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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 *BAD*1 virulence gene. An internal standard control, pTJV2, 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 pTJV2 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

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 *BAD*1 [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.

Table 1 List of organisms tested for cross-reactivity with Blasto I:Blasto II primer set.

Species	PCR Result
Fungi	
Onygenales:	
Onygenaceae:	
Histoplasma capsulatum	Negative
Emmonsia crescens	Negative
Spiromastix grisea	Negative
Polytolypa hystricus	Negative
Coccidioides immitis	Negative
Arthrodermataceae:	
Trichophyton rubrum	Negative
Trichophyton mentagrophytes	Negative
Microsporum gypseum	Negative
Other fungi:	
Aspergillus flavus	Negative
Penicillium chrysogenum	Negative
Candida albicans	Negative
Cladosporium carionii	Negative
Malassezia furfur	Negative
Bacteria	
Bacillus megaterium	Negative
Pseudomonas flourescens	Negative
Streptomyces griseus (actinomycetes)	Negative
Nocardia brasiliensis (actinomycetes	Negative
Natural samples	
Marsh	Negative
Forest	Negative

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 on 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 crescens* was obtained from the ATCC. *Spiromastix grisea* and *Polytolypa hystricus* were obtained from Wendy Untereiner from Brandon University (Manitoba, Canada). *Trichophyton mentagrophytes, Cladosporium carionii*, and *Malasezzia furfur* were obtained from Gundersen 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.

Construction of the pTJV2 control plasmid

Blastomyces dermatitidis ATCC 26199 was used for all assays and procedures. The primers, Blasto I (5′-AAGTGGCTGGGTAGTTATACGCTAC-3′) and Blasto II (5′-TAGGTTGCTGATTCCATAAGTCAG G-3′), synthesized by Integrated DNA Technologies, (Coralville, Iowa) were used to amplify a 363-bp region of the *BAD*1 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 *YPS3* gene of *Histoplasma* [11]. PCR amplifications were set up using 25 pmol of each primer, 2.5 mM $MgCl_2$, 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 *Sma*I digested pGEM-3Z DNA (Promega, Madison, WI), and then transformed into *E. coli* strain DH5α

[15]. 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 *BAD*1 gene fragment, plasmid DNA isolated from pTJV1 using a commercial extraction kit (Qiagen, Valenica, CA) was PCR amplified with the following oligonucleotide primers: pTJV2 *Not*I F: 5′-ATATTGCGGCCGCGT GCTGCTGCACCTTGATTC-3′ pTJV2 *Not*I R: 5′-ATTCCGCGGCCGCATTTTGCACCCATCCTCTC T-3′ These were designed to have a *Not*I site at the 5' end of each primer to allow the linear PCR amplification product to be cleaved with *Not*I and ligated together to create a new plasmid with a 100-bp deletion within the *BAD*1 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 *Not*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 pTVJ1 (data not shown). One of the resulting plasmids, labeled pTJV2, PCR amplified a 263-bp product with the Blasto 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 $MgCl_2$ 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 (Triton 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 aqueous 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 72 h, and then autoclaved once again for 20 min. Soil samples from a presumptive endemic area for *Blastomyces* were collected from the Iroqouis 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 Ultra-Clean 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 *Not*I 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

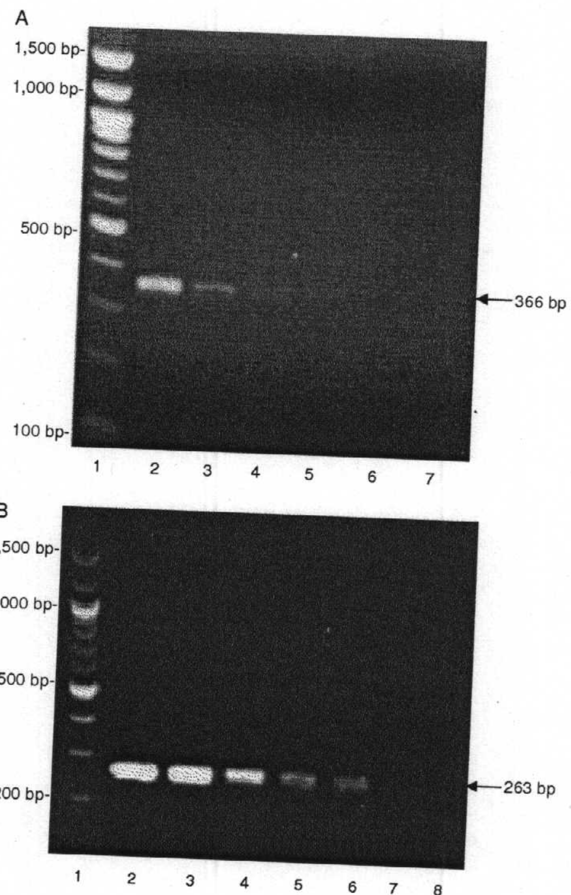

Fig. 1 (A) Sensitivity of the *Blastomyces dermatitidis* PCR using genomic fungal DNA. The PCR amplification products were loaded onto a 1.5% agarose gel. Lane 1: 100-bp DNA ladder; Lane 2–6: ATCC 26199 *B. dermatitidis*; Lane 2: 50 pg; Lane 3: 10 pg; Lane 4: 1 pg; Lane 5: 500 fg; Lane 6: 100 fg; Lane 7: Negative control (sterile water). (B) Sensitivity of the *Blastomyces dermatitidis* PCR with the internal standard pTJV2 plasmid. The PCR amplification products were loaded onto a 1.5% agarose gel. Lane 1: 100-bp DNA ladder; Lane 2–7: pTJV2; Lane 2: 50 fg; Lane 3: 10 fg; Lane 4: 1 fg; Lane 5: 500 ag; Lane 6: 100 ag; Lane 7: 50 ag; Lane 8: Negative control (sterile water).

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

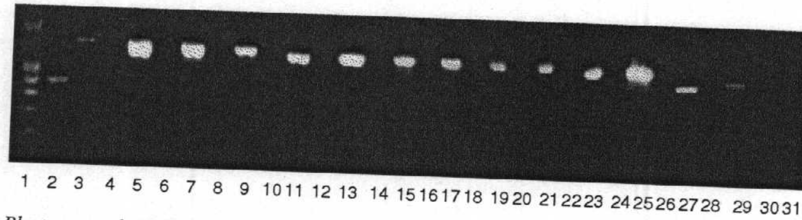

Fig. 2 Specificity of the *Blastomyces dermatitidis* PCR against other fungal species. The PCR products were run on a 1.5% agarose gel. Lane 1: 100-bp DNA ladder; Lanes 2–31: even lanes Blasto I:Blasto II, odd lanes ITS1:ITS4; Lanes 2,3: ATCC 26199 *B. dermatitidis*; Lanes 4,5: *Histoplasma capsulatum*; Lanes 6,7: *Emmonsia crescens*; Lanes 8,9: *Spiromastix grisea*; Lanes10,11: *Polytolypa hystricus*; Lanes12,13: *Coccidioides immitis*; Lanes 14,15: *Trichophyton mentagrophytes*; Lanes 16,17: *Trichophyton rubrum*; Lanes 18,19: *Aspergillus flavus*; Lanes 20,21: *Penicillium notatum*; Lanes 22,23: *Candida albicans*; Lanes 24,25: *Malasezzia furfur*; Lanes 26,27: *Cladosporium carionii*; Lanes 28,29: *Microsporum gypseum*,; Lanes 30,31: Negative control (sterile water).

lower limit of 10 fg or 304 copies (Fig. 3). Next, yeast cells at 8.5×10^6 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 8.5×10^3 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 Iroqouis 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 *BAD*1 amplification, the positive samples were purified and concentrated using the Qiagen MiniElute 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.

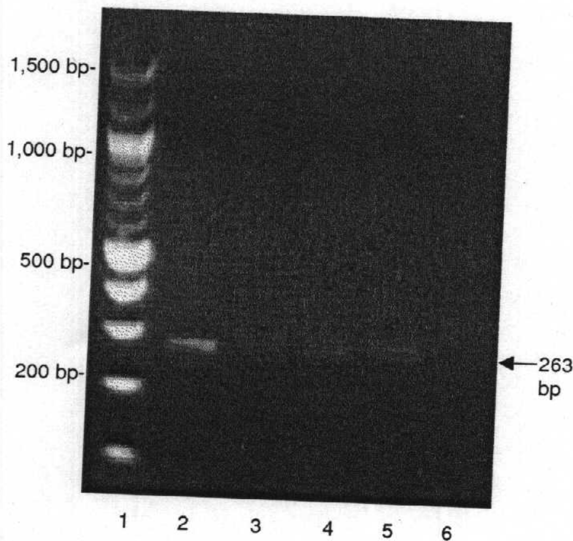

Fig. 3 Sensitivity of the *Blastomyces dermatitidis* PCR using pTJV2 extracted from sterile soil. DNA concentrations relate to amount originally spiked into soil. Lane 1: 100-bp DNA ladder; Lane 2: 10 pg pTJV2 diluted 1/40, 5 µl template added; Lane 3: 100 fg pTJV2 diluted 1/40, 5 µl template added; Lane 4: 10 fg pTJV2 diluted 1/40, 25 µl template added; Lane 5: 1.0 fg pTJV2 diluted 1/20, 25 µl template added; Lane 6: 0.1 fg pTJV2 diluted 1/20, 25 µl template added.

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.

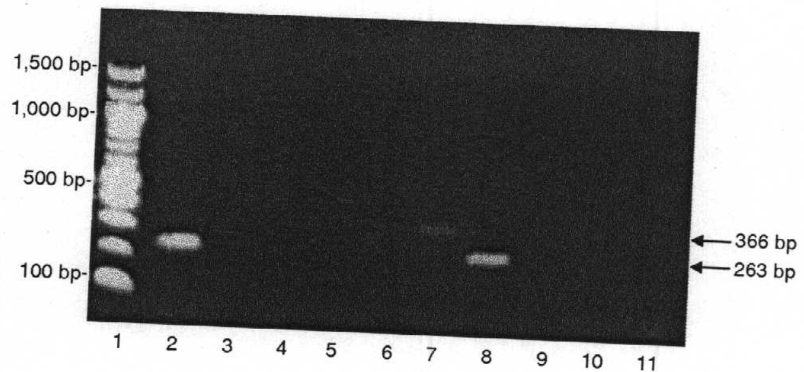

Fig. 4 PCR amplification of soil extracted *Blastomyces* yeast cells at a 1/80 dilution (positive sample highlighted by arrow). The PCR products were loaded and visualized on a 1.5% agarose gel. Lane 1: 100 bp DNA ladder; Lanes 2–10: even lanes contain 10 fg pTJV2; Lane 2: 10 fg pTJV2; Lanes 3,4: *Blastomyces* yeast cell extraction at 8.45×10^5; Lanes 5,6: *Blastomyces* yeast cell extraction at 8.45×10^4; Lanes 7,8: *Blastomyces* yeast cell extraction at 8.45×10^3; Lane 9,10: *Blastomyces* yeast cell extraction at 8.45×10^2; Lane 11: Negative control.

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 Unterеiner [14], a suitable list of organisms from the Onygenales was compiled and tested, including the closest known relatives of *Blastomyces*: *Spiromastix*, *Polytolypa*, and *Emmonsia 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 BAD*1 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 *BAD*1 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

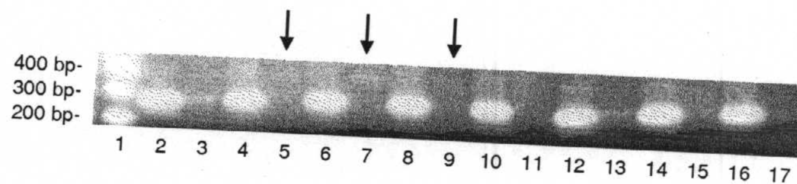

Fig. 5 Detection of *Blastomyces dermatitidis* DNA from natural soil samples. Lane 1: 100-bp DNA ladder; Lanes 2–16: even lanes contain 10 fg pTJV2; Lanes 3,4: Soil sample 1; Lanes 5,6: Soil sample 2; Lanes 7,8: Soil Sample 3; Lanes 9,10: Soil Sample 4; Lanes 11,12: Soil sample 5; Lanes 13,14: Soil sample 6; Lanes 15,16: Soil sample 7; Lane 17: Negative control. All soil samples diluted 1/40, 25 μl template added.

*BAD*1-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 nuclei per cell, which theoretically improves the sensitivity to about 76 cells required for extraction. The drop in sensitivity in soil was not necessarily a result of a lack of the recovery of DNA, but rather the necessity to dilute the DNA to remove soil PCR inhibitors. When extracting live yeast cells, the drop in sensitivity was about 100-fold (8.5×10^3 cells) compared to when pTJV2 DNA alone was spiked into the soil extraction. Future studies are warranted that need to 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 Legendre, 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 the 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*.

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