CLINICAL AND VACCINE IMMUNOLOGY, May 2010, p. 802–806 Vol. 17, No. 5
1556-6811/10/$12.00 doi:10.1128/CVI.00043-10

Detection of Latin American Strains of *Histoplasma* in a Murine Model by Use of a Commercially Available Antigen Test▽

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Received 31 January 2010/Returned for modification 3 March 2010/Accepted 23 March 2010

During a *Histoplasma* outbreak in a colony of fruit bats at a southern United States zoo, it was observed that although *Histoplasma* was recovered in culture from multiple sites at necropsy, none of the samples collected from those bats tested positive for *Histoplasma* antigen (HAg). Five of the *Histoplasma* isolates from the bats were subsequently identified as Latin American (LA) clade A, restriction fragment length polymorphism (RFLP) class 6. These observations raised concern as to whether the commercially available HAg test could detect *Histoplasma* antigen not of the North American clade upon which the HAg test had been developed. To evaluate this concern, a murine model of disseminated histoplasmosis was established, and mice were infected with multiple LA *Histoplasma* isolates, including clinical isolates recovered from Brazilian AIDS patients (RFLP class 5 and class 6) and isolates recovered from the bats during the outbreak (RFLP class 6). *Histoplasma* antigen was detected in all infected mice in our experiments, even when *Histoplasma* was not recovered in culture. Because the currently available HAg test is able to detect *Histoplasma* antigen in mice infected with Latin American isolates, this suggests that bat host factors rather than differences among *Histoplasma* RFLP classes were responsible for the inability to detect HAg in infected bats.

Histoplasmosis is the most common endemic pulmonary and systemic fungal infection in the United States. Human infection is established when microconidia are inhaled into the lungs, with dissemination occurring via draining lymph nodes and hematogenous spread throughout the reticuloendothelial system. Most cases of histoplasmosis are mild or asymptomatic; however, with the advent of the AIDS epidemic, symptomatic (especially disseminated) infection has become more commonly seen in clinics, especially in the large metropolitan cities of the midwestern United States (11).

Classically, *Histoplasma capsulatum* has been described as comprising three distinct varieties (*capsulatum*, *duboisii*, and *farciminosum*) that differed in morphology, pathogenicity, clinical manifestations, and geographical distribution. However, molecular techniques are revising our understanding of the current distribution of *Histoplasma* species in the world, as well as allowing further discrimination and typing among *Histoplasma* strains. Recent DNA analysis of four independent protein-coding genes suggests that *Histoplasma* comprises at least seven phylogenetic species that began diverging 3 to 13 million years ago in Latin America (7). Containing four to seven chromosomes, *Histoplasma* has also been divided into six classes based on restriction fragment length polymorphism (RFLP) pattern analysis of the *yps-3* locus, a yeast-phase-specific nuclear gene (8). North American (NA) isolates are predominantly typed as class 2, while Latin American (LA) isolates are predominantly typed as class 5 or 6 by this method. NA and LA strains of *Histoplasma* appear to demonstrate differences in organ tropism, histopathology, disease chronicity, and mortality in experimentally infected mice (2), as well as in clinical presentation and seropositivity for anti-*H. capsulatum* antibodies in AIDS patients (6).

In addition to classic serologic tests, culture, and histopathology, an antigen test is available to help rapidly diagnose histoplasmosis in patients with compatible clinical findings. This test has become commonly used in the United States, especially in HIV-infected patients with disseminated disease.

Many mammals, including bats, are also susceptible to *Histoplasma* infection. An outbreak of histoplasmosis in bats at a southern U.S. zoo afforded the opportunity to examine the utility of the *Histoplasma* antigen (HAg) test to aid in diagnosis. The first cases recognized during the outbreak were of a progressive, wasting illness highlighted by diffuse gastrointestinal involvement which was eventually fatal. The surviving bats in the original colony were treated with itraconazole supplied in their drinking water. Six months after completion of treatment, a subset of 45 bats from the colony, 40 of which were the Egyptian fruit bats, were sacrificed to assess whether the itraconazole treatment had eradicated the disease. While able to suppress overt signs of infection, itraconazole did not eradicate the *Histoplasma*, and necropsy of healthy-appearing bats revealed subclinical disease; 23 of the 40 Egyptian fruit bats had positive cultures from lung and/or spleen. However, HAg tests of these tissue homogenates all returned negative whether or not they produced *Histoplasma* growth in culture. In light of these findings, the decision was made to sacrifice the entire remainder of the colony, which consisted of 116 bats. The

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▽ Published ahead of print on 31 March 2010.

Histoplasma isolates obtained by culture from the tissues of these bats were typed as Latin American clade A by protein sequence analysis and as class 6 by *yps-3* RFLP analysis (Robert Zarnowski, personal communication). Of note, these bats were all descendants of 12 bats that were born in an Ohio zoo and were obtained by the affected zoo in 1987.

Because none of the bats with either overt or subclinical infection had positive HAg testing despite subsequent recovery of *Histoplasma* in culture, this observation raised concern as to whether the currently available test, developed using an NA class 2 clinical isolate, would be able to detect *Histoplasma* infections produced by non-North American strains. We describe the establishment of a murine model of disseminated histoplasmosis to examine the correlation between fungal burden and *Histoplasma* antigen (HAg) detection in infections produced by Latin American strains.

(This work was presented in part at the 45th Annual Meeting of the Infectious Diseases Society of America, San Diego, CA, 4 to 7 October 2007.)

MATERIALS AND METHODS

Fungal burden in naturally infected bats. Serum, urine, lung, and spleen samples were collected from 40 Egyptian fruit bats. Organs were weighed and then ground in Ten Broeck tissue grinders containing 2 ml of RPMI 1640 medium. Homogenates were diluted and plated on brain heart infusion (BHI) agar containing 10% sheep blood. A 0.1-ml aliquot of the 2 ml of undiluted homogenate sample was cultured, representing a detection limit of 20 CFU per organ. Dilutions of the organ homogenates (1:10 for spleen and 1:100 for lung) were made to fall within the working range of the antigen assay. Plates were incubated for 10 days at 30°C, and colony counts were determined. Quantitative culture data were expressed as CFU per gram by dividing the CFU per organ by the organ weights. *Histoplasma* antigen was measured by enzyme immunoassay (EIA) in serum, urine, and the organ homogenates, with the results expressed as EIA units (EU). EIA units were determined by dividing the mean value obtained for each organ by 1.5 times the mean value of the negative controls. Results of ≥1.0 EU are considered positive.

Fungal burden in experimental mouse model. Murine experiments were conducted with 4-week-old female CD-1 (ICR) mice (Harlan Sprague Dawley, Indianapolis, IN) weighing approximately 18 to 22 g, which were housed in six groups of six for each experiment, and given food and water *ad libitum*. This work was performed under a protocol approved by the local institutional animal care and use committee.

Experiment 1: establishment of inoculum size. Yeast-phase inocula were prepared according to previously published procedures (1, 9). Briefly, isolates were grown in human milk macrophage (HMM) medium in a 37°C incubator with shaking at 150 rpm for 48 h. The yeast culture was centrifuged and washed with Hanks' balanced salt solution containing 20 mM HEPES. The inoculum was adjusted with a hemacytometer. Groups of 6 mice (36 total) were infected by intraperitoneal (i.p.) injection using a 22-gauge needle with 0.5 ml containing 10^5, 10^6, or 10^7 yeast cells of an isolate from an infected bat or a North American human clinical control isolate (designated IU-CT, an RFLP class 2 isolate maintained at MiraVista Diagnostics, Indianapolis, IN). On day 14 post-i.p. injection, all mice were anesthetized with inhaled isoflurane and euthanized by exsanguination via cardiac puncture for blood collection. Lungs and spleen were removed aseptically and placed in sterile containers. Fungal burden was assessed by quantitative culture, and antigen levels were determined on lung and spleen as described above.

Experiment 2: comparison of LA versus NA isolates. Based on the results obtained in the first experiment, an i.p. inoculum of 10^7 yeast cells was employed to produce infection in 6 groups of 6 mice each in the second experiment. Infection in this experiment was produced using 6 strains: three Latin American bat isolates (all class 6; previously designated 89, 102, and 137), two Latin American human clinical isolates (one class 5 and one class 6; designated H201 and H381, respectively), and the North American clinical control isolate (class 2; IU-CT). Fungal burden assessment by quantitative culture and antigen level in lung and spleen was performed as described above.

TABLE 1. Antigen levels in tissue, serum, and urine of 40 bats naturally infected with Latin American class 6 *Histoplasma* grouped by organ culture results

Organ culture result (no. of bats)[a]	Antigen testing result (EU) in[b]:		
	Tissue	Serum	Urine
Spleen positive (20)	0.85 ± 0.30	0.61 ± 0.03	0.72 ± 0.13
Spleen negative (20)	0.83 ± 0.20	0.59 ± 0.02	0.67 ± 0.08
Lung positive (10)	0.54 ± 0.09	0.60 ± 0.02	0.72 ± 0.15
Lung negative (30)	0.55 ± 0.12	0.60 ± 0.03	0.69 ± 0.09

[a] The lower limit of detection by culture is 20 CFU per organ.
[b] Antigen testing results are reported as EIA units (EU) ± 1 SD. An EU result of ≥1 is positive.

RESULTS

Fungal burden in naturally infected bats. Samples collected from lung and spleen were labeled as culture positive or culture negative. Antigen levels were determined for lung and spleen homogenates along with serum and urine. Comparisons of antigen levels in culture-positive versus culture-negative organs were not different (Table 1). All organ samples, whether culture positive or culture negative, were negative by antigen testing. Antigen testing was also performed on both serum and urine collected from the 40 bats. Of the 40 bats, 20 had a positive spleen culture, 10 had a positive lung culture, and 7 had both positive spleen and lung cultures. Twenty-three bats had either a positive spleen or lung culture, while 17 bats had both negative spleen and lung cultures. HAg levels in serum and urine were not different between bats with spleen-culture-positive or -negative samples, between bats with lung-culture-positive or -negative samples, or between bats with either the spleen or lung culture positive versus bats with both spleen and lung cultures negative (Table 1).

Fungal burden in experimental mouse model. (i) Experiment 1: establishment of inoculum size. The dose response of inocula was determined by both quantitative culture and HAg level in the spleen (Table 2). Within the lung, no significant difference existed between LA and NA strains by antigen level or by culture (CFU/g) at any inoculum. Within the spleen, no significant difference existed between the isolates by antigen level or by culture at an inoculum of 10^5. At an inoculum of 10^6, a significant difference between the isolates existed by antigen level but not by culture. Using an inoculum of 10^7 yeasts, fungal burden by antigen level and culture was significantly higher with the North American isolate than with the Latin American isolate. Colony counts (CFU/g) for lung and spleen tissue homogenates correlated well with HAg levels (Fig. 1) (NA isolate, $R^2 = 0.768$; LA bat isolate, $R^2 = 0.877$; combined data, $R^2 = 0.741$).

(ii) Experiment 2: comparison of LA versus NA isolates. Having established an inoculum size of 10^7 in experiment 1, experiment 2 examined additional LA bat isolates and LA clinical isolates. The plots for quantitative culture (CFU/g) with corresponding antigen levels for the 6 different isolates used for infection in mice are shown in Fig. 2. For both the lung and the spleen, antigen was detectable in all mice regardless of isolate source, whether NA versus LA or human versus bat isolates. Furthermore, antigen was detectable in those sam-

TABLE 2. Fungal burden in mice experimentally infected by i.p. injection with an LA class 6 isolate recovered from naturally infected bats versus an NA class 2 clinical isolate of *H. capsulatum*

Inoculum	Median fungal burden in[a]:											
	Lung						Spleen					
	HAg (EU)		*P*	Culture (CFU/g organ)		*P*	HAg (EU)		*P*	Culture (CFU/g organ)		*P*
	LA	NA		LA	NA		LA	NA		LA	NA	
10^5	0.92	1.07	0.699	0	170	0.180	0.56	1.30	0.132	286	3912	0.180
10^6	0.66	0.82	0.818	197	48	0.589	0.51	2.42	0.002	1233	1984	0.589
10^7	0.77	1.31	0.180	401	820	0.937	1.96	11.85	0.026	6697	35235	0.041

[a] Data are shown as median values from 6 mouse groups. The Mann-Whitney rank sum test was performed to determine *P* values.

ples that were below the limit of detection for quantitative culture (20 CFU/organ).

DISCUSSION

The outbreak of histoplasmosis in a colony of bats at a southern United States zoo provided an opportunity to examine potential differences between genetically distinct RFLP classes or clades of *Histoplasma*. Antigen detection did not permit identification of subclinical infection among bats in the colony, despite subsequent moderate to heavy growth of *Histoplasma* in culture after sacrifice. The *Histoplasma* antigen detected by the immunoassay is cell wall galactomannan, and the test was developed using a North American class 2 isolate (IU-CT) obtained from an immunosuppressed patient with disseminated histoplasmosis. Initially, it was thought that the strain of *H. capsulatum* infecting the bats had a unique antigen composition that was not recognized by the antibodies used in this assay. Subsequent analysis of the *Histoplasma* isolates recovered from the bats revealed that they were Latin American clade A by protein sequencing analysis and class 6 by *yps-3* RFLP and sequencing analysis, and this seemed to support the initial hypothesis that perhaps antigenic differences were responsible for the inability of the HAg test to detect antigen.

However, our experiments suggest that bat host factors produced negative antigen testing rather than the test itself not being able to detect LA strains. Although infection killed some of the bats in the described colony, it appears many inhibit proliferation of *Histoplasma* but do not kill the yeast, representing a latent infection state in which the bats remain in-

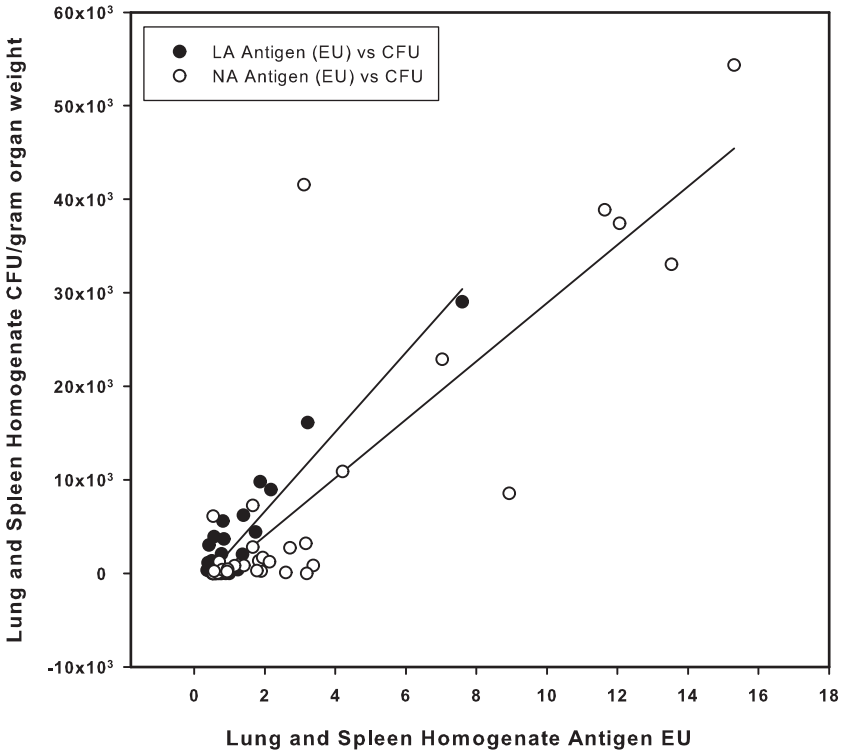

FIG. 1. Correlation of lung and spleen homogenates (CFU/g) and HAg in mice infected with a North American (NA) isolate (open circles) or a Latin American (LA) isolate (black circles). Data are from 36 animals divided into groups of 6 mice infected with three inocula (10^5, 10^6, and 10^7) of each isolate. The regression line reads to the left from the LA data.

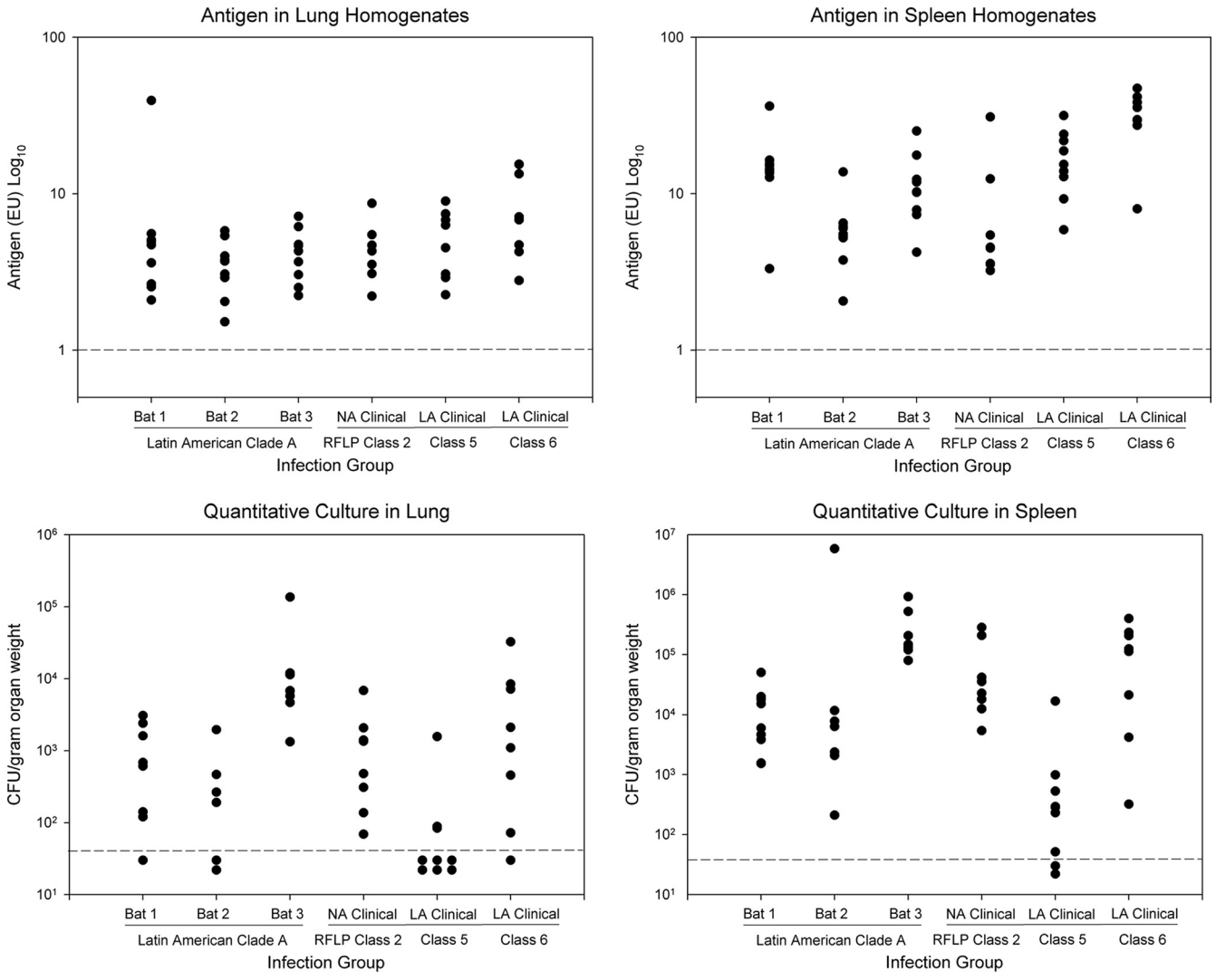

FIG. 2. Quantitative culture and antigen level in mouse lung and spleen produced by 6 isolates used to produce infection with a 10^7-CFU inoculum (groups of 6 mice). The dashed lines on the antigen (upper) and culture (lower) graphs represent the cutoff for a positive antigen value (1 EU) and the lower limit of quantitative culture detection (20 CFU/g), respectively. Note that the line and the points beneath the line of detection are not to scale and have been separated so they may be easily seen. NA, North American isolate; LA, Latin American isolate.

fected but otherwise healthy. Although its pathogenesis in bats remains obscure, *Histoplasma* has several unique features in bats. A wide variation in infection rates occurs among different genera of bats, among different colonies of the same species, and within the same colony in a single harborage over time (10). During natural infection, the intestines appear more extensively involved than other organs. Interestingly, tissue reaction in these bats was minimal or absent and no granulomatous lesions were seen on microscopy in the intestines, lung, liver, or spleen (5). As with natural infection, experimental infection in bats by the intranasal route produced slight tissue reactions of chronic inflammation or focal histiocytosis without granuloma formation (3). Several studies have confirmed that gastrointestinal involvement is a late manifestation of disease and that viable yeast cells can be excreted in bat feces (3). How the bats at the southern U.S. zoo acquired Latin American class 6 *Histoplasma* is unclear. The colony was reported to have been born and raised within the United States.

Human infection with RFLP class 6 isolates has been described, including in AIDS patients with disseminated histoplasmosis from Brazil (6) and Panama (8). Although histopathology, quantitative culture, and seropositivity for anti-*H. capsulatum* antibodies were included in these reports, *Histoplasma* antigen testing was not. Our experience with negative HAg testing in the infected zoo bats and the evolving knowledge that *Histoplasma* likely consists of at least 7 phylogenetic clades or species (7) have raised concern that HAg testing might only be effective in the detection of North American class 2 *Histoplasma*.

Our experiments have shown that in a murine model of *Histoplasma* infection, Latin American class 5 and class 6 *Histoplasma*, although genetically distinct from North American

class 2 *Histoplasma*, is not different enough antigenically to escape detection by an HAg EIA. More recent testing performed with the 3rd generation quantitative *Histoplasma* EIA assay on both urine and serum from 21 AIDS patients in Panama with progressive disseminated histoplasmosis found excellent detection of infection (4). Two isolates recovered from patients (both positive for HAg in serum and urine) in this study were analyzed by RFLP and found to be class 6. This data further supports a hypothesis that the HAg test is effective in the detection of both North American and Latin American infection with *Histoplasma* and that bat host factors were most likely responsible for the initial observation of negative HAg testing in the setting of positive *Histoplasma* cultures.

ACKNOWLEDGMENTS

L. J. Wheat, P. A. Connolly, and M. M. Durkin are employees of MiraVista Diagnostics and MiraBella Technologies, the company that developed and performs the MVista *Histoplasma* antigen EIA.

We thank Robert Zarnowski for performing RFLP analysis of the *Histoplasma* isolates.

The views expressed herein are those of the authors and do not reflect the official policy or position of the Department of the Air Force, Department of the Army, Department of Defense, or the U.S. Government. The authors are employees of the U.S. Government.

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