# POLYMERASE CHAIN REACTION-BASED IDENTIFICATION AND GENOTYPING OF *ANOPHELES* MOSQUITOES WITH A 96-PIN BACTERIAL REPLICATOR

CRISTINA S. RAFFERTY, SCOTT R. CAMPBELL, ROBERT A. WIRTZ, AND MARK Q. BENEDICT

*Centers for Disease Control and Prevention, Division of Parasitic Diseases, Atlanta, Georgia; Suffolk County Department of Health Services, Arthropod-Borne Disease Laboratory, Yaphank, New York*

*Abstract.* A simple method for rapid identification of large numbers of *Anopheles* mosquitoes was developed based on polymerase chain reaction (PCR) amplification of the rDNA intergenic spacer and internal transcribed spacer 2. By means of previously described primers for the *Anopheles gambiae* and *An. quadrimaculatus* species complexes, rDNA was amplified simultaneously from 96 whole mosquitoes or parts. No homogenization or individual DNA preparation was necessary, and transfer of 96 samples to PCR reactions was performed simultaneously with a bacterial replicator. Control reactions indicate that the level of cross-contamination is negligible, and false-negative findings are rare. The method was tested on larvae, pupae, adult heads, whole adult males and females, and single tarsi. All parts except tarsi provided satisfactory template. Fresh, ethanol-preserved, dried, and frozen adults were also tested with similar results. The method was also tested for amplification of a single-copy gene, *white*. Results were generally positive, although some false-negative findings were observed. This method allows rapid analysis of large numbers of mosquitoes without robotic equipment and should enable rapid and extensive PCR analysis of field-collected samples and laboratory specimens.

## INTRODUCTION

Morphologically indistinguishable *Anopheles* often consist of genetically distinct sibling species in which one member of the species complex is a significant malaria vector, although others are not. Entomological assessment of risk of malaria transmission therefore depends on exact identification of the *Anopheles* species present. Various methods have been used to distinguish sibling species: karyotype, isozymes, and most recently polymerase chain reaction (PCR) amplification of rDNA, as reviewed by Collins and Paskewitz.[1] This method has the following advantages: small parts of any life cycle stage provide sufficient DNA for amplification; preserved material can be identified; and the analysis is straightforward and can be performed with relatively little training. However, unless DNA sequence analysis is performed on each specimen, this method requires preliminary morphological identification of the species complex to which the mosquito belongs in order to select the appropriate primers and to correctly interpret the results. Primers for species identification have been developed for numerous species complexes, including *An. gambiae*,[2] *An. punctulatus*,[3] *An. dirus*,[4] and *An. quadrimaculatus*.[5] The existence of other species complexes is suggested by rDNA divergence within *An. oswaldi*[6] and *An. nuneztovari*.[7] Therefore, rDNA PCR should also be a useful approach to distinguish members of these species complexes as well.

In order to simplify the PCR method used to identify vector species, we have developed a method to rapidly analyze large numbers of individual mosquitoes without individual DNA purification. We show that suitable template to amplify both rDNA and a single-copy gene can be obtained from fresh and preserved specimens by use of a simple process. This is then followed by simultaneous transfer to 96 PCR reactions with a bacterial replicator.

## MATERIALS AND METHODS

Mosquitoes were cultured according to the method of Benedict.[8] *Anopheles gambiae* G3 and *An. quadrimaculatus* Q2 were used for experimental stocks, positive controls, or both. Field-collected *An. quadrimaculatus* were frozen at −80°C, and the head and thorax were separated on ice from the abdomen, which was used for PCR. Desiccated specimens were prepared by placing a single adult into 2-mL Sarstedt tubes, placing the tubes in a sealed container of Drierite, and capping the tubes after 3 days. Ethanol-preserved specimens were prepared by placing a single adult into 200 μL of 80% ethanol and capping the tubes. Desiccated and ethanol-preserved specimens were stored at room temperature (~ 22–24°C) for 2 months before analysis.

To determine the approximate quantity and uniformity of fluid transfer, 3 clean, dry replicators were sequentially dipped into a 96-well plate, 32 wells of which contained 50 μL each of 1.0% Coomassie blue G-250 in denaturing buffer (10 mM Tris-HCl 8.2, 1 mM ethylenediaminetetraacetic acid, 50 mM NaCl, 0.1% Triton-X 100). The buffer is a variation of "*Drosophila* squishing buffer" used by Gloor and Engels (personal communication), but with Triton-X 100 added to improve the consistency in wetting volume of the pins. Each replicator was then dipped into a second 96-well Immulon flat-bottomed plate, the corresponding 32 wells of which contained 200 μL of 1× PCR reaction buffer. This volume results in approximately the same 6-mm fluid depth as the sample plate. A dilution series standard and 8 negative controls were also placed in each of these 3 plates. The absorbance of each well was read at 600 μm, converted to μL equivalents, and statistically analyzed for the mean and sample standard deviation of each plate. Error was ± 2 sample standard deviations, and statistical significance was considered to be $P < 0.05$.

Templates were prepared as follows: an individual mosquito or part was placed into 50 μL of denaturing buffer in a 96-well PCR plate, covered with a foil adhesive (Marsh AB-0626, Rochester, NY) and heated for 15 min at 94°C in a thermal cycler. After briefly centrifuging the plate to collect the sample, the cover was removed, and a sterile 96-well replicator (Boekel, Feasterville, PA) was dipped into the buffer and transferred to a second 96-well plate, each well of which contained 25 μL of PCR reaction mixture. This replicator consists of parallel stainless steel combs mounted in grooves of an aluminum support plate. (A similar device could be constructed easily by a machinist by using inert materials that could withstand decontamination. We are familiar with simi-
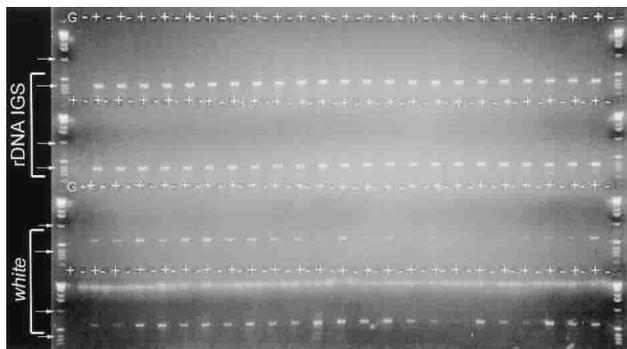
FIGURE 1. Agarose gel electrophoresis of false-positive and false-negative multicopy and single-copy target experiments. Alternating rows of a 96-well plate contained fresh non-blood-fed *Anopheles gambiae* females in buffer (+) or buffer alone (−). After heating the samples, 2 replicators were dipped into the plate and used to inoculate 2 sets of polymerase chain reactions. The top 2 rows show agarose gel electrophoresis of 8 μL of rDNA products. The bottom 2 rows show amplification of the *white* gene from the same samples. Alternating samples are positive or negative controls or genomic DNA (G). The first and last lanes contain a 1-kb ladder, with 396- and 1,018-bp marker bands indicated (**arrows**)
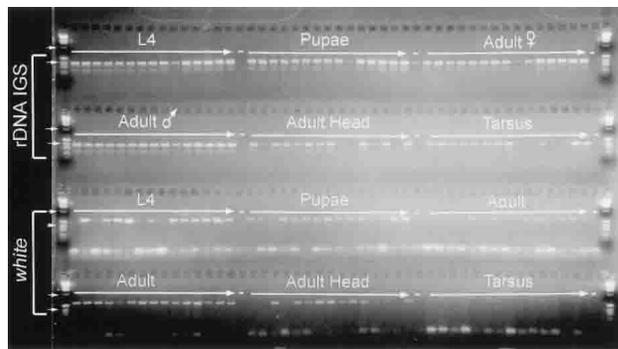


FIGURE 2. Amplification of multicopy and single-copy template from various mosquito stages, sexes, or parts. Eleven specimens of each type were tested along with a negative control (except those marked with an asterisk, into which an adult was accidentally placed). The first and last lanes contain marker as in Figure 1. Note that lanes 9 and 10 of the rDNA have consistently low amounts of product.

lar devices in which small rods are inserted into an aluminum or stainless steel backing plate with a grid of suitable-sized holes drilled in it.)

The reactions consisted of the following concentrations for both rDNA and *white* gene amplification: 1× *Taq* reaction buffer containing 15 mM MgCl$_2$, 2 mM dNTPs, and 1 U Promega *Taq* polymerase per 25 μL. Primer sequences to amplify rDNA have been published for the An. gambiae IGS[2] and *An. quadrimaculatus* ITS2.[5] Primers for the *white* gene were AACACGGACGACCAGTATG and TGT-TGTCGGGCTTCATTATC and are the same as those used by Benedict et al.[9] The rDNA PCR reactions were supplemented with 0.3 mM Mg$^{++}$. The plate was sealed again with a foil top, and thermal cycling for all reactions was performed on a Perkin-Elmer 9600 (Norwalk, CT). Conditions were the same for all samples: 94°C for 5 min, then 30 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec, then 72°C for 10 min. Five microliters of 6× orange-G loading dye in Tris-EDTA buffer was added to each sample through the foil cover with a multichannel pipette, and 10 μL of each sample was loaded onto a 200-well 2% agarose 1× Tris-borate EDTA gel with the same pipette.

Replicators were cleaned by soaking in Liquinox solution, dried, dipped into ethanol, and flamed over a gas burner. Occasionally, replicators were exposed to ultraviolet light in a hood for 4 hr as a supplemental DNA decontamination method.

## RESULTS

In 3 experiments performed to determine the approximate volume of fluid transferred to each PCR reaction, mean values of fluid transferred were 0.62, 0.94, and 1.5 μL (± 14, 17, and 23%, respectively). Significant differences in the mean volumes were therefore observed among the experiments. However, the variation in volumes transferred within each experiment was small. We also found significant differences in the volumes transferred between plate columns within each experiment (data not shown).

We performed an experiment to determine whether a multicopy locus (rDNA) or single-copy locus (*white*) could be amplified. This experiment also determined whether cross-contamination between reactions was prevalent and whether the rate of false-negative findings was such that the method would not be suitable for routine screening. Samples were placed in alternate rows of the plate in order to maximize the probability of cross-contamination. Figure 1 illustrates that both rDNA and the *white* gene could be consistently amplified, although *white* amplification was less consistent than multicopy rDNA. In this experiment, no sample cross-contamination was evident, and product sizes were as expected.

Because species identification or genotyping is often performed on various stages or in conjunction with analysis of another part of the same mosquito, amplification of template from various mosquito parts and stages was also evaluated. Again, all mosquito stages and parts tested provided template suitable for amplification of rDNA (Figure 2), except that the *white* gene amplified poorly with a tarsus, and adult females provided less suitable template than males. In Figure 3, lanes 9 and 10 of the rDNA amplifications were consistently weak or negative. Because of the manner in which the gel was loaded and because of the temperature cycler plate pattern, we suspect that the thermal-cycler plate temperature distribution was not consistent.

Fresh and frozen specimens are often unavailable for analysis, and one must rely on ethanol-preserved or desiccated material. A third experiment was therefore performed to determine whether the technique was sufficiently robust to analyze preserved material. We performed similar experiments with specimens that had been preserved for 2 months by desiccation or storage in 80% ethanol. We found that rDNA and the *white* gene amplified similarly whether prepared from fresh or preserved material (Figure 3). However, neither fresh nor preserved material amplified as abundantly in this particular experiment as we have observed in other experiments with fresh material (e.g., Figures 1 and 2).

Finally, we determined whether the method would be suitable for analysis of field-collected material. Adult mosquitoes that had been collected in the vicinity of 2 autochthonous cases of *Plasmodium vivax* malaria in New York in August of 1999[10] were identified on the basis of morphology as *An.*
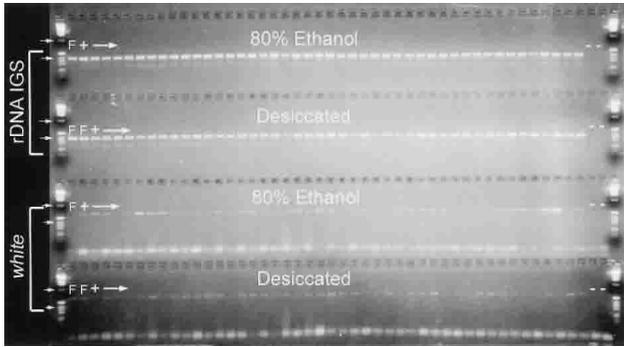
FIGURE 3. Polymerase chain reaction analysis of preserved specimens. Adult females preserved in either 80% ethanol or desiccated were compared with fresh females for amplification of template. The first and last lanes contain marker as in Figure 1.
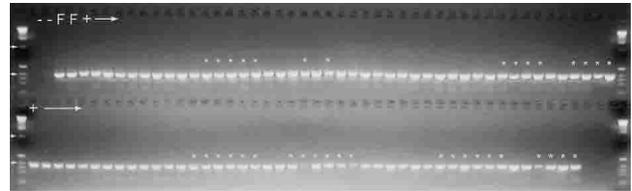


FIGURE 4. Amplification of rDNA from field-collected specimens. Abdomens of adults were handled as other experimental material, and the ITS2 of *Anopheles quadrimaculatus* s.l. was amplified and compared with fresh (F) material and negative controls (−). Lanes containing product from blood-fed females are marked with an asterisk. The first and last lanes contain marker as in Figure 1.

*quadrimaculatus* s.l. When 237 specimens were subjected to rDNA analysis as described above and compared with *An. quadrimaculatus* s.s. Q2 stock mosquitoes, all were positive, except for 20 samples from which no amplification was observed (Figure 4). No relationship between amplification and either sex or presence of bloodmeal was observed. In all positive reactions, the mobility of the product was indistinguishable from that of *An. quadrimaculatus* s.s.[11]

## DISCUSSION

We have shown that this method is suitable for amplification of rDNA and that the rate of false-positive and false-negative findings is acceptable for large-scale screening. Neither mosquito stage nor preservation method was a significant variable in our ability to amplify template. We found that the most time-consuming aspects of the procedure are placing the mosquito samples in the plates and the time required for thermal cycling. All other liquid and sample transfers were completed quickly by use of the replicator or multichannel pipette. When multiple loci or plates were analyzed, the limiting factor was the number of thermal cyclers available.

The volume of template solution transferred to each well by means of this method was similar to the volume typically pipetted by means of the traditional method: ∼ 1 μL. Although we did not determine the DNA concentration, it is clearly adequate for most PCR applications. Given this volume of fluid, it should be possible to run at least 25 PCR reactions from each sample.

Because it is possible to amplify single-copy loci by means of this method, it should also be possible to rapidly screen preserved or fresh specimens for the presence of epidemiologically relevant genes (e.g., those relating to insecticide resistance) without running biochemical assays or bioassays. For example, a PCR diagnostic assay for a significant form of pyrethroid resistance has been identified as a single-base change in a sodium channel gene, resulting in the kdr form of resistance in *An. gambiae*.[12] We have used this method to distinguish *white* mutant alleles in 2 different An. gambiae stocks and for amplification of a microsatellite locus. In all cases, satisfactory results were obtained (data not shown). Given the inconsistent results we observed on amplification of the *white* locus, optimization of the reaction conditions will

be required to obtain consistent results in instances where detection of all samples positive for the target sequence is essential.

In summary, we have demonstrated that the quality and quantity of template prepared and transferred by this method is suitable for PCR amplification of both multicopy and single-copy genes. The cost of the PCR reagents (excluding primers) agarose, and disposable items in our laboratory for this analysis is $0.26 per locus, and the reduced labor required provides significant savings relative to typical individual sample preparation. The speed and ease of the process should allow more rapid and extensive analysis of field collections and more robust evaluation of the vector aspects of malaria transmission.

## REFERENCES

1. Collins FH, Paskewitz SM, 1996. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. *Insect Mol Biol 5:* 1–9.

2. Scott JA, Brogdon WG, Collins FH, 1993. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. *Am J Trop Med Hyg 49:* 520–529.

3. Cooper L, Cooper RD, Burkot TR, 1991. The *Anopheles* punctulatus complex—DNA probes for identifying the Australian species using isotopic, chromogenic, and chemiluminescence detection systems. *Exp Parasitol 73:* 27–35.

4. Walton C, Handley JM, Kuvangkadilok C, Collins FH, Harbach RE, Baimai V, Butlin RK, 1999. Identification of five species of the *Anopheles dirus* complex from Thailand, using allele-specific polymerase chain reaction. *Med Vet Entomol 13:* 24–32.

5. Cornel AJ, Porter CH, Collins FH, 1996. Polymerase chain reaction species diagnostic assay for *Anopheles quadrimaculatus* cryptic species (Diptera: Culicidae) based on ribosomal DNA ITS2 sequences. *J Med Entomol 33:* 109–116.

6. Marrelli MT, Malafronte RS, Flores-Mendoza C, Lourenco-de-Oliveira R, Kloetzel JK, Marinotti O, 1999. Sequence analysis of the second internal transcribed spacer of ribosomal DNA in *Anopheles oswaldoi* (Diptera: Culicidae). *J Med Entomol 36:* 679–684.

7. Onyabe DY, Conn JE, 1999. Intragenomic heterogeneity of a ribosomal DNA spacer (ITS2) varies regionally in the neotropical malaria vector *Anopheles nuneztovari* (Diptera: Culicidae). *Insect Mol Biol 8:* 435–442.

8. Benedict MQ, 1997. Care and maintenance of anopheline mosquito colonies. Crampton JM, Beard CB, Louis C, eds. The Molecular Biology of Insect Disease Vectors. New York: Chapman and Hall, 2–12.

9. Benedict MQ, Besansky NJ, Chang H, Mukabayire O, Collins FH, 1996. Mutations in the *Anopheles gambiae pink-eye* and *white* genes define distinct, tightly linked eye-color loci. *J Hered 87:* 48–53.

10. CDC, 2000. Probable locally acquired mosquito-transmitted *Plasmodium vivax* infection—Suffolk County, New York, 1999. *MMWR 49:* 495–498.

11. Reinert JF, Kaiser PE, Seawright JA, 1997. Analysis of the *Anopheles* (Anopheles) *quadrimaculatus* complex of sibling species (Diptera: Culicidae) using morphological, cytological, molecular, genetic, biochemical, and ecological techniques in an integrated approach. *J Am Mosq Control Assoc* Suppl 13S:1–102.

12. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Berge JB, Devonshire AL, Guillet P, Pasteur N, Pauron D, 1998. Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae* s.s. *Insect Mol Biol 7:* 179–184.