Dengue Virus and Yellow Fever Virus Detection Using Reverse Transcription–Insulated Isothermal PCR and Comparison with Real-Time RT-PCR

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Abstract. Real-time reverse transcriptase PCR (rRT-PCR) is the most accurate method for the detection of dengue virus (DENV) and yellow fever virus (YFV) in acute illness. However, performing rRT-PCR is not feasible for many laboratories in regions of endemicity. The current study compared new reverse transcription–insulated isothermal PCRs (the POKKIT DENV and YFV reagent sets) with laboratory-developed rRT-PCRs for both viruses using clinical samples and viral strains from different endemic regions. Sensitivity and specificity of the POKKIT DENV Reagent Set were 87.2% (68/78 samples) and 98.2% of samples (54/55), respectively. The YFV reagent set demonstrated sensitive detection of YFV RNA from six viral strains down to an estimated concentration of 2.5 log₁₀ copies/mL and proved to be specific for YFV. Although the POKKIT assays require RNA extraction, they may provide accurate and less-complex options for molecular testing in laboratory settings where rRT-PCR is not practical.

The genus *Flavivirus* includes dengue virus (DENV), the most common human arbovirus worldwide, and yellow fever virus (YFV), which remains a significant public health threat despite the availability of an effective vaccine.^{1–4} Both pathogens present diagnostic challenges in regions of endemicity.^{3–6} Real-time reverse transcriptase PCR (rRT-PCR) is the most accurate method for DENV and YFV detection during acute infection, although viral RNA may only be detectable in serum or plasma for 5–7 days after symptom onset.^{4,6} In resource-limited settings, few laboratories have the capacity to perform high-complexity molecular testing, and results from national or regional reference laboratories are not available in a clinically relevant time frame. As a result, patients may receive a clinical diagnosis that is inaccurate for differentiating DENV or YFV from many other causes of acute febrile illness.^{2–4} Timely diagnosis and initiation of management for dengue improve clinical outcomes.² Similar data are not available for yellow fever, where early detection may be particularly important, given the high case-fatality rate for severe cases.⁴ Rapid diagnostics are available for the detection of DENV nonstructural protein 1 (NS1) and anti-DENV IgM, but these are significantly less sensitive than rRT-PCR during acute infection.^{6,7} Testing for anti-YFV IgM is often limited to reference laboratories. For both DENV and YFV, IgM is not reliably detectable until 5 days after symptom onset, and significant cross-reactivity occurs between flaviviruses.^{3,4,6,8}

Recently, a hydrolysis probe–based reverse transcription–insulated isothermal PCR has been developed for the detection of DENV in serum (POCKIT DENV Reagent Set, GeneReach Biotechnology, Taichung City, Taiwan).^{9–13} This assay can be performed on a handheld battery-powered instrument that is inexpensive relative to the costs of real-time PCR instruments. Although assay comparisons between the

DENV reagent set and an FDA-cleared DENV rRT-PCR have been reported,^{9,10} independent evaluations of this assay have not been published. In addition, no data have been published regarding the performance of the POKKIT YFV Reagent Set. The objectives of the current study were to evaluate both reagent sets using a diverse set of clinical samples and/or reference viral strains from different regions of endemicity and to compare assay performance with sensitive laboratory-developed rRT-PCRs for each pathogen.^{14,15}

Evaluation of the POKKIT DENV Reagent Set was performed using pre-collected clinical samples from sites in Guatemala (DENV-2 and -3), Paraguay (DENV-1 and -4), and Sri Lanka (DENV-2). Samples had been obtained from patients with a suspected arboviral illness within the first 7 days of symptom-onset as part of ongoing arboviral studies (Guatemala and Paraguay) or routine clinical care (Sri Lanka). Sera were collected in Paraguay and Sri Lanka, whereas plasma and whole blood samples were collected in Guatemala. This research protocol was reviewed and approved by the Emory University Institutional Review Board (Protocol 91162). All statistical analyses were performed using GraphPad software (v. 8.1.1, GraphPad, San Diego, CA).

Total nucleic acids were extracted on an eMAG instrument (bioMérieux, Marcy-l'Étoile, France) according to the manufacturer-recommended protocols. Samples were initially screened using an rRT-PCR for Zika virus, chikungunya virus, and DENV (the “ZCD assay”).¹⁶ Dengue virus–positive samples were then confirmed in a quantitative, serotype-specific DENV rRT-PCR.¹⁵ Nucleic acids were stored at –80°C until tested once with the POKKIT DENV Reagent Set on a Micro Plus Nucleic Acid Analyzer according to the manufacturer-supplied protocol.⁹ Samples with discordant rRT-PCR and POKKIT results were tested a second time with the serotype-specific DENV rRT-PCR for confirmation.

One hundred thirty-three samples were tested for DENV (serum/plasma, $n = 118$; whole blood, $n = 15$). Overall results are shown in Table 1. Four samples (3.0%) yielded “undetermined” results, which display as a “?” on the nucleic acid

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TABLE 1

Comparison of the POKKIT DENV Reagent Set with a laboratory-developed rRT-PCR

DENV rRT-PCR Result	N	Viral load Median (range)*	Results, POKKIT DENV	
			Detected, n	Sensitivity (%)
DENV positive	78	5.2 (2.7–10.8)	68	87.2
DENV-1	24	5.3 (2.7–9.3)	20	83.3
DENV-2	33	4.4 (2.8–10.8)	29	87.9
DENV-3	18	6.8 (4.2–9.5)	16†	88.9
DENV-4	3	4.8 (4.8–5.5)	3	100
DENV negative	55	–	1‡	–

DENV = dengue virus; rRT-PCR = real-time reverse transcriptase PCR.

* Determined by rRT-PCR, expressed as log₁₀ copies/mL of serum.† One eluate each from plasma and whole blood tested negative (viral loads 4.2 and 5.4 log₁₀ copies/mL, respectively).

‡ Specificity, 98.2%.

analyzer. Undetermined samples included 3 DENV-positive samples (DENV-1 and DENV-2, $n = 2$) and 1 DENV-negative sample. These were retested with the POKKIT assay: 3/4 yielded negative/undetermined results and one sample yielded one positive and one negative result. All undetermined samples were thus classified as negative for further calculations.

The POKKIT assay demonstrated a high level of agreement with reference rRT-PCR results (kappa statistic 0.83, 95% CI 0.74–0.93), and the sensitivity was 87.2% (68/78). Seven samples were negative in the POKKIT assay but positive by rRT-PCR. When combined with undetermined samples, 8/10 samples that tested positive by rRT-PCR but negative/undetermined in the POKKIT had viral loads less than 4.0 log₁₀ copies/mL serum (DENV-1, $n = 4$; DENV-2, $n = 4$). Two DENV-3 samples gave false-negative results and included one plasma and one whole blood sample with viral loads of 4.2 and 5.4 log₁₀ copies/mL, respectively. Therefore, the POKKIT assay detected 96.2% of samples with viral loads ≥ 4.0 log₁₀ copies/mL (50/52). Specificity of the POKKIT assay was 98.2% (54/55), which resulted from one positive serum sample that was repeatedly negative in both the ZCD and DENV serotype-specific rRT-PCRs.

Evaluation of the POKKIT YFV Reagent Set was performed using dilutions of genomic RNA from six reference YFV strains representing five genotypes (Table 2). Dilutions were tested in duplicate across a range of concentrations extending from 1.0 to 9.3 log₁₀ copies/mL of sample. Viral load was quantified in a laboratory-developed rRT-PCR.¹⁴ For dilutions with viral loads ≥ 2.5 log₁₀ copies/mL (~ 1 copy/ μ L of eluate), RNA

detection in the POKKIT YFV Reagent Set (43/48, 89.6%) was similar to that in the laboratory-developed rRT-PCR (44/48, 91.7%). Of samples with viral loads below this level, 1/12 (8.3%) was detected in the POKKIT assay compared with 5/12 (41.7%) by rRT-PCR. To evaluate assay specificity, genomic RNA was tested from related flaviviruses (Table 2).¹⁴ In addition, 12 DENV-positive and 12 DENV-negative serum samples from Paraguay were tested. All samples in the specificity analysis tested negative except for one DENV-1 sample that yielded an indeterminate result. This sample tested negative for YFV by rRT-PCR and was negative in the POKKIT YFV Reagent Set on repeat testing.¹⁴

In this study, the POKKIT DENV and YFV reagent sets were compared with laboratory-developed rRT-PCRs that have proven to be more sensitive than common comparators for each pathogen.^{14,15,17} The POKKIT DENV assay demonstrated a sensitivity of 87.2% and detected 96.2% of DENV-positive samples with viral loads ≥ 4.0 log₁₀ copies/mL. Viral loads in this range are common among patients who present within the first 5 days of symptom onset,⁷ such that assay use could be targeted to a high-yield population based on information that is readily available at presentation. Notably, sensitivity is higher than that of a rapid NS1 assay used to test patients in Paraguay (71.4%).⁷ Given the rapidity of assay performance (setup time, ~ 5 –10 minutes; run time, 45 minutes), this could provide a sensitive testing option for use near the point of patient care.

Assay performance of the YFV reagent set has not yet been published, but in this initial evaluation, the assay proved to be as sensitive as rRT-PCR down to viral loads of 2.5 log₁₀ copies/mL using genomic RNA from six YFV strains (five genotypes). A single DENV-1 sample gave an indeterminate result in the YFV assay, although this was negative on repeat testing. Given the high significance of detecting even a single yellow fever case, which can trigger vaccination campaigns and the issuance of travel medicine advisories, reference YFV testing with rRT-PCR testing will need to be available to confirm results.^{3,4,8} However, this test may provide an option for sensitive molecular testing in remote and/or resource-limited regions disproportionately affected by YFV.^{3,4} As such, it warrants further evaluation with acute-phase clinical samples.⁴

Currently, the manufacturer only recommends the use of serum with these two reagent sets, although one previous

TABLE 2
Evaluation of POKKIT YFV reagent set

Sample	Viral load, range*	YFV real-time reverse transcriptase PCR	POKKIT yellow fever
		Detected, n/total (%)	Detected, n/total (%)
Sensitivity evaluation	≥ 5.5	22/22 (100)	22/22 (100)
	3.25–5.49	14/14 (100)	14/14 (100)
	2.5–3.24	8/12 (66.7)	7/12 (58.3)
	1.0–2.49	5/12 (41.7)	1/12 (8.3)
Specificity evaluation			
Flaviviral strains†	7.2–8.8	0/8	0/8
DENV-positive samples§	5.1–9.3	0/12	0/12
DENV-negative samples	–	0/12	0/12

DENV = dengue virus; YFV = yellow fever virus.

* Expressed as log₁₀ copies/mL of sample, estimated for YFV strains and calculated for DENV serum samples.

† Yellow fever virus strains: Asibi-Ghana, 1927 and Senegal ArD 149194, 1996 (West Africa II genotype); Couma-Ethiopia 1961 (East/Central Africa); Angola SH 281788, 2016 (Angola); INHRR 10a-10 – Venezuela 2010 (South America I); and FVB 0196 – Bolivia 2006 (South America II).

‡ Genomic RNA from West Nile virus (two strains), tick-borne encephalitis virus, Japanese encephalitis virus, St. Louis encephalitis virus (two strains), Zika virus, and DENV-4.

§ DENV-1 ($n = 11$) and DENV-4 ($n = 1$); a single DENV-1 sample yielded an initial indeterminate POKKIT YFV result that was negative on repeat testing (DENV viral load, 8.1 log₁₀ copies/mL of serum).

publication also used plasma.⁹ It is unclear if this difference in the sample matrix affects performance, but notably, the DENV-3 samples that tested negative in the POKKIT assay were plasma and whole blood. The POKKIT assay still requires nucleic acid extraction, which in turn requires the availability of skilled personnel and relatively sophisticated laboratory facilities that can maintain molecular workflow. However, additional instruments are available for automated RNA extraction and sample-to-answer detection based on this technology.^{11,12} In conclusion, the POKKIT DENV and YFV reagent sets compared favorably with sensitive rRT-PCRs for each pathogen, and these may provide accurate and less-complex options for molecular testing in certain laboratory settings.

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