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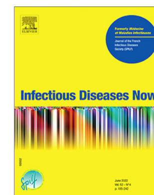

Short communication

Usefulness of an *in vitro*-transcribed RNA control for the detection and quantification of *Yellow fever virus* through real-time reverse transcription-polymerase chain reaction

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A B S T R A C T

Introduction: Unvaccinated individuals in endemic areas with proven enzootic transmission of *Yellow fever virus* are at risk of infection due to a dramatic shift in the epidemiology of the disease over recent years. For this reason, epidemiological surveillance and laboratory confirmation of cases have become mandatory.
Objective: To develop and test a control RNA for YFV detection through real-time RT-PCR.
Methods: A 437-bp insert containing the T7 promoter and the target sequences for two different in-house protocols was designed in the context of the pUC57 vector and obtained through gene synthesis. After T7-driven *in vitro* transcription, standard curves were developed for Log10 serial dilutions of the YFV control RNA with 8 replicates.
Results: A dynamic range of quantification of 10 orders of magnitude was observed with a limit of detection of 6.3 GCE/μL (95% CI, 2.6 to 139.4 GCE/μL).
Conclusion: The plasmid construct is available for YFV molecular test validation on clinical, entomological, and epizootic samples.

1. Introduction

Yellow fever is a febrile, icteric, and hemorrhagic illness native to Sub-Saharan Africa [1]. The disease is caused by the *Yellow fever virus*, the first member of the genus *Flavivirus*, family *Flaviviridae*, with non-human primates (NHP) and sylvatic mosquitoes serving as amplifying hosts and vectors, respectively, in the enzootic cycle. The virus first appeared in humans several centuries ago in urban areas of Africa. It then spread and established itself in other tropical areas, including the Americas and the Caribbean, where it was transmitted between humans by an anthropophilic mosquito species, *Aedes aegypti* [2]. Having entered the human population, the virus led to significant reported outbreaks and epidemics [3], up until the time when an effective vaccine was created and an aggressive vector control program was implemented, at least in the Americas. After registering the last human cases of urban transmission in the Americas during the 1930′s and 1940′s, epizootics with sporadic human cases among individuals exposed to the sylvatic vectors were reported, without major epidemiologic consequences [4]. Since 2016, however, the epidemiology of this disease changed dramatically in Brazil, where densely populated cities with low vaccine coverage and close to forest areas with active YFV enzootic circulation have been repeatedly exposed to the virus, entailing 2240 human cases and 760 deaths during 2016–2019 [5]. The diagnosis of yellow fever can be assessed by closely monitoring the clinical course and carrying out a number of virological and serological tests, which can be confirmed only through conventional or real-time RT-PCR and immunohistochemistry (in tissue samples from fatal cases suspected of yellow fever) [6]. Altruistic initiatives have been of help in the Americas (e.g., development of the Tariki YFV kit for IgM antibody detection), and the Pan-American Health Organization (PAHO) currently rec-

Abbreviations: GCE, Genome copy equivalents; NHP, Non-human primates; NTC, No Template Control; PAHO, Pan-American Health Organization; YFV, *Yellow fever virus*; RFLP, Restriction Fragment Length Polymorphism; RT-qPCR, Reverse Transcription-Polymerase Chain Reaction.

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ommends two RT-qPCR protocols for YFV detection and case confirmation in the viremic phase of the disease [7,8]. However, the difficulty of accessing positive samples limits the standardization of in-house protocols for routine surveillance and research on virus activity. In the present study, we designed and evaluated plasmid control for *in vitro* transcription of the YFV genomic regions targeted by the primers and probes reported by Domingo *et al* [9] and Jhonson *et al* (unpublished) [8], thereby demonstrating its usefulness in the implementation of sensitive fluorogenic assays.

2. Materials and methods

2.1. Design of the YFV control plasmid

The *in-silico* design of the control plasmid was performed with Geneious® 9.1.8 software (Biomatters Inc., San Diego, CA, USA) and consisted in the cloning of a 437-bp insert through the *Eco* RI and *Hind* III restriction sites in the multiple cloning site of the pUC57 plasmid. The target region comprised the nucleotide positions 14–115 of the 5′ untranslated region (UTR) from the reference sequence NC_002031. In the middle of the insert, the *Bam* HI restriction site was artificially introduced, which enabled differentiation of a positive YFV sample (STAMARIL YFV vaccine (Sanofi Pasteur) from the *in vitro*-transcribed YFV control RNA, by using Restriction Fragment Length Polymorphism (RFLP) on agarose gel electrophoresis (Fig. 1a). The plasmid was generated by gene synthesis (Macrogen Inc., Seoul, Rep. of Korea).

2.2. In vitro transcription for YFV control RNA synthesis

The plasmid was linearized with the 3′-overhang restriction enzyme *Sap* I, and used for the T7 promoter-driven run-off *in vitro* transcription, through the T7 RiboMAX™ Express Large Scale RNA Production System (Promega Inc., Madison, WI, USA), as previously described [10]. Transcribed RNA was treated with 2U RQ1 DNase I (Promega Inc., Madison, WI, USA) for the elimination of the template plasmid DNA, and the RNA was subsequently purified with the QIAamp Viral RNA mini kit (Qiagen Inc., Chatsworth, CA, USA), quantified in a Qubit 4 fluorometer (Thermo Fisher Scientific Inc., MA, USA) using the Qubit RNA Broad Range assay kit, stored at −80 °C, and subsequently serially diluted and used as control for the YFV RT-qPCR assays.

2.3. Determination of the limit of detection

Genome copy equivalents (GCE) were calculated for the RNA extract assuming a transcript of 406-bp comprised between the position + 1 (the transcription start site) and the cutting site for the linearizing endonuclease *Sap* I, as previously described [11]. To assess dynamic range and analytical sensitivity, standard curves were performed for Log10 serial dilutions of the YFV control RNA with 8 technical replicates [10,12], as part of two independent and complementary experiments covering the 10^{-1} to 10^{-11} dilution range, through the qScript™ XLT One-Step RT-qPCR ToughMix® (Quanta BioSciences, Inc., MA, USA) in the CFX96 Touch Real-Time PCR Detection System (BioRad Laboratories, Inc., CA, USA). A No Template Control (NTC) reaction was included for every dilution series. The limit of detection (LoD) was determined as the last dilution for which 95 % of replicates were positive (CT < 38), was calculated using the probit analysis algorithm (IBS SPSS Statistics v.18.0, USA) and expressed in GCE/μL and GCE per PCR reaction (GCE/Rxn).

3. Results

3.1. Successful synthesis of the YFV control RNA from the plasmid

The YFV control plasmid was designed to contain the T7 promoter sequence at the 5′ region, followed by target sequences for the primers and probes and the SP6 promoter at the 3′ region (Fig. 1b). The RFLP analysis displayed the expected results, with a single band of around 89–102 bp for YFV control RNA uncut and YFV vaccine + *Bam* HI; and two bands of around 57–58 and 32–44 bp for YFV control RNA + *Bam* HI (Supplementary Fig. 1), as a secondary control to be used in cases where cross-contamination with the positive control was suspected or needed to be discarded. The T7-driven *in vitro* transcription contributed to production of a large amount of the YFV RNA control (6.3 μg), from which a DNase I-treated RNA extract at a concentration of 17.2 ng/μL was obtained.

3.2. Standard curves from the YFV control RNA demonstrates very high sensitivity of two recommended RT-qPCR protocols for YFV diagnostics

The standard curves obtained for the dilution series of the positive control RNA showed amplification efficiencies of 117.0 % and 109.5 % for the Domingo *et al* (Fig. 2a) and Jhonson *et al* (Fig. 2b) assays, respectively. A wide dynamic range was observed in both assays, with linearity through at least six orders of magnitude (Fig. 2), corresponding to a wide range of concentrations (7.8 to 784,982 GCE/μL) and Ct values of 37.2 to 21.4. While this range comprises the expected concentration of YFV RNA in clinical samples [9], higher concentrations were also tested in triplicates, displaying the same linear behavior and extending the dynamic range to ten orders of magnitude (7.8 to 7.8×10^9 GCE/μL) and Ct values of 37.2 to 6.1. The LoD for both assays was 6.3 GCE/μL (95 % CI, 2.6 to 139.4 GCE/μL) or 31.7 GCE/reaction (Fig. 3), as the same control RNA was used for serial dilution, and the same number of positives was obtained per each dilution.

4. Discussion

YFV is a reemerging virus that has extended to at least seven countries in Africa and to South American populations with low vaccine coverage (Brazil, Peru, French Guiana…), with a public health impact justifying active and integrated surveillance in humans [13], NHPs [14] and mosquito vectors [15]. The enzootic transmission of the virus is commonly accompanied by severe illness and death in new world primates, motivating routine monitoring as an early-warning strategy [16], with molecular detection in samples obtained from dead NHPs [17].

While several conventional methods based on RT-PCR for YFV detection have been aplied over the past decades [18], they have been largely replaced by RT-qPCR protocols [19], which are recommended by health authorities [8] due to their higher sensitivity, lower propensity to amplicon cross-contamination, and to the widespread availability of equipment (even in low complexity laboratories). Implementation of the current strategy for the generation of YFV control RNA through *in vitro* transcription has been characterized by amplification and detection of viral genome copies applying the two one-step RT-qPCR methods recommended by PAHO [8] to a wide linear range of concentrations, reaching a LoD of 6.3 GCE/μL. This LoD should enable detection of the virus in RNA extracts obtained from several sources with variable viral loads, including those expected from clinical samples [9,20].

The global 2017–2026 Eliminate Yellow Fever Epidemics (EYE) strategy, launched by WHO (a) to coordinate actions to protect at-risk populations, (b) to prevent cross-border spread and (c) to

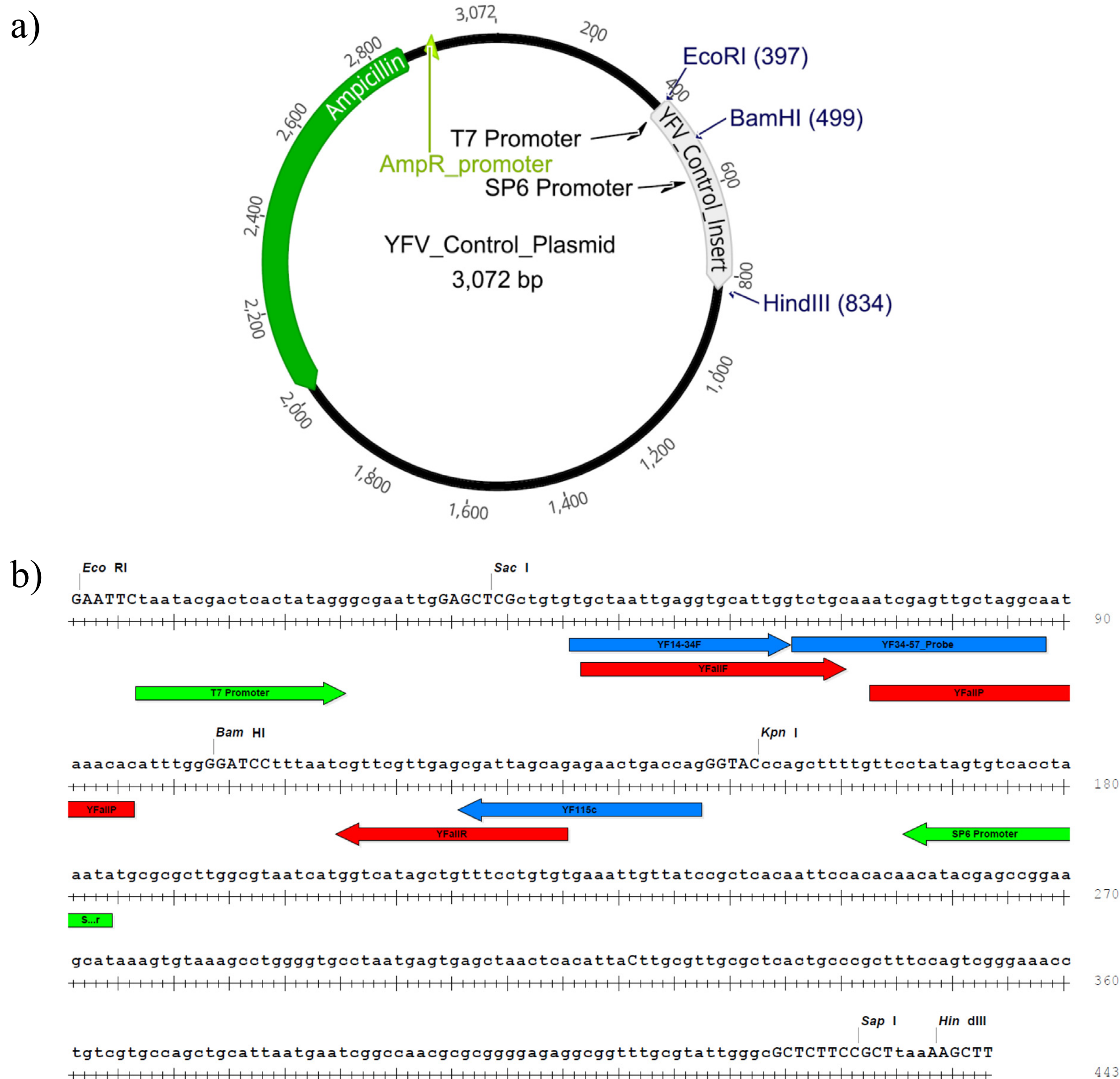

Fig. 1. Yellow fever virus positive control plasmid design. a) The pUC57 plasmid was used as vector for cloning of a 437-bp insert from the YFV 5′ UTR into the *Eco* RI-*Hind* III region. b) The YFV control insert contained the T7 and SP6 promoters flanking the primers, and probed targets for two widely used real-time RT-PCR assays directed to the YFV 5′ UTR, and the *Bam* HI restriction site as a molecular marker to distinguish between a YFV positive sample and the positive control.

rapidly early contain outbreaks [21] underscores the importance of diagnostic capacity. Once molecular assays are properly implemented for laboratory confirmation of YFV infection at regional laboratories in areas of potential or active YFV circulation, more robust surveillance and early response can be achieved.

5. Conclusions

During *in vitro* diagnostics, *in vitro*-transcribed RNA enables simultaneous control of the reverse transcription and PCR amplification steps. With a high level of transcribed RNA, it is possible, in diagnostic laboratories in endemic regions, to generate multiple aliquots of the working dilution for routine use in one-step RT-qPCR protocols. The positive YFV plasmid DNA control presented here is available for research laboratories planning to perform molecular detection of YFV as part of their basic research and public health activities.

Ethic statement

According to the national law 9/1979, decrees 786/1990 and 2323/2006, the Instituto Nacional de Salud is the reference lab and health authority of the national network of laboratories and in cases of public health emergency or those in which scientific research for public health purposes as required, the Instituto Nacional de Salud may use the biological material for research purposes.

Authors' contributions

Katherine Laiton-Donato: Performed the *in-silico* design, analysis and writing of the manuscript. Paula Quintero-Cortés and Juan Pablo Franco-Salazar performed the *in vitro* transcription, RT-qPCR assays and results analysis, Dioselina Peláez-Carvajal, María-Cristina Navas, Sandra Junglen, and Gabriel Parra-Henao made a

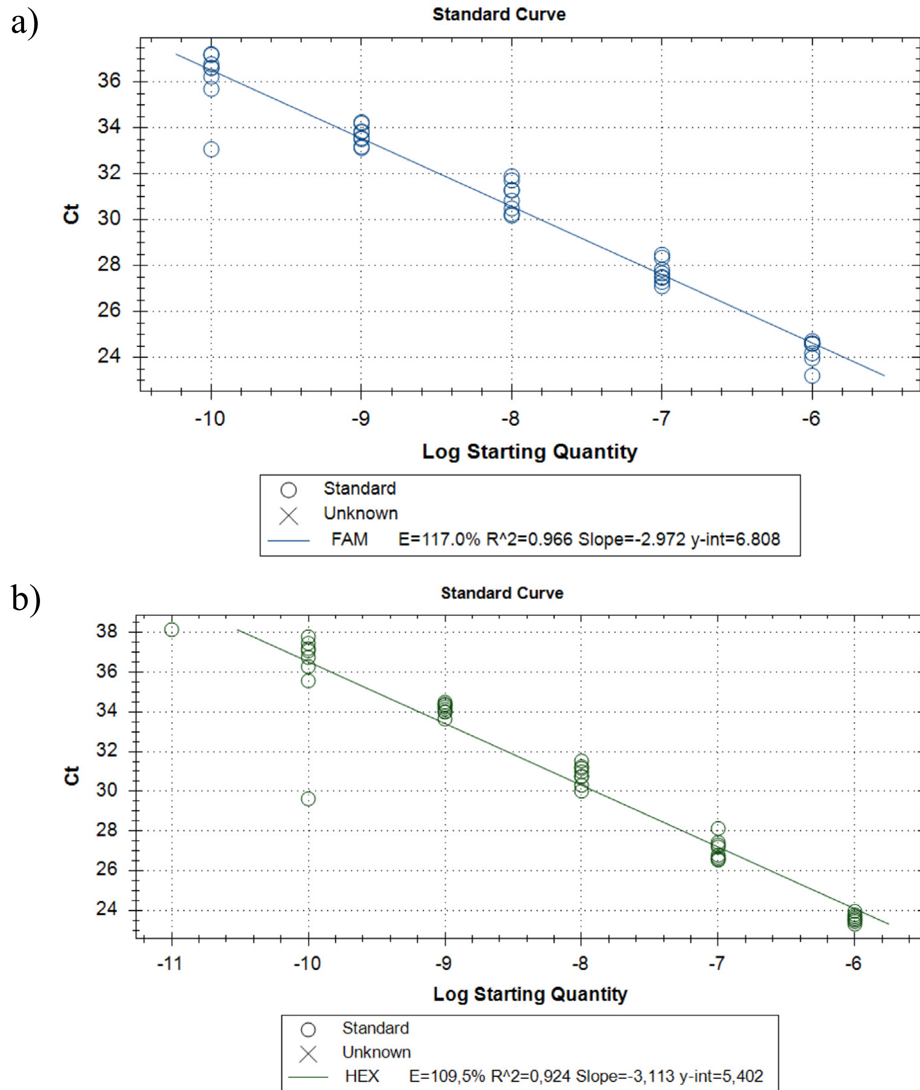

Fig. 2. Standard curve for YFV amplification using two different real-time RT-PCR assays. a) Standard curve for the Domingo et al., assay. b) Standard curve for the Jhonson et al., assay.

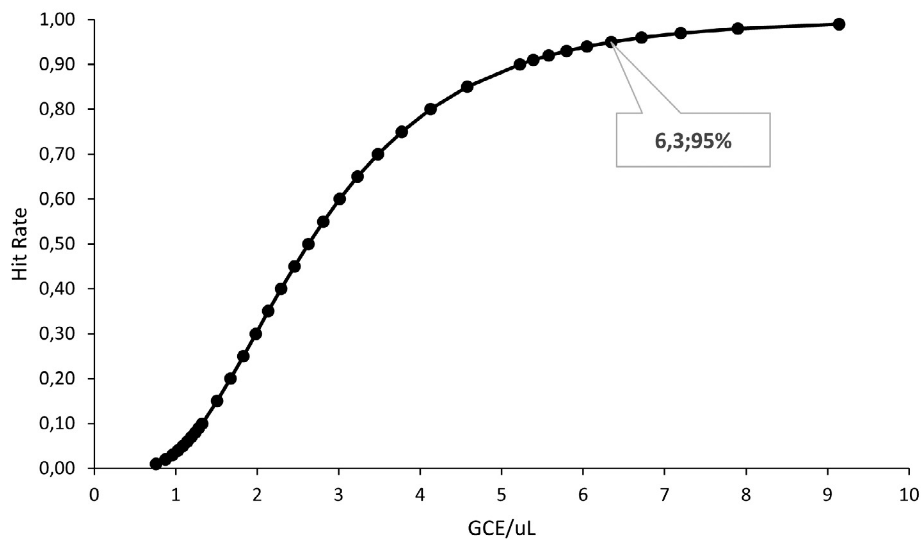

Fig. 3. Limit of detection for the two real-time RT-PCR assays for the detection of YFV with 95% confidence.

critical review of the manuscript, Jose A. Usme-Ciro conceived the study, and performed the *in-silico* design, analysis and writing of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idnow.2023.104654.

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