Evaluation of two new enzyme immunoassay reagents for diagnosis of histoplasmosis in a cohort of clinically characterized patients

Chen Zhang1, Guang-Sheng Lei1, Chao-Hung Lee1 and Chadi A. Hage2,*

1Department of Pathology and Laboratory Medicine and 2Thoracic Transplantation Program, Indiana University Health, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN

*To whom correspondence should be addressed. Chadi A. Hage, MD, Assistant Professor of Medicine, Indiana University, Thoracic Transplantation Program, Indiana University Health, Methodist Professional Center-2, 1801 North Senate Boulevard, Suite 2000, Indianapolis, IN 46202, Tel: +317-962-5820; E-mail: chage@iu.edu

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Abstract

The performance characteristics of the recently available analyte-specific reagent based enzyme immunoassay (ASR-EIA) and in vitro diagnostic (IVD) kit for urine Histoplasma antigen detection were evaluated in a cohort of 50 clinically characterized patients with histoplasmosis and 50 control patients. Overall sensitivity and specificity of the ASR-EIA were significantly improved compared with those of the IVD kit (sensitivity 72% vs. 22%, P < .001, specificity 98% vs. 84%, P = .014). Fourteen specimens from patients with clinically characterized histoplasmosis (five with pulmonary histoplasmosis and nine with progressive disseminated histoplasmosis) were falsely negative by ASR-EIA. All 10 specimens from patients with severe symptoms of progressive disseminated histoplasmosis were positive by ASR-EIA, although the average reading value of these 10 specimens was not significantly different from that of others with positive results. Compared to the MiraVista antigen assay, both the IVD kit and the ASR-EIA were significantly less sensitive in detecting Histoplasma antigen in the urine of patients with histoplasmosis. The ASR-EIA and MiraVista assay had comparable specificity. In conclusion, the ASR-EIA has improved performance compared with the IVD kit in the detection of Histoplasma antigen in the urine. However, users should be aware of the potential for false negative results using the currently recommended cutoff value.

Key words: histoplasmosis, EIA, Histoplasma galactomannan.

Introduction

A multifaceted approach is recommended by various medical societies for the diagnosis of histoplasmosis [1,2]. Parameters to be considered include clinical symptoms, radiographic findings, treatment responses, and laboratory tests such as histopathological examination, culture, and antigen and antibody detection [3–6]. Histopathological examination and culture have limited utility in the rapid diagnosis of histoplasmosis due to their relatively low sensitivity and the long incubation times required for culture (up to 4 weeks). A positive antibody test can be due to prior and resolving infection, usually appears 3–4 weeks after the initial infection [7] and is often not observed in immunocompromised patients [8,9]. Molecular methods using nucleic
acid amplifications are useful in detecting *Histoplasma* in biopsies and in expediting culture confirmation of *Histoplasma*, but are not sensitive in detecting *Histoplasma* from the noninvasive clinical samples such as urine and serum [10,11]. *Histoplasma* antigen detection is the most commonly used laboratory test for the rapid diagnosis of histoplasmosis, especially in acute and disseminated infections [12,13]. Enzyme immunoassay (EIA) using polyclonal antibodies against *Histoplasma* galactomannan developed by MiraVista Diagnostics (Indianapolis, IN) has been widely studied and is used for diagnosis of the disease and monitoring the response to treatment [14]. The clinical sensitivity and specificity of this assay have been well characterized in various patient populations [9,14–17]. The major limitation of the MiraVista assay is that it is not commercially available and sending out the samples for testing increases clinical turnaround time.

The in vitro diagnostic (IVD) assay for *Histoplasma* antigen (IMMY, Norman, OK) was approved by the Food and Drug Administration (FDA) and has been commercially available since 2007. This assay uses polyclonal antibodies and has been developed into a standardized analytical kit format. However, publications to date suggest that the sensitivity of the IVD assay is low, limiting its utility in diagnosis of histoplasmosis and monitoring response to treatment [18]. An addition to the IVD kit, analyte-specific reagents (ASR) using monoclonal antibodies against purified *Histoplasma* galactomannan have recently become available (IMMY, Norman, OK). Two recently published studies [18,19] showed that the ASR based EIA assay results correlated well with those in the MiraVista (MVista) *Histoplasma* antigen test. The performance of the ASR-EIA has not been systematically evaluated in specimens from patients with confirmed histoplasmosis. Theel et al. [19] compared results of the ASR-EIA with those of the MVista EIA in a large number of retrospectively collected specimens and showed an overall agreement of 97.6% (979/1,003 samples). However, the determination of sensitivity and specificity of the ASR-EIA were compromised due to the lack of clinical information for the specimens. A subsequent study [20] by the same group investigated the clinical significance of low-positive *Histoplasma* urine antigen results. The performance characteristics of the ASR-EIA were further evaluated in their recent prospective study with limited numbers of clinically confirmed histoplasmosis cases (17 of 150 cases) [21]. Zhang et al. [18] published a similar study that demonstrated an overall agreement of 95% between ASR and MVista EIAs. Since only 21 histoplasmosis cases were evaluated in this study, the analytical statistics were less convincing.

In the current study, we evaluated the performance of the FDA-approved IVD kit and the ASR-EIA for *Histoplasma* antigen detection in the urine of a cohort of confirmed and clinically characterized patients with histoplasmosis as well as control cases.

### Materials and methods

#### Study cohort

Fifty previously reported patients with histoplasmosis [9] were included in the study. Urine specimens were obtained from patients evaluated at Indiana University Health Medical Center from November 2005 through December 2009, and stored at −80°C until used. All the specimens were tested for *Histoplasma* antigen at MiraVista Diagnostics during the period of 2005–2009 and retested in 2009 for a multicenter study, which confirmed the original results [9]. The samples were retested in the MiraVista assay in 2014 when this study was performed. All of the positive results were reproducible and there was no evidence of antigen degradation. Results of the other diagnostic tests including culture, cytology and/or histopathology examination, and antibody detection using immunodiffusion (ID) and complement fixation (CF), were obtained by review of medical record. The CF uses both the yeast and mycelial phase antigens. These tests were performed at the originating institution or other commercial laboratories. The study protocol was approved by the Indiana University institutional review board.

The criteria for diagnosis included a positive test result of culture, antigen, histopathology, cytology, or *Histoplasma* antibody tests demonstrating H or M precipitin bands by ID or titers of CF antibodies greater than eight and compatible clinical and radiographic findings. Positive culture, cytology, or histopathology results demonstrating yeast-like structures characteristic of *Histoplasma capsulatum* were required for classification as proven disease, whereas positive antigen or antibody test results were required for classification as probable histoplasmosis, combined with compatible clinical and radiographic findings [1,4,22].

Cases were excluded if there was no clinical information available, if histoplasmosis was not diagnosed according to the medical record, or if the specimens were not stored properly.

Control specimens were obtained from 50 patients evaluated at Indiana University Health Medical Centers in whom the diagnosis of fungal infection was excluded. Two other groups of controls included urine samples from 13 cases of clinically confirmed aspergillosis with positive results for *Aspergillus* galactomannan in serum or bronchoalveolar lavage fluid and 10 cases of clinically confirmed blastomycosis were also included.
Measurement of urine Histoplasma antigen using IMMY IVD

The FDA-cleared IVD assay kit (Alpha Histoplasma Antigen EIA) was purchased from Immuno Mycologics, Inc. (IMMY, Norman, OK). This kit utilizes a rabbit polyclonal antibody for both capture and detection and is approved for use on urine specimens. The assay was performed according to manufacturer’s instructions without any modifications, and all data were generated from assays that met quality control (QC) criteria specified in the product insert.

Measurement of urine Histoplasma antigen using IMMY H. capsulatum GM ASR EIA

The ASR and related EIA reagents were purchased from IMMY (Norman, OK). Undiluted urine (0.1 ml) was added directly to microtiter wells coated with monoclonal antibody for H. capsulatum galactomannan (GM). After incubation and washing, horseradish peroxidase (HRP)-conjugated anti-GM monoclonal antibody was added and incubated. Excess conjugate was removed, and the bound HRP-conjugated antibody was detected with the addition of 3,3,5,5-tetramethylbenzidine (TMB). The optical density (OD) of the reaction mixture was measured at dual excitation wavelengths of 450/620 nm on a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA). The OD results were compared to a standard curve generated with seven calibrator solutions (0.4, 0.8, 1.6, 3.2, 6.3, 12.5, and 25 ng/ml of GM) to determine the quantitative value and to obtain the associated qualitative interpretation. According to manufacturer’s recommendation, the linearity of this assay ranged from 0.4 ng/ml to 25 ng/ml, and values ≥ 0.5 ng/ml were considered positive. The quantitative values below 0.4 ng/ml were calculated using the standard curve formula, in order to allow comparison with previous publication by Theel et al. [21] in which an “indeterminate” category was introduced for specimens with values falling between 0.11 ng/ml and 0.49 ng/ml.

Statistical analysis

Sensitivity and specificity were calculated and compared between two tests using the chi square test. Continuous variables were compared between groups using Student t-test. An overall significance level of alpha = .05 was used for all comparisons.

Results

Patient characteristics

Of the 50 histoplasmosis cases, 22 were classified as proven histoplasmosis, including 18 cases with positive culture on blood, bone marrow, or body fluids, and four cases with H. capsulatum-like yeasts on histopathology and/or cytology examinations. The remaining twenty-eight cases were classified as probable histoplasmosis according to the aforementioned criteria. Based on clinical presentations, 12 cases were pulmonary histoplasmosis without signs of progressive dissemination or severe symptoms; 38 cases were classified as progressive disseminated histoplasmosis (PDH), and 10 of the 38 PDH cases were clinically severe.

Histoplasma antigen was detected in 48 of the 50 urine specimens in the MiraVista assay (sensitivity 96%). None of the negative controls tested positive in the MiraVista assay (specificity 100%). One of 13 Aspergillosis controls had a positive reaction with a value of below limit of quantification (BLQ) in MiraVista assay. Seven of 10 blastomycosis controls tested positive in MiraVista assay.

Performance characteristics of the IMMY ASR EIA and IMMY IVD kit

The results of the IMMY ASR EIA and IVD assays are summarized in Table 1. Eleven of the 50 histoplasmosis cases (sensitivity 22%), five of 22 proven cases (22.7%), and six of 28 probable cases (21.4%) were positive in the IVD assay. Forty-two of the 50 control specimens were negative (specificity 84%). The positive and negative predictive values for the IVD assay were 58% and 52%, respectively.

Table 1. Performance characteristics of ASR-EIA and IVD kit.

<table>
<thead>
<tr>
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<th>ASR-EIA</th>
<th>IVD kit</th>
<th>MVD</th>
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<tbody>
<tr>
<td></td>
<td>Overall</td>
<td>72% (36/50)*</td>
<td>96% (48/50)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>Proven</td>
<td>81.8% (18/22)†</td>
<td>22% (5/22)†</td>
</tr>
<tr>
<td></td>
<td>Probable</td>
<td>64.3% (18/28)†</td>
<td>21.4% (5/24)†</td>
</tr>
<tr>
<td>Specificity</td>
<td>Overall</td>
<td>98% (49/50)*</td>
<td>84% (42/50)*</td>
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*P<.05, compared with IVD kit result in the same category.
†P<.05, compared with MVisa test result in the same category.

Abbreviations: ASR-EIA, analyte-specific reagent based enzyme immunoassay; IVD, in vitro diagnostic, MVD MiraVista Diagnostics.
The sensitivity of the IVD kit was 30% (3/10) for specimens from patients with severe PDH, and 8% (1/12) for those from patients with pulmonary histoplasmosis.

Specimens from 36 of 50 histoplasmosis cases (sensitivity 72%), 18 of 22 proven cases (81.8%), and 18 of 28 probable cases (64.3%) tested positive in the ASR EIA. Forty-nine of 50 control specimens were negative in the ASR EIA (specificity 98%). The positive and negative predictive values for ASR EIA were 95% and 79%, respectively.

The sensitivity of the ASR test was 100% (10/10) for specimen from severe PDH patients, 68% (19/28) for specimen from nonsevere PDH patients, and 58% (7/12) for those from patients with pulmonary histoplasmosis. The ASR-EIA values of the specimen from 10 patients with severe PDH (27.9 ± 29.5 ng/ml) were not significantly different from those of the other positive specimens (13.5 ± 19.4 ng/ml, P = .09) or average value of the overall cohort (17.5 ± 23.1 ng/ml, P = .24).

None of 13 aspergillosis controls, and 6 of 10 blastomycosis controls tested positive in ASR-EIA. The IVD kit was not evaluated in the aspergillosis and blastomycosis controls, since a previous study [18] showed that the analytical specificities against related pathogenic fungi, including Blastomyces and Aspergillus, were the same in the ASR-EIA and IVD kit.

Overall sensitivity and specificity of ASR-EIA were significantly improved compared with those of the IVD kit (sensitivity 72% vs. 22%, P < .001, specificity 98% vs. 84%, P = .036). Compared to the MiraVista antigen assay, both the IVD kit and the ASR-EIA were significantly less sensitive in detecting Histoplasma antigen in the urine of patients with histoplasmosis (P < .001 for IVD and P = .003 for ASR-EIA). The ASR-EIA and the MVista EIA had comparable specificity. Both the ASR-EIA and MVista EIA had high specificity in samples from aspergillosis patients (100% in ASR and 92% in MVD, P = .84) and frequent cross-reactions (60% in ASR and 70% in MVD, P = .18) in samples from blastomycosis patients. The IVD was significantly less specific than the MVista EIA (P = .009).

Patient characteristics of the false negative cases by IMMY ASR-EIA

Clinical information of the 14 false negative cases is summarized in Table 2. Four biopsy or culture proven histoplasmosis cases and 10 probable cases with positive antibody and/or antigen tests combined with clinical and radiographic evidences were falsely negative. Five of the 14 cases were pulmonary histoplasmosis without evidence of dissemination, and nine cases were PDH (none with severe symptoms). Two specimens from these 14 cases were also negative in the MVista assay, and two other specimens were below the limit of quantification by the MVista Histoplasma antigen test. The MVista Histoplasma urine antigen from the other 10 patients ranged from 0.41 to 6.16 ng/ml (median 1.08 ng/ml, mean 2.14 ng/ml). Eleven of 12 discrepancy cases between ASR-EIA and MVista had
low antigen levels between 0.1 and 0.4 ng/ml by ASR-EIA, and one was due to failure to detect antigen by ASR-EIA.

Discussion

Our study evaluated the performance of the newly available ASR-EIA and the FDA-approved IVD kit for the detection of urine Histoplasma antigen, using stored specimen from a cohort of clinically characterized cases of histoplasmosis and controls. We found that the new ASR-EIA had improved sensitivity and specificity in the detection of Histoplasma antigen in urine, compared to the FDA cleared IVD kit (Table 1). Both IVD and ASR-EIA were significantly less sensitive than the MVista test in detecting Histoplasma antigen in the urine of patients with histoplasmosis. These findings are in agreement with those of Zhang et al. [18], although the sensitivities for ASR EIA and IVD kit were higher in their study (90.5% and 61.9%, respectively). This discrepancy may be caused by the variation in sample size and patient characteristics. The study by Zhang et al. was based on 14 clinically confirmed cases of histoplasmosis, including nine with acute histoplasmosis and 12 cases of histoplasmosis tested as part of treatment follow-up. The sensitivities of the ASR EIA and IVD kit in the 12 follow-up patients were 83.3% and 41.6%, respectively. Although the exact measurement values and reference lab values were not provided, it is conceivable that antigen concentrations in the follow-up specimens were generally lower than in those of acute stage specimens. It is also likely that the sensitivities of both assays are low in specimen from patients with lower levels of urine antigen. Our study cohort consisted of a larger group of clinically characterized patients with histoplasmosis. Twelve of them were diagnosed as pulmonary histoplasmosis without signs of dissemination and 10 had severe PDH. The diverse distribution of disease spectrum in our study cohort reflects the more common scenario in real life clinical practice. Our finding of low detection sensitivity of the IVD kit reflects its limited clinical utility in the diagnosis of histoplasmosis.

Compared with the IVD kit, the ASR-EIA showed significantly improved sensitivity and specificity. This could be due to the different antibodies used for the detection of Histoplasma antigen in the two tests. The IVD kit uses a polyclonal antibody that detects a Histoplasma polysaccharide antigen (HPA) and the ASR EIA uses a monoclonal antibody that detects galactomannan antigen. Despite the improved performance of the ASR-EIA compared with the IVD kit, there were 14 specimens from patients with histoplasmosis that were falsely negative. These patients had either pulmonary histoplasmosis without signs of dissemination (n = 5) or PDH without severe symptoms (n = 9). Two specimens from these 14 cases were also negative by the MVista Histoplasma antigen test. The MVista Histoplasma antigen levels of the other 12 cases ranged from below the limit of quantification (BLQ) to 6.16 ng/ml. This result suggests that the ASR-EIA has limited sensitivity for specimen with relatively low levels of Histoplasma antigen. Similar observations were made by Theel et al. [19]; they reported 10 IMMY ASR-negative/MVista positive samples with MVista values of BLQ. The clinical significance of these BLQ values was not effectively explored in that study.

Another study [20] was published by the same group evaluating the clinical significance of low-positive Histoplasma urine antigen results. This study showed that over half (13 of 25, 52%) of patients with positive but BLQ urine antigen levels were confirmed to have histoplasmosis based on other clinical data. Accordingly, the authors in a recent study [21] suggested to define a range of values (0.11-0.49 ng/ml) that were lower than the ASR suggested cutoff value (0.5 ng/ml) as indeterminate, and to perform further testing of the indeterminate specimens with an alternative assay or repeat testing with a fresh urine specimen. This approach may increase the detection sensitivity but may also delay the diagnosis in a significant proportion of patients (12 of 150 samples in their study). Lowering the cutoff value will increase sensitivity but decrease specificity of the test. For example, lowering the cutoff value to >0.15 ng/ml will decrease the specificity (96% to 70% in negative controls, 100% to 85% in aspergillosis controls) in current study, since 14 of 50 negative controls and two of 13 aspergillosis controls had a value of 0.1-0.4 ng/ml (supplemental data). A more definitive cutoff value that offers improved sensitivity without sacrificing specificity is needed before the ASR-EIA can be generally utilized as a screening test for histoplasmosis.

The ASR EIA test showed excellent sensitivity for specimen from patients with severe PDH (100%), suggesting that it could be used to diagnose histoplasmosis in clinically suspicious patients with severe symptoms, although the number of such patients in current study is too low (n = 10) to draw a definitive conclusion. The average ASR-EIA reading value of these 10 specimens was not significantly different from the overall average value or that of the other specimens with a positive reading value. The currently available information is inadequate to assess the ability of the ASR-EIA to predict disease severity. Recent study by Theel et al. [21] reported four patients in whom two or three specimens were tested using the ASR-EIA. The currently available information is inadequate to assess the ability of the ASR-EIA to predict disease severity.

Limitation of our study includes the common limitations of a retrospective study, such as selection bias and information errors. A prospective study with a large number of patients is needed for further characterization of the tests.
In summary, our study showed that the newly available ASR EIA has improved performance characteristics compared to the IVD kits for the detection of *Histoplasma* antigen in the urine, particularly in patients with severe histoplasmosis. However, users should be aware of the potential for false-negative results with the currently suggested cutoff value, especially in those with lower infectious burden.

Declaration of interest

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

Supplementary Material

Supplementary material is available at Medical Mycology online (http://www.mmy.oxfordjournals.org/).

References