Performance Characteristics of a Polyclonal Enzyme Immunoassay for the Quantitation of Histoplasma Antigen in Human Urine Samples

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Abstract

An antigen detection assay was prepared with rabbit anti-Histoplasma antibodies to detect and quantitate Histoplasma capsulatum antigen in urine samples. By using a 4-parameter curve fit, the assay calibration ranges from 2 to 1,000 enzyme immunoassay (EIA) units. We compared results for 99 urine samples with those of a reference laboratory, half of which tested positive or equivocal by that reference laboratory. Performance characteristics were further defined by studying the assay linearity, precision, percentage of positive agreement, and percentage of negative agreement. An acceptable correlation with the reference laboratory (R^2=0.7174) was obtained with results ranging from less than 2 (negative samples) to 132 EIA units by the new method. Compared with the reference laboratory, the percentage of agreement for positive samples, excluding equivocal samples, was 92% and for negative samples was 98%. Cross-reactivity occurred with culture filtrates of Paracoccidioides brasiliensis, Coccidioides immitis, and Blastomyces dermatitidis. No cross-reactivity was observed with Candida albicans or Aspergillus fumigatus culture filtrates. The current EIA for the detection and quantitation of H. capsulatum antigen in urine specimens is a valid assay that agrees well with results from an established reference laboratory.

The disease histoplasmosis occurs worldwide and is caused by the dimorphic fungus Histoplasma capsulatum. Pulmonary infection occurs after inhalation of the spores found in soils of river valleys. In the United States, endemic areas have been reported in the Ohio and Mississippi river regions. The disease is not contagious, and the fungus cannot be transmitted from one person to another. Opportunistic infection can occur in susceptible people, specifically people who are immunocompromised, resulting in disseminated disease.

Laboratory diagnosis of histoplasmosis can be accomplished with histopathologic examination, culture, serology, and antigen detection. Histopathologic examination is not very sensitive and often requires an invasive procedure for sampling of the organ involved. Cultural isolation and identification of the fungus is definitive, using a laboratory diagnostic feature of the morphologic change into a yeast form on raising the environmental temperature to 35°C to 37°C. Culture of H. capsulatum from human samples, however, usually takes 2 to 4 weeks for identification, and sensitivity varies according to sample quality. An alternative to culture is serology; however, antibodies to H. capsulatum are only detected in approximately 71% to 77% of disseminated disease because most of these cases occur in immunocompromised people. Also, long-term elevation of antibody titers makes it difficult to diagnose recurrent infections and monitor ongoing treatment of infections. Histoplasma antigens can be detected in the urine of infected patients with good sensitivity for diagnosis and monitoring of disseminated histoplasmosis.

We developed and evaluated a Histoplasma antigen assay using polyclonal antibodies produced by Immuno-Mycologics (IMMY; Norman, OK). The assay performance was compared...
with that of a reference laboratory whereby testing had previously been correlated with clinical information in patients with histoplasmosis. Quantitative results are produced to allow clinicians to monitor patients being treated for histoplasmosis.

**Materials and Methods**

**Antibody Generation**

Antibodies were developed in New Zealand rabbits immunized with a mixture of 3 patient isolates of yeast-phase *H. capsulatum* cells (IMMY). Each of the isolates was cultured using brain-heart infusion broth (Becton Dickinson, Franklin Lakes, NJ) at 35°C.

**Assay Development**

The enzyme immunoassay (EIA) was designed using a 2-step sandwich-type assay involving a capture antibody (rabbit anti-*Histoplasma* IgG antibody) immobilized onto wells of a flat-welled microplate (IMMY) available for binding antigen in clinical samples. A detection antibody was prepared using the same capture antibody that had been biotinylated and was used to bind any *Histoplasma* antigen bound to the microplate. By using a streptavidin-biotin detection system (IMMY), the blanked absorbance (450-630 nm) is proportional to the concentration of *Histoplasma* antigen present in the sample. A set of 6 calibrators from 2.0 to 1,000 EIA units (units arbitrary) was developed using dilutions of *H. capsulatum* mycelial-phase culture filtrate antigens. The calibrators were used to generate a calibration curve using a 4-parameter log-logit fit of the blanked absorbance of the calibrators vs the assigned values. A typical *Histoplasma* antigen calibration curve is represented in Figure 1.

![Figure 1](image1.png)

**Figure 1** Calibration curve using a 4-parameter curve fit and 6 calibrators generated from culture filtrate of *Histoplasma capsulatum* cultures from human specimens. EIA, enzyme immunoassay; OD, optical density.

**Assay Validation**

The *Histoplasma* antigen EIA was validated using 99 split samples tested by a reference laboratory whereby a polyclonal EIA is also performed (MiraVista Diagnostics, Indianapolis, IN). Samples had been frozen for up to 2 years after having been tested by the reference laboratory and before being analyzed in the present study. Half of the samples tested were positive and half were negative by the reference laboratory. Data obtained from both laboratories were plotted logarithmically and then analyzed by power regression.

*Histoplasma* antigen detection linearity was studied by diluting with EIA wash buffer (IMMY), the high calibrator (1,000 EIA units) to produce various expected levels of analyte. The same linearity studies were also performed using a positive human urine sample as shown in Figure 2. Measured values were then plotted on a logarithmic scale, and power analysis was performed. A positive sample tested neat and diluted 1:10,000 with negative urine was tested by the reference laboratory’s assay.

Cross-reactivity was studied by using several dilutions of culture filtrate antigens generated from cultures of the dimorphic fungi *Blastomyces dermatitidis, Coccidioides immitis,* and *Paracoccidioides brasiliensis.* The same studies were carried out with culture filtrate antigens of *Candida albicans* and *Aspergillus fumigatus.*

To monitor day-to-day precision of the assay, quality control samples were generated from mixtures of known positive urine samples and diluted to generate high-, medium-, and low-level positive control samples. Negative urine control samples were used with single donor urine whereby the donor had been tested negative by serology and urine antigen.

![Figure 2](image2.png)

**Figure 2** Linearity studies of the *Histoplasma* enzyme immunoassay using dilutions of a positive urine sample (triangles, solid line; \( y = 56.96x^{0.3616} \), \( R^2 = 0.9602 \)) compared with dilutions of the highest calibrator (circles, dotted line; \( y = 1.2905x^{0.3383} \), \( R^2 = 0.974 \)).
Sample Use

The use of samples was undertaken in compliance with human subject research regulations and was approved by the University of Utah Institutional Review Board.

Results

A significant but not unexpected finding was the nonlinearity of the assay with human urine samples (Figure 2). This can be expected given the polyclonal-polyclonal nature of the assay using culture filtrate as calibrating material. Linear dilutions of the high (1,000) calibrator showed an $R^2$ of 0.974, a power slope of 1.2905, and a power exponent of 0.9383 (ideally, both would be 1.0). Although the urine sample dilutions had an $R^2$ of 0.9602, the urine specimen was markedly nonlinear (power slope, 56.964; and power exponent, 0.3616). This nonlinearity is most likely caused by the variable epitopes on a broad spectrum of urine antigens detected by the assay. The 1,000 calibrator solution is composed of mycelial-phase culture filtrate and, therefore, appears linear on dilution. Urine samples likely contain a differing spectrum of antigenic epitopes from *H capsulatum* compared with the 1,000 calibrator.

To confirm that the reference laboratory’s EIA is also nonlinear, a positive urine sample was diluted 1:10,000 with negative urine and sent for testing. The undiluted specimen result was approximately 41 EIA units. The EIA units of the highly diluted specimen decreased only to approximately 26 EIA units. Given the nonlinear nature of the assay with urine samples, it is extremely difficult to monitor patients with high positive values for treatment progress unless prior specimens are saved for parallel analysis with future specimens.

Positive control material, prepared from human urine specimens, was analyzed with each test run throughout the study. Between-run precision showed coefficients of variation of 13.2%, 6.6%, and 4.3% for the high, medium, and low levels of urine control samples, respectively. Also a coefficient of variation of 8.6% was obtained with between-run values of an antigen control sample generated using the same matrix as the calibrators.

A split-specimen comparison study was completed comparing results obtained using the current EIA with results of the reference laboratory EIA. All samples were obtained from patients suspected of experiencing active disease. A total of 50 negative, 38 positive, and 11 equivocal samples (by the reference laboratory) were compared. Of the negative samples, 49 (98%) were in agreement, and 35 (92%) of the positive samples were in agreement. A plot of the current EIA results vs the reference laboratory EIA results produced an $R^2$ of 0.7174 (Figure 3). Although somewhat nonlinear, the results are highly correlated between the 2 polyclonal-polyclonal EIAs.

The reference laboratory interprets a negative result to be less than 1.0 EIA unit; weak positive, between 1.0 and 2.0; questionably positive, between 2.1 and 4.0 EIA units if inconsistent with clinical findings; and positive, greater than 4.0 EIA units regardless of clinical evidence of the disease. Results from the reference laboratory showed 6 samples between 1 and 2 EIA units and 5 samples between 2 and 4 EIA units, whereas all 11 of these “equivocal” samples were less than 2 EIA units and deemed negative by the current assay. No clinical information for these equivocal samples was known. The 4-parameter curve shows the optical density does not change significantly up to 2.0 EIA units. The optical density then increases between 2.0 and 1,000 EIA units. Furthermore, comparison of lower values with the reference laboratory values using patient urine samples validated the use of 2.0 as an interpretive cutoff value.

Cross-reactivity was observed with each of the dimorphic fungal culture filtrate antigens *B dermatitidis*, *C immitis*, and *P brasiliensis*. No cross-reactivity, however, was observed with culture filtrates of the monomorphic fungi *C albicans* and *A fumigatus*, which are common organisms that may be found in human urine specimens.

Discussion

The polyclonal antibodies (capture and detection) used in the current assay were directed toward a pool of clinical isolates of *H capsulatum* yeast cells. The assay performs similar to that of a reference laboratory from which samples were compared for evaluation. Monoclonal antibodies toward *H capsulatum* have been generated and tested in the past but are...
currently not favored for use owing to poor sensitivity.\textsuperscript{18,19} Assays using polyclonal antibodies have been shown to be sensitive but not specific owing to cross-reactivity to other dimorphic fungal pathogens such as \textit{B dermatitidis} and \textit{P brasiliensis}.\textsuperscript{2,12,14} Additional studies to determine cross-reactivity using disease-state urine specimens are desired to assess the true nature of cross-reactivity. Owing to the lack of existence of a sensitive and specific monoclonal assay, the current polyclonal EIA is the best antigen detection assay available. The reference laboratory performs an EIA of similar design (ie, polyclonal capture antibody and polyclonal detection antibody), but analytic performance characteristics are unknown. Clinical correlation, however, has been reported by the reference laboratory through previous studies.\textsuperscript{12} The present study fully reports the performance characteristics of an assay of similar design to that of the reference laboratory.

Given the polyclonal nature of the assay antibodies, it is not surprising to see cross-reactivity with other dimorphic fungi. We have shown that antigens generated against \textit{B dermatitidis}, \textit{P brasiliensis}, and \textit{C immitis} cross-react with the current assay. The reference laboratory has reported cross-reactivity with \textit{B dermatitidis}, \textit{P brasiliensis}, \textit{Penicillium marneffei},\textsuperscript{20} and more recently with \textit{C immitis},\textsuperscript{21} using samples from patients diagnosed with the respective mycosis. For purposes of monitoring patients being treated for disseminated histoplasmosis, cross-reactivity may be a concern for clinicians treating patients who may be infected with multiple types of fungi. A specific assay for the detection of histoplasmosis without cross-reactivity would be ideal but currently is not available at the required level of sensitivity. For purposes of making an original diagnosis, cross-reactivity needs to be considered and confirmatory results should be obtained by culture, serology, or histopathologic examination.

Although specimens were analyzed from patients for whom a clinician ordered \textit{Histoplasma} urine antigen testing, we do not have specific clinical histories of the patients and cannot determine positive and negative predictive values. Also, because there is no “gold standard,” we do not believe sensitivity and specificity can accurately be calculated. We instead assessed agreement between the current assay and an assay of similar design performed by the reference laboratory using correlation analysis. A potential limitation is that stability studies have not been reported for urine samples and require further research. The current assay used samples that had been frozen and, therefore, could contribute to differences in correlation. However, comparable results are observed with periodic split-sample testing with the reference laboratory using fresh samples for both tests (data not shown).

The current EIA for the detection of \textit{Histoplasma} antigen in human urine samples is an acceptable quantitative assay for use in the diagnosis and monitoring of patients with histoplasmosis. The assay design is comparable to that used by a popular reference laboratory that has been previously evaluated for usefulness in monitoring patients’ progress during treatment.\textsuperscript{14} Without knowing the method details of the assay of the reference laboratory (ie, calibration strategy, including curve fitting technique, quality control protocols), it is difficult to apply confidence in the assay results, although clinical correlation has been shown.\textsuperscript{13} We have described the method details in the current assay, and our studies show that it has acceptable performance characteristics for a polyclonal assay. We have extended the calibration curve to 1,000 EIA units to allow a large measurable range, which is desirable in a nonlinear assay from which sample dilutions cannot be used to bring result into the measurable range.

Quality control material generated from positive human samples is necessary to evaluate assay performance because \textit{Histoplasma} antigen from human urine samples is quantitated in a nonlinear manner. Urine samples are the sample of choice for diagnosis and monitoring of disseminated histoplasmosis\textsuperscript{12} because the samples are easily collected and do not contain immune complexes to interfere with the assay. Currently, urine is the only specimen source validated for use with this \textit{Histoplasma} antigen EIA. For acute and chronic pulmonary histoplasmosis, testing of respiratory samples by antigen detection or polymerase chain reaction may be more sensitive for diagnosis.

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References

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