



# Comparative analysis of three methods from dried blood spots for expeditious DNA extraction from mosquitoes; suitable for PCR based techniques

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## Abstract

The objective of this work was to compare the quality, purity and quantity of DNA isolated from dried blood spots (DBS) by three methods (Chelex-100, QIAamp DNA mini kit, and TE (Tris EDTA)-Buffer). Sample collection was performed in six districts in Odisha, India and screened for cases of clinical malaria and dengue and vector density. Mosquito abdomens were spotted on Whatman 3MM (MERCK) Filter paper and dried for 10 min at room temperature. DNA was isolated from DBS using three methods (Chelex-100, QIAamp DNA mini kit, and TE-Buffer), and PCR was used to determine the feeding behaviours of vector mosquitoes. DNA was quantified using a UV-spectrophotometer, and q-PCR was used to determine the target gene copy number to compare the methods. The QIAamp DNA mini kit method was used as the reference method. The yield and purity of DNA extracted with Chelex-100 and TE were 14–72 ng/μl and 1.51–1.85 and 9–50 ng/μl and 1.68–2.1, respectively. DNA extracted using the Chelex-100 method was stored for over 1 month at – 20 °C and was suitable for later use. The Chelex-100 method had a sensitivity of 99.5% and specificity of 78%. A Bland–Altman plot suggested that the Chelex-100 method was similar to the QIAamp DNA mini kit method for determining the feeding behaviours of vector mosquitoes. The Chelex-100 method is simple, cost-effective, and safe and requires minimal time for DNA extraction from dried blood spots. In malaria and dengue research, detecting the feeding behaviours from mosquito DNA from dried blood spots on filter paper by PCR is an easy, minimally invasive and inexpensive molecular technique that can be performed in remote areas.

**Keywords** Chelex-100 · TE-Buffer · Dried blood spots · Anopheles · Aedes

## Introduction

Dengue and malaria are major public health problems in India. In spite of different control measures, malaria is a major public health issue in rural areas in the central, eastern and north-eastern states in India [1]. Approximately 60% of malaria cases in India are confined to tribal areas

[2]. *Anopheles culicifacies* and *Anopheles fluviatilis* are efficient vectors in tribal-dominated areas [3]. Dengue was previously considered an urban and peri-urban disease, but dengue infections are also being reported in rural areas [4]. *Aedes aegypti* is found in rural areas in Andhra Pradesh, Karnataka, Maharashtra and Gujarat [5]. *Aedes albopictus* is a rural vector for dengue that is now spreading quickly to urban and semi-urban areas. To design appropriate vector control programs and to understand the epidemiology of malaria and dengue, it is necessary to know the vector species composition and human biting preference of mosquitoes in disease-prevalent areas [6].

When performing epidemiological surveys in remote areas, it is difficult for facilities to preserve and process mosquito blood samples; therefore, dried blood spot methods (DBS) are commonly applicable [7]. Filter paper has been evaluated for the easy preservation and room temperature storage of various biological specimens for the analysis of

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their proteins and nucleic acids [8, 9]. Dried blood spots show strong advantages compared to the other conventional collection methods for the analysis of plasma and blood samples. Thus, the recently usage of DBS has been increasing. Apart from proper storage and transportation, this method provides an easier way to process collected blood with significantly lower volumes compared to RDTs or microscopy. Moreover, the entire process can be performed in a time- and cost-effective manner [10]. DBS do not require cold storage and allow for retrospective PCR analysis [11]. Studies on DBS stored over a long period of time have shown varying results. When stored beyond 5 years, one study showed diminished sensitivity [12], though another study showed increased sensitivity after 4 years [13]. The preservation of the DNA in the DBS totally depends on the storage conditions, including temperature and humidity, as well as the type of filter paper used [14].

As an integral part of control programs, endemic countries are increasingly adopting molecular techniques for the efficient identification of vector mosquitoes and surveillance against malaria parasites [15]. There are different molecular methods, including the phenol–chloroform method, TE method, saponin, and PBS method, which can extract DNA from dried blood spots. The Chelex method is superior to the previously mentioned methods, as they require more time to extract DNA and their storage and yield capacities are not great [16, 17]. DNA isolation from filter paper with Chelex-100 provides many advantages that can remarkably improve DNA extraction efficiency, especially in DNA barcoding studies. Because of practical difficulties and methodological problems, it is necessary to optimize the conditions to maximize the purity and yield of DNA obtained from different types of samples using various methods [18].

Chelex-100 is an ion exchange resin. Chelex is composed of styrene divinylbenzene copolymers that contain paired iminodiacetate ions that act as chelating groups for binding polyvalent metal ions such as magnesium ( $Mg^{2+}$ ). Nucleases are inactivated and the DNA is protected by removing the

$Mg^{2+}$  from the reaction. This method requires only one heating step at 99 °C; therefore, this method is less labour-intensive [19]. Research groups have used different Chelex-100 methods to extract DNA from filter paper, which is time consuming and requires more chemicals to perform [16]. However, in our modified protocol, we used Chelex-100 throughout the entire process.

The aim of this study was to extract DNA from filter paper and compare this method with other DBS methods in terms of time, storage temperature, buffer stability and DNA yield, which are important for later PCR techniques.

## Methodology

### Collection and storage of mosquito samples

Sample collections were carried out in remote areas in six districts, including Jaipur, Jagatsinghpur, Balasore, Malkanagiri, Kalahandi and Kandhamal, in Odisha state, India from January to December 2017 (Table 1).

Mosquitoes were collected with the help of mouth aspirators and torches in the summer, rainy and winter seasons. Adult mosquitoes were collected from indoor and outdoor resting habitats from 6:00 a.m. to 9:00 a.m. and in the evening from 6:00 p.m to 10:00 p.m from human dwellings, cattle sheds and mixed dwellings. The collected adult mosquitoes were labelled and morphologically identified using standard keys developed by Christopher [20] in the field and brought to the laboratory for processing [21].

Immediately after identification, each individual mosquito was separated into two parts. The abdomen was spotted onto Whatman 3MM (MERCK) Filter paper and dried for 10 min at room temperature. The samples were stored in a plastic zip lock bag. Desiccant was added to each bag before placing the filter paper samples in the plastic zip lock bag, and then the bags were brought to the laboratory for processing at room temperature (20–25 °C). Samples being

**Table 1** Geographical, socioeconomic and disease endemic information of the study districts [Source: NVBDCP, Odisha]

Location	Geo-physiographical zone	Coordinates	Population	Malaria				Dengue			
				2016		2017		2016		2017	
				API	Death	API	Death	Total +ve sample	Death	Total +ve sample	Death
Kandhamal	Eastern Ghat	20.47°N 84.23°E	7,31,952	38.07	9	43.02	3	11	0	14	0
Kalahandi	Eastern Ghat	20.083°N 83.2°E	1,573,054	28.67	3	19.64	0	9	0	7	0
Mayurbhanja	Northern plateau	21.933°N 86.733°E	2,51,3895	10.15	2	10.76	1	52	0	51	0
Jajpur	Coastal Belt	20.85°N 86.33°E	3,74,58	0.41	0	0.32	0	554	0	392	0
Balasore	Coastal Belt	21.49°N 86.93°E	1,44,373	0.23	0	0.35	0	468	0	1327	2
Jagatsinghpur	Coastal Belt	20.266°N 86.166°E	1,058,894	0.13	0	0.11	0	368	2	234	0

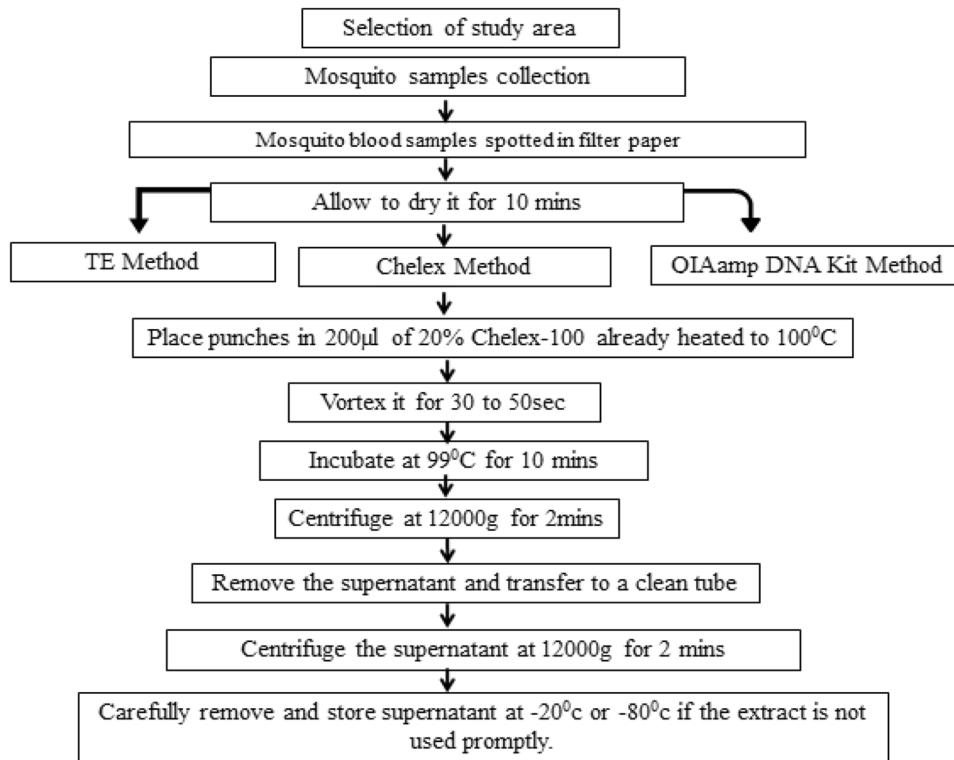
processed within 4 weeks were kept in a refrigerator at 4 °C, and samples for longer storage were kept at – 20 °C.

### DNA isolation from DBS

Only *A. culicifacies*, *A. fluviatilis*, *A. aegypti* and *A. albopictus* were used for molecular evaluation. Chelex-100 (Himedia), TE buffer and the QIAamp DNA mini kit (Qiagen,

in saponin overnight, (ii) impregnating in PBS overnight and (iii) no impregnation. We followed the third method with some necessary modifications, which gave the best results with higher sensitivity and specificity.

The detailed procedures used for the Chelex-100 method are described in the flow chart below.



Germany) methods were tested to extract DNA from all DBS. Eight punches from the individual DBS were used for the extraction of DNA for all three methods.

#### Tris -EDTA buffer-based extraction

The TE buffer method was performed per Bereczky et al. [22]. Briefly, the DBS punches were placed in 65 µl TE buffer (10 mM Tris, pH 8.0, and 0.1 mM EDTA in distilled water) in a clean Eppendorf, incubated at 50 °C for 15 min and then heated to 97 °C for 15 min. The tube was then centrifuged at 12,000 rpm for 10 s and the supernatant was removed and placed in a clean Eppendorf.

#### Chelex-100 based extraction

We used the following three different Chelex-100 methods [16] to extract DNA from filter paper DBS: (i) impregnating

#### DNA yield and purity

The DNA yields using the three different extraction methods were evaluated with a UV–vis spectrophotometer.

#### q-PCR for *Anopheles* and *Aedes* mosquito blood-meal analysis and quantification

For the quantitative determination of the PCR template copy numbers (DNA), SYBR Green q-PCR measurements were performed using the Applied Biosystems 7500 Fast Real Time PCR system, V 2.0.3 software. This assay was used to quantify the blood meal genome copy number in the purified DNA samples. The mitochondrial CO-II genes from human, cow and goat were used as q-PCR standards. The three sets of DNA generated from each sample by the three respective methods were analysed together in the same

q-PCR experiment. To determine the host feeding behaviours of *Anopheles* and *Aedes*, q-PCR was performed with primers specific to human, goat and cow [23]. The q-PCR tests were performed in triplicate and average numbers were used for the calculations and analysis. The thermal profile used for the q-PCR reaction was as follows: (1) 6 min at 94 °C and (2) 35 cycles of 30 s at 94 °C, 45 s at 55 °C, and 1 min at 72 °C. Each reaction contained 3 µl of template DNA, 14.5 µl SYBR Green, 1 µl of each primer and 5.5 µl of DNase-free water in a final volume of 25 µl. All q-PCR assays were run with appropriate controls including a non-template control (NTC). The detection limits of the three methods were assessed using 10-fold serial dilutions (10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg per 3 µl) of genomic DNA from each animal species.

### Data analysis

A t-test was applied to determine the differences in the anthropophilic index for *Anopheles* and *Aedes* mosquitoes in indoor and outdoor spaces.

The sensitivity and specificity of the Chelex-100 and TE buffer methods were calculated [24].

A Bland–Altman plot was drawn to determine the differences between the Chelex-QIAamp and TE-QIAamp methods designed to measure the same property. The QIAamp DNA mini kit method was used as the reference method.

### Ethical clearance

This study was approved by the Institutional Human and Animal Ethics Committee of the ICMR, Regional Medical Research Centre, Bhubaneswar with IRB No. ECR/911/Inst/OR/2017. Villagers were informed about the aims and objectives of the study through group meetings.

## Results

### Species composition

A total of 812 female *Anopheles* species and 338 female *Aedes* species were collected. Among them, 702 *Anopheles* species and 289 *Aedes* species blood meal-fed mosquitoes were captured from the study district from January 2017 to December 2017. The most common species were *A. culicifacies* 31.7% ( $n = 258$ ,  $n_{\text{indoor}} = 197$ ,  $n_{\text{outdoor}} = 61$ ), *A. fluviatilis* 22.7% ( $n = 185$ ,  $n_{\text{indoor}} = 154$ ,  $n_{\text{outdoor}} = 31$ ), *A. albopictus* 51.4% ( $n = 174$ ,  $n_{\text{indoor}} = 69$ ,  $n_{\text{outdoor}} = 105$ ) and *A. aegypti* 39.9% ( $n = 135$ ,  $n_{\text{indoor}} = 48$ ,  $n_{\text{outdoor}} = 87$ ). We did not find any variation in the anthropophilic index for *Anopheles* mosquitoes between the indoor and outdoor populations ( $t = -0.14$ ,  $p < 0.05$ ). There was a significant variation in

the anthropophilic index for *Aedes* mosquitoes between the indoor and outdoor populations ( $t = -0.02$ ,  $p < 0.05$ ).

### DNA yield and purity

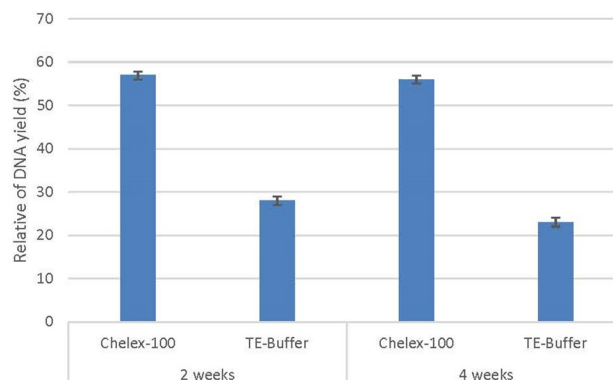
DNA extraction using Chelex-100 and TE-Buffer resulted 14–72 ng/µl and 9–50 ng/µl genomic DNA/isolation, respectively. The mean dsDNA concentration using the Chelex-100 method was 56.8 ng/µl with a standard deviation of 10.38 ng/µl. In contrast, the mean dsDNA concentration from the TE method was 27.7 ng/µl with a standard deviation of 11.74 ng/µl. Storage of the extracted DNA with Chelex-100 and TE for > 15 days and 1 month at room temperature,  $-20$  °C and  $-80$  °C did not affect PCR performance.

### Quantification of DNA extracted from DBS with the different buffers

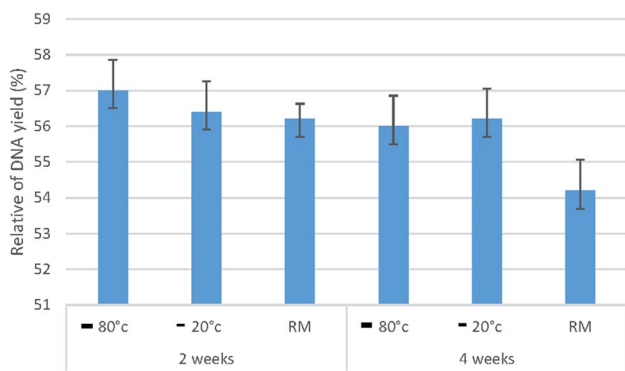
The extraction yield with Chelex-100 and TE buffer were compared in Fig. 1. Neither buffer produced significantly different yields for the different sample storage times. In contrast, the DNA yield with Chelex-100 was approximately two times higher than with TE buffer. Therefore, Chelex was more suitable than TE for DNA extraction.

### Yield of DNA from DBS (Chelex extraction method) at different storage temperatures and times

The DNA yields from DBS (Chelex extraction method) were compared at different storage temperature ( $-80$  °C,  $-20$  °C and room temperature) and times. There was a 1% difference in the DNA yields between 2- and 4-week storage at  $-80$  °C. In contrast, there was a 2% difference in the DNA yields between 2- and 4-week storage at room temperature, while there was 0.2% difference in the DNA yields from samples stored at  $-20$  °C for the same period. Therefore, storage at  $-20$  °C was best for the samples on filter paper (Fig. 2).



**Fig. 1** Quantification of DNA extracted from dried blood spots with the Chelex-100 and TE buffer methods



**Fig. 2** Yield of DNA from dried blood spots using the Chelex method stored at – 80 °C, – 20 °C and room temperature

**Blood feeding pattern of *Anopheles* and *Aedes* vector species: a comparative q-PCR result obtained from three methods**

Species-specific q-PCR assays for human, cow and goat DNA were successfully developed using the CO-II gene.

The sensitivity of this assay ranged from 99 to 100%. Each q-PCR successfully amplified the DNA of the target species using the different methods. No amplification was obtained with the NTC. Of the 752 *A. culicifacies*, *A. fluviatilis*, *A. aegypti* and *A. Albopictus* mosquito vectors collected from the indoor and outdoor spaces, 300 samples were tested for abdominal blood meal analysis. In total, 198, 217 and 216 reacted positively to the TE- Buffer, QIAamp DNA kit and Chelex-100 methods, respectively (Table 2).

The detection limit for the QIAamp DNA kit and Chelex-100 and TE-Buffer methods ranged from 10 fg to 10 pg, with CT-values ranging from 20.8 to 25.6, 21 to 26.3 and 25.3 to 31.3, respectively (Table 3).

**Blood meal analysis of *Anopheles* and *Aedes* collected from indoor (HD/CS) and outdoor study districts (PCR templates result obtained using the Chelex-100 method)**

The human blood feeding rate was higher in *A. fluviatilis*, *A. albopictus* and *A. aegypti*. The anthropophilic index for *Aedes* mosquitoes was higher in the cattle shed. All species showed an inclination for multiple feedings (Table 4).

**Table 2** Comparative analysis of the three methods for PCR for positive feeding behaviours of *Anopheles* and *Aedes* vector species collected from indoor (HD/CS) and outdoor spaces in the study districts

Collection site	Species	Sample tested	No. of sample +ve for three methods		
			TE-Buffer	QIAamp DNA Kit	Chelex-100
HD	<i>A. culicifacies</i>	25	19	19	19
	<i>A. fluviatilis</i>	25	20	21	21
	<i>A. Albopictus</i>	25	17	19	19
	<i>A. Aegypti</i>	25	20	22	22
CS	<i>A. culicifacies</i>	25	23	23	23
	<i>A. fluviatilis</i>	25	13	18	18
	<i>A. Albopictus</i>	25	18	19	18
	<i>A. Aegypti</i>	25	16	16	16
OUTDOOR	<i>A. culicifacies</i>	25	14	16	16
	<i>A. fluviatilis</i>	25	9	12	12
	<i>A. Albopictus</i>	25	17	18	18
	<i>A. Aegypti</i>	25	12	14	14

HD human dwelling, CS cattle shed

**Table 3** Summary of the q-PCR results obtained from blood meal analysis of mosquitoes

	Human			Cow			Goat		
	Ct value	2 <sup>-(ΔΔct)</sup>	Detection limit (3 μl)	Ct value	2 <sup>-(ΔΔct)</sup>	Detection limit (3 μl)	Ct value	2 <sup>-(ΔΔct)</sup>	Detection limit (3 μl)
QIAamp method	20.8	8.574188	1 fg	26	5.656854	10 fg	25.6	9.189587	10 fg
Chelex method	21	7.464264	10 fg	26.3	4.594793	1 pg	26	6.964405	100 fg
TE method	25.3	0.011842	10 pg	30.6	0.008974	1 pg	31.3	0.005524	10 pg

**Table 4** Blood meal analysis of *Anopheles* and *Aedes* specimens collected from indoor and outdoor spaces in the study districts

Species name	+ve for host	District name											
		Kandhamal		Kalahandi		Mayurbhanja		Jajpur		Balasore		Jagatsinghpur	
		Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor
<i>A. culicifacies</i> (n = 75)	Human	2.6	1.3	4	2.6	2.6	1.3	2.6	1.3	1.3	1.3	4	2.6
	Cow	6.6	2.6	4	1.3	5.3	4	8	1.3	4	1.3	1.3	-
	Goat	-	1.3	-	1.3	-	-	-	-	-	-	-	-
<i>A. fluviatilis</i> (n = 75)	Multiple host	2.6	-	1.3	-	1.3	-	-	-	1.3	-	-	-
	Human	8	2.6	5.3	1.3	6.6	4	2.6	1.3	5.3	2.6	4	-
	Cow	4	1.3	2.6	-	4	-	2.6	-	1.3	1.3	4	1.3
	Goat	-	-	-	1.3	-	-	-	-	-	-	-	-
<i>A. aegypti</i> (n = 75)	Multiple host	-	-	1.3	-	-	-	-	-	-	-	-	-
	Human	4	1.3	5.3	-	2.6	2.6	10.6	4	12	2.6	8	1.3
	Cow	-	-	-	1.3	-	-	1.3	2.6	1.3	-	1.3	1.3
	Goat	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. albopictus</i> (n = 75)	Multiple host	-	-	-	-	-	-	2.6	-	1.3	-	-	1.3
	Human	2.6	1.3	1.3	-	5.3	1.3	13.3	5.3	9.3	6.6	8	4
	Cow	1.3	-	-	-	2.6	1.3	4	1.3	1.3	1.3	-	1.3
	Goat	-	-	-	-	-	-	-	-	-	-	-	-
Multiple host		-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-

**Table 5** Comparative results prepared using the TE Buffer, QIAamp DNA isolation Kit and Chelex-100 DNA extraction methods from filter paper

Status	Chelex-100	QIAamp DNA isolation Kit	TE buffer
+ve for feeding behaviour	216	217	198
–ve for feeding behaviour	84	83	102
Sensitivity	99.5%	–	91.2%
Specificity	78%	–	73.3%

**Statistical comparative results obtained for the three methods** The sensitivity and specificity of the Chelex-100 and TE buffer DNA extraction methods were evaluated against the QIAamp DNA isolation kit.

The Chelex method had a sensitivity of 99.5% and a specificity of 78%. The TE buffer method had a sensitivity of 91.2% and a specificity of 73.3% (Table 5).

The Bland–Altman plot for the differences between the Chelex-QIAamp method and the mean of the two measurements suggested a bias of 0.08 (mean) units representing the gap between the X-axis, which corresponded to zero differences and a parallel line at the X-axis at 0.08 units. The limits of agreements are narrow (95% CI – 0.48 to 0.64); thus, the two methods are essentially equivalent (Fig. 3a).

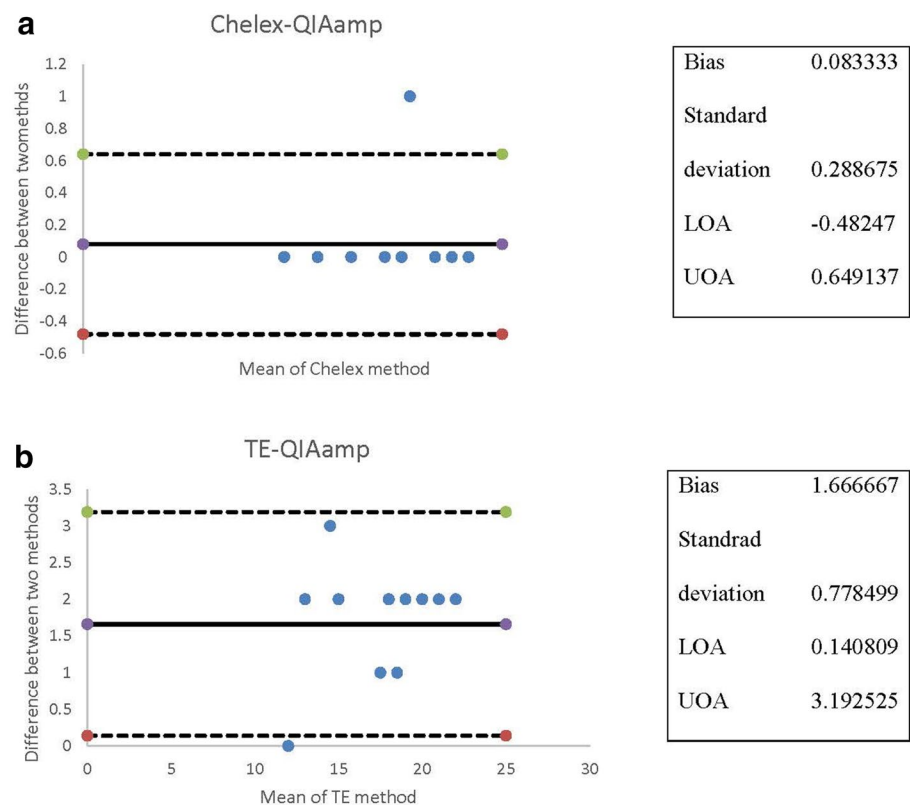
The Bland–Altman plot for the differences between the TE-QIAamp method and the mean of the two measurements suggested a bias of 1.66 (mean) units representing the gap between the X-axis, which corresponded to zero differences and a parallel line at the X-axis at 1.66 units. The limits of agreements are wide (95% CI 0.14–3.19); thus, the results from the two methods showed dissimilarity (Fig. 3b).

## Discussion

This study aimed at improving tools for malaria and dengue research in malaria-endemic countries and to identify a sensitive, rapid and cost-effective method for the extraction of mosquito DNA from filter paper.

DBS is a very simple alternative sample collection procedure that collects much smaller volumes of blood and has simpler storage and transportation conditions compared to other conventional plasma sampling procedures [25]. The Chelex procedure is simple, rapid, does not involve any harmful chemicals and does not require multiple steps for most samples. Chelex DNA preparation is stable and can be stored at 4 °C for 3–4 months after boiling [17]. Chelex-100 Molecular Biology Grade Resin can be used for easy and fast DNA purification from many sample types, including blood, forensic, mammalian

**Fig. 3** **a** Bland–Altman plot showing the differences between the Chelex-100 and QIAamp methods. **b** Bland–Altman plot showing the differences between the TE and QIAamp methods



cells, bacteria and mosquito eggs, to identify the vector approach for population genetics studies [26].

The majority of *Anopheles* mosquitoes were collected from districts in which the annual parasite index is > 10, while the majority of *Aedes* mosquitoes were collected from districts where the incidence of dengue-positive cases are high. The *Anopheles* vector density was higher indoors than outdoors, while the *Aedes* vector density was more often found outdoors. DNA extraction using the Chelex-100 method was found to be suitable for PCR techniques. The present study also revealed the feeding patterns of *Anopheles* and *Aedes* mosquitoes. Based on these results, 300 blood meals collected from all sites were identified for specific hosts. The remaining 84 (28%) were not identified; most likely, the mosquitoes fed on hosts not included in our assays or the results failed due to partially digested blood meals. The *A. culicifacies* density was greater than the other vector species and it is considered as an opportunistic species that takes blood meals from a variety of domestic animals as well as human beings. We found that *A. fluviatilis*, *A. albopictus* and *A. aegypti* predominantly feed on human hosts. We also found human blood meal samples from mosquitoes resting in CS, which means that after feeding on human blood, the mosquitoes used animal sheds to rest to help complete the gonotrophic cycle. All mosquitoes have multiple blood feeding behaviours, but the frequency of multiple feedings on human, cattle and goat was very high for *A. culicifacies*. The HBI of *A. albopictus* and *A. aegypti* was higher in the Jaipur, Jagatsinghpur, and Balasore districts, whereas the HBI of *A. culicifacies* and *A. fluviatilis* was higher in the Kandhamal, Kalahandi and Mayurbhanja districts, which are endemic to malaria. *Anopheles* species having a higher HBI indicates that it can function as a malaria vector [27]. *A. culicifacies*, *A. fluviatilis*, *A. albopictus* and *A. aegypti*, which are efficient malaria vectors in Odisha, were found to rest and bite humans in outdoor places from both malaria and dengue endemic and nonendemic areas. The biting tendency and human blood index of malaria and dengue vectors in outdoor places indicates that bed nets or other devices used indoors may not provide enough protection from vectors [28, 29].

In the present study, a SYBR Green-based q-PCR method was also developed to quantify a target gene to compare the three methods and the ratio of the blood meal hosts. Comparison of the results for the three methods showed that the Chelex-100 method allowed the detection of as little as 100 fg per reaction mixture (i.e., 3 µl) from the host DNA (i.e., human), whereas the TE-Buffer method only allowed the detection of 10 pg per reaction mixture (i.e., 3 µl) from the host DNA (i.e., human). Blood meal analysis from both methods (i.e., Chelex-100 and TE-Buffer) suggest that the

human gene is expressed at levels in mosquitoes than the cow and goat genes.

The DNA yield determined using the UV-spectrophotometer was 14–72 ng/µl for Chelex-100 and 9–50 ng/µl for TE-buffer, which is nearly two-fold higher when compared with conventional methods. Additionally, in the q-PCR analysis, the DNA concentrations using the Chelex-100 method were approximately 10-fold higher than the TE-Buffer method. The purity and yield of extracted DNA are also dependent on the researcher's handling procedure [18]. The concentration of DNA was significantly greater when using the Chelex-100 DNA extraction method.

Harty et al. [30] have reported that DNA storage can reduce DNA yield; however, PCR amplification of samples from long-term storage were performed successfully. Some studies showed that DBS can be stored at room temperature for more than 1 year and that genomic DNA can be extracted successfully from these samples [31]. The extracted DNA from all samples stored at – 20 °C were suitable for further study purposes. The DNA isolated using the Chelex-100 method was stored for over 1 month stored at – 20 °C and was suitable for later use.

Most studies report the sensitivity and specificity of dried blood spots collected from remote areas as > 94% and > 99%, respectively, for the diagnosis of parasitic infections [7]. In this study, both extraction methods have a sensitivity > 90% and a high specificity (> 70%). However, the Chelex-100 extraction method displayed a sensitivity > 99.5%, a high specificity (> 78%) and appeared to be able to detect feeding behaviours of mosquitoes. The Bland Altman Plot suggests that the average difference between Chelex-100 and QIAamp methods is 0.08 units, whereas the average difference between the TE-Buffer and QIAamp methods is 1.66 units. The limits of agreements are narrow; thus, the Chelex-100 and QIAamp methods are essentially equivalent, and this comparison is shown to have better correlation than the TE-Buffer and QIAamp method correlation.

Cox-Singh et al. reported that the InstaGene matrix method was superior to Chelex-100 [32], as it is claimed to be more effective than the conventional Chelex-100 resin to remove PCR inhibitors. The QIAamp DNA mini kit costs approximately \$4.50 US per sample, while the Chelex-100 method is much cheaper, with an estimated cost of < \$0.16 US per sample. This kit was also one of the more labour-intensive methods. The TE buffer method was the cheapest method but it was performed poorly in the current study. Berczky et al. [21] described that the TE buffer DNA extraction method was superior to the methanol and Chelex methods in terms of sensitivity and reproducibility when using dried blood spots. However, in this study, we found that the sensitivity and reproducibility of the Chelex method was better than the TE buffer method.



This study has the following limitations. This standardized method required trained and skilled manpower to perform the PCR reaction along with facilitated infrastructure. In remote areas, particularly in our country, it's difficult to use PCR due to lack of the availability of these facilities. Therefore, we standardized our protocol in such a way that it will be easier to collect specimens for long-term storage on filter paper until it is ready for further processing. Finally, filter paper has been shown to be an asset for increasing accessibility, affordability, robustness, and sensitivity and making specific diagnostic testing available to patients in remote settings. Its use in the surveillance of important tropical diseases targets their elimination, which makes the DBS technique an important tool in international health.

The filter paper used in this study has not been validated; however, several articles we reviewed regarding the use of filter paper (Whatman 3MM) emphasized the evaluation of nucleic acids with various serological assays for the diagnosis of infectious diseases using dried blood spots and validated Whatman 3MM filter paper as well as other types of filter paper [22, 25, 33]. Whatman 3MM filter paper was a potential filter paper in this study due to its practical use in terms of cost, time, and DNA sensitivity and stability [34]. These cellulose filters (Whatman 3) are used for the assay of qualitative analytical techniques to determine and identify materials, which promote an improved flow rate and increased loading capacity compared to other equivalent filter papers.

## Conclusion

We studied blood feeding behaviours of *Anopheles* and *Aedes* mosquitoes in vector-borne disease areas in Odisha using three comparative DNA extraction methods from filter paper. In malaria and dengue research, detecting the feeding behaviours from mosquito DNA from dried blood spots on filter paper by PCR is an easy, minimally invasive and inexpensive molecular technique for use in remote areas.

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**Author contributions** BBP and RKH conceived the study and participated in its design and co-ordination; BBP and RKH carried out the field studies; BBP and NP drafted the manuscript, performed the statistical analysis; BBP, NP and RKH critically revised the manuscript for intellectual content; All authors read and approved the final manuscript; BBP and RKH are guarantors of the paper.

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## Compliance with ethical standards

**Conflict of interest** The author declares that they have no conflict of interest.

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