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Safety and immunogenicity of 17DD attenuated yellow fever vaccine in howler monkeys (*Alouatta* spp.)

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Abstract

Background: *Alouatta* spp. are highly susceptible to yellow fever (YF) infection and develop an often fatal disease. The threat posed by an outbreak started in 2016 leads us to investigate vaccination as a potential tool in preventing YF in non-human primates (NHP).

Methods: Susceptible howler monkeys were immunized with three different concentrations of the human Brazilian commercial YF17DD vaccine. Post-vaccination viremia/RNAemia, immunogenicity, and safety were characterized.

Results: The vaccine did not produce YF clinical manifestations in any of the NHPs. After immunization, all animals seroconverted demonstrating the ability of the YF vaccine to induce humoral response in *Alouatta* species.

Conclusions: The present work has demonstrated the safe and immunogenic profile of the existing YF 17DD vaccine in howler monkeys. This knowledge may support further studies with other susceptible monkey species and provide a possible solution for controlling epizootics and preventing the devastation of endangered species.

KEYWORDS

Alouatta, Atelidae, epizootic, howler monkey, monkey immunization, sentinel monkey, vaccine efficacy, virus, yellow fever

1 | INTRODUCTION

Yellow fever (YF) is a viral hemorrhagic fever transmitted by yellow fever virus (YFV) and is considered the most severe mosquito-borne infection ever to circulate in the Americas, with a case fatality rate from 20% to 60%.[1]

The virus is maintained in nature mainly by an enzootic cycle between mosquito vectors such as *Haemagogus* spp. And *Sabethes* spp. and non-human primates (NHPs) with humans cases occurring incidentally in forested areas.[1-3] However, the transmission can also occur through an urban cycle involving *Aedes aegypti* mosquitoes and humans.[2]

The last Brazilian outbreak started in November 2016, and two waves of transmission were recognized: one during the seasonal period of 2016-2017 with 778 human confirmed cases, 262 deaths, and 1655 non-human primate cases, and another during the 2017-2018 period with 1376 confirmed human cases, 483 deaths, and 865 non-human primate cases. In 2018-2019 seasonal period (July 2018 to March 2019), 75 confirmed human cases, 17 deaths, and 33 non-human primate cases were reported.[4-6] During the monitoring period started in July 2019, 46 non-human primate cases were confirmed, and 320 are still under investigation; 2 human cases were confirmed, and 72 are under investigation.[7]

Viral spread has reached areas not initially included in the YF vaccination program and was facilitated by the low vaccination coverage of potential risk areas. The rapid spatial dissemination of the virus to the southeastern and southern regions of the country, in fragments of the Atlantic Forest close to populous peri-urban areas of megacities such as São Paulo and Rio de Janeiro, has led to an exponential increase in the number of cases of YF during this last epidemic.[8] Although no YF urban transmission has been documented since 1942 in Brazil, the occurrence of outbreaks in the states of the southeast region close to urban areas as well as the detection of YFV in *Aedes* spp. mosquitoes raises concern on the potential risk of reestablishment of urban cycle transmission in the Americas.[4,9]

In Brazil, the surveillance of epizootics in wild NHPs started in 1999 and became compulsory in 2006.[10-12] The observation of epizootics is an important tool in the detection of YFV circulation and allows rapid preventive strategies as vaccination of human susceptible population in surrounding areas.[10] On the other hand, the immunization of NHPs could reduce virus circulation in risk areas and, therefore, the chances of human infection. This strategy could also contribute to the preservation of endangered monkey populations.[13]

African NHP species are more resistant to YFV and rarely develop fatal disease, while neotropical species are more susceptible and vulnerable to YF, probably for having evolved in the absence of the virus.[14,15] Although among New World monkey species the virulence is variable across taxa, howler monkeys (*Alouatta* sp.) have been associated with severe and usually fatal disease.[13,14] In Brazil, previous studies have demonstrated the circulation of YFV in five neotropical NHP families: Aotidae, Atelidae, Callitrichidae, Pitheciidae, and Cebidae. However, in recent studies howler monkeys and marmosets have been recognized as the most frequently infected NHPs..[9,13,16]

Alouatta is a genus of NHP belonging to the family Atelidae, subfamily Alouattinae, popularly known as howler monkey. Howler monkeys are folivorous-frugivorous, arboreal primates that generally do not come to ground to feed and live in the forest canopy.[17] The distribution of howler monkeys extends from Southern Veracruz State in Mexico, through Central and South America to northern Argentina and represents the most widely distributed New World primate genus. In Brazil, they inhabit the ecoregions of Chaco, Pantanal, Cerrado, and the Atlantic and Amazon forests.[18,19] Due to the fact that *Alouatta* spp. are highly susceptible to YFV and are present in the largest YF endemic area of the country, these monkeys are the ideal host target for monitoring the disease in Brazil.[20-22]

YF vaccine was developed in the 1930s by Max Theiler and colleagues and has been successfully used for more than 80 years to control YF disease. It has proved to be safe and to provide lifelong immunity. The vaccine is produced in specific pathogen-free embryonated chicken eggs using a seed lot system implemented in the early 1940s that had no significant changes, hitherto.[23] The commercial YF vaccine produced in Brazil by Fiocruz uses the 17DD strain while other manufacturers use the 17D strain with no significant variation in safety and immunogenicity.[23] Every year, 20-60 million doses of YF vaccine are distributed all over the world, but despite the availability of the YF vaccine for humans, thousands of cases of YF still occur every year, in travelers to and residents of endemic areas.[3]

The main objective of this study is to test the safety and immunogenicity of 17DD YF attenuated virus in monkeys of *Alouatta* species. Our encouraging results can provide important insights on the use of the human YF vaccine for immunization of NHPs living in parks, sanctuaries, natural reserves areas, and zoos, contributing to the preservation of endangered NHP species and biodiversity.

2 | MATERIAL AND METHODS

2.1 | Animal ethics and guidelines

The study protocol (number 470/2018) was approved by the Institutional Ethical Committee for Use of Animals (CEUA-UNIFESO), and an environmental license (ICMBio-SISBIO number 60511-2/2018) was provided by Instituto Chico Mendes de Conservação da Biodiversidade from the Brazilian Ministry of Environment. The study followed the guidelines from the National Council for the Control of Animal Experimentation and from the International Primatology Society and complied with all applicable Brazilian laws.[24,25]

2.2 | Vaccine

YF17DD live attenuated commercial vaccine, batch 167VFA022Z produced in Bio-Manguinhos/Fiocruz/Brazil, was used for animal

immunization. To calculate monkey vaccine doses, virus titers determination was performed by plaque assay in Vero cells as described previously.[26]

2.3 | Animals and experimental procedures

Ten flavivirus-naïve male and female adult howler monkeys from different *Alouatta* species (*Alouatta guariba clamitans*, *Alouatta caraya* and *Alouatta discolor*) kept in captivity in the Centro de Primatologia do Rio de Janeiro (CPRJ)/Instituto Estadual do Ambiente (INEA), Rio de Janeiro, Brazil, were used in this study. The animals are housed in outdoors enclosures of 9 m^2, built of masonry, closed with wire mesh on two sides, and partially covered with a roof top to allow sunlight incidence. All enclosures have a resting platform, a floor covering made of natural substrate (earth and dry leaves) and wood trunks and branches forming natural paths that allow movement throughout the space. Monkeys receive twice a day a commercial diet (Nuvilab Primates, Quimtia) supplemented with fresh fruits, vegetables, and greens. During this study, all animals remained in pairs, except for monkeys number 3636, 3622, and 3723 that were housed individually. Public visitation is not permitted.

The animals were randomly allocated into three groups as follows: Gr1 = 3, Gr2 = 4 and Gr3 = 3 (Table 1). Prior to vaccination or blood drawing, monkeys were anesthetized with ketamine 10% (10 mg/kg, maximum volume injected 0.85 mL, Dopalen—Ceva Saúde Animal Ltda.) and midazolam 5 mg/mL (1 mg/kg, maximum volume injected 1.7 mL, Dormonid—Roche Farmacêutica) by intramuscular injection into the quadriceps muscle, in the upper thigh. For vaccination, monkeys were inoculated by subcutaneous (s.c.) route in the hypogastric region with a single dose of YF 17DD vaccine (0.5 mL) containing either 2.7 $\log_{10}$ plaque-forming units (PFU)—low dose (Gr1), 3.0 $\log_{10}$ PFU—intermediate dose (Gr2), or 3.7 $\log_{10}$ PFU—high dose (Gr3). There was an interval of 15 days between the immunization of each group, beginning with the lowest dose for the determination of safety and to endorse the immunization of the next group. During the 15 days periods, animals were observed for assessment of characteristic clinical signs produced by YF virus infection such as fever, vomiting, prostration, dehydration, myalgia, hemorrhagic, and neurological manifestations. No slight suspected YF symptoms were observed in any of the ten monkeys. Blood samples were collected by venipuncture of the right and left femoral veins, before immunization, at days 2, 4, 7, 14, 30, 60, and over one year after immunization (Table 2). The monkeys were daily observed by veterinarians and animal care staff for well-being assessment. Before the end of the study, monkey 3230 from Gr2 escaped the cage and had the last blood sample drawn on day 60.

2.4 | Viremia

Viremia was assessed on fresh serum samples corresponding to pre-immunization and days 2, 4, and 7 after vaccination. Non-diluted and serial dilutions (1:3, 1:30) of each monkey serum were added to Vero cell monolayers previously seeded into six-well plates and incubated for 1h at 37°C. The serum samples were then replaced by maintenance medium supplemented with 2% carboxy methyl cellulose (CMC). After 7 days at 37°C in 5% CO_2, cells were fixed

TABLE 1 Non-human primate distribution by group

Groups	NHP	Species	Sex	Age[a]	Weight (kg)
G1	2443	*Alouatta guariba clamitans*	Male	Adult (10 Y)	8
	2799	*Alouatta guariba clamitans*	Female	Adult (7 Y)	3
	3636	*Alouatta guariba clamitans*	Male	Adult (4 Y 8 M)	6
G2	3230	*Alouatta guariba clamitans*	Male	Adult (4 Y 6 M)	5, 8
	3234	*Alouatta guariba clamitans*	Male	Adult (4 Y 6 M)	7
	3556	*Alouatta guariba clamitans*	Male	Adult (4 Y)	5, 1
	3622	*Alouatta guariba clamitans*	Female	Adult (4 Y 8 M)	3,1
G3	3723	*Alouatta guariba clamitans*	Female	Adult (3 Y 8 M)	2, 6
	2576	*Alouatta caraya*	Male	Adult (8 Y 7 M)	8, 5
	3273	*Alouatta discolor*	Female	Adult (5 Y)	5, 5

[a]Age in brackets refers to time in captivity. At the time of the study, all animals were adults but due to their free-living origin, the date of birth is unknown.

TABLE 2 Blood collection for experimental procedures

Experimental procedure				
	Viremia	**RNAemia**	**Humoral immunity**	**Cell immunity**
Analytical method	Immunofocus	qPCR	$PRNT_{50}$, ELISA	ELISPOT
Days of collection	0, 2, 4, 7	0, 2, 4, 7	0, 30, 60, >365	0, 7, 14, >365
Analytical material	Serum			PBMC[a]
Blood volume	3 mL			4 mL
Tube type	Clot activator			heparin

[a]Peripheral blood mononuclear cells.

with a 5% formaldehyde solution followed by YF virus focus detection using a horseradish peroxidase HRP-conjugated YF monoclonal antibody (in-house production) and incubation with TrueBlue (KPL – Kirkegaard & Perry Laboratories). YF focus were counted with the naked eye, and infectious virus titers were expressed as focus-forming units (FFU/mL).

2.5 | RNAemia

RNAemia was quantified by quantitative real-time polymerase chain reaction (RT-qPCR). Viral RNA was extracted from 140 µL of monkey serum samples corresponding to pre-immunization and days 2, 4, and 7 after vaccination, using the commercial kit QIAamp Viral RNA Mini Kit (QIAGEN, GmbH) according to the manufacturer's instructions. The positive strands were reverse-transcribed using High-Capacity cDNA Reverse Transcription Commercial Kit. The cDNAs synthesized were amplified and quantified by real-time PCR for NS5 region of the YFV genome. For each PCR run, a master mix was prepared with TaqMan Universal PCR Master Mix (Applied Biosystems) primers and probe (300 nM each primer, forward—5′ GCA CGG ATG TAA CAG ACT GAA GA 3′ and reverse—5′ CCA GGC CGA ACC TGT CAT 3′, 150 nM probe 5′ FAM-CGA CTG TGT GGT CCG GCC CAT C—measured by fluorometric analysis and compared to the clinical samples as previously described.[27] The assay was performed in duplicate, and final results were achieved taking into account the sample dilution factor and the lower limit of detection (LOD = 50 copies/rxn, 3.63 $\log_{10}$ genome copies/mL). Results below the LOD were considered negative, and results above the LOD were considered positive.

2.6 | Cells

Vero cells (ATCC, CCL 81—American Type Culture Collection, Gaithersburg, MD, USA) were grown at 37°C in a humidified 5% CO_2 incubator in Medium 199 with Earle's salts (E199), buffered with sodium bicarbonate, and supplemented with 5% fetal calf serum and antibiotics.

2.7 | Micro-plaque reduction neutralization test (micro-PRNT)

In order to assess YF neutralizing antibody levels, monkey sera corresponding to pre-immunization and 30, 60, and over 365 days after immunization were run in micro-$PRNT_{50}$ (96-well plates) as described previously.[28] Briefly, 30 YFV plaque-forming units (PFU) (in 50 µL) were mixed with equal volumes of serially diluted monkey sera for 1 hour at 37°C. The mixtures received a Vero cells suspension and after 3 hours at 37°C in 5% CO_2 atmosphere, replaced by maintenance medium supplemented with 3% CMC. Six days later, cells were fixed with a 5% formaldehyde solution followed by staining with crystal violet. PFU were counted on BioSpot (CTL—Cellular Technology Limited) and the PRNT titer was defined as the reciprocal of the serum dilution that reduced the number of plaques by 50% relative to the PFU counted with the virus control. Titers above 1:5 were considered positive. Statistical analysis was performed using Kruskal-Wallis with Student's *t* test (GraphPad Prism version 7.0—GraphPad Software). Differences were considered significant when *P* values were less than 0.05 ($P < .05$).

2.8 | ELISA

Microtiter plates (Maxisorp NUNC—44 204, Thermo Fisher Scientific) were coated with 0.125 µg/well of YFV in carbonate-bicarbonate buffer and incubated overnight at 4°C. Each well was washed with PBS-Tween and then blocked for 1 hour at 37°C with PBS-Tween-BSA-FBS milk (BDS). After a washing step, serial 4-fold dilutions starting at 1:20 of monkey serum samples corresponding to pre-immunization and 30, 60, and over 365 days after immunization were added to each well and incubated for 1 hour at room temperature (RT). Serial 2-fold dilutions ranging from 0.015 to 1 IU/mL of Monkey Anti-Yellow Fever Reference Serum (NIBSC—The National Institute for Biological Standards and Control, Potters Bar, UK) were used to compose the standard curve. The plate was again washed before the addition of Anti-Monkey IgG conjugated with HRP (A2054—Sigma-Aldrich) diluted 1:5000 in BDS and incubated for 1 hour at RT. The plates were finally washed before the

addition of TMB plus (Kementec Solutions/Bio-Connect Diagnosis BV) for 30 minutes in the dark, at RT. The colorimetric reaction was stopped with H_2SO_4 2 M, and optical densities (OD) measured at 450 nm in a plate reader VersaMax (Molecular Devices). The ODs of the sera dilutions were plotted on the standard curve, and the antibody titers were calculated by a four parameter logistic (4PL) regression using the software SoftMax Pro (Molecular Devices) and expressed as IU/mL relative to the reference monkey antiserum. Statistical analysis was performed using Kruskal-Wallis with Student's *t* test (GraphPad Prism version 7.0—GraphPad Software). Differences were considered significant when *P* values were <0.05 ($P < .05$).

2.9 | IFN-γ ELISPOT assay

The frequency of interferon gamma (-IFN-γ) secreting cells in peripheral blood mononuclear cells (PBMCs) from *Alouatta* sp in groups vaccinated with 2.7 $\log_{10}$ PFU, 3.0 $\log_{10}$ PFU or 3.7 $\log_{10}$ PFU was analyzed using the ELISPOT assay as described elsewhere.[29] PBMCs were obtained using the Histopaque density gradient (Sigma-Aldrich) according to the manufacturer's suggestions and resuspended in supplemented Roswell Park Memorial Institute (RPMI) 1640 medium after the lysis of residual red blood cells using ACK solution (Invitrogen/Thermo Scientific). The cell suspensions were plated (2×10^5 cells/well) into pre-coated IFN-γ ELISPOT plates (Mabtech AB) according to the manufacturer's protocol and cultured for 20 hours in the presence or absence of 20 μg of YFV envelop protein (YFE). As positive control, cells were incubated with 2 μg/well of Concanavalin A (Sigma). After incubation, cells were washed and incubated with a biotinylated anti-IFN-γ antibody for 2 hours at RT followed by incubation with alkaline phosphatase-conjugated streptavidin for 1 hour at RT. The spots of IFN-γ-secreting cells were visualized using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate and counted using the ImmunoSpot image analyzer (CTL - Cellular Technology Limited). After the subtraction of background, results were compared using Kruskal-Wallis with Dunns post hoc test (GraphPad Prism version 7.0—GraphPad Software). Differences were considered significant when *P* values were <.05 ($P < .05$). The cutoff value was determined as the mean spot number from all non-stimulated cells plus the standard deviation (26.7 Spot Forming Cells (SFC)/10^6 PBMC).

3 | RESULTS

3.1 | Post-vaccination viremia and RNAemia

Viremia and RNAemia days to detection, duration, and peaks are shown on Figure 1 and Table 3. Immunization of YF 17DD vaccine at low dose (G1) resulted in detectable viremia and RNAemia in only 1 out of 3 NHPs, both on the 4th and 7th days. With the intermediate dose (G2), 3 out of 4 NHPs had detectable viremia and RNAemia. One animal from G2 (NHP 3556) did not show detectable viremia or RNAemia in any day post-vaccination. This animal was revaccinated with the same dose on day 263. Viremia was present in all animals immunized with high dose (G3), mainly on the 4th day, and RNAemia was detectable both on the 4th and 7th days post-immunization. Viremia and RNAemia were detected in 70% of vaccinated animals and ranged from 0.87 to 2.88 $\log_{10}$ FFU/mL and from 4.51 to 7.01 $\log_{10}$ copies/mL, respectively. Although viremia and RNAemia were analyzed only in the 2nd, 4th, and 7th days post-infection, the peak for both parameters was clearly between the 4th and 7th days.

3.2 | Immunogenicity of YF 17DD vaccine

To characterize the elicited YFV 17DD antibody responses, sera collected before immunization (day 0) and at 30, 60, and over 365 days after immunization were tested for neutralizing antibodies (YF-nAb) by micro-$PRNT_{50}$ (Figure 2A) and for total IgG antibodies by ELISA (Figure 2B) (Table 4).

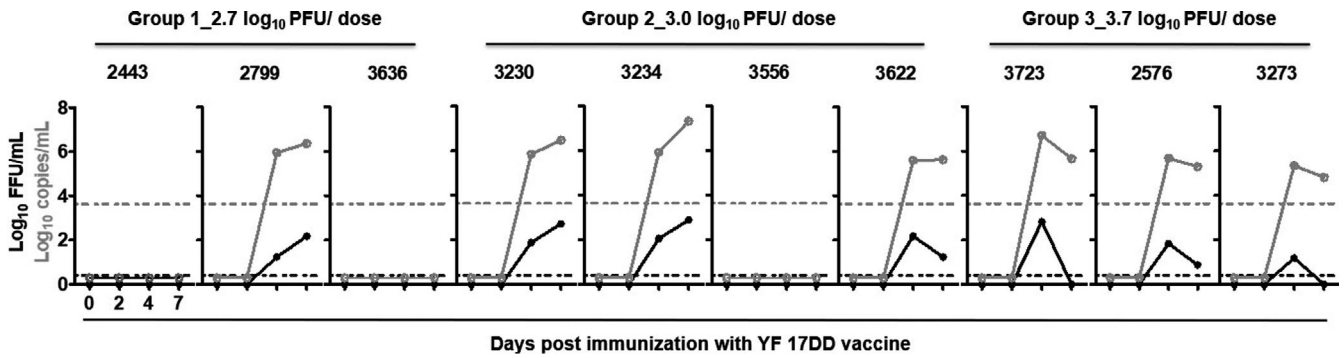

FIGURE 1 Viremia and RNAemia detected after vaccination with YF vaccine.*Alouatta*spp. monkeys were subcutaneously inoculated with a single dose of YF 17DD vaccine (0.5 mL) containing either 2.7 $\log_{10}$PFU—low dose (Gr1), 3.0 $\log_{10}$PFU—intermediate dose (Gr2), or 3.7 $\log_{10}$PFU—high dose (Gr3). Sera were collected previous to vaccination (d 0) and on days 2, 4, and 7 post-immunization and tested by focus assay for their infectious content, that is, viremia, expressed as $\log_{10}$(FFU)/mL and by real-time RT-PCR for their YF genome copies content, RNAemia, expressed as $\log_{10}$genome copies/mL. Shown are the individual viremia and RNAemia. Horizontal dashed lines indicate the threshold of detection for the focus assay and the real-time RT-PCR assay, that is, 0.40 $\log_{10}$FFU/mL and 3.63 $\log_{10}$genome copies/mL

TABLE 3 Viremia and RNAemia in non-human primates following vaccination with a single dose of YF 17DD vaccine

Groups	Vaccine dose (log_{10} PFU)	NHP	Viremia (log_{10} FFU/mL)				RNAemia (log_{10} copies/mL)			
			Pre-immune	2 dpi	4 dpi	7 dpi	Pre-immune	2 dpi	4 dpi	7 dpi
G1	2.70	2443	-	-	-	-	-	-	-	-
		2799	-	-	1.24	2.18	-	-	5.65	6.06
		3636	-	-	-	-	-	-	-	-
G2	3.00	3230	-	-	1.88	2.72	-	-	5.54	6.17
		3234	-	-	2.05	2.88	-	-	5.61	7.01
		3556	-	-	-	-	-	-	-	-
		3622	-	-	2.18	1.24	-	-	5.30	5.33
G3	3.70	3723	-	-	2.83	-	-	-	6.40	5.36
		2576	-	-	1.83	0.87	-	-	5.37	5.00
		3273	-	-	1.18	-	-	-	5.04	4.51

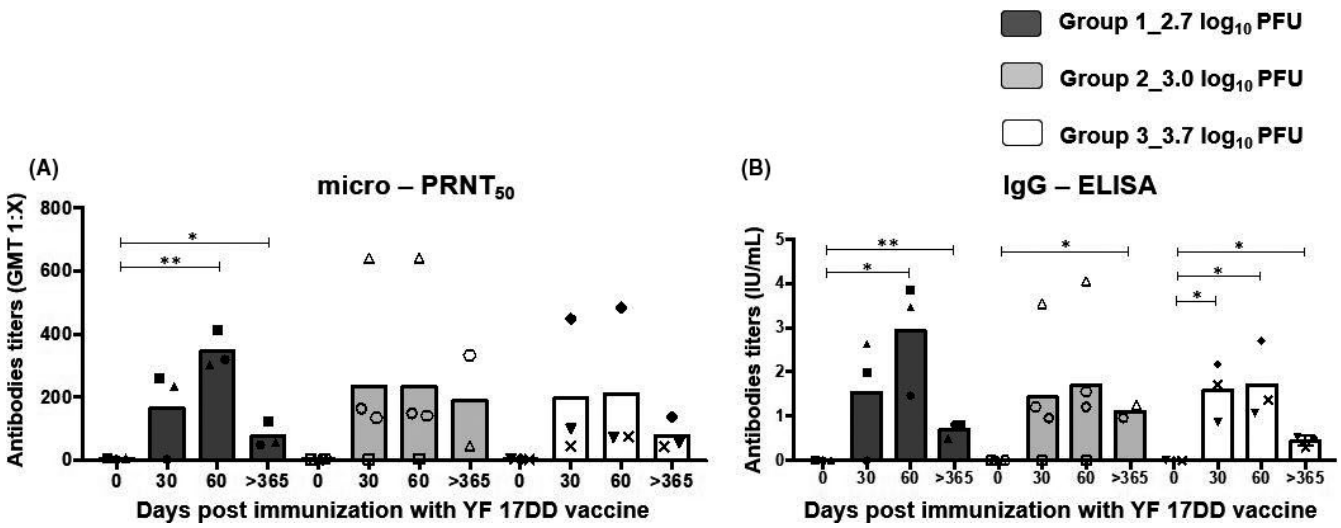

FIGURE 2 Antibody responses detected after vaccination of*Alouatta*spp. monkeys with three different doses of YFV 17DD vaccine. Sera collected before vaccination (day 0) and at 30, 60, and over 365 days post-immunization were tested for their neutralizing activity and for their total antibody content by ELISA against YFV. (A) Neutralizing antibodies shown as geometric mean titers (GMT) of individual serum dilutions associated with 50% reduction in virus plaque control counts and (B) Total IgG antibodies measured by an in-house ELISA where values >0 IU/mL are considered as positive. Differences between days were calculated using Student'sttest ($P < .05$)

All tested vaccine doses elicited YF-nAb, presenting neutralizing antibody titers at 60 days post-immunization (dpi), except for animal 3556 (G2), that did not develop detectable neutralizing antibodies at any time point. At 263 dpi, NHP 3556 received a booster dose and developed YF-nAb that could be detected 14 days after the second dose.

Total IgG antibody levels demonstrated the ability of the three YF vaccine concentrations to induce humoral response in *Alouatta* monkeys, and significant differences between time 0 and >365 dpi were observed for all groups ($P < .05$). In the group of NHPs receiving the highest dose (Gr3), at 30 dpi, the animals showed increased total antibody levels ($P < .05$), suggesting early seroconversion.

3.3 | YF-specific cellular response in NHPs immunized with different doses of YF 17DD vaccine

To evaluate the vaccine-induced cellular responses, frequency of IFN-γ-producing cells upon stimulation with YFE was assessed using the ELISPOT assay. Animals immunized with YF 17DD vaccine at low dose presented high levels of IFN-γ-secreting cells at 7 dpi; however, this response was transient and declined at 14 dpi. On the other hand, the number of IFN-γ-secreting cells in the groups immunized with intermediate or high dose gradually increased, achieving higher levels at 14 dpi. Despite the limited sample size, animals immunized with the high dose presented a statistically significant increase in IFN-γ-secreting cells comparing 0 and 14 dpi ($P < .05$) (Table 5). No significant changes

TABLE 4 Neutralizing and total IgG antibody titers by groups immunized with the YF 17DD vaccine

		Neutralizing antibodies GMT (1:X)				Total IgG antibodies (UI/mL)			
Groups	NHP	Pre-immune	30 dpi	60 dpi	>365 dpi	Pre-immune	30 dpi	60 dpi	>365 dpi
G1	2443	<5.00	<5.00	319.26	48.74	0.00	0.00	1.47	0.82
	2799	6.30	233.83	302.07	57.00	0.00	2.64	3.47	0.51
	3636	6.10	258.61	414.37	122.00	0.00	1.99	3.86	0.80
G2	3230	<5.00	163.69	148.86	NC[a]	0.00	0.96	1.21	NC[a]
	3234	<5.00	640.00	640.00	45.00	0.00	3.55	4.05	1.25
	3556	<5.00	<5.00	<5.00	NA[b]	0.00	0.00	0.00	NA[b]
	3622	<5.00	134.55	140.78	333.00	0.00	1.21	1.56	0.97
G3	3723	<5.00	448.05	484.02	137.00	0.00	2.18	2.70	0.51
	2576	<5.00	44.43	74.95	43.00	0.00	1.72	1.37	0.31
	3273	<5.00	98.93	71.38	55.00	0.00	0.86	1.07	0.53

Abbreviation: dpb, days post-booster; GMT, geometric mean titer; NA, not applicable; NC, not collected.

[a]NHP 3230 had the last blood sample drawn on day 60.

[b]This sample was not collected at this time. NHP 3556 did not develop detectable neutralizing and total IgG antibodies at any time point (30 and 60 dpi) after immunization. At 263 dpi, this animal received a booster dose and developed YF-nAb and IgG antibodies that were detected at 14 dpb (640.00/0.29, respectively), 34 dpb (640.00/3.08), and 242 dpb (311.00/3.22).

TABLE 5 Frequency of IFN-γ-secreting cells upon stimulation with YFE by ELISPOT assay by groups immunized with the YF 17DD vaccine. Data are represented as mean ± SE

		IFN-γ ELISPOT (SFC/10^6 PBMC)			
Groups	Vaccine dose (log_{10} PFU)	Pre-immune	7 dpi	14 dpi	>365 dpi
G1	2.70	17.93 ± 14.89	96.27 ± 52.07	3.33 ± 3.33	10.33 ± 10.33
G2	3.00	11.90 ± 2.81	17.20 ± 8.05	29.04 ± 10.95	5.16 ± 2.62
G3	3.70	3.33 ± 3.33	10.87 ± 1.83	37.93 ± 18.45	34.83 ± 34.83

were found between baseline frequency of IFN-γ-secreting cells and cells collected after one year of immunization.

3.4 | Correlation between humoral and cellular responses in NHPs immunized with different doses of 17DD vaccine

The correlation between humoral and cellular responses after vaccination was evaluated comparing results of neutralizing antibodies with total IgG antibodies, neutralizing antibodies with ELISPOT and total IgG antibodies with ELISPOT (Figure 3). In all groups analyzed, the levels of neutralizing antibodies and total IgG antibodies presented a significant positive correlation. Animals immunized with the intermediate dose presented results that suggest a positive correlation between IFN-γ-secreting cells and total IgG antibodies.

4 | DISCUSSION

The impact of the largest sylvatic YF outbreak in the last 80 years in Brazil with wide epizootics may be devastating for endangered monkey species such as *Alouatta* spp.[8,30] Most experimental studies with YF vaccine were performed with Old World monkeys, and experimentation with New World species was done only using wild YFV.[31] Our study aimed to assess the safety and the immune response of the human YF 17DD vaccine in howler monkeys. While rhesus monkeys were used as the safety and protection model to evaluate vaccine candidates in the development of the attenuated 17D vaccine strain,[32,33] it was the first time that a human YF vaccine was tested in *Alouatta* spp. With the three doses investigated, no adverse reactions nor suspected YF or other untoward clinical signs were observed in the animals during the study period.

The restricted availability of vaccines for YF is a central issue for expanding vaccine coverage in the world population.[34] In this context, fractional dosing, a dose-sparing strategy, was tested with the YF 17DD vaccine in humans and proved the non-inferiority of the response.[35] Here, our results with fractional doses (2.7 log_{10}_low, 3.0 log_{10} intermediate and 3.7 log_{10} high dose), in addition to covering the safety concern of immunization with attenuated vaccine in these sensitive species, can endorse the future use of the vaccine in NHPs, bypassing potential criticism of this approach based on vaccine shortages.

Previous studies of the 17DD vaccine in rhesus monkeys demonstrated low and transient levels of viremia with peaks at 4 dpi and

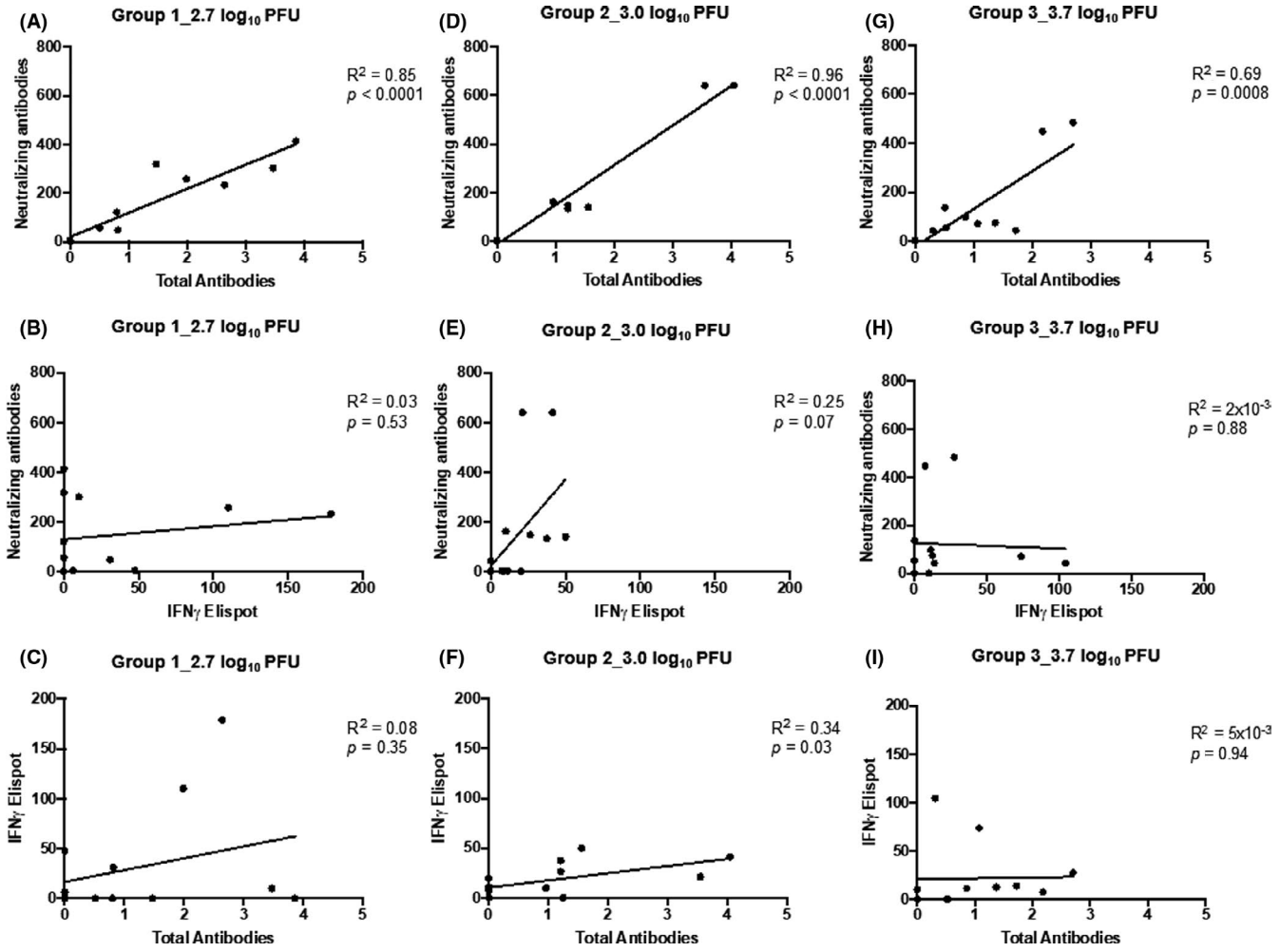

FIGURE 3 Correlation between humoral and cellular responses in*Alouatta*spp. monkeys immunized with YFV 17DD vaccine. Monkeys were subcutaneously inoculated with a single dose of YF 17DD vaccine (0.5 mL) containing either 2.7 $\log_{10}$PFU—low dose (Gr1), 3.0 $\log_{10}$PFU—intermediate dose (Gr2) or 3.7 $\log_{10}$PFU—high dose (Gr3). Sera collected before vaccination and at 30, 60, and over 365 days post-immunization were tested for their total IgG antibody content by ELISA and for their neutralizing activity against YFV by micro-$PRNT_{50}$. Blood samples collected before vaccination and at 7, 14, and over 365 days were tested for the frequency of IFN-γ-secreting cells. Results from all times of collection were correlated using the Spearman test, considering$P < .05$

RNAemia at 6 dpi that is consistent with our findings where viremia and RNAemia peaked between the 4th and 7th days.[23] Furthermore, viremia and RNAemia were observed in 100% animals of G3, in 75% animals of G2 and 33,3% animals of G1, suggesting that the highest dose is more suitable for inducing infection. It is important to point out that one animal from G2 (3556) failed to develop viremia, RNAemia and immune response. In G1, even though two animals did not show detectable viremia and RNAemia, antibody response was elicited by vaccination suggesting that some failure in inoculation or response might have occurred with the monkey from G2.

The evaluation of neutralizing antibody response in rhesus monkeys inoculated with graded doses of the 17D vaccine showed that the vaccine induced a high degree of immunogenicity in those animals.[36] Likewise, the immunization of *Alouatta* spp. monkeys with the human attenuated vaccine elicited the production of neutralizing antibodies against YFV as previously demonstrated by studies with the 17DD strain in rhesus monkeys.[27] Seroconversion at 60 dpi was observed for all animals, except for monkey 3556 that seroconverted after receiving a booster dose. The decrease in neutralizing antibody titers after 365 dpi does not reflect waning of immunity, and a low neutralization titer of 1:10 has been shown to be protective in humans.[37]

The most successful vaccines are part of a combination of humoral response, consisting of persistent and high levels of specific antibody by memory cells, with cellular response.[38] Previous studies have demonstrated that early IFN-γ response is crucial for the magnitude of neutralizing antibodies response in YF vaccination in rhesus monkeys as well as in humans, achieving high production 15 days after vaccination.[38-41] Here, the frequency of IFN-γ secreting cells in pre-immune samples within vaccination groups varied with a high standard deviation and could reflect the heterogeneity and the small number of animals. It is also important to consider that these non-human primates are housed in outdoors enclosures, exposed to mosquito bites in an area where the circulation of flaviviruses as zika and dengue, but not YF, is prevalent. We could speculate that the high pre-immune levels

could be due to cross-reactivity with circulating flaviviruses, although all animals from group 1 were negative by PRNT in screening tests for dengue 1, 2, 3, 4, and zika. In addition, since these animals were all wild-caught chances are they might have been exposed to known or unknown viruses which may have modulated their immune system accounting for the observed variability in number of IFN-γ-secreting cells pre-immunization. It was also observed that immunized *Alouatta* spp. presented an IFN-γ cellular response after YF 17DD vaccination despite the dose. Still, animals immunized with the intermediate and high doses (3.0 $\log_{10}$ PFU and 3.7 $\log_{10}$ PFU, respectively) showed an increasing frequency of IFN-γ secreting cells with peak on 14 dpi, consistent with the expected IFN-γ production kinetic.[41] The observation of this trend, added to the achievement of seroconversion in both groups, suggests that the intermediate and the high dose could be equally suitable candidates for vaccination. However, in order to determine the adequate dose, additional studies involving a statistically significant sample size would be necessary to better characterize the immune response to YF 17DD vaccine in *Alouatta* spp.

In view of the current expansion of the geographical distribution of YF in Brazil, the search for initiatives to protect NHPs is extremely relevant. Nonetheless, vaccinating monkeys is a matter of debate due to their role as sentinels in early detection of virus circulation and to launch control measures such as vaccination in the affected areas. In this regard, a recent epizootics survey has demonstrated YFV transmission in areas previously considered to be free of virus circulation and thus where vaccination was not recommended.[16] On the other hand, vaccination of these animals as a contingency measure for viral circulation in the vicinity of areas with high demographic density as well as the protection and preservation of the species may be much more relevant than their role as sentinels.

The proposal of immunization of NHPs in monitored areas such as parks and forest reserves close to urban areas, research centers and zoos can contribute to disease control not only by avoiding the participation of these animals as virus reservoirs and source of infection for mosquitoes, but also contributing to the preservation of endangered species.

5 | CONCLUSION

In conclusion, our results support the potential use of the YF17DD vaccine for *Alouatta* spp., but additional studies on more distantly related New World monkey species like marmosets or tamarins might be needed to endorse the vaccination of NHPs threatened by YF infection.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

ATSF, SBM, LPG, ECC, RCG, RSM, AP and MSF contributed to concept and design. ATSF, LPG, and MSF contributed to project administration. ATSF, MS, ACRAC, JHRL, VOS, RCP, MPBG, JFA, RTS, SMBL, CBF, LNT, JS, TACB, AMVS, JGM, and APDAB contributed to protocol development, acquisition, and analysis and interpretation of data. ATSF, LPG, PCCN, and MBB contributed to writing—original draft. ATSF, LPG, PCCN, MBB, MS, ACRAC, APDAB, TACB, JGM, SBM, AP, and MSF contributed to writing—critical review and editing. All authors read and approved the final manuscript.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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