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THE POLYMERASE CHAIN REACTION METHOD AS A TOOL FOR IDENTIFYING MEMBERS OF THE *ANOPHELES GAMBIAE* COMPLEX (DIPTERA: CULICIDAE) IN NORTHEASTERN TANZANIA

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ABSTRACT. Polymerase chain reaction (PCR) primers developed at the Centers for Disease Control in Atlanta for the identification of members of the *Anopheles* (*Cellia*) *gambiae* Giles complex were tested on material collected in the Bagamoyo and Muheza districts of northeastern Tanzania. Part of the sample from Bagamoyo was chromosomally identified and correlated with the PCR identifications. This sample contained 170 *Anopheles arabiensis,* 328 *An. gambiae,* and 58 *Anopheles merus,* of which 121, 237, and 54 specimens, respectively, were identified with both PCR and chromosomes. Three specimens identified chromosomally as *An. merus* gave only the PCR fragment characteristic for *Anopheles quadriannulatus,* but on retesting gave the correct result. The Muheza sample consisted of 771 *An. arabiensis,* 852 *An. gambiae,* 43 *An. merus,* and 4 specimens producing the fragment characteristic for *An. quadriannulatus.* Because *An. quadriannulatus* has never been recorded from mainland Tanzania and due to the high number of specimens that produced no result (193), it is probable that DNA degradation led to misidentification of *An. merus* specimens as *An. quadriannulatus.* The overall probability of correct identification by PCR was 99.685% at first testing, which compares favorably with other genetic methods currently in use.

INTRODUCTION

The *Anopheles* (*Cellia*) *gambiae* Giles complex of mosquitoes consists of 6 morphologically indistinguishable species that vary in their importance as vectors (Service 1985, Gillies and Coetzee 1987). The mosquitoes differ in their feeding behavior as well as in their resting behavior after feeding (White 1974), resulting in different responses to residual insecticides. These differing responses have important implications for malaria control programs and it is therefore important to correctly identify the species present in any given area (Coluzzi 1970, 1984; White 1974). Up to 4 species occur in sympatry in some malarious areas, with at least 2 in most areas (Coetzee et al. 1993).

The most reliable and frequently used method for identification of the *An. gambiae* complex up to now has been the use of banding patterns on the giant polytene chromosomes (Coluzzi and Sabatini 1967, 1968, 1969) found in the female ovarian nurse cells. These chromosomes are obtained from half-gravid females (*ca.* 36 h at 24°C after a mated female has had a blood meal) (Hunt 1973). This means that only a percentage of collected specimens are identified because many females die before they become half-gravid. Interpretation of the banding patterns also requires a high level of expertise, which is not readily available in most malarious countries.

Isoenzyme analysis using electrophoresis is also used frequently (Mahon et al. 1976, Miles 1979). Storage requirements for collected specimens are cumbersome, requiring that specimens be stored in liquid nitrogen, which makes collection in field conditions very difficult. Also, genetic overlap due to polymorphism in diagnostic electromorphs can result in misidentifications at some localities (Miles 1979).

Other methods for identification, such as analysis of cuticular hydrocarbons (Carlson and Service 1980) and synthetic DNA probes have either proved less successful or have not yet been adequately tested in the field (Collins et al. 1987, Gale and Crampton 1987, Hill and Crampton 1994).

With the advances made in the field of molecular biology and the development of the polymerase chain reaction (PCR) method it has become possible to use this technique for the identification of insect vectors of disease. The PCR method for identifying mosquitoes was originally developed by Paskewitz and Collins (1990); Scott et al. (1993) published a protocol using oligonucleotide primers to identify 5 members of the *An. gambiae* complex. The method utilizes species-specific DNA sequences in the IGS region of the ribosomal RNA genes. The primers consist of one universal primer that is complimentary to all 5 species and 4 species-specific primers for *Anopheles arabiensis* Patton, *Anopheles gambiae* Giles, *Anopheles quadriannulatus*

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Table 1. Polymerase chain reaction identification of specimens of *Anopheles* collected in the Bagamoyo and Muheza districts of northeastern Tanzania.

Locality	*An. arabiensis*	*An. gambiae*	*An. merus*	*An. quadriannulatus*?	No result
Muheza	771	852	43	4	193
Bagamoyo					
Chromosomally correlated	121	237	54	0	0
Uncorrelated	49	91	4	0	0
Total	170	328	58	0	0

Theobald, and the *Anopheles melas* Theobald/*Anopheles merus* Dönitz combination. The universal primer binds in conjunction with one of the species-specific primers to produce a DNA fragment of distinctive length for each species. The results are easy to interpret and this method can be applied to any life stage and both sexes of the mosquitoes. Storage requirements are also much simpler than for any of the other techniques. This gives the PCR method a distinct advantage over the other methods of identification but testing of field-collected specimens from a wide range of localities is needed to establish the accuracy and usefulness of the PCR method. Tests like these have been done by Paskewitz et al. (1993) and Bredenkamp and Sharp (1993) on specimens collected in southern Africa. Both papers reported the method to be accurate and practical. The present paper reports on the use of this technique for the identification of large numbers of *An. gambia s.l.* mosquitoes collected in northeastern Tanzania.

MATERIALS AND METHODS

Mosquitoes were collected over a 5-year period (1991–95) in the Bagamoyo (6°26′S, 38°55′E) and Muheza (5°10′S, 38°48′E) districts of northeastern Tanzania. Hand, light trap, larval, human bait, and animal bait collections were used. Blood-fed specimens from Bagamoyo were allowed to develop to the half-gravid stage before being dissected and the ovaries stored in Carnoy's fixative (3 parts absolute ethanol, 1 part glacial acetic acid). The Muheza sample was stored directly in 70% isopropanol. Chromosomally identified specimens from Bagamoyo were correlated with PCR reactions carried out on the cuticular tissue attached to the ovaries. The chromosomal identifications were done according to Hunt (1973, 1988).

The following modifications to the protocol of Scott et al. (1993) were made. One leg per mosquito was placed in a microcentrifuge tube and placed on ice; 12.5 μl of a mastermix containing 1.25 μl 10× PCR buffer; 125 μM of each nucleotide; 1 mM $MgCl_2$; 0.3 μM each, universal, *An. arabiensis, An. gambiae,* and *An. melas/An. merus* primers; 0.15 μM *An. quadriannulatus* primer; and 0.5 units of Taq polymerase enzyme (Advanced Biotechnologies) were added. The tubes were then placed in a microcentrifuge and spun at 13,000 rpm for 1 min to release template DNA from the tissues. Cuticular tissues from the ovaries were homogenized with a pipette tip and treated as for the legs. The reaction mix was overlaid with 35 μl of mineral oil and placed in a Hybaid thermal cycler for 30 cycles consisting of 94°C denaturing for 30 sec, 50°C annealing for 30 sec, and 72°C extension for 30 sec. An autoextension step of 72°C for 10 min was added. Ten microliters of the amplified product was loaded onto a 2.5% agarose gel, stained with ethidium bromide, submerged in TBE buffer, and run electrophoretically at 120 V for ±1.5 h. Each gel contained 4 control specimens from insectary colonies as well as a negative control. The gel was viewed on an ultraviolet transilluminator and photographed on polaroid film. The amplified fragment sizes are 315 basepairs (bp) for *An. arabiensis,* 390 bp for *An. gambiae,* 153 bp for *An. quadriannulatus,* and 464/466 bp for *An. melas/An. merus*; these were compared to a 1 kilobase DNA ladder (BRL). *Anopheles melas* and *An. merus* are allopatric in distribution and are separated depending on where they were collected. DNA extractions for retesting some of the specimens were done as in Collins et al. (1987).

RESULTS AND DISCUSSION

A total of 2,419 specimens was tested using PCR (Table 1). The Bagamoyo sample contained 412 specimens identified both chromosomally and by PCR. All of the *An. merus* specimens produced a fragment equal in size to the *An. quadriannulatus* fragment (153 bp) in addition to the specific *An. merus* fragment (466 bp), but this as a rule did not interfere with positive iden-

tification. Three of these specimens, however, identified chromosomally as *An. merus*, produced the 153 bp fragment only. Only after retesting did they produce the correct *An. merus* fragment (466 bp). All *An. merus* colonies in our insectary that were tested also produced both fragments, but the *An. merus* fragment was always brighter than the nonspecific band. The double band in *An. merus* was also reported in Paskewitz et al. (1993) and Scott et al. (1993), but it was noted that the specific band was always brighter. The failure of the specific band to amplify in some of our specimens could be ascribed to DNA degradation due to long-term storage. In such a case the longer DNA fragment would probably be at a disadvantage for amplification compared to the shorter (153 bp) fragment. The 144 specimens from the Bagamoyo sample (49 *An. arabiensis*, 91 *An. gambiae*, and 4 *An. merus*) were identified with PCR but were not identified chromosomally because the ovaries were not at the correct stage of development needed for chromosomal identification. Our success rate with the correlated Bagamoyo sample was 99.27%, comparing positively with previously reported identifications (Paskewitz et al. 1993, Scott et al. 1993).

The sample from the Muheza district (Table 1) contained 4 specimens that produced the *An. quadriannulatus* fragment only, even after retesting. These specimens were also tested in the absence of the *An. quadriannulatus* primer, but no bands were produced (the shorter fragment disappeared in our laboratory colonies of *An. merus* whenever the *An. quadriannulatus* primer was excluded, suggesting that this primer finds a homologous region somewhere in the *An. merus* genome to produce the nonspecific fragment). When *An. quadriannulatus* primer was added again, the 153-bp fragment was the only one amplified. The bodies of these mosquitoes were then used for DNA extractions, but this DNA did not produce any bands at all, despite our control specimens producing results. Because of the failure to amplify *An. merus* fragments in these specimens, the possibility of them being *An. quadriannulatus* cannot be excluded. However, *An. quadriannulatus* has never been recorded from mainland Tanzania and the collection of more specimens from this area is necessary. The problem with the *An. merus* double band has to be taken into consideration, though, and it is quite possible that these specimens were *An. merus* in which the DNA was so degraded that longer fragments failed to amplify. A total of 193 specimens (10.4%) from this sample did not produce any DNA bands at all, indicating the poor quality of the DNA. Up to 50 mosquitoes had been stored per 1 ml of isopropanol, ideal conditions for DNA degradation because of the dilution effect so many mosquito bodies would have on the preservative. Post et al. (1993) did studies on the preservation of *Simulium damnosum s.l.* for DNA analysis. They found that liquid nitrogen, silica gel drying, or storage in 100% ethanol at 4°C provided the best conditions for DNA preservation. Storage in Carnoy's or propanol gave a lower yield and quality of DNA. DNA degradation increased over time with all the storage methods tested. In our study, most of the material from the Bagamoyo sample, preserved in Carnoy's fixative, was PCR analyzed more than a year after collection. Adequate amplification of DNA was still achieved in every case. The fact that only short pieces of DNA (<500 bp) need to be amplified with these primers may explain the high success rate. The material from Muheza, stored in 70% isopropanol, was tested within months of collection. The critical factor in our study thus seemed not to be storage medium or duration of storage, but rather the number of specimens stored per volume of preservative. Only one, or at most a few specimens should be stored per 1 ml of preservative. This should minimize the chance of *An. merus* specimens being misidentified as *An. quadriannulatus.*

Despite the difficulties experienced because of poor storage technique, the method still proved to be accurate, with only 4 of the 1,670 specimens (0.24%) identified from Muheza giving results that were suspect. We recommend that all specimens producing only the *An. quadriannulatus* fragment be retested in the absence of the *An. quadriannulatus* primer. This will maximize the chances of the *An. merus* band being amplified if the specimens are *An. merus.* This retesting especially should be done for samples coming from areas where *An. quadriannulatus* is not known to occur and in studies where an accurate estimate of species proportion is required.

Provided storage conditions are optimal, PCR is a useful and accurate tool for identifying members of the *An. gambiae* complex. The advantage of this method over the chromosomal method was demonstrated in the Bagamoyo sample where 144 specimens could be identified by PCR, but not chromosomally. In the Muheza sample a large percentage of the specimens were males collected in a pit trap and these could not have been identified chromosomally, but they posed no problem for PCR identification. The use of nonextracted DNA proved to be a rapid and simple method for routine identification, with up to 100 specimens being processed by a single person in one working day. Compared to chromosomal identification, PCR is quicker and

simpler with less expertise needed for interpretation of the results.

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