FTA Cards Facilitate Storage, Shipment, and Detection of Arboviruses in Infected *Aedes aegypti* Collected in Adult Mosquito Traps

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Abstract. The utility of applying infected *Aedes aegypti* to Flinders Technology Associates (FTA®) cards for storage, transport, and detection of dengue, Zika, and Barmah Forest viruses was assessed in laboratory-based experiments. The mosquitoes had been removed from Gravid *Aedes* Traps maintained under conditions of high temperature and humidity. RNA of all viruses could be detected in infected mosquitoes on FTA cards either individually or in pools with uninfected mosquitoes, and stored for up to 28 days. Importantly, there was only a minimal decrease in RNA levels in mosquitoes between days 0 and 28, indicating that viral RNA was relatively stable on the cards. FTA cards thus provide a mechanism for storing potentially infected mosquitoes collected in the field and transporting them to a central diagnostic facility for virus detection.

The global burden of arboviruses continues to increase. Zika virus (ZIKV) and chikungunya virus (CHIKV) have emerged across multiple continents in the last decade, whereas the dengue viruses (DENVs) remain the leading cause of arboviral disease in tropical and subtropical regions.^{1–3} These viruses are predominantly transmitted by the peridomestic mosquitoes *Aedes aegypti* and *Aedes albopictus*.

As part of a comprehensive surveillance and control program, arbovirus detection in mosquito populations can incriminate vector species before and during outbreaks, provide information on the geographical distribution and intensity of virus transmission, and provide a template for virus genotyping. A number of adult traps have been developed to sample *Ae. aegypti* and *Ae. albopictus*, including sticky ovitraps, Biogents (BG)-sentinel traps (Biogents AG, Regensburg, Germany), and Gravid *Aedes* Traps (GATs).^{4–6} DENVs have been detected in mosquitoes removed from these traps in either a field or laboratory setting.^{7–9} However, during recent dengue outbreaks in the Torres Strait, northern Australia, and the Solomon Islands, we were unable to detect DENVs in *Ae. aegypti* and *Ae. albopictus* specimens, despite apparently intense virus transmission as evidenced by human cases. It is likely that mosquitoes may not have been infected, but it was also suspected that viral RNA may have degraded in samples due to suboptimal storage of the samples before dispatch.

A potential solution to these issues is storage of mosquitoes on nucleic acid preservation cards, such as Flinders Technology Associates (FTA®; GE Healthcare Life Sciences, Pittsburgh, PA) cards, which inactivate the virus and bind the liberated RNA within a fiber matrix. Although FTA cards have previously been used to store other viruses, including arboviruses, their utility for storing infected mosquitoes has not been assessed.^{10–12} Herein, we report laboratory-based experiments undertaken to evaluate the ability of FTA cards

to preserve DENV, ZIKV, and Barmah Forest virus (BFV; an Australian alphavirus related to CHIKV) RNA in individual mosquitoes and in pools of uninfected mosquitoes up to 28 days after being removed from GATs.

Virus strains used for the experiments were DENV type 2 (DENV-2) strain ET300 isolated from a traveler from East Timor in 2000, ZIKV strain TS17-2016 isolated from a traveler from Tonga in 2016, and BFV isolated from a patient from Queensland, Australia, in 1989. Laboratory-reared *Ae. aegypti* (Innisfail strain, northern Australia), were inoculated with approximately 217 nL of inoculum containing approximately 10^{5.0}, 10^{5.5}, and 10^{7.3} tissue culture infectious dose (TCID₅₀/mL) of DENV-2, ZIKV, and BFV, respectively, using a Nanoject II (Drummond Scientific, Broomall, PA). Mosquitoes were then maintained at 28°C, high humidity, and a 12:12 light:dark (L:D) photoperiod within an environmental growth cabinet (Sanyo Electric, Gunma, Japan), and offered 15% honey water as a nutrient source.

Seven days postinoculation, mosquitoes were anesthetized with CO₂, before their heads were removed and placed in 2-mL tubes containing a 5-mm stainless steel bead and 1 mL of growth media (Opti-MEM; Gibco, Invitrogen Corporation, Grand Island, NY) supplemented with 3% fetal bovine serum, antibiotics, and antimycotics. Tubes were stored at –80°C. Heads were retained if required for retrospective analysis of mosquito infection status.

Large GATs (Biogents AG) were assembled according to the manufacturer's instructions, and placed in an environmental growth cabinet set at 28°C, high humidity, and 12:12 L:D photoperiod to simulate field conditions, as per the method of Ritchie and others.⁹ Twenty-four hours later, mosquito bodies minus their heads were placed individually or with nine dead uninfected *Ae. aegypti* on the nylon gauze of the catch bag within the GAT. The mosquitoes were removed after 7 days. Fifteen mosquitoes were squashed individually with a polypropylene pestle (Sigma-Aldrich, St. Louis, MO) directly onto FTA cards and the excess chitin, legs, and wings were removed. Five other individual mosquitoes were placed into separate 1.5-mL microfuge tubes and homogenized in 0.5 mL of RNase-free water with a polypropylene pestle before another 0.5 mL of RNase-free water being added to provide a final volume of 1 mL. Eight pools of 10 mosquitoes were processed as described

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TABLE 1

Detection of DENV-2, ZIKV, and BFV RNA in mosquitoes stored for up to 28 days on FTA cards

	Day*	DENV-2	ZIKV	BFV
Individual mosquitoes	0	20.7 ± 1.4‡	17.8 ± 0.6	17.3 ± 0.8
	7	19.3 ± 1.1	18.1 ± 0.7	20.0 ± 5.2
	14	19.2 ± 1.0	17.8 ± 1.4	15.2 ± 1.0
	28	19.0 ± 0.3	18.2 ± 0.2	16.7 ± 1.5
Pools†	0	24.2 ± 0.6	20.9 ± 1.0	19.4 ± 1.5
	7	24.7 ± 1.2	22.5 ± 1.2	22.1 ± 1.2
	14	24.9 ± 1.7	22.7 ± 0.8	20.6 ± 1.6
	28	25.6 ± 1.2	23.6 ± 0.6	21.8 ± 1.1

BFV = Barmah Forest virus; DENV-2 = dengue virus type 2; FTA = Flinders Technology Associates; ZIKV = Zika virus. All mosquito samples were positive by real-time TaqMan reverse transcription polymerase chain reaction.

*Day after mosquito samples applied to the FTA cards.

†One infected and nine noninfected mosquitoes.

‡Mean ± standard deviation cycle threshold (C_t) values of five individual mosquitoes or eight pools; a C_t value < 40.00 is equal to a detected result, whereas C_t values > 40.00 are equivocal to not detected.

above. The pools were briefly centrifuged in a mini personal centrifuge (Tomy Kogyo Co., Ltd., Tokyo, Japan) before 150 μ L of homogenate was applied to the center of the marked sample circle of three separate FTA cards, so that the same pools could be sampled at different time points postapplication. The day 0 individual and pooled mosquito homogenates were stored at -80°C . All FTA cards were allowed to air-dry for 1 hour before being stored in an envelope at room temperature.

To test the stability of the viral RNA, five individual mosquitoes and one aliquot of each of the eight pools were sampled 7, 14, and 28 days after being applied to the FTA cards. Nucleic acids were eluted from the cards using the method of Hall-Mendelin and others¹² with the modification of cutting out each mosquito sample, which was then cut into 2–4 strips depending on the size of the area of dried mosquito material. Viral RNA was extracted from 140- μ L samples of the eluates using a Bio Robot Universal System (Qiagen, Hilden, Germany) and the QIAamp[®] Virus Bio-Robot[®] MDx Kit (Qiagen, Clifton Hill, Australia) according to the manufacturer's instructions. Viral RNA was detected using real-time TaqMan reverse transcription polymerase chain reaction (RT-PCR) assays specific for DENVs, ZIKV, and BFV.^{13–15} Controls for each assay included positive and negative extraction controls, synthetic primers and probes, and no-template controls. The threshold cycle number (C_t) was determined for each sample and a positive result, in which viral RNA was detected, corresponded to any C_t value which was < 40 cycles.

Viral RNA was detected in all mosquito samples removed from GATs and stored on FTA cards at room temperature irrespective of the virus or the day sampled. The RNA on the cards remained stable during this time, with only a minimal increase in C_t values, representing a decrease in RNA copy number, for all three viruses in the pools observed between 0 and 28 days (Table 1). Although the virus may have degraded slightly during storage, relatively small fluctuations in C_t values may also be due to differences between the initial virus titer in infected mosquitoes, loss of material during application to the FTA cards, elution, extraction manipulations, or the slight variation in efficiency of the TaqMan assays between runs. Regardless of these parameters, the highest mean C_t value was 25.6, more than 14 C_t values lower than the threshold C_t value for equivocal or nondetection (≥ 40). At the infectious doses of virus used to

inoculate the mosquitoes in our study, we have so far not identified any factors hindering the detection of any of these viruses in mosquitoes stored on FTA cards.

A number of studies have previously demonstrated the ability of RT-PCR to detect arbovirus RNA in laboratory-infected mosquitoes stored under different temperature regimes or in field-collected mosquitoes.^{7,8,16–18} We have extended these studies by providing a simple and highly effective method for preparation, storage, and transport of mosquito specimens removed from traps by field workers in locations geographically isolated from diagnostic facilities. We deliberately used rudimentary methods for preparing and applying the mosquito samples to the FTA cards which do not require specialized equipment. The FTA cards can then be shipped without a cold chain in regular post from remote locations.

Our results suggest that infected mosquitoes can be stored on FTA cards either individually or in pools for at least 28 days without losing the ability to detect viral RNA. However, in a field context, cards would most likely be processed sooner than 28 days so that a control response can be initiated with minimum delay. Furthermore, the FTA cards inactivate virus on contact,¹² so the quarantine or biosecurity risk associated with the importation of potentially infected insects is reduced. Finally, eluted nucleic acids can serve as a template for genome sequencing of viruses or for mosquito species identification.^{14,19}

Received December 15, 2016. Accepted for publication January 14, 2017.

Published online March 6, 2017.

Acknowledgments: We thank Scott Ritchie for providing *Aedes aegypti*. We also thank Jane Cameron, Bruce Harrower, and Sean Moody for technical assistance, and Jamie McMahon for initial discussions relating to the study.

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