



RESEARCH ARTICLE

Laboratory evaluation of molecular xenomonitoring using mosquito excreta/feces to amplify *Plasmodium*, *Brugia*, and *Trypanosoma* DNA [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

Background: Results from an increasing number of studies suggest that mosquito excreta/feces (E/F) testing has considerable potential to serve as a supplement for traditional molecular xenomonitoring techniques. However, as the catalogue of possible use-cases for this methodology expands, and the list of amenable pathogens grows, a number of fundamental methods-based questions remain. Answering these questions is critical to maximizing the utility of this approach and to facilitating its successful implementation as an effective tool for molecular xenomonitoring.

Methods: Utilizing E/F produced by mosquitoes or tsetse flies experimentally exposed to *Brugia malayi*, *Plasmodium falciparum*, or *Trypanosoma brucei brucei*, factors such as limits of detection, throughput of testing, adaptability to use with competent- and incompetent-vector species, and effects of additional blood feedings post parasite-exposure were evaluated. Two platforms for the detection of pathogen signal (quantitative real-time PCR and digital PCR [dPCR]) were also compared, with strengths and weaknesses examined for each.

Results: Experimental results indicated that high throughput testing is possible when evaluating mosquito E/F for the presence of either *B. malayi* or *P. falciparum* from both competent- and incompetent-vector mosquito species. Furthermore, following exposure to pathogen, providing mosquitoes with a second, uninfected bloodmeal did not expand the temporal window for E/F collection during which pathogen detection was possible. However, this collection window did appear longer in E/F collected from tsetse flies following exposure to *T. b. brucei*. Testing also suggested that dPCR may facilitate detection through its increased sensitivity. Unfortunately, logistical obstacles will likely make the

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large-scale use of dPCR impractical for this purpose.

Conclusions: By examining many E/F testing variables, expansion of this technology to a field-ready platform has become increasingly feasible.

However, translation of this methodology from the lab to the field will first require the completion of field-based pilot studies aimed at assessing the efficacy of E/F screening.

Keywords

molecular xenomonitoring, excreta/feces, lymphatic filariasis, malaria, human African trypanosomiasis, mosquito, surveillance

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Introduction

Due largely to renewed commitments and coordinated efforts between local leaders, government officials, non-government organizations, international donors, and pharmaceutical companies, tropical disease control, elimination, and eradication efforts are making unprecedented gains¹⁻⁵. Combined approaches, integrating chemotherapies, vector control strategies, education and outreach, and improvements to infrastructure are all contributing to significant programmatic successes. These successes are generating lofty goals for future interventions and expanding belief in the possibility of elimination of some tropical vector-borne diseases^{3,6-10}. However, as successes mount, new challenges arise, including an increasingly pressing need for capable surveillance tools. Following suspected transmission interruption, a failure of surveillance to identify and quickly react to possible incidences of disease recrudescence has significant potential to result in the forfeiture of hard-fought gains. For years, pharmaceutical partners and non-government organizations have supported programmatic efforts with substantial financial commitments, making such gains possible¹¹. However, insufficient oversight or inadequate follow-through may result in substantial disease rebound. Should such recrudescence occur in locations where transmission interruption or elimination efforts were previously believed to have succeeded, the remobilization of significant economic resources may not occur. Given these stakes, the need for low cost, non-invasive, high throughput surveillance methods is paramount to the realization of long-term programmatic goals.

Despite facing many challenges, the Global Programme for the Elimination of Lymphatic Filariasis (GPELF) continues to make remarkable progress in its efforts to meet its ambitious targets. Through the incorporation of novel strategies, such as triple drug (Ivermectin, Diethylcarbamazine, and Albendazole (IDA)) therapy¹²⁻¹⁷, global intervention efforts are beginning to realize more rapid successes. These accelerated accomplishments are allowing a growing number of countries to aspire towards World Health Organization (WHO)-sanctioned certification of lymphatic filariasis (LF) elimination. Currently, transmission assessment surveys (TAS) are used as the primary tool for measuring the successes of programmatic interventions^{18,19}. However, pilot studies are demonstrating that TAS surveys may not be well-suited to surveillance and monitoring in IDA settings, and the need to re-examine monitoring and evaluation strategies under triple-drug interventions has been recognized²⁰. This has prompted the organization of operational research (OR) efforts aimed at developing an appropriate monitoring and evaluation strategy for triple drug stopping decisions. (Please see www.ntdsupport.org/cor-ntd/ntd-connector/term/lymphatic-filariasis for examples.) Such efforts may benefit from novel and innovative diagnostic screening methods. Of further concern, recent modeling efforts of helminth infection suggest that even in conventional treatment settings, the potential for recrudescence of infection, particularly when systemic non-compliance with mass drug administration (MDA) is significant, is likely greater than previously believed²¹. Applying the findings of these predictive models to filarial infection, the threat of rebound likely extends for a period of many years past the WHO-suggested timeline for the completion of post-intervention TAS surveys.

Warning signs of infection rebound, resulting from pockets of sustained focal transmission, are also being identified with increased frequency as “successful” LF elimination programs become further removed from the cessation of MDA²²⁻²⁶. These discrepancies between defined programmatic timelines and the modeled potential/empirical evidence of recrudescence suggest there exists a post-TAS “black box” period, during which infection rebound is possible but appreciable monitoring efforts have ceased. Accordingly, integrated, non-invasive, low cost, high throughput approaches to surveillance, capable of providing a “first alert” warning during such periods are critically lacking^{27,28}.

Similar to the needs of the LF community, requirements for improved malarial surveillance are growing. Largely due to the expansion of coordinated interventions under the WHO’s Global Malaria Programme (GMP), examples of successful elimination are becoming more commonplace²⁹⁻³¹, and many additional elimination efforts have been established or revitalized³²⁻³⁴. While encouraging, such successes also breed new challenges and raise new concerns. Recognizing the dangers associated with bestowing a “malaria-free status” upon a population, the WHO has cautioned against reallocating surveillance funding following programmatic achievement, advising of the need to retain adequate surveillance systems to detect recrudescence and facilitate a rapid response in the event that such rebound occurs³⁵. These statements warn of the potential for complacency that naturally follows success, resulting in the prioritization of more immediate resource needs and potentially erasing years of progress due to insufficient post-interruption monitoring activities³⁵.

Insufficient surveillance also has the potential to threaten the developing momentum of human African trypanosomiasis (HAT) elimination efforts. With 2016, 2017, and 2018 each marking record lows in reported global cases of HAT³⁶⁻³⁸, belief in the elimination of this disease as a public health concern is increasing. While gains realized through intervention have been significant and encouraging, monitoring efforts have relied heavily upon human sampling, an approach that is commonly met with increased resistance as infection prevalence declines³⁹. Further complicating matters, the causative agents of HAT, *Trypanosoma brucei* spp., are vectored by the tsetse fly. These flies are notoriously difficult to trap, and vector control strategies continue to reduce their numbers⁴⁰⁻⁴². While interventions aimed at decreasing fly numbers are an increasingly important component of transmission reduction efforts^{41,43}, declining vector populations make supplemental xenosurveillance strategies increasingly impractical. Accordingly, as aspirations for elimination grow, the importance of alternative approaches to surveillance will continue to increase.

The molecular testing of mosquito excreta/feces (E/F) for the presence of pathogens provides one approach that is a potential solution to the growing surveillance challenges plaguing GPELF, as well as global malaria and HAT elimination efforts. Previously, we described the capacity for mosquito E/F testing to vastly improve the throughput of surveillance for filarial parasites⁴⁴. Similarly, we demonstrated the capacity of this

novel molecular xenomonitoring (MX) approach to facilitate the detection of the human malaria-causing parasites *Plasmodium vivax* and *Plasmodium falciparum*^{44,45}, and demonstrated proof-of-concept for the “cross-vector” detection of *Trypanosoma brucei brucei* in non-vector mosquitoes⁴⁵. However, the expanded utility of this method will require the fine tuning of sampling strategies, centering upon the identification of appropriate target mosquito populations. We have therefore performed a series of proof-of-concept experiments aimed at further evaluating the practicality of E/F testing in preparation for field trials. Exposing laboratory-reared mosquitoes and tsetse flies to various pathogens, we have endeavored to more fully understand the variables impacting parasite signal detection within E/F collected following parasite exposure.

Methods

Insect rearing and blood feeding

Mosquitoes. Both *Anopheles gambiae* and *Aedes aegypti* mosquitoes were internally-sourced from laboratory colonies maintained at the Liverpool School of Tropical Medicine. Mosquitoes were reared from eggs to adults and housed in BugDorm-1 insect rearing cages (Megaview Science, Taiwan; Catalogue #DP1000) at 26–27 °C with 70–80% relative humidity. Experimental exposures were performed as previously described⁴⁵. Briefly, adult female mosquitoes, aged 3–7 days, were sugar-starved for 18 hours prior to blood exposure in order to facilitate blood feeding. For experiments involving exposures to *Brugia malayi* or *P. falciparum*, mosquitoes were provided with either a standard human bloodmeal (obtained from the local blood bank), or a human bloodmeal spiked with a known concentration of parasites. Exposures to *B. malayi* were conducted using a Hemotek feeding system (Hemotek Ltd, Blackburn, UK; Catalogue #SP6W1-3), while *P. falciparum* exposures were performed using a glass feeder (Chemglass Life Sciences, Vineland, NJ; Catalogue #CG-1836). For experiments involving mosquito exposures to *T. b. brucei*, mosquitoes were provided with a Hemotek feeding system-supplied bloodmeal of defibrinated horse blood (TCS Biosciences, Buckingham, UK; Catalogue #HB030), with or without parasites.

Tsetse flies. Flies were reared from larvae and housed in internally-made cages, constructed of lengths of plastic piping covered at each end with netting, at 27 °C ± 2 °C with a relative humidity of 65–75%. Adult flies were fed on defibrinated horse blood, with or without parasites. Feedings occurred by placing blood on an aluminum tray heated to 37 °C. Fly cages were then placed on a silicon membrane positioned directly above the blood, allowing flies to feed through the membrane.

Parasites

B. malayi. Microfilaria (mf) were generously provided by the anit-*Wolbachia* Consortium, generated as part of their maintenance of the *B. malayi* lifecycle⁴⁶. Harvested parasites were added to human blood at the appropriate concentrations to generate experimentally desired parasite densities as described below for individual applications.

P. falciparum. Red blood cells containing trophozoites (3D7 strain) were combined with uninfected human serum to produce

experimentally desired parasite concentrations as described below for individual applications.

T. b. brucei. The bloodstream form of *T. b. brucei*, strain AnTat 1.1 90:13⁴⁷, was used for all experimental feedings. Parasites were cultured in HMI-11 medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO₂. Parasite densities were determined microscopically using a hemocytometer.

Collection of excreta/feces

***B. malayi* experiments.** All experiments involving *B. malayi* were performed in accordance with the previously described superhydrophobic cone collection method⁴⁵. Briefly, sheets of A4 printer paper were used to create cone-shaped funnels, which were coated in NeverWet (Rust-Oleum, Durham, UK). Cones were placed inside of mesh-covered un-waxed paper beverage cups, with mosquitoes housed above the cones, allowing E/F produced by the mosquitoes to travel down the walls of the cones and pool at the base of each funnel. For these collections, GenSaver DNA Cards (GenTegra, Pleasanton, CA; Catalogue #GSD4-100) were used in place of the 1.7 mL microcentrifuge tubes that were employed when this method was previously described⁴⁵.

***P. falciparum* experiments.** When performing experiments involving *P. falciparum*, E/F was again collected in accordance with the previously described superhydrophobic cone collection method⁴⁵ briefly described above. For all experiments involving *P. falciparum*, E/F samples were collected into 1.7 mL microcentrifuge tubes as previously described⁴⁵.

***T. b. brucei* experiments.** For all experiments involving *T. b. brucei*, flies/mosquitoes were housed in 50 mL conical tubes allowing for direct deposition of E/F onto the walls of the holding vessel. During the experimental housing of vectors, tubes were covered with mesh netting, and flies/mosquitoes were transferred to new vessels at experimentally specified time intervals. While in tubes, tsetse flies were removed from tubes for feeding on uninfected defibrinated horse blood every second day as described above.

Extraction of DNA from excreta/feces

Following superhydrophobic cone collections onto GenSaver DNA Cards. All samples were excised from GenSaver DNA Cards using a standard paper punch (0.64 cm round). For each sample, three punches were placed into a 2.0 mL microcentrifuge tube and the sample was recovered using the GenSolve DNA Recovery Kit (GenTegra; Catalogue #GVR-113) in accordance with the manufacturer’s suggested protocol. Following recovery, each sample was added to a MinElute column (Qiagen, Germantown, MD) for sample binding. Sample washes and DNA recovery procedures occurred utilizing the manufacturer’s recommendations. After recovering the eluate, the total volume of eluate was re-loaded onto the column a second time and again spun through the matrix to maximize sample recovery.

Following superhydrophobic cone collections into microcentrifuge tubes. DNA was extracted from all samples utilizing the

QIAamp DNA Micro Kit (Qiagen; Catalogue #56304) following a modified version of the manufacturer's suggested protocol. Briefly, 180 μ L of Buffer AL was added to each E/F sample and tubes were vortexed on a shaking platform for 1 hr. 20 μ L of Proteinase K was then added, and samples were incubated at 56 °C for 1 hr with shaking at 1,400 RPM. Following incubation, 200 μ L of Buffer AL (containing 5mM carrier RNA) was added to each sample, and samples were incubated at 70 °C for 10 min. Column binding and washing steps were then performed in accordance with the manufacturer's specifications. Following washes, elution of DNA occurred in 50 μ L of Buffer AE. As described above, following the elution of DNA in 50 μ L of Buffer AE, eluate was re-loaded onto the column to maximize recovery.

Following collection into 50 mL conical tubes. E/F was eluted from tubes through the direct addition of 7.5 mL of nuclease free water. Following the addition of water, samples underwent agitation on a vortexing platform for 30 min at 56 °C to facilitate the complete resuspension of material. Tubes were then spun at 5,000 RPM for 5 min, and the supernatant was removed from each sample. Pelleted material was resuspended in the residual volume of liquid. Following recovery, each sample underwent DNA isolation in the same manner as described above for superhydrophobic cone-based collections into microcentrifuge tubes.

Isolation of tsetse fly midguts and preparation for DNA extraction

Tsetse fly midguts were prepared for DNA extraction following the protocol previously described by Cunningham, *et al.*⁴⁸. Briefly, following dissection, midguts were placed in 60 μ L of 100% ethanol. 70 μ L of nuclease free water was then added to each sample and samples were centrifuged at 13,000 RPM for 15 sec. Following centrifugation, 100 μ L of supernatant was aspirated from each sample, and samples underwent three sequential washes with 100 μ L of nuclease free water to remove residual ethanol.

Extraction of DNA from mosquitoes and tsetse flies

In preparation for DNA isolation, 20 μ L of Proteinase K, 180 μ L of Buffer ATL and a 4.5 mm ball bearing were added to all carcass and midgut samples. Samples were then mechanically homogenized at a setting of 30.0 1/S for 5 min using a TissueLyser II (Qiagen). All DNA extractions were then performed using the DNeasy Blood and Tissue Kit (Qiagen; Catalogue #69581) following the extraction plate procedure. All extractions were conducted in accordance with the manufacturer's suggested protocol.

Real-time PCR

Quantitative real-time PCR (qPCR) testing for the presence/absence of *B. malayi* occurred using the StepOnePlus Real-Time PCR System (ThermoFisher Scientific, Waltham, MA) and was performed using primers and probe previously described for use with the Bm HhaI real-time PCR assay⁴⁹. Cycling conditions consisted of an initial hold at 50 °C for 2 min, followed by a 95 °C incubation for 10 min. These incubations were followed by 45 cycles of sequential denaturation and annealing/extension steps at 95 °C for 15 sec, and 60 °C for 1 min respectively.

Testing for the presence of *P. falciparum* also occurred using the StepOnePlus Real-Time PCR System and employed the recently described *Pf* TR1 assay in accordance with suggested reagent concentrations and cycling conditions⁵⁰. Cycling conditions for *P. falciparum* detection were identical to those described above for *B. malayi* detection. All reactions for *B. malayi* and *P. falciparum* detection were performed in 25 μ L total volumes with 5 μ L of template. Each reaction was conducted using 12.5 μ L of TaqPath ProAmp Master Mix (ThermoFisher Scientific; Catalogue #A30867) and reactions were allowed to proceed for 45 cycles. Depending upon the experiment, samples were tested in duplicate or triplicate reactions and mean Cq values were reported as was the number of positive replicates.

Testing for the presence/absence of *T. b. brucei* was performed using the Rotor-Gene Q Instrument (Qiagen) and made use of the previously described Tb117 assay primers at concentrations of 400 nM⁴⁸. All reactions for the detection of *T. b. brucei* were performed in 10 μ L volumes, using 5 μ L of Type-it HRM PCR Master Mix (Qiagen; Catalogue #206542) and 4 μ L of DNA template. Cycling conditions consisted of an initial hold at 96 °C for 5 min, followed by 35 cycles of 95 °C for 15 sec, 60 °C for 30 sec, and 72 °C for 10 sec. As this assay makes use of a saturating fluorescent dye (similar to SYBR Green assay chemistry) a dissociation step was then performed utilizing a temperature gradient gradually increasing from 55 °C to 95 °C. All *T. b. brucei* testing occurred in duplicate and both mean Cq values and the number of positive replicates were reported.

Digital PCR

All digital PCR (dPCR) reactions were performed on the QuantStudio 3D Digital PCR instrument using V2 chips (ThermoFisher Scientific; Catalogue #A26359). Reactions were conducted using the same *P. falciparum* primer-probe pairings selected for qPCR with identical working concentrations. All reactions were prepared in 15 μ L volumes, with 14.5 μ L of this prepared reaction mix loaded onto each chip for analysis. Individual reaction mixes contained 7.5 μ L of QuantStudio 3D Digital PCR Master Mix v2 (ThermoFisher Scientific; Catalogue #A26316), the appropriate concentrations of primers and probe, and 5 μ L of template. Cycling conditions consisted of two initial holds at 96 °C for 10 min and 50 °C for 30 sec. These holds were followed by 39 cycles of 60 °C for 2 min, 98 °C for 30 sec, and 60 °C for 2 min. Two replicate chips were analyzed when testing each sample. For each iteration of samples tested, two no template control (NTC) chips containing nuclease-free water in place of template were analyzed alongside experimental samples. For a given iteration, NTC results were used to determine positivity by setting the fluorescence threshold for the entire sample set at 125% of the fluorescence reading generated by the NTC well producing the greatest level of background. When visualizing QuantStudio 3D output graphically, signal-producing wells containing true positives should be located in positions along the x-axis directly above the population of wells that failed to amplify. For this reason, as well as for consistency, and for the maintenance of a conservative approach to positivity determination, only wells with a fluorescence unit values of -240 to 240 along the x-axis were analyzed.

Limits of detection for parasite signal from pooled E/F

B. malayi. Utilizing previously published temporal collection windows⁴⁵, infected blood exposures were conducted in order to evaluate the capacity to detect *B. malayi* signal in the E/F of both competent (*A. aegypti*) and incompetent (*A. gambiae*) vectors. To evaluate limits of detection, mosquitoes were exposed to either 2,000 *B. malayi* mf/mL, or 5,000 *B. malayi* mf/mL. For each species of mosquito, either 10 or 11 replicate exposures were performed and the accumulated E/F was collected at the 48- and 72-hour time points post-exposure. An additional 5 mosquitoes were provided with naïve bloodmeals to serve as uninfected controls, and collections from naïve mosquitoes occurred at the same post-exposure time points. All collections were performed using superhydrophobic cones and E/F was collected onto GenSaver DNA Cards. Following collection, DNA was isolated from all E/F samples and the resulting extracts were analyzed using qPCR.

P. falciparum. As was done to evaluate limits of detection for *B. malayi*, the capacity to detect *P. falciparum* signal in the E/F of mosquitoes exposed to varying blood concentrations of parasite was examined. Exposures of individually housed *A. gambiae* mosquitoes occurred at 5,000 trophozoites/ μ L (0.1% parasitemia), 500 trophozoites/ μ L (0.01% parasitemia), and 50 trophozoites/ μ L (0.001% parasitemia), with between nine and 14 mosquitoes successfully undergoing exposure at each experimental concentration. An additional five mosquitoes were provided with a parasite-naïve bloodmeal for control purposes. Following exposure, all mosquitoes were individually housed in paper cups facilitating superhydrophobic cone-based collections of E/F into 1.7 mL microcentrifuge tubes. At the 48-hour time point, and again at 72 hours post-exposure, mosquitoes were transferred to new cups and all deposited E/F was prepared for qPCR analysis. In order to investigate whether dPCR could be used as a means of extending detection windows, E/F samples also underwent analysis by dPCR.

T. b. brucei. Previous work has demonstrated the successful detection of *T. b. brucei* from the E/F produced by pools of 10 mosquitoes following exposure to parasites⁴⁵. However, the capacity for detection of *T. b. brucei* signal from the E/F of individual mosquitoes has not yet been evaluated. The capacity for tsetse fly E/F to similarly allow for *T. b. brucei* signal detection has also yet to be appraised. To investigate these possibilities, *A. gambiae* and *Glossina morsitans* were exposed to defibrinated horse blood containing either “high dose” (10^5 trypanosomes/mL) or “low dose” (10^3 trypanosomes/mL) concentrations of parasites. Following exposure for 24 hours, individual flies and mosquitoes were transferred into 50 mL conical tubes for the collection of E/F. In total, E/F samples from 25 flies and 25 mosquitoes exposed to each dose of parasite were evaluated. An additional five flies and five mosquitoes provided with a bloodmeal that was naïve for parasite were included for control purposes. Following an initial 48-hour housing, flies/mosquitoes were transferred to new tubes and soiled tubes were collected for molecular analysis. This collection process was repeated at 96 hours post-exposure,

again at 144 hours post-exposure, and finally at 192 hours post-exposure. DNA was then extracted from all collected samples and real-time PCR analysis was performed. Following the 192-hour time point, flies and mosquitoes were sacrificed, and both fly midguts and mosquito carcasses underwent DNA extraction and qPCR analysis.

Demonstration of high throughput detection of *P. falciparum* signal from Pooled E/F

Prior experimentation has revealed the improved throughput of detection for *B. malayi* using E/F⁴⁴. To investigate if throughput would also improve when detecting *P. falciparum*, pools of 49 *A. gambiae* mosquitoes were provided with a parasite-naïve bloodmeal and E/F from each pool was allowed to collect into a single microcentrifuge tube for 72 hours using a hydrophobic cone. Following 72 hours, this tube was transferred to the collecting position beneath a new cone, allowing for the collection of E/F from a single mosquito exposed to *P. falciparum* at a parasitemia of 0.1%. Accumulation of E/F from this single exposed mosquito continued until the 72-hour post-exposure time point, after which the tube was removed for downstream DNA extraction and qPCR analysis. All samples were tested in triplicate, and positivity was defined as the occurrence of a positive result in two or more reactions with a Cq value ≤ 40 . Ten replicate pools were prepared. Additionally, E/F from 10 individual mosquitoes, also exposed to *P. falciparum* at the same 0.1% parasitemia, were collected for comparative purposes.

Effects on parasite detection of a second blood feeding with pathogen-naïve blood

To evaluate whether the provision of a second bloodmeal following an initial infected blood exposure would facilitate an extended window of parasite detection, three pools of 10 *A. gambiae* mosquitoes were exposed to *P. falciparum*-containing blood at a parasitemia of 0.01%, and an additional control pool, also containing 10 *A. gambiae* mosquitoes, was provided with a parasite-naïve bloodmeal. Using a superhydrophobic cone, E/F from each pool of mosquitoes was collected into a microcentrifuge tube for a 72-hour period following exposure. Mosquito pools were then transferred to new cones, and E/F was allowed to accumulate for an additional 72 hours into a new microcentrifuge tube. At six days post-feeding, mosquitoes were again transferred to new cones/tubes and a naïve bloodmeal was provided. Following this second blood exposure, an additional 72-hour collection was performed. All collected samples then underwent DNA extraction and triplicate testing by qPCR (Figure 1).

Statistical analysis

Statistical differences between groups were determined by means of a Student's two-tailed *t*-test performed using GraphPad's “*t* test calculator” freely available from graphpad.com. A *p* value < 0.05 was considered to be statistically significant ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Where appropriate, confidence intervals were calculated using the previously described E. B. Wilson method^{51,52} utilizing software freely available at <http://vassarstats.net/propl.html>.

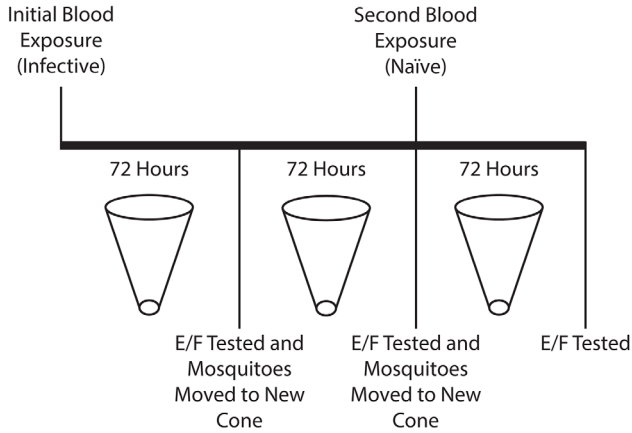


Figure 1. Schematic of the re-exposure experiment. Following an initial exposure to *Plasmodium falciparum*-positive blood, excreta/feces (E/F) was collected from mosquito pools for 72 hours. Following this time period, E/F samples were collected for qPCR analysis and mosquitoes were transferred to new superhydrophobic cones, where they were held for an additional 72 hours. At the conclusion of this period, E/F samples were again collected for qPCR analysis and mosquitoes were provided with a second bloodmeal, this time naïve to parasite. Mosquitoes were then transferred to a third superhydrophobic cone, where excretion continued for an additional 72 hours. Following this final incubation period, E/F was again collected for qPCR analysis.

Results

Raw qPCR and dPCR data underlying the below results are available as underlying data⁵³.

Limits of detection for parasite signal from pooled E/F

B. malayi. Individual competent vector (*A. aegypti*) and incompetent vector (*A. gambiae*) mosquitoes were exposed to *B. malayi* at blood concentrations of 2,000 mf/mL or 5,000 mf/mL. E/F collection occurred at the 48- and 72-hour post-exposure time points. Irrespective of time point, qPCR analysis resulted in the detection of parasite signal from the E/F of 10 of 11 *A. aegypti* mosquitoes exposed at a parasitemia of 5,000 mf/mL, and from 9 of 11 *A. aegypti* mosquitoes exposed at a parasitemia of 2,000 mf/mL (Figure 2A). Results for *A. gambiae* exposures were similar, with positive detection occurring in 8 of 10 pools produced from mosquitoes exposed at a parasitemia of 5,000 mf/mL and in 10 of 11 pools produced following exposure at a parasite density of 2,000 mf/mL (Figure 2B). Consistency of detection across time points was greater when testing E/F produced by *A. aegypti*, occurring for six mosquitoes following exposure at 5,000 mf/mL, and five mosquitoes following exposure at 2,000 mf/mL. In E/F produced by *A. gambiae*, detection across multiple time points occurred from only two mosquitoes and one mosquito following exposures to *B. malayi* at 5,000 mf/mL and 2,000 mf/mL respectively. Unsurprisingly, mean Cq values were lower, suggesting greater concentrations

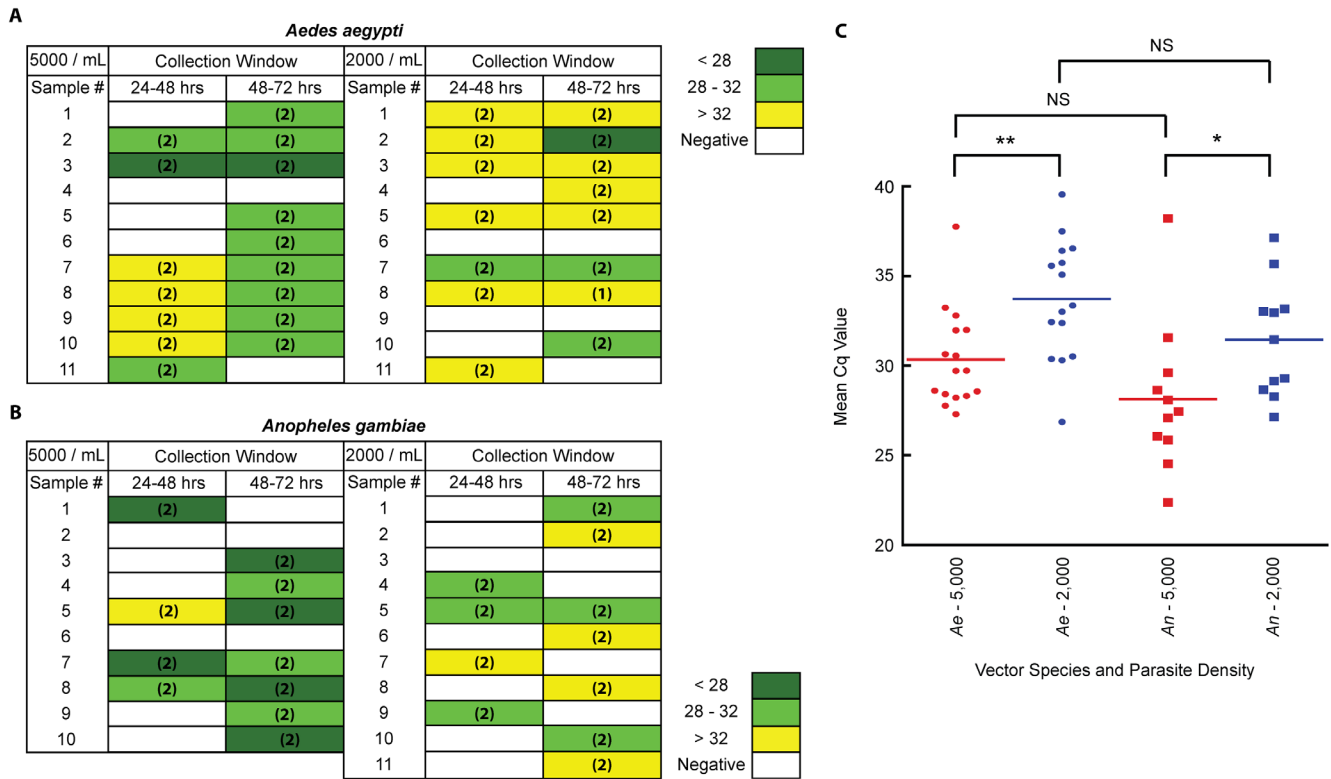


Figure 2. Detection windows for parasites in the excreta/feces (E/F) of mosquitoes following a *B. malayi*-containing bloodmeal. Individual (A) *Aedes aegypti* (competent vector) mosquitoes and (B) *Anopheles gambiae* (incompetent vector) mosquitoes were exposed to a *Brugia malayi*-containing bloodmeal at a parasitemia of either 5000 mf/mL or 2000 mf/mL. E/F was then collected from each mosquito, individually, in 24-hour time blocks. Collected E/F samples were then tested in duplicate qPCR reactions. Colors represent Cq values, and numbers in parenthesis indicate the number of positive qPCR replicates. (C) A comparison of the mean Cq values from all positive E/F samples produced by both mosquito species at both parasitemias. Significance, as determined by the results of unpaired *t* tests, is provided. **p* < 0.05, ***p* < 0.01, NS = not significant.

of target DNA, in the E/F produced by mosquitoes exposed to higher blood concentrations of *B. malayi* (Figure 2C). Of note, a single negative control mosquito, not exposed to *B. malayi*, did give a positive signal. Contamination, resulting in amplification, likely occurred either during mosquito rearing or during DNA extraction. The use of no-template negative controls during PCR suggests that the contamination was unlikely to have occurred during the PCR.

***P. falciparum*.** Following exposure of individual *A. gambiae* mosquitoes to *P. falciparum* at parasitemias of 0.1% (5000/μL), 0.01% (500/μL), and 0.001% (50/μL), E/F was collected at the 48-hour and 72-hour time points. As measured by qPCR, all nine mosquitoes exposed at 0.1% produced E/F that gave positive results: four samples were positive at the 48-hour time point, while six were positive at the 72-hour time point. Following exposure at 0.01% parasitemia, 13 of 14 mosquitoes produced

E/F that gave positive qPCR results: 12 were positive at the 48-hour time point, while only one sample was positive at the 72-hour time point. Exposures at 0.001% parasitemia resulted in positive detection from the E/F produced by seven of 11 mosquitoes: four were positive at the 48-hour point, while three were positive at the 72-hour point (Figure 3A). Interestingly, only one mosquito produced sample that was detectable at both collection time points (0.1%, sample 2). This result was in sharp contrast with findings for *B. malayi* (Figure 2A, B). Taken together, these results may mean that deposition of parasite material occurs largely as the result of a solitary excretion event. When signal detection occurs across time points, it may be that this event spans collection intervals, resulting in multiple positive time points from an isolated excretion occurrence. Whether the duration of this excretion event is longer following a *B. malayi* exposure, or these findings are chance results, remains an open question.

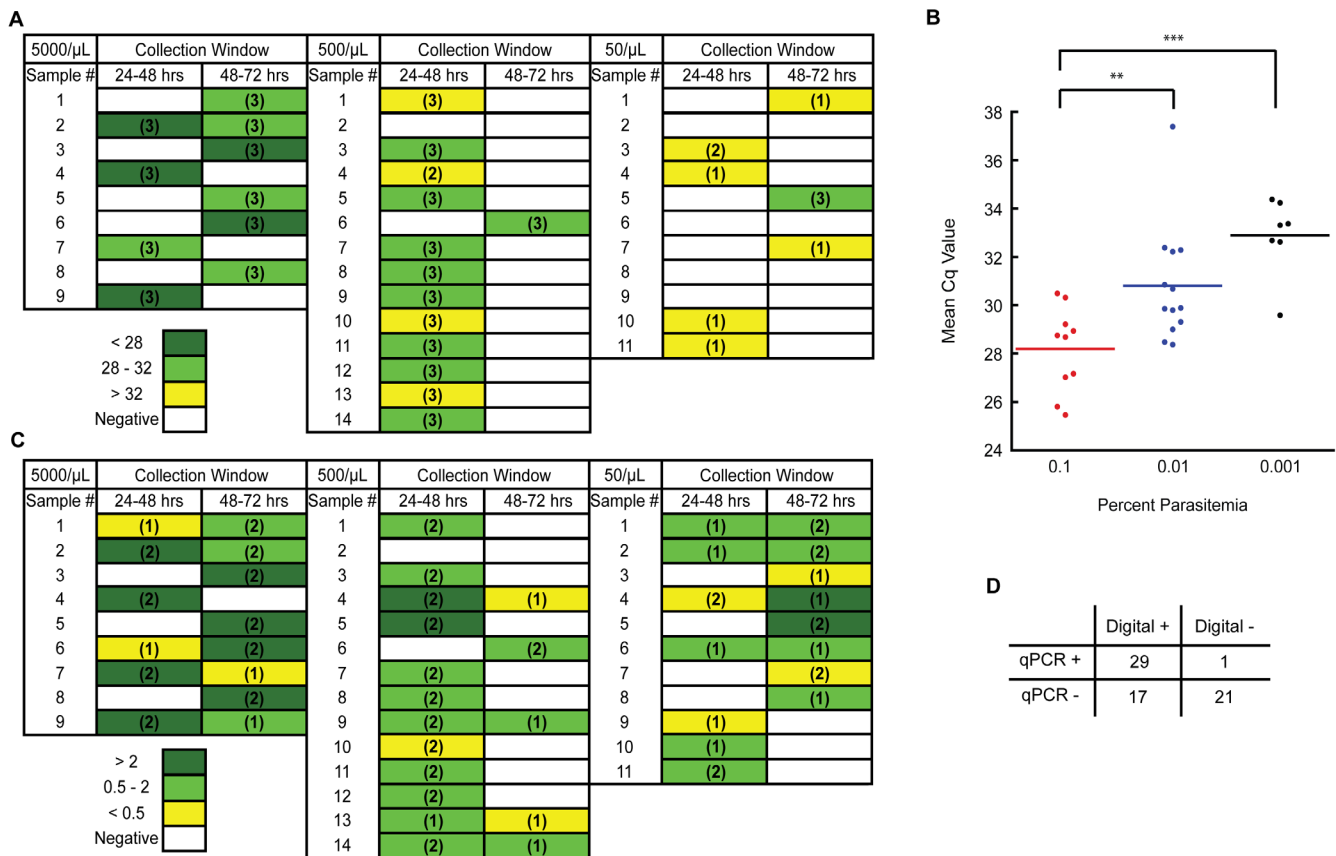


Figure 3. Detection windows for parasites in the excreta/feces (E/F) of mosquitoes following a *Plasmodium falciparum*-containing bloodmeal. (A) Individual *Anopheles gambiae* mosquitoes were exposed to a *P. falciparum*-containing bloodmeal at a parasitemia of 5000 trophozoites/mL (0.1% parasitemia), 500 trophozoites/mL (0.01% parasitemia) or 50 trophozoites/mL (0.001% parasitemia). E/F was then collected from each mosquito, individually, in 24 hour time blocks. Collected E/F samples were then tested in triplicate qPCR reactions. Colors represent Cq values, and numbers in parenthesis indicate the number of positive qPCR replicates. (B) A comparison of the mean Cq values from all qPCR-positive E/F samples (irrespective of time point) produced by mosquitoes at each tested parasitemia. Significance, as determined by the results of unpaired *t* tests, is provided. ***p* < 0.01, ****p* < 0.001. (C) Testing, in duplicate dPCR reactions, of the same samples collected in panel A. Colors represent the number of positive sample wells per reaction, and numbers in parenthesis indicate the number of positive dPCR replicates. (D) A comparison of E/F sample positivity as determined by qPCR and dPCR results from panels A and C.

As expected, and as seen following *B. malayi* exposures, Cq values increased with declining numbers of parasites, suggesting greater amounts of template in the E/F produced by mosquitoes exposed to higher concentrations of pathogen (Figure 3B). Digital PCR analysis of samples resulted in the improved overall sensitivity of detection, as more positive results were seen at the 48–72-hours time point compared to the qPCR results (Figure 3A, C). In total, across all parasite concentrations, 17 qPCR negative samples demonstrated positivity when tested by dPCR, while only 1 sample which was qPCR positive produced a negative result by dPCR (Figure 3D).

***T. b. brucei*.** Following exposure to *T. b. brucei*, E/F was collected from individually housed *G. morsitans* and *A. gambiae* at 48 hour intervals. Within 96 hours of exposure to “high dose” *T. b. brucei* (10^5 trypanosomes/mL), 23 of 25 individually housed *G. morsitans* had produced at least one E/F sample that was qPCR positive for parasite (96%). In contrast, only six of the 25 individual *A. gambiae* mosquitoes exposed to the “high dose” produced E/F which was qPCR positive for *T. b. brucei*. Following “low dose” exposures (10^3 trypanosomes/mL), six out of 25 tsetse flies produced parasite-positive E/F (24%) within 96 hours of exposure, while a single mosquito out of the 25

exposed was *T. b. brucei* positive by qPCR (4.0%) (Figure 4A). Interestingly, E/F-based *T. b. brucei* detection from E/F produced by *G. morsitans* readily occurred at the 192-hour time point following both “high dose” (70.8%) and “low dose” (20.8%) exposures to parasite (Table 1). In contrast, only the E/F produced by a single mosquito gave positive *T. b. brucei* qPCR detection at a time point later than 96 hours post-infection (4.2%), and this sample was derived from an individual of “low dose” exposure (Table 1).

Following sacrifice at the 196-hour time point, qPCR analysis of DNA extracted from *G. morsitans* midguts and *A. gambiae* carcasses was performed. Testing revealed *T. b. brucei* positivity in 15 of 20 midgut-derived samples from *G. morsitans* subjected to “high dose” exposures, and in 3 of 14 samples collected from “low dose” individuals. Neither “high” nor “low dose” mosquitoes produced a single *T. b. brucei*-positive carcass (Figure 4B).

Demonstration of high throughput detection of *P. falciparum* signal from pooled E/F

To investigate the capacity for high throughput sampling when testing mosquito E/F for the presence of *P. falciparum* by qPCR,

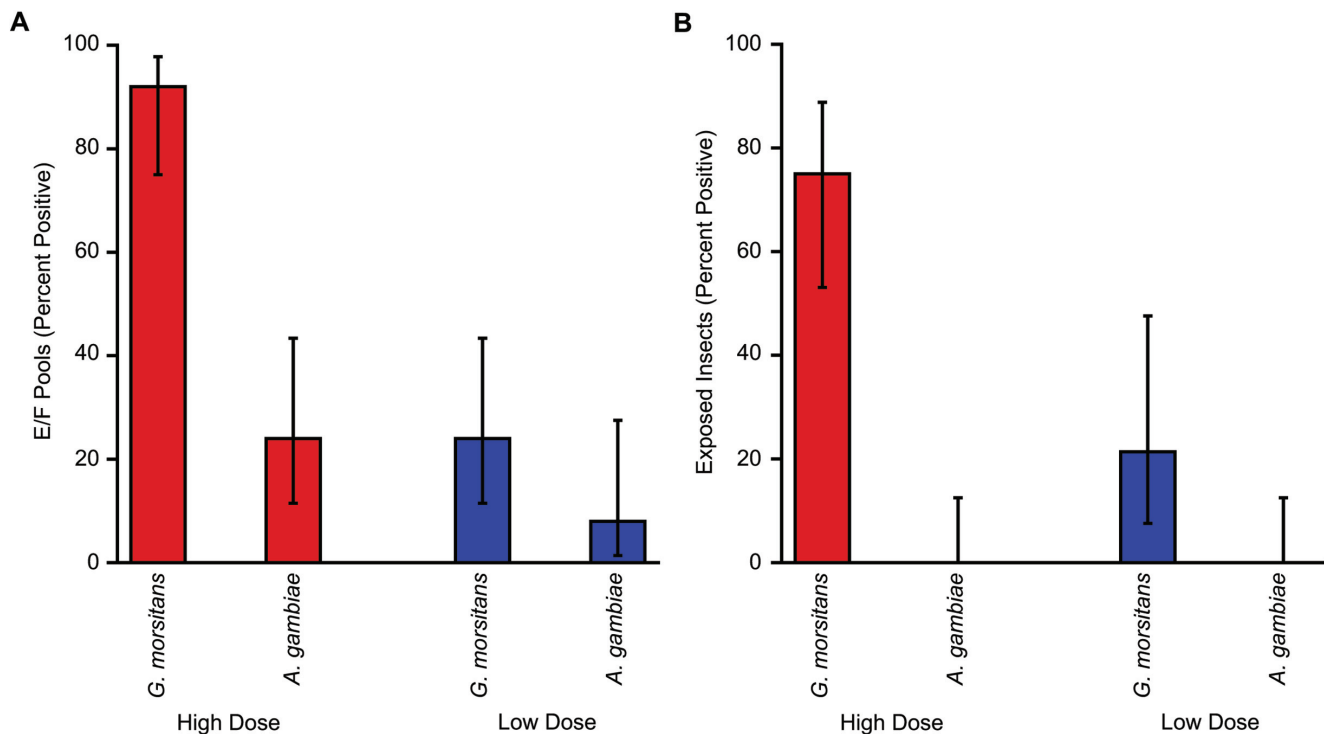


Figure 4. Levels of qPCR positivity in excreta/feces (E/F) pools and exposed insects following a *Trypanosoma brucei brucei*-containing bloodmeal. (A) Twenty-five *G. morsitans* and 25 *A. gambiae* were provided with a *T. b. brucei*-positive bloodmeal at either high (10^5 trypanosomes/mL; red) or low (10^3 trypanosomes/mL; blue) parasitemias. E/F was collected from individual insects and tested by qPCR for *T. b. brucei*. Results are shown as percentages of positive E/F pools \pm 95% CI. (B) Following high- or low-dose exposures to *T. b. brucei* and subsequent collection and testing of E/F (depicted in panel A), DNA was extracted from *Glossia morsitans* midguts and *Anopheles gambiae* carcasses. Extracts were tested for pathogen presence by qPCR and results are shown as percentages of exposed insects testing positive by qPCR \pm 95% CI.

comparative analysis of samples containing the pooled E/F from 50 mosquitoes (49 unexposed and 1 *P. falciparum*-exposed) and mosquitoes individually exposed to *P. falciparum* was performed. Eight of 10 samples containing pooled E/F gave positive qPCR results with a mean Cq value of 31.37 for all positive samples. By comparison, nine of 10 control samples containing the E/F from individually exposed mosquitoes resulted in the detection of *P. falciparum* signal, with a mean Cq value of 28.33 for all positive samples (Figure 5).

Effects on parasite detection of a post-exposure second blood feeding with pathogen-naïve blood

Following an initial exposure to an infected bloodmeal, detection of *P. falciparum* signal in the E/F from all three experimental pools of 10 mosquitoes occurred. As expected, at the 144-hour post-exposure time point, signal detection was no longer possible. Following the provision of a second, uninfected

bloodmeal, signal remained undetectable, indicating that such an exposure was not capable of extending, or re-initiating the post-parasite exposure collection window (Table 2).

Discussion

Proof-of-concept work has previously demonstrated the capacity for mosquito E/F to serve as a novel, high throughput testing medium for various parasitic and viral pathogens^{44,45,54-58}. However, the future utility of E/F testing will depend upon an ability to effectively and efficiently collect and test E/F from the appropriate mosquito source populations. Having previously described a novel methodology facilitating the high throughput collection of mosquito E/F⁴⁵, the work described here aimed to identify the characteristics of such mosquito populations through the definition of amenable mosquito species, and the determination of optimal pathogen densities, and sample pool sizes.

Table 1. Percentage of qPCR-positive excreta/feces (E/F) Pools collected from *Glossina morsitans* and *Anopheles gambiae* following exposure to both “high dose” and “low dose” concentrations of trypanosomes.

Post-Exposure Time Point (Hrs)	High Dose (10 ⁵ trypanosomes/mL)		Low Dose (10 ³ trypanosomes/mL)	
	<i>G. morsitans</i> % Positive (95% CI)	<i>A. gambiae</i> % Positive (95% CI)	<i>G. morsitans</i> % Positive (95% CI)	<i>A. gambiae</i> % Positive (95% CI)
48	56.0 (35.3 – 75.0)	24.0 (11.5 – 43.4)	0.0 (0.0 – 13.3)	0.0 (0.0 – 13.3)
96	50.0 (31.4 – 68.6)	0.0 (0.0 – 13.3)	0.0 (0.0 – 13.3)	4.0 (0.7 – 19.5)
144	45.8 (27.9 – 64.9)	0.0 (0.0 – 13.8)	8.7 (2.4 – 26.8)	4.2 (0.7 – 20.3)
192	70.8 (50.8 – 85.1)	0.0 (0.0 – 13.8)	20.8 (9.2 – 40.5)	0.0 (0.0 – 13.8)

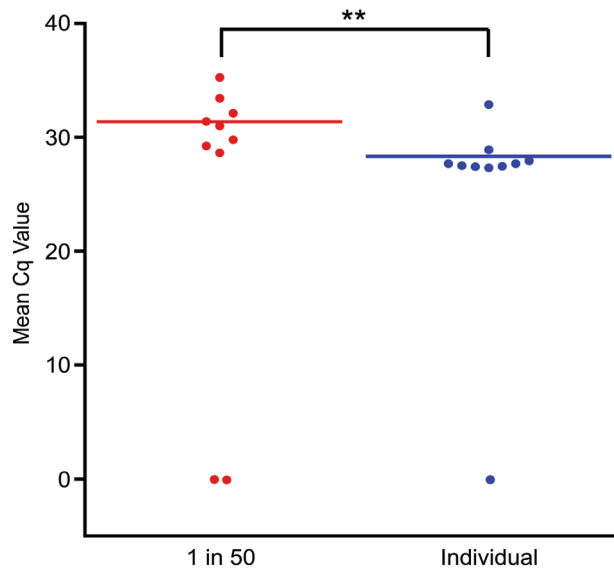


Figure 5. High throughput detection of *Plasmodium falciparum* signal from pooled excreta/feces (E/F). Individual *Anopheles gambiae* mosquitoes were exposed to *P. falciparum* trophozoites at a parasitemia of 0.1%. E/F was then collected from mosquitoes, either individually, or following pooling with 49 additional *A. gambiae* mosquitoes having been exposed to a parasite-naïve bloodmeal. All E/F samples were then tested by qPCR, in triplicate reactions, for the presence of *P. falciparum* signal. Mean results from the testing of each pool are indicated. Significance, as determined by the results of unpaired *t* tests, is provided. ***p* < 0.01.

Table 2. Mean Cq values for *P. falciparum* detection in the excreta/feces (E/F) of pools of 10 exposed mosquitoes provided with a second, pathogen-naïve bloodmeal.

Sample	72 hr post 1° Exposure	144 hr post 1° Exposure	72 hr post 2° Exposure
Negative Control Pool	Undetected	Undetected	Undetected
Experimental Pool #1	33.51	Undetected	Undetected
Experimental Pool #2	31.02	Undetected	Undetected
Experimental Pool #3	36.09	Undetected	Undetected

For the detection of *B. malayi*, the testing of E/F from both competent- and incompetent-vectors consistently allowed for pathogen detection. As *A. gambiae* mosquitoes do not support *B. malayi* development, it logically follows that the ingested pathogens should undergo rapid expulsion from the mosquito in the E/F of these non-vector hosts. In contrast, as *A. aegypti* mosquitoes allow for *B. malayi* development, we initially hypothesized that pathogen detection in competent vector E/F may be more difficult. However, even following the exposure of an efficient vector species to mf, the percentage of ingested worms that mature to the L3 stage remains relatively low⁵⁹. Therefore, while possible differences in expulsion rates for parasite-derived material from competent and incompetent vector species could be anticipated, these disparities would likely be modest. Accordingly, when individual competent and incompetent vector mosquitoes were provided with a *B. malayi*-containing bloodmeal during the determination of detection limits, E/F produced by both *A. aegypti* and *A. gambiae* mosquitoes gave positive qPCR results from a high percentage of parasite-exposed mosquitoes, demonstrating the amenability of both populations to E/F-based collection and testing.

To an even greater extent than occurs during filarial infections of mosquitoes, the majority of parasites obtained by mosquitoes during a *P. falciparum*-containing bloodmeal are trophozoites, a lifecycle stage that is incapable of developing in the mosquito host^{60,61}. Since trophozoites reach a developmental dead-end following a mosquito bloodmeal, it can be anticipated that they are rapidly expelled in the E/F. Likely for this reason, detection of *P. falciparum* appeared strong and consistent at both the 5,000 and 500 trophozoite/ μ L concentrations. It should be noted that experimental exposures to *P. falciparum* were performed using exclusively trophozoites, effectively rendering bloodmeals non-infective. For this reason, exposures were only performed using *A. gambiae* mosquitoes. Given their limited numbers within the overall *P. falciparum* population, absence of gametocytes would be unlikely to dramatically change expulsion rates of parasite-derived material. However, additional experiments in conjunction with future field-based testing will be conducted to conclusively evaluate this supposition.

Given the absence of developmental capacity of *T. b. brucei* within a mosquito, the limited detection of trypanosome signal

in the E/F of exposed mosquitoes was unexpected. Since *T. b. brucei* is not believed capable of developing within the mosquito, expulsion would presumably be complete and rapid. Nonetheless, detection of *T. b. brucei* showed significantly greater promise when testing the E/F from tsetse flies, the pathogen's vector, than when testing E/F shed by mosquitoes. Furthermore, the window for consistent *T. b. brucei* detection from fly E/F extended well past the 72-hour time point observed for pathogen detection from mosquitoes. However, improved detection in tsetse fly E/F may have simply been a result of increased bloodmeal volume (approximately 20 μ L of blood per tsetse fly feeding⁶² vs. 2–4 μ L per *Anopheles* bloodmeal⁶³). Future work will aim to evaluate other “cross-vector” pathogen detection capacities to determine whether this limitation is unique to the *T. b. brucei*-mosquito pairing, or whether it is an inherent property of the “cross-vector” screening approach.

Previous work has demonstrated that the testing of mosquito E/F for the detection of *B. malayi* allows for a higher throughput of screening when compared with standard mosquito-based approaches to molecular xenomonitoring⁴⁴. As expected, experimental detection of *P. falciparum* demonstrated high throughput capability as well. While a direct comparison of E/F samples collected from pooled and un-pooled mosquitoes did result in a significant difference, these differences were marginal and the consistency of detection was similar (Figure 5). Additional testing, with larger replicate numbers and increased mosquito pool sizes should help to further elucidate the true extent of this increased capacity for high throughput screening.

Previous testing of E/F produced by laboratory-reared competent and incompetent mosquito species exposed to *B. malayi*, *P. falciparum*, or *T. b. brucei* has strongly suggested that the principal collection window for the detection of all tested pathogens occurs within the first 72 hours post-blood exposure⁴⁵. Results of re-feeding experiments, during which a second, parasite-naïve bloodmeal was provided to mosquitoes following an initial exposure, did not allow for an expansion of this window (Table 2). Taken together, these results strongly suggest that when testing for the presence of parasite signal, E/F derived from blood-fed, resting mosquitoes should be targeted as the preferred sample population. Recent work has suggested that collection window constraints may be less important when

utilizing E/F for the monitoring of viral pathogens^{54,55}. This broadening of the collection window likely results from the ability of many viruses to replicate within the mosquito host. However, through the implementation of proper collection and trapping strategies, the surveillance of E/F for eukaryotic pathogens also becomes a viable option.

Despite the improved throughput of testing enabled by E/F, diagnostic sensitivity remains critical for drawing accurate conclusions from population surveys, and all possible means of maximizing sensitivity of detection should be evaluated. Accordingly, we assessed the use of dPCR as a possible methodology for improving the sensitivity of detection and for expanding temporal detection windows by comparing dPCR to standard qPCR in the evaluation of E/F samples produced by *P. falciparum*-exposed mosquitoes. While dPCR did expand the capacity for pathogen detection at reduced parasitemias, testing using the QuantStudio 3D dPCR platform is time-intensive and more costly than qPCR analysis. As such, analysis with the QuantStudio 3D dPCR platform is likely not a practical option in most E/F testing environments. However, exploration of other digital PCR platforms, and/or technological improvements may facilitate its future use, and further exploration is warranted.

While unlikely to replace the need for human sampling, or to completely eliminate the utility of more traditional MX approaches, E/F testing has the potential to serve as a complementary tool, filling gaps and expanding the surveillance capabilities of monitoring efforts. In addition to its possible utility as an early warning “first alert” system for detecting recrudescence or residual pathogen in post-intervention settings, the utility of E/F testing could be expanded to fill other operational gaps. In the context of LF, the rapid clearance/sterilization of adult female worms occurring under IDA is leading to questions regarding the suitability of traditional TAS surveys²⁰, as rapid pathogen clearance results in many individuals who are parasite negative but antigen positive. MX has been suggested as a possible solution to such shortcomings, as pathogen presence in the mosquito population would provide real-time evidence of recrudescence or remaining infection “hotspots”. In conjunction with such efforts, the high throughput nature of E/F testing could facilitate its usefulness as a pre-screening tool, channeling the allocation of resources for traditional MX to populations of mosquitoes demonstrating E/F positivity. One could also envision E/F testing as a mechanism facilitating *Culex* spp. monitoring for LF in urban settings, where focal transmission can occur despite the passage of TAS criteria. The relative ease of *Culex* capture in passive traps, coupled with the high throughput nature of E/F testing, could facilitate the detection of residual infections, allowing for the rapid re-introduction of intervention and establishment of appropriately targeted human surveys.

In the context of other disease settings, should E/F testing prove useful for cross-vector monitoring, envisioning its use as a mapping tool for concomitant filarial infections may also become possible. In regions of the world at risk for severe

adverse events due to the presence of multiple filarial pathogens, E/F pre-screening efforts could be employed to identify the presence of parasites such as *Loa loa*, helping officials to determine where appropriate precautions such as test-and-treat strategies would be required. Coupling the high throughput nature of E/F-based testing with the growing number of examples of E/F-derived viral surveillance possibilities^{54–57}, the potential for integrated viral/parasite monitoring efforts also becomes easy to envision. With the capacity to facilitate resource sharing and maximization, such integrated efforts are worthy of further consideration/exploration.

Having successfully identified the characteristics of amenable mosquito populations and appropriate temporal windows for pathogen detection, the capacity for E/F testing must now be evaluated under field conditions. Ongoing work is aiming to evaluate both collection strategies and the potential for parasite detection in an operational setting. These studies will ultimately help to identify suitable use cases for E/F surveillance, facilitating deployment in appropriate situations and maximizing the utility of this novel vector screening approach.

Data availability

Underlying data

Open Science Framework: Laboratory evaluation of molecular xenomonitoring using mosquito excreta/feces to amplify *Plasmodium*, *Brugia*, and *Trypanosoma* DNA. <https://doi.org/10.17605/OSF.IO/EWRTJ53>

This project contains the following underlying data:

- Compiled results_Bm_LOD_realtime.xlsx. (Raw qPCR data underlying *B. malayi* LOD experiments)
- Compiled results_Pf_LOD_realtime.xlsx. (Raw qPCR data underlying *P. falciparum* LOD experiments)
- Compiled results_Pf_re-feed_realtime.xlsx. (Raw qPCR data underlying *P. falciparum* re-feed experiments)
- Compiled results_Tbb_realtime.xlsx. (Raw qPCR data underlying experiments involving *T. b. brucei*)
- Raw data_Pf_LOD_Digital.xlsx. (Raw dPCR data underlying *P. falciparum* LOD experiments)

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

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Hans-Peter Fuehrer 

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Pilotte *et al.* describe the use of excreta/faeces of mosquitoes and tsetse flies after experimental exposure to *Brugia malayi*, *Plasmodium falciparum* and *Trypanosoma brucei brucei* for molecular xeno-monitoring.

The manuscript is of very good quality and of interest. Only few modifications are recommended:

- Title: Maybe include tsetse-flies in the title.
- Methods: Please name “strains” or “origin” of the vectors.
- Page 4: *Brugia* experiments: Change „the“ to „then“ – at: „Cones were then placed...“.
- Entire manuscript: Please use Ae. for *Aedes* and An. for *Anopheles* instead of A. (e.g. Figure 5).
- Discussion: It is recommended to add a sentence that “vector competence” is not a proof for true vector capacity in the field.

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?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Mosquitoes and mosquito borne diseases; Molecular xeno-monitoring

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

Reviewer Report 28 January 2020

<https://doi.org/10.21956/gatesopenres.14242.r28423>

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Ana L. Ramirez 

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This paper by Nils Pilotte and co-authors describe the evaluation of using mosquito excreta/feces for xenomonitoring applications. The use of mosquito excreta as a tool for molecular xenomonitoring of parasitic diseases has clear applications for disease surveillance and the evaluation of intervention programmes. The authors evaluated key factors that can have an effect once the methodology is deployed to the field, such as the limits of detection, throughput of testing and the effect of subsequent bloodmeals after exposure using different vector/parasite models. Additionally, this is the first study evaluating molecular detection of *T. b. brucei* in excreta from tsetse flies. The article is well written, and the methods are generally clear. All the data generated from the study has been deposited in a data repository. However, some issues in the results need to be addressed (clarification of pooled/individual samples and checking the consistency between data presented in figures/tables and in-text).

Introduction

Consider removing acronyms that are only used once (i.e. in the second paragraph of the introduction “operational research (OR)”).

Methods

Most of the information in the methods section is presented in a clear and detailed manner. A few observations:

- Page 4 - Tsetse flies. “Flies were reared from larvae..” Add species at the first mention “*Glossina morsitans* flies were reared from larvae”.

- Page 4 - Were parasite densities determined for *B. malayi* and *P. falciparum* too or just for the trypanosomes?
- Page 4 - For the collection of excreta/feces: were the GenSaver DNA cards used whole or did you cut a smaller piece?
- Page 5 - Real-time PCR: “Quantitative real-time PCR testing for the presence/absence..” --> mention what was tested by qPCR (DNA extracts from E/F, fly midguts and whole mosquitoes and flies?). Same for Digital PCR (only DNA extracts from E/F).
- Page 5 - Mention the controls used for each qPCR assay and the Cq used as a cut-off.
- Page 6 - Limits of detection for parasite signal from pooled E/F: How many mosquitoes were used to collect pooled E/F?
- Page 6 - Both hours and days are used through the manuscript (i.e “144 hours post-exposure”, then “at six days post feeding”. Consider sticking to hours or days for consistency.

This is just a suggestion but given that the methodology has different components it could be re-structured to make it easier to follow by first describing the insects and parasites, then moving to the exposure, then describing the design of each experiment and finally describing how the samples were tested. Something like:

- Insect rearing
 - Mosquitoes
 - Tse tse flies
- Parasites
 - *B. malayi*
 - *P. falciparum*
 - *T. b. brucei*
- Blood feeding
 - Move here the sections where you describe the exposure for all of the above (i.e “experimental exposures were performed as previously described. Briefly, adult female mosquitoes...”
- Collection of excreta/feces (keep it as it is).
- Limits of detection for parasite signal from pooled E/F.
- Demonstration of high throughput detection of *P. falciparum* signal from pooled E/F.
- Effects on parasite detection of a second blood feeding with pathogen-naïve blood.
- Isolation of tsetse fly midguts and preparation for DNA extraction.
- DNA extraction from E/F.
- DNA extraction from DNA from mosquitoes and tsetse flies.
- Real-time PCR.
- Digital PCR.
- Statistical analysis.

Results

- Page 7 - Limits of detection for parasite signal from pooled EF: Check that the numbers in text match the info in the figures/tables. “Consistency of detection across time points (...) and FIVE mosquitoes following exposure at 2,000 mf/mL”. In the table, there are SIX mosquitoes with detection at both time points. “In E/F produced by *A. gambiae* detection across multiple time points occurred from only TWO mosquitoes and one mosquito following...” in the table there are 3

mosquitoes with detection at both time points.

- Page 7 - Limits of detection for parasite signal from pooled EF: The text in this section references Figure 2. However, the caption indicates that “E/F was collected from each mosquito”. It is not clear if results from E/F from individual mosquitoes is being shown or if correspond to pooled samples. If it is pooled, indicate how many and change the figure. If it is from individual mosquitoes removed “pooled” from the section.
- Page 7 - Limits of detection for parasite signal from pooled EF: “unsurprisingly, mean Cq values were lower, suggesting greater concentration of target DNA..” --> “unsurprisingly, for both species, Cq values were lower, suggesting...”
- Page 7 - Figure 2C: consider changing the x-axis labels to horizontal and add units (Vector species and parasite density (mf/mL).
- Page 8 - *P. falciparum*: Stick to either percentage or trophozoites/mL. In the text it is shown mostly as percentage so maybe change the column headings in Figure 3A and 3C to match.
- Page 8 - “Interestingly, only one mosquito produce sample that was detectable at both collection time points” change to “Interestingly, regardless of concentration, only one mosquito produce sample that was detectable at both collection time points”
- Page 8 - The following statement is not clear “When signal detection occurs across time points, it may be that this event spans collection intervals, resulting in multiple positive time points from an isolated excretion occurrence”. Is the event the act of excreting parasite?
- Page 8 - Figure 2B shows a comparison of mean Cq values irrespective of timepoints, was a comparison done of Cq values between time points?
- Page 9 - First paragraph: consider showing the percentage of samples with positive results from qPCR vs dPCR.
- Page 9 - *T.b.brucei*: The results from E/F collected after 48 and 144 hours are not mentioned in text (they appear in Table 1).
- Table 1: check the numbers from the table, as they don't match the results shown in text (96 h. Also add N. In the title change “trypanosomes” to “*T. b. brucei*”.
- Page 9 - add percentages when describing the data to be consistent “In contrast, only six of the 25 individual *A. gambiae*...”--> “In contrast, only six of the 25 individual *A. gambiae* (24%)...”
- Page 9 - Figure 4. The legend says “qPCR positivity in excreta/feces POOLS and exposed insects” however in the text it is mentioned “individually housed *G. morsitans*”. Again, it is not clear if results from E/F from individual flies is being shown or if correspond to pooled samples. If it is pooled, indicate how many and change the figure. If it is from individual mosquitoes removed “pooled” from the section.

- Page 9 - Figure 4. Mention what time point is being shown (i.e. “samples collected after 96 hours of exposure”). Also, consider rotating the labels in x-axis to either horizontal or 45 degrees.
- Page 9 - “Following sacrifice at the 196-hour time point” add percentages to “15 out of 20” and “3 out of 14” (75%, 21%).
- Page 10 - First paragraph: Is the difference between mean Cq values from positive samples pooled with negative ones and control samples significant?
- Page 10 - Figure 5: change “E/F” was then collected from mosquitoes, either individually, or following pooling with 49 additional *A. gambiae* mosquitoes having...” to “E/F” was then collected from mosquitoes, either individually, or following pooling with THE E/F FROM 49 additional *A. gambiae* mosquitoes having...”

Discussion

- Page 11 - Paragraph 3: The authors observed that for flies, the window of detection extended past 72 hours. It is known that digestion of bloodmeals by mosquitoes generally is finalized by 72 hours. How long does it take for a tsetse fly to digest its bloodmeal? If it's longer, perhaps this would explain why the parasite is still detectable in their excreta after 72 hours.
- Page 11 - Paragraph 5: The authors state that previous studies have suggested that the collection window for detection of *P. falciparum* occurs within 72-hours post-exposure. However, in a study conducted by our group using competent *An. stephensi* mosquitoes, we have observed excretion of *P. falciparum* genetic material from day 4 to 14 post-exposure (intermittently) and continually from day 15 to 19 (Ramirez 2019)¹.

General comments:

- Check that controls are mentioned in the methodology.
- Make sure to make a clear distinction when pooled excreta and the excreta from individual insects is being used. Check figure titles, legends and in-text results. Also, when pooled excreta is being used, indicate the pool size.
- Keep it consistent with the percentages.
- Check the data in Figure 2 and Table 1.

References

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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.

Reviewer Expertise: Vector biology, virology, arbovirus surveillance.

I confirm that I have read this submission and 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.
