UPPSALA
UNIVERSITET

Safety and Stability of Samples Stored on Filter Paper for Molecular Arbovirus Diagnosis

Emelie Bringeland

Master Degree Project in Infection Biology, 30 credits.Spring 2021
Department: Medical Biochemistry and Microbiology
Supervisors: Johanna Lindahl and Jiaxin Ling

Table of Contents

Abstract

Expanding urbanization, climate change, and population growth contribute to increased transmission and spread of arthropod-borne viruses (arboviruses), many of which cause severe disease in humans. Pathogenic arboviruses include dengue, Zika, tick-borne encephalitis, and sindbis viruses, which together threaten more than half the global population. Thus, there is a constant need for safe, specific, and sensitive molecular tests to identify early-stage infections for accurate diagnosis and molecular epidemiological data for disease prevention and control. The study tested the biosafety of using FTA™ cards when working with pathogenic arboviruses by conducting an infectivity assay using sindbis virus. Conditions for RNA extraction and storage of arboviruses on FTA were analyzed by measuring viral RNA (vRNA) stability using a SYBR-Green, Pan-Flavi RT-qPCR method composed of degenerate primers able to detect a variety of flaviviruses. Data from a Pan-Flavi RT-qPCR study comprising of 222 clinical blood and serum samples collected from a 2018 dengue virus outbreak in Hanoi (Vietnam) was analyzed to establish applicability of FTA for molecular epidemiology and diagnosis. Results showed that sindbis virus infectivity was inhibited by FTA-adsorption. FTA-adsorbed arboviruses were extracted with the highest yield using Trizol extraction and were preserved at storage at 4-20ºC for up to 30 days. The results showed that clinical blood samples acquired higher yields of vRNA for molecular testing than serum samples and that it may be possible to perform sequencing for genomic analysis. The study suggests that FTA cards may facilitate the storage and transportation of adsorbed arboviruses for downstream molecular epidemiological and diagnostic tests.

Keywords and Abbreviations: Arthropod-borne viruses (arboviruses), alphaviruses, flaviviruses, dengue virus (DENV), Japanese encephalitis virus (JEV), Tick-borne encephalitis virus (TBEV), Usutu virus (USUV), Zika virus (ZIKV), Flinders Technology Associates™ card (FTA), Biosafety, Vietnam

Popular Summary

The COVID-19 pandemic showed the world the importance of measures needed for disease prevention and control. Observations from the last decades show that growing human populations, increased urbanization, and climate change, all contribute to increased risk of spreading infectious diseases by dispersal of arthropods such as mosquitoes and ticks. Viral infections spread by arthropods are referred to as arthropod-borne viruses (arboviruses) and contribute to a significant proportion of infectious diseases globally. Dengue virus (DENV) is the most prevalent arbovirus, with half the global population at risk of infection and causes approximately 40,000 deaths annually. The emergence of previously uncommon diseases such as Zika and tick-borne encephalitis are becoming more frequent and widespread causing concern for public health.

Accurate and rapid diagnostic tests facilitate measures for disease prevention and control. However, many arboviruses are highly pathogenic and thus require specialized personnel and facilities to work with them safely. Therefore, there is a need for safe, specific, and sensitive tests which can identify viral infection to allow accurate diagnosis and facilitate epidemiological studies. Flinders Technology Associates™ cards (FTA) are specialized papers that allow preservation of a variety of biological samples, including viruses, stored at freezing and room temperature for downstream tests. However, there is a lack of information regarding arboviral samples and suitable parameters for storage and sample extraction.

This study wanted to investigate the potential of using FTA cards as a tool for arboviral sample preservation for downstream tests that can identify specific pathogens on a molecular level and provide genetic information. In order to establish the suitability of FTA cards for arboviruses, the infectivity of samples dried on FTA was investigated and different extraction and storage conditions were tested. Clinical blood and serum samples stored on FTA from hospitalized dengue patients in Hanoi (Vietnam), were analyzed to investigate the applicability of FTA for arboviral molecular tests.

This study found that arboviruses can be inactivated by drying on FTA cards and that storage at refrigeration and room temperature effectively preserved some arboviral samples. The patient samples indicate that blood samples can be stored on FTA cards for later molecular diagnostic tests to identify the specific cause of infection. Thus, the study suggests that FTA cards can be used for arboviral samples to facilitate safer sample handling and transportation while providing important diagnostic information, such as specific causes of infection that may assist in disease prevention and control.

Introduction

Arthropod-borne viruses, or arboviruses, are transmitted by arthropod vectors such as mosquitoes or ticks and contribute to an increasing public health concern.[1–3] Among some of the most notable are flaviviruses such as dengue virus (DENV) or Japanese encephalitis virus (JEV) which contribute to widespread morbidity and mortality. Alphaviruses are also seen as an increasing threat which alongside flaviviruses are referred to as emerging or re-emerging diseases, posing a threat for future epidemics or pandemics.[4,5] Both are enveloped, positive-sense single-stranded RNA viruses (+ssRNA), where the flavivirus genus consists of more than 70 different viruses causing from asymptomatic, mild to severe illness, whereas alphaviruses such as Chikungunya (CHIKV) and Sindbis (SINV) viruses, cause acute to chronic arthritis.[6,7,8]

Many pathogenic flaviviruses are steadily on the incline in different regions and occasionally cause outbreaks in new locations.[5] One of the most concerning and well-known is DENV which is estimated to infect up to 400 million people every year.[9] The distribution of DENV can in part be explained by the vast dispersal of its main vector the *Aedes* mosquito which facilitates the transmission of DENV when introduced to new areas. Once introduced, susceptible vectors need to be present and competent, meaning that transmission of the virus is successful from vector to host. This is often dependent on the vector species, where *Aedes aegypti* is regarded as a more competent vector for DENV transmission than *Aedes albopictus*, for instance.[10] Furthermore, *A. aegypti* is anthropophilic and is highly adapted to breeding and living in urban dwellings, assisting circulation and transmission of disease.[5,11] Additionally, travel, globalization, and increased urbanization account for the geographical distribution of vectors, allowing further spread and introduction to new areas. These global changes have been apparent for other flaviviruses such as Zika (ZIKV), Japanese encephalitis (JEV), West Nile (WNV), and yellow fever (YFV) viruses, which can cause outbreaks or are spreading to new parts of the world. Climate change is also considered a contributing factor, where more Northern regions of Europe and Asia are seeing a steady incline of tick-borne encephalitis virus (TBEV) transmitted by the tick *Ixodes ricinus* and Usutu virus (USUV) by the mosquito *Culex pipiens*.[2,3] This is of particular concern in Sweden, where TBEV is endemic, but cases have increased by 5% since 2005 and the incidence being six times higher than the overall incidence in all of Europe.[12,13] Thus, flaviviruses pose a constant and increasing socioeconomic and public health concern, and focus on molecular epidemiology of flaviviruses in affected areas is required for continuous efforts of disease prevention and control.

Molecular Characterization of Flaviviruses

Flaviviruses are characterized as small +ssRNA viruses around 50 nm in size.[6,7] The genome size ranges from 9-13 kb, consisting of three structural (C, M, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. They encode a single open reading frame, translated at the endoplasmic reticulum into a polyprotein which then is cleaved by viral and host cell proteases. Since flaviviruses are +ssRNA viruses, they require an RNA-dependent RNA-polymerase (RdRp), corresponding to the NSP5B protein, an ideal target for antiviral activity.[7] The NS3 and NS5 proteins are highly conserved in flaviviruses and are therefore ideal for targets of flavivirus identification.[14]

Dengue virus in Vietnam

Since the 1960s, dengue transmission has expanded in correlation to the dramatic urban growth that was seen in cities in tropical developing countries where millions of susceptible people migrated from rural areas.[15] Dengue is endemic in over 100 countries, with Vietnam being one of the most burdened and causing regular outbreaks in correlation with the monsoon season.[2,3] In 2017, the largest DENV outbreak to date was recorded in Vietnam with subsequent over

81,000 cases and 32 deaths.[16] More than 8 million people reside in the capital of Vietnam, Hanoi, posing a huge threat for DENV outbreaks. The health burden of dengue can be estimated in terms of Disability Adjusted Life Years (DALYs), which accounts for a sum of healthy life years lost due to premature mortality or productive life lost due to disability. A study from 2016 estimated that dengue in Vietnam was responsible for nearly 40 thousand DALYs per year, which represents an economic burden of US$94.87 million per year.[17]

Dengue Serotypes and Disease

There are four serotypes of DENV (DENV 1-4) with several genotypes of each, posing a risk of transmission and infection in humans.[15] The E-protein due to its critical role in host cell receptor-mediated endocytosis and fusion, is a primary target for neutralizing antibody (NAb) formation.[15,20] Genetically, there is a degree of variability in the E-protein among DENV serotypes, with up to 37% amino acid divergence which results in them being antigenically distinct from each other. The infection of one serotype gives lifelong immunity but only temporary cross-protective immunity against heterotypic serotypes. It has been observed that the conserved regions are similar enough to produce temporary cross-protection between serotypes but also contribute to increased risk of developing severe disease upon secondary infection of a different serotype.[7,21,22] This is due to the process of antibody-dependent enhancement (ADE), which is the result of viruses being able to evade the effects of NAbs to cause more severe infection.[23] A theory is that the viral replication is facilitated during ADE through the internalization of the NAb-virus complex in white blood cells such as macrophages, acting as viral replication sites. Though DENVs are divided into different serotypes, they are considered a single species due to shared geographical distributions, similar host and vector associations, and disease manifestation.[15] Therefore, the complexity of DENV serology is a major challenge in the treatment of secondary infection and vaccine development.

Dengue is a mostly self-limiting infection and febrile disease but in 1-5% of cases causes severe illness or even death.[3] Humans are natural reservoirs for DENV and the virus is most commonly transmitted through the *A. aegypti* or *A. albopictus* mosquitoes.[11] It has also become apparent that the mosquitoes are capable of transovarial or sexual transmission of DENV.[18,19] Thus, there is an urgent need for vector control and infection prevention.

Flavivirus Epidemiology and Control

The large and expanding global distribution of flaviviruses sets populations in all regions in the world at risk.[2,24] The infection can be asymptomatic or cause febrile illness, hemorrhagic fever, hepatitis, encephalitis, birth defects, or death.[1,11,20] During the last decades, there has been a dramatic increase in incidence, disease severity, and spread of DENV, YFV, JEV, and WNV. For instance, dengue is endemic in large parts of the tropics and subtropics with regular outbreaks occurring in correlation to increased mosquito populations after the yearly monsoon period. However, an alarming expansion has been observed with sporadic outbreaks occurring in non-endemic areas where mosquito vectors are present.[15] Similarly, ZIKV was relatively unknown before 2007 but following its introduction to Brazil, led to the devastating outbreak that occurred in 2015-2016.[17]

Despite an increase of outbreaks in both endemic and nonendemic areas, there have been numerous efforts and successes to prevent flavivirus disease. The live-attenuated YFV vaccine has been used for the last 50 years and has shown to be one of the most successful to prevent viral disease but requires iterative boosters to maintain protection. Similarly, boosters are required for the TBEV and JEV vaccines.[2,11,23] Thus, widespread immunity through vaccination quickly becomes expensive.

A major difficulty is the extensive cross-reactivity seen between flavivirus-immune sera, making it very difficult to develop and use diagnostics to track and manage outbreaks as well as produce effective and useful vaccines that would not induce such a reaction. In 2016, the tetravalent, live-attenuated vaccine, Dengvaxia (Pasteur-Sofi), was licensed and developed to elicit simultaneous immunity against four DENV serotypes. However, serological studies quickly showed that it was most protective if the patients had already been infected by one serotype before vaccination and that vaccination with no prior infection induced an increased risk for severe disease.[23,26]

Efforts to control infection in the mosquito vector have also shown potential in disease control.[23] For instance, a study looked at using *Wolbachia*-infected *A. aegypti* and showed reduced DENV transmission due to *Wolbachia* infection increasing viral RNA (vRNA) degradation inside the mosquito.[27] However, many concerns are raised when considering releasing genetically engineered mosquitoes into the wild.

Diagnosing Flavivirus Infections

Due to the ambiguity of symptoms for flavivirus infection, clinical diagnosis is not reliable and laboratory tests are required to identify the specific pathogen. The current gold standard for flavivirus infection diagnosis is primarily viral isolation which is dependent on the patient being in a viremic state upon sample collection. This is particularly hazardous as a biosafety facility is required, due to that many pathogenic flaviviruses belong to biosafety level (BSL) 2 and 3. Typically, it takes 2-7 days from disease onset to reach the viremic phase whilst the duration varies between different viruses. After the viremic phase, 5-7 days after onset of disease, the host immune system typically starts producing specific antiviral antibodies, which can last up to several months to years. Many diagnostics labs use serological tests, such as ELISA for the detection of virus-specific antibodies.[28–30] However, this method is not reliable in the early stages of infection as specific antibodies will not have been produced yet and are subject to cross-reactions of other flavivirus antibodies and thus becomes less specific. In contrast, molecular techniques, such as PCR can detect viral particles in the viremic state of a patient and have been shown to be highly specific, sensitive, and less time-consuming than serological assays. Many studies have focused on and have tested flavivirus-specific molecular assays to be able to detect and differentiate between flaviviruses by targeting conserved regions of the genome.[31–33] This serves as very beneficial for diagnostics, as it allows simultaneous detection of multiple flaviviruses quickly and efficiently.

Flinders Technology Associates™ Cards

A difficulty when working with either clinical or field samples, is access to proper storage and transportation conditions for optimal preservation, especially in more rural or economically challenged areas. Flinders Technology Associates™ (FTA) cards are chemically treated filter papers that have the potential to replace the cold chain, facilitate easier transportation, and provide appropriate preservation of a variety of biological samples for later analyses.

When samples are placed on FTA cards, cell membranes and organelles are lysed, and proteins denatured. This allows the fixation of nucleic acids and prevents degradation and loss of genomic information.[34] Nucleic acids are immobilized and stabilized within the fibers of the matrix on FTA and are protected from nucleases, oxidative stress and UV damage. The components of FTA cards are patented, though it is known that they contain chaotropic salts, which disrupt hydrogen bonds and van der Waals forces, consequently leading to a denaturing effect on proteins, lipids, and nucleic acids.[35] To date, genomic DNA has been stored up to 14 years at room temperature on FTA and has allowed successful PCR amplification.[34]

There have been numerous studies evaluating different extraction methods of samples from FTA and comparisons to conventional methods of preservation. Among them, it has been found that there are suitable methods for DNA extraction and that FTA allows for maintaining and extracting genomic DNA from samples including whole blood, cell, tissue, and bacterial cultures, and even mosquito saliva.[36–40] One of the typical uses for FTA is to collect and transport plasma or serum samples, providing dried blood and serum spots (DBS) which can allow the later analysis of contents. For example, the WHO recommends using DBS as an alternative for the collection and transportation of plasma for retroviral samples and has been shown to successfully allow downstream tests.[41,42] This facilitates the diagnosis and treatment of HIV-infected patients.

The FTA cards are marketed as a tool for sample collection and preservation of field samples for PCR analysis but are not specified to be used for RNA samples. Despite this, increasing research has been focused on and has shown success in applications of vRNA, in addition to DNA samples.[43–45] However, individual viral families and techniques require more knowledge to evaluate applicability and there is a lack of studies focusing on the important alpha- and flaviviruses.

Handling Pathogenic Samples: A Biosafety Concern

A major concern when working with pathogenic material is the biosafety for the handler, especially when special biosafety facilities are not easily accessible. An alternative is to work with inactivated viruses, making them safer to work within a laboratory setting. Multiple research groups have demonstrated that inactivation of vRNA occurs upon contact and drying for at least 1 hour on an FTA card.[32,43] However, the inactivation of alpha- and flaviviruses have not been established yet.

Viral RNA Stability on FTA™ Cards

The stability of samples is dependent on the conditions of transportation and storage, where sub-optimal conditions increase the risk for degradation or low-quality samples. This also applies to vRNA samples, both from enveloped and non-enveloped viruses. A study found that the enveloped, -ssRNA, rabies virus in suspension could be stored on FTA for up to 30 days in ambient temperatures with minimal decrease in quality during downstream molecular tests.[32] Additional studies have used different sample types and optimized protocols to evaluate the stability of infectious bursal disease virus (a dsRNA virus), and more recently, SARS-CoV-2 from saliva samples, which like flaviviruses and alphaviruses is a +ssRNA virus.[46–48] Therefore, it has been previously shown that FTA cards can effectively be used for -ssRNA, +ssRNA, and dsRNA viruses. However, there is no optimized protocol or directive for use of flavivirus samples on FTA and downstream tests.

Aim

The study aims to evaluate the use of Whatman™ FTA™ Classic cards for the collection and sampling of flaviviruses and alphaviruses for downstream tests.

i. **Evaluate the biosafety of using FTA™ cards for arbovirus samples.**
ii. **Evaluate stability of viral RNA samples placed on FTA™ cards.**
iii. **Evaluate the application of using FTA™ cards for molecular tests.**

Material and Methods

Viruses

Five different virus strains were used to represent alphaviruses, flaviviruses, and BSL-2 and -3 infectious agents, respectively (see **Table 1)**. Virus isolates were provided in Vero cell culture medium in DMEM (Gibco, 41966) supplemented with 10% heat-inactivated FBS (Gibco, USA) and 1X Penicillin-Streptomycin (Sigma-Aldrich, PA333). Virus stock dilutions were stored at -80°C before FTA card placement and RNA extraction.

Virus strains used for Study		
Alphavirus	**Strain**	**Biosafety Level (BSL)***
Sindbis virus, SINV-1	*09-M-358 strain*	2
Flavivirus		
Japanese encephalitis virus, JEV	*Nakayama strain*	3
Tick-borne encephalitis virus, TBEV	*European strain*	3
Usutu virus, USUV	*Merula strain*	2
Zika virus, ZIKV	*African strain MR-766*	2

Table 1 *BSL classification according to the Swedish Work Environment Authority (Arbetsmiljöverket).

Infectivity of viral RNA on FTA™ card

To test if the virus is inactivated upon contact with the FTA™ Classic card (GE Healthcare UK Limited) an infectivity assay was performed comparing the toxicity of the FTA and SINV-1 infection on Vero cells. A dilution of SINV-1 with 1.15 $*10^6$ PFU/ml was used and 125 µL transferred to FTA discs and left to dry for two hours in the hood. The disc with SINV-1 and a blank disc was cut out and placed in 1 ml PBS at 4°C overnight. The supernatant of the discs in PBS was extracted and used for 1:10 dilution series using DMEM (with 2% heat inactivated Fetal Bovine Serum, FBS) medium. SINV-1 from a stock solution was also used for a dilution series as a control. The plate was incubated for 1 h containing 100 µL of dilution sample in each well before replacing the supernatant with 200 µL DMEM (with 2% heat inactivated FBS) medium and incubated at 37°C for 2 days for observation.

Comparing RNA Extraction Methods from FTA™ Cards

To establish the most efficient RNA extraction method from samples placed on FTA, six different RNA extraction methods and kits were tested: Trizol (Invitrogen), Trizol LS (Invitrogen), QiAmp viral RNA Mini Kit (Qiagen), RNeasy Mini Kit (Qiagen) and Direct-zol (Zymogen) using Trizol and Trizol LS. An amount of 250 µL SINV-1 virus stock dilution (1.15 $*10^6$ PFU/ml), was placed on an FTA and left to dry for 2 h under the hood. One-eighth of the FTA disc was cut out and RNA extraction methods were tested to compare the efficiency of RNA extraction from samples placed on FTA. Individual protocols were followed as per kit and eluted in 35 µL of DNase and RNase-free water.

FTA™ card Sample Preparation

To analyze the RNA stability of different alpha- and flaviviruses on FTA, virus dilutions of SINV-1, ZIKV, and USUV were placed on FTA™ Classic cards and stored in different temperatures up to 30 days. JEV and TBEV were prepared in a BSL-3 facility by an authorized person. A 125 µL of a virus stock 1:10 dilution with DMEM (with 2% FBS) was dropped on the FTA which was left to dry under the hood for 2 hours before sealing them in plastic bags and placing them in different temperatures (+4°C, +25°C, or +37°C). Time points for collection of cards were 1, 7, 15, and 30 days, where cards were placed at -80°C until analysis.

RNA Extraction from FTA™ Cards

All RNA extractions were performed using a Direct-zol™-96 RNA kit (Zymo Research). Briefly, one-fourth of an FTA disc was cut out, placed in 300 µL Trizol and briefly vortexed before incubating at 4°C overnight. All RNA extractions were performed the following day and according to the kit protocol. Elution was performed using 35 µL DNase and RNase-free water and stored at -80°C.

Complementary DNA Synthesis

Complementary DNA (cDNA) synthesis was performed using the iScript™ cDNA Synthesis kit (BioRad). About 2 µL RNA was used in a 20 µL reaction also containing, 4 µL 5x iScript Reaction Mix and 1 µL iScript Reverse Transcriptase. Running conditions consisted of annealing at 25°C for 5 min and then 46°C for 20 min and inactivation at 95°C for 1 min. The cDNA was stored at -20°C until real-time PCR analysis.

Pan-Flavi RT-qPCR

A two-step Pan-Flavi RT-qPCR was run using the iScript™ cDNA Synthesis kit (BioRad) and SsoAdvanced™ Universal SYBR® Green Supermix kit (Roche) according to the instructions of the manufacturer. The master mix consisted of 2 µL cDNA, 10 pmol of degenerate primers Flavi all sense (S), Flavi all antisense 2 (AS2), and Flavi all antisense 4 (AS4) designed by Patel et al. (2013) (see **Table 2**).[49] The qPCR running conditions were as follows: denaturation at 95°C for 3 min and 45 cycles of 95°C for 10 s, 60°C for 30 s and then 65°C for 31 s, 65°C for 5 s. Amplification was performed on a BioRad qPCR system (CFX96™ Real-Time System) and data was analyzed using CFX Maestro™ Software.

The Pan-Flavi degenerate primers target conserved flavivirus nonstructural protein 5 (NS5) gene nucleotide region 267 and produce an amplicon product of ~260 bp.[49] Degenerate primer sequences designed by Patel et al. (2013), share the conserved NS5 region and have been shown to exhibit a good efficiency in detecting a broad range of flaviviruses.[49] All flaviviruses used in this study have been shown to be detected well using the Pan-Flavi assay.[49,50]

Pan-Flavi RT-qPCR Degenerate Primers	
Primer	**Sequence**
Flavi all sense (S)	5′-TACAACATGATGGGGAARAGAGARAA-3′
Flavi all sense 2 (S2)	5′-TACAACATGATGGGMAAACGYGARAA-3′
Flavi all antisense 4 (AS4)	5′-GTGTCCCAGCCNGCKGTRTCRTC-3′

Table 2 Primers (S, S2 and AS4) designed by Patel et al. (2013)[49] all target nonstructural protein 5 (NS5) region 267-nucleotide. Degenerate bases: R= (A/G), K=(T/G), Y=(C/T), N=(A/G/T/C).

RNA Extraction and RT-qPCR of Patient Dried Blood and Serum Spots

Dried blood and serum spots were provided from patients in Bach Mai Hospital (Vietnam), consisting of 98 confirmed dengue patient cases and 99 control Hepatitis B patients. The selection of patients and study design has been described in Nguyen-Tien et al. (2021).[51] RNA extraction from blood and serum spots on FTA was performed using RNeasy Mini Kit (Qiagen), using one-fourth of a dried blood or serum spot. The discs were cut and placed in lysis buffer RLT (Qiagen) provided by the kit and were stored at 4°C overnight. All RNA extractions were performed the next day and following the kit protocol. Extracted RNA samples were stored at -80°C until PCR analysis was performed. One-step qPCR was performed using QuantiTect® SYBR® Green RT-PCR Kit (Qiagen) according to the instructions of the manufacturer.

Sequencing of Dengue virus Extracted from FTA™ cards

Sequence data was provided from Illumina 2-pair end sequencing results of extracted positive blood and serum dengue samples supplied by Bach Mai Hospital, Vietnam. The selection of patients and study design has been described in Nguyen-Tien et al. (2021) and sequencing conducted according to Cruz et al. (2016).[51,52] The given sequence was aligned against a DENV-1 reference genome (GenBank Accession Number KY672941.1) using Geneious and BLASTed (megablast) to find highly similar sequences, to then form a phylogenetic tree with the most confident matches.

Results

Infectivity of viral RNA on FTA™ cards

To further establish if FTA cards are suitable for transporting SINV-1, the infectivity of the virus on FTA and toxicity of the card components were tested. The infectivity assay showed that the FTA card is toxic to Vero cells and that dilution 10^{-3} removes the toxic effect. The dilution series containing SINV-1 on FTA showed a similar result of cells thriving at the 10^{-3} dilution. The cells exposed to SINV-1 showed visible cytopathogenic effects (CPE) up until the 10^{-4} dilution, indicating infection of cells. This suggests that vRNA from SINV-1 is inactivated upon contact with FTA. The cells were observed 24, 48, and 72h post-exposure to the supernatant solutions. Infectivity assay results are summarized in **Figure 1.**

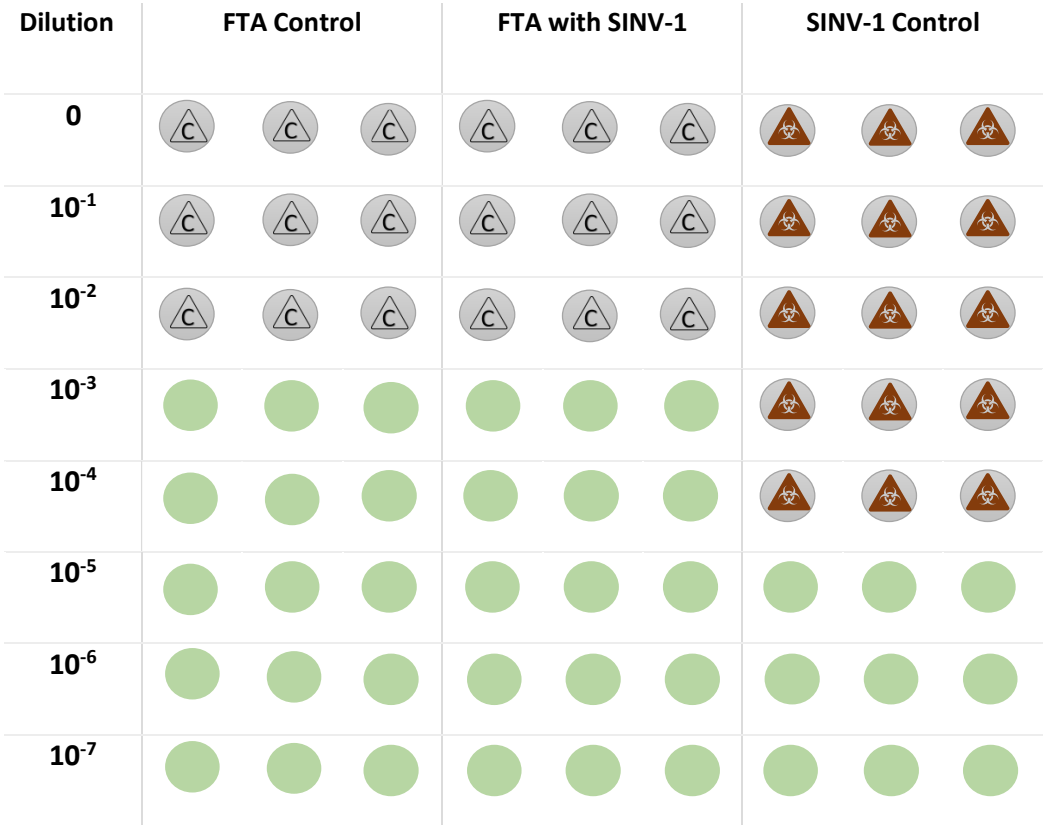

Figure 1. Infectivity assay after 72h using Vero cells and serial dilutions of blank FTA card (FTA Control), SINV-1 placed on FTA card, and SINV-1 (SINV-1 Control), where green represents wells containing live cells and grey representing dead cells due to cytotoxicity marked with "C" and observed cytopathogenic effect (CPE) marked with and orange triangle with the biological hazard symbol.

Viral RNA Stability on FTA™ cards

To further establish if FTAs are suitable for preservation of flavivirus RNA and later molecular testing, the RNA stability of JEV, TBEV, USUV, and ZIKV were measured after 1, 7, 14, 15, and 30-days incubation at 4, 20-25 and 37ºC. A Pan-Flavi RT-qPCR was performed on all the flaviviruses, and average Ct values were calculated of the viral dilution before placement on FTA compared to after in different environments, referred to as "before" and "after" respectively. **Table 3** summarizes the viral strains and the melting temperatures (Tm) observed during Pan-Flavi RT-qPCR.

Flavivirus strains used for Pan-Flavi RT-qPCR		
Flavivirus	**Strain**	**Pan-Flavi RT-qPCR Tm (ºC)**
JEV	*Nakayama strain*	85
TBEV	*European strain*	85
USUV	*Merula strain*	85
ZIKV	*African strain MR-766*	80

Table 3. Melting temperatures (Tm) observed during RT-qPCR using degenerate primers designed by Patel et al (2013).[49]

RNA Extraction Methods from FTA™ cards

To evaluate the most suitable RNA extraction kit to use for vRNA samples, six different RNA extraction kits were tested using a SINV-1 dilution placed on an FTA card compared to the same dilution before placement on FTA. An RT-qPCR was performed to compare relative Ct value change before and after virus dilution placement on FTA. All samples showed melting peaks of 83-84ºC (data not shown), indicating similar contents within the samples. The average Ct values were calculated and compared to SINV-1 virus dilution before placement on the FTA, and the fold change was calculated using the equation $2^{\Delta Ct}$. The Trizol extraction showed a higher average Ct value after sample placement on FTA as opposed to a decrease seen in all other extraction methods. Trizol, Direct-zol using Trizol and Trizol LS, and Trizol LS alone, showed a fold change of 0, 4, 22, 75, and 98, respectively. The QiAmp viral RNA Mini kit and RNeasy Mini kit showed a fold change of 210 and 171, respectively. Average Ct, standard deviation values, and fold change are summarized in **Table 4**, and visualized in **Figure 2**.

Average Ct values of SINV-1 before and after FTA adsorption					
Extraction Method	**Average Ct before**	**± SD**	**Average Ct after**	**± SD**	**$2^{\Delta Ct}$**
Trizol	22.5	0.18	22.6	0.56	1
Direct-zol Trizol	20.7	0.14	25.2	0.66	22
Direct-zol Trizol LS	19.9	0.05	26.2	0.48	75
Trizol LS	25.5	0.80	32.1	0.58	98
RNeasy Mini Kit	18.1	0.07	25.6	0.45	171
QiAmp viral RNA Mini Kit	21.7	0.46	29.4	0.85	210

Table 4. Fold change calculated by $2^{\Delta Ct}$.

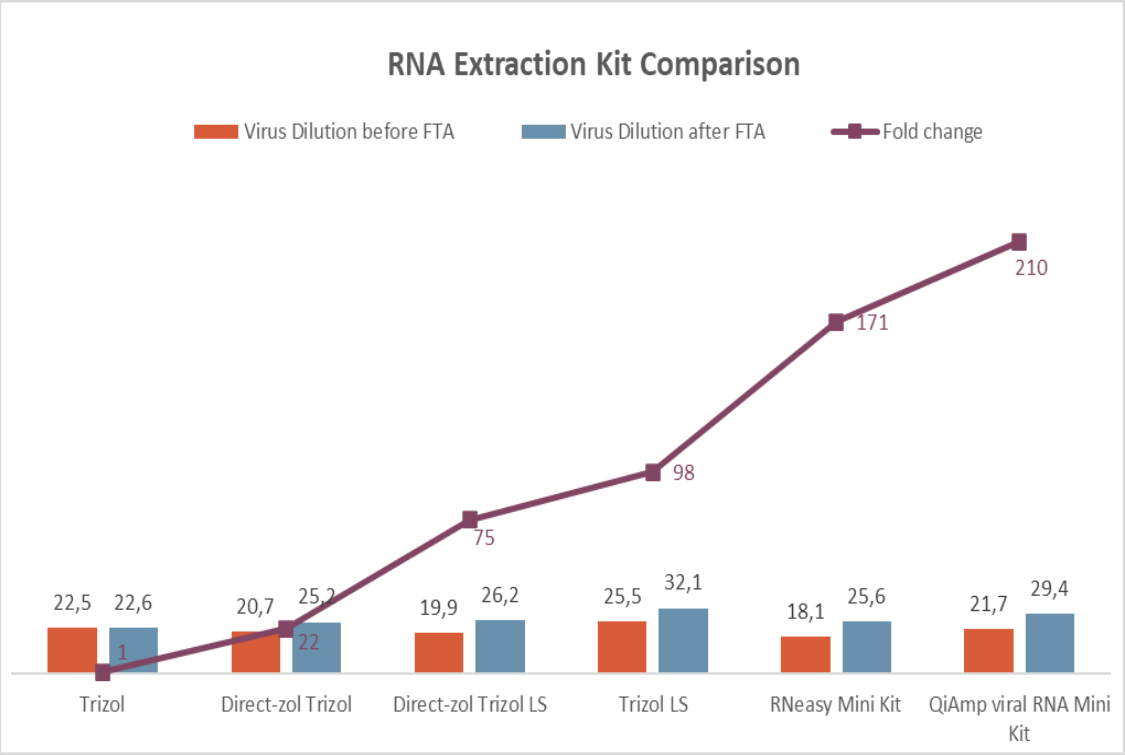

Figure 2. RNA extraction performed on virus dilution samples of SINV-1 before and after placement on FTA cards. The purple line represents the fold change of SINV-1 RNA yield using different kits and comparing before and after placement on FTA card using different extraction methods: Trizol (Invitrogen), Direct-zol (Zymo), Trizol LS (Invitrogen), RNeasy Mini Kit (Qiagen), QiAmp viral RNA Mini kit (Qiagen).

The coefficient of determination (R^2) was calculated for the temperature exposure over time for each of the viruses, JEV, TBEV, USUV, and ZIKV. The R^2 for JEV at 4, 20, and 37ºC were 28%, 20%, and 88% respectively. TBEV scored R^2 values of 50%, 3% and 99% for 4, 20, and 37ºC, respectively. USUV scored R^2 values of 8%, <1% and 4% for 20-25, and 37ºC, respectively. ZIKV had scores of 2%, 20%, and 50% for 4, 20, and 37ºC, respectively. The starting Ct value for JEV and TBEV was 22-23 and 32 for USUV and ZIKV. The Ct change over time and temperatures are summarized in **Figures 3A-B** for JEV and TBEV, respectively and for USUV and ZIKV in **4A-B**, respectively.

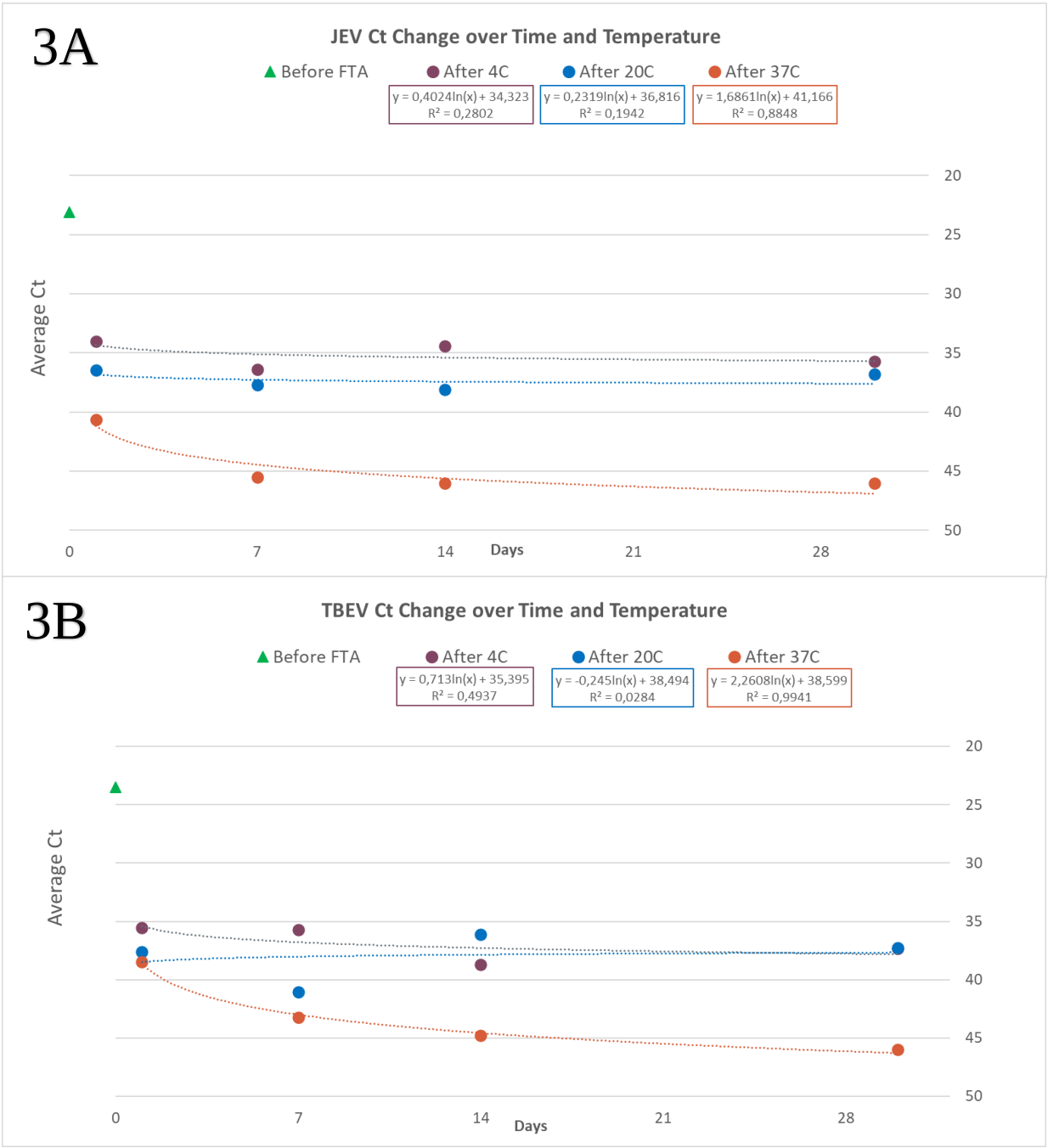

Figure 3A-B. Average Ct values recorded after 1,7,14, and 30 days of incubation at 4, 20 and 37ºC. The initial virus dilution is marked with a green triangle as "before FTA", the different temperature exposures are marked as purple for 4ºC, dark blue for 20ºC, and light blue for 37 ºC. **A**) Average Ct value change over time at different temperatures for JEV. **B**) Average Ct value change over time at different temperatures for TBEV.

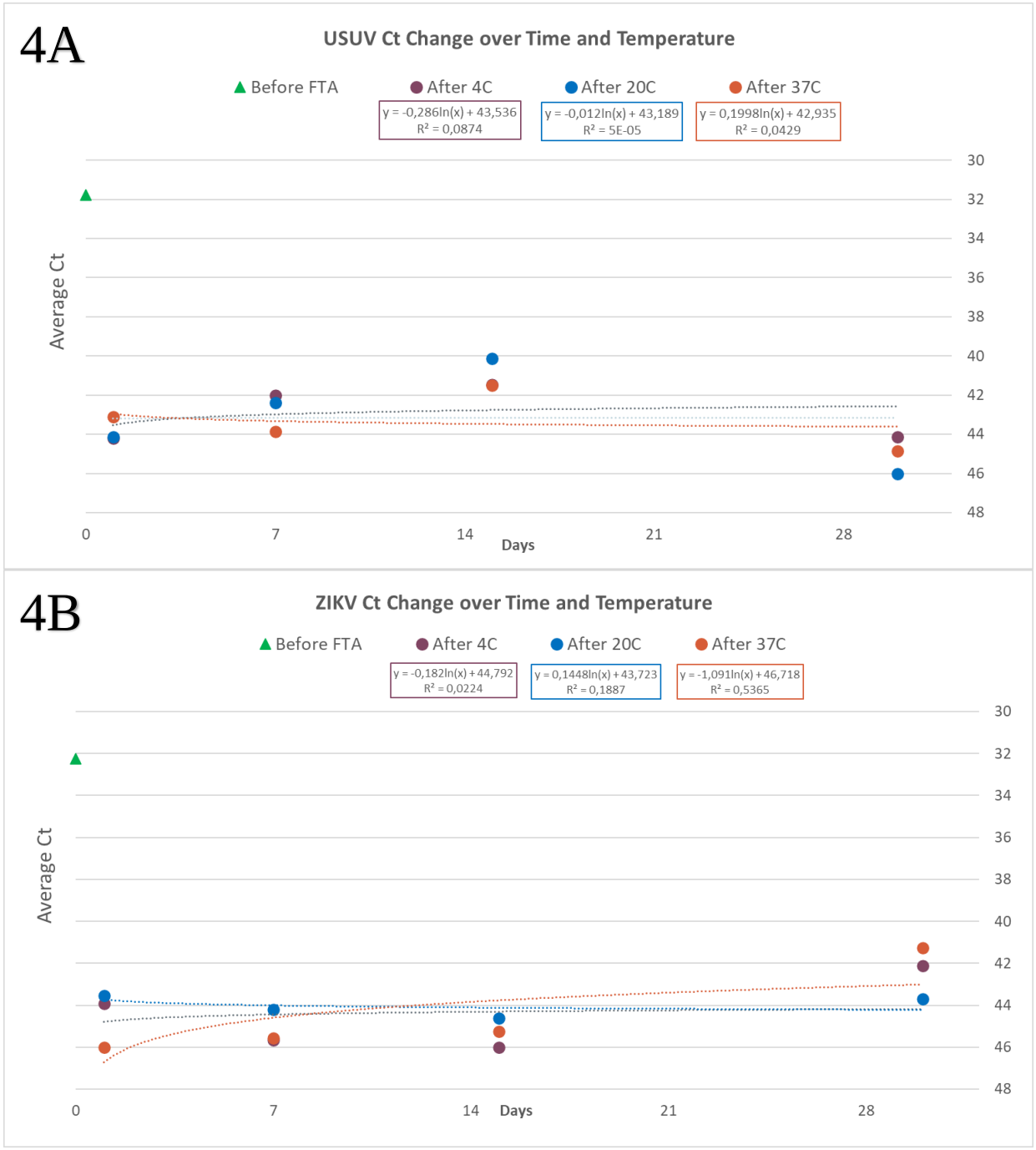

Figure 4A-B. Average Ct values recorded after 1,7,15, and 30 days of incubation at 4, 20 and 37ºC. The initial virus dilution is marked with a green triangle as "before FTA", the different temperature exposures are marked as purple for 4ºC, dark blue for 20ºC, and light blue for 37 ºC. **A**) Average Ct values over time at different temperatures for USUV. **B**) Average Ct values over time at different temperatures for ZIKV.

Dengue virus Detection on FTA™ cards

The data of Pan-Flavi RT-qPCR was analyzed from 222 FTA adsorbed blood and serum spots from Vietnamese patients. A Ct value below 38 was regarded as positive with high certainty while above 40 was regarded as negative, based on sensitivity calculations of Pan-Flavi assay by Patel et al. (2013).[49] Out of the 113 blood samples, 22 were regarded as positive (19.5%) and 11 out of 109 serum samples were regarded as positive (10%) (see **Figure 5**). Most of the blood and serum samples were above Ct value 42 (60%), and 56 samples were valued at Ct between 38-41 (25%).

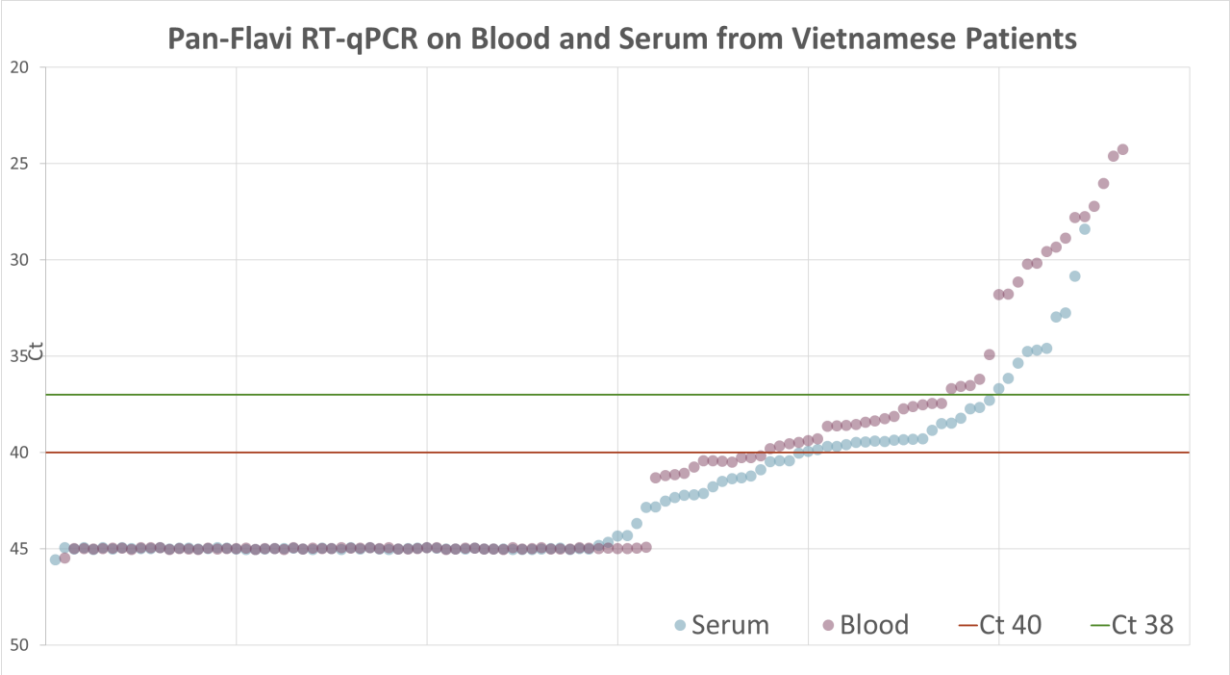

Figure 5. The Pan-Flavi RT-qPCR results from FTA card with dried blood (*N=113*) marked in dark pink and serum (*N=109*) marked in blue (N_{tot}*=222*). Threshold cut-off at Ct 38 marked in green and cut-off 40 marked in red, where values below the threshold are regarded as negative.

The 22 paired DENV positive samples of FTA adsorbed blood and serum spots were compared and showed an average Ct of 32.9 ± 5.3 for blood samples and 41.0 ± 5.0 for serum samples. A paired T-test could confirm that on average, blood samples had a lower Ct by 8.1 ± 5.0 ($P < 0.05$). See summarized data in **Figure 6**.

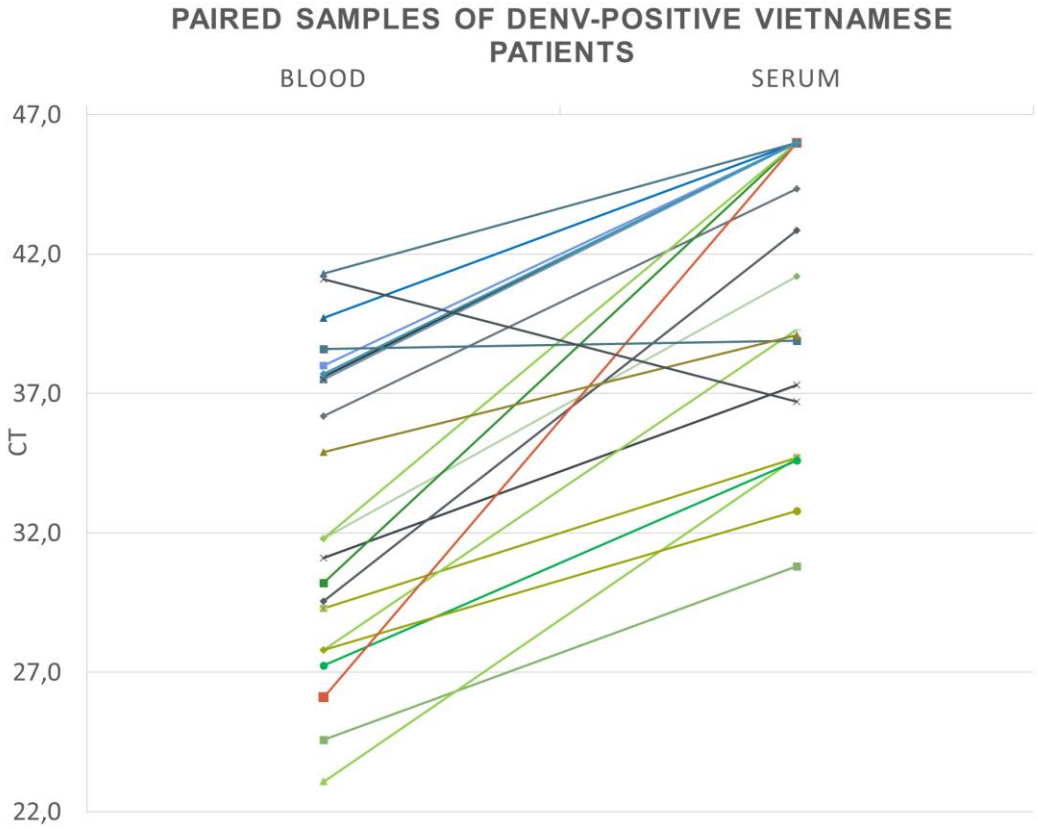

Figure 6. Paired samples (N=22) of dried blood and serum spots positive for DENV. Sequenced sample X marked in red. T-test P-value of <0.001 (CI=95%).

Sequencing of Dengue virus extracted from FTA™ cards

The provided concatenated sequence of sample X, seen in **Figure 6** (marked in red), was aligned with the DENV-1 reference genome (GenBank Accession Number KY672941.1) using Geneious software. The sequence consisted of 40% of the genome, being 4145 base pairs (bp) long, where coverage and map to the reference sequence are shown in **Figure 7**.

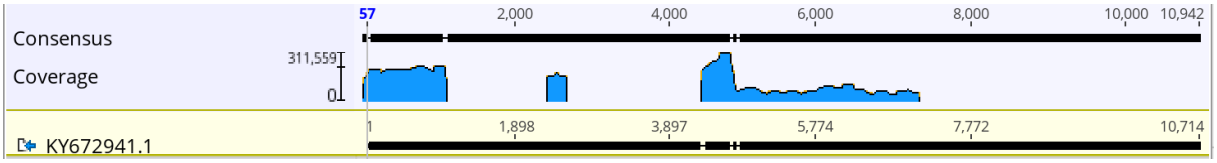

Figure 7. DENV-positive partial NS5 sequence alignment of sample X to reference genome KY672941.1. Alignment performed using Geneious software.

The sequence was run through BLASTn (NCBI) for high similarity sequences which gave matches to the DENV-1 polyprotein gene with 94-96% identity (see **Figure 8**). Upon closer inspection of the top sequence matches, it was found that they were isolated in Vietnam of which the top 8 matches (LC428077.1, LC428063.1, LC428075.1, LC428061.1, LC428076.1, LC428067.1, LC428065.1, LC428064.1) were isolated from the DENV-1 outbreak in Northern Vietnam from 2017 (unpublished data by Hasebe, F. et al. (2018)). The following matches were also isolated from Vietnam but were not associated with the 2017 outbreak, but to sequences from 2011 (JQ045666.1, JQ045665.1), and 2019 (MN621140.1) DENV-1 isolate originally from 2013, from 2020 (MW364630.1) DENV-1 isolate NewCaledonia-2014 and from 2018 (MG877555.1) DENV-1 isolate Ncal2013 originally from 2017.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Dengue virus 1 01-TN-088 gene for polyprotein, complete cds	Dengue virus 1	4863	7054	94%	0.0	98.65%	10179	LC428077.1
Dengue virus 1 03-HD-019 gene for polyprotein, complete cds	Dengue virus 1	4863	7049	94%	0.0	98.65%	10179	LC428063.1
Dengue virus 1 03-HD-002 gene for polyprotein, complete cds	Dengue virus 1	4857	7043	94%	0.0	98.61%	10179	LC428075.1
Dengue virus 1 03-HD-009 gene for polyprotein, complete cds	Dengue virus 1	4857	7043	94%	0.0	98.61%	10179	LC428061.1
Dengue virus 1 03-HD-014 gene for polyprotein, complete cds	Dengue virus 1	4852	7038	94%	0.0	98.57%	10179	LC428076.1
Dengue virus 1 01-TN-078 gene for polyprotein, complete cds	Dengue virus 1	4852	7016	94%	0.0	98.57%	10179	LC428067.1
Dengue virus 1 01-PK-90-L2 gene for polyprotein, complete cds	Dengue virus 1	4852	7021	94%	0.0	98.57%	10179	LC428065.1
Dengue virus 1 09-HD-019 gene for polyprotein, complete cds	Dengue virus 1	4852	7021	94%	0.0	98.57%	10179	LC428064.1
Dengue virus 1 isolate DENV1/Vietnam/10dx-241-802-Placebo-24hrs polyprotein gene, complete cds	Dengue virus 1	4774	7066	96%	0.0	98.06%	10669	JQ045666.1
Dengue virus 1 isolate DENV1/Vietnam/10dx-241-801-Placebo-24hrs polyprotein gene, complete cds	Dengue virus 1	4769	7060	96%	0.0	98.02%	10672	JQ045665.1
Dengue virus 1 isolate 2013/NC/0566 polyprotein (POLY) gene, complete cds; and sfRNA1 lncRNA g...	Dengue virus 1	4763	7054	96%	0.0	97.99%	10723	MN621140.1
Dengue virus 1 isolate NewCaledonia-2014-114035331-455-C6/36 polyprotein (POLY) gene, complet...	Dengue virus 1	4763	7051	96%	0.0	97.99%	10732	MW364630.1
Dengue virus 1 isolate Ncal2013, complete genome	Dengue virus 1	4758	7051	96%	0.0	97.95%	10719	MG877555.1

Figure 8. Top scores of partial NS5 sequences of sequence X (4145bp) BLASTn (NCBI). Results from 23/05/2021.

A phylogenetic tree was constructed with the top BLASTn matches and showed that the sample was most closely related to the isolates taken from the 2017 Vietnam outbreak and less so to the other aforementioned isolates (see **Figure 9**).

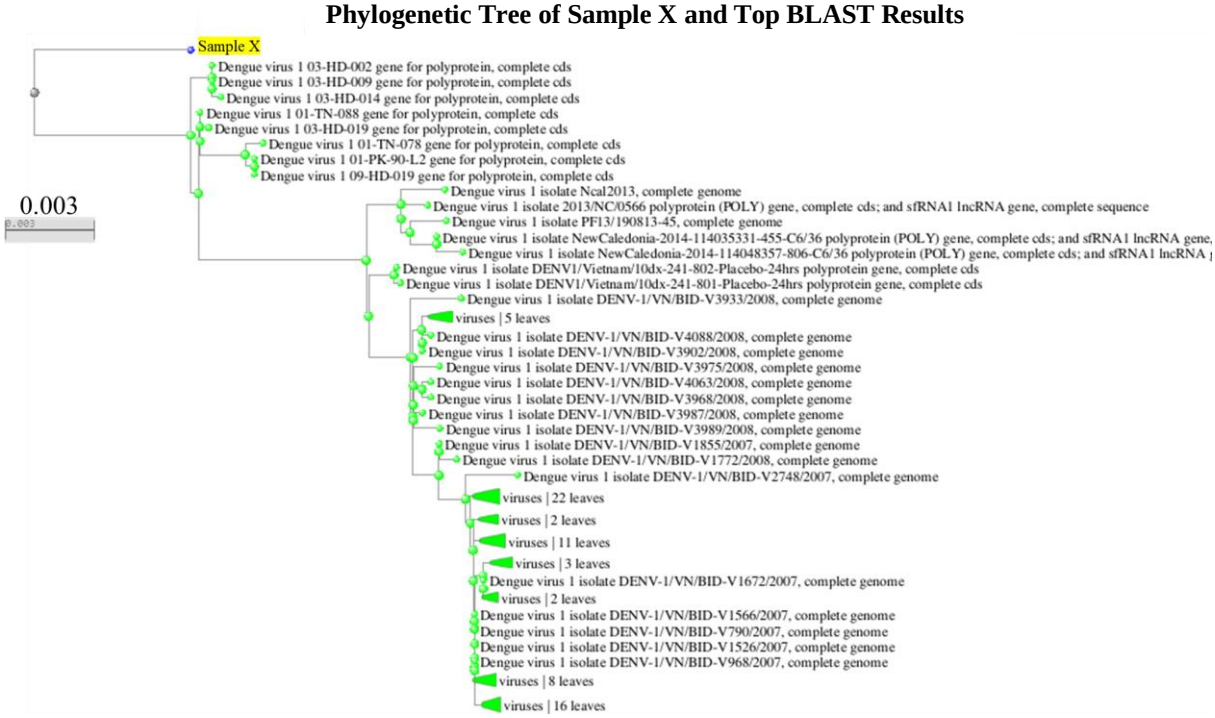

Figure 9. Phylogenetic tree for DENV-positive sample X based on partial NS5 sequences and top matches from BLASTn. Made using Blast Tree View function (NCBI).

Discussion

This study revealed that FTA-adsorbed samples inhibit SINV-1 infectivity of Vero cells (see **Figure 1**). The infectivity assay showed that cells exposed to SINV-1 exhibited CPE, while wells containing FTA-adsorbed SINV-1 did not exhibit CPE, but instead signs of cell death by cytotoxicity. This could be explained by the toxic effect of FTA itself since observations indicated that FTA cards are toxic to Vero cell growth from 24- to 72h post-exposure, and that a 1000-fold dilution reduces the toxic effect. This also seems likely due to the known lytic components of FTA cards, which cause protein degradation and cell lysis.[34] Thus, the results support the hypothesis that FTA-adsorption of SINV-1 inhibits the viral infectivity of cells.

These findings correspond to other studies which have shown that other +ssRNA viruses such as infectious bronchitis virus (IBV), porcine reproductive & respiratory syndrome virus (PRRSV), foot & mouth disease virus (FMDV), and infectious bursal disease virus (IBDV) become inactivated after adsorption to FTA cards.[44,53–55] Similarly, Jóźwiak et al. (2015), found that two avian influenza virus (IAV) strains of low and high pathogenicity were inactivated after adsorption to FTA, though the time varied between 1 to 24 h for the different strains.[56] A variety of different clinical samples from bursal allantoic fluid, infected tissue biopsies, and blood spots have previously been investigated, showing virus inactivation upon FTA adsorption. However, the time for samples to dry on the FTA, as noted in Jóźwiak et al.'s (2015) study, may be an important factor to consider for different sample and virus types.[55–57]

Our results in addition to other studies, imply that adsorption of virus-containing samples on FTA can inactivate vRNA, reduce the biosafety level needed to handle certain pathogenic viruses, and thus increases the possibility of working with them in certain settings. The results also showed that at least 2 h are needed for the inactivation of SINV-1. However, an evaluation of different sample drying times on FTA was not evaluated or compared, nor further evaluation of the vRNA in the infectivity assay. Thus, an RT-qPCR and gel-electrophoresis could be run to confirm the presence of vRNA. To further test the inactivation of SINV-1, a study with RNAse treatment as performed by Cant et al. (2020), could be beneficial to establish the presence or loss of the viral envelope when samples are placed on FTA, indicating infectivity in combination with other tests.[45]

This study also revealed that Trizol and Direct-zol with Trizol were suitable methods for high yield extraction of vRNA from FTA cards. The results showed that Trizol extraction could give the same vRNA before and after placement on FTA whilst Direct-zol with Trizol extraction showed a 22-fold loss of sample RNA during the process (see **Table 4** and **Figure 2**). The 22-fold loss of RNA using Direct-zol with Trizol compared to Trizol by itself may be due to the addition of a column in the extraction method. The reduced yield when using a column may be due to inefficient elution, or blockage within the column due to components of the FTA card. Trizol LS and Direct-zol using Trizol LS also showed a decrease in RNA yield of 75- and 98-fold, respectively, whilst RNeasy Mini and QiAmp Viral RNA Mini kits showed a significant decrease in sample yield ranging from 171- to 210-fold decrease, respectively.

These results align with previous studies that have evaluated different RNA extraction methods for samples placed on FTA, which have found that Trizol gives higher yields than kits such as RNeasy and High Pure RNA Isolation (Roche Diagnostics) kits.[57–59] Wex et al. (2003) found that Trizol effectively stabilizes RNA of biopsy samples up to 6 months and yields higher RNA levels than the RNeasy kit, though typically only by 1µg.[59] Thus, the difference that Wex et al. (2003) found between the different methods was not seemingly significant in terms of RNA yield. Perozo et al.(2007) compared the RNA yield of Newcastle disease virus (NDV) placed

on FTA and found that higher RNA levels were detected using Trizol compared to the column-based High Pure RNA Isolation kit.[58] These results correspond to the findings of Skonieczna et al. (2016), which investigated RNA isolation methods from FTA-adsorbed blood spots and also found that Trizol was more efficient than the High Pure RNA Isolation kit.[57] Despite these findings, the use of RNeasy Mini, QiAmp Viral RNA Mini, and MagMAX™ Viral RNA Isolation kits (Applied Biosystems, Inc.), are widely used as they are less toxic (not requiring chloroform nor Trizol), produce higher quality RNA, and are adequately efficient for different types of samples and downstream tests.[32,43,47,60]

In addition to the extraction method used, the elution of samples from FTA cards is subject to large discrepancies among different studies. Sakai et al. (2014) compared five different eluates and time for elution of rabies vRNA and found that the conditions could significantly impact the RNA extraction efficiency.[43] They concluded that Tris-EDTA (TE) buffer was the best eluent for RNA extraction from FTA compared to Trizol, nuclease-free water, RNA rapid extraction solution (Ambion), and Buffer AVL (Qiagen), although most methods for elution were considered adequate for use. This suggests that the elution method is a factor that is important to consider for downstream tests, as it may affect the resulting sample quantity and quality. Therefore, the elution and RNA extraction method should be established and tested to suit the needs of downstream tests. In the case of FTA-adsorbed samples, they may be extracted using Trizol-based methods for higher yields of RNA, though the benefits of such should be assessed against working with the toxic substance and the possibility of acquiring a lower quality of RNA.

The viral stability of different flaviviruses was tested by comparing the relative virus concentration before and after FTA-adsorption stored up to 30 days at different temperatures. Pan-Flavi RT-qPCR was performed on FTA card eluates and the relative Ct values were compared to the initial concentration before FTA-adsorption. The results showed that average Ct values for JEV and TBEV increased at exposure to 37ºC, indicating decreased vRNA stability (see **Figures 3-4**). The R^2 values for JEV (88%) and TBEV (99%), indicate a high correlation between decreased RNA stability and increased storage time in 37ºC. As for ambient and colder temperature exposure, the Ct values for JEV and TBEV were constant and showed R^2 values <5%, indicating low correlation between RNA stability and storage temperature, meaning that the RNA stability remains invariable over time. The Ct values for both USUV and ZIKV were observed to be above 40 for all FTA-adsorbed samples, thus there is a high uncertainty for the interpretation of data, and they have been categorized as inconclusive results. The uncertainty may be a result of lower initial titers used for USUV and ZIKV, indicated by a starting Ct of 32 compared to 22-23 for JEV and TBEV, though other causes cannot be excluded. Lastly, all three temperature exposures indicated a loss of RNA, likely in the process of RNA extraction (as seen in **Figure 2**) and RT-qPCR, resulting in 0.5-0.6-fold decrease in Ct for ambient and colder temperatures and between 0.8-0.9-fold decrease at 37ºC. The increased loss of RNA for the 37ºC exposure compared to ambient and colder temperatures, is likely due to the loss of vRNA stability due to the increase in temperature, supported by the R^2 coefficient (**Figures 3-4**).

The results are supported by the findings of other studies which have also found that vRNA can remain stable in ambient temperatures from several days to weeks and that loss of vRNA can occur when stored at 37ºC or warmer.[45,55,58,61,62] However, in the case of Madhanmohan et al. (2016), FTA cards containing FMDV stored up to 49 days at temperatures ranging between 21-45ºC remained at detectable levels for downstream amplification tests, whilst Perozo et al. (2006) found that storage of NDV at room temperature reduced the sensitivity of RT-PCR after

only 14 days.[54,58] Linhares et al. (2012) tested serum and oral fluid samples containing PRRSV stored on FTA and observed a significant loss of sample and sensitivity compared to direct testing, also supporting the findings of our study.[53] This suggests that the type of sample and virus may play a significant role in the vRNA stability. Additionally, as previously discussed, the elution and extraction methods used may also affect the final RNA yield and lead to a difference of results between studies. This also suggests that the results obtained in this study may not apply specifically to other viruses, stressing the importance of verifying a method when performing in a different laboratory setting.

Therefore, our results suggest that flavivirus vRNA may remain stable at ambient temperatures and be subject to loss of stability at warmer temperatures. Also, that the use of FTA may lead to some loss of sample during the process of extraction. However, the uncertainty of correlation between storage conditions and vRNA stability for USUV and ZIKV should be reassessed. The low starting titers may have impeded detection due to the process of eluting samples from FTA or other processes that may affect vRNA yield. Therefore, a reevaluation should be performed using higher initial titers for USUV and ZIKV. In addition, standard curves for all four viruses could be evaluated to establish the minimum titer of detection, relevant for the application to clinical samples.

This study also analyzed data from clinical blood and serum patient samples collected during a 2018 DENV outbreak in Hanoi, Vietnam. One-step RT-qPCR results were provided in data form. A general trend of lower Ct values for blood compared to serum samples was observed (**Figure 5**). When comparing 22 paired blood and serum samples from 22 patients, there was a significant difference between Ct values, where blood had a lower Ct of about 8.1 ± 5.0 ($P < 0.05$) compared to serum samples (**Figure 6**).

The lower Ct values observed in the RT-qPCR results for blood samples compared to serum, suggests that the blood samples may contain higher titers of DENV during an active infection since samples were taken from patients admitted to the hospital and positive infection was confirmed using conventional PCR methods before adsorption on FTA (data not shown). Thus, the results suggest that blood samples may be more suitable than serum samples for molecular diagnostics of an active infection. However, due to the course of DENV infection, a false negative result may occur when there is antibody production which ultimately lowers the viral titers in the blood.[28] In order to prevent false negatives, follow-up sampling could be beneficial to give a clearer view of the clinical picture of each patient in combination with serological testing. Prospective studies could focus on combining the serological data obtained from the samples with the molecular tests performed on the paired blood samples to give a more comprehensive clinical picture of the patient.

Following amplicon-based PCR of patient blood and serum, some positive samples were full-genome sequenced using an Illumina platform, and data of sample X was provided for analysis. Preliminary electrophoresis results indicated that the sample had the most abundant DNA input and PCR confirmed that the sample contained DENV-1 (data not shown). The sequencing data revealed that sample X most likely was DENV-1 with an origin in Vietnam (see **Figures 7-9**). Despite the sequence being concatenated, it could be aligned to 40% of a DENV-1 reference genome and matched with multiple DENV-1 isolates using BLAST.

Previous studies have shown that it is possible to serotype viral samples stored on FTA using PCR methods.[44,58] Thus, in addition to other studies, the sequencing results suggest that it may be possible to isolate enough viral isolate from FTA-adsorbed blood and serum to perform

molecular testing.[44,53,57] However, in this case, the analysis could not confirm that the sample contained DENV-1 purely from the sequencing result of a 40% match, but required data from previously performed PCR tests. In future sequencing and alignment studies, it would be beneficial to align a larger part of the genome and especially conserved regions to confirm a more secure match.

Thus, FTA-adsorbed blood and serum spots may facilitate clinical diagnosis by allowing molecular tests such as PCR and sequencing which could also provide valuable molecular epidemiological information. However, the difficulty lies in that patient samples will vary according to the biology of the individual and the stage of disease, among other factors which can alter viral titers.[28] Thus, the threshold to specify the specificity and sensitivity for DENV infection will have to be further investigated to establish a concrete method of diagnosis.

In conclusion, this study found that FTA sample-adsorption inhibits infectivity of SINV-1, detection using Pan-Flavi RT-qPCR for JEV and TBEV is possible after 30 days storage at ambient temperatures, and that loss of vRNA stability is expected after one-day of storage at 37ºC. Furthermore, the study showed that FTA-adsorbed blood samples of DENV-infected patients give higher RNA yields and may allow molecular tests for serotyping by RT-qPCR and possibly sequencing. Thus, FTA-adsorbed flavivirus may be beneficial for future molecular epidemiological and diagnostic research by reducing biosafety requirements needed to process samples and facilitates preservation and transport due to known vRNA stability conditions and optimal extraction methods.

Acknowledgments

I would like to give my gratitude and thanks to my supervisors Johanna Lindahl and Jiaxin Ling which have given me the opportunity to partake in this study. Special thanks to Janina Krambrich for your guidance and help with BSL-3 classified samples. Thank you to Emma Dahl who also provided me with samples needed to perform my experiments.

Thank you to my fellow classmates and colleagues at the Zoonosis Science Center for your support.

Finally, thank you to my family for your never ending love and encouragement, for always supporting me in reaching my dreams and aspirations!

References

1. Solomon, T. & Mallewa, M. Dengue and Other Emerging Flaviviruses. *Journal of Infection* **42**, 104–115 (2001).
2. Pierson, T. C. & Diamond, M. S. The continued threat of emerging flaviviruses. *Nature Microbiology* **5**, 796–812 (2020).
3. Rothe, C. & Jong, E. C. Emerging Infectious Diseases and the International Traveler. *The Travel and Tropical Medicine Manual* 27–35 (2017) doi:10.1016/B978-0-323-37506-1.00003-9.
4. Wilder-Smith, A. *et al.* Epidemic arboviral diseases: priorities for research and public health. *The Lancet Infectious Diseases* **17**, e101–e106 (2017).
5. Liang, G., Gao, X. & Gould, E. A. Factors responsible for the emergence of arboviruses; strategies, challenges and limitations for their control. *Emerging Microbes & Infections* **4**, 1–5 (2015).
6. Simmonds, P. *et al.* ICTV Virus Taxonomy Profile: Flaviviridae. *Journal of General Virology* **98**, 2–3.
7. Payne, S. Chapter 15 - Family Flaviviridae. in *Viruses* (ed. Payne, S.) 129–139 (Academic Press, 2017). doi:10.1016/B978-0-12-803109-4.00015-5.
8. Holmes, A. C., Basore, K., Fremont, D. H. & Diamond, M. S. A molecular understanding of alphavirus entry. *PLOS Pathogens* **16**, e1008876 (2020).
9. Dengue and severe dengue. https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue.
10. Chen, W. J., Wei, H. L., Hsu, E. L. & Chen, E. R. Vector competence of Aedes albopictus and Ae. aegypti (Diptera: Culicidae) to dengue 1 virus on Taiwan: development of the virus in orally and parenterally infected mosquitoes. *J Med Entomol* **30**, 524–530 (1993).
11. Gould, E. A. & Solomon, T. Pathogenic flaviviruses. *The Lancet* **371**, 500–509 (2008).
12. TBE-annual-epidemiological-report-2018.pdf.

13. Tick Borne Encephalitis (TBE) – sjukdomsstatistik — Folkhälsomyndigheten. http://www.folkhalsomyndigheten.se/folkhalsorapportering-statistik/statistik-a-o/sjukdomsstatistik/tick-borne-encephalitis-tbe/.
14. Bollati, M. *et al.* Structure and functionality in flavivirus NS-proteins: Perspectives for drug design. *Antiviral Research* **87**, 125–148 (2010).
15. Murugesan, A. & Manoharan, M. Dengue Virus. *Emerging and Reemerging Viral Pathogens* 281–359 (2020) doi:10.1016/B978-0-12-819400-3.00016-8.
16. Tun, M. M. N. *et al.* Clinical, Virological, and Cytokine Profiles of Children Infected with Dengue Virus during the Outbreak in Southern Vietnam in 2017. *The American Journal of Tropical Medicine and Hygiene* **102**, 1217–1225 (2020).
17. Hung, T. M. *et al.* The Estimates of the Health and Economic Burden of Dengue in Vietnam. *Trends in Parasitology* **34**, 904–918 (2018).
18. Rosen, L., Shroyer, D. A., Tesh, R. B., Freier, J. E. & Lien, J. C. Transovarial transmission of dengue viruses by mosquitoes: Aedes albopictus and Aedes aegypti. *Am J Trop Med Hyg* **32**, 1108–1119 (1983).
19. Rosen, L. Sexual transmission of dengue viruses by Aedes albopictus. *Am J Trop Med Hyg* **37**, 398–402 (1987).
20. Guzman, M. G., Gubler, D. J., Izquierdo, A., Martinez, E. & Halstead, S. B. Dengue infection. *Nature Reviews Disease Primers* **2**, 1–25 (2016).
21. Guzmán, M. G. *et al.* Enhanced severity of secondary dengue-2 infections: death rates in 1981 and 1997 Cuban outbreaks. *Rev Panam Salud Publica* **11**, 223–227 (2002).
22. Alvarez, M. *et al.* Dengue hemorrhagic Fever caused by sequential dengue 1-3 virus infections over a long time interval: Havana epidemic, 2001-2002. *Am J Trop Med Hyg* **75**, 1113–1117 (2006).

23. Whitehead, S. S., Blaney, J. E., Durbin, A. P. & Murphy, B. R. Prospects for a dengue virus vaccine. *Nature Reviews Microbiology* **5**, 518–528 (2007).

24. Kading, R. C., Brault, A. C. & Beckham, J. D. Global Perspectives on Arbovirus Outbreaks: A 2020 Snapshot. *TropicalMed* **5**, 142 (2020).

25. Garcia Serpa Osorio-de-Castro, C., Silva Miranda, E., Machado de Freitas, C., Rochel de Camargo, K. & Cranmer, H. H. The Zika Virus Outbreak in Brazil: Knowledge Gaps and Challenges for Risk Reduction. *Am J Public Health* **107**, 960–965 (2017).

26. Hamdy, R. F. Efficacy of a Tetravalent Dengue Vaccine in Children in Latin America. *Journal of the Pediatric Infectious Diseases Society* **4**, 389–392 (2015).

27. Thomas, S., Verma, J., Woolfit, M. & O'Neill, S. L. Wolbachia-mediated virus blocking in mosquito cells is dependent on XRN1-mediated viral RNA degradation and influenced by viral replication rate. *PLoS Pathog* **14**, (2018).

28. Diamond, M. S. & Pierson, T. C. Molecular Insight into Dengue Virus Pathogenesis and Its Implications for Disease Control. *Cell* **162**, 488–492 (2015).

29. Ludolfs, D., Schilling, S., Altenschmidt, J. & Schmitz, H. Serological Differentiation of Infections with Dengue Virus Serotypes 1 to 4 by Using Recombinant Antigens. *J Clin Microbiol* **40**, 4317–4320 (2002).

30. Raafat, N., Blacksell, S. D. & Maude, R. J. A review of dengue diagnostics and implications for surveillance and control. *Trans R Soc Trop Med Hyg* **113**, 653–660 (2019).

31. Lanciotti, R. S. Molecular amplification assays for the detection of flaviviruses. *Adv Virus Res* **61**, 67–99 (2003).

32. Picard-Meyer, E., Barrat, J. & Cliquet, F. Use of filter paper (FTA) technology for sampling, recovery and molecular characterisation of rabies viruses. *J Virol Methods* **140**, 174–182 (2007).

33. Vina-Rodriguez, A. *et al.* A Novel Pan-Flavivirus Detection and Identification Assay Based on RT-qPCR and Microarray. *Biomed Res Int* **2017**, (2017).
34. Reliable extraction of DNA from Whatman™ FTA™ cards. *Sigma-Aldrich* https://www.sigmaaldrich.com/technical-documents/articles/biology/whatman-reliable-extraction-of-dna.html.
35. Salvi, G., Rios, P. D. L. & Vendruscolo, M. Effective interactions between chaotropic agents and proteins. *Proteins: Structure, Function, and Bioinformatics* **61**, 492–499 (2005).
36. Birnberg, L. *et al.* Viromics on Honey-Baited FTA Cards as a New Tool for the Detection of Circulating Viruses in Mosquitoes. *Viruses* **12**, 274 (2020).
37. Melanson, V. *et al.* Improving vector-borne pathogen surveillance: A laboratory-based study exploring the potential to detect dengue virus and malaria parasites in mosquito saliva. *J Vector Borne Dis* **54**, 301 (2017).
38. Hashimoto, M. *et al.* Nucleic acid purification from dried blood spot on FTA Elute Card provides template for polymerase chain reaction for highly sensitive Plasmodium detection. *Parasitology International* **73**, 101941 (2019).
39. Caza, F., Joly de Boissel, P. G., Villemur, R., Betoulle, S. & St-Pierre, Y. Liquid biopsies for omics-based analysis in sentinel mussels. *PLoS ONE* **14**, e0223525 (2019).
40. Boué, F. *et al.* Use of FTA® card methodology for sampling and molecular characterization of Echinococcus granulosus sensu lato in Africa. *Experimental Parasitology* **173**, 29–33 (2017).
41. WHO | Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection. *WHO* http://www.who.int/hiv/pub/arv/arv-2016/en/.

42. Yacouba, A. *et al.* Whatman FTA® cards versus plasma specimens for the quantitation of HIV-1 RNA using two Real-Time PCR assays. *bioRxiv* 748061 (2019) doi:10.1101/748061.

43. Sakai, T. *et al.* Establishing conditions for the storage and elution of rabies virus RNA using FTA® cards. *The Journal of Veterinary Medical Science* **77**, 461–465 (2015).

44. Moscoso, H., Raybon, E. O., Thayer, S. G. & Hofacre, C. L. Molecular Detection and Serotyping of Infectious Bronchitis Virus from FTA® Filter Paper. *Avian Diseases* **49**, 24–29 (2005).

45. Cant, N., Ayub, M., Ma, S., Gulbicka, A. & Kavanagh, I. Elute FTA cards as a collection device for SARS-CoV-2 saliva samples. 6.

46. Purvis, L. B., Villegas, P. & Perozo, F. Evaluation of FTA® paper and phenol for storage, extraction and molecular characterization of infectious bursal disease virus. *Journal of Virological Methods* **138**, 66–69 (2006).

47. Ponce-Rojas, J. C. *et al.* A Fast and Accessible Method for the Isolation of RNA, DNA, and Protein to Facilitate the Detection of SARS-CoV-2. *bioRxiv* 2020.06.29.178384 (2020) doi:10.1101/2020.06.29.178384.

48. Fukumoto, T. *et al.* Efficacy of a novel SARS-CoV-2 detection kit without RNA extraction and purification. *bioRxiv* 2020.05.27.120410 (2020) doi:10.1101/2020.05.27.120410.

49. Patel, P. *et al.* Development of one-step quantitative reverse transcription PCR for the rapid detection of flaviviruses. *Virol J* **10**, 58 (2013).

50. Barrio-Nuevo, K. M. *et al.* Detection of Zika and dengue viruses in wild-caught mosquitoes collected during field surveillance in an environmental protection area in São Paulo, Brazil. *PLoS One* **15**, (2020).

51. Nguyen-Tien, T. *et al.* Risk factors of dengue fever in an urban area in Vietnam: a case-control study. *BMC Public Health* **21**, 664 (2021).

52. Cruz, C. D., Torre, A., Troncos, G., Lambrechts, L. & Leguia, M. Targeted full-genome amplification and sequencing of dengue virus types 1–4 from South America. *Journal of Virological Methods* **235**, 158–167 (2016).

53. Linhares, D. C. L., Rovira, A. & Torremorell, M. Evaluation of Flinders Technology Associates cards for collection and transport of samples for detection of Porcine reproductive and respiratory syndrome virus by reverse transcription polymerase chain reaction. *J VET Diagn Invest* **24**, 328–332 (2012).

54. Madhanmohan, M. *et al.* Evaluation of the Flinders Technology Associates Cards for Storage and Temperature Challenges in Field Conditions for Foot-and-Mouth Disease Virus Surveillance. *Transbound Emerg Dis* **63**, 675–680 (2016).

55. Moscoso, H., Alvarado, I. & Hofacre, C. L. Molecular Analysis of Infectious Bursal Disease Virus from Bursal Tissues Collected on FTA® Filter Paper. *avdi* **50**, 391–396 (2006).

56. Jóźwiak, M. *et al.* Application of FTA® Cards for detection and storage of avian influenza virus. *J Vet Res* **2016**, 1–6.

57. Skonieczna, K. *et al.* RNA isolation from bloodstains collected on FTA cards – application in clinical and forensic genetics. *Arch Med Sąd Kryminol* **66**, 244–254 (2017).

58. Perozo, F., Villegas, P., Estevez, C., Alvarado, I. & Purvis, L. B. Use of FTA® filter paper for the molecular detection of Newcastle disease virus. *Avian Pathology* **35**, 93–98 (2006).

59. Wex, T., Treiber, G., Lendeckel, U. & Malfertheiner, P. A Two-Step Method for the Extraction of High-Quality RNA from Endoscopic Biopsies. *Clinical chemistry and laboratory medicine : CCLM / FESCC* **41**, 1033–7 (2003).

60. Eisen, A. K. A. *et al. Comparison of Different Kits for SARS-CoV-2 RNA Extraction Marketed in Brazil*. http://biorxiv.org/lookup/doi/10.1101/2020.05.29.122358 (2020) doi:10.1101/2020.05.29.122358.

61. Tam, K. I. *et al.* Evaluation of BBL™ Sensi-Discs™ and FTA® cards as sampling devices for detection of rotavirus in stool samples. *J Virol Methods* **222**, 41–46 (2015).

62. Abdelwhab, E. M., Lüschow, D., Harder, T. C. & Hafez, H. M. The use of FTA® filter papers for diagnosis of avian influenza virus. *J Virol Methods* **174**, 120–122 (2011).