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RESEARCH ARTICLE

Detection and quantification of *Anopheles gambiae* sensu lato mosquito larvae in experimental aquatic habitats using environmental DNA (eDNA). [version 1; referees: 2 approved, 2 approved with reservations]

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Abstract

Background: Growing insecticide resistance and changes in biting and resting behavior of malaria vectors threaten efficacy of insecticide treated nets and indoor residual spraying. Larval source management (LSM) is a promising approach that can target mosquitoes irrespective of their behavior as adults. However, the use of traditional monitoring methods for immature stages of *Anopheles* mosquitoes is a major challenge to LSM due to the variability in their breeding habitats. We evaluate the use of an environmental DNA (eDNA) analysis technique in monitoring *Anopheles gambiae* sensu lato larvae in experimental aquatic habitats.
Methods: eDNA was simultaneously sampled and extracted from different volumes of water, number of larvae, and occupation time. Larval presence was detected using PCR and eDNA concentration in samples from 1 L habitats quantified using an *IGS* and *cyt b* TaqMan assays. The limit of detection of the two assays was tested and larval density correlated with eDNA positivity.
Results: 74% of replicates in the 50 mL habitats were PCR positive with at least 6h required to get a signal from a single larva (0.02 larvae/mL). All 12 replicates where 1 L of water was used were positive with stronger PCR bands than replicates with the same larval density in 50 mL for 24 h. There was a correlation between larval densities and eDNA detection in both assays: *IGS*, $r = 0.503$, $p = 0.047$; and *cyt b*, $r = 0.558$, $p = 0.025$. There was stochasticity in eDNA detection rates, using both PCR and qPCR across all the dilutions.
Conclusion: This study has demonstrated the potential use of eDNA analysis for detection and quantification of *An. gambiae* s.s. mosquito larvae in aquatic habitats. The stochasticity observed in eDNA detection suggest that this technique is best for monitoring aquatic habitats with many larvae at low densities.

Keywords

Environmental DNA (eDNA); An. gambiae s.s.; An. arabiensis; larvae; aquatic habitats; monitoring.

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Introduction

Malaria remains actively transmitted in about 55 countries in sub-Saharan Africa (World Health Organization, 2017). An upscale in distribution and usage of vector control interventions combined with prompt case detection and treatment has seen malaria cases drop significantly over the past decade, with vector control accounting for almost 80% of this reduction (Bhatt *et al.*, 2015). However, growing insecticide resistance (Ranson & Lissenden, 2016) and behaviors such as outdoor biting and resting (Gatton *et al.*, 2013; Killeen, 2014) threaten the efficacy of insecticide treated nets and indoor residual spraying necessitating the requirement of additional vector control tools (Hemingway, 2014), preferably targeting different parts of the mosquito life cycle (Killeen, 2014).

Larval source management (LSM) is a promising approach (Tusting *et al.*, 2013) that can target mosquitoes irrespective of their behavior as adults, and with a greater range of pesticides available, can potentially overcome problems of adult insecticide resistance (Fillinger & Lindsay, 2011). LSM has historically been employed to eliminate malaria in many countries across the globe (Killeen *et al.*, 2002) However, the uptake of this intervention is impeded by the lack of knowledge of aquatic habitats that are the most preferred; if better understood, this would permit a spatially targeted intervention of the larvae sites (Gu *et al.*, 2008). The success of an LSM strategy requires accurate and replicable methods for monitoring the aquatic stages of disease vectors.

Monitoring the immature stages of *Anopheles* is traditionally done by sampling eggs, larvae, or pupae (Silver, 2008). This can be challenging due to the highly variable nature of the breeding sites ranging from tiny, easily-sampled pools (e.g. hoof prints and puddles) to large and complex water bodies such as river or lake margins, and rice fields (Minakawa *et al.*, 2004), which can lead to sampling biases and under/overestimation of larval densities in different habitats (Silver, 2008). Despite broad-scale ecological niche differences the most important malaria vectors within the *Anopheles gambiae* s.l. species complex frequently share larval habitats and are morphologically indistinguishable (Chen *et al.*, 2008), which means that traditional ecological sampling approaches must be supplemented by DNA-based analyses to identify and quantify the different species in a given habitat.

Environmental DNA (eDNA) analysis is an increasingly popular method used for ecological surveillance of both aquatic and non-aquatic habitats that can detect the presence of organisms, and potentially estimate density without direct physical sampling of the organisms (Thomsen & Willerslev, 2015). eDNA is the residual DNA shed by all aquatic organisms in the form of faecal waste, urine, dead skin, gametes or via post-mortem degradation. This residual DNA can be detected by molecular techniques allowing inference of the presence of the organisms from habitat samples (Ficetola *et al.*, 2008). In natural habitats, eDNA concentration is affected by several factors such as: the density of the target species, temperature, microbial activity in the habitat, DNA depurination (nucleic acid degradation) and exposure to ultraviolet light (Barnes *et al.*, 2014; Pilliod *et al.*, 2014). eDNA concentration under experimental sunny conditions has been shown to reduce by 80% after just one day and up to 98% in two days (Pilliod *et al.*, 2014). Hence most DNA detection is expected to indicate a current or recent colonization of the habitat (Piaggio *et al.*, 2014), making it a suitable method for contemporary surveillance of aquatic populations. eDNA analysis has been used for detection of a variety of aquatic animals and, where compared usually correlates well with presences/absence quantification using conventional sampling methods (Dejean *et al.*, 2011; Minamoto *et al.*, 2012; Spear *et al.*, 2015).

Only one aquatic survey with eDNA analysis has been performed in mosquitoes to date in which three *Aedes* species that are invasive in Europe (*Aedes albopictus*, *Aedes j. japonicus*, and *Aedes koreicus*) were monitored in small human container habitats: typical breeding sites for *Aedes* spp. (Schneider *et al.*, 2016). The highest probability of larval detection came from eDNA analysis using quantitative PCR (96%), with both a more complex shotgun sequencing-based method and traditional larval sampling surveys showing lower sensitivity (86% and 89%, respectively), confirming the efficiency of the relatively simple and inexpensive qPCR technique.

The current study is a pilot work conducted under controlled laboratory conditions that applies eDNA analysis in *Anopheles* species for the first time. We evaluated the detection and relative quantification of eDNA of *Anopheles* larvae using two qPCR assays, one of which was also capable of simultaneous differentiation of the two key East African malaria vectors *An. gambiae s.s.* and *An. arabiensis.* This latter method appears very promising for application in field eDNA studies, and we highlight the strengths and limitations of the technique.

Methods

Initial eDNA PCR primer design and validation

We designed eDNA PCR primers as an initial step to test performance of ChargeSwitch® Forensic DNA Purification Kit, Invitrogen in eDNA isolation and to ensure that the biological negative controls gave negative results. The primers were designed from the *An. gambiae* s.l. *cytochrome b* gene (*cyt b*) obtained from the full mitochondrial DNA (mtDNA) sequence of *An. gambiae* s.s. (VectorBase; L20934.1). The region 10413–11549 was screened against a nonredundant database using NCBIs Primer-BLAST tool (Ye *et al.*, 2012) restricting the product size for 70–120bp, to provide product sizes suitable for efficient qPCR amplification of degraded DNA (Thomsen & Willerslev, 2015). Two primer pairs; 1) Forward 5’ TCCTAGCTATACACTATGCCGC3’, Reverse 5’ ATTTGTCACGCTAACGGAGCT3’ and 2) Forward 5’AGCTATACACTATGCCGCAGAT3’, Reverse 5’AAGCTCCGTTAGCGTGACAAA3’ were validated by PCR with separate serial dilutions of 1:10 and 1:100 of *An. gambiae* s.s. control gDNA.

Each reaction consisted of 17.8 µL PCR water, 2.5 µL 10X Dream Taq Green Buffer, 0.5 µL of 10 mM dNTP mix, 0.2 µL Dream Taq DNA Polymerase, 0.5 µL of each primer pair and

3 µL of DNA template. The thermocycler conditions were 1 cycle 95°C for 5 minutes followed by 35 cycles of 95°C for 1 minute, 60°C for 1 minute, 72 °C for 1 min and a final extension at 72 °C for 5 minutes and held at 10°C. PCR products were cleaned using ExoSAP-IT® (Affymetrix, UK) and sequenced commercially (SourceBioscience, UK) to ensure that they only amplified *An. gambiae* s.l. *cyt b* gene. Sequences then aligned against reference *An. gambiae* mitochondrial *cyt b* sequence using CodonCode Aligner software (Version 4.2.7). Primer pair 1 showed consistent amplification at both dilutions and was chosen for further PCR analysis and probe design (see results section).

Larval water preparation

We used laboratory-reared second instar *An. gambiae* s.s. larvae (G3 strain). The larvae were reared in plastic trays (20 × 18 × 7 cm) under controlled insectary conditions of temperatures 26–28 °C, relative humidity 70–80 % and 12:12 hour light: dark cycle and fed once daily on finely-ground TetraMin® fish food.

In a first experiment, we tested 12 different conditions by adding 50 mL of distilled, autoclaved water to 12 sterile 50 mL falcon tubes. We performed three biological replicates with three different larvae densities: 1, 3 and 6 larvae. One tube from each of the three densities was then sampled simultaneously at intervals of 1, 6, and 24 hours. Three negative control experimental habitats with no larvae were run in parallel for each condition.

In the second experiment, we added 1 L of distilled, autoclaved water to five 1 L glass bottles. This was followed by adding 2, 5, 10 and 20 larvae into each bottle with no larvae in the control bottle and left them standing in the enclosed environment of the PCR workstation for 24 hours and sampled three biological replicates from each condition.

For each replicate, larvae were first rinsed with distilled water to reduce chances of any carry over of eDNA from rearing tray water into the experimental habitats. All replicates were set up in a PCR workstation in a room separate from the main molecular laboratory to avoid contamination from aerial mosquito DNA.

Extraction of eDNA from water samples

eDNA from the water samples was concentrated following a precipitation method (Ficetola *et al.*, 2008). We sampled 15 mL of water into a sterile 50 mL falcon tube and immediately added 1.5 mL of 3 M sodium acetate solution followed by 11 mL of absolute ethanol and stored overnight at -20 °C. Samples were then centrifuged at 5000 rpm at 6°C for 1 hour. The supernatant was discarded, and the pellet retained for eDNA extraction. eDNA was extracted using ChargeSwitch® Forensic DNA Purification Kit, Invitrogen which is a magnetic bead-based system that isolates DNA based on changes in pH of the surrounding extraction buffers. The eDNA extraction followed manufactures instructions with some modifications including an overnight incubation at 4°C with 1 mL of lysis buffer, and 10 µL of Proteinase K, a lysis step at 56°C for 90 min., and samples eluted with 60 µL of the proprietary elution buffer.

Quantitative PCR assays

In addition, novel *cyt b* TaqMan primer-probes that would distinguish the *An. gambiae* complex mosquitos from other species was designed and optimized for the primer pair 1 above. The primer probes had product length of 150 bp and on position 1114 at the 3' end of the gene; double dye FAM labelled probes 5'- CCCACCCTTTAATTAGAATCGCTAA-3' and 5'-CGGCATAGTGTATAGCTAGGAATAAT-3' (PrimerDesign, UK). The probes were blasted against all known sequences in the NCBI database to confirm their specificity to *An. gambiae* s.l. complex mosquito species.

To detect eDNA from *An. gambiae* s.s. and *An. arabiensis* we used an existing TaqMan quantitative PCR protocol with probes targeting species-specific polymorphisms in the ribosomal DNA from the 3' 28S to 5' intergenic spacer region (*IGS*) of the genome (Walker *et al.*, 2007); hereafter '*IGS* TaqMan'.

The qPCR standard curves for the two assays were performed by conducting a fivefold dilution series of genomic DNA from (a) *An. gambiae* s.s., (b) *An. arabiensis* and (c) a mixture from both species. Prior to this, Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher) was used to determine the DNA concentration of the starting template for each series. From these standard curves, we aimed to determine: 1) linearity (on a log scale) of the qPCR across DNA concentrations; 2) detection limits for the assay; 3) quantitation equations for DNA present in an unknown sample.

For the experimental aquatic habitats, due to limiting reagents and time, we only conducted qPCR analysis on eDNA samples from 2, 5, 10, and 20 larvae in 1 L habitats. To test the limit of detection of the two assays, we conducted a fivefold dilution (1/5, 1/25 and 1/125) and ran *IGS* and *cyt b* assays for each with four technical replicates for each condition. Samples used in determining the standard curves and two no-template controls were included in each qPCR plate for the 1L habitats eDNA samples. The unknown eDNA samples were scored as positive if their Ct value fell within same range as the standard curve samples.

The total qPCR reaction volume was 20 µL and consisted of 1 µL primer probe pair (PrimerDesign, UK), 10 µL TaqMan® Gene Expression Master Mix (Applied Biosystems, USA), 4.5 µL nuclease free water and 4.5 µL eDNA template. The samples were then run on an Agilent Mx3005P qPCR System using the thermal profile: 95 °C for 10 minutes followed by 50 cycles of 92 °C for 15 seconds and 57 °C for 60 seconds. In the *IGS* assay, fluorescence was recorded through the FAM and VIC channels, with the FAM dye indicating *An. Arabiensis* detection and VIC indicating *An. gambiae* s.s. detection. In the *cyt b* assay, fluorescence was recorded through the FAM channel, and indicated the presence of *An. gambiae* s.l.

Results

Initial testing and validation

The eDNA PCR primers were designed as an initial step to test the extraction method and to ensure that the biological negative controls gave negative results. Both eDNA primers showed 100% amplification on control gDNA from *An. gambiae* s.s. (N=4 samples) at 1:10 concentration. At 1:100 dilutions, primer pairs 1 and 2 showed 100% and 75% amplification respectively with zero amplification in the negative controls. Based on this clear difference in performance and time convenience, primer pair 1 was chosen for subsequent PCR analysis and qPCR probe design. Sequencing results from the PCR positive samples in the two dilutions revealed that the primers were only binding to *An. gambiae* s.s. *cyt b* gene. The ChargeSwitch-extracted DNA showed 100% (N=8) amplification with the An. *gambiae* s.l. primer pair 1 with all negative controls giving showing no band.

A total of 74% replicates in which larvae were maintained in 50 mL of water (0.02, 0.06, and 0.12 larvae/mL) produced PCR bands. 86% of negative results were in replicates with a single larva (0.02 larvae/mL) that required at least 6 hours for the first positive result (1/3 replicates) and presented inconsistent detection at 24 hours (2/3 replicates; Table 1). All 12 replicates where 1 L of water was used (0.002 to 0.02 larvae/mL) were positive with stronger PCR bands than replicates with the same larval density in 50 mL for 24 h (e.g. 20 larvae in 1L vs. 1 larva in 50 mL). Moreover, replicates of the lowest larvae density in 1 L (2 larvae, 0.002 larvae/mL) gave a more consistent positive eDNA signal for 24 h than replicates with higher density (×10) in 50 mL (0.02 larvae/mL). The comparison between replicates of 1 L *vs.* 50 mL indicates a potential stochastic effect for eDNA detection giving more importance to the number of larvae than density for eDNA detection in small portion of water. All the four biological negative controls (with zero larvae) for each test condition showed no bands in the PCR.

IGS and cyt b qPCR Standard curves

The *cyt b* standard curve exhibited linearity in regression down to 0.776 picograms (pg) followed by a visually outlying quantification at the lowest dilution, 0.156 pg, Ct 39.65 (Figure 1). The equation used for calculating concentration of downstream samples therefore excluded this lowest dilution (Figure 1, red equation). From the fitted curve, the lower limit of detection was therefore taken as 0.776 pg hence any quantification below this might be downward-biased.

The *IGS* TaqMan exhibited a linear association between Ct values and DNA concentration within the concentration range tested. Standard curves for the probe targeting each species (i.e. *An. gambiae* s.s. and *An. arabiensis*) showed close similarity, which allowed data to be combined in a single model to produce a predictive equation, with more data points, for DNA detection from subsequent samples (Figure 2). The *IGS* assay readily detected DNA at the lowest dilution of 0.156 pg and quantitation also appeared accurate throughout the range of dilutions.

Table 1. PCR detection of eDNA samples from containers with different volumes of water, numbers of larvae and occupation times.

No. of larvae	Vol. of water	Larvae density (larvae/mL)	Hours	PCR results	Band strength
1	50 mL	0.02	1	0/3	No band
3	50 mL	0.06	1	3/3	Weak
6	50 mL	0.12	1	3/3	Weak
0	50 mL	0	1	0/1	No band
1	50 mL	0.02	6	1/3	Weak
3	50 mL	0.06	6	3/3	Weak
6	50 mL	0.12	6	2/3	Weak
0	50 mL	0	6	0/1	No band
1	50 mL	0.02	24	2/3	Weak
3	50 mL	0.06	24	3/3	Weak
6	50 mL	0.12	24	3/3	Mid
0	50 mL	0	24	0/1	No band
2	1 L	0.002	24	3/3	Weak
5	1 L	0.005	24	3/3	Weak
10	1 L	0.01	24	3/3	Mid
20	1 L	0.02	24	3/3	Strong
0	1 L	0	24	0/1	No band

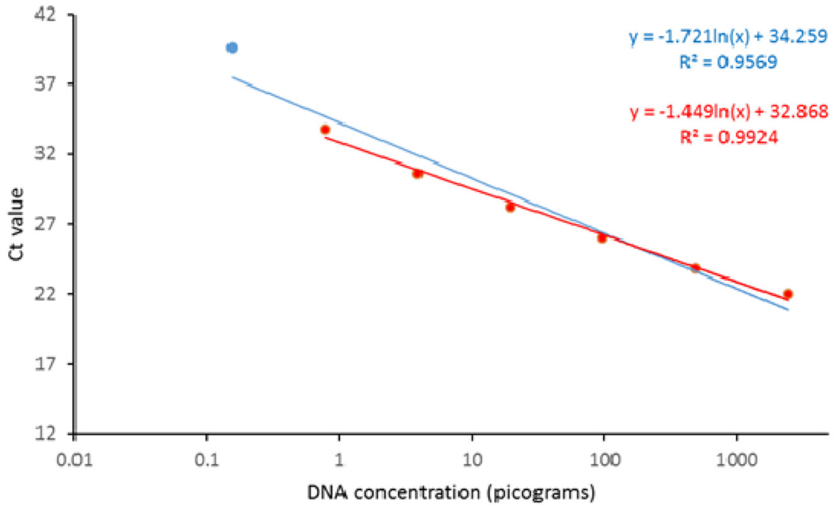

Figure 1. ***Cyt b*** **TaqMan standard curve for across a fivefold dilution series of gDNA from** ***An. gambiae*** **s.s. The blue line shows a regression line fitted through all points, with associated equation in blue font (upper equation).** The red line shows a line plotted through points excluding the lowest concentration sample, with associated equation in red font (lower equation).

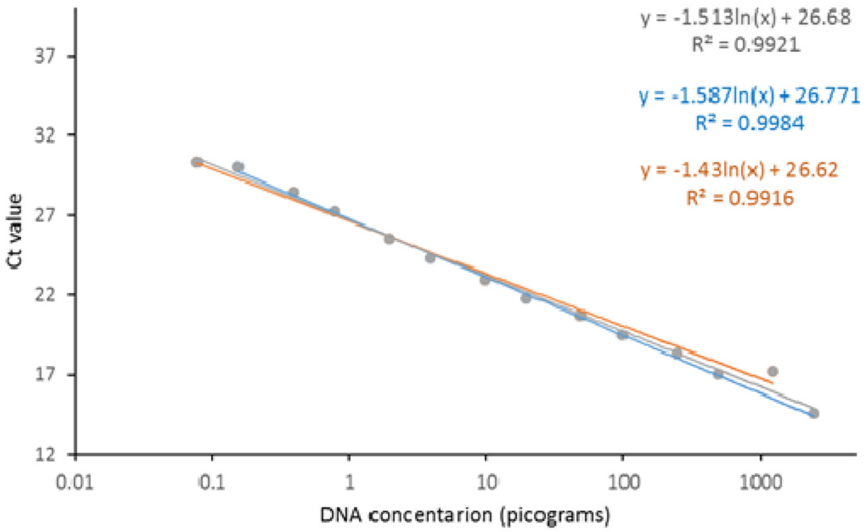

Figure 2. ***IGS*** **TaqMan standard curve for across a fivefold dilution series of gDNA from** ***An. gambiae*** **s.s (blue line and equation),** ***An. arabiensis*** **(orange line and equation) and both (grey line and equation).**

qPCR detection of larval presence from eDNA

Both the *IGS* and the *cyt b* TaqMan assays could detect *An. gambiae* s.s. eDNA in the undiluted samples of 2, 5, 10 and 20 larvae (Table 2). All dilutions with 2 larvae (0.4 - 0.016 larvae/L) were negative, while all dilutions with 20 larvae were positive (0.2 - 4 larvae/L). For 10 larvae, both assays identified the second and third dilutions (0.08 - 0.4 larvae/L) with low consistency (~1/4 positive). Moreover, *IGS* TaqMan presented a better performance with 5 larvae, with positive detection (2/4) in the third dilution (0.04 larvae/L), than *cyt b* assay (only detected d1, 1 larvae/L).

There was a correlation between larval densities and eDNA detection (38 (59%) positive samples for *IGS* and 32 (50%) for *cyt b* - Table 2) in both assays: *IGS*, $r = 0.503$, $p = 0.047$; and *cyt b*, $r = 0.558$, $p = 0.025$. However, there was also stochasticity in eDNA detection across the samples which reflected larval number in addition to larval concentration (Table 2). For example, 0.2/L was readily detected from an initial sample containing 20 larvae but not in one originating from 5 larvae. Similarly, 0.08/L was undetectable in water originating from a sample with 2 larvae but was detectable in majority of replicates from the 10 larvae samples. This experiment

Table 2. Comparison of results from *IGS* and *cyt b* TaqMan assays showing the number of positive samples in each of the four replicates and the average concentrations (pg) for each sample. The larval density was a fivefold dilution series (d1,2,3) for the initial number of larval in water sample, C. Dilutions whose eDNA concentration was not determined are noted as n.d. Minimum sample dilutions at which eDNA was detected are highlighted in bold.

2 larvae						**5 larvae**					
		Density	**Positives**	**Av Conc**	**Std Dev**			**Density**	**Positives**	**Av Conc**	**Std Dev**
IGS	C	**2**	**2/4**	0.0002	0.0002	**IGS**	C	5	4/4	0.006	0.002
	d1	0.4	0/4	n.d	n.d		d1	1	3/4	0.003	0.002
	d2	0.08	0/4	n.d	n.d		d2	0.2	0/4	n.d	n.d
	d3	0.016	0/4	n.d	n.d		d3	**0.04**	**2/4**	0.003	0.008
	NTC	0	0/2	n.d	n.d		NTC	0	0/2	n.d	n.d
cyt b	C	**2**	**2/4**	0.0306	0.0422	***cyt b***	C	5	4/4	0.0885	0.0244
	d1	0.4	0/4	n.d	n.d		d1	**1**	**1/4**	0.05101	0
	d2	0.08	0/4	n.d	n.d		d2	0.2	0/4	n.d	n.d
	d3	0.016	0/4	n.d	n.d		d3	0.04	0/4	n.d	n.d
	NTC	0	0/2	0	0		NTC	0	0/2	0	0
10 larvae						**20 larvae**					
		Density	**Positives**	**Av Conc**	**Std Dev**			**Density**	**Positives**	**Av Conc**	**Std Dev**
IGS	C	10	4/4	0.0078	0.0035	**IGS**	C	20	4/4	9.0527	1.0964
	d1	2	4/4	0.0017	0.0010		d1	4	4/4	1.7046	0.0821
	d2	0.4	2/4	0.0004	0.0002		d2	0.8	4/4	0.3758	0.0884
	d3	**0.08**	**1/4**	0.0002	0		d3	**0.16**	**4/4**	0.0818	0.0182
	NTC	0	0/2	0	0		NTC	0	0/2	0	0
cyt b	C	10	4/4	0.7848	0.4672	***cyt b***	C	20	4/4	27.2678	5.5316
	d1	2	3/4	0.2078	0.1813		d1	4	4/4	6.8140	1.8393
	d2	0.4	1/4	0.1270	0*		d2	0.8	4/4	1.7338	0.2231
	d3	**0.08**	**1/4**	0.0346	0		d3	**0.16**	**4/4**	0.3709	0.1233
	NTC	0	0/2	0	0		NTC	0	0/2	0	0

suggested that depending on starting larval numbers, density of up to 0.04 larvae/L could be detectable.

Discussion

Our study demonstrates that eDNA analysis using *IGS* and *cyt b* qPCR assays can be used to detect presence/absence and quantify *An. gambiae* s.l. mosquito larvae in a controlled laboratory experiment. The slightly better performance by the *IGS* assay suggests that the intergenic spacer region is a suitable target for eDNA primer/probe design. By virtue of the species specificity of polymorphisms in this genomic region, the *IGS* assay has the advantage to distinguish between *An. gambiae* s.s. and *An. arabiensis,* which may allow individually monitoring these primary malaria vectors by eDNA analysis. Assays targeting this region have the potential to distinguish among key species in other species complexes, which can be difficult using mtDNA.

We observed stochasticity in eDNA detection rates, using both PCR and qPCR, across the dilutions in the 50 mL and 1 L laboratory experimental habitats reflecting larval numbers in addition to density. For example, using PCR, replicates of the lowest larval density in 1 L (2 larvae, 0.002 larvae/mL) yielded a more consistent positive eDNA signal for 24 h than replicates with higher density (x10) in 50 mL (0.02 larvae/mL). Similarly, using qPCR, 0.4 larvae/L could be detected in the 10 larvae habitats but not in the 2 larvae habitats. Accurate detection and quantification in natural habitats may depend of a minimum number of larvae without a direct correlation with density. This could translate to organisms not being detected when they are present in small numbers (Ficetola *et al.*, 2008). Such stochasticity can be reduced through replication by either using multiple environmental samples collected at different points around the habitat for pooled DNA extraction or multiple amplification of the extracted DNA (Jerde *et al.*, 2011;

Thomsen & Willerslev, 2015) though this will increase costs and risk of false positives. Time of occupancy as well as biomass of the target species influences eDNA concentration and detection probability.

Based on this laboratory experiment, we extrapolate that mosquito surveillance by eDNA analysis would be better for detecting larvae in habitats with many larvae at low densities than in aquatic habitats with few larvae and higher densities. Since our quantifications were based on a dilution series from an initial larval density that could be higher than those found in natural mosquito habitats, it is not clear how these results can be extrapolated to quantify mosquito biomass in natural breeding habitats necessitating further evaluations on field applicability.

This study has some limitations. First with the dilution series used for our standard curves in which the lowest limit of eDNA detection was high making any quantification below this limit to be extrapolated from the standard curve equation which could potential introduce a bias. Secondly, we used *An. gambiae* s.s. and *An. arabiensis* gDNA for the standard curves instead of eDNA. Thirdly, the laboratory habitat experiments were only conducted using *An. gambiae* s.s. larvae. Since *An. gambiae* s.s. and *An. arabiensis* occur sympatrically in natural habitats (Chen *et al.*, 2008), further validations should include experiments where the two species are occupying the same habitat. However, this did not affect our overall conclusion as a qPCR result from DNA mixture of the two-species had a similar linear relationship as for each on its own.

Further studies need to be done to determine the applicability of eDNA analysis on detecting larvae belonging to the *An. gambiae* complex under field conditions. Evaluation of this tool in monitoring malaria vector species which are difficult to detect in breeding sites, such as *An. funestus* (Gimnig *et al.*, 2001), should also be carried out especially in habitats where traditional sampling methods are logistically difficult such as rice fields.

Conclusion

We have successfully detected and quantified *An. gambiae* s.s. mosquito larvae using eDNA from water samples. The stochasticity observed in eDNA detection suggest that mosquito surveillance by eDNA analysis would be better for detecting larvae in habitats with many larvae at low densities than in aquatic habitats with few larvae at higher densities. The *IGS* assay previously designed for identifying wild-type *An. gambiae* s.s. and *An. arabiensis* can distinguish aquatic stages of these primary malaria vectors using extracellular DNA extracted from water collected in the breeding habitats. This is important as eDNA detection to species level rather species complex level is achievable which could potentially save costs and time employed in monitoring these primary malaria vectors.

Data Availability

Dataset available from OSF: http://doi.org/10.17605/OSF.IO/FRH28 (Odero *et al.*, 2018)

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

Competing interests

No competing interests were disclosed

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Open Peer Review

Current Referee Status: ? ✓ ? ✓

Version 1

Referee Report 06 June 2018

doi:10.21956/wellcomeopenres.15439.r33150

✓ **Iliana Bista**
Wellcome Trust Sanger Institute, Hinxton, UK

Odero and co-authors use eDNA analysis to detect *Anopheles gambie* in laboratory environment, through the use of direct PCR and qPCR methodology. They target IGS and cyt b regions, at different timepoints and varying larvae densities. The work demonstrates positive detection of species in laboratory conditions. This is a good proof of concept study for PCR based detection of *A. gambiae*, with interesting possibilities for field application, since improving detection of *A. gambiae*, which is an important malaria vector, could be beneficial for mosquito management in afflicted areas.

The paper is generally well written, with good use of language and mostly clear descriptions of methodology and discussion. A small number of unclear points exist in methodology, as well as interpretation of results, and final conclusions. More detailed comments are provided below.

1. In Methods section “Larval water preparation”, please clarify the description of conditions and biological replicates. It is my understanding that the 50ml set-up was replicated 3 times, while the 1L bottle experiment was based on a single bottle per larva number (no biological replicates). Yet, at the end of 3rd paragraph it says “…sampled three biological replicates for each condition”. This doesn’t apply to the second experiment. Collection of multiple samples/PCRs per treatment constitutes technical, not biological replicates.

2. In methods section: “All replicates were set up in a PCR workstation in a room separate from the main molecular laboratory to avoid contamination from aerial mosquito DNA. “. eDNA detection is sensitive to contamination, especially through amplified DNA products. Was amplified mosquito DNA handled previously in that PCR workstation? Potential contamination from PCR products could occur by setting up the experiment in that space. The good thing is that the negative controls appear negative in all cases, but there was only one technical replicate for the negatives compared to 3 for other treatments. Please clarify why only one PCR was run for negative controls.

3. The authors refer to calculated larvae density throughout the text. Please explain how was the size of the larvae biomass calculated in order to estimate larval density. Were the larvae weighed or measured prior to being placed in the experimental set-up?

4. At the end of Introduction, beginning of Discussion, and in Conclusions, the authors claim to have established an assay “…capable of simultaneous differentiation of the two key East African malaria vectors *An. gambiae s.s*. and *An. arabiensis*.”. Nevertheless, no direct comparison of eDNA from the two species is performed in experiments. The capability of the IGS region to discriminate

between the two species, if already documented in the literature, could provide an advantage for use of this assay in the field. But the experiments conducted herein don't directly prove this claim. It would be more appropriate to provide this as a suggestion rather than a finding. Instead, the authors could augment the Discussion section by referencing and discussing related literature which supports the ability of the IGS region to make species specific detection.

5. As a general note, conducting such experiments in laboratory environment, using sterile water, is probably far from sampling a natural environment and hard to generalize. Suggestions for future work should include testing this assay in field conditions or even mesocosms. A number of environmental factors can influence detection, by affecting degradation rates, or interfering with PCR, which should be taken into consideration for field applications.

6. The ability of eDNA based methodologies to effectively reflect biomass/ abundance is still a controversial issue in the eDNA field, with the majority of the literature reporting inaccurate detection, mostly related to PCR bias. On the other hand, presence/absence detection is more accurate and reliable, especially when technical replicates are used. Consider rephrasing text in relation to accuracy of eDNA based quantification of species detections.

7. Consider enhancing the discussion section by incorporating related literature.

Minor points:

- Table 2 legend – "larval in water" change to "larvae in water".
- "For the experimental aquatic habitats, due to limiting reagents", change to limited.
- "Time of occupancy as well as biomass of the target species influences eDNA concentration and detection probability." Please provide reference.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Molecular Ecology, Biomonitoring, NGS sequencing

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 30 May 2018

doi:10.21956/wellcomeopenres.15439.r33151

Mathew Seymour
School of Biological Sciences, Molecular Ecology and Fisheries Genetics Laboratory (MEFGL), Bangor University, Bangor, UK

Odero *et al* presents a clearly written study using eDNA based methods to detect mosquito larvae, which as the authors indicate could be a highly relevant application. The study and experiment seem in order, but some clarity on the specificity of the primers and comparison with existing eDNA based biomonitoring programs would be advisable. Additionally, the conclusion are a bit confusing at present, particularly regarding the link between species detection and density derived from the experiment.

Introductions

1) Please provide an explanation for larval source management (LSM). The method is not common knowledge, at least for readers such as myself.

2) Quantifying numbers of individuals/biomass using eDNA is still being validated in the field. The authors cite Thomsen and Willerslev 2015[1], which is a review article. Studies that have assessed the relationship between eDNA and community numbers include Doi, H. *et al.* (2015)[1] and Iliana, B. *et al.* (2018)[3].

3) There are other key factors influencing eDNA degradation that could be mentioned. Environmental DNA degradation dynamics are also influenced by pH as shown in semi-natural and laboratory conditions Seymour, M. *et al.* (2018)[4] and Strickler, K. M *et al.* (2015)[5].

4) The effects of natural sunlight on eDNA degradation is less supported as there are also studies that suggest natural UV levels have no effect on eDNA degradation
Mächler, E et al. (2018)[6] and Andruszkiewicz, E. A et al. (2017)[7].

5) Please also consider that sedimentation will also influence detection of eDNA[8].

Methods

6) Please consider including all relevant MIQE information (could be an appendix), which will help with promoting the application of the proposed primers for eDNA based approaches as suggested in Goldberg et al. 2016[9].

Results

7) Sections of the results are interpretative and should be moved to the discussion or rewritten.
“The comparison between replicates of 1 L vs. 50 mL indicates a potential stochastic effect for eDNA detection giving more importance to the number of larvae than density for eDNA detection in small portion

of water. All the four biological negative controls (with zero larvae) for each test condition showed no bands in the PCR."
"However, there was also stochasticity in eDNA detection across the samples which reflected larval number in addition to larval concentration (Table 2). For example, 0.2/L was readily detected from an initial sample containing 20 larvae but not in one originating from 5 larvae. Similarly, 0.08/L was undetectable in water originating from a sample with 2 larvae but was detectable in majority of replicates from the 10 larvae samples. This experiment suggested that depending on starting larval numbers, density of up to 0.04 larvae/L could be detectable."
8) Table 2. Please consider rewording 'density' to 'dilution' as 'density' might imply number of larvae.
9) Consider rewriting the opening to the second paragraph of the discussion to clarify the main point. My understanding is that lower concentrations/densities increased stochasticity in detection rates, which is expected based on other studies that use similar methods for biomonitoring programs
Biggs, J. *et al.* (2015)[10] and Jerde, C. L. *et al.* (2013)[11].

10) Please consider comparing the findings with existing eDNA based biomonitoring programs with the findings presented here. It is a bit of an elephant in the room when reading the discussion.

11) Were in silico analyses performed to validate the specificity of the primers? Lab based specificity test would be the next step before field applications, but can anything be said about the initial primer selection procedures based on the blast assessment?

12) Could this sentence be rephrased please? In the context of the previous sentence, it is unclear how the similarity in the linear relationships helps validate the specificity of the primers.

13) "However, this did not affect our overall conclusion as a qPCRresult from DNA mixture of the two-species had a similar linear relationship as for each on its own."
The second sentence in the conclusion is confusing. Could the statement be rewritten to simply state that eDNA based detection is less stochastic when sampling from higher density populations?

14) Please specify what the 'this' in the last sentence is referring to.

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Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Referee Report 20 April 2018

doi:10.21956/wellcomeopenres.15439.r31723

✓

Hideyuki Doi
Graduate School of Simulation Studies, University of Hyogo, Kobe, Japan

I enjoyed reading the manuscript and found that the paper was technically sound. However, the qPCR methods was not according to MIQE checklist[1], e.g., R2 and PCR effciency of the standard curves, limited of detection, and others. Also, ethanol volume for precipitation method was quite small (11 ml), generally using 33 mL for 15 mL of sampled water.

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 06 April 2018

doi:10.21956/wellcomeopenres.15439.r31725

?

Stefano Mariani
School of Environment and Life Sciences, University of Salford, Salford, UK

This is a clearly written work reporting on a clearly designed experiment.
The authors provide novel molecular resources for the detection and quantification of trace DNA for two major malaria vectors.
In general, the work is sound, but it would benefit from a couple of modifications and some downscaling of

the conclusions.
My major comments are:
1) the authors extrapolate and conclude that the method devised will suit larger waterbodies with many larvae at low densities, rather than smaller environments with fewer larvae at possibly higher densities. I do not think the data support this conclusion. The data may perhaps suggest something along those lines, but mostly because the study focused on two different sets of artificial habitats, one smaller and one larger, but not fully comparable with one another (neither in terms of replication or stocking densities) and only loosely comparable with real habitats where Anopheles larvae can be found. I am not a mosquito expert, but I am familiar with the complexity of larger aquatic habitats, and stochasticity, patchiness, physico-chemical variability, microbiological activity, etc. will all introduce an array of complications, which may in fact yield a greater negative/misses in larger habitats than in smaller ones, if densities are low. I believe this is a major aspect that the authors need to mitigate and rearrange. The concluding statements should focus more on the efficiency of the molecular tools, their species-specific effectiveness, and their good quantitative performance.
2) The authors never mention explicitly why it is so important to distinguish between the two species of Anopheles. I work on biodiversity conservation and not on infectious diseases, but I presume that the need to identify different species will vary depending on the key management need. At least a paragraph on this should be added.
3) The authors explain quite clearly that targeting larval assemblages may be a more efficient way to control these vectors. However, they mention the "wider spectrum of pesticides" as an effective tool; but from an environmental conservation point of view, this sounds somewhat reckless. I appreciate that malaria is a huge problem in many parts of the world, but I would like to see some caveats about the indiscriminate use of pesticide in aquatic habitats, as their pernicious effects, on both ecosystems and human health, have been widely documented.

I also list below a few minor points to be amended:
- please remove the word "an" before "IGS" in the methods paragraph within the abstract.
- in the methods, it would be nice to explain more clearly that the larvae that were reared in the trays were then transferred to the falcon tubes, which then represented the actual "small experimental habitat".
- I note that autoclaved/distilled water is always used in the experiments. This provides a medium that will depart remarkably from natural conditions. When extrapolating, the authors should caution about this, for the benefit of anyone trying to used these tools in a natural setting, where humic substances, chemical inhibitors, microbiological activity, etc. will place these molecular probes in a very challenging, unchartered territory...
- In the results, it would be nice to reword the sentence mentioning the "86% of negative results..." changing it into "x out of y negative results..." because earlier there is another percentage and it may cause confusion.
- also in the results, it isn't very clear how eDNA "detection" can be correlated with "density". Detection is a binary entity (presence/absence)... do the authors mean "concentration"?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: molecular ecology & conservation genetics of aquatic systems

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.