ONE-STEP REVERSE TRANSCRIPTION QUANTITATIVE PCR ASSAY FOR SIMULTANEOUS DETECTION OF CHIKUNGUNYA, DENGUE AND ZIKA VIRUS BLOOD SAMPLE SPOTTED ON FILTER PAPER

Maria F Avila Mejia[1], Shih-Hung Lin[2], Pei-Yun Shu[2], Yueh-Hsin Ping[3] and Dar-Der Ji[1,4,5]

[1]International Health Program, National Yang-Ming University; [2]Center for Diagnostics and Vaccine Development, Centers for Disease Control, Ministry of Health and Welfare; [3]Department and Institute of Pharmacology, [4]Department of Tropical Medicine, [5]One Health Research Center, National Yang-Ming University, International Health Program, Taipei, Taiwan ROC

Abstract. A standardized method using multiplex SYBR Green RT quantitative PCR for direct rapid simultaneous detection of Dengue (DENV), Chikungunya (CHIKV) and Zika (ZIKV) viruses in dried blood spotted on filter paper was developed for potential field application. A standard curve was constructed using virus-specific primer pairs and same thermocycling conditions of ten-fold serial dilutions of each stock virus solution in double distilled water or whole blood spotted on filter paper, which were allowed to dry and RNA directly extracted for quantification. Samples prepared immediately and after a 2-month-storage at ambient temperature showed limit of detection (LOD) of DENV, CHIKV and ZIKV in blood following immediate application on Whatman 903 filter paper of 16 PFU/ml for all three viruses and, after storage, 16, 160 and 160 PFU/ml, respectively, while on Whatman 3MM filter paper LOD was 16, 160 and 1,600 and 160, 1,600 and 16,000 PFU/ml, respectively. In conclusion, virus-spiked blood sample applied onto Whatman 903 paper was suitable for direct PCR quantification of DENV, CHIKV and ZIKV, with no change in detection sensitivity of DENV after a 2-month storage but a 10-fold decrease for the other two viruses. This method should prove useful for virus detection of field blood samples in regions where there are co-circulation of DENV, CHIKV and ZIKV.

Keywords: chikungunya virus, dengue virus, zika virus, blood sample, detection, filter paper, RT-quantitative PCR

INTRODUCTION

Dengue virus (DENV), Chikungunya virus (CHIKV) and Zika virus (ZIKV) are well-known arboviruses affecting public health as their rapid transmission have resulted in a worldwide distribution (Gould *et al*, 2017). Because these three arboviruses share similar clinical manifestations, clinical diagnosis by itself is not reliable in distinguishing among these arboviruses thus making laboratory confirmation a necessity (Mardekian and

Correspondence: Dar-Der Ji, Department of Tropical Medicine, National Yang-Ming University, No. 155, Sec. 2, Li-Nong St. Taipei 112, Taiwan ROC
Tel: +886 2 28267076
E-mail: darder.ji@gmail.com

Roberts, 2015; Rodriguez-Morales, 2015). Without confirmatory testing, actual prevalence of each disease cannot be accurately estimated and is likely to result in either an under- or over-estimation of cases. One of the main problems of arboviral infection surveillance is a lack of appropriate facilities in remote or undeveloped endemic areas to perform accurate diagnostic tests. Appropriate storage and shipping of samples from those areas to a diagnostic laboratory are not only costly but logistically challenging.

Filter paper has shown to be an economic and efficient method of collecting samples, such as blood and serum, for molecular or immunological detection of pathogens, antibodies and various other proteins associated with human and animal diseases (Smit *et al*, 2014). Although several studies used Whatman 3MM (W-3MM) and Nobuto filter papers for detection of DENV and CHIKV (Matheus *et al*, 2007; Matheus *et al,* 2008; Smit *et al*, 2014), there has been no report to date on the use of filter papers for ZIKV detection. In studies of HIV-1 and HCV detection, HIVDR genotyping and other epidemiological applications, dried blood spots (DBSs) were applied onto Whatman 903 (W-903) filter paper, a US Food and Drug Administration approved Class II medical device (Rottinghaus *et al*, 2013). Another factor is the small DBS volume applied to filter papers making sensitivity and specificity of diagnostic method critical issues (Michaud *et al*, 2007; Snijdewind *et al*, 2012; Smit *et al*, 2014). Currently there is no standard method in the detection of these three arboviruses.

Here, we describe a standardized one-step SYBR Green RT-quantitative (q)PCR assay for accurate and sensitive detection of DENV, CHIKV and ZIKV using the same DBS sample and the same thermocycling protocol.

MATERIALS AND METHODS

Viruses and cell culture method

DENV-2 strain 454009 (Taiwan Centers for Disease Control (TCDC), Taipei, Taiwan) was grown in a mosquito cell line C6/36 (CRL- 1660; ATCC, Manassas, VA) in RPMI medium containing 1% fetal calf serum (FBS) (Gibco/BRL, Life Technologies, Burlington, Canada) at 28°C for 7 days under a humidified atmosphere containing 5% CO_2. CHIKV strain 9700032 (TCDC) and ZIKV strain PRVABC59 (TCDC) were grown in a Vero cell line (CCL-81; ATCC, Manassas, VA) in DMEM medium containing 1% FBS and Penicillin/Streptomycin (PS) (10,000 U/ml) (Gibco/BRL, Life Technologies, Burlington, Canada) at 37°C for 4 to 7 days as described above. Viruses (Dengue, Chikungunya, and Zika) were propagated in Vero cells, and viral titer was determined by a plaque-forming assay.

Virus-spiked dried spots preparation

For each virus, viral supernatant was 10-fold serially diluted from 10^7 to 10^{-1} PFU/ml (determined by a plaque assay) (Eckels *et al*, 1976) in double-distilled water (ddH_2O) [viral supernatant dilutions (VSDs)]. A 100 μl aliquot of each VSD was spotted onto a circular (15 mm in diameter) W-903 or W-3MM filter paper (GE Healthcare Life Sciences, Princeton, NJ) [water-diluted spots (WDSs)]. Another set of dried spots was generated using the same volume of whole blood from a healthy donor (tested negative for all three arboviruses) spiked with 10^7 to 10^{-1} PFU/ml of each VDS [blood-diluted spots (BDSs)]. Each WDS and BDS samples were prepared in duplicate and stored in plastic zip bags for two months at ambient temperature.

RT-qPCR assay

RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). For generation of standard dilution curve of each virus, a 100 μl aliquot of each virus diluant was used for RNA extraction and collected in 70 μl of buffer. For the dried WDS and BDS samples, a 6-mm diameter disk was punched by an office puncher (No. 4711734081000; SDI, Nantou, Taiwan) from each dried filter, placed in 560 μl Avl Buffer of the extraction kit and RNA then extracted as described above. A volume of 16 μl of WDS and BDS in the 6-mm filter paper disc was calculated based on the sample diameter on the original 15-mm disc. In order to avoid cross-contamination and false-positive results, the puncher was flame sterilized after each use and rinsed with RNase-free water.

Primer sets targeting core region (C) specific to the four DENV serotypes (Shu *et al*, 2003) and envelope protein 1 region of CHIKV and ZIKV (Chang *et al*, 2010; Pastorino *et al*, 2005 and this study) are shown in Table 1. Reaction solution (25 μl) comprised 5 μl of RNA, 0.3 μM, 0.3 μM and 0.4 μM DENV, CHIKV and ZIKV primer pairs, respectively and an aliquot of QuantiTect SYBR Green RT-qPCR Kit (Qiagen, Hilden, Germany) solution, and thermocycling was carried out in a LightCycler 96 instrument (Roche, Mannhein, Germany) as follows: 50°C for 30 minutes; 95°C for 15 minutes; 45 cycles of 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 20 seconds; with a final step of 77°C for 20 seconds. Subsequent melting curve was generated by heating at 95°C for 60 seconds, followed by 68°C for 30 seconds and a gradual increase in temperature (1°C/30 seconds) to 90°C during which fluorescence was measured

Table 1
Primers used in the study.

Primer	Sequence 5′→3′	Genomic region	Amplicon size (bp)	GenBank accession no.
DENV-F	CAATATGCTGAAACGCGAGAGAAA	C (135-158)	200	AF038403
DENV-R1-3	CCCCATCTAACCAATATTCCTGCT	C (169-193)		AF180817, AF038403, M93130
DENV-R4	CCCCATCTGTTCAGTATCCCTGCT	C (170-194)		M14931
CHIKV-F	AAGCTYCGCGTCCTTTACCAAG	E1(210-231)	209	EU192142, EU192143
CHIKV-R	CCAAATTGTCCYGGTCTTCCT	E1(398-418)		EU192142, EU192143
ZIKV-F	GCAACATGGCGGAGGTAAGAT	E (1126-1147)	240	KU321639
ZIKV-R	GCTCTYGGTGAATTRGGCGT	E (1504-1525)		KU321639

R: (A/G); Y: (C/T).

continuously. A sample was considered positive for a virus if threshold cycle (Ct) is <40 (based on negative control). Specificity of detection was confirmed by a melting temperature of 80.2, 81.7, 80.5, 83.0, 81.0 and 82.0°C for DENV-1, DENV-2, DENV-3, DENV-4, CHIKV and ZIKV, respectively. A standard curve for each virus was generated by plotting Ct versus viral concentration using a least-square fitting software (GraphPad Prism Software; GraphPad, San Diego, CA).

Validation using clinical samples

For validation of the assay, nine clinically positive random samples stored at -80°C were used, consisting of four DENV serum samples, three CHIKV serum samples and two ZIKV urine samples. A 100 μl aliquot of each sample was spotted on W-903 filter paper and processed for virus quantification as described above.

The study protocol involving human volunteer and samples was approved by the Taiwan CDC Institutional Review Board (approval no. 104122). Prior written informed consent requirement was waived by the Review Board.

RESULTS

Estimation of limit of detection (LOD)

The one-step SYBR green RT-qPCR assays was designed for simultaneous detection of DENV, CHIKV, and ZIKV from RNA extracted from the same sample spotted on W-903 and W-3MM filter papers. A calibration curve was constructed by plotting Ct versus virus concentration using RNA extracted from the same volume of VSD as that of WDS and BDS. LOD is defined as the lowest dilution with positive detection, *ie* Ct <40. LOD of DENV serotype 2 was 1, 1.6 and 16 PFU/ml for VSD, WDS and BDS on W-903 filter paper and 16 PFU/ml for both WDS and BDS on W-3MM filter paper; that of CHIKV 10 PFU/ml for VSD and 16 PFU/ml for both WDS and BDS on W-903 filter paper, and 160 PFU/ml for the latter samples on W-3MM filter paper; and that of ZIKV 10 PFU/ml for VSD and 16 PFU/ml for both WDS and BDS on W-903 filter paper, and 1,600 PFU/ml for the latter samples on W-3MM filter paper (Fig 1). A similar set of experiments performed with DENV serotype 1, 3 and 4 samples spotted on W-903 filter paper and using the same group-specific primers yielded the same result as DENV serotype 2 (1, 1.6 and 16 PFU/ml for VSD, WDS, and BDS respectively) (Fig 2).

LOD of stored viral WDS and BDS samples

In order to evaluate the stability of the three viral WDS and BDS samples on W-903 and W-3MM filter papers, LOD was determined as described above of the samples after storage in plastic zip bags at ambient temperature for two months. Overall, LODs of DENV, CHIKV and ZIKV in WDS and BDS were reduced 10 and 100 folds on W-903 and W-3MM filter paper respectively compared to those detected immediately after application on the filter papers (Fig 3). On W-3MM filter paper stored DENV-spiked BDS was 10-fold more stable than that of WDS. This phenomenon was not observed with CHIKV and ZIKV, the former demonstrating better stability of WDS than BDS on W-3MM filter paper following storage, while there was equal loss of stability for the latter in both types of samples.

Detection of DENV, CHIKV, and ZIKV in clinical samples

Serum samples from patients with verified DENV and CHIKV infection and urine samples from those with ZIKV

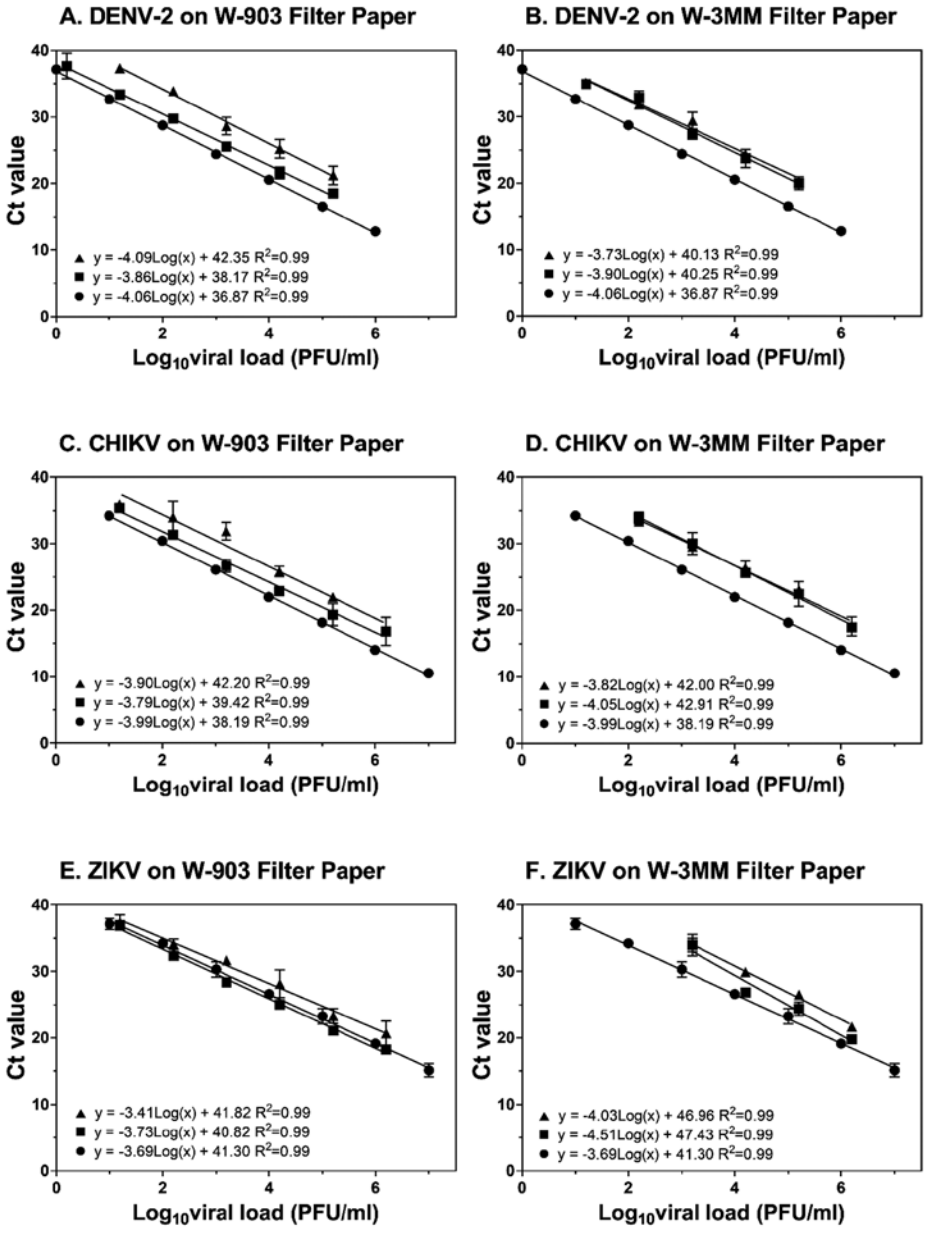

Fig 1-Standard curves of SYBR Green one-step RT quantitative (q)PCR arbovirus detection assay.

A 100 μl aliquot of known arbovirus concentration (determined by plaque assay) was spotted on 15-mm diameter circular Whatman (W) filter paper, allowed to dry at ambient temperature, then a 6-mm diameter sample was excised for RNA extraction and subsequent determination of threshold cycle (Ct) value in a multiplex SYBR Green RT-qPCR assay using arbovirus-specific primers. Negative Ct cut-off >40.

● Viral supernatant; ■ On double distilled water; ▲ On whole blood; CHIKV: Chikungunya virus; DENV: Dengue virus; ZIKV: Zika virus; Ct value: cycle threshold; PFU/ml: plaque-forming unit/milliliter.

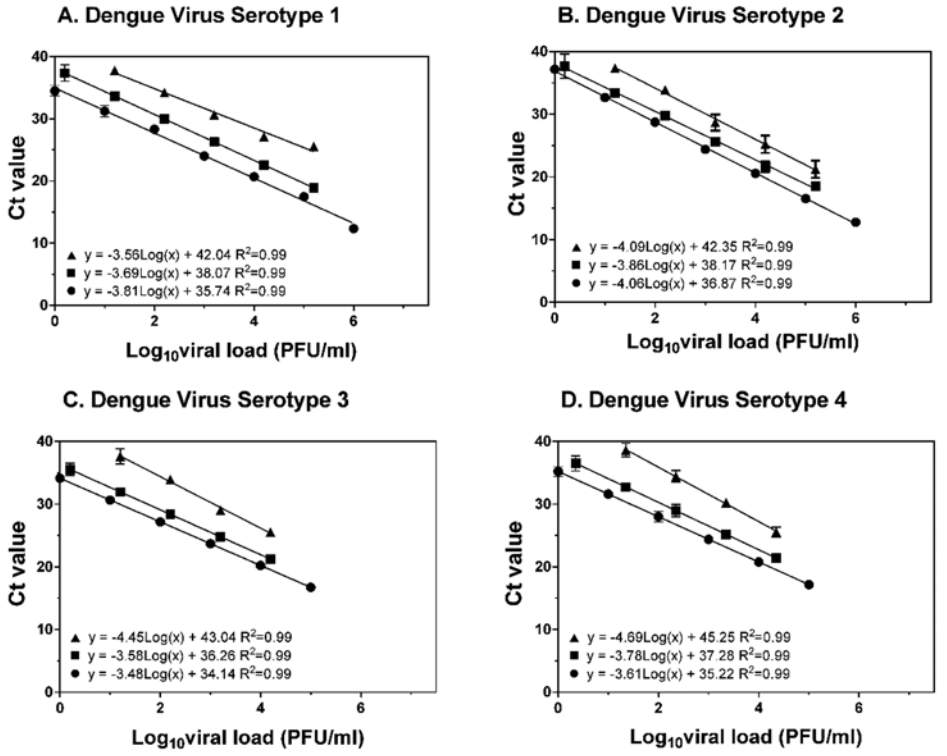

Fig 2-Standard curves of SYBR Green one-step RT quantitative (q)PCR detection assay of dengue virus (DENV) serotypes.

Samples were processed as described in legend to Fig 1 except samples were spotted on Whatman 903 filter paper.

● Viral supernatant; ■ On double distilled water; ▲ On whole blood; Ct value: cycle threshold; PFU/ml: plaque-forming unit/milliliter.

infection (all stored at -80°C) were assayed directly and from samples applied to W-903 filter paper. All samples directly assayed yielded positive results (Ct of 20-29) but sensitivity of detection decreased 4-512 folds when eluted from filter paper (Table 2).

DISCUSSION

Recently, two or even three of the arboviruses CHIKV, DENV and ZIKV were reported to be co-endemic in many countries, especially in Central and South America, such as Honduras and Brazil (Martinez *et al*, 2019). A method that can easily and stably transport collected blood samples from remote areas or in low-income countries and provide simultaneous detection with requisite sensitivity from low sample amount is urgently needed for surveillance of these three arboviruses. We established a multiplex one-step SYBR Green RT-qPCR assay to simultaneously detect these three arboviruses from the same spiked water or blood sample spotted on W-903 filter paper (that was superior

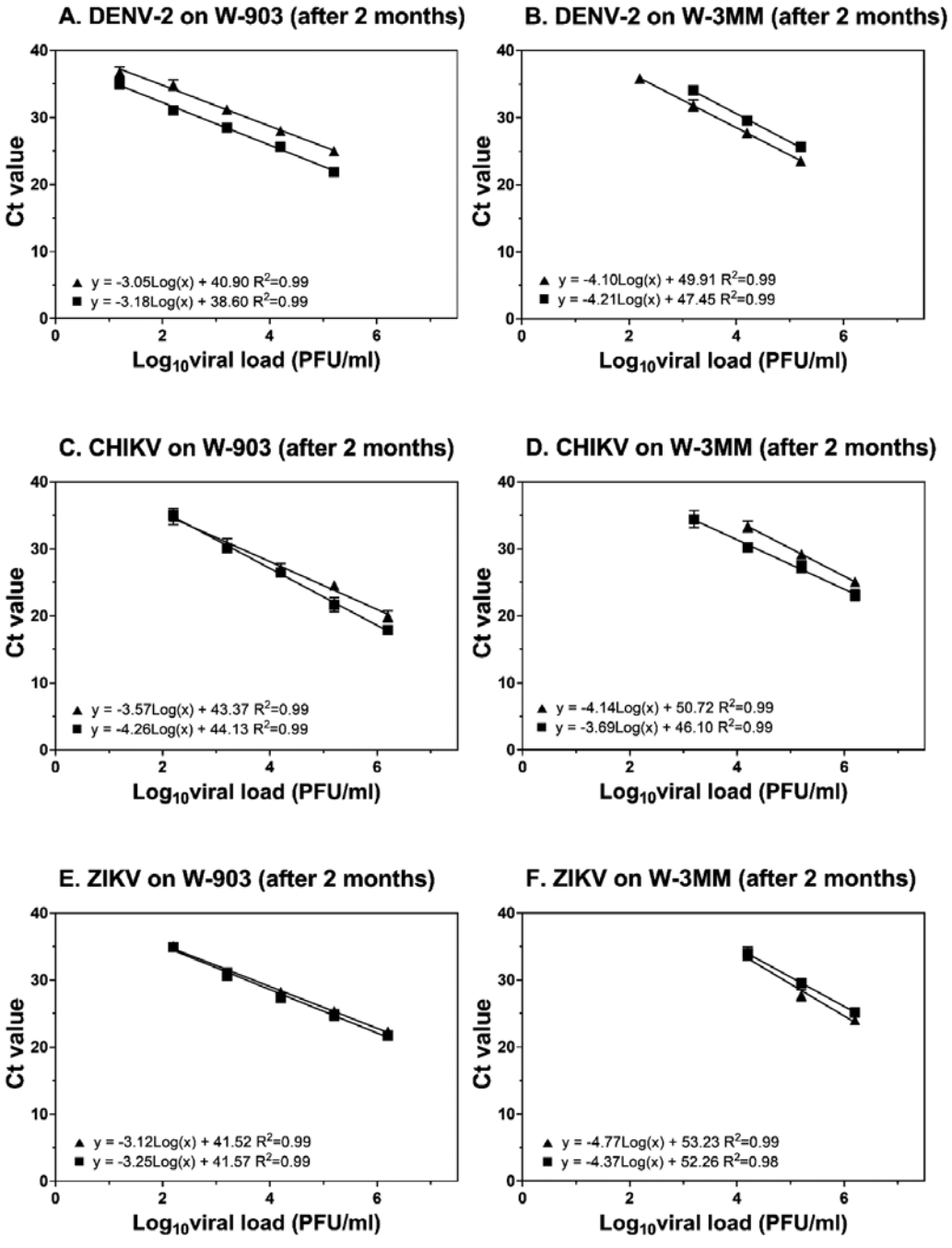

Fig 3-Standard curves of SYBR Green one-step RT quantitative (q)PCR arbovirus detection assay of samples on Whatman (W) filter papers after a 2-month storage at ambient temperature.

Samples were processed as described in legend to Fig 1.

■ On double distilled water; ▲ On whole blood; CHIKV: Chikungunya virus; DENV: Dengue virus; ZIKV: Zika virus; Ct value: cycle threshold; PFU/ml: plaque-forming unit/milliliter.

Table 2
Arbovirus-targeted SYBR Green RT quantitative PCR threshold cycle (Ct) values of clinical samples.

Virus	Serotype/Genotype	Sample Ct*	Spotted on Whatman 903 filter paper Ct*
DENV[a]	Serotype 1	20	24
DENV[a]	Serotype 1	28	33
DENV[a]	Serotype 2	25	31
DENV[a]	Serotype 1	29	34
CHIKV[a]	Asian	24	30
CHIKV[a]	Asian	26	35
CHIKV[a]	Asian	21	24
ZIKV[b]	Asian	28	30
ZIKV[b]	Asian	28	33

*Negative cut-off value >40; [a]Serum sample; [b]Urine sample; CHIKV: Chikungunya virus; DENV: Dengue virus; ZIKV: Zika virus.

than W-3MM filter paper). Sensitivity of the assay decreased 10 to 100 folds after a 2-month storage at ambient temperature depending on virus species and type of sample.

RT-qPCR LOD for DENV utilizing a 6-mm diameter filter paper disc (containing 16 μl of blood) was comparable to LOD of a nested PCR method from a 100 μl blood sample spotted on a Nobuto filter paper (252 and 0.9 PFU for DENV serotype 2 and 3 respectively (Prado *et al*, 2005). LOD of DENV-2 in DBS from Nobuto paper is reduced 100 folds using serotype-specific primer nested PCR) after a 9-week storage at room temperature compared to storage at -70°C (Prado *et al*, 2005), while in the present study there was no change in LOD of DBS on W-903 filter paper after an 8-week storage when detected using group-specific primers in RT-qPCR assay. Although the other three DENV serotypes were not evaluated after storage, given their similar LOD values to that of DENV serotype 2 from assays conducted soon after application on filter paper, similar results could be assumed for stored samples. Shu *et al* (2003) using the same set as in the present study of DENV serotype-specific primer pairs in One-step SYBR Green I RT-qPCR obtained from 10 μl of virus sample LOD of 10, 4.6, 4.1, and 5 PFU/ml for DENV serotypes 1, 2, 3, and 4, respectively indicating the RT-qPCR assay developed in the present study had comparable sensitivity but requiring a smaller volume of sample.

Although LODs of CHIKV in DBS and VSD on W-903 filter paper were comparable, Andriamandimby *et al* (2013) reported a 10-fold reduction in LOD of CHIKV in DBS on W-3MM filter paper compared to that obtained directly from blood sample. As regards detection of ZIKV, both whole blood and urine samples were amenable to detection by the RT-qPCR assay developed.

It is worth noting for samples (serum and urine) stored at -80°C, direct assay of thawed samples gave better results compared to the same samples following application on filter paper. There is no clear explanation for these observations, but perhaps thawing of specimens might have rendered virus more susceptible to physical damage from the application to and subsequent extraction from filter paper matrix. If low temperature storage of clinical samples is not immediately available, storage on filter paper at ambient temperature is the preferred method.

The method established in this study is both reliable and reproducible. Using a simple commercially available paper puncher to obtain BDS is quicker and easier than excising a small sample using scissors or blades. Use of a standardized commercial RNA extraction kit and a one-step multiplex RT-qPCR reduce variations from an in-house RNA extraction protocol and need of separate reaction for each arbovirus of interest. Robustness of the experimental protocol was tested by carrying out the experiments on different days without sigificant deviations of the results (data not shown).

In conclusion, a convenient multiplex SYBR Green RT-qPCR assay was developed for simultaneous detection of six types of arboviruses (four DENV serotypes, CHIKV and ZIKV) from blood samples collected on small filter paper discs, with acceptable sensitivity after a 2-month storage at ambient temperature. This method should find application for timely surveillance in remote regions where two or more arboviruses of interest are circulating and allow development of appropriate control and elimination strategies for these debilitating vector-borne viral diseases.

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REFERENCES

Andriamandimby SF, Heraud JM, Randrianasolo L, Rafisandratantsoa JT, Andriamamonjy S, Richard V. Dried-blood spots: a cost-effective field method for the detection of Chikungunya virus circulation in remote areas. *PLoS Negl Trop Dis* 2013; 7: e2339.

Chang SF, Su CL, Shu PY, *et al*. Concurrent isolation of chikungunya virus and dengue virus from a patient with coinfection resulting from a trip to Singapore. *J Clin Microbiol* 2010; 48: 4586-9.

Eckels KH, Brandt WE, Harrison VR, McCown JM, Russell PK. Isolation of a temperature-sensitive dengue-2 virus under conditions suitable for vaccine development. I*nfect Immun* 1976; 14: 1221-7.

Gould E, Pettersson J, Higgs S, Charrel R, de Lamballerie X. Emerging arboviruses: why today? *One Health* 2017; 4: 1-13.

Mardekian SK, Roberts AL. Diagnostic options and challenges for dengue and chikungunya viruses. *Biomed Res Int* 2015; 2015: 834371.

Martinez JD, Garza JAC, Cuellar-Barboza A. Going viral 2019: zika, chikungunya, and dengue. *Dermatol Clin* 2019; 37: 95-105.

Matheus S, Meynard JB, Lacoste V, Morvan J, Deparis X. Use of capillary blood samples as a new approach for diagnosis of Dengue virus infection. *J Clin Microbiol* 2007; 45: 887-90.

Matheus S, Meynard JB, Lavergne A, *et al*. Dengue-3 outbreak in Paraguay: investigations using capillary blood samples on filter paper. *Am J Trop Med Hyg*

2008; 79: 685-7.

Michaud V, Gil P, Kwiatek O, *et al*. Long-term storage at tropical temperature of dried-blood filter papers for detection and genotyping of RNA and DNA viruses by direct PCR. *J Virol Methods* 2007; 146: 257-65.

Rodriguez-Morales AJ. Zika: the new arbovirus threat for Latin America. *J Infect Dev Ctries* 2015; 9: 684-5.

Rottinghaus E, Bile E, Modukanele M, *et al*. Comparison of Ahlstrom grade 226, Munktell TFN, and Whatman 903 filter papers for dried blood spot specimen collection and subsequent HIV-1 load and drug resistance genotyping analysis. *J Clin Microbiol* 2013; 51: 55-60.

Pastorino B, Bessaud M, Grandadam M, Murri S, Tolou HJ, Peyrefitte CN. Development of a TaqMan RT-PCR assay without RNA extraction step for the detection and quantification of African Chikungunya viruses. *J Virol Methods* 2005; 124: 65-71.

Prado I, Rosario D, Bernardo L, *et al*. PCR detection of dengue virus using dried whole blood spotted on filter paper. *J Virol Methods* 2005; 125: 75-81.

Shu PY, Chang SF, Kuo YC, *et al*. Development of group- and serotype-specific one-step SYBR green I-based real-time reverse transcription-PCR assay for dengue virus. *J Clin Microbiol* 2003 ;41: 2408-16.

Smit PW, Elliott I, Peeling RW, Mabey D, Newton PN. An overview of the clinical use of filter paper in the diagnosis of tropical diseases. *Am J Trop Med Hyg* 2014; 90: 195-210.

Snijdewind IJ, van Kampen JJ, Fraaij PL, van der Ende ME, Osterhaus AD, Gruters RA. Current and future applications of dried blood spots in viral disease management. *Antiviral Res* 2012; 93: 309-21.