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Short communication

Real-time RT-PCR for Mayaro virus detection in plasma and urine

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ABSTRACT

Background: Mayaro virus (MAYV) causes an acute febrile illness which can be difficult to differentiate from dengue or chikungunya. MAYV RNA can be detected in plasma during the first 3–5 days of illness, but only a single rRT-PCR has been fully evaluated in the literature.
Objectives: To develop an rRT-PCR for MAYV and evaluate assay performance using human plasma and urine samples spiked with different MAYV strains.
Study design: A MAYV rRT-PCR targeting a region of the 5′UTR and nsp1 gene was designed from the alignment of all complete-genome MAYV sequences to be compatible with existing laboratory protocols. The assay was evaluated using human samples spiked with six MAYV strains, including strains from each of the three genotypes.
Results: The linear range of the MAYV rRT-PCR extended from 1.0 to 8.0 $\log_{10}$ copies/μL, and the lower limit of 95% detection was 8.2 copies/μL. No detection was observed when the MAYV rRT-PCR was tested with genomic RNA from related arboviruses. The assay demonstrated linear amplification of all 6 MAYV strains when spiked into human plasma samples as well as 2 strains spiked into urine.
Conclusions: We report the design and evaluation of an rRT-PCR for MAYV. Given the concern for MAYV emergence in the Americas and the few molecular tests that have been evaluated in the literature, this assay should provide a useful diagnostic for patients with an acute febrile illness.

1. Background

Mayaro virus (MAYV) is a member of the *Alphavirus* genus (family Togaviridae) that is primarily transmitted by tree-dwelling *Haemagogus* species mosquitoes [1–3]. Although originally isolated in Trinidad in 1954 [1,4], human cases have predominantly been detected in South America [1–3,5–8]. Three genotypes of MAYV have been identified (D, L, and N), with genotype D viruses causing the majority of cases [3,5]. Genotype L has almost exclusively been detected in Brazil, and only one strain of genotype N virus has been identified, in Peru [3,5]. In 2015, MAYV was isolated from a symptomatic child in rural Haiti [9], raising concern for the emergence of MAYV into new areas as the virus has proven transmissible by *Aedes aegypti* in the laboratory [10].

Human infections with MAYV result in an acute febrile illness that is difficult to differentiate from dengue or chikungunya [1,4,6–9]. Patients present with fever, headache, myalgias and arthralgias; a non-pruritic rash develops in the majority of patients [4]. The diagnosis is often confirmed by serological testing [4,5,7,8]. Virus is detectable by culture or reverse-transcription PCR (RT-PCR) within the first 3–5 days of illness [4,7,11]. However, only a few real-time RT-PCRs (rRT-PCRs) have been described in the literature [6,8,10,12], and the analytical evaluation of only a single rRT-PCR has been reported to date [12].

2. Objectives

The objectives of the current project were to develop a sensitive and specific rRT-PCR for MAYV and evaluate assay performance using human plasma and urine samples spiked with different MAYV strains.

3. Study design

3.1. MAYV rRT-PCR design and optimization

All MAYV sequences > 11 kb and available in Genbank (n = 35

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sequences) were aligned using MegAlign software (DNASTAR). Primers and probes were designed using Primer3 software to target highly conserved genomic regions and to be compatible with existing laboratory protocols [13–15]. All rRT-PCRs were performed using 5 µL of nucleic acid template in 25 µL reactions of the SuperScript III Platinum One-Step qRT-PCR Kit (Thermo Fisher Scientific) on a Rotor-Gene Q instrument (Qiagen). Cycling conditions, assay set-up, and interpretation were performed as previously described [13,15].

3.2. Analytical evaluation

Analytical performance of the MAYV rRT-PCR was evaluated according to published recommendations and previous evaluations in our laboratory [13–16]. Dynamic range and lower limit of 95% detection (95% LLOD) studies were performed using quantitated, synthesized single-stranded DNA (ssDNA) containing the consensus MAYV target sequence from the aforementioned alignment, which matched strain BNI-1 (genotype D, 100% identity) [6]. The dynamic range was evaluated by testing 4 replicates of serial 10-fold dilutions from 8.0 $\log_{10}$ copies/µL to 1 copy/µL of eluate. To establish the 95% LLOD, 10 replicates of 5, 2-fold dilutions extending from, and including, the lower limit of the dynamic range were tested on a single run.

Specificity of the MAYV rRT-PCR was evaluated by testing genomic RNA from the following viruses: DENV-1 Hawaii 1944, DENV-2 New Guinea C strain, DENV-3 strainH87, and DENV-4 strain H241, CHIKV (strain R80422a provided by the CDC Division of Vector Borne Diseases and the S27 Petersfield strain from Vircell Microbiologists, Granada, Spain), West Nile (4 strains), Japanese encephalitis, tick-borne encephalitis, yellow fever (17D and Asibi strains), Saint Louis encephalitis, Zika (MR766 strain), Semliki forest, Ross river, Getah, Barmah forest, and Una [14].

3.3. MAYV strains and spiked human samples

Lyophilized culture supernatants from six MAYV strains were kindly provided by the World Reference Center for Emerging Viruses and Arboviruses (Table 1). Strains were reconstituted in 1 mL of culture media and diluted 500-fold. Diluted stocks were used to spike MAYV-negative human plasma and urine and prepare four, serial 10-fold dilutions in these specimen types. Total nucleic acids were extracted from 200 µL of the spiked sample using an eMAG instrument (bioMerieux) with a 50 µL elution volume. Plasma samples were extracted in duplicate, and urine was extracted in triplicate. Each eluate was tested in the MAYV rRT-PCR immediately. The concentration of RNA in the eluates was calculated from a 4-point standard curve (8.0, 6.0, 4.0, and 2.0 $\log_{10}$ copies/µL).

Table 1
MAYV strains tested using the MAYV rRT-PCR.

Strain	Accession Number[a]	Country (State)	Year	Genotype	Reference
ARV 0565	DQ487397, KP842800	Perú (San Martin)	1995	D	[3,5]
BE H 256	DQ487381, KP842819	Brazil (Pará)	1955	L	[3,5]
BEH 342912	DQ487387	Brazil (Pará)	1978	D	[3]
FCB 0587	NA	Bolivia (Nuflo de Chavez)	2007		[10]
FMD 3213	KP842812	Perú (Madre de Dios)	2010	N	[5]
INHRR 11a-10	KP842795	Venezuela (Portuguesa)	2010	D	[5]

[a] Genbank accession number, not available for FCB 0587.

Table 2
Primers and probe sequences for the MAYV rRT-PCR.

Name	Sequence (5′ → 3′)	Concentration[a]	Location[b]
MAYV Forward	AAGCTCTTCCTCTGCATTGC	300 nM	51–70
MAYV Reverse 1	TGCTGGAAACGCTCTCTGTA	300 nM	141–160
MAYV Reverse 2	TGCTGGAAATGCTCTTTGTA		
MAYV Probe[c]	GCCGAGAGCCCGTTTTTAAAATCAC	200 nM	116–140

[a] The concentration of each oligonucleotide in the final reaction mixture.

[b] Genomic locations are provided based on the reference sequence "Mayaro virus isolate BR/SJRP/LPV01/2015, complete genome" (Genbank: KT818520.1).

[c] The 5′ fluorophore and 3′ quencher on the MAYV probe were Cal Fluor Orange 560 and BHQ-1, respectively.

3.4. Statistics

Basic statistics and linear regressions were performed using Excel software (IBM). Probit analysis was performed using SPSS (IBM) to determine the 95% LLOD of the MAYV rRT-PCR.

4. Results

4.1. Analytical evaluation

Primers and probes designed to different genomic regions were first tested *in silico* using BLASTn to query the NCBI nucleotide database and identify potential cross-reactions with other alphaviruses or pathogens that may cause a similar clinical presentation. Selected primer and probe sets were then compared side-by-side using genomic RNA from the 11a-10 strain of MAYV (Table 1). The most sensitive assay, which targets a region of the 5′ untranslated region and non-structural protein 1 gene, was selected for further development (Table 2). Primer and probe concentrations were optimized and concentrations used in the final reaction mixture are shown in Table 2.

The linear range of the MAYV rRT-PCR extended from 1.0 to 8.0 $\log_{10}$ copies/µL (Fig. 1A and B). The 95% LLOD of was calculated to be 8.2 copies/µL of eluate (95% confidence interval, 5.7–18.5). Assay exclusivity was confirmed by testing genomic RNA from a panel of related viral pathogens. No amplification in the MAYV rRT-PCR was observed for any of these viruses. Additionally, a set of serum (n = 72) and plasma (n = 40) samples from patients without a history of travel to South America was tested, and no amplification was detected.

4.2. Spiked human samples

Dilution series of spiked plasma samples were prepared for each MAYV strain (Table 1) with estimated concentrations ranging from 1.5 to 6.9 $\log_{10}$ copies/µL. Dilution series in MAYV-negative human urine were also prepared using the 11a-10 and ARV 0565 strains. Estimated MAYV RNA concentration ranged from 1.9–4.9 $\log_{10}$ copies/µL for the 11a-10 strain and from 2.9–5.9 for ARV 0565. Amplification in the MAYV rRT-PCR was linear for each tested strain and specimen type (R^2 for each linear regression > 0.99).

5. Discussion

The MAYV rRT-PCR demonstrated analytically sensitive MAYV detection and similar performance for strains of each MAYV genotype [5]. Assay sensitivity may be particularly important for MAYV detection as viremia is only detectable for 3–5 days following symptom onset [4,7,10]. Quantified serum viral loads in set of 22 patients ranged from