Detection of *Blastomyces dermatitidis* and *Histoplasma capsulatum* from Culture Isolates and Clinical Specimens by Use of Real-Time PCR

N. Esther Babady, Seanne P. Buckwalter, Leslie Hall, Kara M. Le Febre, Matthew J. Binnicker, and Nancy L. Wengenack*

Division of Clinical Microbiology, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota 55905

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*Blastomyces dermatitidis* and *Histoplasma capsulatum* are dimorphic fungi that often cause self-limited respiratory infections. However, they may also cause severe disseminated disease, depending on the level of the exposure to the organism and the host immune status. In addition, patients with infections caused by these fungi may have very similar clinical presentations. Although microbiologic culture is a standard method for detecting these pathogens, their recovery may require days to weeks, and the manipulation of cultures presents a significant safety hazard to laboratory personnel. Therefore, the goal of this study was to design a rapid, real-time PCR assay to detect and differentiate *B. dermatitidis* and *H. capsulatum* from culture isolates and directly from clinical specimens. Primers and fluorescence resonance energy transfer hybridization probes were designed to target the histidine kinase and glyceraldehyde-3-phosphate dehydrogenase genes of *B. dermatitidis* and *H. capsulatum*, respectively. The analytical sensitivity of the assay was determined to be 100 copies/μl for both fungi. From culture isolates, the assay demonstrated 100% specificity and 100% sensitivity for *B. dermatitidis* and 100% specificity and 94% sensitivity for *H. capsulatum*. Detection directly from 797 clinical specimens demonstrated specificities and sensitivities of 99% and 86% for *B. dermatitidis* and 100% and 73% for *H. capsulatum* compared with the results for culture. This real-time PCR assay provides a rapid method for the detection of *B. dermatitidis* and *H. capsulatum* from culture isolates and directly from clinical specimens.
B. DERMATITIDIS AND H. CAPSULATUM PCR

TABLE 1. Primer and probe sequences

<table>
<thead>
<tr>
<th>Primer/probe Type</th>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer for B. dermatitidis</td>
<td>BD1</td>
<td>GCGATGGTAAAGCGAGTTT</td>
</tr>
<tr>
<td>Reverse primer for B. dermatitidis</td>
<td>BD2</td>
<td>ACCCTCTTTGTGGGAAAAA</td>
</tr>
<tr>
<td>Forward primer for H. capsulatum</td>
<td>HC1</td>
<td>AAATTTGCTGGTGACCGA</td>
</tr>
<tr>
<td>Reverse primer for H. capsulatum</td>
<td>HC2</td>
<td>GTTCTCAGCTAGCTCAG</td>
</tr>
<tr>
<td>Donor probe for B. dermatitidis</td>
<td>BD3</td>
<td>CGGATACATTGAAATTTTGTGTAAGFL</td>
</tr>
<tr>
<td>Acceptor probe for B. dermatitidis</td>
<td>BD4</td>
<td>LC RED 705-CCTTCGAATCCCTTTATTAACCTCTTTT-PH</td>
</tr>
<tr>
<td>Donor probe for H. capsulatum</td>
<td>HC3</td>
<td>CGAGGAGTGCAGGAGTGAGA-FL</td>
</tr>
<tr>
<td>Acceptor probe for H. capsulatum</td>
<td>HC4</td>
<td>LC RED 640-CTCAGTGACGATAATTGGGG-PH</td>
</tr>
</tbody>
</table>

* FL, fluorescein label; PH, phosphate; LC Red 640, LightCycler Red 640-nm dye label; LC Red 705, LightCycler Red 705-nm dye label.

Meeting of the American Society for Microbiology, Philadelphia, PA, 2009 [1].

MATERIALS AND METHODS

Isolates. Strains of B. dermatitidis (n = 60), H. capsulatum (n = 54), and other fungi (n = 65) identified and archived in our laboratory between 1989 and 2009 were used for assay validation. Isolates were freshly subcultured onto inhibitory mold agar (IMA) (BBL, Sparks, MD). Culture isolates were confirmed to be B. dermatitidis and H. capsulatum using FDA-approved nucleic acid hybridization probes (AccuProbes; Gen-Probe, San Diego, CA) specific for both organisms. Culture isolates were lysed by placing a 1-μl loopful of the organism into a 2.0-ml tube containing 500 μl of sterilized water, 0.1-mm silica glass beads, and 2.4-mm zirconia beads (BioSpec Products, Inc., Bartlesville, OK). The tubes were heated at 95°C for 10 min and then placed on a Disruptor Genie (Scientific Industries, Bohemia, NY) for 2 min to mechanically lyse the organisms and release the nucleic acid.

Clinical specimens. Clinical specimens (n = 797) were collected over a 12-month period from patients with suspected fungal infection and were analyzed immediately. These included specimens from bronchial washings (n = 346), bronchoalveolar lavage (BAL) fluid (n = 212), pleural fluid (n = 157), tracheal secretions (n = 35), tissue (n = 14), sputum (n = 13), lung washes (n = 6), blood (n = 4), bone marrow (n = 5), peritoneal fluid (n = 3), and other body fluids (n = 2).

Culture. Respiratory specimens were cultured by inoculating 500 μl of the specimen onto an IMA plate supplemented with chloramphenicol (125 μg/ml) (Remel, Inc., Lenexa, KS), a brain heart infusion (BHI) blood agar plate supplemented with chloramphenicol (50 μg/ml) and gentamicin (40 μg/ml) (BHI III; Remel), and a BHI agar plate supplemented with chloramphenicol (50 μg/ml) and gentamicin (40 μg/ml), and cycloheximide (0.5 mg/ml) (BHI IV; Remel). Sterile body fluids were cultured by inoculating 500 μl of the clinical specimen onto an IMA plate with chloramphenicol and a BHI III plate. Tissues were homogenized and cultured by inoculating 500 μl of the specimen onto an IMA plate with chloramphenicol and a BHI III plate. Plates were incubated at 30°C and held for up to 24 days. Blood and bone marrow specimens were cultured by inoculation onto an IMA plate and a Sabouraud’s dextrose agar plate (SAB) (Emmons’ modification, pH 7 and 2% dextrose; Remel), followed by incubation at 30°C for 30 days. The identity of culture isolates resembling B. dermatitidis and H. capsulatum was confirmed using the AccuProbe DNA hybridization probes.

Specimen extraction (nontissue specimens). Respiratory specimens and body fluids were processed prior to extraction using proteinase K (100 μl) and incubation on a Thermomixer (Eppendorf, Hauppauge, NY) at 55°C and 1,400 rpm for 15 min. The liquefied specimens were heated at 95°C and mechanically lysed using a mixture of 0.1-mm silica glass beads and 2.4-mm zirconia beads on a Disruptor Genie for 2 min. Two hundred microliters of the processed specimen was then extracted on a MagNa Pure instrument (Roche Diagnostics, Indianapolis, IN) using the total nucleic acid isolation kit (Roche Diagnostics, Indianapolis, IN) without any preprocessing steps. Cerebrospinal fluid specimens were processed by incubating 1 ml of sterilized water, 0.1-mm silica glass beads, and 2.4-mm zirconia beads (BioSpec Products, Inc., Bartlesville, OK). The tubes were heated at 95°C for 10 min and then placed in a Thermomixer (Eppendorf, Hauppauge, NY) at 55°C and 1,400 rpm for 15 min. The following day, 200 μl of the digested tissue was transferred into a MagNa Pure compact tube and extracted as previously described (3).

LightCycler PCR assay. The real-time PCR assay was developed on the LightCycler 2.0 instrument (Roche Applied Sciences, Indianapolis, IN). Primers and fluorescence resonance energy transfer (FRET) hybridization probes were designed to target a 174-bp region of the histidine kinase (DRK-1) gene of B. dermatitidis and a 192-bp region of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene of H. capsulatum. These targets were chosen because of their highly conserved nature within each organism and the availability of sequences within public nucleotide databases (e.g., GenBank, http://www.ncbi.nlm.nih.gov/). Primers and probes were synthesized by TIB MolBiol (primer-probe set 353) (Adelphia, NJ), and their sequences are provided in Table 1. The acceptor probe for B. dermatitidis was labeled with red 705 dye, while the acceptor probe for H. capsulatum was labeled with red 640 dye. Labelling the acceptor probes with 2 different dyes allows simultaneous detection and differentiation of B. dermatitidis and H. capsulatum within a single PCR assay using two different detector channels. The PCR assay was performed using the LC FastStart DNA master hybridization probe kit (Roche Applied Sciences, Indianapolis, IN), with each reaction mixture containing 3 mM MgCl2, 1× Roche LC FastStart mix, 0.5 μM each forward primer, 0.7 μM each reverse primer, and 0.5 μM each labeled probe. The total volume per reaction mixture was 20 μl (15 μl master mix plus 5 μl extracted nucleic acid). PCR amplification with real-time detection was performed using the following cycling parameters: 1 template-denaturing cycle at 95°C for 10 min, followed by 45 amplification cycles at 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. Following amplification, melting curve analysis was performed by measuring the fluorescent signal during the following cycling parameters: 95°C for 0 s, 59°C for 20 s, 45°C for 20 s with a 0.2°C/s transition, and 85°C for 0 s with a 0.2°C/s transition.

Generation of positive-control plasmids. Positive control plasmids were constructed for B. dermatitidis and H. capsulatum using the primers listed in Table 1 and American Type Culture Collection strains ATCC 28305 for B. dermatitidis and ATCC 38904 for H. capsulatum. The amplified targets were cloned into the pCR 2.1 vector using the pCR 2.1 TOPO TA cloning kit (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer’s instructions. The plasmid constructs were then purified using the High Pure plasmid isolation kit (Roche Applied Sciences, Indianapolis, IN). The expected size of the recombinant plasmid was confirmed with 2% agarose gel electrophoresis. Subsequent Sanger sequencing confirmed the inserts to be derived from the DRK-1 and GAPDH genes of B. dermatitidis and H. capsulatum, respectively.

Analytical sensitivity and specificity. The analytical sensitivity of the assay was determined by testing a dilution series of the B. dermatitidis and H. capsulatum control plasmids. A 10-fold dilution (105 copies/μl serially diluted to 105 copies/μl) of each plasmid was performed in TE buffer, and each dilution was then tested in triplicate. The analytical sensitivity in buffer was determined to be the highest dilution whereby all three replicates were positive. The analytical sensitivity in the specimen matrix was determined by spiking at least 30 analyte-negative specimens (i.e., respiratory specimens) with plasmid control at the limit of detection (LOD) and testing by PCR. The specimen matrices tested were respiratory specimens (e.g., sputum, tracheal secretions, and BAL fluid), CSF, tissue (e.g., lung), body fluids (e.g., pleural and peritoneal), and blood.

The analytical specificity was determined by performing a BLAST search of each primer, each probe, and the entire amplicon sequence using the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). In addition, a panel of nucleic acid extracts from bacteria (n = 83), other fungi (n = 66), viruses (n = 23), parasites (n = 5), and human DNA (n = 1) were also tested for cross-reactivity to both sets of primers and probes (see the supplemental material). Amplification and Sanger dye sequencing of either 16S (bacteria) or D2 LSU (fungi) ribosomal DNA (rDNA) was utilized to confirm the presence of amplifiable nucleic acids in the specificity panel.
H. capsulatum and B. dermatitidis.

RESULTS

Analytical sensitivity and specificity. The analytical sensitivity was determined to be 100 copies/μl for B. dermatitidis and H. capsulatum. The B. dermatitidis probe is read at 705 nm and demonstrates a melting peak ($T_m$) at 66°C ± 0.36°C (mean ± standard deviation [SD]), while the H. capsulatum probe is read at 640 nm and has a $T_m$ at 61°C ± 0.29°C (Fig. 1). The analytical sensitivity of the assay in all specimen matrix types tested was confirmed to be 100 copies/μl (see Table 4). The BLAST search of the primer, probe, and target sequences for both B. dermatitidis and H. capsulatum did not yield any cross-reacting sequences. In addition, testing of nucleic acids from 179 potentially cross-reacting microbes demonstrated no cross-reactivity with other organisms (see the supplemental material).

Sensitivity and specificity from culture isolates. The sensitivities of the assay from culture isolates were 100% (60/60) and 94.5% (51/54) for B. dermatitidis and H. capsulatum, respectively (Table 2). In order to investigate the 3 discordant H. capsulatum isolates, the D2 large ribosomal subunit and intergenic spacer (ITS) regions of the discordant isolates were sequenced, and the isolates were confirmed to be Histoplasma species using both sequencing targets. Sequencing identified one of the three discordant isolates as H. capsulatum var. farciminosum (a horse pathogen consisting of diverse clades) (15), while the other two isolates were identified as H. capsulatum var. capsulatum. The sequencing results revealed a deletion under the RED 705 probe in the H. capsulatum var. farciminosum isolate and base pair mismatches in the region targeted by the probe in the two H. capsulatum var. capsulatum isolates.

The specificity of the assay was 100% for both organisms. All members of the specificity panel, which included Chrysosporium sp. and Sepedonium sp. (phenotypic homologues to B. dermatitidis and H. capsulatum, respectively) were negative when tested by the real-time PCR assay.

Clinical sensitivity, specificity, and inhibition. A total of 797 clinical specimens were tested concurrently by fungal culture and the real-time PCR assay to assess clinical sensitivity and specificity. The sensitivity and specificity of the B. dermatitidis PCR were 86% (12/14) and 99.4% (778/783), respectively (Table 3). The overall sensitivity and specificity of the assay for H. capsulatum were 73% (11/15) and 100% (782/782), respectively (Table 3).

Additional studies using analyte-negative clinical samples spiked with the B. dermatitidis plasmid control at the LOD for the assay were positive in 60/60 respiratory, 30/30 CSF, 120/120 tissue, 60/60 body fluids, and 29/30 blood specimens. The spiking studies, therefore, demonstrated a sensitivity of 100% for B. dermatitidis for all specimen types except blood, which showed a sensitivity of 96.6% (29/30). In addition, H. capsulatum...
Validation studies demonstrated that the sensitivity and specificity of the *B. dermatitidis* assay from culture isolates were each 100%. Similarly, the specificity of the *H. capsulatum* assay from culture isolates was 100% (125/125); however, the sensitivity was 94% (51/54). The three discordant *H. capsulatum* culture isolates were re-extracted and repeated as negative by PCR. Sequencing of both the ITS and ribosomal D2 regions identified two of these isolates as *H. capsulatum* var. *capsulatum* and one isolate as *H. capsulatum* var. *farciminosum*, which causes lymphangitis in horses and mules (19). Sequencing of the GADPH gene of all three isolates showed either a deletion or mismatches in the complementary sequence targeted by the PCR probes (data not shown). These data underscore the possibility that a small number of *Histoplasma capsulatum* strains may carry variations in the region targeted by this PCR assay directly from clinical specimens were 73% (11/15) and 100% (778/783), respectively. Among the 5 discordant specimens, three (2 tissue and 1 sputum) were PCR positive, culture negative, but yeast resembling *B. dermatitidis* was visualized during the direct microscopic examination, suggesting that the PCR results were likely to be true positives.

The sensitivity and specificity for detection of *H. capsulatum* directly from clinical specimens were 73% (11/15) and 100% (782/782), respectively. The lower overall sensitivity was due to the fact that only 33% (2/6) of culture-positive BAL fluid specimens were also positive by the PCR assay. Interestingly, 2 of the 4 patients with culture-positive, PCR-negative results from BAL fluid had another specimen source that tested positive by the *Histoplasma* PCR assay. The sensitivity from all clinical laboratories using this method should follow up on highly suspect isolates testing negative by PCR with morphological and DNA probe analyses or DNA sequencing.

Compared to routine fungal culture, the sensitivity and specificity of the *B. dermatitidis* PCR assay directly from clinical specimens were 86% (12/14) and 99% (778/783), respectively. Among the 5 discordant *B. dermatitidis* specimens, three (2 tissue and 1 sputum) were PCR positive, culture negative, but yeast resembling *B. dermatitidis* was visualized during the direct microscopic examination, suggesting that the PCR results were likely to be true positives.

The sensitivity and specificity of the *H. capsulatum* assay from clinical specimens were 73% (11/15) and 100% (782/782), respectively. The lower overall sensitivity was due to the fact that only 33% (2/6) of culture-positive BAL fluid specimens were also positive by the PCR assay. Interestingly, 2 of the 4 patients with culture-positive, PCR-negative results from BAL fluid had another specimen source that tested positive by the *Histoplasma* PCR assay. The sensitivity from all other specimen types (e.g., tissue and other respiratory samples) was 100% (5/5). Therefore, the lower sensitivity of PCR in BAL fluid may reflect a low concentration of organisms in this specimen type, where the volume of saline instilled and the volume of BAL fluid obtained during the procedure can vary widely across institutions (9, 10). Although the results of our study suggest that a negative PCR from BAL fluid does not adequately rule out the presence of *H. capsulatum*, a positive PCR result from BAL fluid provides the health care provider with a rapid (~4 h) diagnosis of histoplasmosis (versus several
weeks for routine culture) and would allow for early initiation of antifungal therapy.

The real-time PCR assay described above is able to reliably identify *B. dermatitidis* and *H. capsulatum* from culture isolates and directly from clinical specimens. Specimen types suggested for this assay based on the above verification studies include respiratory specimens, fresh tissue specimens, blood, bone marrow, CSF, and body fluids. Currently, urine is not considered to be a good specimen source for this assay. Previous literature (25) and unpublished studies performed in our laboratory indicate that *Histoplasma* nucleic acid is not routinely found in urine of patients with disseminated histoplasmosis. Therefore, the *H. capsulatum* antigen assay is the recommended test for urine at this time. Although the PCR assay provides a rapid result, the data presented in this report suggest that routine culture should also be performed on all clinical specimens to enhance sensitivity.

In summary, the real-time PCR assay described in this report offers a rapid, sensitive, and specific method for the detection of antifungal therapy.

**REFERENCES**


