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An interferon gamma release assay specific for *Histoplasma capsulatum* to detect asymptomatic infected individuals: A proof of concept study

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Abstract

Histoplasmosis is the most common endemic mycosis in the Americas. Currently, there is no laboratory test capable to detect subclinical or latent infections by *Histoplasma capsulatum* (*Hc*), which might develop as severe infections in immunocompromised individuals. For the first time to our knowledge, we explore the suitability of an interferon gamma release assay (IGRA) to detect latent *Hc* infection in asymptomatic individuals. A cohort of 126 volunteers was enrolled in the study, 13 of which underwent a *Hc* infection in the past, and 93 of them showing risk factors for this infection. The remaining 20 participants did not refer any risk factors of *Hc* infection, but eight of them showed evidences of infection with *Mycobacterium tuberculosis*. All participants were recruited in Medellin, Colombia, between January 2014 and December 2017. Whole blood samples were cultured with four different *Hc* crude antigens and phytohemaglutinin as positive control. The interferon (IFN)-γ released by T lymphocytes upon antigen stimulation was quantified by ELISA. A defined cutoff value of 20 pg/ml for the IFN-γ concentration allowed us to distinguish between the group with documented past infections and the group of noninfected individuals with high sensitivity (70–92%) and specificity (85–95%), for the four tested antigens. Positive 82–95% and negative 77–92% predictive values were also very high, comparable to those reported for commercially available IGRAs. The new test constitutes a promising screening method to detect individuals with latent *Hc* infection, even decades after the primary infection, as evidenced in this study.

Key words: interferon gamma release assay, *Histoplasma capsulatum*, histoplasmosis, latent infection.

Introduction

Histoplasmosis, caused by the primary fungal pathogen *Histoplasma capsulatum* (*Hc*), is the most common endemic systemic mycosis in the Americas[1] and is responsible for numerous deaths in Latin America.[2] Prevalence estimations show that a third of the Latin American population has been exposed to this fungus. However, *Hc* infection is generally neglected, being often misdiagnosed as tuberculosis.[2]

Immunocompromised individuals are especially at risk of suffering a disseminated *Hc* infection. In addition, other factors such as the use of immunosuppressive drugs and biologic therapies have contributed to the high burden of this disease.[3,4]

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This phenomenon is associated not only to primary infections in immunocompromised individuals but also to the reburst of previous infections that leave latent foci of the fungus.[5–7]

Historically, the histoplasmin skin test, which measures delayed-type hypersensitivity to *Hc*, was essential in defining the areas where this fungus is endemic and in revealing the unsuspected high frequency of asymptomatic infection in those areas.[8,9] This test, however, has important practical drawbacks and is not being used any more in the clinical practice. On the other hand, and despite ongoing developments, at present there is no laboratory test able to efficiently detect subclinical or latent *Hc* infections remaining after several years from the primary infection, which would help to prevent a potential development of active and severe forms of histoplasmosis.

A very appealing method to be tried to detect latent *Hc* infections is the interferon gamma release assay (IGRA). This type of test is based on the fact that individuals infected with a microorganism that stimulates the cell-mediated immunity develop specific effector or memory T cells. When restimulated *in vitro* with the same specific antigens, these lymphocytes release interferon (IFN)-γ, which can be quantified with an immunoassay.[10]

The first reported IGRA test (QuantiFERON-TB) was developed to detect infection by *Mycobacterium tuberculosis* (*Mtb*), and several commercial versions have been released since the first Food and Drug Administration (FDA) approval in 2001.[11–13] Other IGRA tests have been developed for different infections. These include Cytomegalovirus (QuantiFERON-CMV – the only nontuberculosis IGRA test approved by the FDA, in 2017)[14]; *Leishmania*[15,16]; Hepatitis B virus[17]; Varicella-zoster virus[18]; *Toxoplasma gondii*[19]; *Mycobacterium leprae*[20]; and human immunodeficiency virus (HIV).[21] In this study we explore, for the first time to our knowledge, the suitability of an IGRA test to detect infection by *Histoplasma capsulatum* in asymptomatic individuals.

Methods

Population sample

A cohort of 126 individuals was included in the study. All the participants were recruited in Medellin, Colombia, between January 2014 and December 2017. The study was conducted at the Corporación para Investigaciones Biológicas (CIB) in Medellin. We looked for people who, because of the nature of their work or recreational activities, might have been at risk of *Hc* infection at least during some periods of their lives, for example, clinical lab technicians manipulating cultures of this fungus, poultry farm workers, or speleologists, who are exposed to the bat guano frequently found in caves. Some of the individuals were identified from the clinical records maintained at CIB, revealing the occurrence of active *Hc* infection and including data related to those infection events, in particular, results from serological tests.

Individuals willing to participate in the study were first evaluated by a physician. After receiving a thorough description of the study, volunteers provided a written informed consent. A structured interview was performed to ascertain if these individuals had been exposed to risk factors of infection by *Hc* and to collect demographic data. They also passed an HIV test. General inclusion criteria were a negative HIV test and age between 10 and 82 years. Exclusion criteria were pregnancy and immunosuppressive conditions. All the participants were subjected to serological tests, as described below, and to chest x-ray radiography.

Individuals were distributed in five groups (see Table I) according to specific clinical laboratory criteria, as well as risk factors for infection with *Hc*. Group A1 (noninfected) included individuals with neither clinical evidences nor risk factors for *Hc* infection. Group A2, defined with the purpose of assessing the specificity of the IGRA test (in addition to group A1), was set to include individuals with the same characteristics as those in group A1, but showing in addition clinical records demonstrating the previous occurrence of an infection caused by either *Toxoplasma gondii*, *Paracoccidioides brasiliensis*, *Cryptococcus neoformans,* or *Mycobacterium tuberculosis*, as determined by high titers of specific antibodies (*T. gondii*), culture (*P. brasiliensis*, *C. neoformans*) or the tuberculin test.

Assignment of individuals to group "B–Proven *Hc* infection" (abbreviated hereinafter as "B-Proven") was based on documented evidences of symptoms compatible with histoplasmosis, and the positive results of at least one of the following mycological tests (either documented in their clinical records or performed within the current study), following EORTC criteria[22] and recent guidelines from the literature[23]: (i) isolation of the fungal pathogen in culture from a specimen obtained from the affected site or from blood, (ii) histopathologic or direct microscopic demonstration of appropriate morphologic forms with a truly distinctive appearance characteristic of this dimorphic fungus (*Hc*), such as the presence of characteristic intracellular yeast forms in a phagocyte in a peripheral blood smear or in tissue macrophages, (iii) detection of *Hc*-specific antibodies in serum, especially precipitin M in an agar gel immunodiffusion (AGID) test, (iv) nested polymerase chain reaction (PCR) in clinical samples, or (v) presence of urinary *Hc* antigen (in this case, at least one of the previous criteria had to be taken into account as well).

Individuals in groups "B–Probable *Hc* infection" (B-Probable) and "B–Possible *Hc* infection" (B-Possible) have in common their exposure to risk factors of *Hc* infection and negative serological tests using histoplasmin. Individuals in the B-Probable group showed, in addition, low antibody titers against yeast extracts in complement fixation (CF) tests.

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Serological assays

Blood samples were obtained from all the participants. Serum was extracted for AGID and CF tests to detect *Hc*-specific antibodies. Histoplasmin was used for the AGID test as described elsewhere.[24] This test was considered positive if either the M or H band, or both, were observed.[23] CF tests were performed as described in Azar and Hage[24], using either histoplasmin or whole yeast cells extracts. Different threshold titer values were used for group classification, as specified in Table 1.

Hc antigens for IGRA

Four different *Hc* strains were used as sources of the antigen extracts assayed in this study: G184AR (ATCC 26027), and native strains CIB1980, 46079 and 48395, isolated at CIB, Medellin, Colombia. In-house histoplasmin (Hmin) was prepared from mycelial form cultures, for strains G184AR and CIB1980. Mycelial cultures were grown in broth HAM F-12 medium supplemented with glucose (18.2 g/l), glutamic acid (1 g/l), HEPES (6 g/l) and cysteine (84 mg/l) and incubated at 37°C in a rotator shaker at 150 rpm for 1 month. Secreted antigens from *Hc* yeast phase were obtained from culture supernatants of *Hc* strains 46079 and 48395, grown in the same medium as the mycelial form, but incubated for 1 week at 37°C. Filamentous and yeastlike phenotypes were monitored for morphology by light microscopy. Control cultures were performed in brain heart infusion (BHI) supplemented with 5% sheep's blood and Sabouraud agar without antibiotics, to check for possible culture contamination.

For both the mycelial and yeast forms, after incubation in broth HAM F-12 medium, the fungus was inactivated with thimerosal at 1:10000 dilution during 7 days at 25°C. Supernatants were separated by centrifugation at 12000 rpm, 18°C for 15 min, and subsequently filtered in two steps using 8 μm and 0.45 μm porosity membranes, respectively. Soluble fractions were lyophilized to reach a 20-fold concentration and then dialyzed with Standard RC Tubing MWCO:6-8 KD (Spectra/Por® 1 Dialysis membrane) against phosphate-buffered saline (PBS), 0.01 M, pH 7.2 to be stored at −70°C, and against 0.025 M citrate buffer, pH 3.5, before cation exchange chromatography (only for histoplasmins).

Soluble fractions from mycelial cell cultures were applied to a carboxymethyl (CM) sepharose CL-6B column (2.0 × 16 cm) (Sigma-Aldrich, St. Louis, MO, USA) previously equilibrated with citrate buffer. The absorbed antigens were eluted at 1 ml/min with a step salt gradient consisting of 0.05 M, 0.5 M, and 1 M NaCl in citrate buffer, yielding M antigen-enriched fractions with the 0.5 M NaCl buffer. Fractions containing the M-antigen, as detected by Western blot,[25] were pooled. The secreted antigens obtained from the *Hc* yeast phase were not chromatographed. Protein concentration was determined using a NanoDrop 2000 equipment (Thermo Fisher, Waltham, MA, USA).

IGRA test

Blood culture stimulation with *Hc* antigens

Approximately 15 ml of blood per individual was collected in vacutainers containing sodium heparin (BD Vacutainer®, Franklin Lake, NJ, USA). Whole blood (500 μl/well) was dispensed in 24-well tissue culture plates (CELLSTAR, Greiner Bio One International, Austria) within 4 h of collection and stimulated with either (i) 10 μg/ml of histoplasmin, for *Hc* strains CIB1980 and G184AR; or (ii) 10 μg/ml of secreted *Hc* antigens from yeast phase, for strains 46079 and 48395. Nonstimulated wells (nonspecific IFN-γ production) and stimulated with 20 μg/ml of the selective T-cell mitogen phytohemagglutinin (PHA) (Sigma-Aldrich, USA) were used as negative and positive controls, respectively. Cultures were incubated at 37°C with 5% CO_2 for 24 h. At the end of the incubation period, supernatants were collected and stored at −70°C until subsequent analysis by ELISA.

Measurement of released IFN-γ

Cytokine production was measured using a commercially available ELISA kit for IFN-γ (Duo-set R&D Systems, Minneapolis, MN, USA), following manufacturer's recommendations. Optical density (OD) was measured with an ELISA microplate reader (Bio-Rad Laboratories Inc., USA) at 450 nm. An 8-point standard curve of known amounts of IFN-γ (starting at 7.8 pg/ml and ending at 1000 pg/ml) was used to determine the level of produced IFN-γ in response to the different *Hc* antigens. Interpolation from the generated standard curve was performed using the Prism 5 statistical package (GraphPad Software, La Jolla, CA, USA). Cytokine measurements are presented as the amount of cytokine produced following stimulation with a correction accounting for the basal level, measured from unstimulated cells from the same individual.

Statistical analyses

Data were compiled in a Microsoft Excel spreadsheet. Statistical analyses were performed using R-project version 3.4.2 with the statistical packages caret, psych, and multcomp. The Shapiro-Wilk normality test was employed to analyze the empirical distribution of the IFN-γ data. None of the data sets showed a normal distribution (highest $P = 1.1E{-}14$). The Levene's test was used to evaluate the homogeneity of the variances among the different groups, showing that the data were no homoscedastic (highest $P = .038$). In consequence, the Kruskal-Wallis non-parametric test and the post hoc Dunn's test were used for multiple comparisons of IFN-γ medians between the groups. A Benjamini-Hochberg correction was applied to control the type I error and p-adjusted (q) values were calculated. The assay

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Table 1. Criteria used for group assignment.

Type of evidence		Group A1 – Non-*Hc* infected ($n = 12$)	Group A2 – Non-*Hc* inf. + *Mtb* ($n = 8$)	Group B – Proven *Hc* infection ($n = 13$)	Group B – Probable *Hc* infection ($n = 30$)	Group B – Possible *Hc* infection ($n = 63$)
Direct	Culture / PCR / *Hc* antigen	No test	No test	Yes	No test	No test
Indirect (serological)	AGID–band M			Reactive		Nonreactive
	AGID–band H		Nonreactive		Nonreactive	
	CF–Hmin	Nonreactive		≥ 1:8		
	CF–yeast		≤ 1:8	≥ 1:32	≥ 1:8	
Risk factors of *Hc* infection		No	No	Yes	Yes	Yes
Past clinical evidences of histoplasmosis		No	No	Yes	No	No
Current clinical evidences of histoplasmosis		No	No	No	No	No
Past evidences of other infection		No	Yes	No	No	No

Group A2: Non-*Hc* infected, but with previous infection with *Mycobacterium tuberculosis* (*Mtb*). Group B-Proven: at least one direct or indirect evidence, accompanied by exposure to risk factors of *Hc* infection. Group B-probable: CF yeast titers <1:32, accompanied by exposure to risk factors. Group B-Possible: nonreactive serology tests AGID and CF, but with exposure to risk factors. AGID, agar gel immunodiffusion; CF, complement fixation; *Hc, Histoplasma capsulatum*; Hmin, histoplasmin.

sensitivity was evaluated as the percentage of positive responses in group B-Proven, while specificity was calculated as the percentage of negative responses in the control groups A1 and A2, taken together. The positive predictive value (PPV) was calculated as the ratio between positives in group B-Proven and the total number of positives in groups A1, A2, and B-Proven. The negative predictive value (NPV) is the ratio between the sum of negatives from groups A1 and A2, and the total number of negatives from groups A1, A2 and B-Proven. Statistical differences were considered significant if p-adjusted (q) < 0.05.

Ethics statement

All study procedures and written consent forms were approved by CIB's ethics review board. The study was approved also by the ethics review boards of the Health Promoting and Provider Companies in Medellin that aided in recruiting some of the individuals participating in the study.

Results

Characteristics of the study population

We recruited 126 asymptomatic individuals (64 women and 62 men) who met the inclusion criteria set for the study. Table 1 shows the numbers of individuals assigned to each group. For group A2, we could only enroll people with previous records of tuberculosis.

Serological reactivities upon enrollment in the study

For group A1, by definition, all the serological tests were negative. In group A2, whose members all showed previous records of tuberculosis, no individual reacted to the AGID tests and only one person showed a very weak reaction (titer 1:8) to the CF test against yeast. In group B-Proven, only a few individuals (5/13) presented a positive reaction in the immunodiffusion test, all of them to antigen M, while the CF tests using histoplasmin yielded positive results for seven individuals (with 1:8 titers). When performed with yeast extracts, the CF tests yielded positive reactions only for six individuals, with different titers: 1:8–2/6, 1:16–1/6 and 1:64–3/6 individuals.

Group B-Probable members showed no reaction in the AGID tests, but they did show reactivity to yeast extracts in the CF test. Most of these individuals presented CF titers between 1:8 and 1:64 (90%), while only 10% showed antibody titers greater than 1:64. Only one individual from this group reacted to histoplasmin. In group B-Possible, on the other hand, all the individuals were characterized by the absence of serological evidences (assignment to this group was based solely on risk factors).

IFN-γ production in response to different *Hc* antigens

Blood cultures were stimulated with four crudes of *Hc* antigens—two histoplasmins and two yeast extracts, obtained from different *Hc* strains, as well as with the T-cell mitogen PHA, serving as a positive control for immunocompetence. The basal IFN-γ levels, obtained in the absence of any stimulation, showed a median value of 0.4 pg/ml. Only six individuals presented basal levels higher than 20 pg/ml (see below the cutoff value definition), with one member of the B-Probable group reaching 87 pg/ml. In all of the individuals, PHA increased their blood IFN-γ levels (median value = 302 pg/ml), which spanned a large range of concentration values and showed a similar behavior for the five groups (Fig. 1).

Figure 2 shows the IFN-γ responses by group for each of the four selected antigens, as well as the results from statistical analyses. Here, the *Hc* antigen-specific levels of IFN-γ

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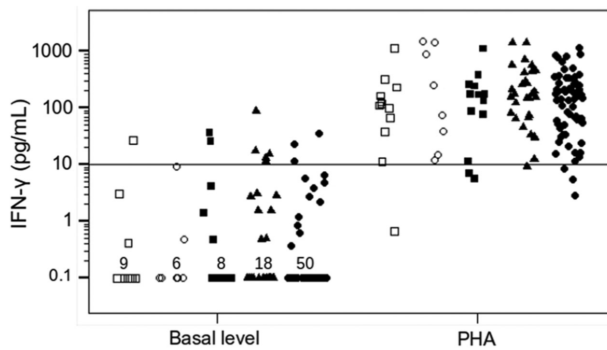

Figure 1. Basal IFN-γ levels and IFN-γ production after stimulation with the T-cell mitogen PHA, for the five population groups. Note that the vertical axis is in logarithmic scale. Symbols □,○,■,▲,● represent individuals in groups A1, A2, B-Proven, B-Probable and B-Possible, respectively. For the basal level, IFN-γ concentrations below 0.1 pg/ml were assigned a value of 0.1 pg/ml to fit into the plot (the numbers of individuals within the 0–0.1 pg/ml range are indicated for each group). The horizontal bar at 10 pg/ml was included to visually distinguish between medium/high and low IFN-γ levels.

obtained for a given individual were adjusted by subtracting his/her basal level of this cytokine. In group B-Proven, the responses to histoplasmins and yeast extracts were above 20 pg/ml in most cases. The two yeast extracts yielded higher IFN-γ responses ({median, [95% CI]}: {223, [15–547]} pg/ml for yeast 46079; {133, [21–530]} pg/ml for yeast 48395) as compared with the two histoplasmins ({47 [24–111]} pg/ml for Hmin CIB1980; {60, [36–107]} pg/ml for Hmin G184AR), although with much higher variability, as reflected in their much wider ranges of confidence intervals.

In groups A1 and A2, both including individuals with no records or exposure to risk factors of *Hc* infection, the IFN-γ responses to the four *Hc* antigens were mostly within low ranges, with medians at the ground level and a relatively low dispersion. For groups B-Probable and B-Possible, the responses to all the antigens showed a high variability, with medians within low response levels.

Definition of an IFN-γ response cutoff value

Despite the statistically significant differences obtained between the B-Proven and non-*Hc* infected groups (A1 and A2), the overlapping of their IFN-γ concentration ranges does not yield an obvious cutoff value to discriminate between positive and negative IFN-γ responses to *Hc* antigens. Aiming to facilitate this analysis we produced scatter plots for the six possible pairwise combinations of the four selected antigen preparations, as

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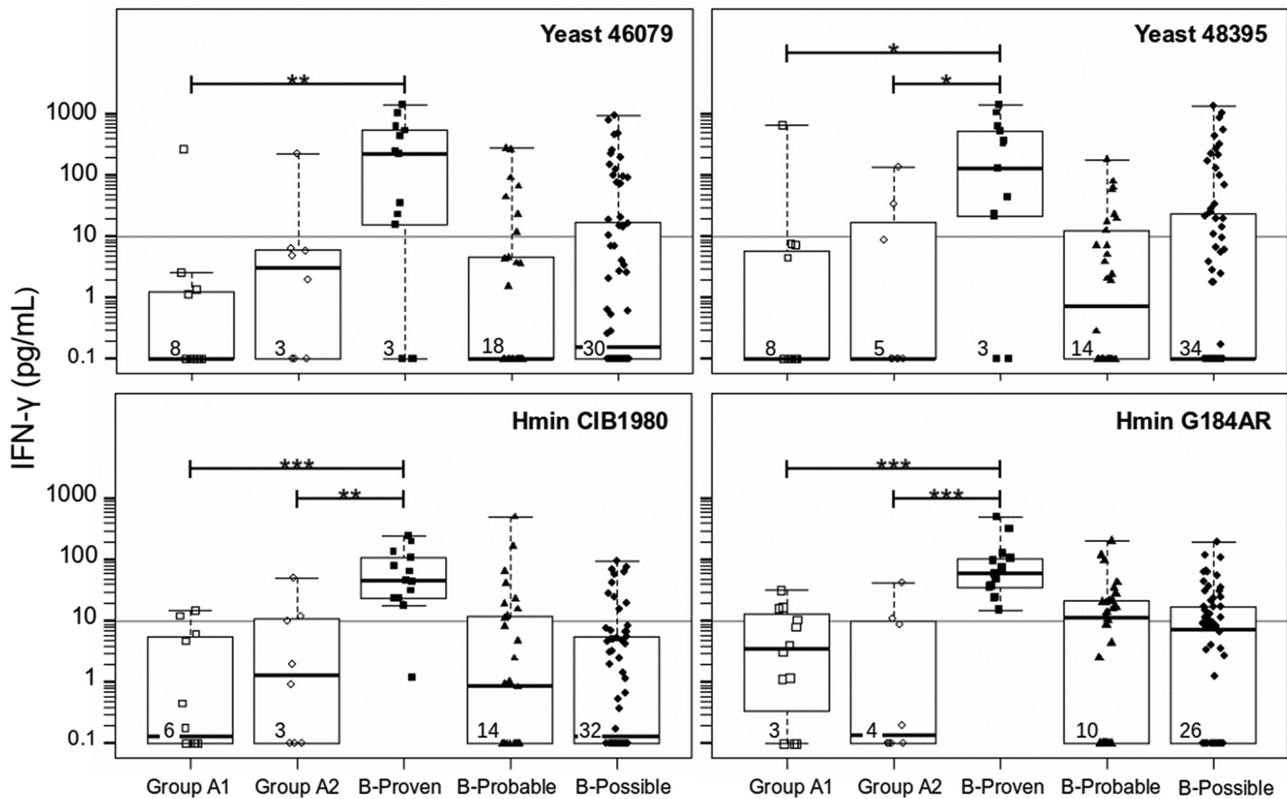

Figure 2. Statistical box plots for IFN-γ production upon stimulation with the different yeast extracts and histoplasmins, calculated for each group of individuals. The vertical axis is in logarithmic scale. Symbols □,○,■,▲,●, represent individuals in groups A1, A2, B-Proven, B-Probable and B-Possible, respectively. IFN-γ concentrations between 0 and 0.1 pg/ml were assigned a value of 0.1 pg/ml to fit into the plot. In each plot, the number of individuals within this range is indicated at the bottom. The upper bars indicate statistically significant differences between pairs of groups: * q<0.05; ** q<0.01, *** q<0.001 ("q" represents adjusted p values).

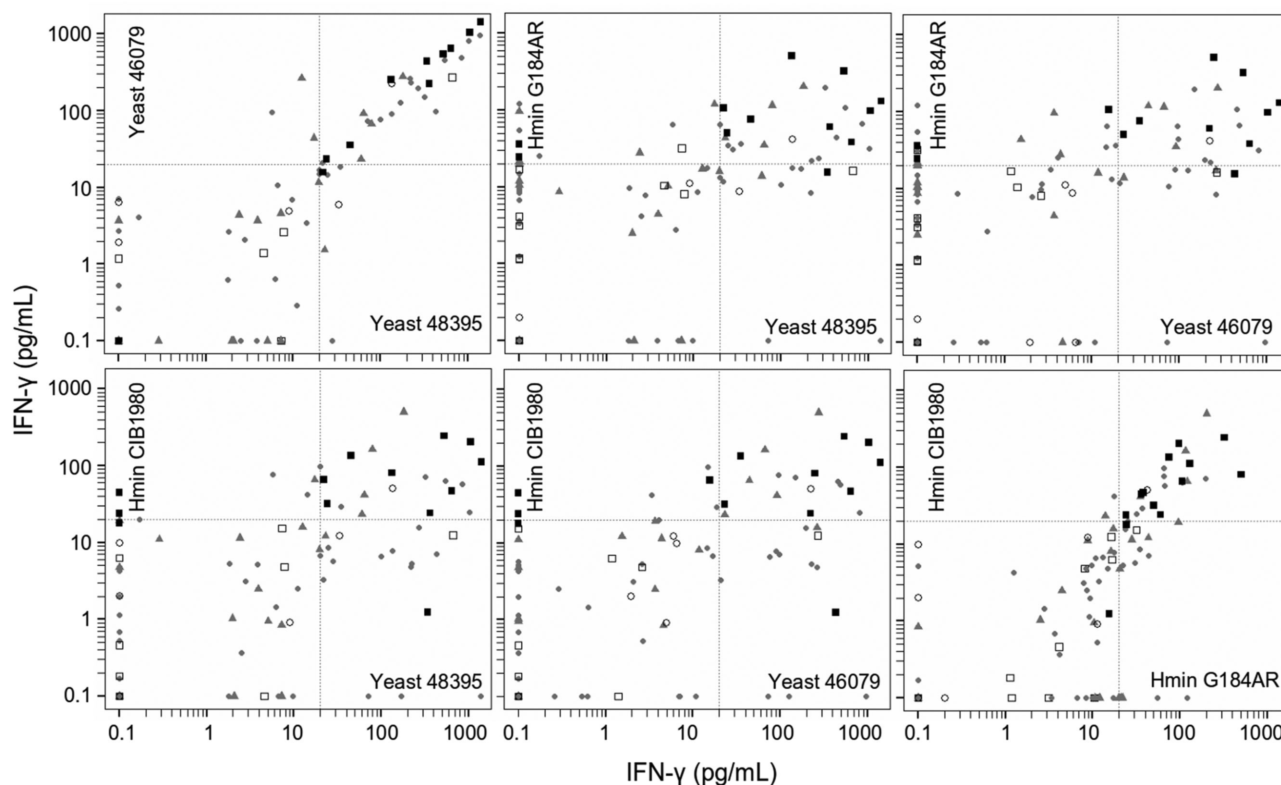

Figure 3. Scatter plots of IFN-γ production for all possible pairwise combinations of the four *Histoplasma capsulatum* antigens. Both axes are in logarithmic scale. The horizontal and vertical dotted lines in these graphs, are placed at the defined IFN-γ concentration cutoff of 20 pg/ml, separating the plots into quadrants. Symbols □,○,■,▲,● are for groups A1, A2, B-Proven, B-Probable and B-Possible, respectively

shown in Figure 3. The horizontal and vertical dotted lines in these graphs, placed symmetrically at the same IFN-γ level on both axes, divide the plots into quadrants. Setting these lines' level at 20 pg/ml separates most of the proven cases of histoplasmosis into the upper right quadrant, while most of the negative controls (groups A1 and A2) remain in the lower left quadrant.

Figure 3 shows also, for group B-Proven, a marked correlation within the right upper quadrant between the IFN-γ responses to the two yeast filtrates (R = 0.96), as well as between the responses to the two histoplasmins (R = 0.91). Similar correlations are evident also for groups B-Probable and B-Possible (not calculated). In contrast, there is no apparent correlation for any of the yeast/mycelium combinations.

Using the chosen IFN-γ concentration of 20 pg/ml as cutoff value to classify the response, we calculated the sensitivity, specificity and discriminating power (positive and negative) of our assay for each of the four antigen preparations. As listed in Table 2, all of the antigens yielded high values for all the calculated parameters, with the two histoplasmins showing a better performance. In groups B-Probable and B-Possible, 40% and 36% of the individuals, respectively, showed positive responses to at least one of the *Hc* antigens.

Table 2. Discriminating power of the IGRA test for the different *Hc* antigens.

Hc antigen	Sensitivity (%)	Specificity (%)	Predictive positive value (%)	Predictive negative value (%)
Hmin-G184AR	92	90	95	86
Hmin-CIB1980	85	95	91	92
Yeast-46079	70	90	82	82
Yeast-48395	77	85	85	77

IGRA versus serological tests

We compared the responses to the serological tests performed in this study with the IGRA responses to the assayed antigens, for all the members of group B-Proven (Table 3). All 13 members yielded at least one positive response in the IGRA test, with eight individuals responding to the four *Hc antigens*. Seven individuals from this group had been infected with *Hc* several years (5.5–36 years) before the beginning of this study. Within this subgroup, a minor proportion presented positive results to the different serological tests, as shown in Table 3. In contrast, the seven individuals yielded positive responses to at least two antigens in the IGRA test, with 4/7 (57%) reacting to the four

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Table 3. IGRA versus serological tests in Group B-Proven.

Number of individuals	Time post infection[a]	Serology[b]			IGRA		
		AGID	CF-Hmin	CF-yeast	Hmin[c]	Yeast[d]	Hmin+Yeast[e]
6	< 1 year	3 (50%)	4 (67%)	4 (67%)	6 (100%)	4 (67%)	4 (67%)
7	5.5–36 years	2 (29%)	3 (43%)	2 (29%)	6 (86%)	6 (86%)	5 (71%)

AGID, agar gel immunodiffusion; CF, complement fixation; *Hc, Histoplasma capsulatum*; Hmin, histoplasmin.
[a]Time elapsed between the reported *Hc* infection and the tests performed in this study.
[b]Individuals with positive responses to the different serological tests performed in this study.
[c]Positive responses to at least one of the histoplasmin antigens.
[d]Positive responses to at least one of the yeast antigens.
[e]Positive responses to at least one of the histoplasmins and one of the yeast antigens.

antigens. A second B-Proven subgroup, composed of six individuals who became infected less than 1 year before the study, showed better reactivities in the serological tests than the "old" subgroup.

Discussion

At present, physicians lack a laboratory test capable of detecting subclinical or latent infections by *Histoplasma capsulatum*. Here we evaluated, to our knowledge for the first time, the performance of an IGRA test in detecting the presence in whole blood cultures of *Hc* antigen-specific T cells, as an evidence of a latent infection in asymptomatic individuals.

We decided to test two types of crude preparations of *Hc* antigens: (i) a mycelium culture filtrate (histoplasmin) and (ii) a yeast culture filtrate.[26] Although these crudes come from two different fungal morphotypes, they contain common antigens,[27] in particular the M and the H glycoproteins,[28,29] characterized by their capability to stimulate both the antibody and T lymphocyte-mediated immune responses.[30] It is worth noting that three of these antigen preparations were obtained from three different *Hc* strains that are autochthonous from Colombia.

Both the histoplasmins and the yeast extracts were found to induce highly specific IFN-γ responses, allowing to differentiate between most of the proven cases of histoplasmosis (group B-Proven) and the majority of individuals not exposed to the fungus (groups A1 and A2). In general, the IFN-γ concentrations obtained by stimulation with the yeast filtrates were greater than those induced by the histoplasmins, but, on the other hand, the responses to the mycelium antigens were more homogeneous, showing a lower dispersion. In all cases, the differences between group B-Proven and the negative control groups A1 and A2 were statistically significant.

The IFN-γ concentration cutoff value of 20 pg/ml allowed us to discriminate between positive and negative control groups with high sensitivity and specificity, which ranged between 70–92% and 85–95%, respectively, as evaluated individually for each of the four antigens. The histoplasmin crudes showed a better sensitivity than the yeast filtrates (85–92% vs 70–77%), whereas the specificities were similar. The positive and negative predictive values were also very high: 82–95% and 77–92%, respectively. All of these values are comparable to those reported for the commercially available IGRAs.[14,31]

IGRA versus serological tests

About 37% of the individuals from groups B-Probable and B-Possible showed positive IFN-γ responses to at least one of the *Hc* antigens, and thus could be classified as infected. At this point, it is worth recalling that individuals in group B-Possible did not show antibody titers neither by immunodiffusion nor in the complement fixation assay, so they would be classified as negative according to these serological tests. Thus, the IGRA test may be revealing a latent infection in these individuals, which cannot be detected with the standard serological tests. Interestingly, the proportion of IGRA-positive individuals in groups B-Probable and B-Possible are somewhat higher, but comparable with the numbers published by Carmona (1971)[8], who describes a prevalence of subclinical infection by *Histoplasma capsulatum* in Medellín of about 25%, as detected by the skin test with histoplasmin.

In this study we observed a clear trend for the serological responses to fade with time, in agreement with previous reports describing the disappearance of *Hc*-specific antibodies in individuals that resolved an active histoplasmosis and remain "healthy" through the years.[32,33,34] It is therefore striking to observe the long persistence of a humoral immune response in some individuals, who showed detectable titers of anti-*Hc* antibodies up to 23 years after infection. Such persistence might be due to the presence of latent foci of the fungus,[5] although we cannot discard that some of these individuals could have been in contact with the fungus in more recent occasions, which may have boosted their immune system without causing a recurrence of the disease.

In the IGRA test, in contrast, cellular immune responses were detected in all the individuals from group B-Proven, regardless of the time elapsed since the primary infection, indicating that

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the cellular immune memory to *Histoplasma capsulatum* persists much longer than the humoral memory. A plausible explanation to this observation comes from the fact that this fungus is an intracellular pathogen and, therefore, activates antigen processing and presentation on class II MHC molecules by phagocytes,[35,36] which might produce a recurrent restimulation of a preexisting *Hc*-specific cellular memory.

The results obtained in this exploratory study are very encouraging and pave the road for a further development of a standardized IGRA test capable of detecting subclinical or latent infections by *Histoplasma capsulatum* in asymptomatic individuals, even after many years from the occurrence of the primary infection. More studies are necessary to validate and introduce this IGRA test in the clinical practice, including larger cohorts of individuals and different antigen preparations. This kind of diagnostic test would be of great help in epidemiological studies aiming to evaluate the real magnitude of the infection by *Histoplasma capsulatum* in populations at risk, as is the case in many regions of Colombia.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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