Journal of the American Mosquito Control Association, 14(1):58–60, 1998
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PRESERVATION OF ANOPHELINE MOSQUITOES FOR DNA PROBE ANALYSIS

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ABSTRACT. *Anopheles (Cellia) farauti* Laveran *sensu stricto* adults were subjected to a range of conditions similar to those experienced by trap-collected mosquitoes prior to processing. Squash blots of these specimens were then analyzed using isotopic DNA probes. Freezing and thawing 6 times and dry storage on silica gel in a vacuum desiccator for up to 180 days did not affect the quality of the DNA. Exposure to conditions of high humidity and high temperature for more than 2 h seriously reduced the quality of the DNA.

KEY WORDS Anopheline DNA, preservation, storage, DNA probe analysis

INTRODUCTION

The appearance of isomorphic species within many of the malaria vector taxa has necessitated the use of alternative techniques for species identification, an essential prerequisite to any field study. Various techniques are available (Cooper et al. 1991); however, for the identification of large numbers of field specimens, the most suitable method we have found is that using squash blots and isotopic DNA probes. This method was applied to members of the Punctulatus Group collected from Papua New Guinea (PNG) (Cooper et al. 1997) using the encephalitis virus surveillance (EVS) light trap, a device similar to the CDC light trap but incorporating CO_2 as bait (Rohe and Fall 1979). Although most specimens were positively identified, there was considerable variation in the strength of the signal, indicating that degradation of the DNA had occurred. With some specimens, the signal was very weak or nonexistent, and a positive identification could not be made.

The DNA in these specimens may have degraded after collection. Because mosquito traps (EVS or CDC) are usually set before 1800 h and not retrieved until 0600 h the following morning, these may contain specimens that died early in the evening and have undergone decomposition for several hours. Material collected during whole-night biting catches may suffer the same fate if the specimens are not preserved at intervals during the night.

DNA degradation may also occur during transport and storage of the specimens prior to processing. In the field, liquid nitrogen (LN_2) or dry ice may not be available, and specimens may go from wet ice to a domestic freezer (−20°C) a number of times while in transit. Power supplies may also be unreliable and intermittent, allowing specimens to thaw out for various periods of time.

In this study, the degradation of mosquito DNA, following the death of the specimen, was examined in mosquitoes exposed to high temperature and humidity, repeated freezing and thawing, and dry storage for long periods.

MATERIALS AND METHODS

Colony specimens of *Anopheles farauti* Laveran *sensu stricto* (= *An. farauti* 1, one of 12 members of the Punctulatus Group) were killed using chloroform and placed individually into Eppendorf tubes to make 25 sets, with each set containing 5 replicate tubes. Fifteen of these sets were maintained at 26°C and 80–85% RH, 2 sets were removed and frozen (−70°C) at ¼-, ½-, 1-, 2-, 4-, 6-, 8-, 10-, 12-, 14-, 16-, 18-, 20-, 22-, and 24-h intervals. Three sets were stored in a silica gel charged desiccator under vacuum and removed and frozen (−70°C) after 30, 60, and 180 days. Five sets were frozen at −70°C; each set was then brought to 26°C (taking approximately 5 min) then frozen again. This treatment was repeated, excluding a set each time, until the last set, at the time of processing, had been thawed and frozen 6 times. The remaining 2 sets were frozen (−70°C) and used as controls.

Following the above treatments, all specimens were squash blotted and probed using the *An. farauti s.s.* genomic probe made by Cooper et al. (1991). The abdomens of the specimens were placed, 1 cm apart, onto a nylon membrane saturated with 10% SDS. Each abdomen was individually squashed using a glass pestle and left for 10 min. The DNA was then denatured in 0.5 M NaOH, 1.5 M NaCl for 5 min; neutralized in 0.5 M Tris, 1.5 M NaCl buffer for 10 min; and then fixed to the membrane by baking at 80°C for 2 h. The membrane was then saturated with 0.1 M NaOH and any chitinous material was rubbed off. The membrane was rinsed in distilled water and prehybridized in 40 ml of phosphate buffer (0.263 M Na_2HPO_4, 1.0 mM EDTA [pH 8.0], 7% SDS, 1% bovine serum albumin) for at least 2 h at 65°C. The membrane was then hybridized using plasmid DNA containing the *An. farauti s.s.* probe labeled by random priming with ^{32}P-dCTP. The probe concentration was 10 ng/ml of the phosphate buffer, and hybridization was run overnight at 65°C. The membrane was then washed in 0.1 × SSC, 0.1% SDS for 1 min at room temperature and then twice for 30 min at 65°C. It was then sealed in plastic wrap and exposed to au-

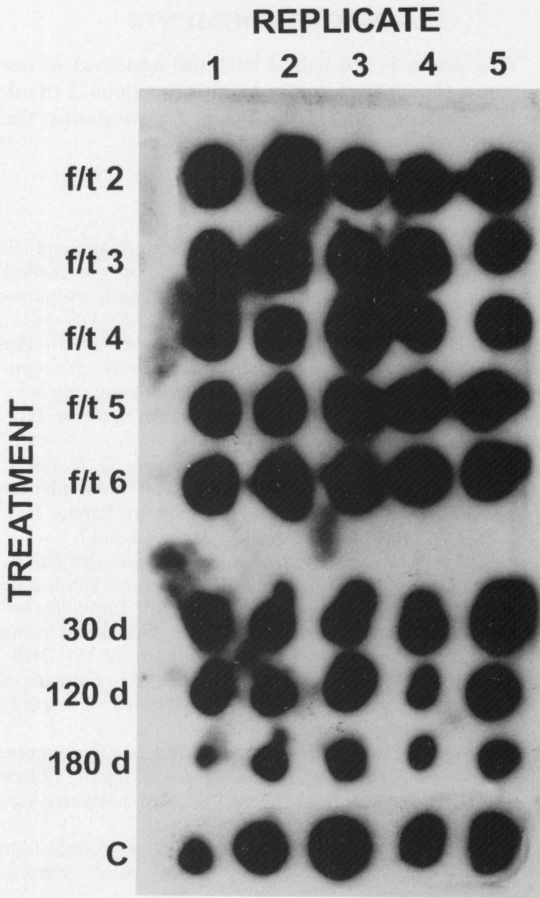

Fig. 1. Results of isotopic probing of *Anopheles farauti* specimens following exposure to repeated freezing and thawing (f/t), and dry storage for 30, 120, and 180 days. C = controls.

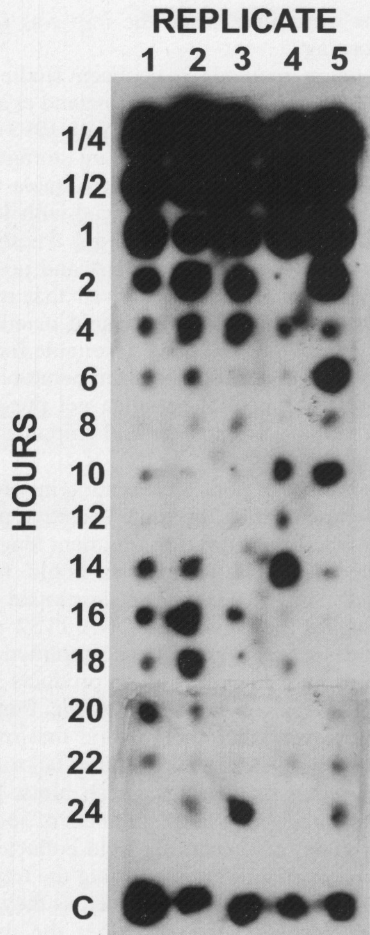

Fig. 2. Results of isotopic probing of *Anopheles farauti* specimens following exposure to 26°C and RH of 80–85% for periods of up to 24 h. C = controls.

toradiographic film in a cassette with intensifying screens at −70°C for 4–16 h. The film was brought to room temperature, developed, and scanned onto a computer. Controls, which had been frozen (−70°C) once, were run on each filter.

RESULTS

Repeated thawing and freezing (up to 6 times) did not appear to affect the quality of the DNA compared to the control signal. Dry storage in a vacuum desiccator for 30 and 120 days also did not appear to affect the quality of the DNA; after 180 days of dry storage, the signal was still strong but not as strong as that of the controls (Fig. 1).

With the specimens maintained under conditions of high temperature and humidity, their DNA rapidly deteriorated. After 2 h, one specimen had no usable DNA, and from 4 h on, the majority of specimens had no signal or one that was greatly reduced compared to the controls. There were, however, one or two specimens which retained DNA comparable to the controls up to 24 h (Fig. 2).

DISCUSSION

Average coastal temperatures in PNG range from 26°C to 27°C and RH is >80% (McAlpine et al. 1983). The results of these experiments indicate that specimens that die early in the night; during either trap or man-biting collections, would be unsuitable for use as squash blots if not preserved until the following morning. With CDC and EVS traps, preservation of the collected material is not possible until the trap is picked up in the morning; however, the use of traps is extremely convenient in situations where numerous collections have to be made over a large area. With the PNG material, the quality of the DNA from trapped specimens was, in most cases, adequate to positively identify the species (Cooper et al. 1997), indicating that most

specimens were alive when the trap was retrieved in the morning.

Preservation methods have been studied for a number of species of Diptera (Copeland et al. 1992, Post et al. 1993, Stevens and Wall 1995). These workers found that low-temperature storage (liquid nitrogen or −70°C) was the most suitable method, although good results were achieved with 100% alcohol and dry storage over silica gel. These studies were conducted on extracted DNA and not squash blots. Cooper et al. (1991) showed that mosquito specimens fixed in 70% isopropanol or ethanol or frozen at −20°C or −70°C were suitable for squash blot analysis. Therefore, if low-temperature storage is not available, alcohol or silica gel preservation would be suitable for storage and shipping prior to processing.

If the problem of poor preservation involves only a few specimens, then the more sensitive polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis could be used. Beebe and Saul (1995) have developed a PCR method where the ribosomal DNA ITS2 (internal transcribed spacer) region, once amplified and digested with *Msp* I, will produce products that can be used to separate members of the Punctulatus Group. However, since this method first requires a DNA extraction step and is more laborious and time-consuming than using squash blots, it is unsuitable for processing large numbers of specimens.

The problem of preserving field-collected material may be overcome by modifying the trap design so that the specimens are preserved as they are collected. McDonald (1970) describes the use of an alcohol light trap to collect *Culicoides,* and McDonald (1980), using a New Jersey light trap (Service 1993) containing 70% methanol rather than a killing agent, successfully collected and preserved several species of mosquito. The EVS traps currently used could be modified so that the specimens are retained in an alcohol-filled jar rather than a collection bag. However, consideration would have to be given to the effect alcohol may have on the specimens with regard to other studies, such as parasite detection, host blood meal source, or morphological studies.

ACKNOWLEDGMENTS

This paper is published with the approval of the Director General of Army Health Services. I thank A. W. Sweeney and Tom Travis for preparing the figures.

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