Vidyodaya J. of Sci. (2010) Vol. 15 No.1 & 2, pp 63-72

# A simplified non radioactive DNA probe technique for the field detection of sibling species A of the *Anopheles culicifacies* (Diptera: Culicidae) complex.

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*Received on : 20 - 10 - 2009*
*Accepted on : 03 - 11 - 2009*

## Abstract

Three cloned highly repetitive DNA sequences Rp36, Rp217 and Rp234 isolated from *An. culicifacies* Giles, *sensu lato* were developed as non radioactive DNA probes by using a biotinylated labeling and colorimetric detection system. These non radioactive DNA probes distinguish sibling species A from species B and C of the *An. culicifacies* complex in a dot blot hybridization assay using single mosquito DNA extracts diluted 50 fold. The biotinylated Rp217 probe was further assayed in a more simple procedure which involves the hybridization of blots prepared from squashed mosquito heads. This technique avoids the separate extraction of mosquito DNA and facilitates a number of samples to be processed rapidly while also allowing several field analyses to be carried out on one mosquito specimen.

**Key words :** *Anopheles culicifacies*, Non radioactive, DNA probes.

## Introduction

*Anopheles culicifacies* Giles, *sensu lato* (Diptera : Culicidae) is considered to be the most important vector of malaria in Sri Lanka (Wickramasinghe & Samarasinghe, 1991). The taxon *An. culicifacies* exists as a complex of four reproductively isolated populations. These have been provisionally designated as species A and B (Green & Miles, 1980), species C (Subbarao et al., 1983) and species D (Subbarao et al., 1988; Vasantha et al., 1991).

Differences in the vectorial capacity, seasonal prevalence, response to insecticides and biting behaviour have been recorded in India among the member species of the *An. culicifacies* complex (Subbarao et al., 1988). Hence, precise identification of this vector species is essential for accurate epidemiological studies as well as for the successful implementation of control programmes.

The four sibling species of *An. culicifacies* complex are currently identified by the analysis of polytene chromosome invertions (Subbarao, 1998), mitotic karyotype (Vasantha et al., 1983, Suguna et al., 1989) or by cuticular hydrocarbon components (Milligen et al., 1986). The current methods of vector identification are laborious, time consuming and also require live, fresh or frozen specimens. Application of DNA probes for the identification of vector species complexes of *An. gambiae* Giles, (Gale & Crampton, 1987a, 1987b ; Collins et al., 1987), *An. farauti* Larveran (Booth et al., 1991), *An.dirus* Peyton & Harrison (Panyim et al., 1988) and *An. punctulatus* Doenitz (Cooper et al., 1991) has been reported previously. These probes offer an attractive alternative to other available taxonomic methods to identify the members of vector species complexes.

We have previously described (Gunasekera et al., 1995) the isolation of three highly repetitive DNA sequences Rp36, Rp217 and Rp234, from *An. culicifacies*, and their development as $^{32}P$ labeled DNA probes to detect sibling species A of the *An. culicifacies* complex. The identification of sibling species A was carried out using a 200 fold dilution of a single mosquito DNA extract in a dot blot hybridization assay. However, the need for extraction and purification of total mosquito DNA, radioisotopes for labeling of probes and the associated risk involved in handling, storage and disposal of radioisotopes, have limited the use of this method in the field. Simplified processes using non radioactive DNA probes have been described previously for the field identification of several other Anopheline species complexes (Hill et al., 1992, Hill and Crampton, 1994). The feasibility of using Rp234, Rp36 and Rp217 DNA probes in the field was investigated in this study. Here, we describe the development of a simplified squash blot technique which utilizes biotinylated DNA probes for the field identification of *An. culicifacies* sibling species A.

## Materials and Methods

Air dried samples of laboratory stocks of *An. culicifacies* sibling species A, B and C were kindly provided by Dr. S. K. Subbarao, Malaria Research Centre, India. *An. culicifacies* B of Sri Lankan origin was obtained from the laboratory stocks maintained in the laboratories of the University of Colombo and the University of Sri Jayewardenepura. The sibling species status of each

laboratory stock has been identified by polytene chromosome analysis by each respective laboratory. All other mosquito samples were obtained from the field and identified by their morphological characteristics.

**Mosquito DNA**

DNA was extracted from the head of single mosquitoes by the method described by Collins et al., (1987).

**Dot Blots**

DNA samples obtained from single mosquito heads were diluted appropriately in TE (10mM Tris, 1mM EDTA) buffer and were denatured by the addition of $1/10^{th}$ sample volume of 3M NaOH and incubating at room temperature for 5 minutes. The samples were neutralized by the addition of an equal volume of ice cold 2M ammonium acetate. The DNA was blotted onto nitro-cellulose filters (0.45µm pore size, Sigma; pre-equilibrated with 1M ammonium acetate) using a Bio-dot microfiltration apparatus (Bio-Rad laboratories, Richmond, California). The filters were baked at 80°C for 90 minutes in a vacuum oven and stored at room temperature.

**Mosquito squash blots**

Mosquitoes were beheaded using a scalpel. Mosquito heads were placed 0.5 cm apart on a nitro-cellulose filter paper lying on a Whatman 3MM filter paper saturated with a 10% solution of sodium dodecyl sulphate (SDS). After 10 minutes, the nitro-cellulose filter was transferred to a dry Whatman filter paper. The mosquito heads were then squashed using a clean glass plate to apply firm downward pressure. The nitro-cellulose filter was then placed on a Whatman filter paper saturated with a solution of 0.5M NaOH/ 1.5M NaCl. After 5 minutes, the filter was blotted dry and transferred to a Whatman filter paper saturated with a solution of 0.5M Tris/ 1.5M NaCl, (pH 7.0). Chitinious head parts were removed using a clean pair of forceps. The nitro-cellulose filter was then blotted dry and baked for 2h at 80°C to bind the DNA to the filter. The filter was gently rubbed off using gloved fingers and rinsed in distilled water.

**Nitro-cellulose filter hybridization**

Radioactive DNA probes were prepared by random priming labeling technique (Feinberg & Vogelstein, 1983) using $^{32}$P-dCTP. Routinely, a specific activity of $10^9$ cpm/ µg DNA was achieved. Non-radioactive DNA probes were prepared by labeling with biotin by the nick translation labeling technique (Rigby et al., 1977).

Prehybridization of nitro-cellulose filters was carried out at 37°C for 6–12 h in a prehybridization solution (100µl/cm$^2$) consisting of 1M NaCl, 50% formamide, 10% dextran sulphate, 1 x 'P' (5 x 'P'= 1% each of ficoll, polyvinylpyrolidone and bovine serum albumin-Pentax fraction 5, 5% SDS, 250mM Tris, pH 7.6) and 100 µg/ml heparin. Denatured radiolabeled DNA or biotin labeled probes were added to the solution for hybridization of filters at 37°C for 12-16 h. The filter washings under low stringency conditions were carried out at 30°C, two rinses each in 2 x SSC (1 x SSC = 0.15M sodium chloride, 0.015M trisodium citrate) containing 1% SDS, 0.1 x SSC containing 0.5% SDS and a final wash in 0.1 x SSC. More stringent filter washings were carried out at 60°C in 0.1 x SSC. The filters hybridized with radiolabeled probes were autoradiograghed at -70°C for 2 h using Kodak X-ray film with an intensifying screen. Biotin labeled probes were detected using a colorimetric detection system (Cool Probe ™Sigma, USA).

## Results

### Simplification of the method of DNA preparation for field use

A preliminary dot blot hybridization assay was carried out separately using the DNA extracted only from the mosquito head of each sibling species A, B and C of *An. culicifacies* and *An. jamesi* (a negative control) with radiolabeled Rp36, Rp217 and Rp234 DNA probes. The hybridization signals given with probes Rp36, Rp234 and Rp217 with total and diluted head DNA extracts of species A were compared with that of B and C. A negative hybridization signal was observed with species A and a positive hybridization signal was observed with both species B and C when the single mosquito head DNA samples were diluted by 10 fold and probed with Rp36, Rp234 and Rp217 DNA probes. The control DNA samples of *An. jamesi* also gave a negative hybridization signal (Fig. 1). To further simplify this method for field use, squash blots of mosquito heads were also prepared. Since the hybridization signal given by the DNA probe Rp217 was found to be lower than that of Rp234 or Rp36 probes with total and diluted DNA samples of species A, $^{32}$P labeled Rp217 DNA probe was selected for the identification of species A by squash blot hybridization technique.

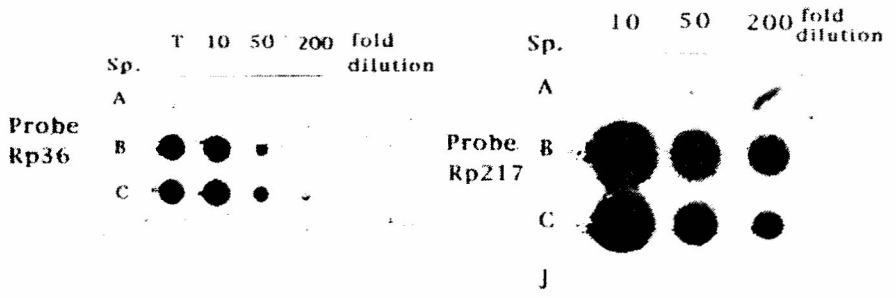


**Figure 1.** Total (T) and diluted DNA samples of the heads of *An. culicifacies* species A, B, C and *An. jamesi* (J) hybridized with $^{32}P$ labeled probes Rp36 and Rp217 (Rp 234 gives an identical hybridization pattern as probe Rp36).

Twelve mosquitoes of *An. culicifacies* B (laboratory stocks, Sri Lanka) and 4 control mosquito samples, including *An. culicifacies* A from India, *An. subpictus*, *An. tessellates* and *Cx. quinquefasciatus* from Sri Lanka were used in this assay. Although weak cross hybridization between Rp217 probe and *An. subpictus*, *An. tessellates*, *Cx. quinquefasciatus* mosquito DNA samples were detected under low stringency washing conditions (0.1 x SSC, 0.5% SDS at 30°C), only *An. culicifacies* B gave a strong hybridization signal at 2 h of exposure and a negative hybridization signal was given by *An. culicifacies* species A (Fig. 2). As shown in Fig. 2 weak hybridization signal given by other mosquito species during low stringency washing conditions disappeared under more stringent washing conditions (0.1 x SSC, 0.5% SDS at 60°C).

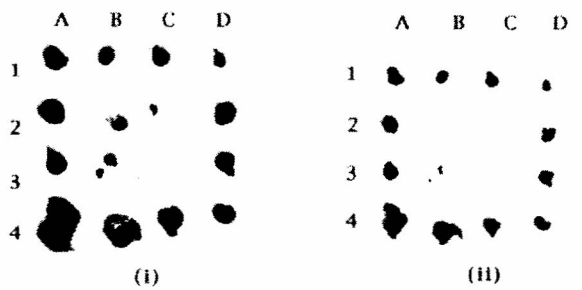


**Figure 2.** Squash blots of mosquito heads hybridized with $^{32}P$ labeled Rp217 probe at 37°C. This shows a simple and quick preparation of mosquito DNA for hybridization assays.

(i) Filter washings carried out at low stringency conditions. (0.1 x SSC, 0.5% SDS at 30°C).
(ii) Filter washings carried out at high stringency conditions. (0.1 x SSC, 0.5% SDS at 60°C).

**Development of non radioactive labeling and detection system for field identification of *An. culicifacies* species A**

The feasibility of using non-radioactive labeling and detection system was investigated. Assays were carried out with species A, B and C using diluted DNA samples as well as squash blots of mosquito heads.

Dot blot hybridization assays were carried out with dilutions of total DNA extracts (10, 50 and 200 fold) of single mosquitoes and biotinylated Rp36, Rp217 and Rp234 DNA probes and detected using colorimetric detection system. As shown in Fig. 3 the results were similar to that obtained with radiolabeled DNA probes (Gunasekara et al., 1995). However, the non radioactive detection system was found to be less sensitive than the radioactive detection system as it required more DNA (a 50 fold dilution of the DNA sample) for species identification.

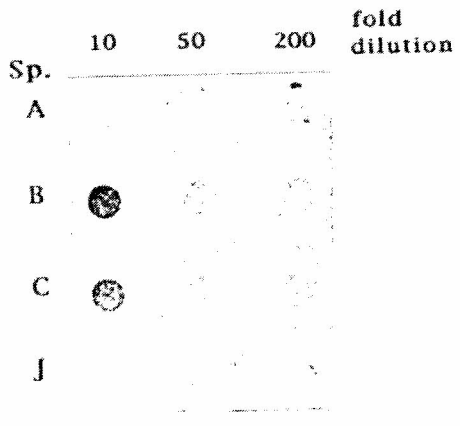


**Figure 3.** Dot blot hybridization of DNA extracted from individual mosquitoes with non-radioactive (biotinylated) DNA probes. Diluted mosquito DNA samples of *An. culicifacies* A, B, C and *An. jamesi* (J) hybridized separately with Rp36 and Rp217 probes (An identical hybridization pattern is also given with probe Rp234).

Squash blot hybridization assays of mosquito heads were then carried out using biotinylated Rp217 DNA probe and detected using colorimetric detection system. A further modification was also made to this assay by changing the hybridization buffer to a less expensive buffer containing 5 x SSC, .05% SDS and 2% skimmed milk. As shown in Fig. 4 the species A of *An. culicifacies* can be distinguished from species B using biotinylated Rp217 DNA probe in a squash blot hybridization assay. Furthermore, any mosquito sample giving a negative hybridization signal with biotinylated Rp217 could be further tested, if necessary, with probes Rp36/Rp234 to detect a positive hybridization signal prior to confirming as species A.

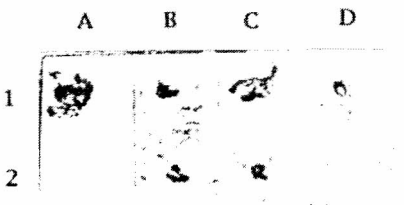


**Figure 4.** Squash blot hybridization of mosquito heads with biotinylated Rp217 probe at 37°C. Filter washings were carried out in 0.1 x SSC, 0.5% SDS at 60°C.

2A - *An. culicifacies* species A (India)

2D - *An. tessellates*

Others *An. culicifacies* species B (Sri Lanka)

## Discussion

For identification of sibling species A, the method described previously (Gunasekara et al., 1995) requires a 200 fold dilution of the total DNA extract from a single mosquito. This method has now been further simplified by preparing a squash blot using a mosquito part (head) thereby allowing a number of field analyses such as detection of life cycle stages of malaria parasite, parity rate etc. to be performed on a single mosquito specimen. The squash blot technique also enables a single investigator to process a large number of mosquito samples in a sample batch process, thereby reducing the time and cost of mosquito DNA preparations. Some of the problems

associated with the use of radiolabeled DNA probes in the field include probe instability, risk involved in handling, storage and disposal and the necessity of special laboratories. These problems are eliminated by the non-radioactive labeling and colorimetric detection system that has been utilized in this study for the detection of *An. culicifacies* species A. Although chemiluminiscent detection systems are found to be more sensitive than colorimetric detection systems (Hill et al., 1991) the colorimetric detection system is simpler, less expensive and can be carried out under field conditions without the requirement of film processing equipment and laboratory facilities. For these reasons colorimetric detection system is more appropriate for developing countries. Hence, a simplified and safe method for field use has been developed for the detection of species A of *An. culicifacies*. Studies have also been initiated in our laboratory to further simplify this method by developing a synthetic non radioactively labeled oligonucleotide probe using the DNA sequence data of Rp217.

## Acknowledgements

This work was supported by Third World Academy of Sciences (TWAS) Grant Nos. 93-133 RG/BIO/AS, 94-417 RG/BIO/AS and Natural Resources Energy and Science Authority of Sri Lanka (NARESA) Grant No. RG/93/BT/2 to M. B. Gunasekara and Swedish Agency for Research and Economic Corporation (SAREC) Grant No. BIL/SRI/HN-01B for Molecular Biology and Gene Technlogy to E. H. Karunanayake. We wish to thank Ms. N. Wimalasiri, Messers K. S. Wijenayake, A. S. P. Abeysinghe, K. H. Weerasena, K. B. A. T. Bandara for technical assistance.

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