RESEARCH PAPER

Development of a TaqMan-Based Multiplex Real-Time RT-PCR Assay for Detection of Mosquito-Borne Viral Diseases

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Abstract

Background: Mosquito-borne viral diseases, including Dengue (DENV), Zika (ZIKV), Chikungunya (CHIKV), Yellow fever (YFV), and West Nile (WNV), represent a significant global health burden. Existing singleplex molecular assays are often time-consuming and resource-intensive. We aimed to develop and analytically validate a one-step multiplex TaqMan real-time RT-PCR assay for the simultaneous detection of these major arboviruses.

Methods: Primers and hydrolysis probes were designed against conserved genomic regions. In vitro transcribed RNA standards were used to establish standard curves, determine PCR efficiency, and calculate limits of detection (LOD). A clinical validation panel comprising 250 samples with pre-determined infection statuses was used to assess diagnostic sensitivity and specificity against singleplex reference assays. Cross-reactivity was evaluated against a panel of related and unrelated pathogens.

Results: The multiplex assay demonstrated linear detection across eight orders of magnitude (10^1 to 10^8 copies/µL) with PCR efficiencies ranging from 94.2% to 102.1% and coefficient of determination (R^2) values ≥ 0.992 for all targets. The 95% LOD ranged from 10 to 50 copies/µL. No cross-reactivity was detected with any of the non-target organisms tested. In the clinical cohort, the assay achieved an overall diagnostic sensitivity of 98.0% (95% CI: 93.8–99.5%) and specificity of 100% (95% CI: 97.5–100%), with complete concordance observed between multiplex and singleplex assays for all targets except one low-titer ZIKV sample.

Conclusion: The developed TaqMan multiplex qRT-PCR assay provides a rapid, sensitive, and highly specific platform for syndromic screening of major mosquito-borne viruses. The analytical and clinical validation framework supports its potential utility in both clinical diagnostics and vector surveillance programs.

Keywords: multiplex RT-PCR; TaqMan; arbovirus; Dengue; Zika; Chikungunya; Yellow fever; West Nile; molecular diagnostics.

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1. Introduction

Mosquito-borne viruses (arboviruses) transmitted by Aedes and Culex species cause hundreds of millions of infections annually [1]. Dengue virus (DENV) alone is responsible for approximately 390 million infections per year, while Zika virus (ZIKV), Chikungunya virus (CHIKV), Yellow fever virus (YFV), and West Nile virus (WNV) continue to trigger regional epidemics across tropical and subtropical regions worldwide [2]. Clinical presentation of these infections is often nonspecific, characterized by fever, arthralgia, myalgia, and rash, making differential diagnosis based on symptoms alone unreliable [3]. Conventional diagnostic approaches such as virus isolation, serological testing, and singleplex real-time RT-PCR are limited by turnaround time, cross-reactivity of antibodies, and the need for multiple discrete reactions to rule out co-infections [4]. The increasing incidence of co-circulation and documented co-infections underscores the necessity for syndromic molecular panels capable of detecting multiple arboviruses in a single reaction vessel [5]. Multiplex TaqMan-based real-time RT-PCR offers a powerful solution by combining reverse transcription, complementary DNA amplification, and target-specific detection into a one-step workflow. By assigning a unique fluorophore to each viral target, these assays

enable simultaneous detection and discrimination without post-amplification processing [6]. In this study, we describe the development, optimization, and analytical validation of a novel one-step multiplex TaqMan qRT-PCR assay targeting DENV (pan-serotype), ZIKV, CHIKV, YFV, and WNV. We present comprehensive results demonstrating the analytical sensitivity, linearity, specificity, and clinical diagnostic performance of the assay using quantified synthetic RNA standards and a representative clinical sample panel.

2. Materials and Methods

2.1 Viral RNA Standards and Controls

In vitro transcribed RNA (IVT-RNA) standards were generated from plasmids containing the target regions for DENV (3' UTR, all four serotypes), ZIKV (NS5 gene), CHIKV (E1 gene), YFV (NS5 gene), and WNV (envelope gene). RNA concentration was determined by spectrophotometry (NanoDrop One), and copy number was calculated based on molecular weight. Ten-fold serial dilutions (10^8 to 10^1 copies/µL) in RNase-free TE buffer supplemented with yeast tRNA carrier (20 ng/µL) were prepared for standard curve analysis.

2.2 Primer and Probe Design

Primers and dual-labeled hydrolysis probes were designed using Primer Express Software v3.0 and BLAST alignment to ensure specificity. The DENV primer set was designed within the conserved 3' untranslated region to detect all four serotypes (pan-DENV). Probes were labeled with distinct reporter dyes across the detection channels: FAM (DENV), VIC (ZIKV), ABY (CHIKV), NED (YFV), and JUN (WNV) [7]. All probes contained a 3' minor groove binder/non-fluorescent quencher (MGB-NFQ). The sequences of all primers and probes are given in Table 1.

2.3 Multiplex qRT-PCR Conditions

Reactions were performed in a 25 µL total volume using the AgPath-ID One-Step RT-PCR Reagents. The optimized final reaction contained: 1× RT-PCR buffer, 3.5 mM $MgCl_2$, 800 µM dNTP mix, 400 nM of each forward and reverse primer, 200 nM of each probe, 1 µL of RT-PCR enzyme mix, and 5 µL of template RNA. Thermal cycling conditions on the Applied Biosystems QuantStudio 5 Flex Real-Time PCR System were: reverse transcription at 45°C for 10 min, initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s and combined annealing/extension at 60°C for 45 s [8].

2.4 Analytical Sensitivity and Linearity

Standard curves for each target were generated in triplicate across five independent runs. Limits of detection (LOD) were determined by probit analysis of 24 replicates per dilution near the predicted end-point. A result was considered positive if the cycle threshold (Ct) value was ≤ 40.0 with an exponential amplification curve [9].

2.5 Specificity Panel

Analytical specificity was challenged using high-titer nucleic acid extracts from closely related flaviviruses (Japanese encephalitis virus, Usutu virus), alphaviruses (Sindbis virus, Ross River virus), human coronavirus 229E, influenza A (H1N1), Plasmodium falciparum, and uninfected human serum [10,11].

2.6 Clinical Validation

A retrospective, blinded panel of 250 clinical serum samples was constructed to represent varying prevalence rates. Sample statuses were predetermined based on singleplex reference assay results and sequencing, and included mono-infections, negative samples, and a subset of co-infections. The multiplex assay was run by a single technician blinded to the expected results. Diagnostic sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using standard contingency tables [12].

3. Results

3.1 Assay Optimization

Multiplex reaction conditions were optimized to minimize primer-dimer formation and cross-channel fluorescence bleeding. A final $MgCl_2$ concentration of 3.5 mM and an annealing temperature of 60°C provided the optimal balance of analytical sensitivity and reaction efficiency for all five targets. No evidence of competitive inhibition was observed when targets were combined at equimolar concentrations.

3.2 Primer and Probe Sequences

Table 1. Primer and probe sequences used in the multiplex qRT-PCR assay.

Target	Gene/Region	Oligo Type	Sequence (5'—3')	Fluorophore/ Quencher
DENV	3' UTR	Forward	GCAGCAAGATGACAAGGATGC	—
DE	3' UTR	Rev	GGT	—

NV		erse	CTC GTG GCA TGT CTT GA	
DENV	3' UTR	Probe	CCG TCT CAG TTA TGT GG	FAM-MGB
ZIKV	NS5	Forward	TCC TGA TGA TGC TGA TGC TGG	—
ZIKV	NS5	Reverse	CCG TCC TCA TAG AGC AGA TCC	—
ZIKV	NS5	Probe	AAC GCA GCA GGA ACA ACA GGA	VIC-MGB
CHIKV	E1	Forward	GCG TAA CGC TGC TCA ACC AA	—
CHIKV	E1	Reverse	AGG CGT GTA CCA AGA GTC CAT	—
CHIKV	E1	Probe	TTC ATC CGC TGG TGG CCA TCA	ABY-MGB
YFV	NS5	Forward	GAC TCA TGG AAG ACA GGA A	
YFV	NS5	Reverse	GCC AAT GGT CTT CGC ATA GGT	—
YFV	NS5	Probe	TGC CCA GCC TTC AAG GTC AA	NED-MGB
WNV	Envelope	Forward	CCA CGG CTC TGG TTC TAA AAA	—
WNV	Envelope	Reverse	CTG GGT CCT CCT GTA GTC ATT	—
WNV	Envelope	Probe	AGC GGG TCG CTC GCT GGA A	JUN-MGB

3.3 Standard Curve Performance and PCR Efficiency

All targets demonstrated strong linearity across the dynamic range, with Pearson correlation coefficients (R^2) ≥ 0.992 and PCR amplification efficiencies within the acceptable 90–110% range. Standard curve parameters and PCR efficiency for each target are presented in Table 2.

Table 2. Standard curve parameters and PCR efficiency for each target in the multiplex assay.

Targ	Slo	y-	R^2	Efficie	Linear

et	pe	Interc ept	Val ue	ncy (%)	Range (copies /µL)
DEN V	−3.2 89	38.42	0.9 98	101.4	10^1 – 10^8
ZIK V	−3.4 21	39.15	0.9 94	96.1	10^1 – 10^8
CHI KV	−3.3 12	38.88	0.9 97	100.7	10^1 – 10^8
YFV	−3.4 52	40.02	0.9 92	94.2	10^1 – 10^8
WN V	−3.3 01	38.71	0.9 99	100.9	10^1 – 10^8

[Figure 1 should be inserted here]

Figure 1. Representative standard curves for the five viral targets in the multiplex qRT-PCR assay. Serial ten-fold dilutions of in vitro transcribed RNA standards (10^1 to 10^8 copies/µL) were analyzed in triplicate.

3.4 Limit of Detection

The multiplex LOD remained within one log dilution of the corresponding singleplex LOD for all targets, indicating minimal loss of analytical sensitivity under multiplex conditions. The limits of detection and hit-rate Ct values for singleplex and multiplex configurations are compared in Table 3.

Table 3. Limits of detection (LOD) and hit-rate cycle threshold values for singleplex and multiplex assay configurations.

Target	Singleplex LOD (copies/µL)	Multiplex LOD (copies/µL)	95% Hit Rate Ct (Mean ± SD)
DENV	10	10	37.8 ± 1.12
ZIKV	10	25	36.4 ± 1.45
CHIKV	10	10	37.2 ± 0.98
YFV	25	50	35.9 ± 1.67
WNV	10	10	37.5 ± 1.21

3.5 Specificity Testing

No amplification was detected for any of the non-target organisms tested, including related flaviviruses and alphaviruses. Mean Ct values for all specificity panel members were undetermined (no amplification by cycle 45), confirming a lack of cross-reactivity.

3.6 Clinical Validation Results

A blinded panel of 250 clinical serum samples was analyzed. The sample distribution and multiplex assay results are given in Table 4 and 5. The single false-negative DENV sample had a singleplex Ct of 39.1, representing a viral load near the LOD. The single false-negative ZIKV sample was similarly borderline (singleplex Ct 38.7) and was not detected in the multiplex reaction. Two samples contained DENV-CHIKV co-infections, and both were correctly identified as positive for both targets by the multiplex assay. The overall diagnostic performance metrics of the multiplex assay are given in Table 4, and the breakdown by individual viral target is presented in Table 5.

Table 4. Diagnostic performance metrics of the multiplex assay against the reference standard.

Assay Parameter	Value (n/N)	Percentage	95% Confidence Interval
Sensitivity	98/100	98.0%	93.8 – 99.5%
Specificity	150/150	100%	97.5 – 100%
PPV	98/98	100%	-
NPV	150/152	98.7%	95.7 – 99.6%
Overall Accuracy	248/250	99.2%	-

Table 5. Breakdown of diagnostic performance by individual viral target.

Virus	True Positives	False Negatives	True Negatives	False Positives	Sensitivity
DENV	45	1	205	0	97.8%
ZIKV	19	1	230	0	95.0%
CHIKV	15	0	235	0	100%
YFV	8	0	242	0	100%
WNV	11	0	239	0	100%

3.7 Reproducibility

Intra-assay coefficients of variation (CV) for Ct values at 10^4 copies/µL ranged from 0.45% to 1.12%. Inter-assay CVs across five independent runs ranged from 1.02% to 2.34%, demonstrating high precision.

4. Discussion

This study describes the successful development of a one-step TaqMan multiplex qRT-PCR assay capable of detecting five clinically significant arboviruses in a single reaction. The assay achieved excellent linearity, high PCR

efficiency, and low limits of detection consistent with requirements for acute-phase clinical diagnostics. Our validation approach allowed for rigorous characterization of analytical performance prior to prospective clinical deployment, following established frameworks for presenting methodological findings in molecular assay development.

The multiplex format did not substantially compromise analytical sensitivity relative to singleplex reactions. The observed minor increases in LOD for ZIKV and YFV (from 10–25 to 25–50 copies/µL) are likely attributable to dynamic competition for reaction reagents and minor fluorescence channel cross-talk, which is a known limitation of high-plex reactions. Nevertheless, these LODs remain well within the range of viremia typically observed during the acute phase of infection, suggesting clinical relevance.

The 100% specificity observed in the clinical panel indicates that probe design strategies targeting conserved yet distinct genomic regions were effective at excluding closely related viruses. The pan-DENV primer/probe set successfully detected all four serotypes without requiring four separate DENV-specific reactions, streamlining laboratory workflow.

From a diagnostic operations perspective, the assay supports sample-to-answer syndromic testing. The ability to detect co-infections in a single tube is particularly advantageous in endemic regions where Aedes aegypti vectors transmit multiple viruses simultaneously. The assay also demonstrated robust reproducibility, with inter-assay variation below 2.5%, supporting its suitability for high-throughput screening during outbreak investigations.

Limitations of this study include the reliance on a retrospective sample cohort; however, the blinded validation design and predetermined reference standard mitigated operator bias. Future work will involve prospective clinical validation in cohorts from multiple endemic regions and incorporation of an internal extraction control to monitor both nucleic acid extraction and PCR inhibition.

5. Conclusion

The developed TaqMan-based multiplex real-time RT-PCR assay provides a sensitive, specific, and reproducible method for the simultaneous detection of DENV, ZIKV, CHIKV, YFV, and WNV. With high PCR efficiencies, low limits of detection, and excellent concordance with singleplex reference methods, this assay represents a valuable tool for arbovirus surveillance, clinical diagnostics, and rapid outbreak response in regions endemic for mosquito-borne viral diseases.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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