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Development of a Duplex Real-Time PCR Assay for the Differentiation of *Blastomyces dermatitidis* and *Blastomyces gilchristii* and a Retrospective Analysis of Culture and Primary Specimens from Blastomycosis Cases from New York (2005 to 2019)

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Mitchell Kaplan and YanChun Zhu contributed equally. Mitchell Kaplan was designated first author, as he devoted full time in the laboratory to do this project while learning many techniques. YanChun Zhu performed many crucial experiments independently as well as with Mitchell Kaplan.

ABSTRACT Blastomycosis due to *Blastomyces dermatitidis* and *Blastomyces gilchristii* is a significant cause of respiratory mycoses in North America with occasional reported outbreaks. We developed a highly sensitive, specific, and reproducible TaqMan duplex real-time PCR assay for the differentiation of *B. dermatitidis* and *B. gilchristii*. The new assay permitted retrospective analysis of *Blastomyces* cultures (2005 to 2019) and primary clinical specimens from blastomycosis cases (2013 to 2019) from New York patients. We identified *B. dermatitidis* as the predominant pathogen in 38 cases of blastomycosis, while *B. gilchristii* was a minor pathogen involved in five cases; these findings expand understanding of blastomycosis in New York. The duplex real-time PCR assay could be implemented in reference and public health laboratories to further understand the ecology and epidemiology of blastomycosis due to *B. dermatitidis* and *B. gilchristii*.

KEYWORDS *Blastomyces*, ecology, epidemiology, molecular assay, validation

Until recently, *Blastomyces dermatitidis* was considered a single species within the genus *Blastomyces* and the sole etiologic agent of pulmonary and disseminated blastomycosis (1). This knowledge base was behind the development of several diagnostic tests (2–5). With the recent advances in fungal biology, several distinct genetic populations were identified as new species within the genus *Blastomyces*. Among the new species are *Blastomyces gilchristii* (6, 7), *Blastomyces percursus*, and *Blastomyces silverae* (8, 9). *Blastomyces helicus* was proposed as a new species responsible for rare blastomycosis cases in the western part of North America. Finally, *B. percursus* was proposed as a new species for blastomycosis cases from Africa (10, 11). In addition, taxonomic revisions assigned *Emmonsia parva* as *Blastomyces parvus* and *Emmonsia helica* as *Blastomyces helicus* within an expanded *Blastomyces* genus (9). *Blastomyces dermatitidis* is the predominant species in North America. *Blastomyces gilchristii* might be the leading cause of blastomycosis in northwestern Ontario and northern Wisconsin areas where the disease is hyperendemic (10). The ecological niche(s) of *B. dermatitidis* and *B. gilchristii* remains undefined. Similarly, the role of remaining rare *Blastomyces* species in the overall incidence and outcome of blastomycosis cases requires further investigations.

All *Blastomyces* spp. are thermally dimorphic fungi, existing as a mold form at ambient temperature in the environment. *Blastomyces* spp. convert to the pathogenic yeast form *in vitro* at 37°C or when a susceptible mammalian host inhales the conidia of the

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mold form. The yeast forms of these pathogens have distinguishable microscopic features: *B. dermatitidis* and *B. gilchristii* produce abundant, large (8- to 20-μm) broad-based budding yeasts, *B. percursus* produces large yeast-like cells from fragmented swollen hyphal cells, *B. helicus* produces variably shaped yeast cells (4 to 5 μm) in short chains, and *B. parva* and *B. silverae* produce thin-walled giant cells and a few broad-based budding yeast-like cells (12). A close examination of the yeast morphology might help in the species differentiation in the primary specimens. However, microscopic diagnosis requires expertise in mycology, which is not available in all laboratories. Culture and histopathology will help diagnose blastomycosis, but molecular methods are needed for accurate identification of newly described *Blastomyces* species. There are currently no molecular or serologic diagnostic tests for the rapid and accurate identification of species within the genus *Blastomyces*. An available commercial test (Gen-Probe, Inc., San Diego, CA) is somewhat limited, as it can be used only with pure cultures of *B. dermatitidis*. Also, culture-based identification is not feasible in all laboratories because of the occupational hazard associated with the fungus mold form. Earlier, we developed a rapid and specific real-time PCR assay to identify and detect the then-known four haplotypes of *B. dermatitidis* (5).

Outdoor exposure and proximity to water have been associated with blastomycosis (13). Blastomycosis is reportable in only five states in the United States, namely, Arkansas, Louisiana, Michigan, Minnesota, and Wisconsin (13). In New York State, blastomycosis is considered an emerging disease (http://www.nycasm.org/Alerts/Notification_101674.pdf). Several recent case series and case reports describe blastomycosis from New York. However, areas in New York where blastomycosis is endemic remain undefined, and it is not included in the list of reportable diseases (14–17). Likewise, in the absence of a skin test, it is difficult to assess the geographic location of likely exposure to the pathogen. Limited publications on canine blastomycosis suggest that the disease is prevalent in the Saint Lawrence River valleys on the New York-Canada border (14). Pneumonia is the most common manifestation of blastomycosis; approximately half of all cases are asymptomatic (18). However, *Blastomyces* infection can lead to a severe and fatal disease, often because of respiratory failure (19). Disseminated infection can involve any organ, often including cutaneous abscesses and osteomyelitis, and is frequently accompanied by fever, weight loss, and night sweats (2).

BAD-1 is an important conserved adhesion-promoting protein and virulence factor of *B. dermatitidis* (20). Two large insertions were noted in the *B. gilchristii* BAD1 gene (21), while this gene was absent in African strains of *Blastomyces* spp. (22). Several conventional PCR assays were designed using the *BAD1* gene (23) for the detection of *B. dermatitidis* DNA from clinical and soil samples (4, 24). Sidamonidze et al. (5) developed a real-time PCR assay targeting the *BAD1* gene to identify *B. dermatitidis* in culture and primary specimens. In the present investigation, we focused on the sequence variations observed within *BAD1* to develop a duplex real-time PCR assay for the differentiation of *B. dermatitidis* and *B. gilchristii*. We present data on the sensitivity, specificity, and reproducibility of the assay. The new assay was used for a retrospective analysis of culture and primary specimens from blastomycosis cases from 2005 to 2019 in New York. Our results highlight the idea that *B. dermatitidis* is the major pathogen in blastomycosis cases in New York, with *B. gilchristii* being involved in few cases.

MATERIALS AND METHODS

Strains, primary specimens, and DNA. Seventy-nine isolates of *Blastomyces* spp. were evaluated in this study. Forty-eight clinical isolates came from the sporadic cases of blastomycosis between 2005 and 2019 from New York. Isolates from canine (6 isolates), feline (1 isolate), sea lion (2 isolates), bat (1 isolate), polar bear (1 isolate), soil (4 isolates), and human (2 isolates) sources were obtained from Gene M. Scalarone (Department of Biological Sciences, Idaho State University). American Type Culture Collection (ATCC) strains used were MYA2586 (soil), MYA2585 (dog), ATCC 56214 (human, Africa), and ATCC 56216 (human, Africa). Five well-characterized strains each of *B. dermatitidis* and *B. gilchristii* were obtained from the Public Health Laboratory, Public Health Ontario, Toronto, Ontario, Canada. The species-level identifications of *Blastomyces* cultures from Canada were confirmed by sequencing of the *ITS2* and *drk1* genes described in an earlier publication (7). Thirty-three primary clinical specimens from New York patients, including 12 paraffin-embedded tissues, six bronchial wash specimens, five lung tissue

samples, three cerebrospinal fluid samples, two skin lesion tissue samples, two bone marrow samples, and one sample each of blood, brain lesion tissue, and sputum submitted for *B. dermatitidis* identification, were also part of this investigation. Additionally, DNA from closely and distantly related fungal pathogens was included as part of a specificity panel (5).

DNA extraction. DNA samples from *Blastomyces* spp. were extracted using a Qiagen DNA minikit on the Qiacube automated extractor. In brief, fungal growth (approximately 5 by 5 mm) was removed from the agar surface using a sterile loop and added to a lysing reagent in a biosafety level 3 (BSL3) laboratory. The fungal suspension was incubated at 90°C for 1 h and then brought to a BSL2 laboratory for further processing. The heat-killed fungal suspension was homogenized three times in a Precellys homogenizer at 6,500 rpm for 15 s each time (program number 5; 6500-3x60-015). The homogenized suspension was transferred to a 2-ml screw-cap tube, leaving behind the beads. DNA from samples was extracted using the Qiagen DNA minikit in the QiaCube semiautomated DNA extractor, resulting in 50 μl of eluted DNA. All extracted DNAs were stored at -80°C. DNA of fungal species (yeasts and molds) other than *Blastomyces* spp. were procured from the Wadsworth Center Mycology Laboratory (WCML) DNA Collection Repository.

The Wadsworth Center Histopathology Core first sectioned DNA from paraffin-fixed tissues, and then the paraffin was dissolved with 1 ml of xylene, followed by two washes using 1 ml of 100% ethanol. The sample was then dried and extracted using the Qiagen DNA minikit as described above with an incubation temperature of 70°C instead of 90°C. For nonfixed tissues, an approximately 5- by 5-mm section of tissue was cut with a sterile blade, and extraction was done using the Qiagen DNA minikit as described for the isolates, with an incubation temperature of 70°C instead of 90°C.

Primers and probes. Primers and probes for the real-time PCR assay were designed from the promoter region of the *BAD1* gene using Geneious R9 9.1.6 software (Biomatters, Inc., San Diego, CA). The choice of *BAD1* promoter was based upon our earlier successful utilization of this region for the singleplex real-time PCR assay for *B. dermatitidis* identification/detection (5). Multiple alignment of *BAD1* promoter sequences revealed unique sequences for *B. dermatitidis* and *B. gilchristii*, which were used for primers and probes design (see Fig. S1 in the supplemental material). Oligonucleotide sequences of the primers and probes for *B. gilchristii* and *B. dermatitidis* are as follows: V2556 (*B. gilchristii;* forward), 5'-ATGGGTGCAAAATCCGCCTA-3'; V2558 (*B. gilchristii*; reverse), 5'-AATCTAGAAGCTGGAGCGCC-3'; V2557 (*B. gilchristii;* probe), 5'-FAM (6-carboxyfluorescein)-CCGTACTCC/ZEN/CTCCCCGGTACTCC-3'IABkFQ (Iowa Black fluorescent quencher); V2559 (*B. dermatitidis*; forward), 5'-GCAAAATCCGCCTACTACTA-3'; V2561 (*B. dermatitidis*; reverse), 5'-AGCTGAACCTGGAAGTATTG-3'; V2560 (*B. dermatitidis*; probe), 5'-Cy5-TCCCTACCC/TAO/CTGGCTACTTTTCT-3'IAbRQSp (Iowa Black red quencher spacer). The internal ZEN and TAO quencher was incorporated between bases 9 and 10 from the 5' end of the probes V2557 and V2560, respectively. This design decreased the distance between the dye and the quencher and was expected to reduce the background signal and achieve an improved dynamic range. The primers and probes were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

Real-time PCR assay. Each DNA sample (isolate or primary specimen) was tested in duplicate in 20-μl reaction volumes using an optical 96-well plate. Each reaction mixture contained 1$\times$ PerfeCTa multiplex qPCR ToughMix (Quanta Biosciences), a 1,000 nM concentration of each *B. gilchristii* (V2556 and V2558) and *B. dermatitidis* (V2559 and V2561) primer, a 250 nM concentration of each *B. gilchristii* (V2557) and *B. dermatitidis* (V2560) probe, and 2 μl of DNA (approximately 1 to 10 ng) extracted from *Blastomyces* spp. or 5 μl of DNA extracted from primary clinical specimens. Each PCR run also included 2 μl (1 ng) of positive extraction control (M808; *B. dermatitidis*), 2 μl (1 ng) of positive amplification controls (M808 [*B. dermatitidis*] and MB97 [*B. gilchristii*]), and 2 μl of negative extraction (extraction reagents only) and negative amplification (sterilized nuclease-free water) controls. The unidirectional workflow kept the reagent preparation, specimen preparation, and amplification and detection areas separate to avoid cross-contamination. Cycling conditions on the ABI 7500 FAST system (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA) were initial denaturation at 95°C for 20 s, followed by 45 cycles of 95°C for 3 s and 60°C for 30 s. Based on limit of detection (LOD), a cycle threshold (C_T) value of $\leq$38 was reported as positive and $>$38 was reported as negative. Specimens were reported as inconclusive if PCR inhibition was observed for the primary specimens.

Data availability. GenBank accession numbers for *BAD1* are MT822768 to MT822773; GenBank accession numbers for the internal transcribed spacer (ITS) genes are MT822762 to MT822767.

RESULTS

Assay sensitivity, specificity, and reproducibility. The duplex real-time PCR assay was highly sensitive, with linearity over 5 logs, a correlation coefficient of $>$0.99, and amplification efficiency of $>$92%. The limit of detection (LOD) of the assay was 1 pg of genomic DNA (gDNA) per PCR within the linear range of the standard curve for *B. dermatitidis* and *B. gilchristii* (Fig. 1 and Table 1). The assay was highly specific, as it did not cross-react with other closely and distantly related fungal pathogens (see Table S2). The duplex real-time PCR assay correctly identified *B. dermatitidis* and *B. gilchristii* from 10 genomic-DNA samples submitted in a blind fashion from Ontario, Canada, confirming the assay validity (Table 2). Additionally, DNA from *B. dermatitidis* and *B. gilchristii* strains run on three different days and within the same day yielded consistent C_T
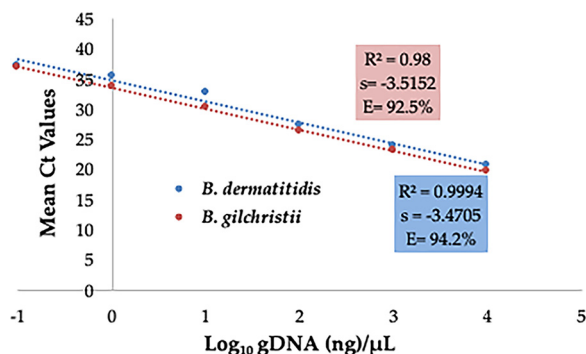
FIG 1 Sensitivity of duplex real-time PCR assay. Serial 10-fold dilution series of gDNA *of B. dermatitidis* and *B. gilchristii* were prepared, and PCR was run in duplicate. The slope (*s*), correlation coefficient (R^2), and amplification efficiency (E) are shown.

values with a cofficient of variance of <5.0, confirming assay reproducibility (Table S3A and B).

Retrospective analysis of *Blastomyces* isolates and primary specimens. The retrospective analysis of 79 isolates of *Blastomyces* spp. revealed 62 to be *B. dermatitidis* and 15 to be *B. gilchristii* (Table 3). One isolate (ATCC 56214), which was identified by singleplex real-time PCR assay as *Blastomyces* spp., was neither *B. dermatitidis* nor *B. gilchristii* by the duplex real-time PCR assay. It was later confirmed to be *B. percursus* by sequencing of the ribosomal genes and BLAST search. One strain of *Blastomyces emzantsi* (ATCC 21516), identified by sequencing, was not identified by the singleplex real-time PCR or the duplex real-time PCR assay, further confirming the specificity of the current duplex real-time PCR assay. Of 15 *B. gilchristii* isolates identified, 5 were well-characterized strains of *B. gilchristii* from Canada, 4 were from the Eagle River outbreak (Wisconsin) involving human, dog, and soil samples, and 6 isolates were recovered from five New York patients. All six New York isolates of *B. gilchristii* were also confirmed by sequencing of the BAD1 and ribosomal genes. Of 62 *B. dermatitidis* isolates identified, 5 were well-characterized strains of *B. dermatitidis* from Canada, 15 were from Illinois, Kentucky, Minnesota, Tennessee, and Wisconsin involving animals or soil and 42 isolates were from 38 patients from New York collected from 2005 to 2019. Of 33 primary specimens analyzed from 2013 to 2019, five specimens (one skin lesion, one bronchial wash, two tissue blocks of nasal masses, and one tissue block of unknown origin) from five patients were positive for *B. dermatitidis* DNA while none were positive for *B. gilchristii* DNA (Table 3).

Blastomycosis in New York. Analysis of 43 cases of blastomycosis from New York (Table S1) revealed one to four cases per year from 2005 until 2016, but that number increased to 7 in 2017, and cases remained high, with 9 identified in 2018 and 8 identified in 2019 (Fig. 2). Men were more frequently infected than women with *B. dermatitidis*, while the number of cases was too small to derive this conclusion for *B. gilchristii*. Older people (over age 40) were found to be more prone to symptomatic infection

TABLE 1 Sensitivity study of *B. dermatitidis* and *B. gilchristii*[a]

DNA concn (ng/μl)	*B. dermatitidis*			*B. gilchristii*		
	C_T 1	C_T 2	Mean C_T	C_T 1	C_T 2	Mean C_T
10	20.73	20.70	20.72	19.85	19.76	19.80
1	23.88	23.87	23.88	23.10	23.08	23.09
0.1	27.25	27.51	27.38	26.54	26.44	26.49
0.01	32.96	32.60	32.78	30.59	30.15	30.37
0.001	35.96	35.27	35.62	33.68	33.64	33.66
0.0001	36.91	37.48	37.20	36.22	37.74	36.98
0.00001	Undet	Undet	Undet	Undet	Undet	Undet

[a]Undet, undetermined.

TABLE 2 Blind panel of DNA of *B. dermatitidis* and *B. gilchristii*[a]

Sample no.	Mean C_T in real-time PCR assay[b]			
	Singleplex	Duplex		Final ID
		B. dermatitidis (Cy5)	*B. gilchristii* (FAM)	
1	23.02	23.48	Undet	*B. dermatitidis*
2	22.4	Undet	21.74	*B. gilchristii*
3	18.52	20.04	Undet	*B. dermatitidis*
4	22.31	22.64	Undet	*B. dermatitidis*
5	19.86	Undet	19.35	*B. gilchristii*
6	22.12	Undet	21.54	*B. gilchristii*
7	23.28	Undet	22.46	*B. gilchristii*
8	19.75	20.15	Undet	*B. dermatitidis*
9	22.23	22.39	Undet	*B. dermatitidis*
10	21.45	Undet	20.39	*B. gilchristii*

[a]DNA was supplied by J.V.K. in a blind fashion.
[b]Undet, undetermined.

irrespective of whether the agent was *B. dermatitidis* or *B. gilchristii*. *B. dermatitidis* was most commonly isolated from respiratory specimens, followed by skin/wounds/subcutaneous tissue and bone, while all six isolates of *B. gilchristii* were isolated from respiratory specimens (Table 4). Geographic distribution of blastomycosis cases revealed that the majority of patients presented with symptoms and lived in Mohawk Valley, Capital District, and Finger Lakes. Few cases were also reported from other regions in New York (Fig. S2).

DISCUSSION

In this study, we developed a duplex real-time PCR assay to differentiate *B. dermatitidis* from *B. gilchristii*. These two closely related species possibly overlap in their endemicity in North America. The duplex real-time PCR assay targeting the putative promoter region of the *BAD1* gene was highly sensitive, specific, and reproducible. *BAD1* and its promoter have been extensively used for the differentiation of *Blastomyces* species using restriction fragment length polymorphism (RFLP), PCR, and real-time PCR assays (5, 21, 24). The polymorphism in *BAD1*, its markedly different sizes (363 bp in *B. dermatitidis* and 663 bp in *B. gilchristii*), and the absence of this gene in African isolates of *Blastomyces* (5, 21, 22) confirmed the choice of this target as a highly specific one for the identification of *B. dermatitidis* and *B. gilchristii* in the present investigation.

The retrospective analysis of *Blastomyces* cultures and primary specimens confirmed 43 cases of blastomycosis in New York from 2005 to 2019 based on the samples received in our facility. We noted an increase in number of cases in the recent years. The reason behind the observed increase in blastomycosis cases in New York are not apparent; our results are in agreement with other investigations describing the

TABLE 3 Validity of duplex real-time PCR assay for culture identification of *Blastomyces* spp. and direct detection from primary specimens

Assay type and singleplex real-time PCR result (*n*)	No. of samples with duplex real-time PCR result			
	B. dermatitidis		*B. gilchristii*	
	Positive	Negative	Positive	Negative
Culture identification				
Positive (78)	62	16	15	63
Negative (60)	0	60	0	60
Direct detection				
Positive (5)	5	0	0	5
Negative (28)	0	28	0	28
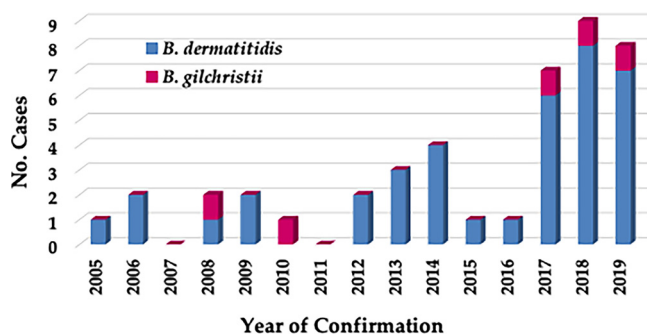
FIG 2 Blastomycosis cases in New York from 2005 to 2019. Blastomycosis cases confirmed earlier by singleplex real-time PCR assay for isolates and primary specimens were analyzed retrospectively with the newly developed duplex real-time PCR assay. Of 43 cases, 38 were confirmed as being caused by *B. dermatitidis* and 5 as *B. gilchristii*. There was a marked increase in blastomycosis cases from 2017 onward.

increase in case counts in New York (15, 17, 25, 26). Of interest was the identification of five of 43 blastomycosis cases due to *B. gilchristii*. Interestingly, patients infected with *B. gilchristii* resided in different parts of New York. Additional studies are needed to determine if these cases are due to patients traveling between regions or a focal niche of *B. gilchristii* in New York. We found that most blastomycosis cases were caused by *B. dermatitidis*, with concentrations of cases in Mohawk Valley and the Capital District, followed by the Finger Lakes. The regional aspect of geographic risk for blastomycosis is not well understood. It is challenging to track the progression from exposure to disease onset in patients. A skin test or another reliable marker of prior exposure is not available, and *B. dermatitidis* is rarely recovered from the environment (10). Furthermore, the recognition of clinical cases in New York is sporadic. Therefore, evidence regarding areas of endemicity in New York is equivocal. Our results are in agreement with other

TABLE 4 Characteristics of Mycology Laboratory (Wadsworth Center)-confirmed blastomycosis cases in New York from 2005 to 2019

Characteristic	Value for group		
	Blastomycosis	*B. dermatitidis*	*B. gilchristii*
No. of patients	43	38	5
Sex			
M	29	26	3
F	14	12	2
Age (yr)			
20–29	4	4	0
30–39	4	4	0
40–49	8	7	1
50–59	11	10	1
60–69	13	10	3
>69	3	3	0
No. of specimens (*n* = 48)			
Bronchial wash	28	22	6
Skin, wound, subcutaneous tissue	15	15	
Bone	4	4	
Brain	1	1	
No. of specimens positive for DNA (*n* = 5)			
Bronchial wash	1	1	
Tissue block—nasal mass	2	2	
Tissue block—source not provided	1	1	
Skin wound fluid	1	1	

reports indicating that *B. dermatitidis* has a broader geographic distribution throughout North America while *B. gilchristii* has a limited distribution in the northern United States and certain Canadian provinces (27).

We know little about the geographic and phenotypic differences between *B. dermatitidis* and *B. gilchristii*. However, preliminary studies suggest that there may be a difference in the clinical manifestation of the diseases between the two species (28). There is also a report of an acute respiratory distress syndrome-related fatal case due to *B. gilchristii* (29). *BAD1* has been implicated as the major virulence factor of *B. dermatitidis* (20). The significant difference in size of the *BAD1* promoter due to two large insertions between *B. dermatitidis* and *B. gilchristii* noted above and the issue of whether these insertions have any influence on *BAD1* expression and virulence need further investigation (21). To the best of our knowledge, no studies are available to demonstrate the comparative virulence of *B. dermatitidis* and *B. gilchristii* in vertebrate or invertebrate model systems. The highly sensitive and specific assay developed in the present study would allow more comprehensive surveillance of blastomycosis to monitor disease incidence. Systematic disease reporting and surveillance efforts will help diagnose new blastomycosis cases. Prompt diagnosis ensures prompt initiation of treatment to decrease illness and death. Finally, public awareness campaigns such as health advisories might be needed for blastomycosis in New York. In summary, the newly developed duplex real-time PCR assay would ensure accurate laboratory diagnosis of *B. dermatitidis* and *B. gilchristii* and help expand the understanding of the ecology and epidemiology of blastomycosis.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.7 MB.

ACKNOWLEDGMENTS

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