PCR-based detection of DNA from the human pathogen *Blastomyces dermatitidis* from natural soil samples

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*Blastomyces dermatitidis* is the dimorphic fungal agent of blastomycosis, a disease that primarily affects humans and dogs. The clinical appearance of this mycosis is well characterized, but there is still little known about its environmental niche, having been isolated from nature only 21 times. We have developed a PCR-based assay to detect *B. dermatitidis* from soil samples using primers specific to a portion of the promoter region of the *BAD1* virulence gene. An internal standard control, pTVJ2, was constructed to validate the results from soil samples. Amplification of this control indicated adequate removal of ambient soil inhibitors. The PCR detection limits for the control plasmid and *B. dermatitidis* genomic DNA were 0.1 and 500 femtograms, respectively. No PCR cross-reactivity was observed against bacteria, actinomycetes, and 13 other fungi that were genetically related or found in the same geographic areas. In spiked soil samples, this method was sensitive to 304 copies of pTVJ2 DNA and 8,450 live *B. dermatitidis* yeast cells. Three of eight natural soil samples from a dog kennel near Lexington, KY in which dogs suffered from blastomycosis were positive using the described method, demonstrating its utility in detecting *B. dermatitidis* in its natural surroundings.

**Keywords** *Blastomyces dermatitidis*, soil sampling, PCR

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**Introduction**

*Blastomyces dermatitidis*, the causal agent of blastomycosis, is one of four important thermally dimorphic fungal pathogens that start as pulmonary infections, often progressing to infect other parts of the human body [1]. *Blastomyces* is also a pathogen of other animals, infecting dogs with a rate as high as 1,420 per 100,000 [2].

The two morphologies of *Blastomyces* include an infectious conidium-producing filamentous form found in the soil (25°C) and an invasive yeast form found in the mammalian body (37°C). Blastomycosis is typically acquired through the inhalation of conidia produced on the mycelium at ambient temperatures in the soil. The conidia germinate in the lungs, causing an infection that resolves spontaneously in the majority of cases. If the infection is not cleared, the onset of blastomycosis is generally indolent and insidious [3].

The majority of cases of blastomycosis occur throughout parts of the midwestern and southeastern United States, including the entire Mississippi River Valley, the Great Lakes Region, and North Carolina [4]. The areas reporting the highest incidence of blastomycosis include Vilas and Oneida Counties in northern Wisconsin and along the Mississippi River in Arkansas, where the occurrence of blastomycosis is about 4 per 100,000 people [5,6]. Since *Blastomyces* was first described in 1894, there have been nearly a dozen large outbreaks of human blastomycosis. The most notable outbreak occurred in June 1984 at an environmental camp near Eagle River in Vilas County, Wisconsin. The common source of infection appears to have been a beaver dam, from which two isolates of *B. dermatitidis* were eventually isolated by animal inoculation [4]. It is thought that *B. dermatitidis* resides in moist, organically rich soils typically near waterways [7].

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Although blastomycosis can be clinically diagnosed with ease, there remains much mystery regarding the ecological niche for *B. dermatitidis*. Most natural isolation attempts have been futile, laborious procedures yielding negative results. Despite countless attempts using animal inoculation, the only available method proven to detect *B. dermatitidis* in nature, *Blastomyces* has only been isolated 20 times from its natural environment [8,9]. Even when positive results have been obtained, re-isolation of the fungus using animal inoculation has only ever worked on a single occasion, demonstrating the ineffectiveness of the procedure [10]. Recently, an in vitro method of isolation has been developed, but it has proven effective in only a single instance [8].

In this study, we have developed an optimized PCR detection method with excellent sensitivity and specificity, using primers specific to a region of the *BAD1* [11] promoter region to detect *Blastomyces* from natural samples. Positive results were obtained from several soil samples around a dog kennel where one third of the dogs had blastomycosis, showing the validity of the method and offering hope for epidemiologists tracking the natural sources for blastomycosis outbreaks.

### Materials and methods

**Strains and culture conditions**

*Blastomyces dermatitidis* strain ATCC 26199 and *Histoplasma capsulatum* DNA was obtained from Tom Sullivan, Department of Pediatrics and Internal Medicine at the University of Wisconsin-Madison. *Blastomyces dermatitidis* was grown at 37°C on Middlebrook 7H10 agar. A list of fungi and bacteria was developed based closely related species according to recent molecular and morphological analysis (Table 1) [13,14]. All other fungal cultures were grown at room temperature on Sabouraud's agar. *Emmonsia crescents* was obtained from the ATCC. *Spiromastix grisca* and *Polyotylpa histricus* were obtained from Wendy Unterine from Brandon University (Manitoba, Canada). *Trichophyton mentagrophytes*, *Cladosporium carinii*, and *Malassezia furfur* were obtained from Gunderson Lutheran Medical Center (La Crosse, Wisconsin). *Coccidioides immitis* DNA was obtained from Gary Cole at the Medical College of Ohio (Toledo, OH). For transformation procedures, *Escherichia coli* strain DH5α cells were grown for 24 h on Luria Bertani agar at 37°C.

<table>
<thead>
<tr>
<th>Species</th>
<th>PCR Result</th>
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</thead>
<tbody>
<tr>
<td>Fungi</td>
<td></td>
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<tr>
<td><em>Histoplasma capsulatum</em></td>
<td>Negative</td>
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<tr>
<td><em>Emmonsia crescents</em></td>
<td>Negative</td>
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<tr>
<td><em>Spiromastix grisca</em></td>
<td>Negative</td>
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<tr>
<td><em>Polyotylpa histricus</em></td>
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<tr>
<td><em>Coccidioides immitis</em></td>
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</tr>
<tr>
<td><em>Arthrodermataceae</em></td>
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<tr>
<td><em>Trichophyton rubrum</em></td>
<td>Negative</td>
</tr>
<tr>
<td><em>Trichophyton mentagrophytes</em></td>
<td>Negative</td>
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<tr>
<td><em>Microsporum gypseum</em></td>
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<tr>
<td>Other fungi:</td>
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<tr>
<td><em>Aspergillus flavus</em></td>
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<tr>
<td><em>Penicillium chrysogenum</em></td>
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<tr>
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<tr>
<td><em>Candida tropicalis</em></td>
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<tr>
<td><em>Malassezia furfur</em></td>
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<tr>
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<tr>
<td><em>Bacillus megaterium</em></td>
<td>Negative</td>
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<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>Negative</td>
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<tr>
<td><em>Streptomyces griseus (actinomycetes)</em></td>
<td>Negative</td>
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<tr>
<td><em>Nocardia brasiliensis (actinomycetes)</em></td>
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<td>Marsh</td>
<td>Negative</td>
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<tr>
<td>Forest</td>
<td>Negative</td>
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</table>

### Construction of the pTIV2 control plasmid

*Blastomyces dermatitidis* ATCC 26199 was used for all assays and procedures. The primers, *Blasto I* (5'-AAGTGGCTGGTAGTTACGCTAC-3') and *Blasto II* (5'-TAGGTGCTGATCCATAAGTCAGG-3'), synthesized by Integrated DNA Technologies, (Coralville, Iowa) were used to amplify a 363-bp region of the *BAD1* virulence gene promoter. These primers were originally designed by Bialek *et al.* [12] for the detection of *Blastomyces* in paraffin-embedded canine tissue and are considered unique to *Blastomyces*, except for a partial similarity with the promoter region of the *YP53* gene of *Histoplasma* [11]. PCR amplifications were set up using 25 pmol of each primer, 2.5 mM MgCl₂, 10 mM dNTPs, 1.5 U *Taq* polymerase (Promega, Madison, WI), and 10 ng of template DNA under the following conditions: an initial denaturation of 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, with a final elongation of 72°C for 5 min [15]. The 363-bp PCR product was concentrated and washed in 10 mM Tris using a Microcon 30 filter (Millipore, Billerica, MA), ligated to *Smal* digested pGEM-3Z DNA (Promega, Madison, WI), and then transformed into *E. coli* strain DH5α.
Transformants were selected on LB agar containing 100 μg/ml ampicillin X-Gal (Roche). One of the resulting transformants, pTJV1, was selected for further processing.

To create the internal standard control plasmid with a truncation of the 363-bp BAD1 gene fragment, plasmid DNA isolated from pTJV1 using a commercial extraction kit (Qiagen, Valencia, CA) was PCR amplified with the following oligonucleotide primers: pTJV2 Nol F: 5′-ATATTCGGCCGAGGTGCTGGTGACCCATTGATCC-3′; pTJV2 Nol R: 5′-ATCCGCGGCGGCAATTTTCGCCATCCTC-3′. These were designed to have a Nol I site at the 5′ end of each primer to allow the linear PCR amplification product to be cleaved with Nol I and ligated together to create a new plasmid with a 100-bp deletion within the BAD1 gene. PCR amplification used 10 ng of pTJV1 template and reagents described above under the following parameters: an initial denaturation of 94°C for 5 min, followed by 35 cycles of: 94°C for 30 s, 53°C for 30 s, 72°C for 3.5 min, with a final elongation of 72°C for 7 min.

The PCR product was concentrated as described previously, cut with Nol I, ligated together, and used to transform E. coli strain DH5α cells. Transformants were selected on LB agar containing 100 μg/ml ampicillin and several had their plasmid DNA extracted, checking for a 100-bp deletion compared to pTJV1 (data not shown). One of the resulting plasmids, labeled pTJV2, PCR amplified a 263-bp product with the Blast I/Blasto II primer pair and sequencing verified that it was the truncated BAD1 region (data not shown).

**PCR optimization**

To optimize the PCR amplification procedure with the Blasto I/Blasto II primer pair, the amount of Taq polymerase was increased to 2.5 U per reaction, the MgCl2 concentration was decreased to 2 mM, and the annealing temperature of the PCR was increased to 55°C.

**Extraction of DNA from pure cultures of fungi and bacteria**

Extractions of fungal DNAs were done using an adaptation of the procedure developed by Van Burik [16]. Briefly, 30–100 mg of mycelial mat was added to 400 μl lysis buffer (Trition X-100 and 1% sodium dodecyl sulfate), 400 μl phenol/chloroform:isoamyl alcohol (24:24:1), and 500 mg glass beads (0.4–0.6 mm). Samples were vortexed for 30 min and the supernatants were phenol/chloroform:isoamyl alcohol extracted again followed by a chloroform extraction. The DNA from the aqeous phase was precipitated at −20°C washed with 70% ethanol, air dried, and finally resuspended in 10 mM Tris pH 8.0.

**Specificity testing of the B. dermatitidis PCR**

Using the optimized PCR procedure noted previously, 100 ng of DNA from each fungus was tested using the Blastomyces-specific primers and pan-fungal primers ITS1 and ITS4, which anneal specifically to a highly conserved region of the 18S rDNA and 28S rDNA, respectively [17]. For actinomycetes and bacteria, the universal primers 8F and 1492R [18] were incorporated.

**Collection of soil samples**

A total of 12 20-g samples were collected from the edge of Myrick Marsh and from a low ground area of Hixon Forest (La Crosse, Wisconsin). Two samples from each location were not treated, whereas the remaining samples were autoclaved for 20 min, incubated at room temperature for 2 h, and then autoclaved once again for 20 min. Soil samples from a presumptive endemic area for Blastomyces were collected from the Iroquois Kennel Club near Lexington, KY, from a location documented to have had a recent outbreak of canine blastomycosis.

**PCR amplification of Blastomyces in soil samples**

To extract DNA from soil samples, the MoBio UltraClean Soil Kit (MoBio Labs, Solana Beach, CA) was used. Extractions took place as outlined in the kit, with several amendments. The samples were vortexed for 30 min, and the DNAs were eluted with 30 μl of S5 solution. Unspiked soil samples were used as a control. In the in vitro experiments, soil spiked with 10-fold dilutions of an initial concentration of 10 pg/μl pTJV2 DNA was used after addition of S2 solution. Additional experiments were run using soil samples with B. dermatitidis yeast cells grown on Middlebrook 7H-10 agar for 2 wk at 37°C. For the B dermatitidis yeast cell assays, two loopfuls of cells were added to 1.0 ml of sterile water and vortexed in microfuge tubes. Ten μl of the suspension was pulled from the tube and placed onto a haemocytometer to count the cells. With the yeast counted, 10-fold serial dilutions of the yeast suspension through 10−6 were made into sterile water and 100 μl of each dilution was added to a soil extraction tube containing 0.75 g sterilized soil. The extraction protocol was followed as described above. Using the optimized PCR procedure, soil samples were diluted 1/40 and 25 μl template was added to two separate reaction tubes. Ten fg of the internal standard.
control plasmid pTJV2 was run in the same tube as the soil extraction template for each reaction. In addition, a separate tube was run containing only the soil extraction template to add to the PCR. Samples that did not amplify were diluted 1/80 or 1/160 to remove any residual soil inhibitors.

Sequencing of PCR samples

Sequencing reactions were performed by the Mayo Clinic sequencing core (Rochester, MN) using the Blasto I and Blasto II primers.

In vitro isolation of natural samples

In vitro isolation was performed at the Medical College of Wisconsin (Milwaukee, WI) as published by Baumgardner using the tested soil isolates [8].

Results

Sensitivity and specificity of the B. dermatitidis PCR with DNA samples

The internal standard control plasmid pTJV2 was constructed to control for the presence of false negatives by inserting a 363-bp portion of the BAD1 gene sequence into pGEM-3Z. Inverse PCR created an internal 100-bp deletion using a NotI digestion (data not shown). Sequencing verified that part of the BAD1 gene was present in pTJV2. To ascertain the sensitivity of the PCR amplification system, B. dermatitidis genomic DNA and pTJV2 plasmid DNA were serially diluted and amplified with the Blasto I/Blasto II primer set. This PCR was shown to be sensitive down to 500 fg or 40 copies for pure Blastomyces DNA (Fig. 1A), and 100 ag or 32 copies for pTJV2 DNA (Fig. 1B).

To evaluate the specificity, DNA was extracted from closely related fungal species and from microbial species that would be typically found in soil samples (Table 1). No PCR amplification of non-Blastomyces fungal DNA was observed with the Blasto I/Blasto II primer set, but a pan-fungal ITS1/ITS4 primer set amplified all of the fungal species tested (Fig. 2, Table 1). Moreover, none of the DNAs from the bacterial species (including actinomycetes) amplified DNA with the Blasto I/Blasto II primer set, yet the universal 8F and 1492R primer set gave rise to PCR products (data not shown), conclusively showing no cross-reactivity with the Blastomyces-specific primers. Additionally, duplicate non-sterile soil samples from the marsh and forest did not show any PCR amplification using the Blasto I/Blasto II primer set, but there were bands representing other soil fungi present when the ITS1 and ITS4 primer set was used (data not shown).

Sensitivity testing of soil samples with spiked pTJV2 DNA or Blastomyces yeast cells

Although the sensitivity of the PCR assay was excellent for purified pTJV2 DNA, this assay was developed in an attempt to detect B. dermatitidis in nature, e.g., in soil samples. To ascertain the sensitivity in natural samples, dilutions of purified pTJV2 DNA were added to sterile soil samples and then PCR amplified with the Blasto I/Blasto II primers. Detection was observed at a...
lower limit of 10 fg or 304 copies (Fig. 3). Next, yeast cells at $8.5 \times 10^4$ cells/ml were serial 10-fold diluted, the diluted cells added to soil, and each soil specimen PCR amplified. Figure 4 shows that as few as $8.5 \times 10^3$ yeast cells could be detected, but PCR was frequently inhibited when a 1/80 dilution of the soil was utilized. However, a 1/160 dilution of the soil sample displayed no inhibitory effects as measured by the internal standard control.

Detection of Blastomyces from natural soil samples

The data above demonstrated that fewer than $10^4$ live Blastomyces cells could be detected in soil samples. To validate the assay, soil was obtained from the Iroquois Kennel Club near Lexington, KY, a site where several dogs had been diagnosed with B. dermatitidis infections. Of eight soil samples extracted and PCR amplified, three produced a weak positive reaction (Fig. 5) using the optimized assay. To confirm BAD1 amplification, the positive samples were purified and concentrated using the Qiagen MinElute Gel Extraction kit (Qiagen, Valencia, CA), and sequencing was performed. A BLAST search of the sequenced amplicon demonstrated it to be the target sequence within the B. dermatitidis virulence gene.

Discussion

Blastomyces dermatitidis has been an elusive fungus to isolate from natural habitats, having only been grown in culture from a natural source 21 times. Although the occurrence of blastomycosis is relatively rare, the repercussions of infection are far greater, considering the significantly toxic effects of long-term anti-fungal treatment and long-term complications resulting from infection. Thus, the best method of treatment for the deep mycoses is prevention from initial infection. To this end, in order to better understand its environmental niche, a significantly improved method of detection for Blastomyces was developed.

Previously, the only method of isolation of Blastomyces from natural samples was through laborious, inefficient animal inoculation techniques. In addition, simple plating of Blastomyces-positive soil will not work due to the presence of other, faster growing, competitive bacterial and fungal species. This study has devised a new method of detection using PCR-based techniques relying on B. dermatitidis-specific primers. In addition to the development of an internal standard control, we have optimized the PCR for sensitivity and specificity as well as determined the levels of detection from soil using DNA and live Blastomyces yeast cells.
Finally, we have confirmed the efficacy of this method by detecting Blastomyces DNA from natural soil samples.

To ensure that all PCR amplified Blastomyces-negative soil samples gathered from studies of Blastomyces are not the result of PCR inhibitors (such as humic and fulvic acids) present in variable concentrations depending on soil type, the internal standard control, pTJV2, was constructed (data not shown). With the pTJV2 internal standard control, it is easy to ascertain the quality of the extraction method used in this technique based on residual levels of inhibitors post-extraction. After optimization of the protocol using the primers initially developed by Bialek [12], the data displayed in this study indicated a good starting point before proceeding with the extraction and amplification of nucleic acids from actual soil samples. Moreover, the applications of this type of control plasmid extend beyond the boundaries of this study. Further investigations may warrant using this internal standard control in clinical diagnosis of blastomycosis.

Specificity of the putative Blastomyces-specific primers was a concern. It was necessary to test many organisms phylogenetically related to B. dermatitidis for cross reactivity, as well as organisms that are frequently encountered in soil specimens. Using molecular and morphological clade analysis based on work performed by Sugiyama and Mikawa [13] and Unterainer [14], a suitable list of organisms from the Onygenales was compiled and tested, including the closest known relatives of Blastomyces: Spiorastix, Polytelypa, and Emmonsona crescens. Also, since the gene being amplified is a B. dermatitidis virulence gene, it was especially necessary to consider other organisms that have similar mechanisms of virulence to Blastomyces, including Histoplasma capsulatum.

As mentioned earlier Histoplasma capsulatum contains an area of partial identity with similar function to that of the Blastomyces BAD1 virulence gene[11], and according to analysis of the LSU rDNA, [13] E. crescens is the closest known relative to Blastomyces, mimicking B. dermatitidis both micro- and macro-morphologically. No PCR amplification was observed for these fungi under the optimized conditions set forth in this study. Of equal importance are the negative results shown for the other soil organisms tested, including all of the bacteria (including actinomycetes) and the ubiquitous fungi such as Aspergillus, Penicillium, and Cladosporium since these are most commonly encountered in soil.

Because there were no cross-reactive species, the BAD1 primers along with use of the internal standard control, pTJV2, demonstrate excellent utility for future molecular studies of Blastomyces, both ecological and clinical. By using a unique gene (BAD1) to generate primers for PCR, it is possible to bypass the problems with cross-reactivity that are often associated with the ITS primers, which in the past were compensated for by confirmatory Southern blot hybridizations. By using
BAD1-specific primers, results can be obtained in a single step that decreases turnover time and allows for processing of a greater number of samples.

This study also determined the lowest level of detection of Blastomyces nucleic acids when both the live yeast cells and pTJV2 plasmid DNA were spiked into a twice sterilized soil sample. In each instance, although there is an inherent drop in sensitivity from optimal conditions, the assay remains practical for application in the field. When extracting the spiked pTJV2 plasmid DNA from the soil sample, there was only about a 10-fold loss in sensitivity from 32 copies to 304 copies required for detection in 0.75 g of soil (Fig. 3). However, Blastomyces yeast cells average 4 nucleic per cell, which theoretically provides the sensitivity to about 76 cells required for extraction. The drop in sensitivity in soil was not necessarily a result of a lack of recovery of DNA, but rather the necessity to dilute the DNA to remove PCR inhibitors. When extracting live yeast cells, the drop in sensitivity was about 100-fold (8.5 x 10^3 cells) compared to when pTJV2 DNA alone was spiked into the soil extraction. Future studies are warranted that address soil inhibitors and ultimately the sensitivity of the test.

The greatest significance for this study was the detection of B. dermatitidis in three soil samples collected from a dog kennel near Lexington, KY in which 35 out of 100 of the animals had contracted blastomycosis within the previous year (Al Legrende, personal communication). This represents the first reported detection of Blastomyces DNA from a natural source. Thus, this method could provide a rapid tool of investigation to epidemiologists returning to the site of a blastomycosis outbreak. By using this method, an investigator can save months in turnaround time screening for the presence of B. dermatitidis, allowing for the rapid identification of potential hotspots. Unfortunately, no live Blastomyces were later detected, only its nucleic acids. To determine the true ecology it will be necessary to devise a better method of isolating B. dermatitidis.

During the first documented environmental isolation of Blastomyces in 1958, more than 600 samples were tested with animal inoculation before a single positive isolate was found [9]. Although this new PCR method has only been tested in one location, the results are encouraging for the eventual replacement of animal inoculation. Additionally, this method could provide a template for the design of environmental detection methods for other dimorphic fungal pathogens or other recalcitrant organisms. Furthermore, the rapid detection of Blastomyces could lead to better culturing techniques, which would aid in a better understanding of the life cycle and ecological niche of this elusive fungus. Ultimately, this method could be utilized to help gain an understanding of the overall ecology of Blastomyces.

Acknowledgements

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