ASSESSMENT OF THE CLINICAL RELEVANCE OF FUNGAL GENOTYPE IN BLASTOMYCOSIS INFECTIONS

By Klaire L. Laux

While blastomycosis is a relatively rare disease throughout the United States at an annual incidence of 1-2/100,000 in humans, it is hyperendemic to Wisconsin. Some counties have reported an annual incidence as high as 41/100,000 in humans and 1,420/100,000 in dogs. The Midwestern region of the United States also has among the highest mortality rates associated with the disease in the country at 43.8% as opposed to 1.7% mortality in the Northeast region where it was initially discovered. Blastomycosis is caused by inhalation of conidia which are produced by soil-inhabiting fungi of the genus *Blastomyces*. Since 1896, it was believed that all cases of blastomycosis were caused by inhalation or inoculation with the species *Blastomyces dermatitidis*. However, in 2013, multi-locus sequence typing revealed another species was involved in these infections named "*Blastomyces gilchristii*."

The research question of this thesis was to assess whether there was any clinical relevance to this recent species distinction. I collaborated with the Marshfield Clinic Research Institute and the University of Wisconsin Madison to establish a database of 141 patients from Wisconsin from 2008-2016 for which a clinical isolate was available to be species typed through a single-nucleotide polymorphism in the *ITS2* region of the fungal genome. Species data were then compared to clinical features associated with blastomycosis abstracted from medical records to determine if a new diagnostic assay would be valuable for clinicians.

Significant differences in distributions were discovered among age groups ($p<0.01$), clinical presentation ($p<0.001$), and physician specialty seen ($p<0.001$) between patients infected *B. gilchristii* and *B. dermatitidis*. No significant differences were found in the non-invasive diagnostic test's ability to detect either pathogen. Additionally, evidence was illuminated to support the use of therapeutic drug monitoring throughout the course of treatment for patients infected with either pathogen. These data may help inform new Infectious Diseases Society of America guidelines for the treatment and diagnosis of blastomycosis, which have not been updated since the recent species distinction.

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Klaire L. Laux

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Member

Member

Member

PROVOST AND
VICE CHANCELLOR
FOR ACADEMIC AFFAIRS

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To my dad because his relentless passion and devotion to the public inspired this work and his relentless encouragement pushed me forward to complete it. To my mom and step-dad for their moral support and always being there to answer the phone and take care of my dog, Marzipan. To my Aunt Kelly and Uncle Mark for making me breakfast on Sundays and encouraging me to finish my degree. To my Grandma Mary and Aunt Beth for cheering me on with their infectious enthusiasm. To my friends: Travis for always getting Pad Thai with me and talking science, Shannon for going first, Nick for being a goof-ball and keeping me company in the laboratory, and Nicole for talking feminism and helping me let loose at Bug Camp. To all my friends, family, fellow interns, committee members, and co-workers unmentioned but equally important. All of your moral support and encouragement were instrumental to my ability to complete this work. Thank you even more if you still speak to me after this work is finished—I hope one day to return the favor.

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Chapter I

Introduction to *Blastomyces* and Blastomycosis Infections

Discovery of *Blastomyces*

In 1894, Dr. Thomas Casper Gilchrist, a dermatologist and researcher at Johns Hopkins Medical School, presented a novel pathogen to the American Dermatological Society (Gilchrist, 1896). Dr. Gilchrist received a skin biopsy from what was initially diagnosed as tuberculosis. Rather than finding the predicted *Mycobacterium* in the diseased tissue, Dr. Gilchrist discovered budding yeast cells that he classified as a protozoan (Gilchrist, 1894). Gilchrist and Stokes determined that the etiological agent of the disease was a fungus in their 1898 paper (Gilchrist and Stokes, 1898). Dr. Gilchrist named the organism *Blastomyces dermatitidis* and the disease it caused blastomycetic dermatitis (Gilchrist and Stokes, 1896). Blastomycetic dermatitis was later known as Gilchrist's Disease, Chicago's Disease, and is currently known as blastomycosis.

This was the first disease in which a parasitic fungus was found to be the etiological agent in a human infection (Gilchrist and Stokes, 1896). Following the discovery, inoculation of infectious spores into dogs, sheep, horses, and guinea pigs demonstrated the formation of nodules in the lungs (Gilchrist and Stokes, 1896). When these nodules were cultured, they were found to contain yeast cells 10-20μm in diameter, which grew as multicellular mold at ambient temperatures (Gilchrist and Stokes, 1896). The presentation of these two morphological forms led to the distinction of this organism as dimorphic (Greek: "twice-shaped").

Taxonomy of *Blastomyces* spp.

Blastomyces spp. are known as dimorphic pathogens within the kingdom Fungi, subkingdom Dikarya, phylum Ascomycota, class Eurotiomycetes, order *Onygenales*, family *Ajellomycetaceae* (Alexopoulos, Mims, and Blackwell, 1996). Within the family *Ajellomycetaceae*, other closely related genera that are also known as dimorphic fungal pathogens of mammals include *Paracoccidioides* spp., *Emmonsia* spp., and *Histoplasma capsulatum* (Alexopoulus *et al.*, 1996). Collectively, dimorphic fungal pathogens are responsible for millions of human infections each year (Gauthier, 2015).

The fact that *Emmonsia parva* is a sister species of *Blastomyces* spp. can be directly visualized through the maximum likelihood tree constructed from 2,062 core genes in Figure 1, which is derived from the work of Munoz, Gauthier, Desjardins, Gallo, Holder, Sullivan, and Gujja, (2015). *Histoplasma capsulatum* strains are sister species to *Emmonsia parva* and *Blastomyces* spp. and all three are dimorphic fungal pathogens with the ability to cause infections in mammalian hosts (Munoz *et al.*, 2015).

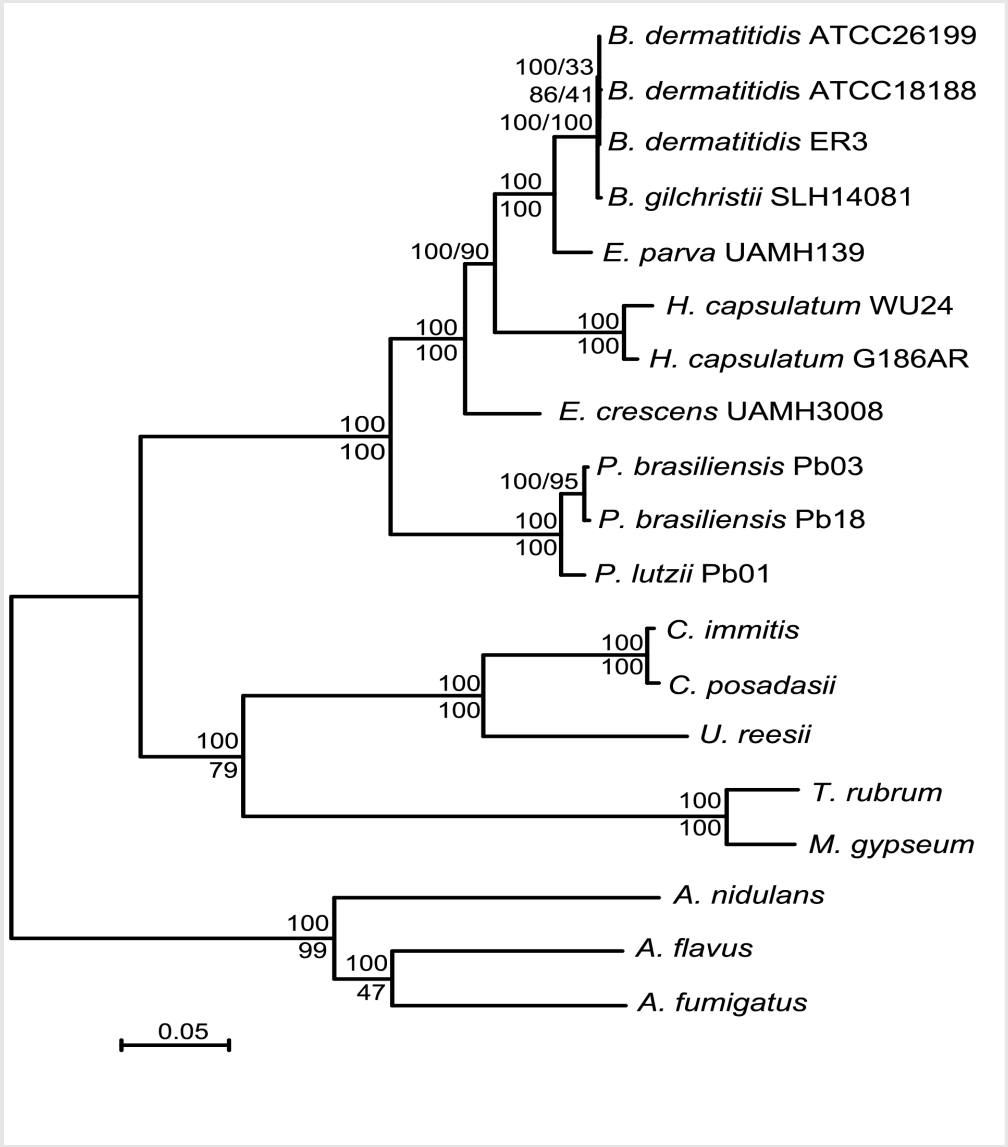


Figure 1. Phylogeny of Blastomyces spp. (Munoz et al., 2015). Phylogeny determined by maximum likelihood tree constructed from 2,062 core genes. The genus *Blastomyces* is closely related to *Emmonsia parva* and *Histoplasma capsulatum*.

Epidemiology of Blastomycosis

Blastomycosis is a regional disease, which afflicts the Midwest, Southeast, Northeast, and Southcentral regions of the United States where infections range from 1-2

per 100,000 each year in humans (CDC, 2017). Blastomycosis is also reported in provinces of Canada that surround the Great Lakes and the St. Lawrence Seaway (Chapman, Dismukes, Proia, Bradsher, Pappas, Threlkeld, and Kauffman, 2008). There is also some controversy about blastomycosis infections outside the North American Continent. McTaggart, Brown, and Richardson (2016) argue that two species of *Blastomyces* that have been isolated from outside North America are either cases where a person was exposed on the North American Continent or the clinical isolate would be better classified as *Emmonsia* spp. In particular, *Emmonsia parva* shows close genetic similarity to *Blastomyces* spp. (Fig. 1) and are known to cause infections in mammals throughout the world (Schwartz, Kenyon, Feng, Govender, Dukik, Sigler, and Botha, 2015).

Within the North American continent, Wisconsin is hyper-endemic to blastomycosis where some counties have had an annual incidence as high as 41.9 per 100,000 in humans and 1,420 per 100,000 in dogs (Reed, Meece, Archer, and Peterson, 2008) showed that Wisconsin has many areas that are predicted to be supportive of the growth of *Blastomyces* spp. in soil (Fig. 2). Reed *et al.* (2008) developed these data through maximum entropy modeling of exposure sites. Areas in Northcentral and Northeast Wisconsin appear to be most impacted by blastomycosis along with the Milwaukee County area, and the fewest exposures in Southwest Wisconsin (Fig. 2).

Klein *et al.* in 1986, showed that *Blastomyces* spp. have a relationship with sandy and acidic soils. Mctaggart *et al.* (2016) also illustrated that *Blastomyces* spp. have an association with fresh water basins and discovered clinical isolates belonging to both

species of *Blastomyces* actively growing in the state of Wisconsin. Reed *et al.* (2008) showed that areas of Wisconsin believed to be most conducive to the growth of *Blastomyces* spp. are areas along the Wisconsin River extending into North Central Wisconsin (Fig. 2). Eagle River has among the highest rates of infection in the country, which is as high as 101.3/100,000 in humans (Cherry, Demmler-Harrison, Kaplan, Steinbach, and Hotez, 2013). Furthermore, Khuu, Shafir, Bristow, and Sorvillo in 2014 found that the Midwest region of the United States reports among the highest mortality rates associated with blastomycosis in the country at 43.8% as opposed to 1.7% in the Northeast region where it was initially discovered.

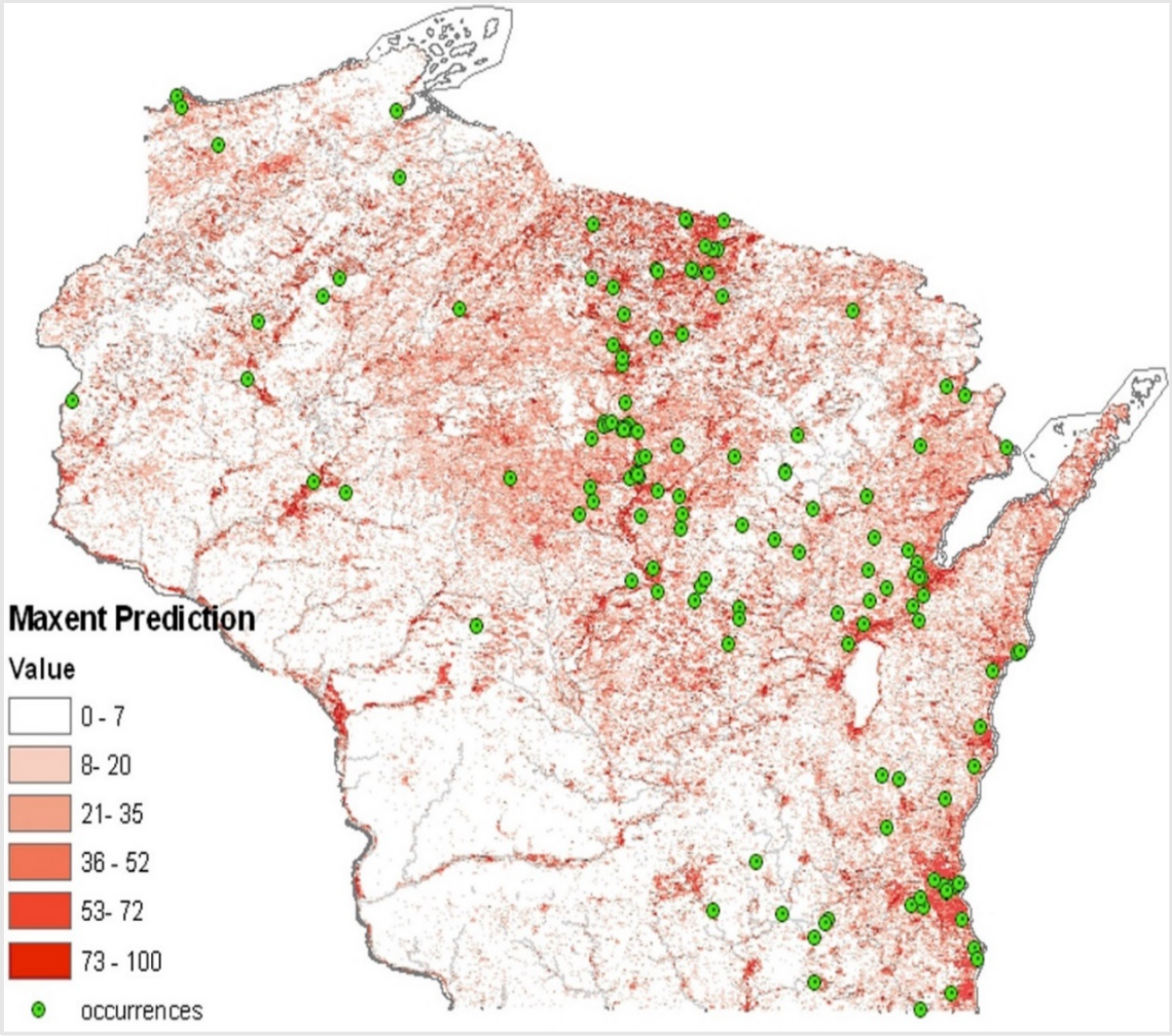


Figure 2. Blastomyces spp. Life Cycle/Ecological Niche/Physiology (Reed et al. 2008). Through maximum entropy modeling of exposure sites, regions in red are shown to be regions of the state conducive to the growth of *Blastomyces*. Green dots demonstrate regions where blastomycosis was contracted by a human or canine host.

The sexual reproduction of the mycelial form of *Blastomyces* spp. is heterothallic, meaning that they require compatible mating types (Mcdonough and Lewis, 1967). The meeting of compatible hyphae results in the formation of cleistothecia with asci, which contain eight ascospores each, as was demonstrated on steamed bone meal and yeast agar

which stimulate both growth and spore production of *Blastomyces* spp. (Mcdonough and Lewis, 1967). Mcdonough and Lewis (1967) went on to demonstrate that these ascospores produce similar infections in animals as do conidia under laboratory conditions.

While the role of *Blastomyces* spp. in the environment has yet to be thoroughly elucidated, it is known that they grow as multicellular, filamentous saprophytes at ambient temperatures where they reproduce asexually to form infectious spores called conidia (Klein, Vergeront, Weeks, Kumar, Mathai, Verkey, Kaufman, Bradsher, Stoebig, and Davis, 1986). Other members of the order Onygenales are known keratin degraders where they are believed to play an important role in the cycling of nutrients in the hair and nails of mammals in the environment (Alexopoulos *et al.*, 1996).

The gene DRK1 was shown to encode a histidine kinase that appears to be involved in the dimorphic switch from mold to yeast as knock-outs of this gene resulted in mold form at 37°C (Nemecek, 2006). It is believed that this gene is also involved in the active expression of virulence factors, which confer the ability of the yeast cells to cause disease, such as the gene BAD-1 (Nemecek, 2006).

The gene SREB was shown to be a transcription factor involved in the conversion from yeast back into mold stages when exposed to ambient temperatures (Gauthier, 2010). While not conclusively demonstrated, this fact alludes to the possibility that, upon the death of the host, the saprophytic life style is activated once again, thereby allowing the fungus to continue its existence at ambient temperatures. Collectively, along with evidence that yeast may be released in fecal material of mammals (Witorsch and Utz,

1968), these data indicate that the parasitism of mammalian hosts may be part of the ecological niche of this organism.

Blastomycosis Infection

Infectious *Blastomyces* spp. spores are dispersed in air from soil disruption events such as excavations, construction, digging, or gardening. Once inhaled by a mammalian host, spores undergo temperature-induced gene transcription that results in the transformation of spores into yeast cells (Krajaejun, Wuthrich, Gauthier, Warner, Sullivan, and Klein, 2010). The yeast cells can also be infectious through dog bites (Guann, Bressler, Bodet, and Avent, 1983), laboratory accidents (Larsh and Schwarz, 1977), environmentally contaminated materials (Gray and Baddour, 2002), sexual transmission (Farber, Leahy, and Meadows, 1968), and possibly through organ transplants (Gauthier, Safdar, Klein, and Andes, 2007).

Once a mammalian host is infected, the incubation period before the onset of disease is estimated to be 33-44 days (Klein *et al*., 1986) but can be as short as a week (Guann *et al.*, 1983) or as long as 106 days (Morgan and Salit, 1996). This variation may be species dependent as there are significant differences in incubation period between species (Meece, Anderson, Gruszka, Sloss, Sullivan, and Reed, 2012). It is estimated that 54% of exposures to *Blastomyces* spp. result in disease (Klein *et al.*, 1986). Further investigation is warranted to determine if this, too, may be species dependent.

Blastomycosis Clinical Presentation

The yeast can persist in the lungs where it can cause fungal pneumonia (pulmonary-only infections) or disseminate to other sites in the body causing skin, eye, or cardiac infections (Langlois, 2013), osteomyelitis (Jain, Singh, Lamzabi, Harbhajanka, Gattuso, and Reddy, 2014), meningitis (Izban, Reyes, and Gujrati, 1995) and/or sepsis (Gilchrist and Stokes, 1896). The majority of blastomycosis infections present as pulmonary-only infections, which can range from pneumonia to acute respiratory distress syndrome (ARDS) (McBride, Gauthier, and Klein, 2017). All of these diseases, if left untreated, have the potential for lethality in the patient. Variations in the gene encoding the BAD-1 protein have also shown significant variation among isolates (Meece *et al.*, 2010) which may both be species dependent as well as account for variations in clinical presentation.

Virulence of *Blastomyces* spp.

The most essential and well-studied virulence factor of *Blastomyces* spp. is the *Blastomyces* adhesion 1 (BAD-1) protein. BAD-1 is released and accumulates on the surface of yeast where it interacts with a chitin receptor on the surface of yeast cells (Brandhorst, Wuthrich, Finkel-Jimenez, and Klein, 2003). Once on the surface of the yeast, it binds to CR3 and CD14 receptors on macrophages (Brandhorst *et al.*, 2003) which subsequently prevents the release of TNFα (Finkel-Jimenez, 2001) and inflammatory cytokines. In this way, BAD-1 allows for the fungus to prevent an initial immune response (Brandhorst *et al.*, 2003). BAD-1 may also interact with T-cells in a

manner similar to CD47 proteins expressed ubiquitously on all human cells, which serves to disguise the fungal pathogen as self to evade immune detection (Brandhorst *et al.*, 2013).

Diagnosis of Blastomycosis

The "gold standard" for diagnosis of blastomycosis is the presence of big, broad-based buds of *Blastomyces* yeast cells visualized by microscopy from patient sputum or from biopsy of infected tissue and growth on culture (Chapman *et al.*, 2008). Direct visualization of yeast from patient specimen facilitates rapid diagnosis (Chapman *et al.*, 2008). In contrast, growth on culture usually requires several weeks of incubation. The development of an antigen test on blood or urine has supplanted the use of serologic testing because the antigen test has substantially improved sensitivity when compared to serological assays (Campbell and Binkley, 1953; Spector, Legendre, Wheat, Bemis, Rohrbach, Taboada, and Durkin, 2008). In addition, blastomycosis should be suspected in patients who live in the endemic region, have risk factors for environmental exposure to blastomycosis, or have an infection (e.g. pneumonia) that does not respond to antibiotics. As a result of the rarity of blastomycosis, confusion over areas of endemicity, and its ability to resemble other diseases, it is often misdiagnosed (McBride *et al.*, 2017).

Treatment of Blastomycosis

When blastomycosis is suspected or confirmed, there are two types of pharmaceutical drugs that are available: polyenes and azoles. The polyene class of

antifungals include Amphotericin B deoxycholate, Amphotericin B lipid complex, Amphotericin B colloidal dispersion, and liposomal Amphotericin (Hamill, 2013). Amphotericin B binds to ergosterol in the fungal cell membrane to form a channel in the membrane, which results in yeast cell death (Brajtiburg, Powderly, Kobayashi, and Medoff, 1990). However, due to structural and functional similarity between ergosterol in fungi and cholesterol in mammalian cells, there are prohibitive side effects to using the drug including but not limited to: nephrotoxicity, infusion reactions (e.g. vomiting, rigors, hyper or hypotension, hypoxia), and electrolyte disturbances (e.g. hypokalemia, hypomagnesemia) (Laniado-Laborin and Cabrales-Vargas, 2009). Therefore, the Infectious Diseases Society of America (IDSA) recommends that treatment with Amphotericin B be guided by physicians with expertise in using this antifungal. Itraconazole is prescribed as initial therapy in persons with mild to moderate blastomycosis, and as a step-down therapy following completion of Amphotericin B treatment (Chapman *et al.*, 2008). In cases where itraconazole cannot be prescribed, fluconazole, voriconazole, posaconazole, ketoconazole, or isavuconazonium sulfate may be used. The mode of action of azoles is to interfere with the biosynthesis of ergosterol in fungal cells by binding to cytochrome P450 enzyme (Odds, Brown, and Gow, 2003). While these drugs are effective at preventing the formation of new fungal membranes, treatment duration is six to twelve months (Chapman *et al.*, 2008). These medications can have side effects (e.g. hepatotoxicity), have multiple drug interactions, and are very expensive.

Post-Diagnostic Monitoring

The Infectious Diseases Society of America (IDSA) in its guidelines for the treatment of blastomycosis in 2008 recommended the use of therapeutic drug monitoring of itraconazole throughout the course of treatment to ensure a therapeutic threshold of the drug is present (Chapman *et al.*, 2008). The therapeutic threshold is defined as >0.5 mcg/mL for localized infections and >1.0 mcg/mL for disseminated infections (Andes, Pascual, and Marchetti, 2008) (Hope, Billaud, Lestner, and Denning, 2008). These minimum guidelines are established for the adequate clearing of the pathogen, but say nothing about upper limits of the threshold. Furthermore, there are currently no accessible guidelines on when or how these doses might be altered and reduced. Extrapolation from the guidelines posted by the British Society for Medical Mycology indicate that a serum concentration level of 3.4 mcg/ml using HPLC might reach the toxic threshold (Ashbee, Barnes, Johnson, Richardson, Gorton, and Hope, 2014). The authors also point out that itraconazole is relatively unpalatable, which illustrates the need to monitor the presence of sufficient levels of itraconazole in patient serum to ensure that patients are taking their medication.

***Blastomyces* spp. Genetics**

Through sequencing of 27 polymorphic microsatellite markers in 112 clinical isolates of *Blastomyces* spp., Meece *et al.* (2012) demonstrated that there were two genetically distinct organisms group 1 and group 2. Through multi-locus sequence typing Brown, McTaggart, Zhang, Low, Stevens, and Richardson, (2013) confirmed that there

was strong enough genetic evidence to distinguish these two groups as two distinct organisms. Group 1 was named *Blastomyces gilchristii* after Dr. Thomas Caspar Gilchrist and Group 2 retained the name *Blastomyces dermatitidis*. The findings in Meece *et al.* (2012) illustrated less sequence homology in microsatellite genotyping in the genome of *B. dermatitidis* than in *B. gilchristii*.

In 2012, Meece *et al.* went on to demonstrate that there were significant differences in clinical presentation between these two species in 227 clinical isolates. In this data set, *B. gilchristii* was found to be more commonly associated with outbreak infections than *B. dermatitidis*. Additionally, the two infections showed significant differences in the onset to diagnosis, symptoms, and the likelihood of patients to have comorbidities (Meece *et al.*, 2012). Through studies into the classification of genetic groups, it was found from an analysis of 360 previously genotyped clinical isolates that sequencing of the internal transcribed spacer 2 (ITS2) region of the fungal genome, a single nucleotide polymorphism (SNP) was enough to distinguish between these two species (Frost, Anderson, Ivacic, Sloss, Embil, and Meece, 2016).

Research Question

The purpose of this work was to explore the clinical relevance of the genetic differences between *Blastomyces gilchristii* and *Blastomyces dermatitidis*. Currently, there are no rapid diagnostic methods for distinguishing between these two species save for the SNP of the ITS2 region of the fungal genome (Frost *et al.*, 2016), which is not accessible to many routine medical laboratories. Assessments were made to significant

differences in the patients infected, the efficacy of the urine antigen test, and the ways in which patients were treated with either disease without physician knowledge of species of etiological agent. It is hoped that these data will contribute to updated IDSA guidelines to assist medical providers to improve care for patients with blastomycosis.

Chapter II

Correlation between Fungal Genotype and Different Clinical Phenotype in Blastomycosis Infections

Background

Blastomycosis is a potentially lethal fungal infection caused by inhalation of spores produced by fungi of the genus *Blastomyces* (Gilchrist and Stokes, 1896). Members of the genus *Blastomyces* grow in association with fresh water and acidic soils where humans and other animals may become exposed to the spores which the fungi produce (McTaggart *et al.*, 2016). Previously, it was believed that the disease was caused by a single species of fungi—*Blastomyces dermatitidis*.

However, in 2011, Meece *et al.* demonstrated that through sequencing of 27 polymorphic microsatellite markers in 227 clinical isolates of *Blastomyces*, there appeared to be two distinct genetic groups of Blastomyces. Then, in 2012, Meece *et al*., discovered that these two groups had significant differences in clinical phenotype. In 2013, Brown *et al*. determined through multi-locus sequence typing that these two groups differed enough to be considered two distinct species--*B. dermatitidis* and *B. gilchristii.* The purpose of this study was to investigate the clinical and demographic features of patients infected with either species of *Blastomyces* using novel analytical methods to search for potentially previously unexplored patterns. Additionally, the purpose of this particular aspect of the investigation was to assess the use of the selected cohort for the purpose of evaluating the sensitivity of the urine antigen test (see Chapter 3).

Materials and Methods

Patient inclusion. Institutional review board approval for a retrospective case series was obtained from both the Marshfield Clinic Research Institute and the University of Wisconsin Madison School of Medicine and Public Health to obtain medical records associated with clinical isolates for cases of blastomycosis. Owing to the fact that the last update to the blastomycosis guidelines were published in 2008, in an effort to assess physician compliance to these guidelines, our cohort only included patients from 2008 until 2017. Furthermore, patients were included in the study if they had a laboratory confirmed case by culture or cytology/histopathology and there existed a clinical isolate available for genotyping.

Isolates. Archived clinical isolates from patients who were treated at the MCRI for blastomycosis were assigned to a fungal species based on a single nucleotide polymorphism in the 19th base pair of the ITS sequence (Brown *et al*., 2013). Clinical isolates from patients who were treated at the UW Madison School of Medicine and Public Health were assigned to a species based on variation in BAD1 promoter region (Burgess, Schwan, and Volk, 2006; Meece *et al*., 2011). Clinical isolates were genotyped either by SNP analysis (Frost *et al.*, 2016) or Sanger sequencing.

Species typing. The internal transcribed spacer 2 (*ITS2*) sequence was amplified through polymerase chain reaction (PCR), and sequenced with Sanger sequencing. A single nucleotide polymorphism (SNP) was used to distinguish between *Blastomyces gilchristii* and *Blastomyces dermatitidis* (Frost *et al.*, 2016). PCR parameters were

consistent with Table 1 and thermocycler reaction parameters were consistent with Table 2. Sequencing parameters were consistent with Table 3.

Table 1

ITS Positive Control PCR Reaction. The total DNA concentration was determined based on Q-bit® quantification and these numbers were used to adjust the amount of added template to achieve a 15-20µl of DNA per reaction.

Reagent	**Volume (µl)**
HotStarTaq® Master Mix	15
ITS 3 F	1
ITS 4 R	1
PCR grade Water	Varied
Template	Varied
Total	30

Table 2

Thermocycler Parameters for amplification of ITS2 in Positive Controls
The thermocycler ran for 45 cycles.

°C	**95**	**94**	**55**	**72**	**72**	**4**
Minutes	15:00	0:30	0:30	1:00	4:00	∞
Reaction	Denaturation	Denaturation	Annealing	Extension	Extension	Hold

PCR products were cleaned up through QIAquick PCR Purification kit® (QIAGEN, Valencia, CA). Buffer PB (50µl) and PCR amplicon solution (10µl) were mixed and added to the QIAquick® column, which was centrifuged for 60 seconds at 13,000rpm. Wash Buffer PE (750µl) were added to the QIAquick® column which was centrifuged for 60 seconds at 13,000rpm. Then 10µl of elution was added to the column and allowed to sit for 5 minutes before centrifugation. The flow-through from the final step was estimated to have a total of 10 ng/µl of amplicon DNA.

The flow-through from the PCR purification step was then used in the sequencing reaction. Sequencing reaction was performed by using BigDye®, PCR grade water, template, and primer (Table 3). Separate sequencing reactions were performed for forward and reverse primers to be aligned together after sequence was obtained. The forward reaction was performed using 1µl ITS 3 F and the reverse reaction was performed using 1µl ITS 4 R as described in primer design. These reactions were then placed in the thermocycler for 25 cycles in accordance with the parameters shown in Table 2.

Table 3

Sequencing Reaction for ITS2 in Blastomyces spp. Clinical Isolates
BigDye® contained a series of dNTPs, fluorescently labeled ddNTPs, buffer, and DNA polymerase. Primer design described earlier. Template obtained through PCR purification.

Reagent	**Volume (µl)**
BigDye®	2
Primer	1
PCR grade water	6
Template	1
Total per reaction	10

Sequencing products were then cleaned up of the excess ddNTP's in preparation of the Sanger sequencer using the DyeEX® 2.0 Spin Kit from Qiagen, (QIAGEN, Valencia, CA). After being spun through the column, sequencing products were dried on the thermocycler for 40 minutes at 70°C and then re-suspended in 10µl of HIDI Formamide before being placed on the sequencer. Sequence data were analyzed with the software package DNASTAR® using the Seqman Pro function for contig assembly, and

the 19th base pair was used to distinguish between *B. dermatitidis* and *B. gilchristii* in accordance with the methods developed by Frost *et al.* (2016).

Database. Research Electronic Data Capture (REDCap) secure software powered by Vanderbilt University and hosted by the Marshfield Clinic Research Institute was used to construct a database, which was developed and managed to hold both fungal genotype and information about each clinical case abstracted from the medical record (Harris, Taylor, Thielke, Payne, Gonzalez, Conde, 2009) (Harris, Taylor, Minor, Elliott, Fernandez, O'Neal, McLeod, Delacqua, Delacqua, Kirby, and Duda, 2019). Information gathered included patient demographic data as well as clinical data about hospital stay, physicians seen, and treatment length

Data analysis. Non-metric multidimensional scaling (NMDS) and principal components analysis (PCA) were performed as exploratory analyses. Specifically, differences in clinical presentation and patient demographics between people who were infected *B. dermatitidis* and *B. gilchristii* within the cohort were searched. The Vegan package in R Studio version 4.3 (2017) was used for NMDS, which had 21 runs before a solution was reached with a distance set to "bray" and a trymax of 200. The Vegan package in R Studio was also used for the Principal Components Analysis. In both cases, data were scaled to account for the difference in values between 0 and 2016 before analysis was performed by using the "scale()" function in R Studio version 4.3 (2017). Not all data gathered were available for every patient. Therefore, the sample sizes may vary depending on the analysis to exclude empty data sets. Additionally, not all patient records were complete in terms of treatment length. An average of all other values (8.54)

was used in its stead for NMDS and PCA to prevent these values from skewing these analyses. However, blank treatment values were omitted for X^2 analyses.

The X^2 tests of independence were used to measure significance in differences among proportions within frequencies of distribution in nominal variables (Ambrose and Ambrose, 2002). These tests were performed in accordance with the rules outlined by Ambrose and Ambrose, 2002. Age groups, for the purpose of the X^2 test of independence, defined by life cycle category by The National Statistics Office of Canada (2017). A Student T-test was also performed on mean ages in R studio version 4.3 (2017).

Definitions. The operational definitions in Table 4 were constructed and used by all those performing abstractions at the Marshfield Clinic Research Institute (MCRI) and University of Wisconsin Madison School of Medicine and Public Health (UW Madison).

Table 4

Definitions Used in Medical Record Abstractions

Term	Definition
Death	Blastomycosis was a contributing factor to death of a patient which occurred anywhere up to a maximum of 6 months after anti-fungal medication termination.
Fatigue	Fatigue reported either by patient or as determined while under physician care.
Fever	Fever reported either by patient/family or as determined while under physician care.
Fungal genotype	Genotype of isolate determined based on SNP assay of ITS2, microsatellite typing, multi-locus sequencing typing or the BAD1 promoter region.
Hospitalization	Patient was considered hospitalized if he/she was admitted to a facility where the patient was under the supervision of a physician for symptoms associated with blastomycosis.
Immunosuppression	Immunosuppression caused by a disease such as acquired immunodeficiency syndrome (AIDS) or severe combined immunodeficiency (SCID) or any other acquired or congenital immunosuppression. Immunosuppressive drugs, obesity, smoking, heavy drinking, drug abuse, and stress were not listed in immunosuppression but were listed in "medications," "other," "smoking status," and "describe" sections.
Musculoskeletal findings	Musculoskeletal findings included fractures, joint pain, body aches, and general malaise.
Night sweats	Night sweats reported either by patient or as determined while under physician care.
Outcome	Outcome at termination of anti-fungal medication. If anti-fungal medication was not terminated, listed as partial resolution.
Physician specialty	Physicians primarily responsible for major decisions in patient treatment, follow-up, and/or diagnosis.
Pre-existing conditions	Medical conditions that existed before the onset of symptoms of blastomycosis and did not include diseases or conditions that began during the onset of blastomycosis or after treatment termination. These included but were not limited to pulmonary diseases, neurological disorders, metabolic disorders, and pregnancy.

Respiratory symptoms	Respiratory symptoms included coughing (productive or non-productive), low O_2 concentration, labored breathing, wheezing, chest pain, exacerbation of asthma symptoms, sneezing, nasal congestion, runny nose, and dizziness. These were either reported by patient or determined under physician care.
Sex	Biological sex of the patient at the time of blastomycosis symptom onset.
Treatment Length	Total length of treatment for blastomycosis defined by the date an anti-fungal medication was prescribed to the date the anti-fungal medication was discontinued.
Weight Loss	Loss of 5 pounds from symptom onset to one month after diagnosis reported by patient, determined, while under physician care or as verified by medical report.

Table 5

List of Abbreviations Used in NMDS and PCA

Abbreviation	**Meaning**
AGE	The age of the patient at the time of diagnosis of blastomycosis.
CHILLS	Chills either described by patient or experienced under physician care.
DAYSHOSP	Days hospitalized as described by Table 1.
DEATH	Case in which patient died from blastomycosis as defined in Table 1.
DERM	Clinical case of blastomycosis caused by infection with *B. dermatitidis*.
DERPHYS	Dermatologist was consulted for blastomycosis.
DISSEM	Case of disseminated blastomycosis. This case could be skin dissemination, bone dissemination, meningitis, etc.
FATIGUE	Fatigue as defined in Table 1.
FEMALE	Patient was female as defined by sex in Table 1.
FEVER	Fever as defined by Table 1.
GIL	Clinical case of blastomycosis caused by infection with *B. gilchristii*.

HEADACH	Headache as reported by patient or experienced while under physician care associated with blastomycosis.
HEMOP	Hemoptysis associated with blastomycosis.
MENT	Other mental health symptoms such as disorientation or delusions associated with blastomycosis illness.
MUSCUL	Musculoskeletal findings as defined in Table 1.
NEURPHYS	Neurologist was consulted for blastomycosis.
OTHSYMP	Symptoms not previously listed associated with blastomycosis disease.
OTHPHYS	Physician specialty consulted for blastomycosis not previously listed.
PCOND	Pre-existing conditions as defined in Table 1.
PULM	Pulmonologist was consulted on case as defined in Table 1.
RESP	Respiratory symptoms as described in Table 1.
SEIZ	Seizure as reported by patient or experienced while under physician care associated with blastomycosis.
SKINLES	Skin lesion associated with blastomycosis.
SURGERY	Patient had surgery associated with blastomycosis either in the process of diagnosis or treatment.
SWEATS	Night sweats as described in Table 1.
TOTPHYS	Total number of physicians consulted for blastomycosis.
TOTSYMP	Total number of symptoms associated with blastomycosis.
TREAT	Total length of treatment as defined in Table 1.
UAGTOT	Total number of urine antigen tests throughout the course of diagnosis and treatment for blastomycosis.
WLOSS	Weight loss as defined in Table 1.
XRAYTOT	Total number of X-rays throughout the course of diagnosis and treatment of blastomycosis.
YOD	Year of diagnosis.

Results

Overview. A total of 141 cases of patients diagnosed with blastomycosis from 2008-2017 were obtained. Patient ages ranged from 3 to 93 years of age with a mean of 40 years of age, 45 (32%) of our patients were female, the average treatment length was 8.9 months and ranged from 6 days to more than three years. Of these patients 40 (28%) experienced disseminated disease presentation, 61 (43%) were infected with *B. dermatitidis* while the remaining 80 (57%) were infected with *B. gilchristii*. For the patients for whom there were available data, a total of 71 (62%) were hospitalized and 6 (4%) died as a direct cause of the disease.

Non-Metric Multi-Dimensional Scaling (NMDS). In NMDS, vectors are visualized for their power of explaining the variation within the data. As vectors merge away from the center of the graph, they hold more power in explaining the variation of the data. In Figure 3, "DERM" (*B. dermatitidis* as etiological agent in clinical case) and "GIL" (*B. gilchristii* as etiological agent in clinical case), had the strongest power in explaining the variability in the data. Species type of etiological agent was closely followed by clinical presentation and biological sex of patient. Alternatively, death, pre-existing condition, patient age, year of diagnosis, and treatment length were near the origin of the graph thereby demonstrating little to no impact on explaining the variability within the dataset.

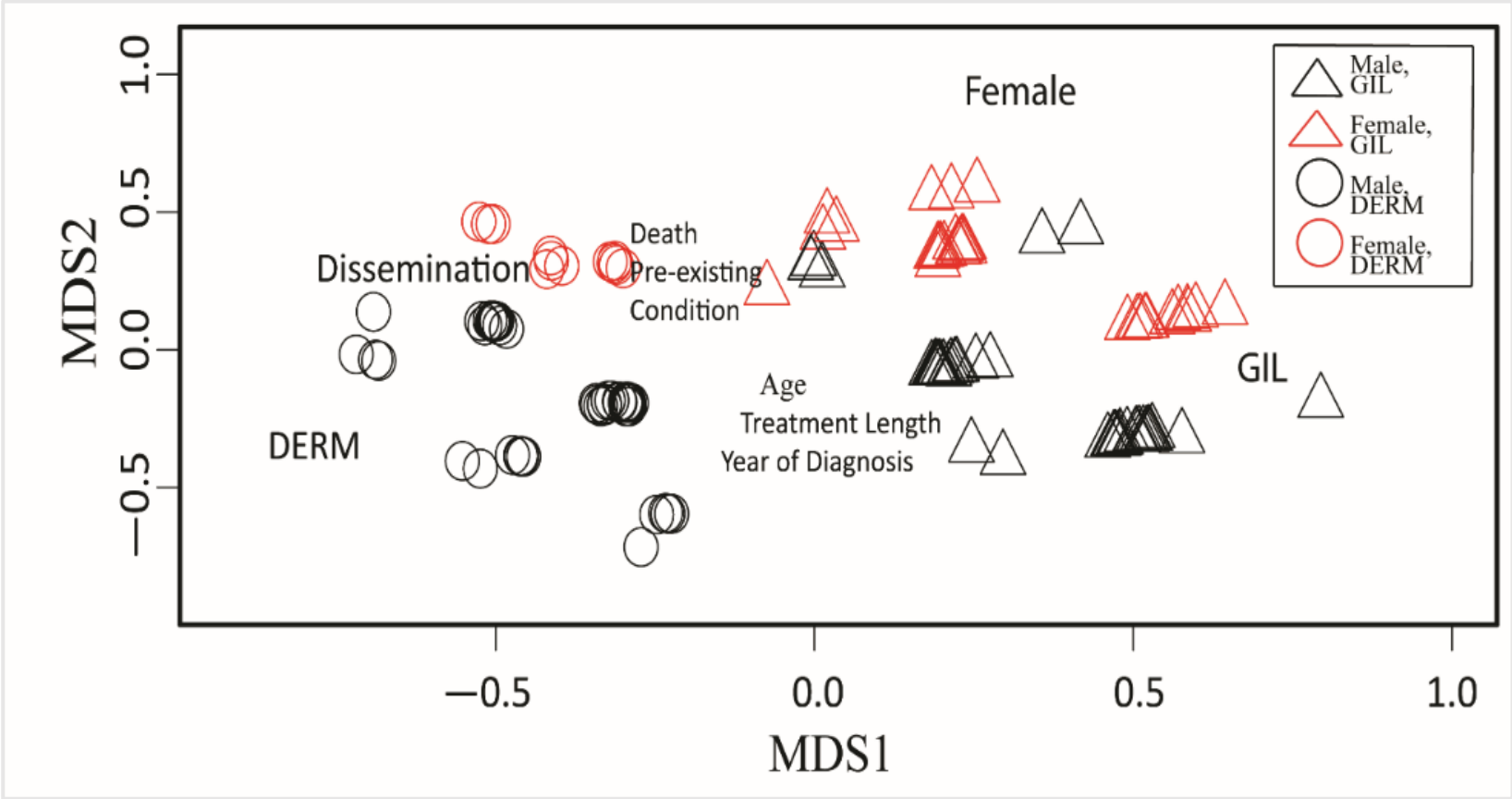


Figure 3

NMDS of Patients from Wisconsin Afflicted with Blastomycosis from 2008-2016 and their Affiliated Clinical Data (n=139) Female patients shown in red. Male patients shown in black. The NMDS ran 28 times before solution was reached. Open triangles are patients infected with B. gilchristii (GIL) and open circles are patients infected with B. dermatitidis (DERM).

Principal Component Analysis. In the Principal Component Analysis vector plot shown in Figure 4, each line is a vector with possible explanatory power over the data set. When two vectors are <90° from one another, this represents a possible correlation between those vectors. Hemoptysis, respiratory symptoms, and *B. gilchristii* as the etiological agent held a possible correlation as they were all within 90° of one another. However, the length of the vector illustrates the strength of that factor in explaining the variability in the data. In this case, *B. gilchristii* as an etiological agent held more explanatory power than respiratory symptoms or hemoptysis in explaining variability in the patient data (Figure 4). Furthermore, *B. gilchristii* and *B. dermatitidis* were close to 180° of one another which indicated that having one or the other etiological agent was inversely correlated (Figure 4).

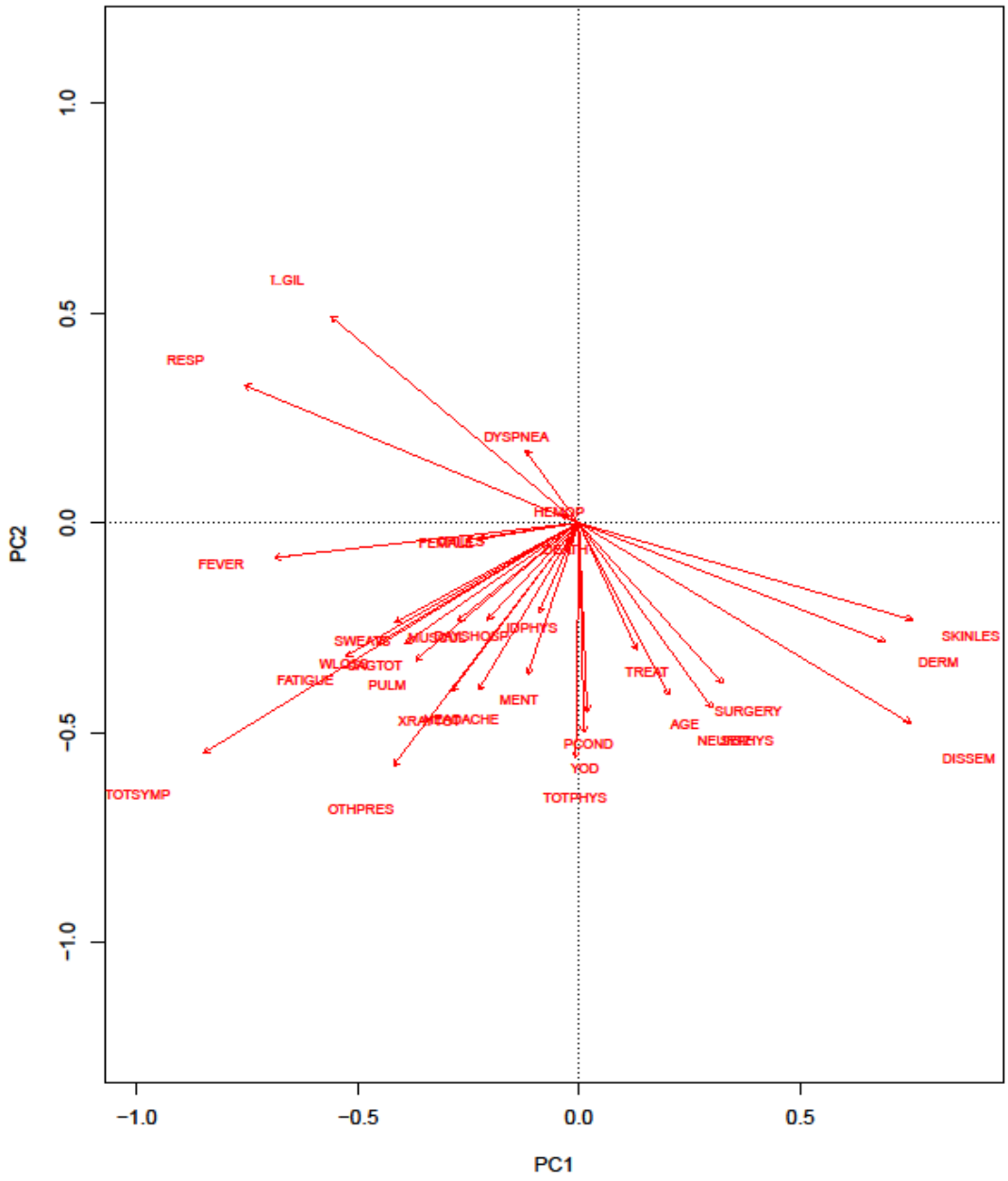


Figure 4

PCA Biplot Vectors of Relationship between Patients from Wisconsin Afflicted with Blastomycosis from 2008-2016 and their Affiliated Clinical Data (n=112)

Comparison of patient demographics and clinical features. Within this cohort, patients infected with *B. gilchristii* were younger both on average (from T-test) and when distributed through age groups based on the biological age groups as defined by the Canadian Department of Biostatistics (2018) (Table 6). Furthermore, within this cohort, patients infected with *B. gilchristii* were more likely to be female, have pulmonary-only disease presentation, have a higher number of symptoms overall, and had longer hospital stays than those infected with *B. dermatitidis* (Table 6).

Table 6

Patient Demographics and Clinical Features in Blastomycosis Cases between B. dermatitidis and B. gilchristii in Wisconsin 2008-2016. Values are compared along rows. Levels of significance determined through a X^2 test of independence between distributions of values between species.

	B. dermatitis	***B. gilchristii***	**Significance (p)**
Patient Demographics			
Mean Patient Age (y)	48	34	<0.01
Pre-existing Condition	75% (46)	56% (43)	<0.001
Female patients	21% (13)	42% (32)	<0.01
Pulmonary-only	54% (33)	87% (67)	<0.001
Treatment			
Days in Hospital (d)	4.6	9.9	<0.001
Length of Treatment (mo)	9.21	8.26	<0.001
Number of X-rays	9.8	10	NS
Amphotericin B	34% (19)	34% (21)	NS
Itraconazole	82% (51)	99% (77)	<0.05
Symptoms			
Weight Loss	21% (8)	27% (20)	NS
Fever	33% (13)	54% (45)	<0.001
Fatigue	46% (18)	38% (28)	NS
Total (4 or more)	23% (9)	51% (37)	<0.01
Specialist Seen			
Pulmonologist	46% (18)	34% (25)	NS
Infectious Disease Specialist	59% (23)	68% (50)	NS
Dermatologist	21% (8)	1% (1)	<0.001

Discussion

The non-metric multi-dimensional scaling analysis of the clinical data for 141 cases, illustrated the importance of species of *Blastomyces* in explaining variations within the data. Previous work by Meece *et al.* illustrated that these genetic groupings impacted patient outcomes and clinical phenotype. The use of this unconventional analysis illustrated another way to support previous findings. Indeed, species of *Blastomyces* had more explanatory power than patient sex, clinical presentation, date of diagnosis, or any other factor taken into account within the confines of the analysis.

The principal components vector analysis showed trends in the dataset previously known intuitively but never previously shown in this specific manner. Again, the strongest vectors that held the largest weight in explaining variability within the dataset were species type. *Blastomyces gilchristii* had a close association with pulmonary-only clinical presentation, hemoptysis, and dyspnea (Figure 4). Whereas *Blastomyces dermatitidis* was more closely associated with disseminated clinical presentation, skin lesions, and having surgery (Figure 4). These results further support the notion that these species result in different clinical presentations.

The X^2 test of independence illustrated the significance of the correlations seen in the NMDS and PCA vector analysis. Patients infected with *B. dermatitidis* were more likely to obtain consultation from a dermatologist which supported the notion that this etiological agent was more closely associated with skin lesions and disseminated infections (Table 6). Conversely, *B. gilchristii* was significantly more likely to result in pulmonary-only clinical presentation and patients infected with this species were more

likely to exhibit a greater number of overall signs and symptoms of infection (Table 6). These data supported the previous findings of Meece *et al.* 2012.

One of the major limitations of this study was the inclusion criteria which required a clinical isolate for genotyping. The X^2 test of independence, for example, also found that patient sex was significantly different in representation between the two species (Table 6). This finding was novel and previously unpublished or seen by other demographic studies. After careful review and analysis of all other potential avenues that might explain this significant difference led to no indirect evidence that would support difference in infectivity between men and women between the species of *Blastomyces*. Furthermore, there is no evidence previously published to support the notion that *Blastomyces* spp. have the ability to respond to endogenous estrogen (Roomiany, 2005). Rather, it appears that this may be due to the inclusion criteria that required a clinical isolate for the purpose of genotyping the etiological agent involved with the infection rather than a true result.

Furthermore, an isolate was not included in the results that tested positive for both *B. dermatitidis* and *B. gilchristii*. Further exploration into the genetics of that particular isolate is warranted. However, for the purpose of this particular investigation, the case was not included in the analysis.

Chapter III

The Urine Antigen Test Detects both Species of *Blastomyces* Equally Well

Background

Blastomycosis is a potentially lethal fungal infection caused by inhalation of spores produced by members of the genus *Blastomyces* (Gilchrist and Stokes, 1896). Once the spores are inhaled, they undergo a thermally regulated dimorphic switch, which leads to the transformation into yeast cells (Klein *et al.*, 1986). In 2013, Brown *et al.* illustrated through multi-locus sequence typing what Meece *et al.* in 2011 illustrated through use of microsatellite markers there were at least two genetically distinct species within the genus *Blastomyces—Blastomyces dermatitidis* and *Blastomyces gilchristii*. Later in 2012, Meece *et al*. showed that the distinct *Blastomyces* spp. are associated with different clinical disease phenotypes in humans. Blastomycosis often resembles influenza or bacterial pneumonia which often results in delayed or mis-diagnosis. Additionally, diagnosis is often complicated by physician reluctance to subject patients to invasive diagnostic methods such as bronchoscopy, which are often required to obtain a fungal culture (McBride *et al*., 2017)

To combat the difficulties associated with obtaining samples for culture and to avoid the extended incubation period for fungal cultures in the laboratory, the non-invasive diagnostic urine antigen test was developed by Mira-Vista (Spector *et al.*, 2008). Through sensitivity testing in human subjects, Mira-Vista has shown up to a 93% sensitivity in the urine antigen test (Durkin *et al*., 2004). The test can be used as a

preliminary diagnostic method when blastomycosis is suspected and shows improved sensitivity when compared to serology methods (Campbell and Binkley, 1953; Spector *et al.*, 2008). Additionally, in 2015, Frost and Novicki demonstrated that the quantitative version of the urine antigen test might be useful for monitoring and treatment of blastomycosis as it allows for an additional measure of disease-clearing within a patient.

The Mira-Vista urine antigen test is a sandwich enzyme immunoassay which detects galactomannan which polysaccharide found in the cell wall of pathogenic fungi (Muszewska, Pilsyk, Perlinska-Lenart, and Kurszewska, 2018) in urine, serum, and other body fluids (Spector *et al*., 2008). Galactomannan is possibly shed during the process of fungal cell growth during the course of infection which allows for detection of the polysaccharide in bodily fluids such as urine from the infected host (Muszewska *et al.* 2018). The sensitivity of the Mira-Vista antigen test had not been evaluated since the recent distinction *Blastomyces* (Brown *et al*., 2013; Meece *et al.*, 2012). Because *Blastomyces dermatitidis* and *Blastomyces gilchristii* differ significantly in their genetic makeup, there remained an unexplored antigenic variation in galactomannan. Galactomannan is at least partially tethered to the fungal membrane with proteins (Muszewska *et al*. 2018) that are affected by differences in genetic content. To assess the ability of the urine antigen test to detect both pathogens, a retrospective case series review of the urine antigen test performance on clinical cases in Wisconsin from 2008-2016.

Materials and Methods

Institutional review board approval for a retrospective case series was obtained from both the Marshfield Clinic Research Institute and the University of Wisconsin Madison School of Medicine and Public Health to obtain medical records associated with clinical isolates for cases of blastomycosis. Patients were included in the study if they had a laboratory confirmed case by culture or cytology/histopathology, there existed a clinical isolate available for genotyping, and they had a urine antigen test performed at least once throughout the course of the disease.

Archived clinical isolates from patients who were treated at the MCRI for blastomycosis were assigned to a fungal species based on a single nucleotide polymorphism in the 19th base pair of the ITS sequence (Brown *et al*., 2013). Clinical isolates from patients who were treated at the UW Madison School of Medicine and Public Health were assigned to a species based on variation in BAD1 promoter region (Burgess, Schwan, and Volk, 2006; Meece *et al*., 2011). Clinical isolates were genotyped either by SNP analysis (Frost *et al.*, 2016) or Sanger sequencing.

Archived medical records associated with each clinical case were examined for information about the patient, the course of treatment, and the clinical manifestation of the disease itself. These data included but were not limited to: patient age, patient sex, medications taken, pre-existing conditions, number and type of physicians seen, and the results of the urine antigen test. These data were then entered into the Research Electronic Data Capture (REDCap) secure software powered by Vanderbilt University and hosted by the Marshfield Clinic Research Institute was used to construct a database

which was developed and managed to hold both fungal genotype and information about each clinical case abstracted from the medical record(Harris, Taylor, Thielke, Payne, Gonzalez, Conde, 2009) (Harris, Taylor, Minor, Elliott, Fernandez, O'Neal, McLeod, Delacqua, Delacqua, Kirby, and Duda, 2019).

All cases of blastomycosis in this study were true cases of blastomycosis as indicated by the presence of a fungal culture viable enough for the determination of genotype and broken up into true positive and false negative groupings. True positive groups were defined as cases in which blastomycosis was both diagnosed and detected by the urine antigen test. False negative groups were defined as cases in which blastomycosis was diagnosed but not detected by the test. The number of true positives and false negatives was compared between species of *Blastomyces* responsible for each infection. Association of *Blastomyces* spp., patient sex, and clinical presentation to false negative results were determined using the X^2 squared test of independence with significance defined as $p \leq 0.05$ in accordance with (Ambrose and Ambrose, 2002). Antigen urine concentration by infecting species were compared with a Wilcoxon Rank-Sum Test to determine differences in the highest values in ng/ml of *Blastomyces* galactomannan antigen within the course of patient treatment. This analysis is visualized using a box and whisker plot that was generated with package in R Studio version 4.3 (2017). highest values in ng/ml for a given patient throughout the course of treatment were compared.

Results

A total of 36 clinical cases of *B. dermatitidis* and 39 clinical cases of *B. gilchristii* were obtained that fit the inclusion criteria (Table 8). Patients ranged in age from 3 to 77 years of age with a mean of 36. Of these patients, 46 (61%) were male and 29 (39%) were female, 50 (67%) had at least one pre-existing condition as defined by Table 4 on page 27, and 33 (44%) patients displayed disseminated disease presentation. For the patients that the data were available, 69% were hospitalized at some point during the course of treatment and 5 (7%) patients died as a direct consequence of the disease. Within our cohort, 77% of the cases displayed a true urine antigen positive, revealing an overall false negative rate of 23% in this study. There were no significant differences in the performance of the urine antigen test to detect the antigen of both *B. dermatitidis* and *B. gilchristii* in patient urine infected with either pathogen (Table 7). There were also no significant differences in the ability of the test to detect pathogen between males and females (Table 8) or in pulmonary-only and disseminated disease presentation (Table 9). We also found no statistically significant differences in the highest value in urine antigen concentration displayed throughout the course of treatment between *B. dermatitidis* and *B. gilchristii* (Figure 5).

Table 7

Blastomyces Antigen Positivity in B. dermatitidis and B. gilchristii
Differences were not significant at p=0.3097. A X^2 Test of Independence was performed to determine if false negative urine antigen test rates differed significantly between B. dermatitidis or B. gilchristii. These differences in proportions were found to not be statistically significant at p<0.05.

	B. dermatitidis	***B. gilchristii***
True positives	26 (72%)	32 (82%)
False negatives	10 (28%)	7 (18%)
Total	36	39

Table 8

Blastomyces Antigen Positivity in male and female patients
Differences were not significant at p=0.3097. A X^2 Test of Independence was performed to determine if false negative urine antigen test rates differed significantly between male and female case patients. These differences in proportions were found to not be statistically significant at p<0.05.

	Male Patients	**Female Patients**
True positives	33 (71%)	25 (86%)
False negatives	13 (29%)	4 (14%)
Total	46	29

Table 9

Blastomyces Anti gen Positivity in Pulmonary-only and Disseminated Cases
Differences were not significant at p=0.3984. A X^2 Test of Independence was performed to determine if false negative urine antigen test rates differed significantly between pulmonary-only and disseminated infections. These differences in proportions were found to not be statistically significant at p<0.05.

	Pulmonary-only	**Disseminated**
True positives	34 (81%)	24 (73%)
False negatives	8 (19%)	9 (27%)
Total	42	21

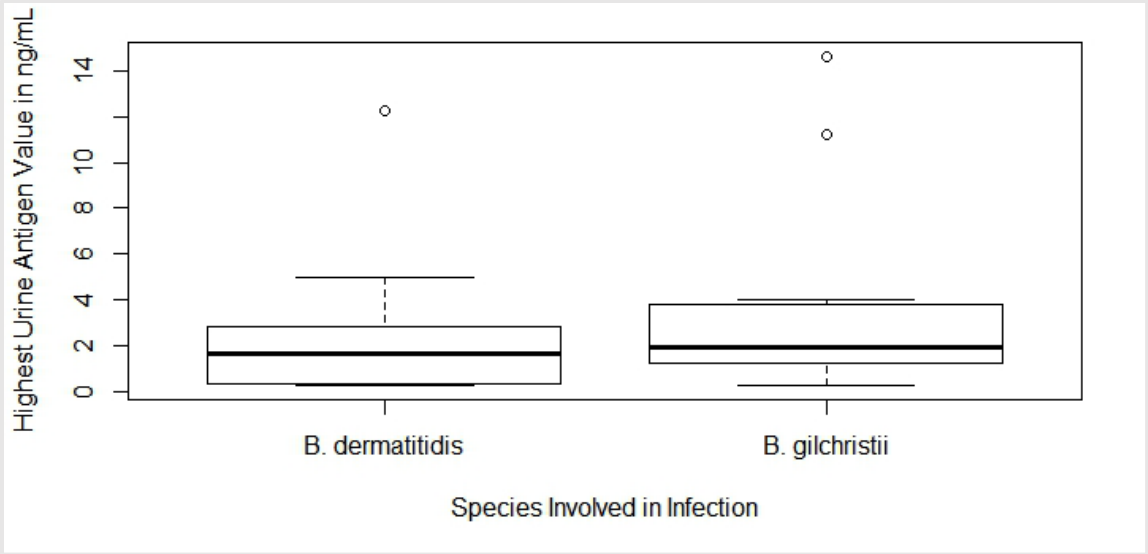


Figure 5.

Urine Antigen Values of B. dermatitidis and B. gilchristii Infections. A Wilcoxon rank-sum test was performed on the highest urine antigen values (ng/ml) for each patient throughout the course of treatment. These values were not significantly different from each other.

Discussion

Despite the usefulness of the urine antigen test, until now the utility of the assay to detect infection of both *Blastomyces gilchristii* and *Blastomyces dermatitidis* had not been evaluated. We aimed to assess the urine antigen assay's sensitivity to *B. gilchristii* and *B. dermatitidis* in a small, retrospective study.

We found that the Mira-vista urine antigen test is capable of detecting both species of *Blastomyces* at about the same sensitivity (Table 7). This may have been expected to due to previously identified cross-reactivity to *Histoplasma capsulatum*. It makes sense that the two species of *Blastomyces* are as similar in antigenicity to each other as they are to *Histoplasma capsulatum*. Nonetheless, this was an important

clinically relevant question that directly impacts the diagnosis of this important fungal disease.

Interestingly, we saw a higher false negative rate than was previously published by Durkin *et al.* in 2004. This significant reduction in sensitivity may be due to subtle differences within our cohort compared to clinical trials such as: specimen collection or quality, shipment methods, laboratory changes, or differences in fungal growth among patients. However, it should be noted that significantly lower field sensitivity compared to clinical trials has been reported elsewhere such as in Frost and Novicki (2015) which found an 80% sensitivity and by Carlos *et al.* (2010) who discovered a 74% sensitivity within their cohort.

We plan to further explore the trend of a higher rate of false negative urine antigen assays in male case patients. Although this finding was not statistically significant, we feel this may be due to our small sample sizes. It is important to understand any differential assay sensitivity that potentially exists between male and female case patients.

This research has several limitations. First, this study was retrospective and relied on the accuracy and completeness of the medical record. Furthermore, our statistical power was limited to assess significance in some comparison due to the small number of cases included in this study. Despite this, we provide the first evaluation of the sensitivity of the MiraVista urine antigen assay since the distinction between *B. dermatitidis* and *B. gilchristii*. Our findings indicate a small, equivalent false negative assay rate to both

species and we would recommend the continued use of this assay for diagnosis and treatment monitoring of blastomycosis.

Chapter IV

Analysis of Therapeutic Drug Monitoring in Blastomycosis

Background

Itraconazole is prescribed for mild to moderate cases of blastomycosis and as a step-down treatment following Amphotericin B formulations in severe cases (Chapman *et al.*, 2008). Itraconazole is also used as a prophylactic anti-fungal therapy in HIV/AIDS patients as well as in cases of histoplasmosis (Nacher *et al.*, 2014; Wheat *et al.*, 2007).

The mode of action of azoles is to interfere with the biosynthesis of ergosterol in fungal cells (Odds *et al.*, 2003). Azoles are metabolized by human cytochrome P450. Evidence suggests that azoles co-catalyze P450-dependent 14α-demethylation of lanosterol which, in turn, depletes ergosterol which results in altered plasma membranes of fungal cells (Hitchcock, Dickinson, Brown, Evans, and Adams, 1990). While effective at preventing the formation of new yeast membranes, these drugs are fungal-static rather than fungal-cidal and are needed for six months or more to ensure thorough eradication of the infection (Chapman *et al.*, 2008). The duration of therapy leads to a treatment dilemma as patients are responsible for taking medication, which may have adverse side effects, for an extended period (Ashbee *et al.*, 2014).

In 2008, the Infectious Diseases Society of America (IDSA) recommended in its guidelines for the treatment of blastomycosis the use of therapeutic drug monitoring throughout the course of treatment to ensure proper eradication of the pathogen (Chapman *et al.*, 2008). The test for itraconazole serum concentration informs physicians

on whether they are within the minimum concentration requirements for patient health and safety but not when these values have exceeded the maximum necessary for therapeutic effect. However, despite being significantly safer than amphotericin B formulations, itraconazole does still come with side effects including but not limited to gastrointestinal distress, neurological problems, and hepatitis (Ashbee *et al.*, 2014). Considering that this drug is used in HIV patients as a means of preventing fungal infections from occurring (Nacher *et al.*, 2014), information to assist physicians in preventing drug related toxicity has larger relevance than solely in blastomycosis infections.

There currently do not exist guidelines on when or how doses of itraconazole might be reduced to ensure patient comfort and compliance. Extrapolation from the guidelines posted by the British Society for Medical Mycology indicate that a serum concentration level of 3.4 mcg/ml using HPLC might reach the toxic threshold and point out that itraconazole is relatively unpalatable, which illustrates the need to monitor the presence of sufficient levels of itraconazole in patient serum to ensure patient compliance (Ashbee *et al*., 2014). The purpose of this study was to investigate how physicians made use of the itraconazole serum concentration test for the purpose of therapeutic drug monitoring during the course of blastomycosis treatment and gain a better understanding of the number of patient serum levels that were within the IDSA recommended range during the course of treatment.

Materials and Methods

Institutional Review Board Approval was obtained for both the Marshfield Clinic Research Institute (MCRI) and the UW Madison School of Medicine and Public Health to perform a retrospective case series report with patient records. Patients were treated at the MCRI or the UW Madison School of Medicine and Public Health for blastomycosis by patient care specialists. Patients were only included in the study if they were diagnosed with blastomycosis via cytology/histology or fungal culture and if they were treated with itraconazole at some point during the course of treatment.

Clinical isolates from the MCRI and the UW Madison School of Medicine and Public Health were obtained for patients treated in Wisconsin from 2008-2016 who suffered from blastomycosis. Clinical isolates were stored at -80°C and were assigned to a fungal species based on a single nucleotide polymorphism in the 19th base pair of the ITS2 intragenic spacer sequence (Brown *et al*., 2013). Clinical isolates from patients who were treated at the UW Madison School of Medicine and Public Health were assigned to a species based on variation in BAD1 promoter region (Burgess, Schwan, and Volk, 2006; Meece *et al*., 2011). Isolates were genotyped either by SNP analysis (Frost *et al.*, 2016) or Sanger sequencing.

Research Electronic Data Capture (REDCap) secure software powered by Vanderbilt University and hosted by the MCRI was used to construct a database which was developed and managed to hold both fungal genotype and information about each clinical case abstracted from the medical record (Harris *et al*., 2009) (Harris *et al*., 2019). Information gathered included patient demographic data as well as clinical data about

hospital stay, physicians seen, and treatment length. Itraconazole values returned from the MAYO Clinic Laboratory, performed by HPLC quantitative test, was used to confirm itraconazole patient serum concentration in mcg/ml. These results were entered into the developed database along with dates of testing.

Itraconazole serum concentration levels were generally compared in a frequency distribution performed in Excel. Next, values were grouped into time periods defined as follows: Period 1 days 1-13 of itraconazole treatment; Period 2, treatment day 14 to 6 months; Period 3 extended from 6 months and one day of treatment and beyond. The itraconazole serum concentration levels for all patients throughout the three time periods were assessed with an analysis of variance (ANOVA) and displayed in a box and whisker plot using R Studio version 4.3 (2017).

To compare the serum concentration levels, the following guidelines were used: below the therapeutic threshold was defined as <0.5 mcg/ml in pulmonary-only infections and <1.0 mcg/ml in disseminated infections, within the therapeutic threshold was defined as 0.5-1.9 mcg/ml in pulmonary-only infections and 1.0-1.9 mcg/ml in disseminated infections (Andes *et al*., 2008) (Hope *et al*., 2008). Opportunity for dose reduction (ODR) was defined as 2.0-3.3 mcg/ml, and above the toxic threshold was defined as value ≥3.4 mcg/ml (extrapolated threshold from the British Society for Medical Mycology, Ashbee *et al*., 2014). The frequency of representation within these groups was then compared for significance with a X^2 test of independence in accordance with Ambrose and Ambrose (2002).

Results

One hundred and thirty-nine patients were included in this study and treated with itraconazole at some point during the course of illness. Of those 139 patients, in only 77 (55%) cases did physicians make use of the itraconazole serum concentration test. This number was higher within the UW Madison School of Medicine and Public Health population with 17 (81%) of physicians making use of the test as opposed to MCRI cases where only 60 (51%) made use of the test. Most patients were male (n=93, 67%), with 88 (63%) of patients having a pre-existing condition. Seventy-three (62%) were hospitalized and 5 (4%) of the cases resulted in death. The course of treatment for the patients within our cohort ranged from 6 days to 3 years with an average of 8 months of treatment. Ages within the general cohort ranged from 3 years of age to 93. Of the 77 physicians that made use of therapeutic drug monitoring, 64% were hospitalized, 43 (56%) were *B. gilchristii*, 28 (36%) of the patients were female, and 42 (54%) had an underlying medical condition.

Two hundred and one serum values from 77 different patients were analyzed. Approximately half (47%) of patient serum concentration levels were within the therapeutic threshold for blastomycosis treatment with a 95% confidence interval (Figure 6). An ANOVA shown through box and whisker plot (Figure 7) displayed significant differences among time periods 1, 2, and 3 of treatment. Patients were significantly ($p<0.05$) more likely to have values below the therapeutic threshold in time periods 1 and 3 as opposed to time period 2 (Table 10).

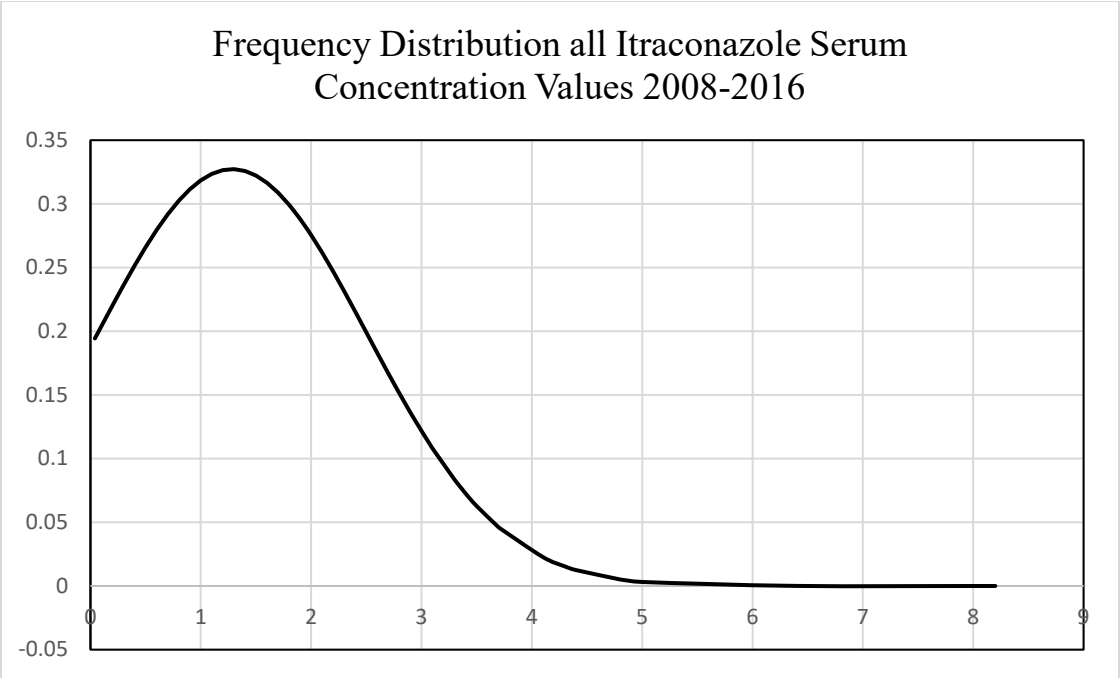


Figure 6.

Frequency distribution for all Itraconazole serum concentration levels. Included in this graph are all the values for itraconazole serum concentration on the x-axis obtained within the patient record from 2008-2016 and the frequency distribution on the y-axis.

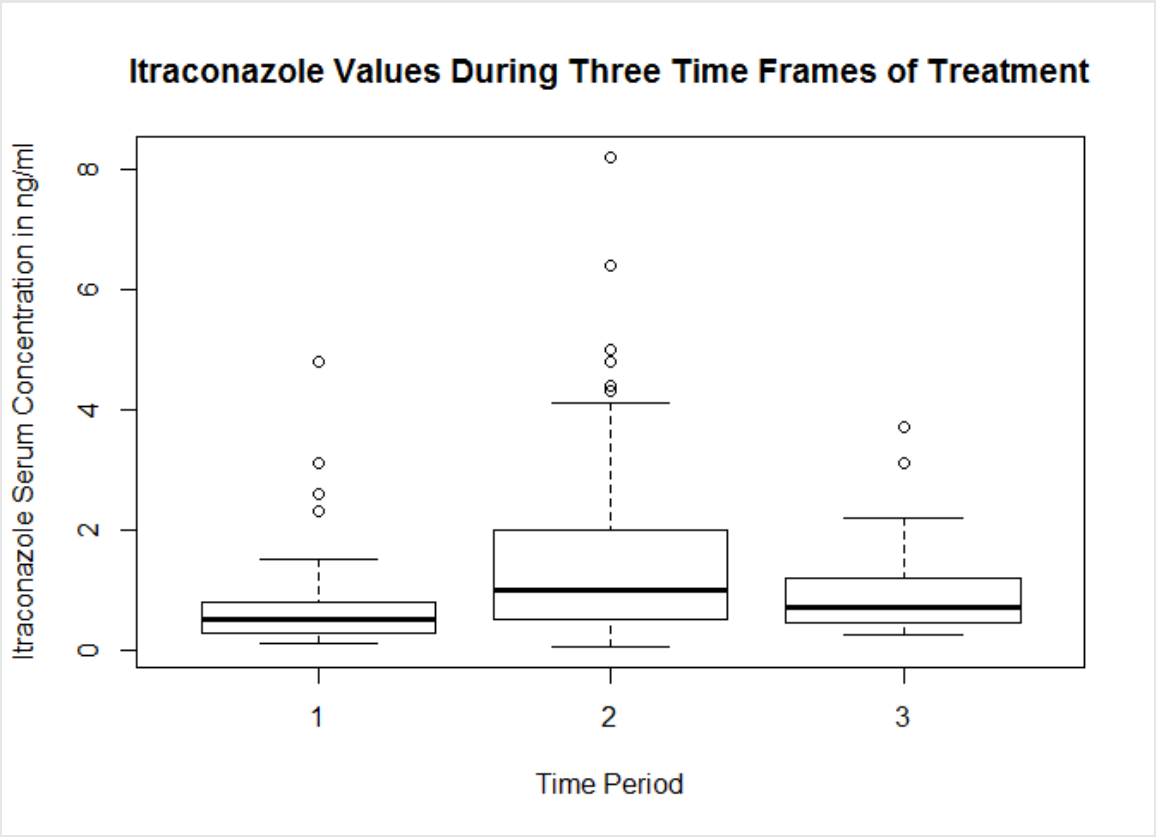


Figure 7.

Itraconazole Serum Concentration Level ANOVA expressed as Box Plot. Differences in values throughout the three time periods were significant with p = 0.0394. In particular, the difference between time periods 1 and 2 were significantly different in TUKEY HSD with a p = 0.0393.

More serum concentration levels were below the therapeutic threshold in time period 1 as opposed to time periods 2 and 3. When a X^2 analysis was run solely on the comparison of distributions of values between below threshold levels and within threshold levels among time periods 1, 2, and 3, there existed significant differences with $p<0.05$. Opportunity for dose reduction and toxic levels were most often seen in time period 2 (Table 9).

Table 9

Distributions of Itraconazole Concentration among Time Periods throughout Treatment. Proportions of values throughout the three time periods were significantly different with $p<0.05$. ODR and above toxic threshold were most common in time period 2. Within threshold values were also most common in time period 2. Below threshold values were most common in time periods 1 and 3.

Value Range	Time Period 1	Time Period 2	Time Period 3
Below Threshold	19 (54%)	36 (24%)	7 (37%)
Within Threshold	11 (31%)	74 (50%)	9 (47%)
ODR	4 (11%)	28 (19%)	2 (11%)
Above Toxicity	1 (3%)	10 (7%)	1 (5%)
Total Values	35 (17%)	148 (74%)	19 (9%)

Discussion

Itraconazole is the most frequently prescribed treatment for blastomycosis and is recommended for mild to moderate cases in addition to being a step-down treatment to amphotericin B formulations. In 2008, IDSA recommended the use of azole serum drug level monitoring to ensure proper dosing and treatment. This study aimed to evaluate physician use of therapeutic drug monitoring and better understand the number of patients falling into sub-therapeutic or toxic ranges.

We found that physicians made use of itraconazole serum monitoring at some point during disease treatment in 55% of cases (n=77). Only 47% of serum values were within the therapeutic threshold according to toxicological guidelines (Andes *et al*., 2008; Hope *et al*., 2008; Ashbee *et al*., 2014). Most sub-therapeutic levels were obtained in time period 1 (1-13 days after the initiation of treatment). IDSA guidelines specify that for both pulmonary and disseminated disease, physicians obtain serum levels after 2

weeks of therapy (Chapman *et al*., 2008). It seems that the physicians in our study were possibly testing too early for the drug to become biologically available and testable within the patient serum. The second most common time period for sub-therapeutic levels was in time period 3, when many of the patients are likely feeling better in terms of their fungal infection and have likely opted to neglect taking their medication. Patient compliance is another avenue of potential study in terms of anti-fungal treatment.

Our data from the category of above toxic serum levels is most concerning and ties in directly to patient compliance. One patient from this study was a 27-year-old male whose serum concentration levels were measured at 5.0 and 4.8 mcg/ml in time period 2 without dose reduction ordered by the physician. In time period 3 the patient's itraconazole serum concentration level dropped below the therapeutic threshold (<0.3 mcg/ml) which indicated that he had taken himself off the drug despite physician orders not to do so.

Pharmacokinetic properties of the drug play a role and these data support the practice of waiting two weeks before TDM begins or at least realizing the limitations of these first tests. The establishment of an upper limit warrants further investigation as 23% of all values fall in excess above the therapeutic range. The use of hydroxyitraconazole levels in itraconazole treatment also warrants further investigation as it appears to be involved as an indication of the metabolism of the drug in question. Whether there may be some pharmacogenetic component to the metabolism of this drug may also be worthy of further consideration.

Because this was a retrospective analysis on cases in which different physicians were involved in treatment decisions rather than a truly case-controlled cohort study, there are some limitations to the data collected. Initial dosage, how values change with and without intervention, and the side effects experienced at different values were limited by what was available in the clinical records. Despite this, our study finds that many patients blood serum levels are falling outside the therapeutic range—either sub-therapeutic or toxic. We recommend better education of physicians on when to perform serum testing and how to interpret test results to lower or increase dosing as needed. We would especially encourage the development of specific IDSA guidelines on itraconazole toxic levels as we had to extrapolate British guidelines for this study.

Chapter V

General Conclusions

The purpose of this study was to evaluate the clinical relevance of the recent distinction between *B. dermatitidis* and *B. gilchristii* which have no visible morphological differences but that significantly differ genetically (Meece *et al.*, 2011; Meece *et al.*, 2012; Brown *et al.*, 2013). These genetic differences lead to differences in clinical presentation, epidemiological patterns, and ostensibly in terms of pathogenic and infectious mechanisms (Meece *et al.*, 2011; Meece *et al.*, 2012). It is believed that these species have different ecological niches in the environment and that these differences in ecological niches are serviced by their differences in genetics (Meece *et al.*, 2011; Meece *et al.*, 2012; McTaggart *et al.*, 2016). Since the advent of this study, two new species, *B. percursus* and *B. helicus* have also been re-categorized as *Blastomyces,* previously *Emmonsia* spp. Despite the fact that infecting *Blastomyces* spp. impacts patient clinical phenotype and outcome, species typing is not routinely performed in a clinical diagnostic setting. This body of work assesses the correlations between species and clinical phenotype in humans, evaluates the utility of the MiraVista urine antigen assay to detect infections with both species, and describes use of itraconazole serum monitoring in order to obtain appropriate therapeutic threshold levels. A better understanding of the impact of *Blastomyces spp*. on the clinical course of disease could directly affect patient outcomes and lead to more personalized treatment.

Previous work by Meece *et al*. showed significant differences exist between the clinical phenotype of *B. dermatitidis* and *B. gilchristii* infections in humans. Patients infected with *B. gilchristii* were more likely to report constitutional symptoms such as fever, night sweats, and chest pain and be diagnosed six weeks or less from onset of symptoms, with pulmonary-only disease. In contrast, patients infected with *B. dermatitidis* were more likely to be diagnosed later, exhibit disseminated disease, have underlying medical conditions and be current smokers. In this dataset of 139 cases, NMDS was used to examine some these same previous associations. Analysis revealed that *Blastomyces* spp. held the largest explanatory power in the dataset, illuminating its impact on clinical factors. Using principal component analysis, *B. dermatitidis* infections showed a strong association with disseminated disease and a negative correlation with respiratory symptoms. The vector analysis showed the strongest statistical power in explaining the variation within the data belonged to species type as opposed to any other observed factor. The X^2 square test of independence reinforced significant differences in patient demographics, treatment, and symptoms between infections caused by *B. dermatitidis* and *B. gilchristii.* These findings support earlier work and reinforce the idea that the wide range of symptoms and severity observed in blastomycosis is partly due to virulence differences between *Blastomyces* spp. We would suggest that species typing of isolates in a clinical setting could help physician's individualize treatment according to the pathogen.

Use of the Mira-Vista urine antigen assay has made testing for blastomycosis easier and faster, due to the ease of obtaining a urine sample and the reduced diagnostic

time as compared to fungal culture. In addition, the test can be used to monitor treatment (Frost *et al*., 2015). Original urine testing in human subjects showed 95% sensitivity (Durkin *et al.*, 2004), but the sensitivity of the assay has not been re-evaluated taking into account *Blastomyces spp*. Since *B. dermatitidis* and *B. gilchristii* differ significantly in their genetic makeup, the urine antigen assay's sensitivity to infections cause by each of the species could differ. In retrospectively assessing the false positivity rate on a group of 75 case patients, we found no significant difference in the assay's ability to detect *Blastomyces* antigen from infections with *B. dermatitidis* or *B. gilchristii*. We did find an overall higher false negative assay rate in our study (23%) than initial evaluations, although more recent by Frost and Novicki (2015) and Carlos *et al.* (2010) showed rate more close to what we report here. We also observed a higher rate of false negative urine antigen assays in male case patients, but it was not statistically significant. Our findings indicate that continued use of this assay for diagnosis and treatment monitoring of blastomycosis is beneficial to patients and equally sensitive to both *B. dermatitidis* and *B. gilchristii*.

The IDSA recommends the use of itraconazole serum level drug monitoring throughout the course of blastomycosis treatment to ensure that proper therapeutic levels are reached (Chapman *et al*., 2008). In this study we investigated how physicians used this testing and the values received from those tests. Of the 139 case patients included in this portion of the study, only 77 (55%) physicians made use of itraconazole serum concentration testing. Most sub-therapeutic levels were obtained in time period 1, which is too early according to IDSA recommendations of waiting after 2 weeks of therapy

prior to testing. Toxic serum levels observed in some cases were the most disturbing finding, with one case being particularly upsetting as the patient reported hair loss and feeling sick and eventually discontinued her meds on her own due to itraconazole toxicity. These findings show that many physicians are failing to monitor itraconazole serum levels at all and those that do, may be testing too early or failing to make dosing changes when it would be indicated. We recommend better education of physicians around the subject of itraconazole therapeutic monitoring and encourage development of specific toxicity level guidelines by the IDSA.

The species of *Blastomyces* responsible for an infection impacts patients directly through the methods infection and potentially in differences in pathogenesis which, in turn, may impact patient care decisions. Improvements in diagnosis and post-diagnostic monitoring can ensure patient comfort, safety, and compliance. This study will hopefully inform new guidelines that improve these three aspects of patient care through imparting improved recommendations on treatment decisions.

APPENDIX

Institutional Review Board Documentation

Marshfield Clinic
Research Foundation

1000 North Oak Avenue
Marshfield, WI 54449-5790

715-387-5241
1-800-782-8581
Fax 715-389-3131

MCRI INSTITUTIONAL REVIEW BOARD

FWA # (FWA00000873) IRB #(IRB00000673)

May 18, 2017

PI: Jennifer K Meece, PHD - 2R2
SP Code: MEE10117 Protocol #:
Title: Analyses of host factors, demographic features, clinical outcomes and Blastomyces sp genotype

The Institutional Review Board (IRB) has reviewed and approved the above-referenced protocol/IRB application dated May 11, 2017 using expedited review on May 16, 2017. This approval is limited to the activities described in the approved protocol narrative and extends to the performance of these activities at each respective site identified in the application for IRB review. IRB approval expires on May 15, 2018. As a courtesy, prior to expiration of this approval, a notice will be sent reminding you to apply for continuing review. However, as the principal investigator, it is your responsibility to apply and receive continuing review for the duration of the study. Any lapse in approval should be avoided to protect the safety and welfare of enrolled subjects.

Based on the information provided in your application, the IRB has waived the requirement to obtain consent from subjects for this study. Regarding the children whose information you will collect as part of this study, the IRB has waived the requirement to obtain parental permission and assent from the minor subjects of this study.

The IRB has found that your request to waive authorization meets waiver criteria as delineated in the Privacy Rule at 45 CFR 164.512(i)(2)(ii) and has therefore waived the requirement for individual authorization for use and/or disclosure of Protected Health Information (PHI). The waiver is for the specific PHI and uses/disclosures described in your request. Any changes to the type of PHI to be collected, used or shared, or to the type of uses and disclosures described in the waiver application will require prior IRB approval.

This IRB review is in compliance with 45 CFR 46, 21 CFR 50 and 21 CFR 56.

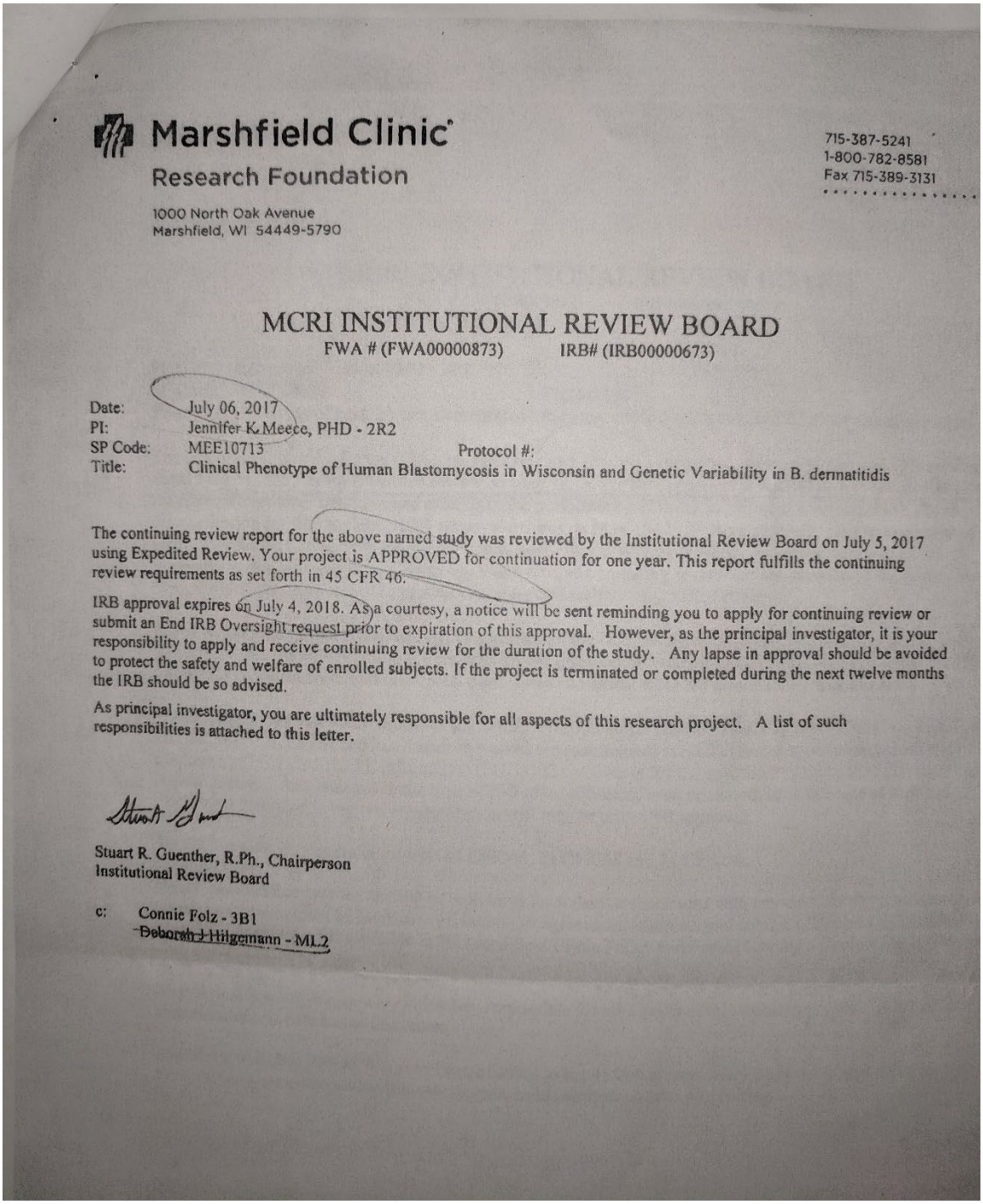
If your research involves the sharing of individual level data or specimens with any external party, an appropriate transfer agreement must be in place. To initiate an agreement, complete and submit a "Request to Transfer Data or Materials" form, which is available in the Marshfield Clinic Policy and Forms Library. Contact Marla Ripp Fischer with any questions.

As principal investigator, you are ultimately responsible for all aspects of this research project. A list of such responsibilities is attached to this letter.

In addition to IRB approval, it is your responsibility to assure that the necessary budgetary, contractual and administrative issues regarding this project have been resolved prior to proceeding.

Stuart R. Guenther, R.Ph., Chairperson
Institutional Review Board

c: Connie Folz - 3B1



Marshfield Clinic
Research Foundation

1000 North Oak Avenue
Marshfield, WI 54449-5790

715-387-5241
1-800-782-8581
Fax 715-389-3131

MCRI INSTITUTIONAL REVIEW BOARD

FWA # (FWA00000873) IRB# (IRB00000673)

Date: July 06, 2017
PI: Jennifer K. Meece, PHD - 2R2
SP Code: MEE10713 Protocol #:
Title: Clinical Phenotype of Human Blastomycosis in Wisconsin and Genetic Variability in B. dermatitidis

The continuing review report for the above named study was reviewed by the Institutional Review Board on July 5, 2017 using Expedited Review. Your project is APPROVED for continuation for one year. This report fulfills the continuing review requirements as set forth in 45 CFR 46.

IRB approval expires on July 4, 2018. As a courtesy, a notice will be sent reminding you to apply for continuing review or submit an End IRB Oversight request prior to expiration of this approval. However, as the principal investigator, it is your responsibility to apply and receive continuing review for the duration of the study. Any lapse in approval should be avoided to protect the safety and welfare of enrolled subjects. If the project is terminated or completed during the next twelve months the IRB should be so advised.

As principal investigator, you are ultimately responsible for all aspects of this research project. A list of such responsibilities is attached to this letter.

Stuart R. Guenther, R.Ph., Chairperson
Institutional Review Board

c: Connie Folz - 3B1
Deborah J Hilgemann - ML2

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