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Protocol optimization for genomic DNA extraction and RAPD-PCR in mosquito larvae (Diptera: Culicidae)

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ABSTRACT

PCR based molecular markers are powerful tools for the analysis of genetic diversity for which isolation of good quality genomic DNA is essential. This paper presents a comparison of three DNA extraction methods for 1, 5 and 10 larvae of Aedes aegypti. First method employing DNAzol® involves simple and one step protocol for DNA isolation where maximum purity (A_{260}/A_{280} - 1.9) was obtained with single larva which decreased by increasing the number of mosquito larvae. DNA isolation with DNeasy® kit according to the procedure recommended by Qiagen provided the most consistent and reproducible results with high A_{260}/A_{280} ratio (>1.9) with single and five larvae but resulted in slight smearing with ten larvae. However, SDS based DNA extraction protocol is cost effective and provides purity level comparable to DNeasy® kit as observed by the genomic DNA profile of 1% agarose electrophoresis. The effect of temperature and incubation time was also investigated on the DNA yield. Moreover, the DNA yield extracted by SDS method was 1.4 times higher than other methods and was found suitable for RAPD analysis which could be used for the identification and genetic diversity evaluation of Aedes aegypti, Anopheles stephensi and Culex quinquefasciatus larvae.

Keywords: Genomic DNA extraction, *Aedes aegypti*, *Anopheles stephensi*, *Culex quinquefasciatus*, RAPD-PCR.

INTRODUCTION

Mosquitoes are the main vectors responsible for the biological transmission of deadly diseases such as, dengue, malaria, filaria, chikungunia, Japanese encephalitis etc. Vector control is still one of the most effective means of disease suppression and larvae are attractive targets and can be easily eradicated at their breeding sites for which an accurate identification of the species is required to determine whether it belongs to a species group that poses a potential risk. Molecular markers such as random amplified polymorphic DNA (RAPD) [1], single strand conformation

polymorphism (SSCP) [2] and restriction fragment length polymorphism (RFLP) [3] are providing new perspectives in the field of medical entomology for the genetic characterization of cryptic species of disease vectors.

Isolation of good quality DNA is a prerequisite for any PCR-based molecular tool therefore, the ability to prepare and isolate the genomic DNA from a variety of sources is an important step in many molecular techniques. The last decade has shown a dramatic departure from the use of traditional DNA purification methods [4]. The use of ready-made kits for the isolation and purification of DNA using pre-made anion-exchange columns packaged with all necessary solutions to lyse the cells and solubilize the DNA, made the process easy. DNeasy® kits (Qiagen) which combines the binding properties of a silica-based membrane with simple microspin technology, is time efficient but very expensive. A new form of DNA isolation using a patented product, DNAzol [5] introduced with a major advantage over many other DNA isolation protocols which can be completed within 30 minutes and it is suitable for nearly all forms of DNA isolation, from small fragments to genomic DNA. Different types of lysis buffers with sodium dodecyl sulphate (SDS) as the prime lysing ingredient were used to isolate DNA from a variety of organisms which is although time consuming but fairly cheaper and yields good quality of DNA [6]. The method relies on the use of SDS extraction buffers and requires overnight lysis followed by phenol/chloroform extraction [7].

Extensive literature survey clearly indicates that various methodologies had been used to isolate genomic DNA from adult insects [8-11], however, no detailed report is available on the isolation of DNA from mosquito larvae. The purpose of this work was to compare all the three methods and to optimize a protocol for extraction of DNA from *Aedes aegypti* larvae based on the quality and the amount of the extracted DNA. Optimized protocol was also followed with *Anopheles stephensi* and *Culex quinquefasciatus* larvae which was subsequently used for RAPD-PCR based taxonomic studies.

MATERIALS AND METHODS

1.Mosquito collections

The mosquito larvae were collected from natural oviposition sites using the standard dipping procedure from district Agra which is situated in the extreme South-West corner of Uttar Pradesh (27º 10’ N and 78º 05’ E, a semi-arid zone of Northern India). Larvae were reared to the fourth instar and were used for DNA extraction.

2. DNA extraction methods

Three methods (Table 1) were used to isolate the genomic DNA from mosquito larvae. For each method, genomic DNA was extracted from single, five and ten freshly emerged IV instar mosquito larvae. Genomic DNA was resuspended in 100 µl of T-10 buffer (10 mM tris-HCl, pH- 8.5) and stored at -20 °C until used except for the DNeasy® kit method where the elution buffer was used to store genomic DNA.

SDS method for DNA extraction was based on the protocol of Ballinger [7] with modifications. Freshly emerged IV instar larvae were ground in 100 µl lysis buffer (100 mM Tris-HCl, pH 8.0;

Table 1 Brief protocol for various DNA extraction methods used for mosquito larvae

Isolation Step	DNAzol method		DNeasy® Kit		SDS method	
	Reagents	**Time of incubation or centrifugation**	**Reagents**	**Time of incubation or centrifugation**	**Reagents**	**Time of incubation or centrifugation**
Lysis	mosquito larvae + 500 µl DNA zol	5 min 8000 rpm	mosquito larvae +180 µl buffer ATL+ 20 µl proteinase K	1h (+55°C)	mosquito larvae +100 µl lysis buffer + 5 µl proteinase K	1h (+ 55°C)
			4 µl RNase A	3 min	5 µl RNase A	20 min
					25:24:1 phenol+ chloroform + Isoamyl alcohol	10 min (+55°C)
						10 min 10,000 rpm
					24:1 chloroform+ Isoamyl alcohol	10 min 10,000 rpm
Precipitat-ion	250 µl ethanol 100%	1-3 min RT 8000 rpm	200 µl buffer AL+ 200 µl ethanol 95%	1 min 10,000 rpm	0.2 volume 5M NaCl + 2.5 volume ethanol 100%	-20°C One hour
						25 min 12,000 rpm
Wash	500 µl ethanol 100 %	1-3 min RT	500 µl buffer AW1	1 min 10,000 rpm	500 µl ethanol 70%	10 min 10,000 rpm
		2 min 8000 rpm	500 µl buffer AW2	3 min 10,000 rpm		
Drying		15-30 s				15-30 min
Elution	100 µl T-10 Buffer		200 µl buffer AE	1 min 10,000 rpm	100,µl T-10 Buffer	

0.5% Sodium dodecyl sulphate; 50 mM NaCl; 100 mM EDTA) and the mixture was treated with 5 µl of proteinase K (20 mg/ml) for one hour at 55℃. To this cell lysate 5 µl of RNAase was added (10 mg/ml) and kept for 20 min for incubation at room temperature. The suspension was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) by heating the contents at 55°C for 10 min. After centrifugation (10,000 rpm) the supernatant was extracted with chloroform and isoamyl alcohol (24:1) once in order to remove every trace of phenol. DNA was precipitated by the addition of 0.2 volumes of 5M NaCl and 2.5 volumes of ethanol at room temperature. The mixture was incubated for one hour at -20°C and spun at 12,000 rpm for 10 min to get pellet which was resuspended in 100 µl of T-10 buffer (10 mM Tris-HCl, pH 8.5) and stored at -20°C until used.

DNAzol® (Molecular Research Center, Inc., Cincinnati, OH, USA) is a complete and ready to use reagent for the isolation of genomic DNA based on the use of a novel guanidine-detergent lysing solution that hydrolyzes RNA and allows the selective precipitation of DNA from a cell lysate. Larvae were homogenized in 500 µl of DNAzol® reagent, precipitated in ethanol and drying procedures were the same as in the SDS method.

DNeasy® tissue kit (Qiagen, Hilden, Germany) uses an advanced silica-gel membrane technology without organic extraction. For lysis 180 µl of buffer ATL was used with 20 µl proteinase K (20 mg/ml). Manufacturer's spin-column protocol for animal tissues was used for getting maximum yield of DNA.

Optimization of temperature and incubation time for DNA isolation for SDS method

SDS method of DNA isolation was optimized with five larvae for the temperature and time of incubation. The samples were incubated at different temperatures and different hours to get the

optimum temperature and incubation time for lysis. Both the conditions were further evaluated with or without proteinase K treatments. The DNA extraction was performed at room temperature, 45°C, 50°C and 55°C and for each temperature the extraction was also performed at 1, 2, 4 and 16 h (overnight). This was followed by DNA extraction and precipitation as described earlier for SDS method.

Quantity, purity and quality of DNA

Quantitative estimation of DNA samples was done by a double beam UV-Spectrophotometer (Shimadzu, UV-2450, Japan) by measuring the DNA concentration at 260 nm and 280 nm. Purity of DNA was checked by means of absorbance ratios A_{260}/A_{280} for protein contamination. Efficiency of DNA extraction methods was compared on the basis of DNA yield from mosquito larvae. Further, the samples were run on 1% agarose electrophoresis to check the quality of DNA [12] along with one kb plus DNA ladder (GeneRuler^{TM}, Fermentas). The bands were visualized under UV light in Gel Doc XR system (Bio-Rad, USA). Data were analyzed statistically using SPSS software (Version 14.0) and results are reported as mean ± S.E.

RAPD – PCR amplification

Polymerase chain reactions for random amplified polymorphic DNA (RAPD) analysis were carried out in 25 µl volume. Each reaction tube contained 20 ng of genomic DNA, 1.0 U of Taq DNA polymerase (Invitrogen), 0.2 mM of each dNTP (Fermentas), 2.5 mM $MgCl_2$, and 10 pmol of a decanucleotide primer (OPA-02: 5'TGCCGAGCTG3', Operon Technologies, Alameda, CA). The amplifications were carried out by using a thermal cycler (MJ-Mini, Bio-Rad, USA) programmed at 94°C for 4 min, followed by 40 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min, a final extension step at 72°C for 5 min and stored at 4°C. The amplified products were resolved and visualized on 1.4% agarose gel and photographed with Gel Doc XR system (Bio-Rad, USA).

RESULTS AND DISCUSSION

DNA yield and absorbance ratios for the three methods of DNA extraction are listed in Table 2. The extraction method had a significant effect on the yield of DNA. The yield by SDS method was significantly higher than those obtained by DNAzol® Reagent and DNeasy® Kit. Modified SDS method resulted in excellent quantity of genomic DNA yielding approximately 9.6% (1 larva), 27% (5 larvae) and 54% (10 larvae, $P<0.05$) higher than DNAzol® method and 5.6%, 38.6% ($P<0.05$) and 55.7% ($P<0.05$) higher than DNeasy® Kit with 1, 5 and 10 larvae respectively. The principle modifications used in the lysis buffer from the original Ballinger-Crabtree method included addition of 100 mM EDTA (instead of 50 mM) and decrease in the concentration of SDS (0.5% instead of 1.0%). Isoamyl alcohol was used to remove phenolic traces. Moreover, RNAase A was used to remove RNA and get purified DNA which was not employed in the previous method. In the precipitation step NaCl (5M) was used instead of 10M ammonium acetate.

The mean absorbance ratios for all the three methods were higher than 1.9. Absorption ratio by DNAzol® was best observed with one larva (1.9) which gradually increased to 2.2 indicating slight protein contaminations. Number of larvae affected the absorption ratio where the

maximum purity was obtained for 1 and 5 larvae by DNeasy® Kit and it dropped with 10 larvae. A similar situation also prevailed for SDS method.

Table 2 DNA yield (µg), absorption ratio and range, cost per isolation and processing time used for DNA isolation from different methods

	DNAzol®			DNeasy® Kit			SDS		
No. of larvae	1	5	10	1	5	10	1	5	10
DNA yield (µg) (mean ±S.E.)	11.3 ±0.66	32.0 ±1.4	41.6 ±4.41	11.8 ±0.44	27.0 ±1.4	40.3 ±2.3	12.5 ±0.86	44.0 ±2.3	91.0 ±9.17
Absorption Ratio (mean ±S.E.) and range	1.9 ±0.11 (1.7-2.1)	2.1 ±0.09 (2.0-2.3)	2.2 ±0.09 (2.3-2.4)	1.9 ±0.06 (1.85-2.05)	2.0 ±0.06 (2.0-2.1)	2.1 ±0.09 (2.2-2.3)	1.9 ±0.06 (1.92-2.1)	1.93 ±0.09 (1.9-2.1)	2.33 ±0.08 (2.0-2.2)
Cost per isolation (USD)	1.67			6.6			0.80		
Processing time	30 min			2 hours			4 hours		

Values represent mean ±S.E. of three replicates.

Effect of temperature and incubation time was also studied on proteinase K treatment and the yield of DNA while optimizing the SDS protocol (Fig.1). Incubation for one hour at 55°C with proteinase K resulted in the highest yield. The data clearly indicates that the modified SDS protocol is more suitable for isolationg genomic DNA from the mosquito larvae than the original

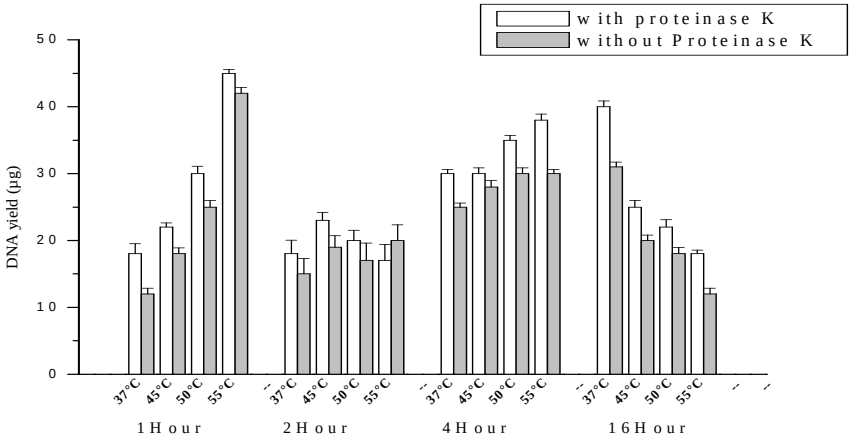

Fig. 1 Effect of incubation time and temperature on DNA yield of mosquito larvae by SDS method. DNA was extracted from five mosquito larvae by incubation with or without proteinase K. Values represent mean ± S.E. of three replicates.

protocol of Ballinger-Crabtree (1992). Overnight (16 h) lysis of the samples at room temperature and incubation at 55°C for four hours were also found suitable for proteinase K treatment however the DNA yields were 11.1% and 15.5% lower than the recommended protocol.

The estimated cost in US dollar (USD) and time in hours for each method is presented in Table 2. SDS protocol was the cheapest requiring manual preparation of buffers although the processing time was slightly higher than commercially available kit protocols. On the other hand, DNeasy® Kit was the costliest of all the DNA isolation methods studied.

The quality of genomic DNA using three extraction methods was visualized on 1% agarose gel (Fig. 2). The main band of DNA was approximately 20 kb in size. Clear bands were observed for one and five larvae whereas smearing was observed with ten larvae indicating slight degradation of genomic DNA. Moreover, PCR amplification with primer OPA-02 was significantly influenced by different extraction methods. Modified Ballinger –Crabtree protocol (SDS method) produced large number of bands in RAPD analysis which were equivalent to the RAPD profile generated from genomic DNA extracted by DNeasy® kit. However, DNAzol® method produced good results with 1 and 5 larvae which were reduced with 10 larvae indicating some impurities with increasing number of larvae (Fig. 3).

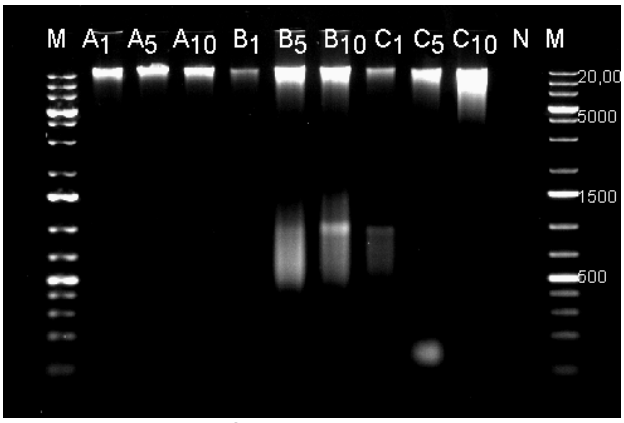

Fig. 2 1.0 % agarose gel showing the quality of mosquito larvae genomic DNA extracted by three methods: A_1, A_5, A_{10} - DNAzol®, B_1, B_5, B_{10} - DNeasy®, C_1, C_5, C_{10} – SDS method, M is molecular marker (1 kb from Fermentas) and N is negative control.

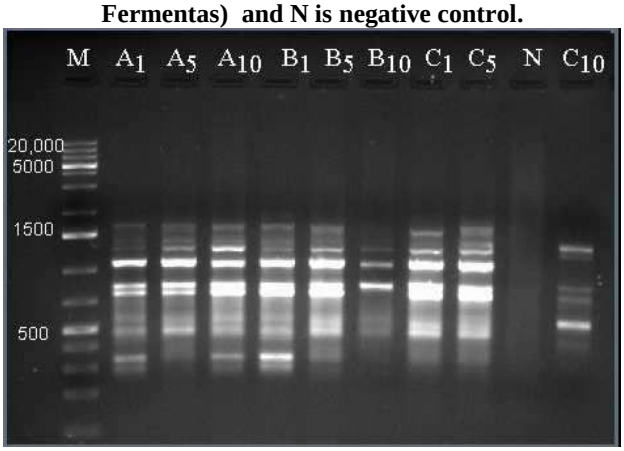

Fig. 3 Agarose gel showing RAPD profile using primer OPA-02 obtained from DNA extracted by DNAzol® method : A_1, A_5, A_{10} , DNeasy®method : B_1, B_5, B_{10} , SDS method : C_1, C_5, C_{10}, M is molecular weight marker (1 kb from Fermentas) and N is negative control.

Although the DNA extracted by the three methods resulted in acceptable DNA concentrations and absorption ratios, DNAzol using one step protocol produced best results with single larvae showing maximum purity (A_{260}/A_{280} - 1.9) which decreased by increasing the number of mosquito larvae. DNA isolation with DNeasy® kit according to the procedure recommended by Qiagen provided the most consistent and reproducible results as indicated by high A_{260}/A_{280} ratios (>1.9) with single and five larvae but resulted in smearing with ten larvae probably because the quantity of tissue exceeded than recommended by the kit protocol. SDS based DNA

extraction protocol is cost effective, time efficient and purity level is comparable to DNeasy® kit as observed by the genomic DNA profile of 1% agarose electrophoresis.

Preliminary PCR optimization for RAPD was performed on *Aedes aegypti* larvae and then evaluated for *Anopheles stephensi* and *Culex quinquefasciatus* species (Fig. 4). The alterations in various PCR parameters influenced the reproducibility and patterns of RAPD amplification. The concentrations of PCR parameters including DNA template, Taq polymerase, primers, $MgCl_2$, dNTPs were selected based on clear and scorable DNA bands produced (Table 3). The best amplification patterns were obtained with 20 ng of template DNA, 1.0 U of Taq DNA polymerase, 2.5 mM $MgCl_2$, 10 pmol of primer and 0.2 mM dNTPs. An increase or decrease in these concentrations resulted in inconspicuous PCR amplifications.

Table 3. Optimization of RAPD-PCR reaction parameters.

PCR parameters	Tested series	Finest conditions
DNA concentration (ng)	10, 20, 40 and 100	20 ng
Magnesium chloride (mM)	1, 1.5, 2.0, 2.5 and 3.0	2.5 mM
dNTPs (mM)	0.1, 0.2, 0.3 and 0.4	0.2 mM
Primer concentration (pmols)	5, 10 and 15	10 pmols
Taq polymerase (U)	0.1, 0.5, 1.0, 1.5 and 2.0	1.0 U
Annealing temperature (°C)	35/38	36°C
Number of cycles	35, 40 and 45	40

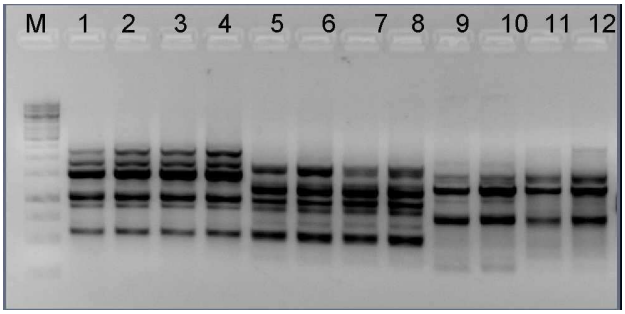

Fig. 4 RAPD amplification pattern of three culicidae larvae with primer OPA-02 (four replicates), lane M with marker; lane 1-4 with *Anopheles stephensi*; lane 5-8 with *Culex quinquefasciatus*; lane 9-12 with *Aedes aegypti*.

The DNA template concentration must be stringently controlled for RAPD analysis [13] as quality and quantity of DNA template is the most common reason for differences in the RAPD profile. In the present study, 20 ng of DNA template was found to be the best with distinct bands and high polymorphism. For most species of organisms, good results have been achieved using 10 to 100 ng of template DNA. However, high amounts of DNA usually inhibit amplification due to competition of primers for template DNA [14].

Magnesium is another PCR parameter which affects the quality of RAPD profiles [15]. It is also known to act as co-factor of the Taq polymerase which influences primer annealing and template denaturation, and formation of primer-dimer artifacts [16]. Moreover, increase in $MgCl_2$ concentration results in the accumulation of non specific PCR products while its deficiency reduces the yield [17]. In the present study 2.5 mM $MgCl_2$ produced reproducible bands.

Primer concentration markedly affected the RAPD profile as distinct polymorphic bands were produced at 10 pico moles however, increase or decrease in concentration either reduced the number of bands or led to complete absence. Different species exhibited varied fingerprinting patterns with primer OPA-02. Optimization of *Taq* polymerase is the most critical parameter in determining the performance of RAPD fingerprinting as the use of different *Taq* polymerases usually results in different amplification patterns on the same target genome [13]. Presently 1.0 U of *Taq* polymerase was found suitable to create clear and reproducible DNA amplification profile. Besides PCR parameters, thermal cycle profiles also influenced RAPD patterns therefore a thermal gradient PCR programmes was run to ensure suitable annealing temperature. It ranged between 35-38°C and best results were produced with 36°C temperature.

The present study on protocol optimization for genomic DNA isolation of high purity and RAPD PCR is the first report in mosquito larvae. This powerful approach will serve as a rapid molecular tool for accurate identification of mosquito larvae of three culicidae without identifying any adult stages in the field conditions and their effective control subsequently.

CONCLUSION

The present study provides a cost effective and sensitive method for genomic DNA isolation from three species of Culicidae i.e. *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti*. DNA extracted by three methods viz. DNAzol method, Qiagen kit method and SDS methods were compared. It was observed that DNA isolated by SDS method produced reproducible RAPD –PCR patterns and comparable with Qiagen method. This is a first report from immature stages of mosquito and would prove useful in the correct identification and genetic diversity study of these mosquito species even from larvae.

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