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Detection of chikungunya viral RNA in mosquito bodies on cationic (Q) paper based on innovations in synthetic biology

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Abstract

Chikungunya virus (CHIKV) represents a growing and global concern for public health that needs inexpensive and convenient methods to collect mosquitoes as potential carriers so that they can be preserved, stored and transported for later and/or remote analysis. Reported here is a cellulose-based paper, derivatized with quaternary ammonium groups (“Q-paper”) that meets these needs. In a series of tests, infected mosquito bodies were squashed directly on to Q-paper. Aqueous ammonia was then added on the mosquito bodies to release viral RNA that adsorbed on the cationic surface via electrostatic interactions. The samples were then stored (frozen) or transported. For analysis, the CHIKV nucleic acids were eluted from the Q-paper and PCR amplified in a workflow, previously developed, that also exploited two nucleic acid innovations, (“artificially expanded genetic information systems”, AEGIS, and “self-avoiding molecular recognition systems”, SAMRS). The amplicons were then analyzed by a Luminex hybridization assay. This procedure detected CHIKV RNA, if present, in each infected mosquito sample, but not in non-infected counterparts or ddH₂O samples washes, with testing one week or ten months after sample collection.

Keywords

Chikungunya virus; cationic (Q) paper; reverse-transcription PCR; Luminex direct hybridization assay; Self-avoiding molecular recognition system (SAMRS); Artificially expanded genetic information system (AEGIS)

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Competing interests

Several of the authors and their institutions own intellectual property associated with this assay, including SAMRS, AEGIS, and the conversion capture technologies. Several of the items mentioned are sold by Firebird Biomolecular Sciences LLC, which employs the indicated authors of the paper.

1. Introduction

Mosquito-borne arboviruses are emerging and re-emerging world-wide as important human pathogens. Their great capacity for evolution (Jenkins et al., 2002) and their consequent ability to rapidly adapt to new vector species makes emergence of these agents a threat to public health (Devaux, 2012; Coffey et al., 2013). The primary method to reduce the risk of transmission of mosquito-borne pathogens is to suppress vector populations. Selective use of suppression tools requires pathogen detection during mosquito surveillance.

For surveillance, tools are needed to collect mosquitoes and transport biological samples. These might be applied for research as well as public health. The nucleic acids (NA) in samples collected with these products must be preserved for use in downstream analyses. This is the case for both individual mosquitoes, and for pools of mosquitoes.

One commercial tool is the Whatman® FTA cards, which is used to collect samples for shipping and downstream analysis. These “Fast Technology for Analysis” paper cards exploit technology licensed from Flinders University (Salvador and De Ungria, 2003; Borman et al., 2010; Kiraz et al., 2015; Lalani et al., 2015). The product combines a weak base, a chelating agent, an anionic surfactant/detergent, and uric acid/urate salt in a cellulose matrix (such as filter paper) to preserve and store the NA (http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-us/products/AlternativeProductStructure_21465). Here, NA adheres to the paper, while the added chemicals lyse cells and denature proteins that might degrade the NAs.

In this study, we asked whether the polyanionic nature of NA might be exploited in an alternative architecture that recovers and stabilizes NA on a cationic paper surface. Such a support would allow a user to (a) place a sample on the cationic paper, (b) adding a drop of a general disruptant (e.g. aqueous ammonia), and (c) allow NA to be pulled into the cationic paper. We expected that the NA would be captured and stabilized by being coulombically bound on the cationic paper.

The cationic moieties could not be the diethylaminoethyl ammonium groups (as found, for example, in certain Qiagen products); these would not maintain their positive charges at high pH. Instead, quaternary ammonium groups are required, such as trimethylammonium alkyl groups. These can be covalently attached to paper by treatment with 2,3-epoxypropyltrimethylammonium chloride (Yang et al., 2013). The paper can be prepared in bulk, and is commercially available as “Q-paper” (www.firebirdbio.com).

In this study, Q-paper was tested for its ability to capture, in a form suitable for downstream analysis, NA from the bodies of mosquitoes that were infected with the chikungunya virus (CHIKV). Successful performance would require:

- a. the simplest of sample preparation, like squishing the body of a potentially infected mosquito on the Q-paper,
- b. releasing and preserving the NA from the virus by an ammonia treatment,
- c. transporting the Q-paper carrying the mosquito to a remote site, and

- d. demonstrating the ability to amplify CHIKV nucleic acids from the sample, to distinguish chikungunya-infected mosquitoes from mosquitoes that were not infected with the virus.

We presented these here in the context of a series of other nucleic acid innovations, reported separately (Glushakova et al., 2015), and also shown in Figure 1 (adapted from Glushakova et al., 2015) that allow sensitive detection of viral PCR-amplicons.

To our knowledge, this is the first application of cationic (Q) paper to a biological object. As advantages of the strategy, it skips the time-consuming and reagent-consuming step of nucleic acid purification from mosquito, increasing the simplicity of sample handling and storage.

2. Material and Methods

2.1 Chikungunya virus (CHIKV)

An isolate of CHIKV from the British Virgin Islands (BVI, Asian lineage, GenBank accession: KJ451624) was originally isolated from an infected human in December 2013, and was responsible for the outbreak in the America's during this time (Diaz et al., 2015). The Indian Ocean strain (IOC) of CHIKV (LR2006-OPY1, GenBank accession: KT449801) was isolated from a febrile patient in France who had been infected in Réunion (Parola et al., 2006) and was responsible for the outbreak in the Indian Ocean region and parts of Europe (Weaver, 2014). CHIKV isolates were passaged twice in cell culture using monolayers of African green monkey kidney (Vero) cells to produce virus stock and viral titer was determined by plaque assay (Reiskind et al., 2008). Freshly harvested CHIKV was combined with blood used in an infection study with *Aedes aegypti* mosquitoes.

2.2 Mosquito infection with the Chikungunya virus

Larval *Aedes aegypti* mosquitoes were collected from containers in residential neighborhoods in Florida in 2014. Mosquitoes used in the infection study were the F₁₋₃ progeny of field-collected parents. The CHIKV infection was performed in the biosafety level-3 virology facility at the Florida Medical Entomology Laboratory in Vero Beach, FL as part of an unrelated study. Subsets of these mosquitoes were used in the current study to evaluate the efficacy of Q-paper as a diagnostic tool for detecting CHIKV.

Mosquito larvae were reared in tap water (1 L) in plastic photo trays (25 cm width, 30 cm length, 5 cm height: Richard MFG Co. Fernandina Beach, FL) on a diet of equal parts of liver powder and lactalbumin. Approximately 150 larvae were reared together in trays in a climate controlled room (26-28°C, photoperiod of 14:10 light:dark). Pupae were placed in water-filled cups and held in 0.3³ meter cages until emergence to adulthood. Adult mosquitoes were provided with 10% sucrose solution and water from cotton wicks.

For infection, 10 to 13-day old adult females were provided with CHIKV infected defibrinated bovine blood (Hemostat, Dixon CA) using an artificial membrane feeding system (Hemotek, Lancashire, United Kingdom) (Alto and Lord, 2016). Virus titers of

infected blood were 8.4 log₁₀ plaque forming unit equivalents/mL. These titers approximate the viral load in patients with symptomatic CHIKV infection (Appassakij et al., 2013).

After the feeding trials, fully engorged females were held in cages and maintained at a 14:10 hour light:dark photoperiod and 30°C for 2, 5-6, or 12-13 days. At this point, mosquitoes were killed and stored at -80°C. Mosquitoes were individually dissected and the legs were tested for individual mosquitoes for the presence of CHIKV RNA by qRT-PCR according to methods described by (Reiskind et al., 2008).

2.3 Nucleic acid isolation from the mosquito tissues

Legs from mosquitoes that ingested CHIKV infected blood and uninfected controls were homogenized with a TissueLyser (Qiagen, Valencia CA) in 2-mL flat-bottom vials containing 1.0 mL of 199 media and two zinc-plated steel BBs. Samples were then centrifuged at 3,148 × g for 4 minutes (4°C). Viral RNA was isolated from a 160 µL sample using the QIAamp viral RNA mini kit (Qiagen, Valencia CA) and eluted in 50 µL of buffer according to the manufacturer's protocol. CHIKV RNA was detected using the Superscript III One-Step qRT-PCR with Platinum® Taq kit by Invitrogen (Invitrogen, Carlsbad, CA) as described previously (Reiskind et al., 2008). Quantitative RT-PCR was performed with the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The presence of CHIKV mRNA in the mosquito legs was determined by a TaqMan real-time RT-PCR using primer/probe sets designed to target a nonstructural polypeptide gene common to both lineages (accession ID of the transcript, KU365292.1) with the following sequences:

Forward Primer, 5'-GTACGGAAGGTAACTGGTATGG-3';

Reverse Primer, 5'-TCCACCTCCCACTCCTTAAT-3';

Probe, 5'-/56-FAM/TGCAGAACCCACCGAAAGGAACT/3BHQ_1/-3'.

Oligos were synthesized by IDT (Integrated DNA Technologies, Coralville IA). The program for qRT-PCR was as follows; 50°C for 30 minutes, 94°C for 2 minutes, 39 cycles at 94°C for 10 seconds and 60°C for 1 minute, and lastly 50°C for 30 seconds. A standard curve method was used to express the titer of CHIKV of mosquito samples by comparing cDNA synthesis for a range of serial dilutions of CHIKV in parallel with plaque assays of the same dilutions of virus, expressed as plaque forming unit equivalents (pfu)/ml. The remaining bodies of mosquitoes whose legs tested positive for the presence of CHIKV RNA were used in development of the Q-paper diagnostic assay.

2.5 Preparation of cationic paper (Q-paper) and measuring its binding capacity

The Q-paper was prepared by a modification of a procedure from (Yang et al., 2013). Briefly, one gram of pre-cut filter paper (paper circles with diameter even to 3.5 cm, Fisher Scientific, Waltham MA) was immersed in 50 mL of 18% NaOH aqueous solution for 10 min for activation. The treated paper was collected by filtration and immersed in 40 mL of (2,3-epoxypropyl) trimethylammonium chloride (Sigma-Aldrich, St. Louis MO) aqueous solution for 24 h at room temperature. The mass ratio of EPTMAC to filter paper was 2.8:1. The cationic paper was collected by vacuum filtration and neutralization with 10% of acetic acid. Then a product was washed three times by ethanol and dried in an oven at 55 °C.

The Q-paper binding capacity was determined with 1,3,5-benzenetricarboxylic acid. For this, a solution of 1,3,5-benzenetricarboxylic acid (2 mM, 5 mL) was neutralized to pH 8 by NaOH solution (3 mM, ca. 2 mL). Then, the solution of sodium benzenetricarboxylate was diluted to final concentration of 1 mM and Q-paper was soaked for 15 min (2 mL of solution for 1 cm² paper). To remove unbound benzenetricarboxylate reagent, 3 washes with 1 mL dd H₂O were done. Acetic acid (aqueous 1 M) was added to release the coulombically from the Q-paper. Total amount of bound benzenetricarboxylate was evaluated by UV measurement. A typical binding capacity was 0.25 μ moles/cm².

2.6 Reverse-transcription PCR (RT-PCR) by the transliteration of dC in the amplicons to dZ

The design and synthesis of the primers and probes sequences were described previously (Glushakova et al., 2015) and listed in Table A1 (Appendix 1). Each RT-PCR was executed in a final volume of 20 μ L according to the Invitrogen protocol for the SuperScript One-Step RT-PCR with Platinum Taq (Life Technologies). The reaction mixture was composed of the 5 μ L of desalted eluate, 0.3 μ M each of Forward and Reverse SAMRS primers; 2.5 units RT/Platinum Taq enzyme Mix. Additional MgSO₄ (1.5 mM) was added to the RT-PCR buffer. Cycling conditions were: one cycle of the cDNA synthesis and pre-denaturation (53°C for 30 min and 94°C for 2 min), 35 cycles of PCR (94°C for 15 s, 53°C for 30 s, and 70°C for 30 s) and final extension at 72°C for 5 min. 4 μ L of PCR were loaded on 2.5% TBE agarose gel and visualized by the ethidium-bromide staining.

To favor incorporation of biotin-tagged reverse primers, to maximize hybridization efficiency and sensitivity, and to convert the dCs in the resulting amplicon to dZs (“transliteration”), reverse primer extension reaction (RPER) was executed with each amplicon. Prior to RPER, excess primers were destroyed and dNTPs were deactivated by treatment with ExoSAP-IT enzyme mixture accordingly to the manufacture’s protocol (Affymetrix, Cleveland Ohio). The RPER (20 μ L) was set up in 1 X ThermoPol Buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton X-100, pH 8.8 at 25°C) with 3 μ L of each ExoSAP-treated RT-PCR product, 5′-biotinylated Reverse primer (0.2 μ M), and Vent (exo-) DNA polymerase (1 unit, per reaction, NEB). For the dZ incorporation into the final amplicon, nucleoside triphosphates (dATP, dTTP, dGTP, and dZTP, final concentration 0.2 mM of each) were added. The reaction mixtures were incubated in BioRad (DNA Engine) Peltier Thermal Cycler at 95°C for 1 min, followed by 20-25 cycles (94°C for 20 seconds, 54°C for 30 seconds, 72°C for 30 seconds). A final incubation was run at 72°C for 1 minute. Reaction mixtures were then quenched with 4 mM EDTA.

2.7 Probe coupling to Luminex MicroPlex carboxylated micro-spheres

Capture probes carrying an amino-C12 linker at their 5′-ends were coupled to Luminex MicroPlex carboxylated microspheres (Luminex, Austin TX) by a carbodiimide-based procedure that followed the manufacturer’s protocol, as previously described (Glushakova et al., 2015). Briefly, for each combination, Luminex beads (ca. 2.5 million) were re-suspended in MES buffer (0.1 M, 50 μ L, pH 4.5, Sigma-Aldrich), with target-specific probe (4 μ L of 0.1 mM stock, 0.4 nanomole final concentration), and twice treated with 1-ethyl-3-[3-dimethylamino- propyl]-carbodiimide hydrochloride (EDC, 5 μ L of a 10 mg/mL solution,

Thermo Scientific/Pierce, Rockford IL) for 30 min (room temperature), rinsed consecutively in Tween 20 (0.02% aqueous solution) and a solution of SDS (0.1%). The beads were then suspended in Tris-EDTA (pH 8.0) to give a final volume of 100 μ L.

2.8 Luminex direct hybridization assay (DHA)

Luminex Direct hybridization assays (DHA) (Dunbar, 2006) were then performed following a “no wash” protocol (<http://www.luminexcorp.com/Support/SupportResources/>) as described previously (Glushakova et al., 2015). Briefly, aliquots (5 μ L) of each reaction were transferred to 96-well plates (PCR thermo polystyrene; Costar Technologies, Coppel TX). Hybridization buffer (25 μ L of 2 X Tm buffer; 0.4 M NaCl; 0.2 M Tris; 0.16 Triton X-100, pH 8.0) each containing 2,500 microsphere types coupled to the target-specific probes. Microspheres were briefly vortexed and sonicated for 20 seconds. The total volume was adjusted to 50 μ L with 20 μ L of ddH₂O. Sample buffer (25 μ L) was then added to each background well (negative control). Hybridization was performed at 55°C accordingly to the Luminex DHA protocol: 95°C for 5 min, cool to 55°C at a speed of 0.1°C/second, 15 min at the hybridization T 55°C. Tm buffer (25 μ L of 1X) containing streptavidin-R-phycoerythrin (2 μ g, PJRS14, PROzyme, Hayward CA) were added to each hybridization mixture, which was then incubated at 55°C for 5 min. Hybridization reactions were done in triplicate, and “no-target” controls were run in replicates of five. Beads were analyzed for internal color and R-phycoerythrin reporter fluorescence using a Luminex 200 analyzer (Luminex xMAP Technology) and the xPonent Software solutions. The median reporter fluorescence intensity (MFI) was computed for each microsphere type in the sample. The instrument’s gate setting was established before the samples were run and maintained throughout the course of the study.

3. Results

3.1 *Ae. aegypti* mosquito’s infection with CHIKV and Q-paper samples preparation

The legs of the infected *Ae. aegypti* females originated from Florida were removed and total NA was extracted from each. These NA samples were used to confirm the presence of CHIKV mRNA in mosquitoes by real-time (TaqMan) RT-PCRs (Table 1). Mosquito bodies were kept at –80°C.

Separately, infected and uninfected mosquito bodies were thawed and then squashed on Q-paper and treated with NH₄OH solution. For this, each mosquito was placed on the top of a piece of Q-paper (one quarter of the round sheet with the diameter 3 centimeters) and squashed with the rounded bases of a sterile 2.0 mL Eppendorf tube (separate for each mosquito). Uninfected mosquitoes were processed in an identical fashion (negative controls). For sterilization and NA capture, drops (totaling 100–200 μ L) of freshly prepared 0.1 or 1 M NH₄OH solution (pH 12–13) were placed on each mosquito sample on Q-paper (Table 1). The wet paper was air-dried in a biological safety cabinet (air flow ~0.5 - 1.0 h). Because in the United States CHIKV is classified as a category C priority pathogen, the biosafety level 3 requirements were fully met during the procedure. Dried Q-paper samples with mosquito bodies were individually packaged in plastic bags in the *Florida Medical Entomology Laboratory* and transported to *Firebird Biomolecular Sciences* BS2 laboratory

via UPS (a small, compact package) on ice and stored at -20°C . The sample preparation workflow is shown in Figure 2.

The information presented in Table 1 was obtained from mosquito legs, to confirm mosquito's infection status. The viral titer in the legs will differ from that of the body, most likely due to different amount of tissue present. There is usually a lower viral titer present in the legs than the body as observed in other studies (e.g. dengue virus, Alto and Bettinardi 2013; chikungunya virus, Richards et al. 2010).

3.2 CHIKV RNA detection in NA eluates from Q-paper samples

Two weeks (samples# 2, 6, 7, 11, 12) or 10 months (samples#6, 8, 13, 14) after the preparation of Q-paper/mosquito samples, the NAs were eluted and the CHIKV RNAs were detected by RT-PCRs and downstream hybridization with a CHIKV specific probe on Luminex microspheres (Figures 4–5).

For these, Q-paper sheets containing squashed mosquito bodies were removed from -20°C , thawed, cut into small pieces, and placed into Eppendorf tubes. The tubes were centrifuged briefly to put the Q-paper on the bottom. To remove unbounded NA from the surface the Q-papers were washed for 20 min to 1 h with ddH₂O (200 μL). To recover the NA from Q-paper surface, aqueous NaCl (1 M) was added to each tube to cover the paper (about 200 μL), and tubes were incubated for 1 hour at 4°C and shaken briefly 3–4 times. Negative controls were: non-infected mosquito samples (C1=3 in each experiment) and water-washes prior elution (C2). NA eluates from Q-paper were desalted by gel filtration using CENTRI-Spin⁻²⁰ columns (Princeton separations, Adelphia NJ).

NA eluates from Q-paper with ammonia-treated mosquitoes and ddH₂O-washes controls were analyzed by PCR-amplification based xMAP Luminex direct hybridization assays (Figures 4 and 5). First, each RNA eluate was amplified by multiplexed RT-PCR with CHIKV specific primers (Table A1) targeting the non-structural protein 4 (NS4). The PCR-amplicons were visualized on 3.5% TBE agarose gel as bands of the desirable size (Figure 4A).

We then sought to capture the amplicons on Luminex beads with high yield, with uniformity in multiplexed capture, and without any potential interference from adventitious NA in the mix. To these ends, we employed a “transliteration” procedure. In transliteration, as the final step, a primer is extended where the guanines (Gs) in a amplicon template are not complemented by cytosine (Cs), but rather by a nucleotide containing a nitroaminopyridone heterocycle (trivially named **Z**); **Z** is part of an “artificially expanded genetic information system”, AEGIS (Glushakova et al. 2015). This creates an amplicon product where the Cs are formally replaced by **Z**. This product can be captured on a Luminex bead where the capture oligonucleotide carries a 8*H*-imidazo [1,2-*a*] [1,3,5] triazin-4-one (trivially **P**) heterocycle; **P** is the complement of **Z** in the expanded AEGIS DNA alphabet. This allows the capture of the amplicon by orthogonal **Z:P** pairing, instead of C:G pairing, which can suffer interference from natural oligonucleotides.

For transliteration in these assays, the CHIKV amplicon was used as a template for the extension of a 5'-biotinylated reverse-primer. Here, however, dZTP replaced dCTP. The final transliterated products were detected, if they were present, by hybridization of biotinylated amplicons with CHIKV-specific probes containing AEGIS P and attached to Luminex microspheres. Each amplicon produced the positive signal on Luminex platform (Figures 4B, 5). The median fluorescence intensities (MFIs) corresponded to the intensities of the amplicon bands obtained by agarose gel electrophoresis. With both amplicon detection methods (gel and Luminex), no signals were observed with non-infected mosquitoes (negative controls C1) and water (negative control C2).

Further, Samples # 8 and #13 were five-fold multiplexed assays performed with CHIKV PCR-specific primers and primers for dengue serotypes 1-4, and target-specific Luminex probes (Table A1). Monoplexed assays were also done for comparison (Figure 5B). The samples from both groups (2 weeks or 10 months after preparations) produced positive CHIKV-specific responses in Luminex DHAs with signals in 1500-to-7,000 MFI-units (median fluorescence intensity) range.

Q-paper sample (#6) was divided into three pieces. One piece was analyzed shortly after sample preparation. Others were kept at -20°C for further analyses; one of these was processed after 10 months. The comparison of these data revealed a decline in assay's (MFI) values after long-term storage (Figures 4-5). This might be an impact either of sample degradation or differences in viral titers in the sample pieces (each might contain different volumes of mosquito body), or even an impact of differences in viral titers in mosquito tissues. The latest two explanations are more likely because, as a part of a different project, some samples were processed almost after 3-years of storage and found to be positive for the CHIKV RNA.

To determine if ammonia concentrations (up to 1 M) could influence the integrity of the viral RNA, Q-paper/mosquito samples were arbitrarily divided into two groups (Table 1) and treated by 1 M or by 0.1 M of the ammonia solution. The comparison of assay data did not reveal significant differences between samples from each group (Figures 4-5).

To confirm this observation, purified CHIKV RNA was directly loaded on a Q-paper and eluates were analyzed by the PCR-amplification-based Luminex DHA (Figure 6). For this, serial dilutions were prepared from the CHIKV RNA stock solution and each dilution was titrated by TaqMan RT-PCR before the experiment. RNA samples were denatured at 65°C for 2 minutes and immediately placed on ice. Aliquots of RNA solutions (1 µL) were loaded on the Q-paper surface (one sixth of the round sheet with the diameter 3 centimeters), treated by 0.1 M NH₄OH reagent (5 µL) and fully dried in the biosafety cabinet. Shortly after sample preparation, the RNA was washed by ddH₂O, and eluted with aqueous NaCl (150 µL, 1 M). The eluates were desalted, collected in 50µL ddH₂O, and the assays were performed with 5 µL aliquots of each RNA solution as described before. The quantities of RNA loaded on the paper and RNA input in RT-PCRs are given in Figure 6 (the top and the bottom of graph bars respectively).

Assuming that the elution efficiency was 100%, the level of detection (LOD) of CHIKV RNA was ~ 8.0 RNA copies per assay. This sensitivity was more than sufficient to detect CHIKV in an infected mosquito. The viral RNA was not detected in ddH₂O washed done prior samples elution by the high salt solution.

3.3 Detection of CHIKV infectivity in Q-paper mosquitos' samples

To assess the biohazard potentials of mosquito bodies prepared in this way, the conditions for full CHIKV inactivation in mosquito's samples on Q-paper were evaluated. Here, sets of samples were prepared in three different ways. First, samples were prepared as described above (Section 3.1). Second, samples were prepared as above, but where the Q-paper sheets carrying the bodies were then placed directly on a heat block at 65°C for 10 minutes. Third, infected and uninfected mosquito bodies were homogenized in Eppendorf tubes with ammonium solutions (adjusted with NaOH to pH 12-13); the homogenates were placed on the Q-paper sheets, which was air dried.

An aliquot (50 µL) of each sample was then inoculated into a tissue culture flask (25 cm²) with a monolayer of Vero cells incubated at 37°C and 5% carbon dioxide atmosphere for 1 hour. After incubation, media (5 mL) was added to each flask and maintained using the same incubation conditions for 4 days to allow for possible CHIKV propagation. Because detection of CHIKV cytopathology in Vero cells is unambiguous, we examined monolayers after 4-days. Afterwards, an aliquot of media from each flask was used in a plaque assay and then confirmed by real time (TaqMan) reverse transcription PCR with the set of primers and probe presented in the Section 2.2 above. For the plaque assay, samples of undiluted media (250 µL) from each flask were plated on Vero cells under complete media solidified with 0.7% agarose, and incubated for 48 hours at 37°C and a 5% CO₂ atmosphere. After incubation, media and agarose were removed and monolayers of Vero cells were stained with crystal violet to visualize cytopathology (e.g., plaques). These assays were qualitatively used to designate samples as infected or uninfected. Positive and negative controls for the plaque assays were stock virus and media, respectively. Lack of cytopathology in cell cultures during propagation and plaque assays indicated virus inactivation in Q-paper samples treated with ammonia solutions (pH 12-13) and additionally heated at 65°C, as well as in samples prepared with mosquito body pre-treated with ammonia solution and then placed on Q-paper (n=9). A t-test comparing body titers pre-inactivation and post-inactivation steps showed no significant differences in the amount of CHIKV RNA present in samples ($t=1.3273$, $df=16$, $p=0.2030$), an indicator that the virus was not propagated in cell culture.

4. Discussion

Chikungunya virus has emerged as a global pathogen, with outbreaks in Africa, the Indian Ocean basin, India, and Europe (Rossini et al., 2016). Phylogenetically, CHIKV strains are classified into three genotypes which form geographic lineages: Asian, West African, and Central/East/South African (ECSA)(Volk et al., 2010; Lo Presti et al., 2016).

In late 2013, local transmission of CHIKV in the Americas was identified in Caribbean countries and territories (Leparc-Goffart et al., 2014). In 2014, there were greater than 2,400

travel-associated cases of CHIKV in the USA and high numbers of locally-transmitted cases reported from U.S. territories of Puerto Rico and the U.S. Virgin Islands, as well as isolated local transmission in Florida (CDC, 2014). The emergence of CHIKV in the America's poses as a threat to public health, especially in regions where dengue, and more recently Zika, are established.

The rapid spread of CHIKV in the Americas is largely due to high contact rates between anthropophilic *Ae. aegypti* and humans (Weaver, 2014). The Asian tiger mosquito, *Ae. albopictus*, is regarded as the most invasive mosquito in the world (Benedict et al., 2007). The first record of an established population of this species in the United States was in Texas in 1985. Subsequently *Ae. albopictus* spread throughout the eastern states, including Florida (Moore et al., 1988), (<http://www.cdc.gov/ncidod/dvbid/arbor/albopic97sm.htm>).

Aedes albopictus is an important vector of chikungunya, dengue, and Zika viruses as well as a laboratory-competent vector of many alphaviruses (e.g., Eastern equine encephalitis and Ross river viruses), flaviviruses (e.g., West Nile, Japanese encephalitis and yellow fever viruses) and bunyaviruses (LaCrosse and Rift Valley fever viruses) (http://www.cdc.gov/ncidod/dvbid/arbor/albopic_new.htm), (Shroyer, 1986; Mitchell et al. 1992). There is a high risk that CHIKV can become endemic in the United States. It was officially classified as a category C priority pathogen in 2008 by the US National Institute of Allergy and Infection Diseases (<http://www.niaid.nih.gov/topics/biodefenserelated/biodefense/pages/cata.aspx>). These factors have increased the needs for improved diagnostic tools for mosquitoes' collection, preservation and downstream detection of CHIKV and other arboviruses in biological samples.

Sampling the adult mosquito population is commonly achieved using mechanical traps (http://mosquito.ifas.ufl.edu/Mosquito_Surveillance.htm), followed by species identification, and screening groups (pools) of mosquitoes for the presence of pathogens. *Ae. aegypti* and *Ae. albopictus* are not efficiently captured by the most commonly used mosquito traps, such as the CDC miniature light trap, CDC gravid trap, or the New Jersey light traps (<https://www.cdc.gov/chikungunya/pdfs/surveillance-and-control-of-aedes-aegypti-and-aedes-albopictus-us>).

The in-field collected mosquitoes must be handled and processed in a way that minimizes the exposure to conditions that degrade the virus (heat, successive freeze-thaw cycles). Optimally, a cold chain (cold packs or dry ice) could be maintained until delivery to the processing laboratory. The pooled samples should be at -70°C for long storage.

The traps that use a fan will eventually induce mortality among mosquitoes attributable to desiccation. However, most mosquitoes collected for arbovirus surveillance will be alive if collected on a 24-hour schedule. This would be a typical collection cycle, as the batteries would need to be replaced for traps that use fans. Traps that do not use fans and provide honey cards will keep mosquitoes alive (Hall-Mendelin et al. 2010).

As alternative capture methods, dead mosquitoes can be placed on sticky cards or dried in ambient temperature for several weeks (Bangs et al., 2001; Mavale et al., 2012). A study demonstrated the persistence of CHIKV viral RNA, although not live virus, in *Ae. aegypti*

mosquitoes for up to 12 weeks at 28°C (Mavale et al. 2012), suggesting that CHIKV viral RNA is robust with respect to degradation at a temperature that approximate field temperatures used to trap mosquitoes.

Recently, FTA Whatman technology combines room temperature sample collection, transportation, and storage with quick NA purification in a single product. Following simple purification steps, NA remains bound to the matrix of FTA cards, which can then be used directly for downstream applications. With FTA Elute cards, NA will be eluted into water after purification and can be used for quantitative analysis (http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-us/products/AlternativeProductStructure_17096/).

In this study, we proposed and exploited the simple technology to collect, preserve and store biological samples (Figure 2). The laboratory-infected mosquitoes were tested on the cationic paper to reveal the CHIKV RNA by downstream PCR-amplification based Luminex direct hybridization assay. These results show that the NAs from mosquitoes can be trapped on Q-paper by a simple workflow that requires only the “squishing” of mosquitoes on the paper and addition of aqueous ammonia. The samples can then be transported or stored in a way that allows their NA components to be later analyzed.

These results also show that this workflow can be applied to chikungunya specifically. In this application, the measured LOD is better than required to allow this workflow to be used by PHS staff collecting mosquitoes in the field. As an added benefit, the same assay can detect four serotypes of dengue, although this was not shown here (but see (Glushakova et al., 2015)).

In this workflow, a mosquito having unknown status with respect to viral infection is squashed on a sample of Q-paper and treated with a drop or two of alkaline (pH 12, with added NaOH) NH₄OH solution to disintegrate the mosquito tissue and virus capsids and release viral RNA. These alkaline conditions also favor the denaturation and deactivation of nucleases, preventing enzymatic degradation of the viral RNAs. Further, the negatively charged viral RNA are not lost in any wash steps; these (as well as mosquito NAs) are coulombically bound to the positively charged paper surface via electrostatic interaction. The NA samples on Q-paper surface are then ready for the immediate pathogen detection, storage, or transport to remote laboratories.

The capture-storage-transportation workflow is compatible with most downstream NA-targeted assays, as the coulombically adsorbed NAs can be released by aqueous salt (1 M NaCl is used in this workflow). In this study, the detection of CHIKV RNA in Q-paper eluates was done by conventional SAMRS PCR combined with transliteration technology and followed by Luminex-based hybridization assay. This is the same procedure that we recently reported was able to detect as many as 22 different arboviruses (Glushakova et al., 2015). Here, SAMRS and AEGIS-based PCR and transliteration technology, allow highly multiplexed without PCR noise detection.

This approach also allows negative results to be reliable. We detected CHIKV RNA only in the infected mosquito samples, but not in non-infected mosquito samples and not in the

negative controls (Figure 4–5). Further, the output is at least semi-quantitative (Figure 6). Further, we could detect CHIKV RNA after testing samples being placed in long-term cold storage (-20°C for at least 10 months).

In fact, many downstream methods, including some field appropriate, can be used to amplify the target (instead of PCR) and to detect the amplicons (instead of Luminex). For example, as a part of separate study of our group (Yaren and Benner, in press), CHIKV RNA from Q-paper samples with infected mosquitoes was amplified by “loop-mediated amplification” (LAMP), an isothermal amplification method, directly from samples of Q-paper with Zika and CHIKV infected mosquitoes. Then, the amplicons were detected by simple fluorescence readout. Here, we did not need to purify nucleic acids from mosquito, permitting lower consumption of both time and reagents compared to kit-based purifications.

In summary, the PCR-Luminex process here is used to prove the assay. Its use does not require these more complex analytical tools.

In this study, the laboratory infected mosquitoes were used to demonstrate the usefulness of Q-paper technology. We carefully controlled the amount of virus provided to mosquitoes using an artificial membrane feeding system. We deliberately chose an amount of virus present in the blood meals that was within the range of CHIKV viral load in patients with symptomatic CHIKV infection (Appassakij et al. 2013). Arbovirus infection, disseminated infection, and transmission potential by mosquitoes are largely dose-dependent (for chikungunya virus, see Pesko et al. 2009). However, viral body titer is usually similar, regardless of the initial dose, for mosquitoes that progress to the point where they can disseminate a pathogen; these were the mosquitoes used in the current study (for West Nile virus, see Anderson et al. 2010, Richards et al. 2007; for St. Louis encephalitis virus, see Richards et al. 2012). Thus, the viral loads in the mosquitoes used in this study would be similar to those found in mosquitoes infected with chikungunya virus disseminating that virus in the field.

Accordingly to (Codeco et al. 2015), larvae also may be used for the surveillance of *Ae. aegypti*. There is evidence for natural vertical transmission of chikungunya virus in field populations in *Ae. aegypti* (Jain et al, 2016), showing that infected females may transmit chikungunya virus to their offspring. However, the utility of using larvae in an arbovirus surveillance program is questionable. Vertical infection occurs at much lower rates than horizontal transmission by bite, making it a minor contribution to chikungunya virus epidemiology. Also, density-dependence in the form of competition is common among container mosquitoes like *Ae. aegypti* (reviewed in Juliano, 2007). Consequently, mortality is high during the larval stages. Therefore, collections of larvae and testing them for the presence of arboviruses may over-estimate actual rates of vertical transmission in mosquitoes that survive to adulthood, the stage that would transmit chikungunya virus by bite to humans.

The Q-paper is a cheap, compact product that might find application not only for mosquito collection/pathogen detection but also in a wide range of forensic, pharmaceutical, biotechnological, research, and screening applications. It can supplement expensive

commercial products, as Whatman FTA/FTA Elute cards, that also provide solutions to collect, preserve, and purify biological samples for downstream screening procedures.

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Appendix A. Supplementary data

Table A1

SAMRS primers and AEGIS probes in this study. All reverse primers are 5'-biotinylated; the probes are 5'-amino -C12- modified. P, AEGIS nucleotide. A*, T*, G*, C*, SAMRS nucleotides.

Primers and probes identity	Oligoes' sequences, 5'-3'	Genome/region (bp)	Gene/region targeting	GenBank Accession No.
CHKV_Foward	ATGGCCACCTTTGC*A*A*G*C	7430-7447	Non-structural protein 4 (NS4)	AHX71796.1
CHKV_Reverse	GTACCTATTTAGGACCGC*C*G*T*A	7493-7514		
CHKV_probe	TTCPAPAAPCTCAPAPPACC	7460-7479		
Dengue1_Foward	GTCTTTCAATATGCTGAAA*C*G*C*G	105-127	Capsid protein C	FJ639679.1
Dengue1,3_Reverse	TGAGAATCTCTTCGCCAAC*T*G*T*G	152-174		
Dengue1_Probe	CPAPAAACCPCTPTCAACT	128-147		
Dengue2_Foward	GAGGCCACAAACCATG*G*A*A*G	10455-10474	3'-UTR	JX073928.1
Dengue2,4_reverse	GGAGGGGTCTCTCT*A*A*C*C	10501-10519		
Dengue2_probe	CPCATPPCTAPTTPACTAP	10480-10499		
Dengue3_Foward	GTCTATCAATATGCTGAAA*C*G*C*G	103-128	Capsid protein C	EU482596.1
Dengue1,3_Reverse	TGAGAATCTCTTCGCCAAC*T*G*T*G	153-175		
Dengue3_probe	APAAACCPPTPTCAACTPP	131-151		
Dengue4_Foward	ATGCGCCACGGAA*G*C*T*G	10363-10379	3'-UTR	GQ199883.1
Dengue2,4_reverse	GGAGGGGTCTCTCT*A*A*C*C	10403-10421		
Dengue4_probe	PCPTPPCATATTPACTAPC	10383-10402		

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Figure 1.

Adapted with permission with re-composition from (Glushakova et al., 2015). (A) An artificially expanded genetic information system (AEGIS) adds up to eight nucleotides that adds four additional base pairs to the four standard nucleotides by strategically rearranging hydrogen bonding patterns. The Z: P pair is exploited in this work. (B) The strategic removal of hydrogen bonding groups gives a self-avoiding molecular recognition system (SAMRS). (C) The “transliteration” strategy allows a template G to direct the incorporation of Z by primer extension using a mismatching between G and deprotonated Z.

Figure 2.
The procedure of CHIKV detection in mosquito on Q-paper. Mosquito is original artwork by LG (a full description is done in the paper text).

Figure 3.

(Left) Overview of the Luminex xMAP assays developed to detect chikungunya viral RNA. (Right) Comparison of Luminex profiles using standard nucleotides (blue) and modified nucleotides from synthetic biology technology (red) (from Glushakova et al., 2015, with permission). The Q-paper procedure included the following stages. Q-paper eluate containing mosquito's nucleic acids and chikungunya virus RNAs were RT-PCR-amplified with set of chikungunya-specific SAMRS primers (Table A1). Each amplicon was subject to reverse primer extension with transliteration (conversion), where dCTP was substituted by artificial dZTP. Finally, identities of biotinylated PCR-amplicons built with dATP, dTTP, dGTP and dZTP were confirmed by Luminex direct hybridization assay with chikungunya-specific probes, containing AEGIS dPTP. MFI, median fluorescence intensities (Luminex units).

Figure 4.

Detection of CHIKV RNA in infected *Ae. aegypti* mosquitoes by Luminex direct hybridization assay, two weeks after sample preparation. (A) 35 cycles of conventional multiplexed RT-PCR with chikungunya virus-specific SAMRS nucleotides built primers (Table A1). Aliquots of the reaction mixture (4 μ L of each 20 μ L) were loaded on a 2.5 % TBE agarose gel and visualized by the ethidium bromide staining. Samples (#) were desalted high salt eluates from Q-paper sheets containing squashed mosquito bodies on the surfaces. First lane from the left, 25 bp PCR markers; CHIKV, chikungunya virus RNA, Luminex positive control; C1, non-infected mosquito control (average of n=3); and C2, ddH₂O-wash of the infected mosquito sample (average of n=5). (B) Luminex direct hybridization assays profiles of samples visualized on TBE agarose by ethidium bromide staining (A). MFI, median fluorescence intensities.

Figure 5.

Detection of CHIKV RNA in infected *Ae. aegypti* mosquitoes' tissues by Luminex direct hybridization assay (LDHA): 10 months after mosquitoes' samples preparation on Q-paper (#8, #13-14). Luminex DHA profiles of biotinylated amplicons, (A) products of conventional monoplexed RT-PCR with chikungunya virus-specific primers; (B) products of five-fold multiplexed RT-PCR executed with CHIKV and dengue1-4 viral-specific primers (Table A1); the panel on the right presents monoplexed Luminex Direct Hybridization Assays (LDHA) executed with the same samples #8 and #13 for a comparison. Positive assay's controls was CHIKV RNA amplicon, negative controls C1 (average of n=3) and C2 (average of n=3) were eluates of the non-infected mosquito NA and ddH₂O -wash from the infected mosquito sample correspondingly. The panel from the left indicates the viral probes identities (dengue1, D1; dengue2, D2; dengue3, D3; chikungunya, CHIKV) annealed to Luminex beads (##29,62,69,87,97). Each sample was run in triplicate. MFI, median fluorescence intensities.

Figure 6.

Luminex DHA profiles executed with PCR–amplicons of CHIKV RNA eluates from Q-paper. The CHIKV pure RNA solution was serially diluted, and each RNA dilution was loaded on the top of Q-paper. RNA copies in each load are given on the top of each bar. The RNA were eluted by high salt solution (150 μ L of 1M NaCl), column-desalted, collected in 50 μ L of water, and 5 μ L aliquots of each sample was PCR – amplified. The numbers on the bars' bottom are the RNA copies in the eluates' aliquots taken into RT-PCRs. They are counted from the consideration that elution efficiency was 100% and RNA were intact. Sim, CHIKV, 85 base pairs amplicon, in vitro produced RNA fragment simulated viral RNA (Luminex DHA positive control). Each sample was run in triplicate. MFI, median fluorescence intensities.

Table 1

Chikungunya virus (CHIKV) infected mosquitos sample on Q-paper sheets

Q-paper samples identity #	NH4OH solution concentration (M)	Volume NH4OH per Q-paper, μL	CHIKV titer in mosquito leg tissues by real time RT-PCR, genomes/mL [*]
2 ^I	1	200	5.1×10^3
6 ^I	1	200	4.37
7 ^I	1	200	4.4
8 ^I	1	200	N/A
11 ^A	0.1	100	28.2×10^3
12 ^A	0.1	100	12.6×10^3
13 ^A	0.1	100	73.4×10^3
14 ^A	0.1	100	24.9×10^4

^{*} N/A, not analyzed. Mosquitoes infected with the Indian Ocean and Asian lineages of CHIKV are denoted by “I” and “A” respectively.