Validation of a real-time RT-PCR based detection system for Yellow Fever Virus specific RNA

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Introduction

Brazil has recently experienced its largest-recorded yellow fever (YF) outbreak in decades, with more than 2000 confirmed cases and over 600 deaths since December 2016. YF is a severe mosquito-borne viral infection in the tropics, responsible for 29000 to 60000 deaths annually in South America and Africa. Yellow fever virus (YFV) is the prototype of the genus *Flavivirus*, which comprises around 70 different arthropod-borne viruses.

No specific treatments have been found to benefit patients with yellow fever, only supportive care to treat dehydration, respiratory failure, and fever. But a YF vaccine is available, which is a live attenuated viral vaccine from the 17D lineage, which elicits a rapid, exceptionally strong, and markedly durable adaptive immune response.

The clinical diagnosis of YF is difficult because of the similarities of the symptoms to those of a wide range of diseases, including dengue fever, other hemorrhagic viral diseases, leptospirosis, viral hepatitis, and malaria; hence laboratory confirmation is essential. Serologic diagnosis is best accomplished using an enzyme-linked immunosorbent assay (ELISA) for IgM. Serological techniques often cross-react among flavivirus infections, thus, the use of real-time reverse transcription polymerase chain reaction (RT-PCR) should be prioritized. Viral RNA can be detected in serum during the first 10 days from symptom onset (viremic phase) using real-time RT-PCR. Samples that test negative by RT-PCR should subsequently be tested by serology.

Here we describe the verification and validation of a new IVD-CE marked real-time RT-PCR assay (RealStar® Yellow Fever Virus RT-PCR Kit; altona Diagnostics) for the detection of YFV specific RNA.

Material and Methods

Here we describe the verification and validation of a newly developed yellow fever virus RT-PCR, which comprised of a specific system for the detection of YFV specific RNA (Figure 1 A) and an Internal Control (IC; Figure 1 B) system that monitors the efficiency of nucleic acid extraction process and possible inhibitory effects during PCR.

The RealStar® Yellow Fever Virus RT-PCR Kit limit of detection was determined using probit analysis after testing replicates of limited dilutions of quantified *in vitro* transcribed RNA containing the RT-PCR target sequence (Table 1). The analytical sensitivity is 0.69 copies/µl [95% confidence interval (CI): 0.41 - 1.56 copies/µl], Figure 2.

The analytical specificity of the YFV RT-PCR was evaluated by testing a panel of genomic RNA/DNA extracted from different pathogens that are related to YFV and/or can cause symptoms similar to YF virus (Table 2).

For the diagnostic validation 45 serum samples, pre-characterized with a reference real-time PCR assay, were used at the Flavivirus Laboratory at Fiocruz which is a Regional Reference for the Brazilian MoH.

Aim of the study

The aim of this study was to compare the newly developed yellow fever virus RT-PCR assay with the yellow fever virus RT-PCR assay established at the Flavivirus Laboratory FIOCRUZ/ Brazil (based on Domingo *et al.,* 2012) on a total of 30 serum samples from patients with yellow fever (YF) virus infection, retrospectively. Nucleic acid was extracted and the eluates were tested in parallel using the two different RT-PCRs. In addition, 15 individual serum samples from non-infected persons were tested.

Results and Conclusion

All 30 positive samples for YF virus RNA with the reference were also tested positive using the newly developed YFV RT-PCR assay. Out of the 15 negative samples for YF virus RNA using the reference assay, 15 samples were tested negative with the altona Diagnostics yellow fever virus RT-PCR (Table 3).

Our verification and validation data of the RealStar® Yellow Fever Virus RT-PCR Kit indicate that the assay is appropriate for the sensitive and specific detection of YFV specific RNA. It can be a useful tool in patient and epidemiological management in YF endemic regions.

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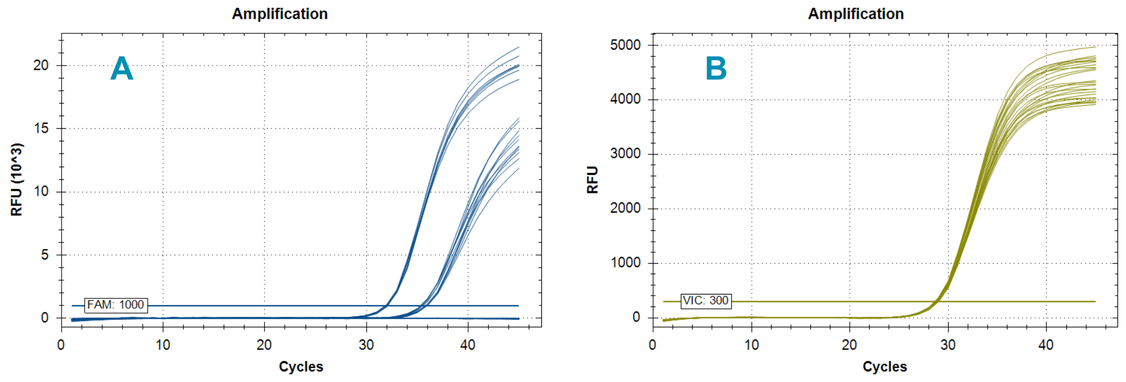

Figure 1. YFV negative samples (no amplification) and different concentrations of YFV specific RNA, detected in the FAM-Channel (A) and the regarding Internal Control signals (IC) detected in the JOE/VIC-Channel (B).

Table 1: RT-PCR results used for the calculation of the analytical sensitivity with respect to the detection of YFV specific RNA

Input Conc. [copies/µl]	Number of Replicates	Number of Positives	Hit Rate [%]
31.600	24	24	100
10.000	24	24	100
3.160	24	24	100
1.000	24	24	100
0.316	24	21	87.5
0.100	24	9	37.5
0.032	24	4	16.7
0.010	24	2	8.3
0.003	24	0	0

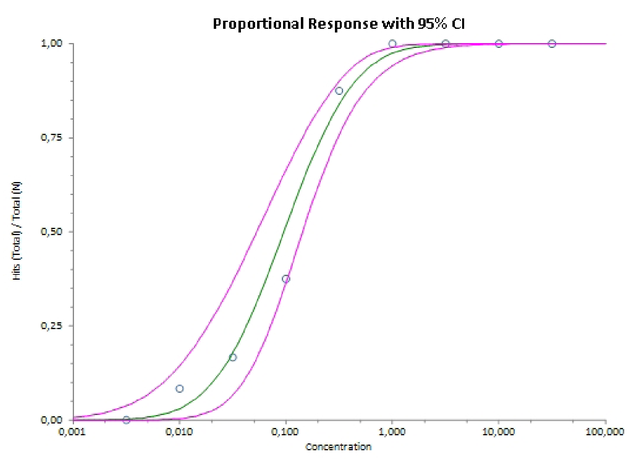

Figure 2. The analytic sensitivity is 0.69 copies/µl [95% confidence interval (CI): 0.41 - 1.56 copies/ µl]

Table 2: Analytic specificity results

Pathogens	FAM (Yellow Fever Virus)	JOE/VIC (Internal Control)
Chikungunya Virus	No Ct	Valid
Crimean-Congo Hemorrhagic Fever virus	No Ct	Valid
Dengue Virus Serotype 1	No Ct	Valid
Dengue Virus Serotype 2	No Ct	Valid
Dengue Virus Serotype 3	No Ct	Valid
Dengue Virus Serotype 4	No Ct	Valid
Ebola Virus	No Ct	Valid
Hepatitis C Virus	No Ct	Valid
Japanese encephalitis virus	No Ct	Valid
Lassa Virus	No Ct	Valid
Marburg Virus	No Ct	Valid
Murray Valley encephalitis virus	No Ct	Valid
Plasmodium falciparum	No Ct	Valid
West Nile Virus	No Ct	Valid
Zika Virus	No Ct	Valid
Positive Control	32.25	Valid
NTC	No Ct	Valid

Table 3: Diagnostic validation

Total number of samples: 45		YF Virus real-time RT-PCR (based on Domingo *et al.*, 2012)	
		POSITIVE	NEGATIVE
RealStar® YFV RT-PCR Kit 1.0	POSITIVE	30	0
	NEGATIVE	0	15