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Research article

Comparison of two DNA extraction methods from larvae, pupae, and adults of *Aedes aegypti*

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ABSTRACT

Mosquitoes are the most important arthropods from the point of view of public health, due to the fact that they can transmit a large number of pathogens which can cause diseases to humans and animals. *Aedes aegypti* (L.) is one of the most important vector species in the world, since it can transmit numerous pathogens such as dengue, Zika, and chikungunya. Therefore, studies involving the molecular aspects of this and other mosquitoes species are currently increasing. In this report, we describe the comparison between two DNA extraction techniques, Chelex and cetyltrimethylammonium bromide (CTAB), for carrying out DNA extraction in larvae, pupae and adult female of *Ae. aegypti*. The Chelex technique was superior in the amount and purity of DNA as compared to the CTAB technique in the three life stages we tested.

1. Introduction

The yellow fever mosquito, *Aedes aegypti* (L.) is one of the most common mosquito species in urban and suburban areas in tropical and sub-tropical regions worldwide. It is native to Africa and is well adapted to urban conditions, becoming one of the most common species in association with humans (Kamgang et al., 2018). *Aedes aegypti* is considered one of the most important species for public health since it is the main vector for a number of arthropod-borne viruses (arboviruses) causing diseases in humans such as yellow fever, dengue fever, chikungunya, and Zika, which are widely distributed across tropical and subtropical regions worldwide (Roldán et al., 2013; Jentes et al., 2011).

The number of molecular techniques available for entomological studies has grown enormously since the conception of the polymerase chain reaction or PCR. The presence of conserved regions in the DNA sequences, such as mitochondrial, ribosomal, and nuclear DNA makes it possible to amplify fragments of organisms whose genome is unknown (Kocher et al., 1989; Fitzpatrick et al., 2010; Albers et al., 2013). The use of a rapid and inexpensive DNA extraction technique that results in high quality DNA is essential for any PCR-based molecular tool. Therefore, the DNA extraction to be used is an essential step in many molecular protocols (Gupta and Preet, 2012).

The use of ready-made kits for the extraction and purification of genomic DNA using pre-made anion-exchange columns packaged with all necessary solutions to lyse the cells and solubilize the DNA makes the process significantly easier (Gupta and Preet, 2012). DNA extracting

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commercial kits of different brands are time efficient, but can be very expensive, especially in resource-poor nations. However, there are some low-cost DNA extraction techniques such as Chelex® 100 (referred to as "Chelex," Bio-Rad® Lab. Hercules, CA, USA) and cetyltrimethylammonium bromide (referred to as "CTAB"). Chelex resins are composed of styrene-divinylbenzene copolymers containing paired iminodiacetate ions, which act as chelating groups in binding polyvalent metal ions (Ip et al., 2015), while CTAB is a cationic surfactant added in the DNA extraction buffer, which dissociates and selectively precipitates DNA from histone proteins (Muhammad et al., 2013). Other rapid techniques for obtaining DNA in vector groups such a blackflies and mosquitoes have been detailed by Hernández-Triana et al. (2012, 2019) in which time and DNA recovery efficiency was assessed. These included adding legs directly into the PCR mix, sonicating legs placed in water, the use of alkaline lysis buffer (Hotshot technique).

Currently, there are a large number of PCR-based studies involving mosquitoes, for which DNA extraction is necessary, for example molecular taxonomy (Hebert et al., 2003; Cywinska et al., 2006), surveillance and detection of pathogens (Snounou et al., 1993; Chanteau et al., 1994; Crochu et al., 2004), forensic studies (Spitaleri et al., 2006), and detection of insecticide resistance genes (Martínez-Torres et al., 2002; Brengues et al., 2003).

Successful DNA amplification depends heavily upon the extraction technique used (De Armas et al., 2005; Sagar et al., 2014; Dai et al., 2017), which might be correlated to the funds available to the researchers. In this study we aimed to compare and evaluate the performance of two DNA extraction methods, Chelex and CTAB, in larvae, pupae, and adults of *Ae. aegypti* for subsequent molecular analyses. These two methods were chosen for they known reliability in keeping the DNA stable for long period of time and they were easy to obtain within our laboratory.

2. Materials and methods

2.1. Mosquito collection

Mosquito eggs of *Ae. aegypti* were collected in September 2017 from ovitraps placed in different locations of Gómez Palacio, Durango, Mexico. Eggs were hatched and reared to the fourth instar stage. A portion of fourth instar larvae and pupae were killed in hot water (60-80 °C), while a portion of the adult females was killed in lethal chambers using chloroform vapors. All specimens were identified to species and immediately transferred to labeled vials with 90% alcohol and frozen at -40 °C until analysis.

2.2. DNA extraction and visualization

A total of 60 specimens were used for DNA extraction: 10 specimens of each stage (larvae, pupae, and adult female) for each DNA extraction technique (Chelex and CTAB). Each specimen was placed in 1.5 ml (Eppendorf®) for DNA extraction and quantification of nucleic acids. The DNA extraction was performed in the Molecular Biology Laboratory of the UAAAN-UL using the methodology of Chelex for the standardized DNA extraction technique described by Musapa et al. (2013), where 300 µl molecular grade chelating resin (Chelex 100 Resin Bio-Rad®) was added to each sample. Following maceration, the sample was centrifuged at 10,000 RPM for 10 s and then the samples were incubated in a heating block at 56 °C for 1 h. The samples were then vortexed to homogenize, and a further incubation was performed in a heating block at 100 °C for 10 min followed by centrifugation at 10,000 RPM for 10 min. Finally, 30–50 µl of supernatant was recovered, which was stored at -20 °C.

The technique of CTAB was performed according to described protocols (Tel-Zur et al., 1999). Briefly, 100 µl of CTAB was added to each sample for maceration and vortexed for 15 s, and then incubated in a heating block at 65 °C during 20 min after which 200 µl of chloroform was added to each sample and mixed by vortexing for 15 s, and centrifuged at 13,000 RPM for 5 min. The aqueous phase was transferred to new vials (1.5 ml), 100 µl of cold isopropanol (-12 °C) was added, and then samples were stored at -30 °C for 15 min. Following the incubation, the samples were centrifuged at 13,000 RPM for 5 min. The supernatant was then decanted, followed by the addition of 100 µl of cold 70% ethanol (-12 °C) and centrifugation at 13,000 RPM for 3 min. The ethanol was then decanted and the pellets containing the DNA were dried in the vial at 24 °C for 24 h. Finally, pellets were re-suspended adding 20 µl of milliQ water and frozen at -20 °C.

The amount of DNA concentration and purity (absorbance values) was measured using a spectrophotometer (Nanodrop, 2000; Thermo Scientific®). Extracted DNA (5 µl of each sample) was visualized using agarose gels (0.8%) with 0.5X TBE buffer solution (Tris base, boric acid, 0.5M EDTA, pH 8.0) in an electrophoresis chamber for 30 min. DNA in the gels was visualized and photographed using a transilluminator (MultiDoc-It Digital Imaging System).

2.3. Statistics

Descriptive statistics of DNA concentrations and absorbance levels (260/280) for qualitative DNA in each specimen were calculated and data were transformed to log10 (x + 1) (Zar, 1999). We performed statistical analyses using a parametric model of Student's T-test to assess differences in the amount of DNA recovered following extraction and DNA absorbance quality levels for all specimens between techniques Chelex and CTAB. To compare the amount of DNA and absorbance levels among life stages tested (larvae, pupae and adult female) for each DNA extraction technique, we performed analyses of variance (ANOVA). All statistical tests were considered significant with $P < 0.05$ and were made in PASW Statistics 18.

3. Results

Extracted DNA in agarose gels for both DNA extraction techniques is shown in Fig. 1. Using the Chelex-based DNA extraction technique, the DNA concentrations from the larvae ranged from 115.4 – 188.9 ng/µl; pupae ranged from 97.8 – 189.2 ng/µl; and adults ranged from 292.4 – 459.7 ng/µl (Table 1). While using the CTAB technique, the concentrations of the larvae were 7.4–21.3 ng/µl; the concentrations of the pupae were 10.8–40.7 ng/µl; and the concentrations of the adults were 30.1–93.1 ng/µl (Table 2).

A significant difference in the amount of DNA from the larvae stage between techniques was observed ($t = 19{,}877$, $df = 18$, $P < 0.001$), where the highest DNA concentration was observed using Chelex extraction ($\bar{x}$= 137.46 ng/µl), while with the CTAB technique the DNA concentration was lower ($\bar{x}$= 15.33 ng/µl) (Fig. 2). However, the DNA absorbance levels between both techniques were similar ($t = 0.395$, $df = 18$, $P = 0.697$) with averages of 1.96 and 1.95, respectively (Tables 1 and 2) (see Fig. 3).

A significant difference in the DNA concentration from the pupa stage between both techniques was observed ($t = 16.142$, $df = 18$, $P < 0.001$). The highest DNA concentration was observed using Chelex ($\bar{x}$= 189.2 ng/µl), while using CTAB, the DNA concentration was lower ($\bar{x}$= 40.7 ng/µl) (Fig. 2). The DNA absorbance levels between both techniques were different ($t = -4.768$, $df = 18$, $P < 0.001$) with averages of 1.81 and 2.04 respectively (Tables 1 and 2).

As in the larvae and pupa stage, a significant difference in the DNA concentration from adults between both techniques was observed ($t = 14.901$, $df = 18$, $P < 0.001$). The highest DNA concentration was observed using Chelex ($\bar{x}$= 377.15 ng/µl), while using CTAB, the amount of DNA concentration was lower ($\bar{x}$= 61.77 ng/µl) (Fig. 2). The DNA absorbance levels between both techniques were similar ($t = 0.036$, $df = 18$, $P = 0.972$) with averages of 2.04 and 2.02, respectively (Tables 1 and 2).

Using the Chelex DNA extraction technique, a significant difference in the DNA concentration among stages was observed ($F = 98.59$, $df = 2$,

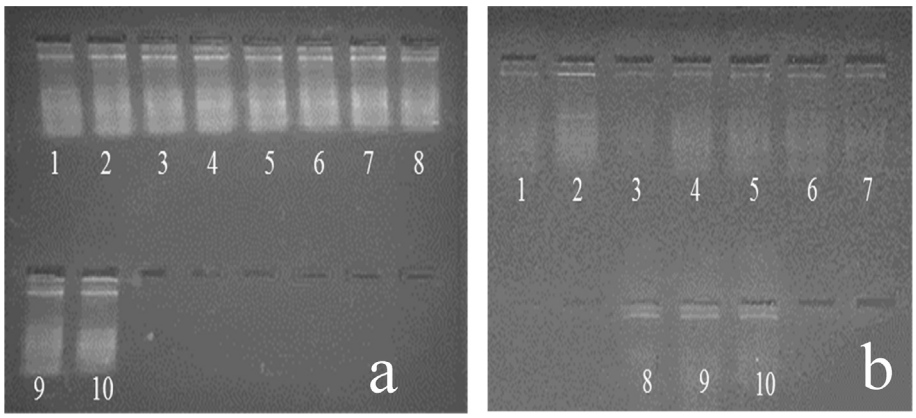

Fig. 1. Amplification of ten adult female samples of *Ae. aegypti* using the DNA extraction technique of Chelex (a), and CTAB (b).

Table 1
Descriptive statistics of DNA concentrations (ng/μl) and absorbance values of A260/A280 using the technique of Chelex. (SD) Standard deviation, (SE) Standard error.

Stage	Chelex					
	DNA			Absorbance		
	$\bar{x} \pm$ SD	SE	Min - Max ng/μl	$\bar{x} \pm$ SD	SE	Min - Max 260/280
Larvae (n = 10)	137.46 ± 23.68	7.48	115.4–188.9	1.96 ± 0.05	0.1	1.87–2.04
Pupae (n = 10)	150.81 ± 32.79	10.37	97.8–189.2	1.81 ± 0.07	0.02	1.69–1.98
Adult (n = 10)	377.15 ± 49.68	15.71	292.4–459.7	2.04 ± 0.08	0.03	1.84–2.1

Table 2
Descriptive statistics of DNA concentrations (ng/μl) and absorbance values of A260/A280 using the technique of CTAB. (SD) Standard deviation, (SE) Standard error.

Stage	CTAB					
	DNA			Absorbance		
	$\bar{x} \pm$ SD	SE	Min - Max ng/μl	$\bar{x} \pm$ SD	SE	Min - Max 260/280
Larvae (n = 10)	15.33 ± 4.40	1.39	7.4–21.3	1.95 ± 0.11	0.03	1.82–2.2
Pupae (n = 10)	16.32 ± 8.88	2.80	10.8–40.7	2.04 ± 0.13	0.04	1.83–2.25
Adult (n = 10)	61.77 ± 21.73	6.87	30.1–93.1	2.02 ± 0.02	0.01	2–2.06

27, $P < 0.001$); adults showed the highest DNA concentration as compared to larvae and pupae. In the DNA absorbance levels, a significant difference was also observed ($F = 22{,}448$, df = 2, 27, $P < 0.001$), where the mean of DNA concentrations from the pupae was lower than larval and adults means. Using the CTAB DNA extraction, a significant difference among stages was also observed ($F = 47.29$, df = 2, 27, $P < 0.001$), with adults producing the highest DNA concentration, as compared to larvae and pupae. In the DNA absorbance levels, no significant difference was observed among stages ($F = 2.44$, df = 2.27, $P < 0.001$).

4. Discussion

A large number of studies comparing DNA extraction techniques have been carried out using different types of organisms (for example Haruyama et al., 2008; Chen et al., 2010; Gutiérrez-López et al., 2014; Ridgeway and Timm, 2014), including mosquitoes of medical importance (Rivero et al., 2004; Gupta and Preet, 2012; Musapa et al., 2013; Sarma et al., 2014; Neiman et al., 2016). One essential point in studies concerning insects of medical importance is the research of pathogenic agents in order to assess their potential vectorial role (Desloire et al., 2006). Given the medical importance of mosquito species such as *Ae. aegypti*, increasing studies involving molecular tools and biotechnological assays are being carried out. Commercial DNA extraction kits as Qiagen DNeasy Blood and Tissue kits are cost-prohibitive, especially for developing countries. Accordingly, it is essential to identify effective and low-cost techniques of DNA extraction of mosquitoes for use in the developing world.

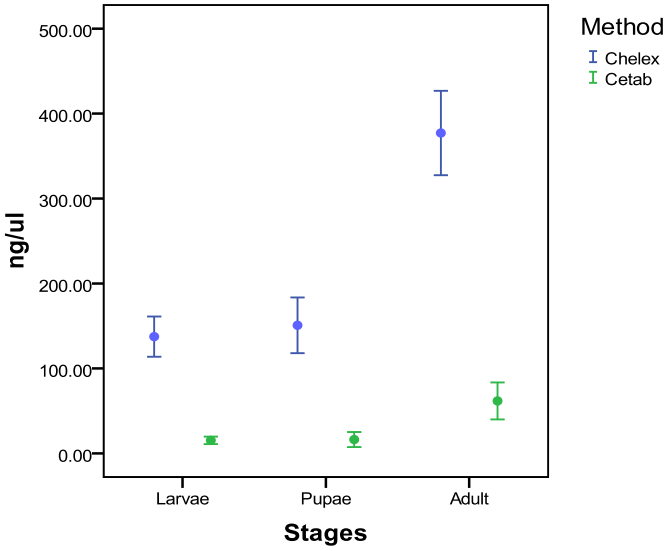

Fig. 2. Mean ± standard deviation of DNA concentration in three different life stages of *Ae. aegypti* using the Chelex and CTEAB DNA extraction techniques. Using Student's t test we found significant difference between methods in larvae ($t = 19{,}877$, df = 18, $P < 0.001$), pupae ($t = 16.142$, df = 18, $P < 0.001$), and adults ($t = 14.901$, df = 18, $P < 0.001$).

Using both DNA extraction techniques evaluated in this study, adult mosquitoes produced higher DNA concentrations as compared to those obtained from larvae and pupae. A possible explanation for this difference is that there were difficulties in the DNA extraction, specifically during manual cell lysis. It is possible that extraction from the different stages resulted in contamination by lipids, polysaccharides and proteins because the earlier stages are difficult to homogenize. In contrast, adult

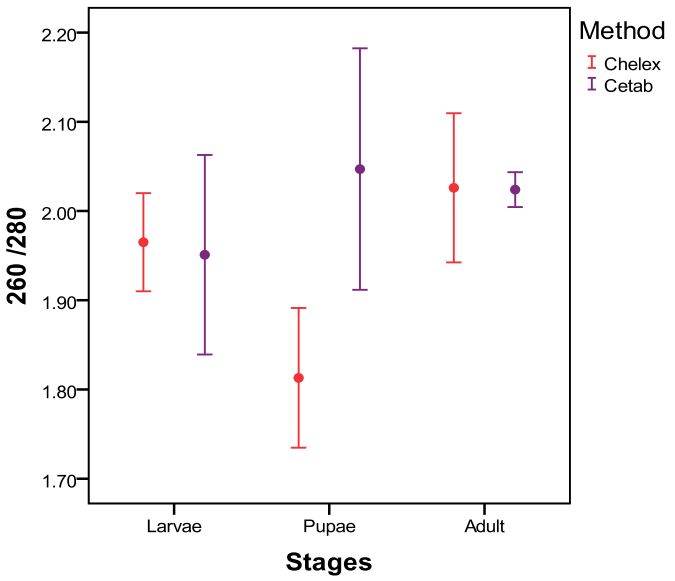

Fig. 3. Mean ± standard deviation of DNA absorbance (260/280) in three different life stages of *Ae. aegypti* using the Chelex and CTAB DNA extraction techniques. Using Student's t test we found no significant difference between methods in larvae (t = 0.395, df = 18, P = 0.697), and adults (t = 0.036, df = 18, P = 0.972); pupae shown significant difference (t = -4.768, df = 18, P < 0.001), with averages of 1.81 and 2.04, respectively.

mosquitoes are small and fragile, with long and thin legs facilitating maceration and the use of reagents for DNA extraction (Asghar et al., 2015; Manuahe et al., 2016).

In previous reports, the DNA extraction technique of Chelex has been recommended over other techniques such as DNAzol, DNeasy, Salting out and prepGEM when studying mosquitoes (Asghar et al., 2015) or other types of arthropods such as bees (Gould et al., 2011) and spiders (Casquet et al., 2011). CTAB DNA extraction has been successfully tested in other organisms such as beetles (Chen et al., 2010), maize (Sahu et al., 2012), and midges (Diptera: Chironomidae) (Wang and Wang, 2012), in which values of 35–42 ng/μl and an absorbance average of 1.83 have been obtained. These results are similar to those acquired in the present study.

When compared to the CTAB technique, the Chelex technique used fewer reagents, proved to be cheaper ($350), safer and produced better results. Also, the processing time was shorter since it only took approximately 30–60 min, depending upon the experience of the technician as detailed in Musapa et al. (2013). In contrast, the standard protocol of the CTAB method used more than five reagents that are difficult to acquire for an average laboratory and its overall cost ($440) was higher than Chelex. Furthermore, the CTAB procedure requires approximately 3 h 30 min and an additional 5–12 h to dry the DNA pellet, and to resuspend it in ultrapure water for downstream use (Tel-Zur et al., 1999). The advantages and disadvantages of both extractions mentioned above indicate that Chelex is preferable for most laboratories in terms of reagent availability, cost, time efficiency and DNA concentration.

According to the two DNA extraction methods compared in this study, we recommend the Chelex technique for the extraction of DNA from *Ae. aegypti* mosquitoes. This technique was superior to CTAB in terms of DNA concentration in all life stages we tested.

Declarations

Author contribution statement

Josué M. de la Cruz-Ramos: Conceived and designed the experiments; Wrote the paper.

Luis M Hernández-Triana: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Cristina Garcí-De la Peña: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Vicente H. González-Álvarez, Américo D. Rodríguez: Performed the experiments.

James Weger-Lucarelli: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Quetzaly Karmy Siller-Rodríguez: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Francisco J. Sánchez Rámos: Analyzed and interpreted the data.

Aldo I. Ortega-Morales: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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