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## Construction and characterization of a recombinant yellow fever virus stably expressing *Gaussia* luciferase

TELISSA C. KASSAR<sup>1,\*</sup>, TEREZA MAGALHÃES<sup>1</sup>, JOSÉ V.J.S. JÚNIOR<sup>1</sup>, AMANDA G.O. CARVALHO<sup>1</sup>,  
ANDRÉA N.M.R. DA SILVA<sup>1,\*\*</sup>, SABRINA R.A. QUEIROZ<sup>1,\*\*\*</sup>, GIOVANI R. BERTANI<sup>2</sup> and LAURA H.V.G. GIL<sup>1</sup>

<sup>1</sup>Departamento de Virologia e Terapia Experimental, Centro de Pesquisas Aggeu Magalhães/CPqAM, Fundação  
Oswaldo Cruz/FIOCRUZ, Av. Professor Moraes Rego, s/n, Cidade Universitária, 50740-465 Recife, PE, Brazil

<sup>2</sup>Departamento de Bioquímica, Universidade Federal de Pernambuco/UFPE, Av. Professor  
Moraes Rego, s/n, Cidade Universitária, 50670-420 Recife, PE, Brazil

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### ABSTRACT

Yellow fever is an arthropod-borne viral disease that still poses high public health concerns, despite the availability of an effective vaccine. The development of recombinant viruses is of utmost importance for several types of studies, such as those aimed to dissect virus-host interactions and to search for novel antiviral strategies. Moreover, recombinant viruses expressing reporter genes may greatly facilitate these studies. Here, we report the construction of a recombinant yellow fever virus (YFV) expressing *Gaussia* luciferase (GLuc) (YFV-GLuc). We show, through RT-PCR, sequencing and measurement of GLuc activity, that stability of the heterologous gene was maintained after six passages. Furthermore, a direct association between GLuc expression and viral replication was observed ( $r^2=0.9967$ ), indicating that measurement of GLuc activity may be used to assess viral replication in different applications. In addition, we evaluated the use of the recombinant virus in an antiviral assay with recombinant human alfa-2b interferon. A 60% inhibition of GLuc expression was observed in cells infected with YFV-GLuc and incubated with IFN alfa-2b. Previously tested on YFV inhibition by plaque assays indicated a similar fold-decrease in viral replication. These results are valuable as they show the stability of YFV-GLuc and one of several possible applications of this construct.

**Key words:** *Gaussia* luciferase, homologous recombination in yeast, reporter gene, yellow fever virus.

Correspondence to: Laura Helena Vega Gonzales Gil  
E-mail: [laura@cpqam.fiocruz.br](mailto:laura@cpqam.fiocruz.br)

Present address: \*Departamento de Medicina Veterinária Preventiva,  
Escola de Veterinária, Universidade Federal de Minas Gerais, Avenida  
Antônio Carlos, 6627, 31270-901 Belo Horizonte, MG, Brazil

\*\*Laboratório de Virologia, Instituto de Ciências  
Biológicas, Universidade Federal do Pará, Rua Augusto  
Corrêa, 01, Guamá, 66075-110 Belém, PA, Brazil

\*\*\*Departamento de Medicina Veterinária, Faculdade de Zootecnia e  
Engenharia de Alimentos, Universidade de São Paulo, Avenida Duque  
Caxias Norte, 225, Jardim Elite, 13635-000 Pirassununga, SP, Brazil

### INTRODUCTION

Yellow fever is an infectious disease caused by the yellow fever virus (YFV), a flavivirus (Family *Flaviviridae*), transmitted to humans by biting infected female mosquitoes of the genera *Aedes*, *Sabethes* and *Haemagogus* (Hanley et al. 2013). YFV is endemic in countries of Africa and South America and is maintained mainly through sylvatic

transmission cycles among nonhuman primates and sylvatic mosquitoes. However, several, often disastrous, urban transmission cycles involving the mosquito *Aedes aegypti* as the main vector have occurred and the risk of urban outbreaks remains a threat, especially in regions where viruses and vectors are found (Hanley et al. 2013).

Yellow fever in humans can be divided into three clinical stages: infection, remission and intoxication (Ishikawa et al. 2014). After an incubation period of 3-6 days symptoms like headache, fever and myalgia may be observed, which corresponds to the viremic period. After the infection period, some patients may develop the remission period, presenting liver and renal failure. An intoxication period may follow, characterized by hemorrhagic fever and organ failure. Approximately 15% of cases develop moderate/severe manifestations. The World Health Organization estimates that 200,000 cases and 30,000 deaths occur annually, mostly in Africa (Ishikawa et al. 2014).

Despite the availability of a human vaccine against YFV, the virus continues to pose a threat to human populations, reaching mortality rates of 20-50% in urban cycles. The currently available vaccine is based on the live-attenuated strain 17D or the substrains 17DD and 17D-204, and although it reaches high protection levels in vaccinees, it may cause rare, severe side effects leading to viscerotropic disease, especially in risk groups (Monath et al. 2015). The other control methods for arthropod-borne viral diseases such as yellow fever are: 1) anti-vector strategies, which have been proven highly difficult in the case of extremely adapted species such as *Ae. aegypti*; and 2) antiviral drugs. For the latter, the antiviral medications available are ineffective against YFV (Monath et al. 2015) and the search for novel effective medications is an active field of research.

The YFV is an enveloped, single-stranded, positive-sense RNA virus, with a genome of approximately 11Kb, capped at its 5' end and non-

polyadenylated at its 3' end. It encodes, within one open-reading frame, three structural proteins at the N-terminal portion (capsid-C, pre-membrane-prM, envelope-E) and seven non-structural proteins at the C-terminal portion (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) of the polyprotein. Additionally, the YFV genome has functional non-coding terminal ends (5' and 3' untranslated regions-UTRs) that are involved in several viral features such as genome replication and protein translation (Lindenbach et al. 2013).

In the 1950s, it was discovered that the nucleic acid of some animal viruses was infectious. Later on, experiments showed that transfection of live, susceptible cells or hosts with the genome of several positive RNA viruses led to the production of infective viruses (Aubry et al. 2015). These discoveries have allowed the development of powerful viral molecular tools for the past decades. Full-length infectious clones are a good example of these tools as they allow the genetic manipulation (e.g. mutations, deletions, insertions) of viral genomes and further studies of the phenotypic effects of such modifications (Aubry et al. 2015).

Since the construction of the first flavivirus full-length clone (Rice et al. 1989), some technical difficulties have been reported, such as the instability of the viral genome in cloning bacterial hosts. To overcome part of the obstacles, several novel molecular techniques have been developed, including low-copy-number plasmids, bacterial artificial chromosomes and yeast-based homologous recombination. In addition, infectious clones or replicon systems expressing reporter genes (e.g. firefly and *Renilla* luciferases, green and yellow fluorescent proteins) have been developed and used mainly on molecular virology studies, to construct viral vectors and on sophisticated antiviral assays (De Baets et al. 2015, Patkar et al. 2009, Roth et al. 2009, Schoggins et al. 2012, van den Worm et al. 2012, Dag et al. 2013, Kato et al. 2014, Ding et al. 2015, Zhang et al. 2015). Among the

reporter genes, *Gaussia* luciferase (GLuc) started to be used in *in vivo* and *in vitro* assays due to some advantages such as high stability and sensitivity, and the fact that it is secreted, facilitating its detection in several experiments (Tannous et al. 2005, Venisnik et al. 2007).

Considering the aforementioned, here we report the construction and characterization of a stable recombinant YFV (made by homologous recombination in yeast using YFV-17D) expressing the GLuc gene. We also show the use of YFV-GLuc in an antiviral assay as one of its several possible applications.

## MATERIALS AND METHODS

### CELL AND VIRUS CULTURE

Baby hamster kidney (BHK-21) cells were maintained at 37°C/5% CO<sub>2</sub> in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin (stock at 10.000UI/mL) and Streptomycin (stock at 10.000µg/mL) (LGC Biotecnologia, Cotia, SP, Brazil) and 0.1% Fungizone (Gibco, Langley, OK, EUA). The YFV-17D virus, previously generated by Gil L.H.V.G. et al. (unpublished data) by pBSC-YFV17D transfection in BHK-21 cells, was amplified in BHK-21 cells, titrated by plaque assay and used as a positive control in experiments. The YFV-17D cloned genome was kindly provided by Galler, Fiocruz/RJ, Brazil.

### CONSTRUCTION OF THE pBSC-YFV-GLuc PLASMID

pBSC-YFV-GLuc plasmid was constructed by homologous recombination in yeast on pBSC-YFV-YFP-DENV1linker, a recombinant virus with the YFP gene inserted in the YFV-17D genome (Fig. 1), previously made by Gil L.H.V.G. and Almeida-Queiroz S.R.A (unpublished data) according to the strategy reported by Bonaldo et al. (2007). For this, pBSC-YFV-YFP-DENV1linker was digested with *NarI* and the GLuc PCR product, containing

terminal sequences homologous to E and NS1 regions of the *NarI*-linearized pBSC-YFV-YFP-DENV1linker, was directly cloned to produce the pBSC-YFV-GLuc recombinant plasmid. Digestion of pBSC-YFV-YFP-DENV1linker and the cloning strategy are shown in Fig. 1.

The GLuc reporter gene was amplified from the plasmid pGLuc-NS (WF10) (kindly provided by Perez, University of Maryland) through PCR with Taq Platinum (Invitrogen, Carlsbad, CA, EUA). PCR conditions were as follows: initial denaturation at 94°C for 30 s; and 32 cycles of denaturation at 94°C for 15 s, annealing at 52°C for 30 s, and extension at 68°C for 1 min. The recombination oligonucleotides (YFV-GLuc-F and YFV-GLuc-R) used to amplify the GLuc sequence are listed in Table I.

Briefly, pBSC-YFV-GLuc plasmid was generated by homologous recombination in *Saccharomyces cerevisiae* strain RFY206 and transformed by a procedure using lithium acetate (Sambrook and Russel 2001). The pBSC-YFV-GLuc plasmid has a coding sequence for tryptophan (trp) as a marker for transformed cells selection on medium without trp. Then, selection of positive colonies was performed using Yeast Nitrogen Base without trp and plasmid DNA extraction was performed using QIAprep Miniprep Kit (Qiagen, Valencia, CA, USA). The DNA of the colonies was sequentially screened by two PCR reactions using Taq Platinum (Invitrogen) and the same PCR conditions mentioned above; oligonucleotide pairs were YFV-GLuc-F and YFV-NS1-2502-R, and YFV-2375-F and YFV-GLuc-R (Table I).

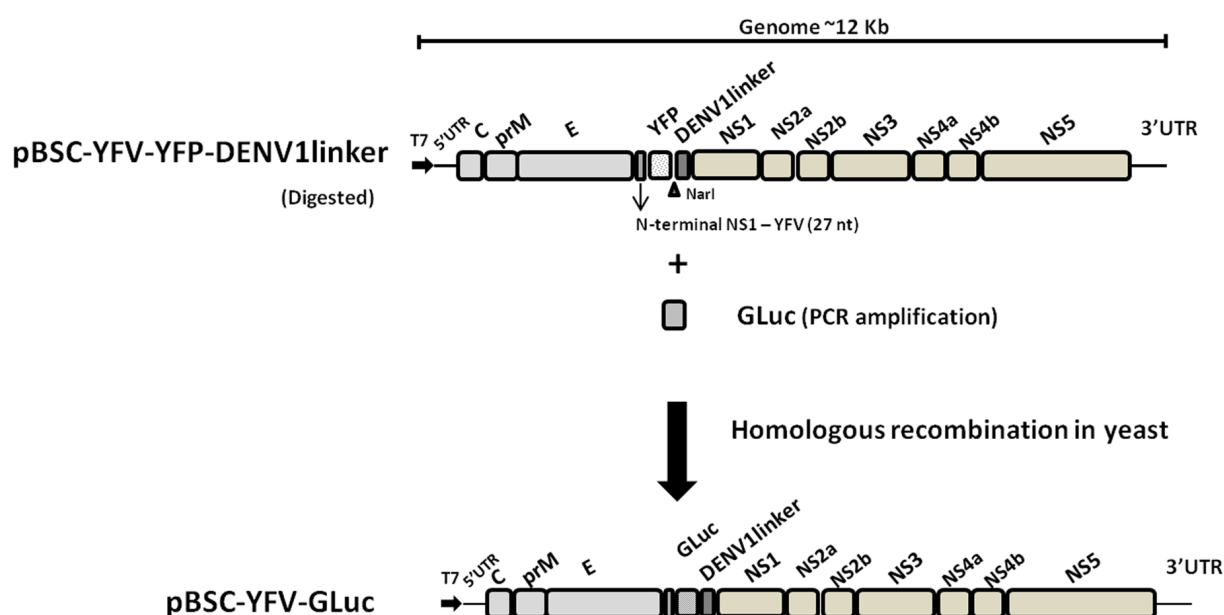
### *In vitro* TRANSCRIPTION AND TRANSFECTION

For *in vitro* transcription, the full-length cDNA recombinant virus genome was amplified by PCR, using the KlenTaq-LA polymerase enzyme (Clontech, Mountain View, CA), and pBSC-RsrII-T7-5'-YFV-F and YFV-3'UTR-R oligonucleotides

**TABLE I**  
**Oligonucleotides used for pBSC-YFV-GLuc construction and *in vitro* transcription.**

Oligonucleotide	Sequence
YFV-GLuc-F <sup>a</sup>	<u>°GACCAGGGCTGTGCAATTAATTT</u> CGGGGGCGCCATGGGAGTCAAAGTTCTGTTTG
YFV-GLuc-R <sup>b</sup>	<u>°CTCGGGCGGTTGCTTCGAACATTTTGGCGCCGTC</u> ACCACCGGCCCCCTTG
PBSC-RSRII-T7-5'-YFV-F	<sup>d</sup> CAAGCATGTAAATATCGTTTGAGTTCGGTCCGTAATACGACTCACTATAGAGTAAATCCTGTGTGCTAATTGAGG
YFV-3'UTR-R	AGTGGTTTTGTGTTTGTTCATCC
YFV-NS1-2502-R	TCTCCGCACTTGAGCTCTC
YFV - 2375 - F	ACAAGAAACATGACAATGTCC

<sup>a</sup>Forward oligonucleotide. <sup>b</sup>Reverse oligonucleotide. <sup>c</sup>Underlined nucleotide sequence corresponds to the region used for homologous recombination in yeast. <sup>d</sup>Bold nucleotide sequence corresponds to the T7 promoter for *in vitro* transcription.



**Figure 1** - Schematic representation of YFV-GLuc virus construction. pBSC-YFV-YFP-DENV1linker was digested with *NarI* restriction enzyme and the *Gaussia* luciferase (GLuc) reporter gene was amplified with recombination oligonucleotides (YFV-GLuc-F and YFV-GLuc-R) (Table I). The GLuc PCR product with homologous terminal sequences to *NarI*-linearized pBSC-YFV-YFP-DENV1linker (E and NS1 region) was cloned to produce the pBSC-YFV-GLuc plasmid. DENV1linker: Stem-anchor E DENV1; UTR: untranslated region; NS: nonstructural; nt: nucleotide.

(Table I). The full-length PCR conditions were as follows: initial denaturation at 95°C for 5 min; 32 cycles of denaturation at 93°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 13 min, with an increase of 10 s in extension per cycle;

and a final extension at 72°C for 20 min. The full-length PCR product, which has the bacteriophage T7 promoter in the 5' terminal sequence, was purified by phenol-chloroform extraction followed by ethanol precipitation, and transcribed using

the MEGAscript T7 kit (Ambion, Foster City, CA, USA) with the addition of the 7-methyl-guanosine cap analog for *in vitro* transcription (Ambion, Foster City, CA, USA). Full-length RNA transcripts were introduced into BHK-21 cells by electroporation as described previously (Santos et al. 2013). Transfected cells were plated in 6-well plates containing coverslips and virus generation was confirmed by immunofluorescence assay (IFA) and expression of the GLuc reporter gene, as described below. For these assays, coverslips or cell supernatants were collected at selected time points for further analyzes.

#### INDIRECT IMMUNOFLOUORESCENSE AND *Gaussia* LUCIFERASE ACTIVITY ASSAY

Five days post-transfection, coverslips containing BHK-21 cells were collected and rinsed with phosphate buffered saline (PBS), fixed with cold acetone at 4°C for 5 min and air-dried. Cells were then incubated at 37°C for 1 h with a polyclonal hyperimmune mouse ascitic fluid raised against group B flaviviruses (kindly provided by Vasconcelos, Instituto Evandro Chagas) diluted 1:100 in PBS. After a washing step with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich, Saint Louis, MO, USA) diluted 1:100 in PBS at 37°C for 1 h. Following a final washing step in PBS, cells were air-dried and the material was mounted on glass slides to allow visualization in a DMI 4000B fluorescence microscope (Leica, Wetzlar, Germany). At the same time point, the GLuc activity was measured in 10 µL of cell supernatant using the Bioluminescence Assay Kit (New England Biolabs, Ipswich, MA, USA) and Mithras LB 940 Multimode Microplate Reader (Berthold, Bad Wildbad, Germany).

#### PHENOTYPIC CHARACTERIZATION

YFV-GLuc (passage 4, p4) was used for phenotypic characterization. The recombinant virus was passaged four times in BHK-21 cells at a multiplicity of infection (MOI) of 1 (titrated by plaque assay) and incubated until 70-80% cytopathic effect was observed (CPE) (7-8 days).

#### PLAQUE-FORMING ASSAY

Plaque assay was performed to evaluate plaques formed by the parental virus (YFV-17D) and recombinant viruses. For this, monolayers of BHK-21 cells were plated in a 6-well tissue culture plate ( $4 \times 10^5$  cells/well) and 24h later inoculated with serial 10-fold dilutions ( $10^{-1}$  to  $10^{-5}$ ) of YFV-17D or YFV-GLuc (p4). Following 1 h of adsorption at 37°C in 5% CO<sub>2</sub>, the inoculum was removed. Cells were washed twice, overlaid with 1 mL of MEM containing 1% agarose and 10% FBS and incubated at 37°C. After 7 days, plates were stained with Thiazolyl Blue Tetrazolium Bromide (MTT) in PBS and plaque morphology and size were analyzed. The staining procedure consisted of adding approximately 100 µL of 5mg/mL MTT in the wells and incubating plates for 2 h at 37°C.

#### REPLICATION KINETICS OF YFV-GLuc VS. YFV-17D AND CURVE OF *Gaussia* LUCIFERASE EXPRESSION

The replication kinetics of YFV-GLuc and YFV-17D were analyzed in BHK-21 cells cultured in 24-well plates ( $8 \times 10^4$  cells/well). Cells were infected with YFV-GLuc (p4) or YFV-17D virus at a MOI of 0.1. The supernatant was collected for 4 days at every 24h and frozen at -80°C. Viral RNA (vRNA) was extracted from 140 µL of BHK-21 cell culture supernatant infected with YFV-GLuc or YFV-17D with the Viral RNA Isolation Kit (Macherey-Nagel, Düren, Germany). vRNA quantification was performed by quantitative real-time PCR (qRT-PCR) with the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA, USA), using a standard



curve of the PCR-targeted gene as described for dengue virus by Carvalho-Leandro et al. (2012). Primers used for YFV qRT-PCR were those used by Dash et al. (2012), targeting the 5' UTR and capsid gene junction of YFV. The positive control was RNA extracted from a known YFV-17D-infected culture. Reactions were performed in duplicate using the Applied Biosystems 7500 Fast Real-Time PCR System. The supernatants collected during the growth curve of YFV-GLuc were also used to perform the curve of GLuc expression. Finally, the relationship between GLuc activity and YFV-GLuc replication was evaluated by linear correlation and confirmed by  $r^2$  value. Both qRT-PCR and GLuc expression were performed twice (two separate biological assays).

#### GENETIC STABILITY OF THE YFV-GLuc RECOVERED VIRUS

To assess the genetic stability of the YFV-GLuc construct, the recombinant virus was successively passed in BHK-21 cells six times at MOI of 1 and incubated until 70-80% CPE was observed (7-8 days). In passage 6 (p6), GLuc expression was evaluated three days post-infection. Moreover, vRNA was extracted from 140  $\mu$ L of supernatant from a BHK-21 cell culture infected with YFV-GLuc using the Viral RNA Isolation Kit and the presence of GLuc gene was confirmed through RT-PCR using the One-Step RT-PCR Kit (Qiagen, Valencia, CA, USA), and YFV-2375-F and YFV-NS1-2502-R oligonucleotides (Table I). The RT-PCR conditions were: reverse transcription at 50°C for 30 min; an initial PCR step (Taq activation) at 95°C for 15 min; and 32 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1 min. The integrity and identity of the RT-PCR products were evaluated by Sanger sequencing. Nucleotide analysis was performed with Ape-A plasmid Editor v1.10.4.

#### ANTIVIRAL ASSAY

In this assay, BHK-21 cells were cultured in 96-well plates ( $10^4$  cells/well) and 24 h later infected with YFV-GLuc (p4) and YFV-17D at a MOI of 0.1. Immediately after adsorption, BHK-21 cells were treated with recombinant human alfa-2b interferon (IFN alfa-2b) (Heber Biotec, Havana, Cuba), a known antiviral molecule (Akira et al. 2001), at 1000 UI/mL by 48 h. The negative controls were cells infected and not treated with IFN alfa-2b. After this step, GLuc activity was assessed according to the protocol described above and viral titer of YFV-17D was evaluated by plaque assay. This experiment was performed in duplicate.

#### STATISTICAL ANALYSIS

To compare the replication kinetics between the YFV-17D and YFV-GLuc viruses, Student's t-test (paired) was performed with the mean value of RNAv molecules/ $\mu$ L (Log10) at each timepoint (24, 48 and 72h) obtained in each experiment. A significance level of 5% was considered.

The correlation between GLuc activity and YFV-GLuc replication was evaluated by calculating the coefficient of determination ( $r^2$ ) after plotting in one graph data from both experiments.

### RESULTS

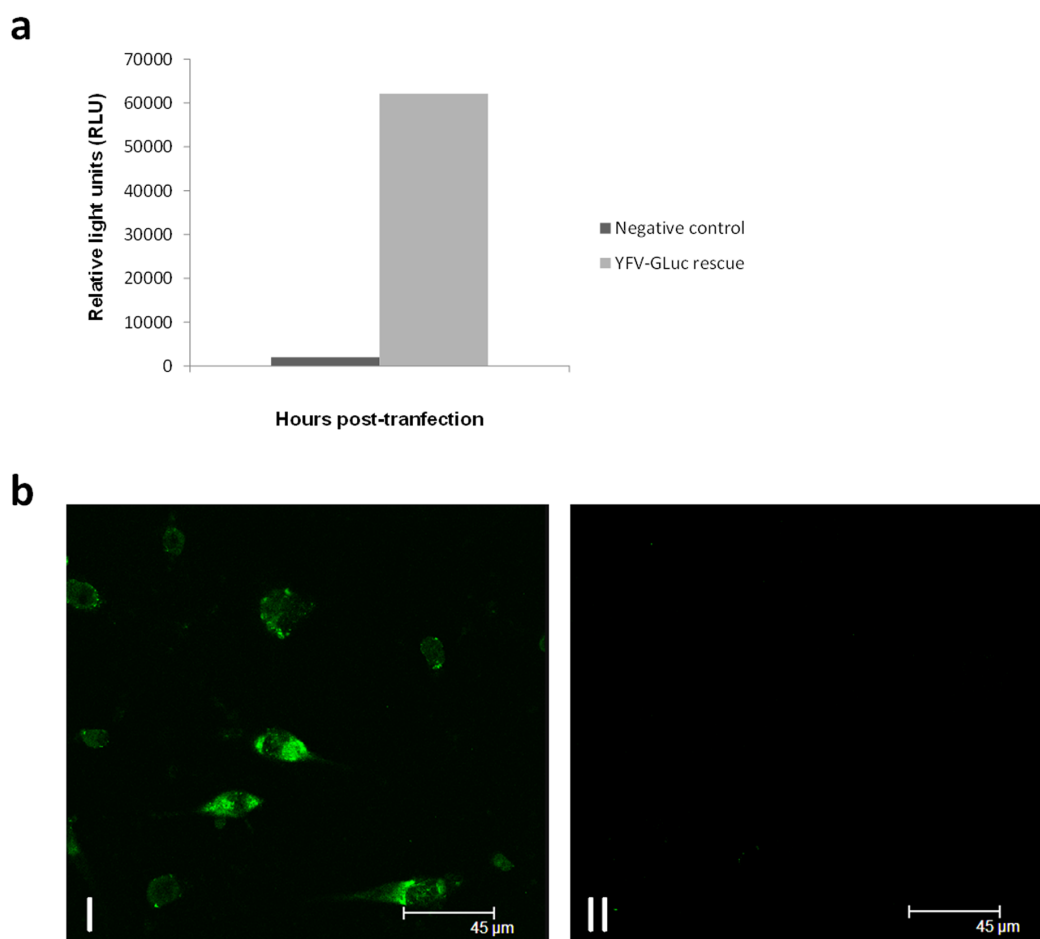
#### CONSTRUCTION OF pBSC-YFV-GLuc PLASMID

The successful construction of pBSC-YFV-GLuc was confirmed by two PCR reactions using YFV-GLuc-F and YFV-NS1-2502-R, and YFV-2375-F and YFV-GLuc-R oligonucleotides (data not shown). The full-length cDNA recombinant virus genome was then amplified and *in vitro* transcribed, and the RNA was introduced into BHK-21 cells. The positive immunofluorescence and GLuc activity assay post-transfection (31-fold increase) showed that the viral protein and GLuc heterologous protein were correctly translated (Fig. 2).

# PHENOTYPIC CHARACTERIZATION OF YFV-GLuc IN CELL CULTURE

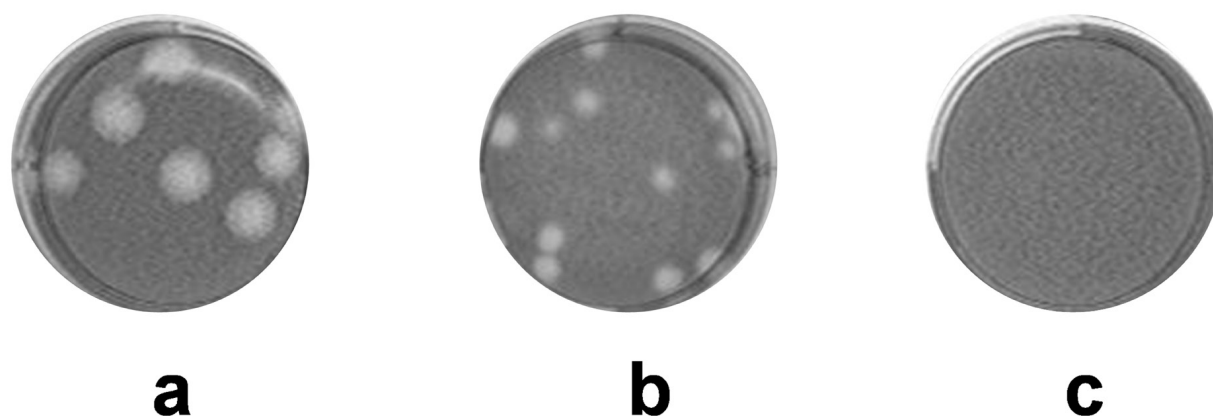
The plaque assay confirmed the efficient replication of the YFV-GLuc rescued virus in cell culture. However, at the  $10^{-5}$  dilution YFV-GLuc plaques (p4) were smaller (5 mm mean diameter; eleven plaques were counted) than those formed by the YFV-17D parental virus ( $10^{-4}$ ) (9 mm mean diameter; seven were counted) (Fig. 3). The

replication kinetics of the YFV-GLuc (p4) vs. YFV-17D showed no statistical significance between the two viruses ( $p < 0.05$ ) (Fig. 4a). The curve of GLuc activity on the supernatant of cells infected with YFV-GLuc (p4) showed a crescent GLuc expression over time (Fig. 4b). Moreover, a direct relationship between GLuc activity and YFV-GLuc replication was confirmed by regression analysis, with an  $r^2$  value of 0.9967 (Fig. 4c).



**Figure 2 - a)** *Gaussia* luciferase activity on the supernatant of BHK-21 cells 120 hours post-transfection with YFV-GLuc RNA. GLuc activity is expressed as relative light units (RLU). Negative control: supernatant of electroporated cells without YFV-GLuc RNA. **b)** IFA (63x magnification) performed with polyclonal hyperimmune mouse ascitic fluid raised against group B flaviviruses (primary antibody) and FITC-conjugated goat anti-mouse IgG antibody (secondary antibody). I) BHK-21 cells transfected with YFV-GLuc RNA at 120 h post-transfection; II) Negative control, mock-electroporated cells at 120 hours post-electroporation.





**Figure 3** - Plaque assay of YFV-17D and YFV-GLuc. BHK-21 cells were inoculated with serial dilutions ( $10^{-1}$  to  $10^{-5}$ ) of YFV-GLuc or YFV-17D, and plaques were revealed 7 days post-infection. **a)** Plaques formed by YFV-17D at  $10^{-5}$  dilution, measuring approximately 9 mm in diameter; **b)** Plaques formed by YFV-GLuc at  $10^{-4}$  dilution, measuring approximately 5 mm in diameter; **c)** Negative control (non-infected cells).

#### GENETIC STABILITY OF THE YFV-GLuc RECOVERED VIRUS

The presence and integrity of the GLuc gene was confirmed after six passages in BHK-21 cells through RT-PCR (Fig. 5) and nucleotide sequencing (data not shown). Moreover, in p6, GLuc activity (130-fold increase) in relation to mock-infected cells confirmed the stability of GLuc expression.

#### ANTIVIRAL ASSAY

Conventional antiviral assays are performed through plaque assays (Green 2008) and are time-consuming and require several steps. Thus, the YFV-GLuc recombinant virus was evaluated about its ability to be used as a novel antiviral assay platform: in this case, GLuc activity is inversely proportional to the antiviral activity of the evaluated substrate.

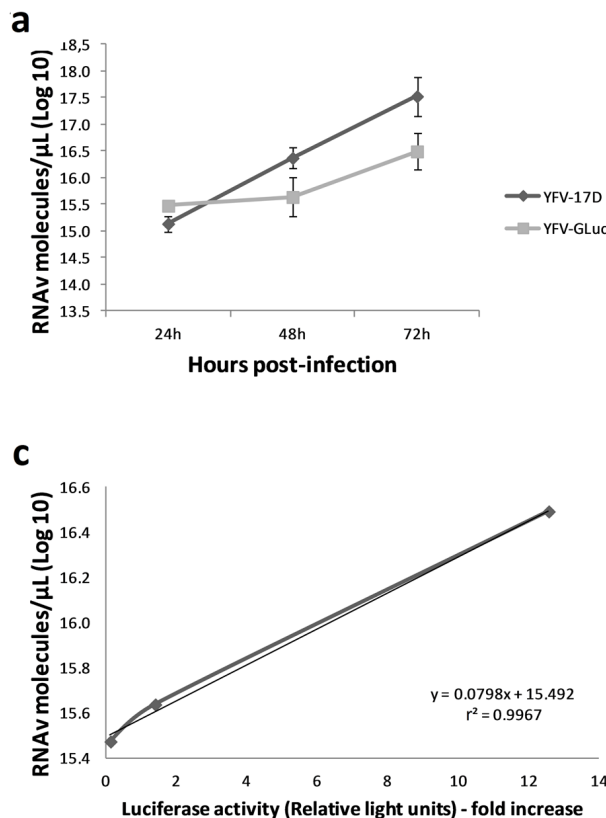
The antiviral assay was performed with IFN alfa-2b at 1000 UI/mL. The result was expressed as the percentage of GLuc inhibition in BHK-21 cells infected by YFV-GLuc. Inhibition of GLuc expression in cells treated with IFN alfa-2b was 60% (similar inhibition results were observed with YFV-17D evaluated by plaque assay). The negative

control consisted of cells infected and not treated with IFN alfa-2b.

#### DISCUSSION

Despite the success of the yellow fever vaccine with attenuated YFV (YFV-17D strain and its substrains YFV-17DD and YFV17D-204), yellow fever still poses a threat to several countries throughout the world. Although several drugs targeting the virus or blocking host responses are being developed, no anti-YFV drug is currently licensed (Julander 2013). Moreover, conventional assays to screen for antiviral compounds are usually performed through plaque assays and thus are laborious and time-consuming (Green et al. 2008). Replicon-based high-throughput screening methods using reporter genes represent important tools to evaluate antiviral compounds against several viruses *in vitro* and/or *in vivo* experiments (Patkar et al. 2009, Kato et al. 2014, Ding et al. 2015). However, replicon systems consist of a viral subgenome containing only nonstructural genes, and antiviral drugs or antibody targeting structural viral proteins cannot be evaluated through this technology (Mayhoub et al. 2011, Umamaheswari et al. 2011). To overcome

Figure 4



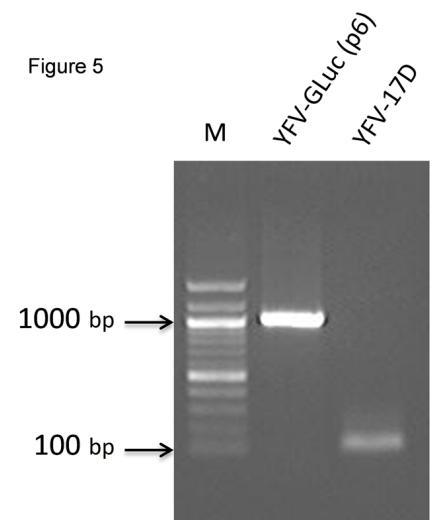
**Figure 4 - a)** Comparison of YFV-GLuc and YFV-17D replication kinetics. BHK-21 cells were inoculated with either virus at MOI of 0.1. At indicated times, the supernatant was collected and viral quantitation was assessed through SYBR Green-based quantitative real-time PCR. Each value represents the mean of two independent experiments; **b)** Curve of GLuc activity on the supernatant of cells infected with YFV-GLuc (MOI of 0.1). At different time points, the supernatant was collected and GLuc activity was measured and expressed as the fold-increase in relation to time zero using the relative light units. Each value represents the mean of two independent experiments. Each value represents the mean of two independent experiments; **c)** Linear correlation between GLuc activity (relative light units) and viral replication (RNAv molecules/μL). The replication kinetics of the YFV-GLuc and curve of *Gaussia* luciferase expression were overlapping and the direct relationship between them was evaluated by linear regression.

this limitation of replicon systems, strategies that use recombinant viruses containing reporter genes may be used.

Recombinant viruses expressing reporter genes (e.g. YFV, GFP, and firefly and *Renilla* luciferases)



Figure 5



**Figure 5 -** RT-PCR of the YFV-GLuc (passage 6, p6). vRNA was extracted from BHK-21 cell culture supernatant infected with YFV-GLuc and YFV-17D parental virus and RT-PCR was performed to confirm the presence of the GLuc gene. The oligonucleotides set used in the amplification are listed in Table I (YFV-2375- F and YFV-NS1-2502-R). The difference between the size of YFV-GLuc (approximately 1000bp) and YFV-17D (approximately 130bp) amplicons is due to GLuc gene and accessory regions (Stem-anchor E DENV1 and NS1 N-terminal-YFV). M: 100bp DNA Ladder.

have been reported for distinct viruses and used for various purposes, e.g. on the evaluation of virus-host interactions, viral tropism, and antiviral and neutralization assays (Roth et al. 2009, Schoggins et al. 2012, van den Worm et al. 2012, Dag et al. 2013, De Baets et al. 2015, Zhang et al. 2015). These studies include the development of recombinant flavivirus expressing reporter genes, such as Dengue virus expressing GFP and firefly

and *Renilla* luciferases (Zou et al. 2011, Schoggins et al. 2012). However, the instability or decreased fitness of the recombinant viruses compared to the parental virus limit their application in some experimental designs, as noted by Aubry et al. (2015). This instability in engineered virus expressing reporter genes was also reported in other flavivirus and DNA virus platforms (Song et al. 2012, Vandergaast et al. 2014, Zhang et al. 2015).

More recently, the constructions of viruses expressing GLuc as reporter gene and their use in several biotechnological applications have been reported. A desired feature of such clones is the stability of GLuc expression to allow their use in long-term studies, but unfortunately such condition has not always been achieved. Zhang et al. (2015) reported the construction of a West Nile Virus (WNV) expressing GLuc and its use in neutralization assays, but the recombinant virus gradually lost stability along the passages, where GLuc activity was no longer detected in passage 3. Vandergaast et al. (2014) also found similar instability in a GLuc-expressing WNV. The lower stability of these recombinant viruses in relation to YFV-GLuc probably is due to the insertion position GLuc gene (within C gene) and/or to the pathogenic strain used as backbone of WNV-GLuc, especially in Vandergaast et al. (2014). Conversely, in our experiments, GLuc gene was inserted into E-NS1 junction of an attenuated virus (YFV-17D). A recombinant poliovirus expressing GLuc retained the complete reporter gene for three passages and after that also gradually lost it (Song et al. 2012).

Our group has recently reported a recombinant bovine viral diarrhea virus stably expressing GLuc (Arenhart et al. 2014) and now reports a recombinant YFV-17D stably expressing this same reporter gene (YFV-GLuc). The choice of GLuc as a reporter gene is mainly due to the following advantages: 1) high stability and sensitivity of GLuc (Tannous et al. 2005); 2) GLuc upon expression is secreted

into the cell culture media or organics fluids/secretions, thus cell lysis to assess GLuc activity is not necessary, making GLuc an ideal reporter gene for time-course studies (Venisnik et al. 2007); and 3) GLuc gene is smaller than other reporter genes, facilitating its manipulation in recombinant technologies.

In the present study, YFV-GLuc was stable after six passages in BHK-21 cells as indicated by RT-PCR (Fig. 5), sequencing integrity (data not shown) and GLuc activity (130-fold increase in comparison to mock-infected cells). The stability of GLuc activity also was evidenced by its increasing expression over time (Fig. 4b). The comparison of plaques formed by the parental and recombinant viruses in BHK-21 cells showed smaller-sized plaques in the latter (Fig. 3). Nevertheless, the replication kinetics of YFV-GLuc vs. YFV-17D showed no statistical significance between the two viruses ( $p < 0.05$ ), although there was a tendency for YFV-GLuc to grow more slowly (Fig. 4a). The similarity between the YFV-17D parental virus and recombinant virus replication has already been reported in YFV platforms expressing heterologous proteins or small heterologous sequences from virus or protozoan at the E-NS1 expression site (Rumyantsev et al. 2010, Franco et al. 2010, Nogueira et al. 2013).

After construction, characterization and stability confirmation, YFV-GLuc was evaluated in an antiviral assay with IFN alfa-2b. Importantly, a direct relationship between GLuc activity and YFV-GLuc replication was confirmed through regression analysis, indicating that GLuc expression may be used to represent viral replication (Fig. 4c). Thus, a decrease in GLuc activity after incubation with IFN alfa-2b reflects, indirectly, a decrease in viral genome replication. For instance, an inhibition of approximately 60% in GLuc activity after incubation with IFN alfa-2b reflects a decrease within the same range in viral replication. The result of the IFN-alfa 2b assay shows the usefulness

of GLuc-YFV construct to be used in further high-throughput antiviral screening assays.

In summary, the genetic manipulation of YFV-17D has proven to be stable in several biotechnological applications, as reviewed by Bonaldo et al. (2014). The present work reports the construction, characterization and additionally the use of a stable recombinant YFV-17D expressing GLuc to antiviral activity, combining in a single strategy the stability of the YFV-17D backbone and the advantages of GLuc as a reporter gene.

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