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Standardization and Prevalence of the Booster Phenomenon: Evaluation Using a Two-Step Skin Test with 43 kDa Glycoprotein in Individuals from an Endemic Region of Paracoccidioidomycosis

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Abstract

Background We estimated the occurrence rate of the booster phenomenon by using an intradermal test with 43 kDa glycoprotein in an endemic area of paracoccidioidomycosis in the central-west region of Brazil.

Methods Individuals who had a negative result on a survey performed by using an intradermal test with 43 kDa glycoprotein in an endemic area of paracoccidioidomycosis underwent a second intradermal test after 10–15 days to determine the presence or absence of the booster phenomenon. Statistical analyses were performed using the Chi-square test, Chi-square for linear trend test, Student's t test, and binomial test; $p < 0.05$ was considered significant.

Results For the first time, we reported the occurrence of the booster phenomenon to an intradermal reaction caused by 43 kDa glycoprotein at a rate of 5.8–8.4%, depending on the test's cutoff point. This suggests that a cutoff point should be considered for the booster phenomenon in intradermal tests with 43 kDa glycoprotein: a difference of 6–7 mm between readings according to the first and second tests, depending on the purpose of the evaluation.

Conclusion The results indicate that the prevalence of paracoccidioidal infection in endemic areas is underestimated, as the booster phenomenon has not been considered in epidemiological surveys for this infection.

Keywords Paracoccidioidic infection · Booster phenomenon · Intradermal tests · *Paracoccidioides brasiliensis* · Paracoccidioidomycosis · gp43

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Introduction

Paracoccidioidal infection is an asymptomatic infection caused by thermo-dimorphic fungi of *Paracoccidioides* genus in individuals that reside or have resided in an endemic area, and it reacts to paracoccidioidin in delayed-type hypersensitivity (DTH) tests

[1]. These tests may also be used to evaluate cellular immunity in patients with paracoccidioidomycosis (PCM), thus allowing physicians to assess the severity and prognosis, as well as the recovery from a specific immune response after antifungal treatment [2–4].

The antigen most commonly used in intradermal tests is paracoccidioidin, a polysaccharide antigen derived from various strains of *Paracoccidioides brasiliensis* [5, 6]. The most widely used paracoccidioidin, the Fava Netto antigen, is prepared from yeast cells, and it only contains antigenic determinants from the fungus. Using several strains promotes a more complete antigenic preparation due to a higher number of different epitopes [7].

The prevalence of paracoccidioidal infection observed in Brazil is quite diverse, as shown by different epidemiological surveys performed since 1966. These variations may be due to the different antigens and dilutions used in different studies [7].

In addition, there is the possibility of cross-reaction with antigens from other fungi such as *Histoplasma capsulatum*, *Coccidioides immitis*, and *Sporothrix schenckii* [8]. For this reason, simultaneous skin tests with paracoccidioidin and histoplasmin, for example, may be useful to estimate whether the infection is caused solely by *H.capsulatum* or *P.brasiliensis* in cases with positivity for just one of the antigens, and in those in which the occurrence of a dual infection or cross-reaction when both reactions are positive is uncertain [9]. These difficulties led other researchers to use purified compounds such as the 43 kDa glycoprotein (gp43) in skin tests to avoid the inoculation of contaminants such as endotoxins that are present in crude antigens [8].

Restrepo and Schneidau [10] suggested that glycoproteins are the key molecules responsible for skin reactivity in a DTH response [8, 10]. Results of a histological evaluation of positive skin tests performed in human volunteers showed that the cell population responsible for a DTH response was similar to Fava Netto paracoccidioidin and gp43. However, the intensity of the DTH in patients with PCM tested with both antigens was higher among those tested with the glycoprotein [4, 8].

Considering that there is a higher prevalence of paracoccidioidal infection than PCM, skin tests are among the best tools for conducting epidemiological surveys [7]. This is also the case for tuberculosis (TB), since this test is considered the main tool for detecting latent *Mycobacterium tuberculosis* [11]. However, there are individuals who present with cutaneous anergy, which decreases the sensitivity of the assay [12–14].

Some factors may account for the decrease in the DTH response, such as malnutrition, immunocompromised conditions, the presence of systemic diseases, co-infection by HIV, measles and mumps, immunosuppressive therapies, the use of corticosteroids, and vaccine administration with an attenuated virus [15–18].

The booster phenomenon is characterized by an increased extent of induration of a skin test after retesting with the same inoculum in the absence of a new infection (i.e., an infected person whose T cell response was low or suppressed when the first skin test was performed who then underwent a second skin test shortly after the first one and became positive due to T cell activation). It has mainly been used for diagnosing latent tuberculosis infection [12, 19–21]. It is thought that this phenomenon is the result of the activation of memory cells of cellular immunity, similar to the anamnestic serologic response [20].

As studies on the booster phenomenon in those with paracoccidioidal infection were not found in the literature, we aimed to estimate the occurrence rate of the booster phenomenon using an intradermal test with gp43 in an endemic area of PCM in the central-west region of Brazil. We also present a new definition of the skin induration measurement, which indicates the presence of the booster effect with a greater accuracy.

Methods

Area of Study

The municipality of Jaraguari has a land area of 2912.822 km^2 and is located at 20°08′30″ south latitude and 54°23′58″ west longitude, 45 km from Campo Grande (MS, Brazil) at an altitude of 589 m [22]. The climate of the municipality is tropical with a dry winter and rainy summer. The rainfall is greater than 750 mm/year, reaching 1800 mm [23].

The municipality has 6341 inhabitants, 72% of whom live in rural areas and perform farming-related activities as their primary economic occupation [22].

Subjects

Individuals living in eight rural settlements located in the municipality of Jaraguari who participated in a previous study on the prevalence of paracoccidioidal infection [24], those who had a negative skin test result with the gp43 antigen (less than 5 mm in diameter), and individuals who agreed to participate in the present study were included. Individuals with anergy to an intradermal test with the candidin antigen were excluded from the study. Two hundred and seventy-five individuals participated in this study.

Ethical Considerations

This study was approved by the Ethics Committee for Research Involving Human Beings of the Federal University of Mato Grosso do Sul State (protocol number: 1303), and all participants agreed to participate in the study by signing the informed consent form.

Skin Tests

Participants in this study underwent a single retesting 10–15 days after the first skin test with gp43 (60 μg/mL) obtained from the strain Pb B-339 [8]; we used an antigen from the same batch with the same concentration, application technique, and test reading used in the previous study [24]. The test was performed in the anterior region of the right forearm, about 5 cm below the crease of the elbow.

The period of 10–15 days between the applications of the two tests was chosen to allow a greater anamnestic response of the cellular immune system of previously sensitized individuals, and because of the low probability of reinfection occurrence.

To set a cutoff point that defined the positivity of the booster phenomenon in the intradermal test with gp43, we used the same criterion used for calculating the cutoff point of the booster effect of tuberculin.

The standard deviation of the mean of the negative results (<5 mm) obtained from the first test was calculated [24]. The second test was considered positive when the values were above the sum of two standard deviations to the result obtained in the first test; thus, we were able to detect at least 95% of positive individuals. This increment of two standard deviations eliminates the limitations of an intradermal test caused by reading errors and individual biological variability [12].

Statistical Analysis

Categorical data were compared using the Chi-square test and Chi-square for linear trend, and continuous variables were analyzed using the unpaired Student's t test. Categorical data in the same individual were analyzed using the binominal test. Prevalence rates were calculated with confidence intervals of 95%. The statistical programs used were Epi Info, version 3.4.3 (Centers for Disease Control and Prevention, Atlanta, GA) and Bio Estat, version 5.0 (Civil Society Mamirauá, Manaus, Brazil).

Results

Of 695 individuals who participated in the previous prevalence study [24], 377 had a negative skin test result (<5 mm). Of these, 275 individuals participated in the present study (Fig. 1). The mean of negative results was 1.32 mm with a standard deviation of ±3.20 mm (Table 1). Considering the booster effect as an increment of at least two standard deviations in the measurement of induration, the cutoff point was calculated as 6.40 mm in the second test when compared to the first test. However, standardized test reading is performed by palpation of the induration, and the skin induration measurement is performed by using a ruler in mm, although without the graduation necessary to measure tenths of a millimeter. Thus, the approximate value is 6 mm; however, for the analyses, two different cutoff points were considered.

By analyzing the results with 6 mm as the cutoff point, 23 (8.4%) of 275 tested individuals had a booster effect on the skin test conducted with gp43 (Fig. 1). The male/female ratio was not different when comparing individuals with and without a booster effect (Table 2). Moreover, the booster effect was predominant among older individuals ($p < 0.001$), as shown in Table 1. We also observed an increase in this effect with increasing age ($p = 0.037$) (Fig. 2).

When using 7 mm as the cutoff point, 16 individuals (5.8%) had a booster effect (Fig. 1), with no difference between sexes ($p = 0.82$), but it occurred predominantly among older individuals ($p = 0.01$), as shown in Table 2.

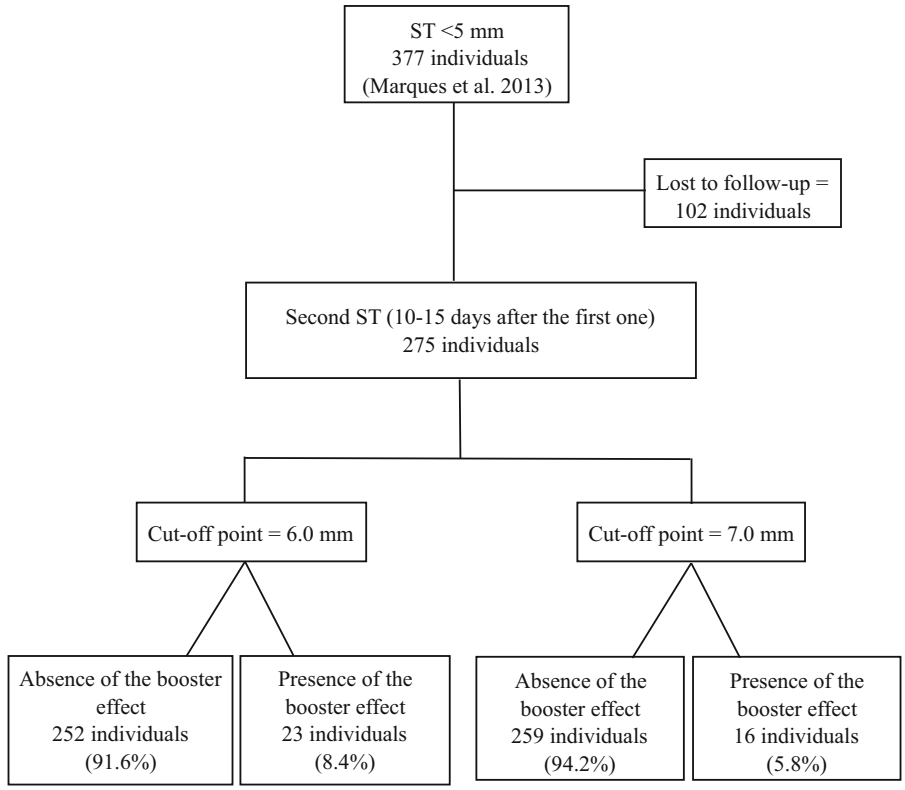

Fig. 1 Booster effect for the two cutoff points among healthy individuals in rural areas of central-west Brazil. *ST* skin test

Table 1 Number of individuals according to the induration size after the first and second skin tests with gp43 ($n = 275$)

Second test (mm)																			
First test (mm)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Total
0	55	12	27	11	9	5	4	4	1	1	–	1	1	1	–	1	1	1	135
1	8	5	3	4	1	–	–	–	–	–	–	–	–	–	–	–	–	–	21
2	12	–	11	12	2	2	–	1	1	–	–	–	–	–	1	–	1	–	42
3	11	2	13	9	7	2	3	3	–	1	–	–	–	1	1	–	–	–	53
4	1	2	3	3	8	1	1	2	1	–	1	–	–	–	–	–	–	–	24
Total	87	21	57	39	27	10	8	10	3	2	1	1	1	2	2	1	2	1	275

gp43, 43 kDa glycoprotein

The prevalence of *P. brasiliensis* infection differed according to the cutoff point: 8.4% when the cutoff point was 6 mm and 5.8% when it was 7 mm ($p = 0.016$).

Among the 275 retested individuals, 143 (52%) did not present with any increase in induration, and 109 (39.6%) presented with an increase in induration of 1–5 mm.

Discussion

Individuals living in rural settlements where they work and are exposed to various occupational diseases have limited access to healthcare facilities; thus, studies similar to ours increase knowledge about the extent of the infection. This information can be used to alert

Table 2 Evaluation of the booster effect using the intradermal test with 43 kDa glycoprotein in 275 individuals from a rural settlement in Mato Grosso do Sul, Brazil

	Cutoff point = 6.0 mm			Cutoff point = 7.0 mm		
	Presence of the booster effect ($n = 23$)	Absence of the booster effect ($n = 252$)	p value	Presence of the booster effect ($n = 16$)	Absence of the booster effect ($n = 259$)	p value
Male/female ratio	1.3	1.1	0.703[a]	1.1	1.0	0.822[a]
Age (mean ± SD)	47.7 ± 18.6	31.6 ± 20.8	<0.001[b]	45.9 ± 18.8	32.2 ± 21.0	0.010[b]

SD standard deviation

[a] Chi-square test

[b] Student *t* test

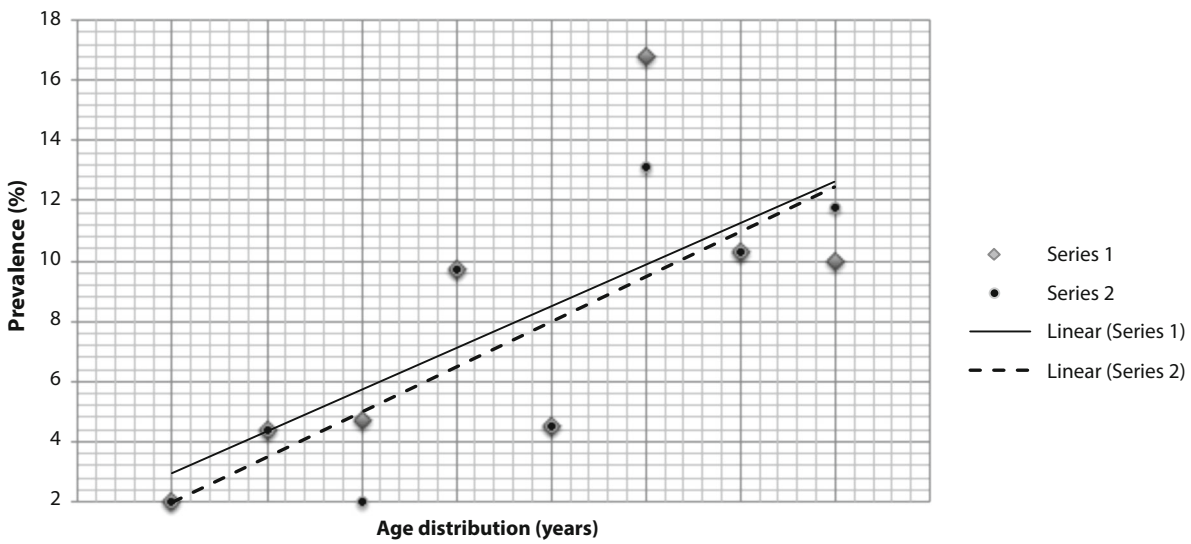

Fig. 2 Booster effect according to the intradermal test with 43 kDa glycoprotein

public health agencies responsible for serving these populations.

PCM and TB have several common immunopathological aspects: The airways are the main route of infection; they form a primary pulmonary complex with parenchymal and lymph node clusters; they have a long latency period and endogenous reactivation of latent foci; the host response to infection occurs through the development of specific cellular immunity, with granulomatous histopathological lesions with a variable pattern according to the degree of suppression of the immune response; and lung involvement in relation to other organs is prevalent [1, 25–28]. However, the etiological agents are diverse for PCM and TB, i.e., thermo-dimorphic fungi and mycobacteria, respectively [1, 17, 25–28].

Intradermal reactions have been used extensively to identify the prevalence of latent *M. tuberculosis* infection in different population groups. This led to the standardization of the composition of the inoculum, volume to be administered, reading of the test, and its interpretation [29, 30]. When intradermal test series are conducted and an increased induration is observed rom the first to the second skin test in the absence of a new infection, the presence of the booster phenomenon can be considered [12, 20, 21, 31].

Several studies have reported the presence of this phenomenon in the Mantoux reaction, along with its frequency and importance [12, 20, 32].

There are several criteria for considering the occurrence of the booster phenomenon in serial tests with tuberculin. Few studies have used criterion to define the booster phenomenon when the induration of the second test is greater or equal to 10 mm, regardless of the increment between the readings. Following this criterion, the prevalence of this phenomenon ranges from 8.0 to 13.8% [33–35]. However, the most widely used criterion considers the phenomenon positive in a tuberculin test when the induration of the second test is equal to or greater than 10 mm, and when there is an increased induration of at least 6 mm [12]. Hence, small increases in the induration size may be caused by the biological variation in the cutaneous response to the antigen, and differences in the administration and test reading. Thus, by evaluating repetitive tuberculin tests, the American Thoracic Society reported that the chance of variation may result in a difference smaller than 6 mm (two standard deviations) in 95% of individuals [32]. In this study, we used the criterion of twofold increased standard deviations in induration for the second test to determine the booster effect, which resulted in 6.4 mm. As a rule, we did not measure to the tenths of an mm, thus the approximated value used was 6 mm. However, considering that the values were between 6 and 7 mm, which are considered borderline values, and the possibility of false-positive results, the analyses were performed with cutoff points of 6 and 7 mm. The choice of the cutoff point varies according to the purpose of the test. For an epidemiological survey, using 7 mm is better to detect true positives, whereas using 6 mm may be better for resource planning in health programs aimed to identify the highest number of possible infected individuals.

Our study was the first to evaluate the booster phenomenon for detecting paracoccidioidal infection by *P. brasiliensis*, which had prevalence rates of 8.4 and 5.8%, depending on a cutoff point of 6 and 7 mm, respectively. These results are similar to those observed by other authors who used the same criteria in the tuberculin test; e.g., the booster phenomenon was observed in 7.8% of healthcare professionals at a university hospital [36], and 6% of patients who had contact with pulmonary tuberculosis [37].

The booster effect is the recall of the hypersensitivity response in the absence of a new infection. Some infected persons present a decreasing antigenic response over time, leading to a false negative or a weak skin test when they receive a new antigenic stimulus. However, it may stimulate the immune system, causing a positive or boosted reaction to a second test, in the called two-step testing [38]. This finding can be explained by few sensitized circulating lymphocytes to produce a significant local response. The later reading, obtained after the second antigen inoculation, seemingly after induction of memory lymphocytes, should be considered the correct one and should be used for decision-making [38].

Conversion is defined as the development of a new DTH to mycobacterial antigens following new infection with *M. tuberculosis*, nontuberculous mycobacteria, or BCG vaccination [20]. The interval between *M. tuberculosis* infection and the conversion in the intradermal test is less than 8 weeks [20, 39]. Thus, to test for the occurrence of the booster effect, the tests should be conducted over a shorter interval. It was observed that the frequency of this phenomenon is higher if the interval between the first and second test varies from 1 to 5 weeks [39, 40], less frequent if the interval is only with in 48 h [12], and rare if the interval is greater than 60 days [46].

Epidemiological studies are rare in areas of infection by fungi of the genus *Paracoccidioides*. Perhaps for this reason, intradermal tests have not been standardized, and antigens with different compositions and dilutions are used [41–43]. This prevents the realization of comparative analyses of the results. Moreover, no assessment of the booster effect has been conducted, which makes our study noteworthy.

In the current study, the second test was performed 10–15 days after the initial application. Study participants retained their residences, professional activities, and personal habits, suggesting that there was no infection after the first application; thus, the observed result is due to the presence of the booster phenomenon. The short period between the tests also ensures that any host factor present on the first day would remain until the last day [12].

The frequency of the booster phenomenon for tuberculin increases with age, and it is prevalent in the population vaccinated with Bacillus Calmette–Guérin [13, 44, 45]. In our study, an increase in this phenomenon was observed with age. However, there is no vaccine for paracoccidioidomycosis, which excludes a possible interference in the development of the phenomenon.

Intradermal tests are also conducted to identify an infection caused by other agents, including protozoa and other fungi such as *H. capsulatum* and *C. immitis* [45–47]. Despite few studies on the topic, the presence of the booster phenomenon has been reported. However, criterion for the definition of positivity, interval between applications, and dilution of the antigen was not found. This hampers the comparison and analyses of our results.

Previous studies on histoplasmin, esferulin, or coccidioidin used the same cutoff point for the first intradermal test and subsequent tests. The period between the tests, i.e., from 3 to 24 weeks, and the dilution of the antigen vary substantially. Despite these differences, the occurrence reported for the booster phenomenon shows little variation. The incidence of the phenomenon observed when compared to histoplasmin ranges from 27.8 to 32.0% [46, 48, 49], whereas in tests that used coccidioidin, it varies between 4.8 and 6.5%. With esferulin, the booster effect occurred in 2.4% of cases [47, 48].

The prevalence rate of 45.8% for paracoccidioidal infection observed in a previous study [24] among residents of rural settlements is underestimated since a second intradermal test demonstrated the existence of the booster phenomenon in 5.8–8.4% of the individuals evaluated, which increased the prevalence of paracoccidioidal infection by *P. brasiliensis* in the region up to at least 54.2%. It should also be highlighted that in the central-west region of Brazil, paracoccidioidomycosis is caused by the species *P. brasiliensis* and *P. lutzii*, the latter of which accounts for 19.8% of cases in the State of Mato Grosso do Sul [50]. It is noteworthy to mention that this fungus does not have the gp-43 gene [51]. Given that the antigen used in this study was gp43, it is possible to assume that we detected only the infection with *P. brasiliensis*, and the prevalence of paracoccidioidal infection in the region, considering the two species, may be even higher than the observed 54.2%.

Regarding tuberculosis, performing two tuberculin skin tests may be an effective way to minimize the booster effect, thus allowing an accurate monitoring of subsequent conversion rates [52]. Similarly, for a paracoccidioidal infection, the use of two skin tests may also decrease the chances of diagnosing false-negative individuals, facilitate future clinical research, and promote greater veracity in epidemiological studies.

The main limitation of this study was the small number of individuals who had a booster effect; among 377 eligible participants, only 275 agreed to receive a second intradermal injection.

The strength of this study is the knowledge of the prevalence of infected individuals, because these people may be potential future patients. Public health services should be alerted about this possibility, mainly because the chronic form of PCM has been misdiagnosed as tuberculosis and the acute/subacute form of PCM has been misdiagnosed as lymphoproliferative diseases, which delays the correct diagnosis and initiation of appropriate treatment. The skin test can also be performed in patients who have been confirmed to have PCM to evaluate the cell-mediated immunity and its progress after initiating treatment.

Studies on skin tests in domestic animals from PCM endemic areas are scarce. The prevalence of positivity was 6.2% in the study of 149 urban dogs inoculated with 6 μg of gp43 [53]. In addition, the intradermal test with 10 μg of gp43 revealed 13.1% of positive results in 61 suburban dogs and 38.1% in 21 rural dogs from the same region, suggesting that the great contact with soil being prepared for plantation plays a role in the inhalation of fungal propagules and the consequent infection [54]. These prevalences cannot be compared due to the different inoculum concentration used. However, the infected dogs can be an indicative of *P. brasiliensis* in the soil. Nevertheless, the booster effect was not evaluated in these publications.

The results of this study showed the occurrence rate of the booster phenomenon by using a skin test with gp43, which indicates the presence of an infection in individuals that were initially identified as non-infected. Thus, the prevalence of paracoccidioidal infection was greater than that previously reported in the studied region. We also proposed two cutoff points as criterion for the booster phenomenon: an increase of 6 and 7 mm, depending on the purpose of the test. Finally, similar studies should be performed in other regions to increase knowledge about the epidemiology of paracoccidioidomycosis.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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