resistant (\textit{r/r}), heterozygote (\textit{r/s}), and partial genotypes (\textit{s/r} or \textit{r/r} or \textit{s/s}) in a mixed M- and S-form population of \textit{An. gambiae} s.s. on Bioko Island, Equatorial Guinea}
\end{table}

\textbf{FIGURE 2.} Example of a 1.5% agarose gel showing the PCR bands characterizing susceptible \textit{s/s} (137 and 293 bps), resistant \textit{r/r} (195 and 293 bps), and heterozygote \textit{r/s} (137, 195, and 293 bps) individuals produced by the multiplex PCR. A 100 bps DNA ladder was run on lane X. A number of samples could not be amplified, and among those amplified many were ambiguous or lacked one of the expected bands suggesting the presence of mismatches in priming regions (here denoted by “?”).

\textbf{FIGURE 3.} Chromatographs generated by Genescan after running the fluorescent PCR products generated by the \textit{kdr} assay. The characteristic allele-specific fluorescent peaks of homozygote susceptible \textit{s/s} (top graph), resistant \textit{r/r} (middle graph), and heterozygote \textit{r/s} individuals (bottom graph) appear at size 110 bps in all cases. A nearby peak of fluorescence-labeled size standard (100 bps) simplifies the identification of the diagnostic peaks.
the substitution linked to kdr resistance in West and East Africa. Successful amplification was achieved even with very old, partially degraded DNA samples. It should also be mentioned that when the assay was run on highly concentrated DNA samples (e.g., DNA produced by whole genome amplification), higher dilution of the fluorescent PCR products was necessary to avoid generating saturation peaks on the fragment analyser. The absence of null-alleles observed here and in samples from other populations (Tripet F, personal observation) is a further improvement over other methods. In our comparison of the new method with the commonly used multiplex PCR,18 the number of successful amplifications increased by ~23% and the estimated kdr frequency increased by ~15%. It appears that in the M-form population from the Island of Bioko, null-alleles seemed to occur more frequently among kdr-resistant individuals than among susceptible ones. We expect the differences in the proportion of amplified individuals and estimated kdr allelic frequency to be substantial in most wild populations. In light of our results, the new assay promises to significantly improve the quality of field surveys aimed at monitoring the changes in distribution of kdr alleles both temporally and spatially.

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Author’s addresses: Frederic Tripet, Jennifer Wright, and Greg Lanzer, Department of Entomology, University of California Davis, 1 Shields Ave., Davis, CA 95616-8584, E-mails: fttripet@ucdavis.edu, jwright@ucdavis.edu and glanzer@ucdavis.edu. Frederic Tripet (new address), Center for Applied Entomology and Parasitology, School of Life Sciences, Keele University, Staffordshire, ST5 5BG, UK.

Reprint requests: Frederic Tripet, Center for Applied Entomology and Parasitology, School of Life Sciences, Keele University, Staffordshire, ST5 5BG, UK, E-mail: fttripet@yahoo.com.

REFERENCES


