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Standardization, validation, and comparative evaluation of a faster and high-performance test for quantification of yellow fever neutralizing antibodies

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ABSTRACT

Although it is considered the reference for quantification of neutralizing antibodies, classical method of the plaque reduction neutralization test (PRNT) is labor intensive, requires specific equipment and inputs, besides a long time for its finalization, even in the micro-PRNT version (in 96-well plates). It has a higher sample throughput, however the smaller wells make the reading of plaques more difficult. With an immunoenzymatic revelation step and a semi-automated reading, the μFRN-HRP (micro Focus Reduction Neutralization - *Horseradish Peroxidase*) is a faster and more efficient test for the quantification of YF neutralizing antibodies. This study aimed to standardize, validate, and compare it with the reference method in 6-well plates (PRNT). Once the execution protocol was standardized, precision, accuracy, selectivity, and robustness were evaluated to validate the μFRN-HRP. In addition, 200 sera of vaccinees were processed by the μFRN-HRP and by the micro-PRNT to compare with the reference test, estimating agreement by Intraclass Correlation Coefficient (ICC). The standardization and validation of the μFRN-HRP was carried out successfully. Weak to moderate agreement was observed between μFRN-HRP and PRNT for titers in reciprocal dilution, while the same comparison between the classical tests resulted in a better ICC. However, titers in milli-international units obtained by μFRN-HRP showed a substantial agreement with PRNT, while the agreement between micro-PRNT and PRNT was inferior. Therefore, μFRN-HRP can be used in the confirmation of natural YF infection and immune response to vaccination, replacing the micro-PRNT, gaining agility, while preserving the specificity of the result.

1. Introduction

Despite the existence of effective vaccines since 1937, Yellow Fever (YF) remains endemic in many tropical regions of Africa and South America, co-circulating with other flaviviruses such as dengue and Zika viruses. Periodically, isolated outbreaks and epidemics of the disease occur in nonendemic and in endemic areas, which may have great impact on public health (Yellow Fever - African Region (AFRO), 2022; Paules and Fauci, 2017).

Vaccination is the most effective way to prevent and control disease dissemination (Watson and Klimstra, 2017). 17D YF vaccines represent the best developed vaccines to date, considered safe and highly immunogenic, inducing the formation of long-term protective antibodies (Barrett, 2020). The efficacy of the YF vaccine has never been determined within a controlled clinical trial in humans, and evidence that it protects against disease is based on its role in disease control. However, studies with different flaviviruses indicated that the protection of animals against viral infection is correlated with neutralizing antibodies (Guirakhoo et al., 2004; Robert Putnak et al., 2005; Tavares da Silva Fernandes et al., 2021) and, therefore, immunogenicity is generally accepted as a proxy of efficacy of the vaccine.

Neutralizing antibodies can be detected ten days after vaccination in almost 90% (Monath et al., 2013) and within 30 days in 99% of vaccinees (adults and children over two years old) (Shearer et al., 2017). Moreover, there is strong evidence that these antibodies mediate long-term protection to the disease and therefore serological tests are fundamental in 17D vaccine efficacy studies (Monath and Vasconcelos, 2015; Pierson et al., 2008).

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Flaviviruses contain cross-reactive epitopes that make it difficult the diagnosis by serological methods for yellow fever virus (YFV) and other species of the genus, especially in regions where several flaviviruses co-circulate (Koraka et al., 2002; Rasulova et al., 2022). Although many useful diagnostic tests are being developed for serological investigation, the Plaque Reduction Neutralization Test (PRNT) (Porterfield, 1959; De Madrid and Porterfield, 1969) is the reference for serological differentiation of *Flavivirus* infections and for protective immune response analysis after vaccination, with superior performance (mainly specificity) to other serological tests (Rasulova et al., 2022; Ferguson et al., 2010; Maeda and Maeda, 2013).

PRNT assess an *in vitro* viral neutralization biological parameter, based on the ability of serum dilutions to prevent the formation of plaques in susceptible cells by known amounts of the virus. The use of the 6-well-plate version – PRNT standard or classic – facilitates visualization and decreases the chances of plaque overlap, allowing more accurate titrations (Roehrig et al., 2008). The micro-PRNT version (in 96-well plates) has higher sample throughput compared with the 6-well-plate version, but the smaller wells make the reading of plaques more difficult. Although it is considered the reference for analysis of immune response to YF natural infection and to vaccination, presenting high specificity to quantify neutralizing antibodies, the standard PRNT is laborious and time-consuming, difficult to execute, requires skilled human resources, equipment and inputs, and has limited reproducibility (Putnak et al., 2008; Simões et al., 2012).

Currently, a new generation of neutralization tests based on modifications in the standard PRNT method is being developed, to optimize, increase throughput and improve determination of neutralizing antibody titers. However, any new approach to quantify neutralizing antibodies will need to be validated against the standard PRNT, which remains the reference test, so that the relationship and equivalence between the two methodologies is fully understood (Rasulova et al., 2022; Roehrig et al., 2008).

The recent epidemiological scenario of YF outbreaks and high co-circulation of different flaviviruses in Brazil and other countries (F.C V, H C, 2016), beyond the need for evaluation of available and developing YF vaccines stimulate the search for a precise, accurate, faster, less laborious, and automated test for the quantification of neutralizing antibodies. The test would meet the need to increase capacity (high throughput) and to improve the quality of sample analysis from patients and clinical and preclinical studies evaluating the vaccine immune response (Rasulova et al., 2022; Whiteman et al., 2018). Considering this, new tests are being developed and validated by groups interested in evaluating more efficiently the vaccine immune response, exploring different readout methods for determining neutralizing antibody titers, as the immunostaining approach.

The present study aimed to standardize, validate, and compare with the reference test (PRNT, in 6-well plates), a faster test for the quantification of YF neutralizing antibodies called μFRN-HRP (micro Focus Reduction Neutralization - Horseradish Peroxidase). Presenting the revelation step based on immunoenzymatic methodology and a semi-automated reading of the FFU, the expectation is to enable the use of a test with superior accuracy and sample throughput and improve data analyses of sera from patients and vaccinees.

2. Materials and methods

2.1. Management of serum samples and ethical considerations

The present study was approved by the Research Ethics Committee of the National Institute of Infectious Diseases Evandro Chagas (INI/Fiocruz – CAAE 52793216.4.0000.5262) where the waiver of the informed consent form was authorized for the two source studies of the sera used. In both retrospective studies, the fundamental ethical principles that guide research involving human beings were considered in the development of the study. Brazilian National standards were followed, and the study team adhered to international scientific integrity and ethical aspects in the design, conduct, recording, and reporting of scientific studies. Consent from donor participants (adults only) had been previously obtained in writing and sample aliquots were immediately coded and stored at −70 °C.

Standard sera (controls of the neutralization tests) were prepared internally, from pools of human sera selected according to data related to previous exposure to *Flavivirus* (disease, vaccine and neutralizing antibody titers for dengue virus and YFV, determined by PRNT and micro-PRNT respectively), from a study carried out in 2013 by our Laboratory "*Protocol for Obtaining and Producing in-House Sera for Plaque Reduction Neutralization Tests (PRNT)*" (unpublished) – Register number CAAE 15120613.4.0000.5262 (INI/Fiocruz). To obtain the positive standard serum (P3), eight sera were selected and pooled before distribution in smaller aliquots for use in the neutralization tests. The negative standard serum (P1/100) was obtained by pooling seven sera. Before distribution in smaller aliquots, the pool was diluted 100 times in medium in order to obtain a control serum with a level of neutralizing antibodies to YF undetectable by PRNT.

Sera were also obtained from the study *"Duration of Immunity After Two or More Doses of Yellow Fever Vaccine in Adults" (*Camacho, *2019)*. This study included only adults who had received a second dose of the yellow fever vaccine 30–45 days before collection of the blood sample, and adults who had been vaccinated with two or more doses. The study protocol was approved by the Research Ethics Committee of the National School of Public Health/Fiocruz (CAAE: 15752013.1.0000.5240). For the performance of the comparative evaluation assay between the neutralization tests, 200 sera from this clinical study were randomly selected, ignoring the neutralizing antibody titers for YFV previously determined, aiming at independent observations and a broad spectrum of immune response.

All laboratory analyses were conducted in the Laboratory of Technology in Virus (LATEV) at Bio-Manguinhos, Fiocruz, which has decades of experience in serological tests for YF.

The sample size of 200 was estimated using the WinPepi program (Abramson, 2004) based on the following parameters: intraclass correlation coefficient (ICC) equal to or >0.8, with a significance level of 5% and range of the confidence interval of 0.1. Expected Kappa of 0.65 or more, considering the frequency of seropositive individuals of 50%, with a significance level of 5% and amplitude of the confidence interval of 0.2.

The *International reference preparation for anti-yellow fever serum (NIBSC code "YF")* was prepared from a pool of sera from three monkeys immunized with Asibi strain of YFV. Each ampoule of this material contains 143 International Units (IU) of anti-YF serum (Krag et al., 1965).

Serum M7 was prepared internally, from rhesus monkeys (*Macaca mulatta*) with a high titer of neutralizing antibodies for YFV induced by a 17DD YF vaccine. *Serum AA61* was prepared internally, from a non-vaccinated rhesus monkey, which did not have antibodies against YFV.

All sera were previously inactivated for 30 min at 56 °C and stored at −20 °C until the tests were carried out.

2.2. Cells

Vero cells ATCC (CCL 81) were used for virus production, titration and neutralization tests. Cell culture flasks (175 cm^2) were maintained in 5% CO_2 atmosphere at 37 °C, in 199 medium with Earle's salts (10×), buffered with aerated sodium bicarbonate 4.4%, supplemented with 5% inactivated fetal bovine serum and antibiotic (gentamicin sulfate 4 mg/mL).

Vero cells NIBSC (access number: 011038), as well as Vero cells ATCC, were evaluated in the μFRN-HRP standardization process. Cell culture flasks (175 cm^2) were maintained in 5% CO_2 atmosphere at 37 °C, in commercial EMEM (Eagle's minimal essential medium).

Routine cell maintenance and the production of plates for

neutralization tests were done with cells between passages 120/09 and 120/21. For the production of a new cell bank the cells are subjected to three passages and then frozen.

2.3. Virus

The vaccine strains 17D-213/77 (WHO) and 17DD (Bio-Manguinhos) were used in the assays of the present study. A batch of 17DD had already been produced in bioreactors and clarified by another group from the Laboratory, with a titer of $10^{6.27}$ PFU/mL. The batch of strain 17D-213/77 was obtained after 3 passages in Vero ATCC cells. For the production of the virus batch, cell culture flasks (175 cm^2) were prepared (density of 6×10^4 cells/cm^2) 24 h before being infected with the 17D strain at multiplicity of infection (moi) of 0.002, followed by clarification (centrifugation 10 min, 2000 rpm, 4 °C – Eppendorf centrifuge 5810 R; 0.22 μm filtration). The titer obtained by plaque assay on Vero cell monolayers in 6-well plates was $10^{8.4}$ PFU/mL.

2.4. Plaque assay – virus titration

The day before the test 6-well plates were prepared with Vero cells ATCC (density of 1×10^5 cells/cm^2). Virus aliquots were randomly chosen and quickly thawed under running water to be diluted in Medium 199 in a serial 2-fold dilution (first dilution 1:10). Then, all the Medium 199 present in the 6-well tissue culture plates was carefully aspirated followed by the addition, in triplicate, of 200 μL/well of each dilution of the virus aliquots. For the adsorption process monolayers were incubated for 1 h (37 °C, 5% CO_2). After this period, the liquid content was replaced by 3 mL/well of the semi-solid medium (carboxymethylcellulose 3%) and the monolayers were incubated again (37 °C, 5% CO_2) for 7 days. Finally, the monolayers were fixed with formaldehyde (5%), washed with water and stained with violet crystal (0.04%). Plaques were counted manually in a transilluminator. The following calculation was performed to determine the virus titer in $\log_{10}$ PFU/mL: $\log_{10}$ of the number of plaques + $\log_{10}$ of the corresponding dilution + $\log_{10}$ 5 (correction factor for 1 mL of 200 μL inoculum).

2.5. Peroxidase-labelled monoclonal antibodies

4G2-HRP (anti-*Flavivirus*) and 2D12-HRP (anti-YF) were produced by the Monoclonal Antibody Technology Laboratory (LATAM / Bio-Manguinhos). The hybridomas were cultivated in Roller bottles (4G2) or Hyperflask® (2D12) using high glucose DMEM supplemented with 10% FBS and 6.4 mM L-glutamine. The Thermo Scientific™ Easy-Titer™ IgG Assay Kit (catalog number 23300) was used to determine the concentration of IgG. Purification and quantification of the total protein by the BCA method (Smith et al., 1985) were carried out by the Macromolecule Laboratory (LAMAM / Bio-Manguinhos). The conjugation to HRP was performed by the Laboratory of Diagnostic Technology (LATED / Bio-Manguinhos) by periodate oxidation method (Pavliuchenko et al., 2019). Immediately before use, the conjugated antibodies were subjected to a dilution (previously defined, varying according to the batch/concentration of the conjugated antibody) in the blocking buffer (5% of BSA 10%; 50% of Blocker Casein in PBS 1%; 45% of distilled water).

2.6. Neutralization tests

2.6.1. PRNT (6-well plates), the reference test

The execution protocol of the PRNT had already been established in the Laboratory (Simões et al., 2012). Briefly, sera were subjected to 6 serial two-fold dilutions (1:20–1:640) and then mixed with the same volume (additional dilution of 1:2) of a viral suspension prepared immediately before use (suspension dilution used gives 90 plaques/well approximately). For the neutralization step, the described mixtures were kept for 1 h in an incubator (at 37 °C with 5% CO_2 atmosphere) and, after that, transferred to 6-well plates with preformed monolayers (3,3 $\times 10^5$ cells/mL). After 1 h at 37 °C with 5% CO_2 atmosphere for the adsorption step, the semi-solid medium (CMC 2.5% at room temperature) was added to the cell monolayers to be incubated again for 7 days (37 °C and 5% CO_2 atmosphere). After this final incubation, fixation (formaldehyde 5%) and staining (0.04% crystal violet), plaques were manually counted at the transilluminator.

2.6.2. Micro-PRNT (96-well plates)

The 96-well plates method had also been established, being used in the routine of assessing the immune response to YF in LATEV (Simões et al., 2012). The first step of the micro-PRNT consists of serial 2-fold dilution of each serum sample and controls. Medium 199 was added to all wells of the microplate followed by the addition of sera, so that the first dilution was started in 1:5 and continued in a serial manner until the dilution 1:640 in the final volume of 50 μL/well. Thus, for each serum, 8 dilutions were obtained. For the neutralization step, sera were subjected to an additional dilution of 1:2, where the same volume of a viral suspension prepared at the time of use was mixed with each dilution of sera. After 1 h of incubation at 37 °C with 5% CO_2 atmosphere, 50 μL of a Vero cell suspension (2.7×10^5 cell/cm^2 density) were added to all wells of the plate and it was incubated again for 3 h (37 °C and 5% CO_2 atmosphere) for the adsorption step of the non-neutralized virus. After that, the inoculum was replaced by a semi-solid medium (CMC 2.5% at room temperature) and the plate was incubated for 6 days (37 °C and 5% CO_2). After this period, cell monolayers were fixed with formaldehyde and stained with crystal violet, so that the formed plaques were counted manually in the BioSpot (CTL Biospot).

2.6.3. μFRN-HRP (96-well plates), the standardization process

In all the method standardization assays, the general steps were as follows: first, diluent medium was added to all wells of the microplate. One column was reserved for virus control (medium + virus suspension) and the border columms for cell control. The remaining columns were reserved for sera. For each serum, 6 dilutions were obtained from serial three-fold dilution (Appendix 1). A virus suspension was prepared at the time of use, at the ideal dilution to obtain approximately 70 FFU/well in the virus control. For the neutralization step, the same volume of virus suspension was added to the same volume of the dilutions of sera or to the medium (virus control). The mixtures were then incubated at 37 °C and 5% CO_2. After incubation, 200 μL of each mixture was transferred to the 96-well plate containing the preformed monolayers for adsorption at 37 °C and 5% CO_2 atmosphere. Subsequently, the monolayers were subjected to fixation for at least 1 h and washes for addition of 100 μL/well of the peroxidase-labelled monoclonal antibody diluted, at the time of use, in blocking buffer. After 2 h of incubation (35 °C and 5% CO_2), the monolayers were washed with DPBS (Dulbecco's Phosphate Buffered Saline) in an automatic washer before adding the substrate (True Blue, KPL). After 15 min in the dark, at room temperature, the monolayers were washed again with distilled water at automatic washer and, finally, photographed automatically in ScanLab. The images generated were transferred to Axiovision for automated YF FFU counting.

The YF μFRN-HRP standardization process has involved all steps of the test (Appendices 2 and 3) and has resulted in its execution protocol obtained from the evaluation of the variables and their respective conditions.

In the first phase of the standardization, where assessment was exclusively qualitative (visual), the fundamental test variables (Appendix 2) were evaluated concomitantly, organized into 4 groups according to a combination of Vero cell and virus substrain: I) Vero cell ATCC (Medium 199) X substrain 17D-213/77; II) Vero cell ATCC (Medium 199) X substrain 17DD; III) Vero cell NIBSC (Medium EMEM) X substrain 17D-213/77; IV) Vero cell NIBSC (Medium EMEM) X substrain 17DD.

In a second phase, other variables were analyzed in a qualitative and/or quantitative way (Appendix 2). For quantitative analysis, each

well of each plate was photographed in the Scanlab II – an automated image acquisition platform, composed of a high-performance camera attached to a microscope with motorized stage, designed to meet the highest quality requirements using automation and robotics to transfer samples (de Lima, 2020). The counting was also automated and fully standardized for YF focus counting with the Axiovision program – a microscope software of digital image processing. The definition of recognition and counting parameters of the YF FFU by the software (called "teaching and reading") was also part of the technique standardization process.

The variables and their respective conditions (Appendix 3) were evaluated from assays in which only the virus was processed, or by testing sera with different levels of neutralizing antibodies (M7; M7/10, diluted 10 times; and AA61), following the predefined rationale for the method steps.

The definition of the μFRN-HRP execution protocol allowed the next tests to be carried out (validation and comparative evaluation with the reference test).

2.6.4. Calculation of the neutralizing antibody titers

The endpoint of 50% (EP50) is the point at which there is a 50% reduction in the number of plaques/focus obtained in virus control. Therefore, the neutralizing antibody titer was defined as the reciprocal of the last serum dilution that reduced the number of plaques by 50%. In reciprocal dilution the titer was calculated through linear regression, by interpolation of the dilutions corresponding to plaque/focus numbers immediately above and below the EP50 value of the test. The transformation of neutralizing antibody titer into mIU/mL was performed considering the antibody titer of the positive internal standard serum (Simões et al., 2012) (P3), which was previously calibrated against the international reference serum for YF *(NIBSC code "YF")* to determine the nominal value in mIU/mL (10,264 mIU/mL for micro-PRNT, 12,811 mIU/mL for μFRN-HRP and 18,180 mIU/mL for PRNT).

2.6.5. Validation of the μFRN-HRP

After its standardization, μFRN-HRP was validated according to the rules established by Brazilian Health Regulatory Agency (ANVISA), with determination of the following parameters: precision, accuracy, selectivity and robustness. Sera from the study "*Protocol for Obtaining and Producing in-House Sera for Plaque Reduction Neutralization Tests (PRNT)*" were selected considering the available data and in order to meet the need for each validation test. The analyses were performed based on the Log_{10} titers of the reciprocal dilution.

2.6.6. Precision (Reliability)

It is defined as the ability of the test to produce similar results from the same serum sample processed several times (Sarzotti-Kelsoe et al., 2014). Intra-assay precision was assessed under minimal variable conditions. The same sample tested several times (replicates) by the same operator, within the same assay, on the same plate and with the same batch of reagents. Six replicates of 16 sera with different levels of neutralizing antibodies were processed in the same assay performed by the same operator (Ministério da Saúde, 2012). The intra-assay precision was determined 3 times by processing these samples by 2 operators. For analysis of the data obtained, geometric mean titer (GMT) of the replicates was determined for each serum. At least 5 (90%) of the 6 titers obtained should not vary >3 times the value of their GMT (± 0.48; Log_{10} 3 = 0.477). The test was considered with intra-assay precision if at least 13 (80%) of the 16 samples tested met this criterion (Timiryasova et al., 2013). Inter-assay precision was determined under different conditions. The variability was measured on different days, by different operators, with the same batch of reagents. To this end, two operators repeated the procedure described above (intra-assay) in three independent tests. Each test was performed without the operator knowing the results previously obtained (Ministério da Saúde, 2012). Two different analyses were performed: I) Inter-assay A (same operator, different assays): from the 12 titers obtained for a serum (6 replicates × 2 assays) the GMT ± 0.48 was determined. Only 1 titer (~ 10%) out of 12 could be outside the range. At least 13 (80%) of the 16 sera tested should have met this criterion to consider the inter-assay precision of the test; II) Inter-assay B (different operators, different assays): from the 18 titers obtained for the same serum (6 replicates × 3 assays) the GMT ± 0.48 was determined. Only 2 (~ 10%) out of 18 results could be outside the titer range determined for that serum. At least 13 (80%) out of the 16 sera tested should have met this criterion (Timiryasova et al., 2013). Complementing the precision, coefficient of variation (CV%) of the replicates (in the log scale) of each serum were determined, which could not exceed 15% (Ministério da Saúde, 2012).

2.6.7. Accuracy (Dilutability)

It determines the degree of agreement between observed and expected titers for a specific serum, based on the simultaneous processing of its undiluted form and submitted to different dilutions. The internal standard serum (P3) and 3 more sera with high titers of YF neutralizing antibodies were processed in the same assay in their undiluted and diluted forms (1:2, 1:4, 1:8, 1:16 and 1:32 for the serum P3; 1:4, 1:8, 1:16 for the other 3 sera) in the negative internal standard serum (P1/100), generating 14 samples (diluted sera) to be processed. Each sample presentation was run five times in the same assay. The expected GMT were determined for each of the 4 sera evaluated: geometric mean obtained from the 5 replicates of the undiluted serum, divided by the proposed dilution factors (1:2, 1:4, 1:8, 1:16 and 1:32). GMTs obtained from the diluted sera were classified as observed. The observed and expected GMT were transformed into Log_{10} to determine the absolute difference between them. This should not be >0.48 in at least 80% (11 out of 14) of the samples tested (Timiryasova et al., 2013).

2.6.8. Selectivity (Specificity)

In the present study, selectivity was defined as the ability of the test to quantify YF neutralizing antibodies in the presence of antibodies against other flaviviruses in the same sample. In Brazil, the arboviruses (genus Flavivirus) of greatest circulation with epidemiological importance today are Dengue and Zika. In this study, due to ethical issues and the volume of serum needed to perform the specificity tests, it was not possible to evaluate this criterion also in seropositive samples for Zika virus or other Flaviviruses besides dengue virus. Therefore, 5 positive sera for YF and negative for 4 dengue serotypes (YF+ / DEN-) were diluted (1:2) in the negative internal standard sera (P1/100) and, in the same way, were also diluted in 3 positive sera for 4 dengue serotypes (DEN +) and negative for YF. The 4 mixtures of each of the 5 sera FA+/DEN- were run in the same assay in triplicate. For analysis, GMT obtained from the 5 sera FA+/DEN- diluted in P1/100 were considered reference for comparison with the GMT obtained from the same 5 sera diluted in the 3 sera DEN+. Respective absolute differences, titers expressed as Log_{10} should not be >0.48 in at least 12 (80%) out of the 15 samples (mixtures FA+/DEN- with DEN+) tested (Timiryasova et al., 2013).

2.6.9. Robustness

It evaluates if the common variations during the execution of the protocol interfere in the generated results (Sarzotti-Kelsoe et al., 2014). Six sera with YF antibody titers were selected (based on volume available) for the evaluation of the following variables: virus concentration, neutralization time, cell density, adsorption time, monoclonal antibody dilution and incubation time (Appendix 4).

Neutralizing antibody titers obtained in standard conditions were compared to the titers obtained in their respective deviation conditions for each evaluated variable. Robustness was determined if at least 80% of the processed samples (5 out of 6) showed an absolute difference between the titers (standard and deviation) <0.48 for comparison of the titers transformed in Log_{10} (Timiryasova et al., 2013).

2.7. Comparative evaluation

Any new methodological approach for quantification of neutralizing antibodies, even validated according to the rules of the regulatory agency, must be validated against the standard PRNT (6-well plates), in a comparative way, to determine the relationship and agreement between the methodologies (Roehrig et al., 2008). In order to determine the agreement between the neutralization tests, 200 sera were selected from the study *"Duration of Immunity After Two or More Doses of Yellow Fever Vaccine in Adults"* (Camacho, *2019*) and processed by the three neutralization tests, but not simultaneously. However, no analyst had access to the results of the other tests. Titers, in Log_{10} of the reciprocal dilution and in mIU/mL, obtained from the index tests (micro-PRNT and μFRN-HRP) were compared to the titers obtained from the same sera carried out in the reference test (PRNT), considering determinations of the ICC and the Pearson's Correlation Coefficient, in addtion to Bland-Altman and scatter plot analysis.

2.8. Statistical analysis

Microsoft Excel 2016 was used for determinations of neutralizing titers (in reciprocal dilution and mIU/mL) and their transformations in Log_{10}, coefficients of variation, replicate averages, variations and absolute differences of the GMT. The statistical program SPSS (Statistical Package for Social Science V.17.0) was used to estimate measurements of agreement (ICC and Pearson's correlation coefficient, *r,* and 95% confidence limits), calculate descriptive statistics (mean, median, standard deviation and interquartile range) and generate scatter plots, Bland-Altman plots and Box-Plots.

3. Results

3.1. Standardization of the μFRN-HRP

μFRN-HRP standardization process started with a qualitative (visual) analysis of the fundamental test variables, using group I only (Vero cell ATCC X substrain 17D-213/77) (Appendix 5). Preparation of preformed cell monolayers at the microplates 24 h before being infected and the revelation step started 48 h after the infection resulted in more satisfactory images, with a more appropriate FFU profile (size, morphology and distribution at the well) when compared to the other time combinations evaluated.

The four groups organized according to the origin of Vero cells and virus substrain were subjected to qualitative tests for the other fundamental variables. The resulting images from these tests (Appendix 6) show the best FFU profile for the 17D-213/77 substrain, with more delimited and adequate phenotype for visualization in microplate wells, compared to the 17DD substrain. Both Vero ATCC and NIBSC cells presented satisfactory monolayers, with good confluence. But the Vero ATCC cells proved to be more appropriate for infection with the 17D-213/77 substrain, obtaining more FFU/well compared to NIBSC cell monolayers. However, the wide variation observed between monolayers from different wells in the same assay and between different assays indicated the need to carry out further tests to determine cell density. Both conjugated antibodies performed as expected to dye YF FFU. The 4G2-HRP seemed to be more efficient (visualization of a larger amount of FFU) when compared to 2D12-HRP and, therefore, was chosen to continue the standardization of μFRN-HRP.

In the following tests, in order to define the ideal virus dilution and cell density, the 17D-213/77 substrain was subjected to serial 10-fold dilution (10^{-2}–10^{-5}) to infect different cell densities: 1×10^5; 2×10^5 and 3×10^5 cells/mL. The steps of μFRN-HRP were followed with the variables already defined (Appendix 7). The density of 2×10^5 cells/mL showed the best performance, resulting in cell monolayers with satisfactory confluence, more homogeneous in the same well, but also with less variation between wells on the same plate and between different tests. Determination of the ideal dilution of the virus still depended on the definition of other variables.

The μFRN-HRP standardization process continued with the evaluation of the best neutralization time. Serial 2-fold dilution of the virus (10^{-3}–$10^{-4.5}$), defined from 10-fold dilutions, were performed to determine the time that results in the least variation in FFU counts between wells on the same plate and between different tests. Considering the importance of the ideal dilution of the 4G2-HRP monoclonal antibody in dyeing and, therefore, FFU visualization and counting, this variable was also evaluated in these titrations by testing four different dilutions. In Appendix 8 images of the same test where each plate was subjected to a different neutralization time. These tests were analyzed quantitatively, in which the images obtained in ScanLab were transferred to Axiovision for the counting of YF FFU. However, the process of "teaching and reading" of the software for YFV had not yet been carried out. The data obtained showed incompatibility between the automated (Axiovision) and manual (visual) counting of the images. Counts generated by the software were underestimated. Therefore, manual counting was considered to determine the best neutralization time.

Counts obtained from all tests were organized in boxplot graphs to visualize dispersion of the quantity of FFU/well, for each virus and antibody dilutions, in different neutralization times (Fig. 1). The time of 2 h for neutralization step resulted in an appropriate amount of FFU/well (between 50 and 100), especially in dilutions $10^{-3.3}$ and $10^{-3.6}$, for the different antibody dilutions evaluated. Similar results were observed for the other neutralization times. However, in the analysis of virus dilutions that resulted in optimal FFU/well counts, the lowest degree of dispersion was observed with 2 h of neutralization when compared to the 1 h and 1 h and 30 min data.

Although these data made it possible to determine not only the best neutralization time, but also ideal dilutions for the virus and the conjugated antibody, these last variables were reevaluated throughout the study (with each new virus production, monoclonal antibody and TB substrate).

Qualitative analysis of the different tests in the μFRN-HRP standardization process, with definition of the fundamental test variables, showed the irregularity in profile and quality of cell monolayers. Considering the importance of cell monolayer (without damage and homogeneous) in the quality of μFRN-HRP, a new cell bank Vero ATCC was produced by the LATEV team, after reviewing the maintenance procedures and passage of cell bottles, but also the preparation of preformed monolayers in 96-well plates. From the new cell bank Vero ATCC, a new lot of 17D-213/77 was produced. In the same period, a new batch of the conjugated antibody 4G2-HRP was also produced, in addition to the availability of a new batch of TB reagent.

Using only serially diluted virus, μFRN-HRP assays were performed to evaluate: ideal dilutions of new productions of 4G2-HRP and 17D-213/77 virus; quality of the Vero ATCC cell monolayers from the newly produced bank; and performance of the new batch of TB reagent. Initially, assays were performed testing 10-fold dilution of the virus and the antibody in 4 dilutions (Appendix 9). From these data, assays simulating μFRN-HRP were run by testing virus subject to 2-fold dilution and 4G2-HRP in 2 dilutions (Appendix 9). The images, which confirmed the results obtained earlier for cell density and neutralization time, showed a considerable improvement in the cell monolayer, with less grume (clusters of cells) and a less heterogeneous profile between wells on the same plate and between wells of different plates or of different tests. Furthermore, images suggested the ideal dilutions of the virus ($10^{-4.9}$) and the antibody (1:1000). However, images processing on Axiovision continued to point out inconsistencies in the counts, despite necessary and possible adjustments for better recognition of YF FFU by the software had been carried out.

The standardization process of the technique continued with the evaluation of use semi-solid medium. The goal was to decrease size and reduce heterogeneity of the YF FFU population so that it was compatible with the area of the microplate wells, which, consequently, would

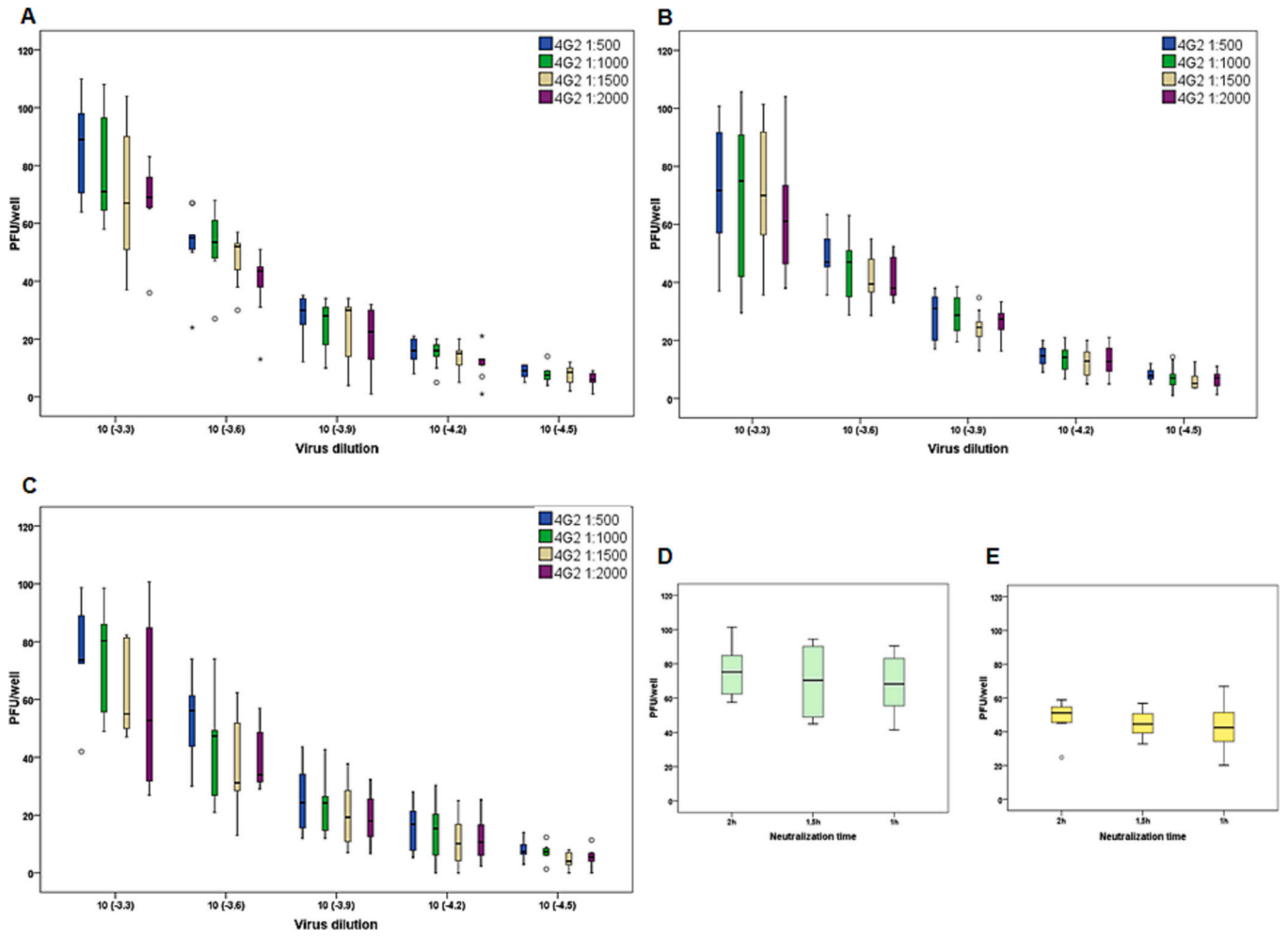

Fig. 1. Boxplots of 2-fold titration of 17D-213/77, simulating μFRN-HRP, in different neutralization times and 4G2-HRP dilution.
(A) quantities of FFU/well obtained from different virus dilutions and in each evaluated dilution of 4G2-HRP (blue = 1: 500; green = 1: 1000; yellow = 1: 1500; purple = 1: 2000) for the neutralization time of 2 h. X-axis: 2-fold dilutions of 17D-213/77 substrain ($10^{-3.3}$–$10^{-4.5}$). Y-axis: quantity of FFU/well (averages of replicates of 10 tests). (B) quantities of FFU/well obtained from different virus dilutions and in each evaluated dilution of 4G2-HRP (blue = 1: 500; green = 1: 1000; yellow = 1: 1500; purple = 1: 2000) for the neutralization time of 1 h and 30 min. X-axis: 2-fold dilutions of 17D-213/77 substrain ($10^{-3.3}$–$10^{-4.5}$). Y-axis: quantity of FFU/well (averages of replicates of 10 tests). (C) quantities of FFU/well obtained from different virus dilutions and in each evaluated dilution of 4G2-HRP (blue = 1: 500; green = 1: 1000; yellow = 1: 1500; purple = 1: 2000) for the neutralization time of 1 h. X-axis: 2-fold dilutions of 17D-213/77 substrain ($10^{-3.3}$–$10^{-4.5}$). Y-axis: quantity of FFU/well (averages of replicates of 10 tests). (D) boxplots of FFU/well obtained from the $10^{-3.3}$ dilution of 17D-213/77 substrain, simulating μFRN-HRP, in different neutralization times and considering the unification of the different dilutions for the 4G2-HRP. X-axis: neutralization times (2 h, 1 h and 30 min and 1 h). Y-axis: quantity of FFU/well (averages of replicates of 10 tests). (E) boxplots of FFU/well obtained from the $10^{-3.6}$ dilution of 17D-213/77 substrain, simulating μFRN-HRP, in different neutralization times and considering the unification of the different dilutions for the 4G2-HRP. X-axis: neutralization times (2 h, 1 h and 30 min and 1 h). Y-axis: quantity of FFU/well (averages of replicates of 10 tests). Graphics from *SPSS V.17.0.*

improve the automated counting by Axiovision.

The first attempt to improve profile and size of the 17D-213/77 FFU population was to carry out the μFRN-HRP method using only diluted virus ($10^{-4.6}$–$10^{-4.95}$), testing different concentrations of the semi-solid medium (0.5%, 1.0% and 1.5%). After the neutralization step and the transfer of the virus + medium mixtures to the plate containing the preformed monolayers, this was incubated for only 1 h so that its contents were replaced by the semi-solid medium and the monolayers were incubated again for 48 h. Qualitative analysis of the images showed that using semi-solid medium prevented the appearance of very small FFUs, which due to their very different profile (in size and color) contributed to the heterogeneity of the FFU population, making it difficult to define the Axiovision's parameters for the accurate counting of YF FFU. On the other hand, due to the need for additional washes to remove semi-solid medium, many damages to the cell monolayers were observed. In addition, the FFU profile remained large and inadequate for the 96-well plate wells (Appendix 10).

Different strategies were evaluated in an attempt to decrease FFU size and preserve the integrity of cell monolayers: decrease revelation time for 36 h and without adding the semi-solid medium; revelation step in 36 or 48 h adding semi-solid medium; evaluation of different washing protocols (1, 2 and 3, detailed in Appendix 11). Assays following the predefined steps for the μFRN-HRP protocol were performed, in which virus was subjected to different dilutions and testing different concentrations of the semi-solid medium (Fig. 2).

Revelation within 36 h (Fig. 2A) without using the semi-solid medium, despite the absence of damage to the cell monolayers, revealed heterogeneous and lighter (blue tones) than expected FFUs. For revelation within 36 h and using semi-solid medium, concentrations of 2.0% and 2.5% resulted in homogeneous FFU population with convenient, smaller and well-defined characteristics, facilitating the individualization of each FFU. The absence of secondary infection FFUs was also observed. However, washing problems for CMC removal remained, with much of the monolayers damaged. Moreover, an additional incubation

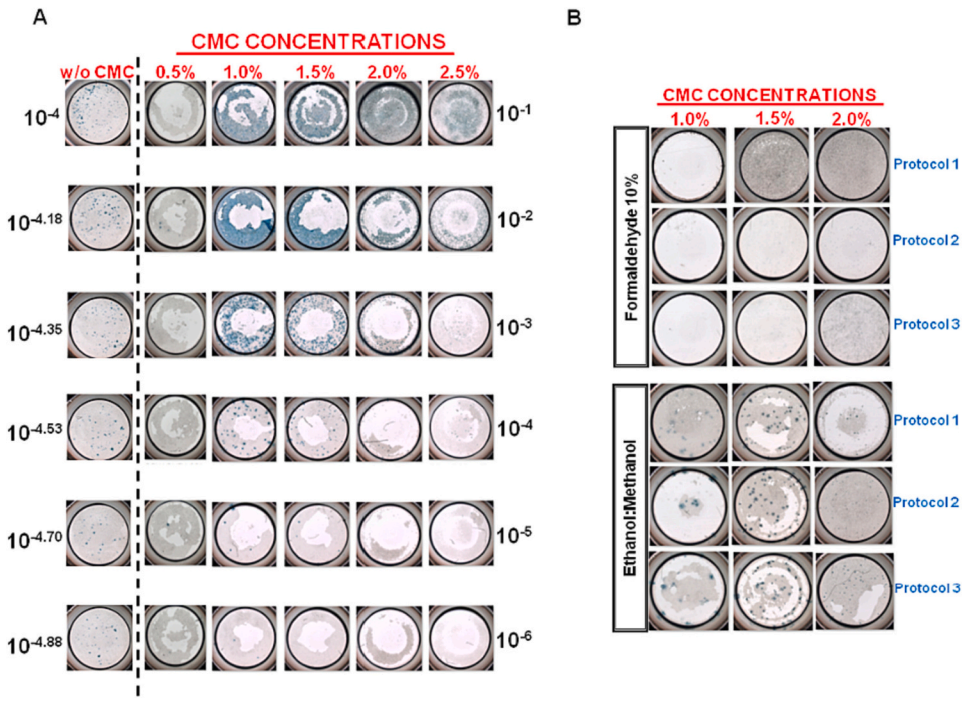

Fig. 2. Representative images from at least 2 μFRN-HRP assays testing different strategies to decrease FFU size.
(A) revelation step in 36 h, without or with addition of CMC to the monolayers (0.5%, 1.0%, 1.5%, 2.0%, 2.5%). Implementation of protocol 1 for washing monolayers for CMC removal. Dilutions of 17D-213/77: 10^{-1}–10^{-6}. (B) revelation step in 48 h, with addition of CMC to the monolayers (1,0%, 1,5%, 2,0%). Protocols 1, 2 and 3 for washing monolayers for CMC removal were evaluated, in addition to two fixing solutions (formaldehyde 10% and ethanol: methanol). Dilution of 17D-213/77: $10^{-4.3}$. Photos from ScanLab.

to the protocol of 1 h before the addition of semi-solid medium and revelation step within 36 h was not very operational. The test became longer, and it was necessary to perform the steps of neutralization, virus infection of the monolayers and adsorption in the afternoon so that it was possible to start the revelation in the morning, after 36 h of incubation.

In order to evaluate the μFRN-HRP with revelation step 48 h after the adsorption step (Fig. 2B), virus 10-fold dilutions ($10^{-3.7}$–10^{-6}) were performed to test not only different concentrations of semi-solid medium, but also 2 types of fixing solution (formaldehyde 10% and ethanol: methanol) and 3 washing protocols for CMC removal (Appendix 11) before starting the revelation step. Visual analysis of the images showed inefficiency of the formaldehyde 10% solution compared to fixation with ethanol: methanol. The monolayers that received semi-solid medium 2.0% and were fixed with ethanol: methanol solution, except for protocol 1, showed better results, with homogeneous FFU population, small and suitable for the microplate well area. However, damage to cell monolayers continued to occur, even in protocols 2 and 3.

In an attempt to improve the washing process for CMC removal, other assays were carried out testing the same conditions (revelation within 48 h with different CMC concentrations and washing protocols) but using for the first time positive (M7 and M7 / 10) and negative (AA61) sera for YF neutralizing antibodies (Appendix 12). Images continued to show the performance of the semi-solid medium 2.0% in obtaining appropriate FFU profile. But damage in the monolayers, although minimized, has not been prevented, even in washing protocols 2 and 3. Although the most efficient washing protocol for the semi-solid medium removal remains undefined and damage on the cell monolayers persists, these assays were used to initiate the "teaching and reading" process of Axiovision for the counting of YF FFU.

To finalize the standardization of μFRN-HRP, it was still necessary to improve washing process to remove the semi-solid medium without damaging cell monolayers. Alternative protocols (Appendix 11) using a hand shower were evaluated, since this accessory was already used in the Lab for successful removal of CMC in other methodological approaches. μFRN-HRP assays were performed using only the virus in a unique concentration. After adsorption step, semi-solid medium was added to the monolayers and, 48 h later, each microplate was subjected to a different washing process (Appendix 13). Protocol 3, as already noted, resulted in small but considerable damage to the monolayers. Plates submitted to the protocols 4, 5 and 6, which were washed with a hand shower after the incubation with the fixing solution, showed a lot of damage in the monolayers, making FFUs count unfeasible. On the other hand, microplates corresponding to the protocols 7, 8, 9 and 10 presented intact and homogeneous monolayers. Protocol 9 drew attention not only for the quality of the monolayers but also the clarity in visualization the YF FFUs, with good contrast between the background (cell monolayers) and the blue dots (dyed FFUs).

In subsequent assays, washing protocols 3, 7, 8, 9 and 10 were repeated (Fig. 3). Steps established during the μFRN-HRP standardization were followed to test different dilutions of the 17D-213/77 substrain and a new batch of 4G2-HRP. The pictures repeated the success observed in previous assays of the washing protocols 3, 9 and 10 (especially protocols 9 and 10), without damage to the monolayers and good FFU dyeing. Qualitative analysis of the images also indicated dilution 1:3000 of the new conjugate and dilution $10^{-4,26}$ of the virus.

Three more assays were performed using unique virus and 4G2-HRP dilutions, but testing the three best washing protocols (3, 9 and 10) (Appendix 14). Images confirmed the better performance of protocols 9 and 10 in obtaining satisfactory monolayers and FFUs for visualization and automated counting of the method in 96-well plates. The ideal dilutions for the virus ($10^{-4.26}$) and for the conjugated antibody (1:3000) were confirmed, resulting in a reasonable amount (good distribution in the well and easy individualization) of approximately 70 FFU/well. Processing of the images referring to protocols 9 and 10 in Axiovision and the careful visual analysis of the count obtained in an automated way demonstrated efficiency of the software in accurate and systematized recognition of YF FFUs.

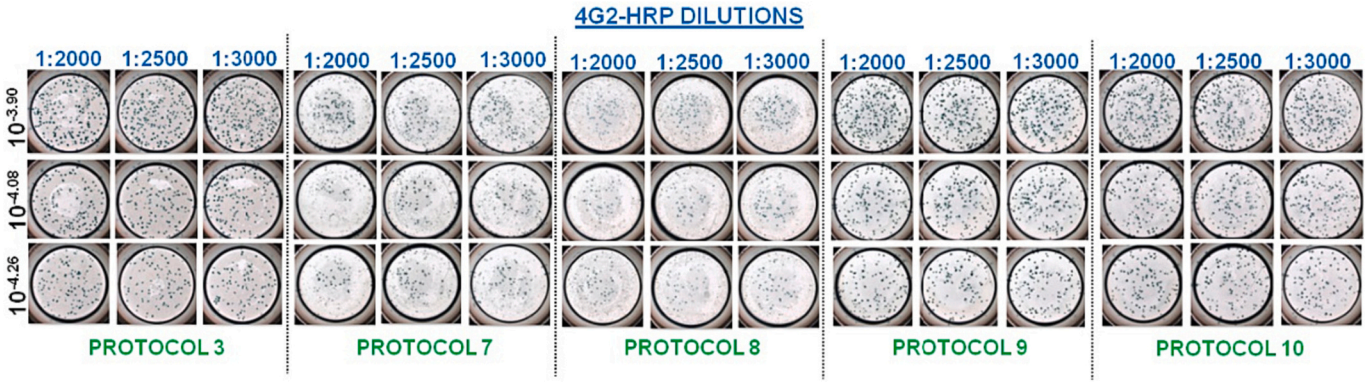

Fig. 3. Representative images from 3 μFRN-HRP assays testing different washing protocols, virus and conjugated antibody dilutions.
Dilutions of the 17D-213/77 substrain (top to bottom): $10^{-3.9}$; $10^{-4.08}$; $10^{-4.26}$. Washing protocols for CMC (2.0%) removal: 3, 7, 8, 9 and 10. Evaluated dilutions of 4G2-HRP: 1:2000, 1:2500, 1:3000. Photos from ScanLab.

From the definition of the execution protocol for YF μFRN-HRP (Appendix 15), with determination of the test variables (Appendix 16), it was possible to carry out the validation and comparative evaluation assays with the reference test.

3.2. Validation of the μFRN-HRP

3.2.1. Precision (reliability)

For precision evaluation, sixteen sera with different neutralizing antibody levels were selected to be processed by two operators, in three independent tests of the μFRN-HRP. GMTs obtained and ranges of sera variation were determined to assess the intra-assay precision (Appendix 17). The data showed that the pre-established criteria were achieved in 100% of the results (16/16).

Inter-assay precision of the μFRN-HRP was assessed from the 12 replicates of the sera processed by the same operator in two independent assays (Appendix 18), but also from the 18 results obtained for each of the 16 sera resulting from 3 assays, varying the operator (Appendix 19). Inter-assay precision of the μFRN-HRP also showed satisfactory results. All the sixteen sera evaluated reached the pre-established criterion.

3.2.2. Accuracy (dilutability)

Data from 14 samples with different neutralizing antibodies levels (resulting from four YF positive sera diluted in negative serum) were used to evaluate the accuracy of μFRN-HRP (Appendix 20). The pre-established criterion was reached showing the accuracy of the test. All absolute differences determined for the diluted presentations of the four evaluated sera were <0.477.

3.2.3. Selectivity (specificity)

Sera with YF neutralizing antibody titers mixed with sera containing antibodies to dengue were processed in a single assay to assess the selectivity of the μFRN-HRP (Appendix 21). Selectivity of the YF μFRN-HRP was proven, since all the absolute differences observed between GMTs of the reference mixtures (sera YF+/DEN- with serum YF-) and those of the test mixes (sera YF+/DEN- with DEN+) were <0.477.

3.2.4. Robustness

Antibody titers of six sera submitted to small and common deviations of different variables of the μFRN-HRP execution protocol were compared to the titers obtained from the respective standard condition for the robustness assessment (Appendix 22). In the evaluation of the variable "cell density", one serum (SIH146) submitted to the condition of 1×10^5 cells/mL (deviation 2), presented titer with an absolute difference >0.477 when compared to the titer of the same serum submitted to the standard condition (2×10^5 cells/mL). However, the criterion established for robustness was reached, as 5 out of the 6 sera evaluated for a given variable had absolute difference between the titers <0.477. In the evaluation of the variable "4G2-HRP time", it was observed that two (SIH199 and SIH146) of the six sera submitted to condition 2 (1 h and 40 min of incubation) presented titers with absolute difference to the titers obtained in the standard condition (2 h) >0.477. These data suggest robustness failure of the μFRN-HRP considering this variable and the importance of following the established incubation time of the conjugated antibody in the test execution. There was no absolute difference >0.477 in the comparison between the titers obtained under the standard conditions and deviations for the other evaluated variables.

3.3. Comparative evaluation

The agreement of YF neutralizing antibody titers of 200 sera submitted to the index test methodologies and the reference test was evaluated from the ICC for results in reciprocal dilution (Table 1) and in mIU/mL (Table 2). Comparison between PRNT and micro-PRNT showed moderate (ICC of single measurement) to substantial agreement magnitude (ICC of average measurement) considering titers obtained in reciprocal dilution and in mIU/mL. PRNT X μFRN-HRP comparative evaluation in reciprocal dilution resulted in weak (single measure) to moderate (average measure) agreement. On the other hand, when the same comparison was made considering the titers transformed in mIU/mL, the agreement estimators showed substantial to almost complete magnitude, superior to the other obtained ICCs.

Linear correlation coefficients were also determined for PRNT X micro-PRNT and PRNT X μFRN-HRP comparisons (Appendices 23 and 24), as well as scatter plots for titers in reciprocal dilution (Appendix 25) and in mIU/mL (Appendix 26) of the 200 sera. The data obtained were very close and showed a moderate correlation ($r > 0.70$) between index and reference tests. Analysis of the scatter plots indicated slightly higher agreement of the micro-PRNT compared to μFRN-HRP, as it presented trend lines with slope profiles closer to 45°.

Complementing agreement analysis, Bland-Altman plots were constructed from titers in reciprocal dilution (Appendix 27) and in mIU/mL (Appendix 28) of the 200 sera processed by the three neutralization tests. The plots showed that titers in reciprocal dilution and in mIU/mL

Table 1
Intraclass correlation coefficients (ICC) for neutralizing antibody titers ($\log_{10}$ reciprocal dilution) of 200 sera for the analysis of agreement between PRNT, micro-PRNT and μFRN-HRP.

Reference test X Index test	ICC (95% CI)	ICC average measure (95% CI)
PRNT X micro-PRNT	0.59 (0.49–0.68)	0.74 (0.66–0.81)
PRNT X μFRN-HRP	0.29 (0.16–0.41)	0.45 (0.27–0.58)

95% CI = 95% confidence interval.

Table 2
Intraclass correlation coefficients (ICC) for neutralizing antibody titers (log_{10} mIU/mL) of 200 sera for the analysis of agreement between PRNT, micro-PRNT and μFRN-HRP.

Reference test X Index test	ICC ($CI_{95\%}$)	ICC average measure ($CI_{95\%}$)
PRNT X micro-PRNT	0.48 (0.37–0.58)	0.65 (0.54–0.74)
PRNT X μFRN-HRP	0.69 (0.61–0.76)	0.82 (0.76–0.87)

95% CI = 95% confidence interval.

generated by the PRNT were systematically higher than those by micro-PRNT. Differences did not show correlation with the averages of the titers. Differently, titers in reciprocal dilution generated by μFRN-HRP were greater than those of PRNT, but differences in the reciprocal of the titers also did not seem to be correlated with the averages of the titers. Differences of the titers in mIU/mL measured by the μFRN-HRP and PRNT were more subtle and did not correlate with the titer averages according to the two methods.

Descriptive statistics of the data (Appendix 29 and Fig. 4) made it possible to evaluate the trend and dispersion profiles of the titers obtained for the different neutralization tests. The observed values for the mean and median were compatible with the trend shown in the scatter and Bland-Altman plots. Titers expressed in reciprocal dilution generated by μFRN-HRP were higher when compared to the reference test. However, transformation of the titers in mIU/mL resulted in a similar trend to the titers obtained for the PRNT and the micro-PRNT.

4. Discussion

The classic PRNT method (or its variations) is extensively used in studies evaluating the immunogenicity of vaccines for YF (Tavares da Silva Fernandes et al., 2021; Camacho, 2019; Martins et al., 2013; Pereira et al., 2015; Wieten et al., 2016; Tottey et al., 2018; Reis et al., 2022), considered the reference for this purpose and for the serological differentiation of infections by Flavivirus. However, there is a lack of uniformity in the methodology used between the different vaccine research and development laboratories (Roehrig et al., 2008). Methodological differences, such as cell lines, dilution factor, semi-solid medium composition, techniques of visualization and reading of plaques/foci, percentage of virus reduction (endpoint), titer calculation, among others, directly impact in the test sensitivity (reflected by the obtained GMT). Consequently, methodological differences are obstacles in the comparison of antibody titers between laboratories or clinical studies, but also to define a value as the true serological correlate of protection (Roehrig et al., 2008).

Successful optimization, standardization/development and validation of a neutralization test are key factors for providing accurate and reliable data, crucial in epidemiological and vaccine studies and to guarantee the reproducibility of the test over long periods (Rasulova et al., 2022; Timiryasova et al., 2013). Although the classic PRNT method has not been validated by most laboratories, the test has been in use for a long time and is widely accepted as a reference standard (Rainwater-Lovett et al., 2012). Therefore, the importance of also determining the agreement between the results obtained by the new methodological approach proposed in this study with those generated by PRNT.

In the past, during the standardization of the micro-PRNT method, a senior virologist with long experience with YF assays reported that the better PFU profile of strain 17D-213/77 compared to strain 17DD, observed during the standardization of μFRN-HRP, was also viewed (Dr. Marcos Freire, personal communication). Although the vaccine administered in Brazil is produced with the 17DD strain and, therefore, clinical studies include participants vaccinated with this strain, it does not substantially affect the data obtained from neutralization tests with the 17D-213/77 vaccine strain. There is a close antigenic relationship between these strains, where both stimulate protection against all genotypes of the YFV (Ferguson et al., 2010).

Quantity of challenge virus and number of replicates (serum or virus) are essential to obtain accurate measures. The amount of virus applied to the test must be modified according to the surface area of the cell monolayer to obtain discernible plaques, minimizing their excess and overlapping (Roehrig et al., 2008). Qualitative (visual) analysis of the different virus dilutions was essential in determining the quantity of FFU/well for the μFRN-HRP test applied to microplate. Previous experience of the GlaxoSmithKline (GSK) team in standardizing the method for dengue virus serotypes indicated the ideal average amount around 75 FFU/well in virus control (personal communication). This amount was also suitable for YF FFU and served as a basis for the standardization assays of the test and the determination of the ideal virus dilution.

Data obtained from all the assays performed ranged from 40 to 120 FFU/well. Validation assays of the μFRN-HRP suggested the success of this range as all predefined criteria for validation were achieved from assays that presented this FFU/well range in virus controls. However, further assays are needed to confirm acceptance range for the amount of FFU/well in the μFRN-HRP virus control, including processing sera with different levels of neutralizing antibodies.

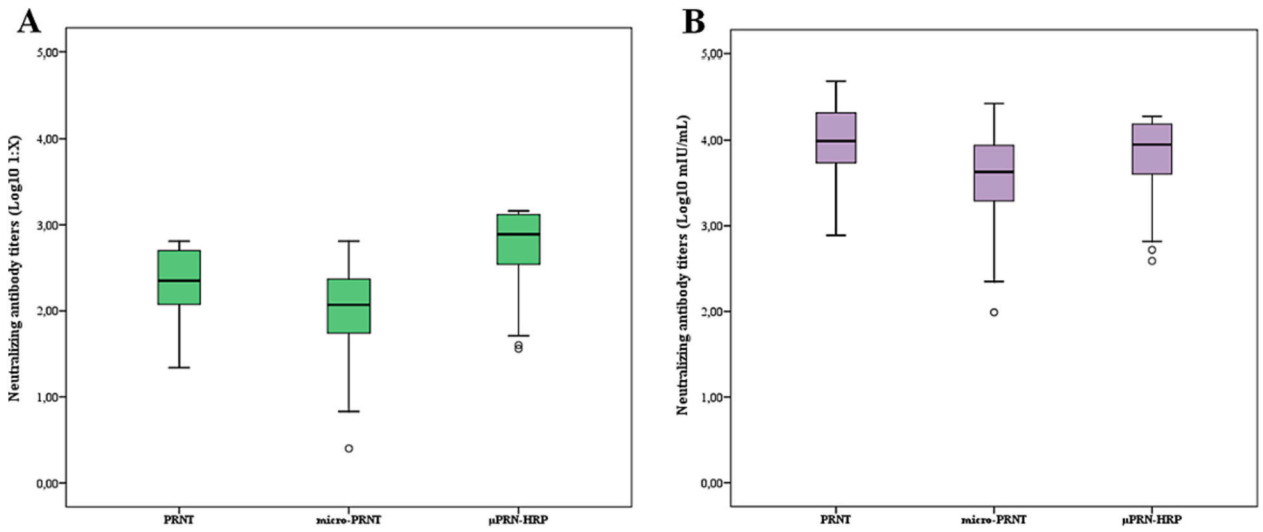

Fig. 4. Descriptive statistics of the 200 sera processed by the three neutralization tests.
X-axis: neutralization tests - PRNT, micro-PRNT and μFRN-HRP. Y-axis: neutralizing antibody titers in (A) reciprocal dilution and (B) in mIU/mL.

Quality of the cell monolayer is critical for development of the plaques/foci and, therefore, for generating accurate results (Roehrig et al., 2008). Production of a new Vero cell bank was fundamental for the success of the μFRN-HRP standardization. Considerable improvement of the cell monolayer was evidenced by the visualization of more homogeneous profile and reduction of grumes.

Standardization of the μFRN-HRP was carried out successfully, with obtainable feasible method and adapted to the automation of YF FFU counting by the Axiovision software.

From the definition of the execution protocol, planning of the validation assays was initially guided by the rules established by current ANVISA's Resolution (Ministério da Saúde, 2012). This provides for the minimum requirements for the validation of bioanalytical methods. However, the analysis criteria established in the Resolution are, in general, inconsistent with the characteristics of the PRNT and its methodological variations. Aiming to carry out a scientific and regulatory validation, the validation plan proposed in this study was drawn from adaptations of the current Brazilian Resolution, based on the work of other groups that also proposed the standardization, optimization and validation of new methods for the quantification of neutralizing antibodies in clinical trials to evaluate the vaccine response (Putnak et al., 2008; Sarzotti-Kelsoe et al., 2014; Timiryasova et al., 2013). Most of those works were carried out in line with the recommendations of the United States Regulatory Agency (Food and Drug Administration - FDA) for the validation of bioanalytical methods (*Bioanalytical Method Validation Guidance for Industry*, 2018). All predefined analysis criteria for the validation assays were achieved by the method proposed in this study.

A new generation of faster tests and with some degree of automation is being developed for the quantification of neutralizing antibodies not only for yellow fever, but also for other viruses of public health importance (Whiteman et al., 2018; Mercier-Delarue et al., 2017; Shambaugh et al., 2017; Masci et al., 2019; Pearson et al., 2020). However, the PRNT remains a reference, against which these new tests must be validated (Roehrig et al., 2008; Whiteman et al., 2018).

The comparative evaluation between the different neutralization tests, using PRNT as reference, showed the impact of methodological differences. The μFRN-HRP showed very low ICC when the titers were compared in reciprocal dilution. However, this low agreement with the reference test was overcome to an almost complete agreement when comparison was made with the results in mIU/mL. The weak agreement between these methods can be explained by the use of immunostaining, but also the use of different dilution factors (factor 3 for μFRN-HRP and factor 2 for PRNT). Comparative evaluation of the micro-PRNT with the PRNT resulted in a satisfactory correlation, even for the titers in reciprocal dilution, justified by methodological similarities, such as the use of the same dilution factor of the sera.

Selection of 200 sera to perform this work was carried out in a blind and random manner, disregarding the antibody titers for YF determined at the time of the study of sera origin. Although the study reached only a population already vaccinated for YF, it was expected to obtain a wide variation in the levels of neutralizing antibodies for the sub-sample used in the present study. Consultation of the data generated by the micro-PRNT for the original study (only after the processing of all sera by the three neutralization tests in the present study) showed the broad spectrum of antibody titers expected for the sub-sample. The median obtained was 1:195 (or 2.29 Log10 of the reciprocal dilution). Of the 200 sera, 27 (13.5%) resulted in titers <1:50; 56 (28%) with titers between 1:50 and 1:149; 74 (37%) with titers between 1:150 and 1:449; and 43 (21.5%) with titers between 1:450 and 1:640. This percentage distribution of the different levels of neutralizing antibodies, as well as the median, were very similar to those observed in the population of the original study (total of 434 samples) and in the reference (PRNT) and micro-PRNT tests in the present study. On the other hand, μFRN-HRP showed considerably higher antibody titers in reciprocal dilution. >60% of the 200 sera had titers >1:450 and no serum titer was lower than the first dilution of this test (1:6). The median observed was 3.5 and 6.7 times greater than the medians obtained for the reference test and micro-PRNT, respectively. However, the transformation of the titers in mIU/mL resulted in a median similar to the medians obtained for the other methods covered in this work, with differences <3 times.

These data showed the importance of turning the titers into reciprocal dilution – considering the endpoint determined for the test and the dilution factor used for each method – in international units (IU), from the calibration of the internal standard serum to the International Reference preparation for anti-yellow fever serum (NIBSC "YF"). This, with a nominal value of 143,000 mIU/mL, was used for the calibration tests of the positive and negative internal control sera (P3 and P1/100, respectively), in which the nominal value of the positive internal standard was determined for each method.

Processing of known negative sera with neutralizing antibody titers <1:20 (first dilution) obtained by PRNT would have allowed determination of the best cut-off point for the μFRN-HRP; that is, the one that provides, at the same time, the best sensitivity, and the best specificity by comparing titers obtained by the reference test and the index tests. Therefore, the selection of a new sera sub-sample with a broad spectrum of immune response, including those without antibodies for YF, will be essential for the definition of cutoff points and the classification of "seropositive" and "seronegative" (dichotomous analysis). Currently, our group uses the cutoff point of 1:50 for the results obtained by micro-PRNT determined in a previous study (Simões et al., 2012) to assess vaccine response. Data observed in this study suggest the need to establish a more restrictive cut-off point (dilution/higher titer corresponding to a 50% reduction in the virus) for μFRN-HRP, giving in sensitivity to achieve greater specificity.

Although there is no consensus on the best percentage for the reduction of plaques between laboratories, the 50% endpoint is preferred for evaluation of sera after vaccination because it provides more accurate results, presenting acceptable sensitivity and specificity. Greater reduction in the plaques count ($\geq$ 80%) is useful for epidemiological studies or for diagnostic purposes as it prevents or reduces the cross-reaction between Flavivirus in endemic areas (Roehrig et al., 2008; Timiryasova et al., 2013).

The methods used in the present study to determine YF neutralizing antibodies were established with a 50% endpoint. LATEV, as a reference for carrying out neutralization tests, collaborates in clinical and pre-clinical studies to assess the immune response induced by 17D vaccines and other vaccines that are being developed at Fiocruz for other flaviviruses. Therefore, the importance of having a validated test capable of efficiently meeting the demand for clinical and pre-clinical studies. However, it does not exclude the use of these methods in differential diagnosis of infections by other flaviviruses, considering that neutralization tests are the most sensitive and specific for YF.

The μFRN-HRP has the same readout mode of FRNT technique which is widely described and used for the quantification of neutralizing antibodies to YF and other viruses (Jirakanjanakit et al., 1997; Vanderheiden et al., 2020; Scheck et al., 2022). All of them have in common the type of revelation, the immunostaining. However, the μFRN-HRP presents advantages over these known FRNTs, especially in the steps of revelation, as we can mention: use of a commercial permeabilization and blocking buffer (meaning one less possible variable in the test) and that is used to dilute the antibody (so in a single step the permeabilization/blocking and antibody labeling is done), as well as the use of a monoclonal antibody already conjugated to the peroxidase (eliminating one more step in the process of developing the test, which would be the timing of the secondary antibody). Added to these peculiarities, we still must highlight the exceptional and automated way of photographing and counting the plates by the Scanlab II and the Axiovision software, respectively, a quality system that saves a lot of time in the process of registering and counting the plates.

Looking at the WHO global strategy launched in 2017 to eliminate YF epidemics by 2026 through immunization with fractional dose of the

17D vaccine (World Health Organization, 2017), we can prospect a large demand for population-based clinical studies, which means the need for processing a huge number of serum samples. The μFRN-HRP could replace the reference test in this purpose, not only because of its high throughput, but especially because it determines YF neutralizing antibody titers faster and efficiently.

5. Conclusions

The μFRN-HRP for YFV meets the growing demand for clinical studies to evaluate the vaccine immune response, justified by being a faster, semi-automated technique with high throughput of samples and high performance in determining neutralizing antibody titers.

Data availability

I have shared the link to my data/code at the Attach File step. https://arcadados.fiocruz.br/dataset.xhtml?persistentId=doi:10.35078/MH1LUL

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2023.113568.

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