

# Improved Diagnosis of Acute Pulmonary Histoplasmosis by Combining Antigen and Antibody Detection

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**Background.** Acute pulmonary histoplasmosis can be severe, especially following heavy inoculum exposure. Rapid diagnosis is critical and often possible by detection of antigen, but this test may be falsely negative in 17% of such cases. Antibody detection by enzyme immunoassay (EIA) may increase sensitivity and permit the measurement of immunoglobulin M (IgM) and immunoglobulin G (IgG) classes of antibodies separately.

**Methods.** Microplates coated with *Histoplasma* antigen were used for testing of serum from patients with acute pulmonary histoplasmosis and controls in the MVista *Histoplasma* antibody EIA. Results for IgG and IgM were reported independently.

**Results.** IgG antibodies were detected in 87.5%, IgM antibodies in 67.5%, and IgG and/or IgM antibodies in 88.8% of patients with acute pulmonary histoplasmosis in this assay, while immunodiffusion, complement fixation, and antigen testing showed sensitivities of 55.0%, 73.1%, and 67.5%, respectively (n = 80). Combining antigen and antibody detection increased the sensitivity to 96.3%.

**Conclusions.** The MVista *Histoplasma* antibody EIA offers increased sensitivity over current antibody tests while also allowing separate detection of IgG and IgM antibodies and complementing antigen detection. Combining antigen and EIA antibody testing provides an optimal method for diagnosis of acute pulmonary histoplasmosis.

**Keywords.** acute pulmonary histoplasmosis; *Histoplasma capsulatum*; serology.

Acute pulmonary histoplasmosis following heavy exposure is characterized by systemic and respiratory symptoms typically beginning 5 days to 3 weeks following exposure. Chest radiograph and/or computed tomographic scanning typically show diffuse interstitial and/or alveolar infiltrates, usually accompanied by hilar or mediastinal lymphadenopathy. The illness is often severe, and sometimes fatal [1–5].

The diagnosis of acute pulmonary histoplasmosis is often suspected when multiple individuals present with similar clinical findings following a common activity involving disturbance of a site contaminated with bird or bat guano. However, diagnosis becomes more complicated if the exposed individuals disperse and are unaware that others have similar illness, if a single individual is exposed, or if the individual was not directly involved in the activity that led to exposure. Once suspected, the diagnosis of acute pulmonary histoplasmosis can usually be established promptly by detection of antigen in the urine

or serum. In one study, antigen was present in 83% of acute pulmonary histoplasmosis cases, but 38% would have been missed if only urine was tested [6]. An antibody assay could assist in the diagnosis of cases with negative antigen results, especially those with mild to moderate disease.

## METHODS

### Patient Samples

The serum specimens were available from patients involved in several small histoplasmosis outbreaks [6–13], which were previously reported in part [6] and are described as “clinical cases” because they sought medical care owing to respiratory illness. Specimens were tested from 2 additional patients who were clinically ill in whom information was provided by the treating physician. The histoplasmosis sera obtained from clinical cases included 13 cases from Costa Rica [8], 5 from Belize [9], 1 from Mexico [10], 1 from Nicaragua [7], 3 from Kentucky [6], 3 from Quebec [11], and 2 from Michigan [13]. These 30 patients were classified as clinical cases.

Specimens also were tested from cases that were recruited for participation in epidemiological investigations of outbreaks that had symptoms and laboratory findings meeting the case definition, but only 10% sought medical care [14, 15]. These 50 patients were classified as epidemiological cases. Cases were only included in the epidemiological studies if they met the following criteria: elevated titers of complement fixation (CF) antibodies ( $\geq 1:8$ ), positive antibodies by immunodiffusion (ID), or positive for *Histoplasma* galactomannan antigen [6–8, 11, 14, 15].

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Of the specimens from the Nebraska outbreak in 2012 [15], 20 had an acute and convalescent pair, obtained between 5–6 and 10–12 weeks after exposure, respectively.

Controls included 25 individuals with culture- and/or pathology-proven blastomycosis [16], and 25 individuals with coccidioidomycosis, of which 7 were classified as proven cases based on culture and/or histopathology and 18 as probable cases based on clinical information from the treating physician and positive tests for anti-*Coccidioides* antibodies. Additional controls included 50 healthy individuals from an area endemic for histoplasmosis (Indianapolis, Indiana), 50 healthy individuals from a nonendemic area (Miami, Florida), 50 clinical controls in whom histoplasmosis was not suspected (Sidney & Lois Eskenazi Hospital, Indianapolis, Indiana), and 48 clinical controls in which antigen testing for histoplasmosis was performed and results were negative (Indiana University Health–Methodist Hospital, Indianapolis). Specimens from healthy controls from Miami were purchased from a company that obtains biological specimens from paid blood donors (Biomedical Resources, Long Island, New York). All specimens had been stored frozen at MiraVista Diagnostics at  $-20^{\circ}\text{C}$  prior to testing.

#### Anti-*Histoplasma* Antibody Standards

Standards containing human anti-*Histoplasma* antibodies were prepared from pooled sera positive for IgG and/or IgM antibodies in the MVista antibody EIA. Dilutions of these pools in Starting Block blocking buffer (Thermo Scientific, Rockford, Illinois) were prepared to obtain standard curves for IgG or IgM testing. Each point of the curve was assigned an EIA unit value ranging from 0 to 80 units to allow for semiquantification.

#### Antibody Immunoassay

Nunc Maxisorp (Thermo Fisher) microplates were coated with a proprietary MVista *Histoplasma* antigen, and the procedure was performed as previously described for antibodies to *Blastomyces dermatitidis* [16]. Results were expressed as EIA units by comparison to the standard curve. Reproducibility was investigated by testing specimens on 2 separate days and precision was determined by the appropriate Clinical and Laboratory Standards Institute protocol.

#### Immunodiffusion and Complement Fixation

Anti-*Histoplasma* antibody results by ID and CF were obtained from the US Centers for Disease Control and Prevention (CDC) during the outbreak investigations, where available. If results were not available from the CDC, ID was performed on residual specimens at MiraVista Diagnostics according to the manufacturer's instructions using commercially available reagents (Meridian Biosciences, Cincinnati, Ohio), and CF was performed at the Indiana University Health Department of Pathology, Indianapolis.

#### Antigen Enzyme Immunoassay

*Histoplasma* antigen in urine and in serum was determined by testing in the MVista *Histoplasma* quantitative antigen EIA

[17], incorporating ethylenediaminetetraacetic acid (EDTA) heat pretreatment of serum to dissociate antigen-antibody complexes and destroy the dissociated antibody, a procedure that improves the sensitivity for detection of antigen in serum [18].

#### Statistical Analysis

SigmaPlot statistical analysis software (Systat Software, San Jose, California) was used for transformation of optical density (OD) values from individual serum samples into EIA unit values based on the standard curve. Receiver operating characteristic (ROC) curve analysis was performed to determine the cutoff for positivity that would give the optimal sensitivity and specificity. Linear regression analysis was used to analyze reproducibility and precision according to the Passing and Bablok method. Paired *t* test was used to compare the paired IgM and IgG acute and convalescent samples.  $\chi^2$  statistical analysis was used to compare diagnostics methods in the clinical and epidemiological cases (MedCalc for Windows, version 12.3.0, Ostend, Belgium). *P* values  $<.05$  were considered statistically significant.

#### Ethical Considerations

The specimens for the patients with histoplasmosis were available from investigations that were previously reported ( $n = 78$ ) or from physicians caring for the patients ( $n = 2$ ). The control specimens were available from studies that were approved by the institutional review board at the participating institution.

## RESULTS

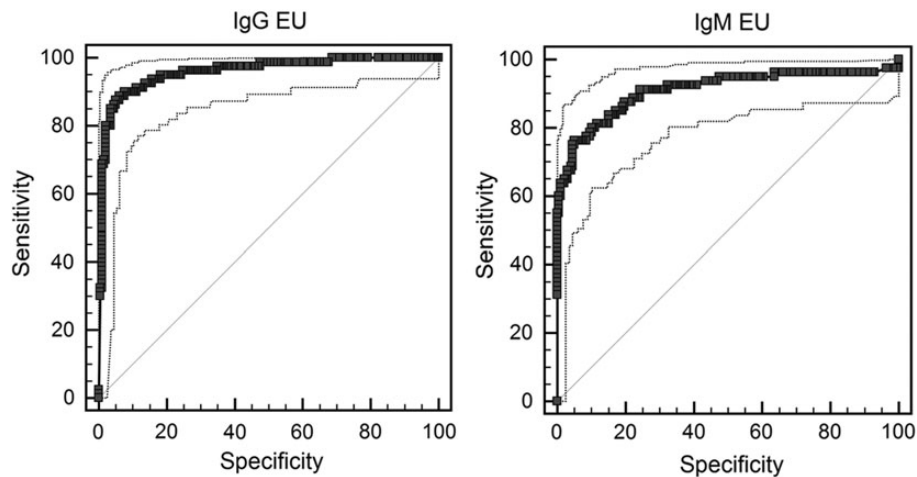
#### Sensitivity and Specificity

ROC analysis determined the optimal cutoff for IgG antibody detection to be an OD of 0.200 (assigned to 10 EIA units). At this cutoff, sensitivity was 87.5% ( $n = 80$ ) and specificity 95.0% ( $n = 198$ ), area under the curve was 0.959 (95% confidence interval [CI], .929–.979), and the standard error was 0.0132 ( $P < .0001$ ). ROC analysis determined the optimal cutoff for IgM antibody detection to be an OD of 0.310 (assigned to 10 EIA units). At this cutoff, sensitivity was 67.5% ( $n = 80$ ) and specificity 97.0% ( $n = 198$ ), area under the curve was 0.910 (95% CI, .870–.941), and the standard error was 0.0243 ( $P < .0001$ ) (Figure 1). When IgG and IgM antibody results were combined, the sensitivity and specificity were 88.8% and 91.9%, respectively.

#### Clinical Cases, Epidemiological Cases, Controls, and Cross-reactivity

Antibody levels in the 80 samples from clinical and epidemiological cases ranged from undetectable to  $>80$  units, with an average of 49.1 units (IgG) and 38.4 units (IgM). IgG antibodies were positive in 70 of 80 samples (87.5%) (Figure 2). IgM antibodies were positive in 54 of 80 samples (67.5%). IgM antibodies were detected in a similar proportion of clinical (63.3%) and epidemiological (70.0%) cases ( $P = .624$ ), but IgG antibodies were detected more frequently in epidemiological (96%) than in clinical (73.3%) cases ( $P = .005$ ).

IgG antibody levels were elevated in 5.0% of the healthy and clinical controls, and IgM antibodies were elevated in 3.5%



**Figure 1.** Receiver operating characteristic (ROC) curve for determination of anti-*Histoplasma* immunoglobulin G (IgG) and immunoglobulin M (IgM) antibody cutoff. The ROC-recommended cutoff optical density (OD) was 0.200 for IgG (assigned to 10 enzyme immunoassay [EIA] units [EU]). At this cutoff, sensitivity for IgG was 87.5% (n = 80) and specificity 95.0% (n = 198), area under the curve (AUC) was 0.959 (95% confidence interval [CI], .929–.979), and the standard error was 0.0132 ( $P < .0001$ ). The ROC-recommended cutoff OD was 0.253 for IgM (assigned to 10 EU). At this cutoff, sensitivity for IgM was 70.3% (n = 80) and specificity 97.0% (n = 198), AUC was 0.879 (95% CI, .833–.916), and the standard error was 0.037 ( $P < .0001$ ).

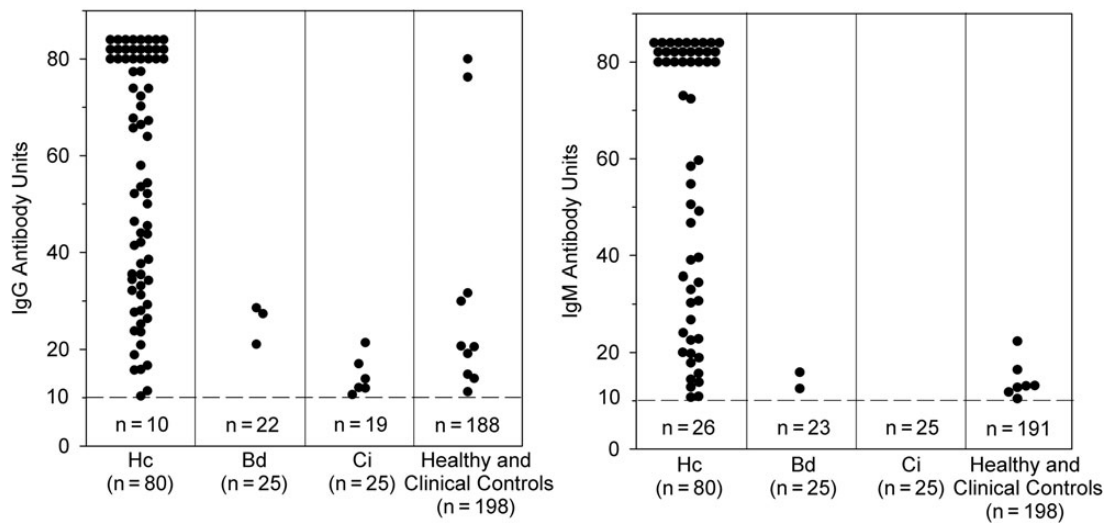
(Figure 2). Two of the endemic controls had high IgG results of 76 and >80 units, neither of whom had positive results for IgM antibodies. Cross-reactivity for IgG antibodies was seen in 12% of patients with blastomycosis and 24% with coccidioidomycosis. Cross-reactivity for IgM antibodies occurred in 8% of patients with blastomycosis but none with coccidioidomycosis.

#### Comparison to Other Methods

Compared to the EIA results, which were positive in 88.8% of cases, ID was positive less frequently, in 55.0% of cases (44 of

80) ( $\chi^2 = 22.54$ ;  $P < .001$ ). CF titers for the yeast or mycelial antigens were positive with titers of  $\geq 1:8$  in 49 of 67 (73.1%) patients, also less frequent than elevated antibody levels by EIA ( $\chi^2 = 5.93$ ;  $P = .015$ ). CF results were unavailable in 13 patients, including 4 in which the serum was anticomplementary and 9 in which volume was insufficient.

*Histoplasma* antigen was detected in the urine in 32 of 75 (42.7%) cases and in the serum in 50 of 79 (63.3%) cases for whom testing was performed. Overall *Histoplasma* antigen was detected in 54 of 80 patients (67.5%), lower than IgG



**Figure 2.** Immunoglobulin G (IgG) and immunoglobulin M (IgM) response in MVista *Histoplasma* antibody enzyme immunoassay. Antibody levels in patients with histoplasmosis (Hc) (n = 80), blastomycosis (Bd) (n = 25), or coccidioidomycosis (Ci) (n = 25), and healthy patients or clinical controls (n = 198). The cutoff for positivity (10 units) is indicated by the broken horizontal line and the numbers below the broken line represent the number of patients with negative results.

**Table 1. Comparison of Clinical and Epidemiological Cases Within Test Methods**

Test	All	Clinical	Epidemiological
MVista <i>Histoplasma</i> IgG Antibody EIA	70/80 (87.5)	22/30 (73.3)	48/50 (96.0)
MVista <i>Histoplasma</i> IgM Antibody EIA	54/80 (67.5)	19/30 (63.3)	35/50 (70.0)
MVista IgG or IgM Antibody EIA	71/80 (88.8)	23/30 (76.7)	48/50 (96.0)
<i>Histoplasma</i> CF Yeast or Mycelial $\geq 1:8$	49/67 (73.1)	12/18 (66.7)	37/49 (75.5)
<i>Histoplasma</i> ID	45/80 (56.3)	11/30 (36.7)	34/50 (68.0)
<i>Histoplasma</i> CF or ID	65/80 (81.3)	19/30 (63.3)	46/50 (92.0)
MVista <i>Histoplasma</i> Urine Antigen EIA	32/75 (42.7)	14/26 (53.8)	18/49 (36.7)
MVista <i>Histoplasma</i> Serum Antigen EIA	50/79 (63.3)	22/30 (73.3)	28/49 (57.1)
MVista Urine or Serum Antigen EIA	54/80 (67.5)	23/30 (76.7)	31/50 (62.0)
MVista Antigen or Antibody EIA	77/80 (96.3)	27/30 (90.0)	50/50 (100.0)

Data are proportion (%) of persons with positive results.

Abbreviations: CF, complement fixation; EIA, enzyme immunoassay; ID, immunodiffusion; IgG, immunoglobulin G; IgM, immunoglobulin M.

and/or IgM antibodies by EIA (88.8%) ( $\chi^2 = 10.57$ ;  $P = .001$ ). When combining antigen and IgG and/or IgM antibody results, positive results were noted in 77 of 80 patient samples (96.3%; Table 1).

The MVista *Histoplasma* antibody EIA was more sensitive in the epidemiological cases (96.0%) than in the clinical cases (76.7%) ( $P = .012$ ). A statistical increase in sensitivity was seen over CF, ID, and antigen in the epidemiological cases ( $P = .003$ ,  $P < .001$ , and  $P < .001$ , respectively) and over ID within the clinical cases ( $P = .002$ ) (Table 1). The MVista antigen or MVista antibody EIA was positive in 27 of 30 clinical (90%) and in all 50 epidemiological cases (100.0%).

#### Precision and Reproducibility

Of the histoplasmosis, blastomycosis, and coccidioidomycosis cases and healthy subject samples, results were reproducibly positive or negative in 195 of 197 instances for IgG (99.0%) and 191 of 197 instances for IgM (97.0%). Comparison of initial and repeat IgG results in 47 histoplasmosis samples by linear regression showed strong correlation with a coefficient of determination ( $R^2$ ) of 0.978, residual standard deviation of 3.638, 95% slope CI of .949–1.032, and  $P < .01$ . Comparison of initial and repeat IgM results in 47 histoplasmosis samples by linear regression showed strong correlation with a coefficient of determination ( $R^2$ ) of 0.994, residual standard deviation of 2.235, 95% slope CI of 1.008–1.021, and  $P < .01$  (Figure 3; Passing and Bablok method).

#### IgM Levels in Acute and Convalescent Pairs

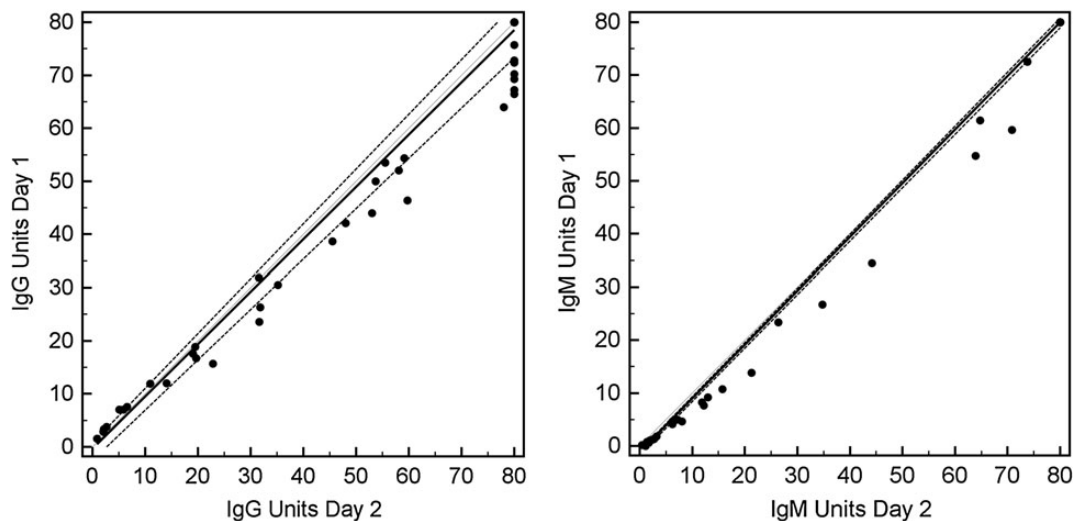
Levels of IgM antibodies were evaluated in acute (5–6 weeks postexposure) and convalescent (10–12 weeks postexposure) pairs from 16 patients with acute pulmonary histoplasmosis. In 15 of 16 (93.8%) patients, the IgM levels dropped significantly in the convalescent sample compared with the level in the acute sample (acute mean,  $64.4 \pm 23.4$  units; convalescent mean,  $20.3 \pm 21.0$  units;  $P < .0001$ ) (Table 2). No detectable decrease was seen in 1 of 16 patients, but the results in the acute

and convalescent samples were both above the upper limit of quantification. Six of the 16 patients had positive IgM levels in their acute sample that became negative in the convalescent sample. An additional 4 patients had negative IgM levels in the acute sample, and all corresponding convalescent IgM levels also remained negative. IgG levels in these acute and convalescent samples demonstrated no significant changes in any of the 20 patients: samples were positive or negative at both time points (acute mean,  $52.6 \pm 25.4$  units; convalescent mean,  $56.0 \pm 24.2$  units;  $P = .529$ ).

#### DISCUSSION

The MVista antibody EIA offers advantages over the currently available tests for the diagnosis of histoplasmosis. In this study, increased sensitivity compared to ID, CF, and antigen testing was shown. This result was demonstrated despite the requirement that patients have a positive result in at least 1 of those tests for inclusion in the study, which could potentially artificially inflate their sensitivities. In addition, this assay offers separate detection of IgG and IgM antibodies. This assay may aid in the diagnosis of acute pulmonary histoplasmosis, which can be difficult to diagnose due to false-negative results in antigen tests, ID, CF, and cytopathology and histopathology of respiratory specimens [6].

The MVista antibody EIA showed high sensitivity (88.8%) in this study. The sensitivity was 76.7% in the clinical cases, which is similar to that reported previously [6]. The current study included cases that were identified during epidemiological investigations of large outbreaks [14, 15]. The sensitivity for detection of IgG or IgM antibody was 76.7% in clinical cases compared with 96.0% in the epidemiological cases [14, 15]. The difference in antibody sensitivity between these 2 groups can likely be attributed to the length of time between exposure and testing and perhaps the severity of infection. Patients exposed to a larger amount of inoculum may present for clinical care sooner, before antibody levels become detectable.



**Figure 3.** Reproducibility by linear regression. Interassay agreement of immunoglobulin G (IgG) unit values obtained from repeat testing of histoplasmosis patient samples showed strong correlation with a coefficient of determination ( $R^2$ ) of 0.978, a residual standard deviation of 3.638, a 95% slope confidence interval (CI) of .949–1.032, and a slope  $P$  value of  $<.01$ . Interassay agreement of immunoglobulin M (IgM) unit values obtained from repeat testing of histoplasmosis patient samples showed strong correlation with a  $R^2$  of 0.994, a residual standard deviation of 2.235, a 95% slope CI of 1.008–1.021, and a slope  $P$  value of  $<.01$  (Passing and Bablok method).

Similarly, antigen sensitivity may also be influenced by exposure to a larger amount of inoculum, which likely causes more extensive pulmonary disease and a higher fungal burden, and thus higher antigen concentrations. The highest sensitivity was achieved by testing for antigen and IgG and IgM antibody—96.3% overall, 90% for clinical cases, and 100% for epidemiological cases—emphasizing the importance of testing for both.

Positivity within healthy or clinical controls and patients with other mycoses occurs in most methods used for the diagnosis of histoplasmosis [19–22]. In this study, 2 control patients from the endemic area showed high IgG results. This is likely attributed to a recent but unrecognized infection within the past 1–2 years, as previous studies have shown background positivity rates by CF of up to 5% [22]. Cross-reactivity in specimens from patients with blastomycosis was 20%, a significant

**Table 2. Comparison of Immunoglobulin M Levels in Acute and Convalescent Pairs**

Patient	Acute			Convalescent		
	Specimen Date	IgM EU	Result	Specimen Date	IgM EU	Result
1	29 June 2012	66.1	+	8 August 2012	4.7	–
2	29 June 2012	>80	+	8 August 2012	6.3	–
3	30 June 2012	>80	+	8 August 2012	22.8	+
4	29 June 2012	22.3	+	8 August 2012	4.0	–
5	29 June 2012	>80	+	9 August 2012	23.7	+
6	29 June 2012	63.4	+	8 August 2012	6.4	–
7	29 June 2012	>80	+	8 August 2012	11.6	+
8	29 June 2012	>80	+	8 August 2012	48.4	+
9	29 June 2012	13.1	+	8 August 2012	2.1	–
10	2 July 2012	57.5	+	8 August 2012	11.1	+
11	29 June 2012	68.7	+	8 August 2012	18.9	+
12	5 July 2012	74.8	+	9 August 2012	15.4	+
13	29 June 2012	>80	+	8 August 2012	>80	+
14	2 July 2012	23.7	+	8 August 2012	7.0	–
15	29 June 2012	>80	+	8 August 2012	17.2	+
16	30 June 2012	>80	+	9 August 2012	45.3	+

Initial histoplasmosis outbreak exposure dates: 21–25 May 2012. Results of  $\geq 10.0$  EU are considered positive.

Abbreviations: EU, enzyme immunoassay antibody units; IgM, immunoglobulin M.

improvement over antigen detection, where cross-reactivity is almost universal [17]. Furthermore, positive results in patients with blastomycosis and coccidioidomycosis were all low, with unit values between 10.6 and 28.6, whereas 78.8% of patients with histoplasmosis had IgG or IgM levels >30 units (Figure 2).

Another concern with antibody detection is differentiation of current and past infection. Antibody levels may persist for several years after clinical recovery, especially if the individual lives in an endemic area and experiences recurrent exposure [23, 24]. A previous study, with follow-up testing of 13 immunocompetent individuals at 1 year postexposure, showed that IgG antibodies persisted in 7, whereas IgM antibodies cleared in all individuals [25]. To our knowledge, this antibody assay is the only available test that detects and reports IgG and IgM antibodies separately to help identify current infections. In this study, 94% of the convalescent samples showed a statistical reduction in IgM levels compared with their acute sample, and 37.5% converted from positive to negative in the 10- to 12-week timeframe for follow-up testing (Table 2).

There are several limitations of this study including limited clinical information, incomplete diagnostic testing, performance of ID and CF at >1 institution, background positivity, and lack of additional specimen for monitoring changes over a longer interval. The antibody EIA results were negative in 11.2% of patients with histoplasmosis. Several factors may contribute to these false-negative results. First, antibody production may require >1 month to reach detectable levels following exposure [22], and most of the samples used in this study were obtained between 1 and 2 months following exposure. Detectable IgM levels may develop earlier; however, in this study, specimens from only 1 of 80 patients with histoplasmosis were positive for IgM but negative for IgG antibodies. Perhaps a greater advantage for IgM antibody detection would have occurred if specimens were obtained earlier following exposure. Second, the *Histoplasma* antigens used in the antibody EIA may not contain epitopes recognized by the antibodies produced in some patients. At least 7 genetically distinct geographical phylogenetic species of *Histoplasma* exist, each of which has different gene expression patterns, which may affect the antibody production of the host [26–29]. Third, the anti-*Histoplasma* antibodies recognized in this assay may be competing with antigens in the specimen and therefore may not be free for detection by EIA. Finally, some patients may not be able to mount an antibody response.

In conclusion, the MVista antibody EIA has the potential to complement antigen detection in the diagnosis of acute pulmonary histoplasmosis, identifying cases that are falsely negative by ID and/or CF, and helping to differentiate histoplasmosis from related mycoses. Furthermore, separate detection of IgG and IgM antibodies may help to distinguish current from past infection. A combination of antibody and antigen testing may provide the highest diagnostic yield for acute pulmonary histoplasmosis.

## Notes

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**Potential conflicts of interest.** L. J. W. is a medical director and owner of MiraVista Diagnostics. S. M. R., M. L. S., M. M. D., and L. J. W. are employees of MiraVista Diagnostics and intend to offer the described test commercially. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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