Immunogenicity of WHO-17D and Brazilian 17DD yellow fever vaccines: a randomized trial

Imunogenicidade das vacinas contra febre amarela WHO-17D e 17DD: ensaio randomizado

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Keywords
Yellow fever vaccine. Randomized controlled trials.

Abstract

Objective
To compare the immunogenicity of three yellow fever vaccines from WHO-17D and Brazilian 17DD substrains (different seed-lots).

Methods
An equivalence trial was carried out involving 1,087 adults in Rio de Janeiro. Vaccines produced by Bio-Manguinhos, Fiocruz (Rio de Janeiro, Brazil) were administered following standardized procedures adapted to allow blocked randomized allocation of participants to coded vaccine types (double-blind). Neutralizing yellow fever antibody titters were compared in pre- and post-immunization serum samples. Equivalence was defined as a difference of no more than five percentage points in seroconversion rates, and ratio between Geometric Mean Titters (GMT) higher than 0.67.

Results
Seroconversion rates were 98% or higher among subjects previously seronegative, and 90% or more of the total cohort of vaccinees, including those previously seropositive. Differences in seroconversion ranged from -0.05% to -3.02%. The intensity of the immune response was also very similar across vaccines: 14.5 to 18.6 IU/mL. GMT ratios ranged from 0.78 to 0.93. Taking the placebo group into account, the vaccines explained 93% of seroconversion. Viremia was detected in 2.7% of vaccinated subjects from Day 3 to Day 7.

Conclusions
The equivalent immunogenicity of yellow fever vaccines from the 17D and 17DD substrains was demonstrated for the first time in placebo-controlled double-blind randomized trial. The study completed the clinical validation process of a new vaccine seed-lot, provided evidence for use of alternative attenuated virus substrains in vaccine production for a major manufacturer, and for the utilization of the 17DD vaccine in other countries.

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Descritores
Vacina contra febre amarela. Ensaios controlados aleatórios.

Resumo

Objetivo
Comparar a imunogenicidade de três vacinas contra febre amarela) das subcepas WHO-17D e 17DD brasileira (diferentes lotes-semente).

Métodos
Trata-se de ensaio de equivalência envolvendo 1.087 adultos no Rio de Janeiro, RJ. As vacinas foram produzidas em Bio-Manguinhos, Fiocruz (Rio de Janeiro, Brasil) e foram administradas seguindo procedimentos adaptados para randomização em blocos, com tipos de vacinas codificados ("duplo-cego"). Anticorpos neutralizantes contra febre amarela foram dosados antes e depois da vacinação. Definiu-se equivalência como diferença nas taxas de soroconversão não superior a cinco pontos percentuais, e razão de títulos médios geométricos superior (TMG) a 0,67.

Resultados
As taxas de soroconversão foram iguais ou maiores do que 98% nos participantes previamente soronegativos. Na coorte completa (incluindo os previamente soropositivos) a soroconversão foi igual ou superior a 90%. As diferenças na soroconversão variaram de -0,05% a -3,02% entre os grupos de comparação. A intensidade da resposta imune também foi semelhante nos grupos: 14,5 UI/mL a 18,6 UI/mL. As razões de TMG variaram de 0,78 a 0,93. Considerando o grupo placebo, as vacinas explicaram 93% da soroconversão. Viremia foi detectada entre os dias três e sete em 2,7% dos participantes vacinados.

Conclusões
A equivalência na imunogenicidade das vacinas contra a febre amarela das subcepas 17D e 17DD foi demonstrada pela primeira vez em ensaio clínico randomizado, duplo-cego, controlado com placebo. O estudo completou o processo de validação clínica do novo lote-semente de vacina, além de ampliar as bases para utilização da vacina brasileira em outros países e de trazer alternativas de subcepas para o produtor da vacina no Brasil.

INTRODUCTION

Yellow fever (YF) is an acute viral disease of public health importance in Africa and South America, and of interest for travel clinics in other areas of the world. There is concern about the potential for urban outbreaks of YF in Latin America with the increase in the distribution of the *Aedes aegypti*, increased intrusion of people into forested areas, and low vaccine coverage.[23,25] In Brazil the number of reported YF cases has been increasing in the last ten years peaking in 2000 (85 cases), and approaching more densely populated areas in the Southeast.

YF vaccines have been available since the1930's and constitute the most important method to control the disease. YF vaccines recommended by the World Health Organization (WHO) are from 17D and 17DD substrains, which have minor differences in nucleotide sequences.[6] Control of viral substrains and serial passage levels through the *seed lot system* were implemented in the 1940's to avert unwanted alterations in biological properties of the vaccine.[4] YF vaccines are now considered safe and highly immunogenic.[16,25] Protective antibodies induced by the vaccine are experimentally correlated to infection resistance, and have long duration, possibly life long.[8,18]

Bio-Manguinhos-Fundação Oswaldo Cruz, which is a WHO-prequalified manufacturer linked to the Brazilian Ministry of Health supplies YF vaccine for Brazil and other countries in South America and Africa. From 2000 to 2004, 26,368,050 doses of YF vaccine have been exported to 50 different countries in South and Central America, Africa and Asia. In Brazil 77,374,755 doses were administered from 1994 to 2002. As the working *seed lot* was used up a new seed lot was prepared and tested, confirming the genetic stability of the virus, its safety in the monkey neurovirulence test (unpublished data), and freedom from avian leucosis infectious virus.[11] As part of the process of clinical validation of the new seed lot, it was compared the immunogenicity and reactogenicity of a vaccine produced from this new seed lot to that of the already licensed vaccine, to the vaccine produced from WHO 17D-203/77 substrain seed lot, and to placebo. This paper reports the results of immunogenicity component of the trial. Data on reactogenicity will be the subject of another publication.

METHODS

A randomized field trial was carried out to test the equivalence of three vaccines in their seroconversion rates for YF and geometric mean antibody titters

(GMT), and to measure the rates of adverse events, abnormalities in liver enzymes and levels of viremia up to 30 days after immunization. The intent was to demonstrate that the vaccine from the new seed-lot of the 17DD substrain was at least as immunogenic as the vaccine from the working seed-lot of the 17DD substrain and a vaccine from the WHO-17D seed-lot. Vaccine and placebo vials were labeled with codes concealed from the participants and the field work team. Participants in the placebo group were vaccinated against YF after the scheduled opening of the codes. The research protocol was approved by Research Ethics Committees from Fundação Oswaldo Cruz and from the Brazilian Army. Placebo was scientifically justified for a precise assessment of the reactogenicity and immunogenicity of the vaccines. It was ethically acceptable since the study area has been free of YF, and immunization was not routinely conducted there. Two independent reviewers examined the study protocol and the preliminary results and reported to the steering committee.

Eligibility assessment, recruitment of participants, administration of vaccines and blood collection were carried out in 21 units of the Army located in Rio de Janeiro, Brazil. Eligibility criteria were age 18 years or more, no previous vaccination against YF, no plans to travel to YF endemic areas during the study period, availability for study follow-up, and signed an informed consent. Vaccination history had to be relied on information from participants. The following conditions constituted exclusion criteria: (1) pregnancy; (2) immunosuppression due to diseases or immunosuppressive therapy; (3) history of hypersensitivity to chicken eggs; (4) administration of any vaccine 30 days before this vaccination; and (5) other severe disease or fever of 38ºC or higher.

Trained nurses adjusted routine immunization procedures to meet the requirements for blinding and randomization. Participants received a single 0.5 ml subcutaneous injection in the deltoid region of one of four products: (*i*) 17DD vaccine (lot 993PFBEXP03; passage 287) produced from new seed-lot; (*ii*) 17DD vaccine (lot 999FB060Z; passage 286; licensed and available at the time of the trial); (*iii*) 17D vaccine (lot # 007FBEX01; passage 239) produced from a secondary seed-lot WHO 213/77/Br 1B/86; and (*iv*) placebo made of chicken embryo juice material without YF virus. All vaccines were produced by the Institute of Immunobiological Technology in Fundação Oswaldo Cruz, Rio de Janeiro. Vaccines were lyophilized, thermostable, and contained ≥1,000 MLD_{50} in each 0.5-ml dose. Vaccines and placebo contained sodium glutamate, sucrose and <5.0 mg of ovalbumin per dose. Distribution, handling and administration of the vaccine followed recommendations from the manufacturer and the Brazilian Program of Immunization.[14] Briefly, freeze-dried vaccines (or placebo) in 50-doses vials were diluted in sterile and apyrogenic saline solution, kept between 2ºC and 8ºC, protected from light, and discarded after four hours.

Vaccine and placebo vials looked identical and were labeled with codes, which were concealed from study participants, field work team, laboratory staff, and data analysts. The computer-generated randomization scheme (block length =12; uniform allocation ratio) was also concealed in opaque envelopes, which were opened in predetermined sequence at the time of vaccine administration.

Seroconversion was defined as doubling of pre-vaccination antibody titters and as seropositivity among individuals with seronegative results in the pre-vaccination test. The antibody titter was measured with the Plaque Reduction Neutralization Test (PRNT).[5,17] Serologic analyses were conducted at the Laboratory of Viral Technology at Fundação Oswaldo Cruz.

Venous blood samples were drawn just before, between Days 4 and 20, and 30 days or more after vaccination. Serum aliquots were coded to conceal the identity of the participant and the timing of collection (pre/post-vaccination). PRNT was conducted in serial two-fold dilutions starting at 1:5, in 50 µl of inactivated serum in 96 well tissue culture plates. Thirty plaque forming units (pfu) of 17D strain of YF virus in 50 µl were dispensed into all wells. Dilutions of virus and serum were performed in 199 medium containing 2.5% 1M HEPES. A positive monkey serum sample with YF antibody content calibrated by a WHO International Reference Preparation was included in each set of test. After incubation at room temperature for 1 hour, 50 µl of a suspension of Vero cells in 199 medium in a density of $1.6x10^5$ cells/well was added to all wells and the plates incubated at 37ºC in 5% CO_2 atmosphere for 3 hours. The medium was discarded and replaced with 199 medium containing 3.5% carboxymethyl cellulose in volumes of 100 µl. After incubation at 37ºC in 5% CO_2 atmosphere for seven days, the monolayers were fixed with formalin, stained with crystal violet and plaques counted at a magnification of x 12.5 on a vertically mounted 35 mm projector with wide angle lens. The $\log_{10}$ dilution of the test and standard serum, which reduced the plaque numbers by 50% relative to the virus control was determined by linear regression. The mean antibody titter at the 50% endpoint of the standard serum was then calculated and added to the $\log_{10}$ endpoint for each sample to give $\log_{10}$ mIU/ml. Seropositivity was defined as YF virus neutralizing

Table 1 - Number and selected characteristics of participants by intervention group (vaccines and placebo). Rio de Janeiro, 2001.

	Intervention				
	17DD-013Z*	17DD-102/84**	17D-213/77***	Placebo	Total
Number recruited	270	273	272	272	1,087
Number with complete follow-up	270	270	270	271	1,081
Age (years)					
Mean	28.1	29.3	28.4	29.3	28.8
Standard deviation	8.6	10.2	8.8	9.4	9.3
% of males	91.5	91.6	94.1	94.5	92.9
Seropositivity to pre-vaccination test (N, %)	61 (22.6)	77 (28.2)	59 (21.7)	71 (26.1)	268 (24.7)

Vaccines produced from *New seed lot; **Current seed lot; ***WHO seed lot

antibody titter equal to or higher than 630 mIU/ml (2.8 $\log_{10}$). The inter-rater agreement of antibody titters was assessed through independent tests performed in duplicate serum aliquots in a 20% sub-sample of participants.

Signs and symptoms experienced after immunization were ascertained in an interview and in diary forms filled in by participants. Serum levels of liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT) and alkaline phosphatase (AP) were measured in all serum samples. Levels of viraemia were measured in the second (intermediate) blood sample collected between Days 4 and 20 using methods described elsewhere.[9]

For a one-sided, non-inferiority equivalence trial it was appropriate to calculate the differences in seroconversion rates among the vaccine groups, and the ratios of GMT, with appropriate 90% confidence interval (90% CI) set by the conventional value of 0.05 for α-error.[3] The vaccines were considered similar if the lower limit of the difference did not exceed -5% and the lower limit of the ratio of GMT were not less than 0.67.

From the comparisons between each vaccine and the placebo it was derived proportions of seroconversion attributable to the vaccine, with 95% CI.[2] Antibody titters from vaccines and placebo were compared in reverse cumulative distribution plots.[19] Agreement between titters in the subset of sera with two aliquots was measured with the intraclass correlation coefficient.[1] Analyses were conducted both for the complete study cohort (intention-to-treat analysis) and for those who met all protocol requirements.

Sample size was based on a null hypothesis of non-equivalence and alternative hypothesis of equivalence. For beta =0.20, alpha =0.05, a difference no higher than 5 percentage points, 95% seroconversion rate and 10% attrition rate, the required number in each group was 240.[1] Epi Info 6.04c and SPSS 10, 1999 were used for data entry and statistical analysis.

RESULTS

From January to May 2001, 1,087 volunteers received vaccine or placebo, donated pre-vaccination blood sample and were interviewed. Six (0.6%) of them did not return for post-vaccination blood collection for reasons unrelated to adverse events. Sera of three individuals were not available for analysis. Three received other vaccines during the follow-up and one was 15 years old. A large proportion of participants were found to be seropositive in pre-vaccination tests (Table 1). They had a balanced distribution across comparison groups, included the same proportion of males, but were older than those with seronegative pre-vaccination tests (median age of 33 and 24 years, respectively).

The age of participants ranged from 14.8 to 67.7 years, with comparable means and standard deviations across groups (Table 1). Young males were highly predominant.

In general, the vaccine was well-tolerated and there were no reports of severe adverse events. A detailed account of the vaccine reactogenicity is reported elsewhere.

Table 2 - Proportion of seroconversion and post-immunisation seropositivity to yellow fever, and geometric mean titter (GMT) by intervention group, and analytical approach. Rio de Janeiro, 2001.

		Intervention			
Endpoint	Analytical approach	17DD-013Z*	17DD-102/84**	17D-213/77***	Placebo
% of seroconversion****					
intention-to-treat*****		90.0	90.8	93.0	9.2
per protocol******		98.0	99.5	99.5	6.4
GMT in the post-vaccination test					
Intention-to-treat*****		14,059	15,107	17,920	94
per protocol******		14,536	15,691	18,649	32

Vaccines produced from: *New seed lot; **Current seed lot; ***WHO seed lot; ****Two-fold increase in pre-vaccination antibody levels; *****Complete cohort; ******Excluding individuals who were seropositive in the pre-vaccination test.

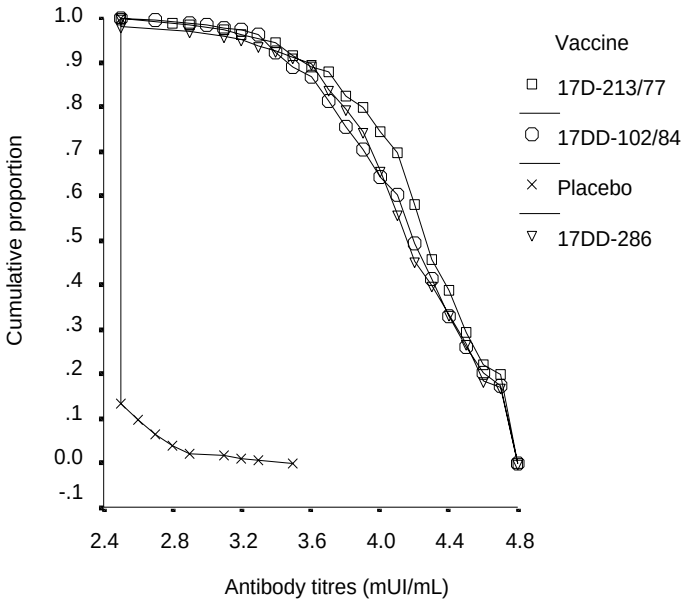

Figure 1 – Reverse cumulative distribution of antibody titers after yellow fever vaccines or placebo, excluding subjects seropositive before vaccination. Rio de Janeiro, 2001.

The proportion seropositive after vaccination was above 97% in the complete cohort as well as in the subset seronegative before vaccination. The immunological response to all three types of vaccine was similar in the whole range of antibody titters (Figure 1). The mean and the median antibody titters were similar for the vaccines (Table 2). More than 90% of those who received one of the vaccines, including the seropositive in the pre-vaccination test, showed at least a two-fold increase in their antibody levels (Table 2).

Excluding participants with seropositive pre-vaccination tests, seroconversion was above 98% among vaccinated groups (Table 2). GMT from the whole cohort were very close to GMT obtained in per protocol analysis.

The intraclass correlation coefficient (ICC) for antibody titters in two aliquots of the same sera was 0.79 (95% CI: 0.74-0.83). The distribution of the differences between titters was symmetrical, with mean 0.12 and median 0 $\log_{10}$ mIU/ml. With seroconversion defined as fourfold increase in pre-vaccination titters, the rates would have been 96.6%, 98.4%, 98.5% and 2.1% for vaccines produced from 17DD-013Z, 17DD-102/84, 17D-213/77 seed lots and placebo, respectively.

The unexpected substantial proportion of individuals seropositive to YF before intervention engendered an analysis of this subset that had not been considered in the protocol. They showed seroconversion rates of 67%, 76%, 77% and 16% in the groups that received the vaccines produced from 17DD-013Z, 17DD-102/84, 17D-213/77 seed-lots and the placebo, respectively. The variation in the placebo group suggested that part of the seroconversion of vaccines was not explained by the vaccine. Contrasting the pool of vaccines with placebo controls, the proportion of seroconversion attributable to immunization was 79% among those with pre-vaccination seropositivity. The post-vaccination GMT was somewhat higher among vaccinated subjects who were seronegative (16.2 IU/ml; 95% CI: 14.7-17.8 IU/ml) before vaccination, compared to those who were seropositive (14.0 IU/ml; 95% CI: 12.0-16.3 IU/ml).

Analysis of the complete cohort indicated that 90% of seroconversion among vaccinated subjects were attributable to the vaccines. Excluding the seropositive before vaccination, study vaccines explained more than 93% of seroconversion of vaccinated subjects.

The differences between the proportions of post-vaccination seropositivity varied from 0.35% to -1.95% with lower confidence limits above -5% (data not shown). Differences in seroconversion ranged from -0.05% to -3.02% (Figure 2). The lower limit of the 90% confidence interval of the difference between 17DD vaccines and the 17D vaccine was beyond the limit of equivalence.

The ratios of post-vaccination GMT ranged from 0.78 and 0.93, and the 90% CI from vaccines pro-

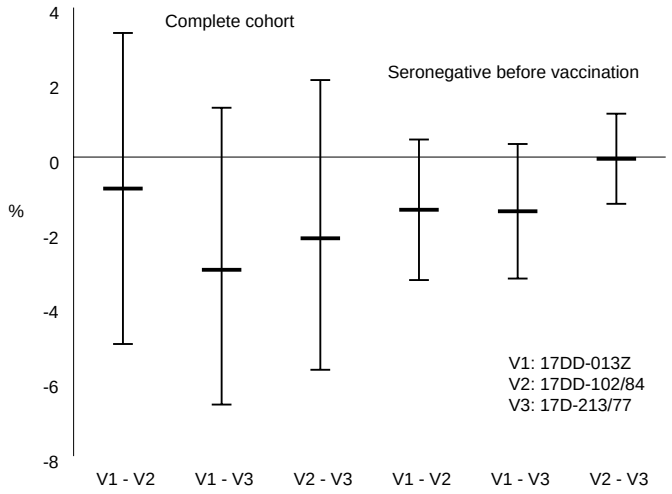

Figure 2 – Differences in proportions of seroconversion (percent and 90% confidence limits) between three yellow fever vaccines, for the complete cohort and for the subset seronegative in the pre-vaccination test. Rio de Janeiro, 2001.

duced from 17DD-013Z and 17D-213/77 seed lots included the 0.67 limit defined as equivalence (data not shown).

Viremia was detected in only 2.7% of vaccinated subjects (2.2% of those who received 17DD and 3.7% with 17D), from Day 3 to Day 7 after vaccination.

Seroconversion rates for the three vaccine groups pooled together differed slightly across age-groups: 30 years or more, 95.4%, 25-29 years, 99.5% and 18-24 years, 99.7%. The GMT for vaccines did not differ substantially across those age groups, though (17.6, 18.0 and 17.7 IU/ml, respectively). Female volunteers (n=54) showed a smaller seroconversion rate compared to men: 95.5% and 99.3%, respectively. Again, the GMT did not differ substantially: 17.9 UI/ml (women) and 17.8 UI/ml (men).

DISCUSSION

YF vaccines have been shown to induce long-lasting protection. Notwithstanding, the immunogenicity of the YF vaccine has been questioned in recent publications.[10,21,25] It was assessed the performance of the YF vaccines under Good Clinical Practice guidelines, as part of the clinical validation of a new seed-lot, used to supply vaccines to the National Immunization Programs in Brazil and other parts of Latin America and Africa. Brazil is the only producer and the major consumer of a 17DD substrain YF vaccine, which had never been compared in controlled studies with the 17D substrain and with placebo. Those elements along with the blinding and the equivalence type of design expanded the possibilities of analyses and increased confidence in the results.

The results indicated that the vaccines prepared with WHO-17D substrain and the Brazilian 17DD substrain induced satisfactory immunological response. High seroconversion rates and GMT in international units were comparable to those reported in other randomized trials,[12,15] and in observational studies in adults, in which neutralization tests were used to assess immunological response and the 17D strain vaccine was used.[16,20,22,24] Comparability with previous results is limited by methodological differences, such as laboratory methods for antibody titration, vaccine dose and method of administration, and age of study subjects. That is the case of three studies involving the 17DD substrain. Lopes et al[13] reported 100% seroconversion with a 600 LD_{50} /dose vaccine; Guerra et al[10] observed 76% seropositivity detected through mouse neutralization tests six months after vaccination with jet injectors; and Stefano et al[21] obtained 78% seroconversion rate among 9-month-old infants.

A substantial proportion of participants in the present study showed pre-vaccination seropositivity probably as a result of previous vaccination, which they ignored or concealed. Previous natural YF infection was less likely as the study site was neither endemic nor epizootic. As PRNT is considered highly specific, previous infections by other flaviruses were not expected to cause false-positive results.[17] There were no striking imbalances in the distribution of pre-vaccination seropositivity across vaccination and placebo groups that could affect their comparability. Most of the subjects with pre-vaccination seropositivity showed immunological response to (re)vaccination, but the differences among comparison groups did not change substantially when they were included in the analysis.

Analysis considering only those seronegative to YF in the pre-vaccination test (per protocol analysis) showed excellent immunogenicity of the vaccines. The differences between proportions of seroconversion were never beyond 3%, but for some contrasts, the 90% CI included values outside the range defining equivalence. This evidence against equivalence should take into account the practical implications of the arbitrary 5% difference in seroconversion rates, and 1.5 ratio in GMT, which are conservative. The lowest confidence limit of -7% in the difference between the new seed-lot and the WHO seed-lot represented the worst scenario of the complete cohort with substantial proportion of individuals less likely to show immunological response to vaccination. For practical purposes, the data provided evidence that there was equivalence between the two seed-lots of the 17DD vaccine in terms of seroconversion and magnitude of immune response.

The apparent "seroconversion" in the placebo group may be including genuine fluctuations in the level of previously existing antibodies and variability of laboratory methods. The effect of unreliability of measurements is attenuation of effect measures and non-differential misclassification,[7] which may lead to underestimation of the proportion of seroconversion attributable to the vaccine compared to placebo. In the present study, a more stringent definition of seroconversion (fourfold increase in pre-vaccination titters) had little impact on the observed rates and differences between vaccines.

The attributable proportion of seroconversion among vaccinated, a measurement analogous to efficacy, estimated the "net effect" (accounting for "seroconversion" in placebo controls) of vaccination, which was greater in the present study than in another study.[10] Seropositivity has been generally con-

sidered to indicate protection against natural infection, although direct evidence is lacking in human beings. Serological correlates of protection against YF are based on data from non-human primates.[8] Efficacy trials have never been performed so that, in addition to pre-clinical data, evidence of effectiveness of the YF vaccine relies on indirect evidence provided by correlation of disease occurrence and vaccination coverage in several endemic areas over the years.[16]

Gender differences in vaccine immunogenicity found in other studies [15] were not confirmed in the present study. Differences in proportion of seroconversion seemed to favor individuals less than 30 years old, but the magnitude of immune response did not confirm the advantage. That converges with results obtained by Monath and colleagues.[15] Generalization of the results to age groups not included in this study, particularly children below two years of age, are not warranted considering the potential interference of maternal immunity and concomitant administration of other vaccines.

The levels and the duration of viremia after YF vaccination are in accordance with previous findings in the literature.[16] Their implications for safety will be analysed in another paper.

In conclusion, the vaccine produced from a new seed-lot generated from a single passage of the 17DD-102/84 seed-lot showed high immunogenicity, and could replace the original seed-lot for the production of the YF vaccine. The similar performance of the 17D and 17DD vaccines is significant from the perspective of vaccine supply in YF endemic countries. The 17DD substrain is produced only in Brazil but is distributed in other Latin American and African countries where 17D vaccines have been utilized. Moreover, the results indicated a choice of vaccine virus for the vaccine manufacturer in Brazil. The placebo group allowed a more precise estimation of the effect of vaccination accounting for concurrent changes unrelated to vaccination, and shortcomings of serologic methods.

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COLLABORATIVE GROUP FOR THE STUDY OF YELLOW FEVER VACCINES

In addition to the paper's authors, members of the Collaborative Group for the Study of Yellow Fever Vaccines: Dr. Anna Yamamura and Mrs. Luciana Lopes of Bio-Manguinhos; Mrs. Fátima Gomes, Mr. Francisco Speranza, Mr. Jaime Ramos, Dr. Marcio Costa and Dr. Monica Almeidaof Brazilian Army; Mrs. Itália Portugal and Mr. Jorge Silva of Hospital Evandro Chagas.

DISCLOSURE

Four of the authors were employed by the vaccine manufacturer (Bio-Manguinhos, Fundação Oswaldo Cruz) and three others worked in other units of Fundação Oswaldo Cruz. Bias from competing interest was prevented by: (1) participation of members of the Army with expertise in infectious diseases, vaccines and laboratory virological methods in the Collaborating Group, which conducted the study; and (2) having two independent university professionals knowledgeable in the field of infectious diseases and study designs and analysis examine the study protocol, the setting for laboratory and data processing and analysis.

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