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PHENOTYPIC CHARACTERIZATION OF *BLASTOMYCES DERMATITIDIS*

A Manuscript Style Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology, Clinical Microbiology Concentration

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We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science in Biology, Clinical Microbiology Concentration.

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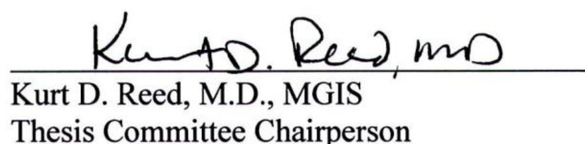

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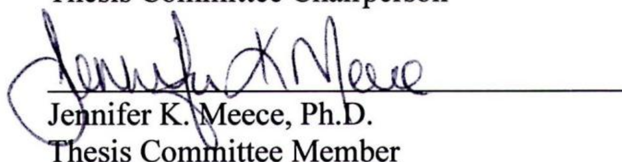

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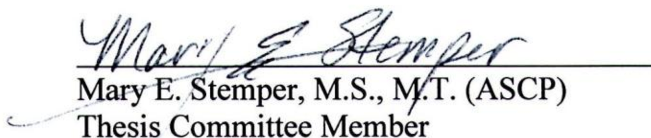

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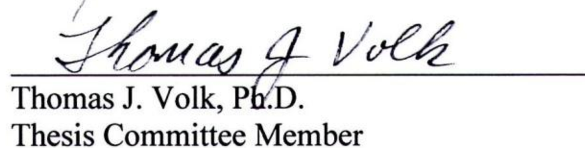

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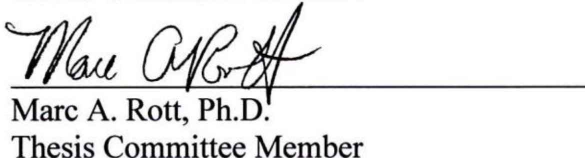

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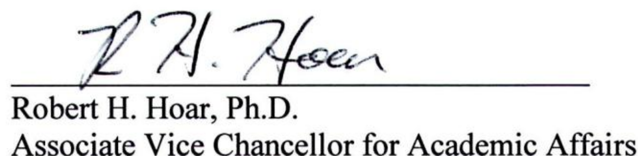

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ABSTRACT

Traore, B.Y. Phenotypic characterization of *Blastomyces dermatitidis*. MS in Biology, Clinical Microbiology Concentration, December 2011, 80pp. (K.D. Reed)

Blastomyces dermatitidis is a dimorphic fungal pathogen associated with severe repiratory and disseminated disease in humans. Recent investigations suggest that the two major genetic groups of the fungus are associated with different clinical outcomes. This study extends that work by exploring how laboratory phenotypic traits (mating locus type, ability to mate, spore production and antifungal susceptibility) might vary by genetic group. Among 100 clinical and environmental isolates, both genetic groups had roughly equal distributions of the α-box and HMG loci. Additionally, crosses between compatible strains of group 1 and group 2 organisms yielded visible cleistothecia and ascospores, suggesting that mating between the two groups could be possible in natural settings. Experiments related to spore production were found to be very difficult to standardize and the results were not interpretable. Although only a small group of isolates were tested for antimicrobial susceptibility testing, results showed that several strains had high MICs to amphotericin B. These results have significant clinical implications and should be confirmed on a larger group of isolates. Also, the new techniques developed as part of this study will provide a foundation for incorporating susceptibility testing of dimorphic fungal pathogens into the clinical laboratory.

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DEDICATION

This thesis and my degree in general are dedicated to my family for their unconditional love and support. To my wife Wassa, thank you for your patience and understanding. To my son Youssouf and my daughter Fatoumata, two years without Dad was hard, but I'm sure that you will understand when you grow up.

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INTRODUCTION

Blastomycosis is an invasive infection of humans and animals caused by *Blastomyces dermatitidis*, one of the thermally dimorphic fungi. It was first described in the United States in 1894 by Gilchrist and acquired the name Gilchrist's disease. North American blastomycosis and Chicago disease are other names used for blastomycosis due to the high number of cases in these areas (56), although other studies have reported worldwide distribution (42). Blastomycosis is endemic in the central and southeastern parts of the United States along the Mississippi and Ohio River basins and around the Great Lakes (55). The inhalation of infectious conidia from soil is the main route of infection. Uncommon routes of infection include direct skin inoculation, laboratory accidents and animal bites (56). The primary site of infection is the lung. After inhalation, conidia deposit in the distal alveoli. At body temperature, the conversion to the yeast form is triggered. The yeast cells then multiply and may disseminate through the blood and lymphatics to other organs such as skin, bone, prostate and brain (4, 25, 64).

In recent years, fungal pathogens have emerged as a public health concern leading to the expansion of research in this area. Recent advances in the development of molecular tools for genetic manipulation of *B. dermatitidis* have enhanced the understanding of the pathogenic mechanisms of this fungus, but further research characterizing phenotypic virulence traits is needed.

Growth Characteristics of *B. dermatitidis* and Other Dimorphic Fungi. The dimorphic fungi comprise six anamorphic genera which are *Blastomyces, Histoplasma, Coccidioides, Paracoccidioides, Sporothrix* and *Penicillium* that cause disease worldwide. *Blastomyces, Histoplasma* and *Coccidioides* are closely related and belong to the same teleomorphic genera. *Blastomyces dermatitidis* grows in the filamentous or mycelial form in the environment and transforms to yeast at body temperature. This phenomenon is called thermal dimorphism. In the laboratory, *B. dermatitidis* can be cultured either as mold or yeast. The preferred culture media for the mycelial form are Sabouraud dextrose agar, potato dextrose agar, and potato flake agar. Media such as brain-heart infusion agar with blood and chloramphenicol, yeast extract phosphate agar, and inhibitory mold agar are also useful, as they are more selective and decrease the growth of saprophytic fungi and/or bacteria (56).

At 25ºC, *B. dermatitidis* can take up to 4 weeks to form colonies that appear white to tan and may become brown with age. Under the microscope, the mycelia exhibit delicate septate hyphae, 1 to 2 µm in diameter, with fruiting bodies, the conidia, resembling lollipops (FIG. 1a). Similar structures can be observed in other molds (e. g., *Pseudallescheria boydii* and *Chrysosporium* species); therefore, conversion to the yeast form is required for confirming the laboratory diagnosis (56). At 37ºC, the hyphae convert slowly into yeast cells. The colonies are typically white to beige, creamy and 0.5 to 3 cm in diameter. Under the microscope, the yeast cells measure 8-12 µm in diameter with a double-contoured thick wall and characteristic broad base attaching the bud to the mother cell (FIG. 1b).

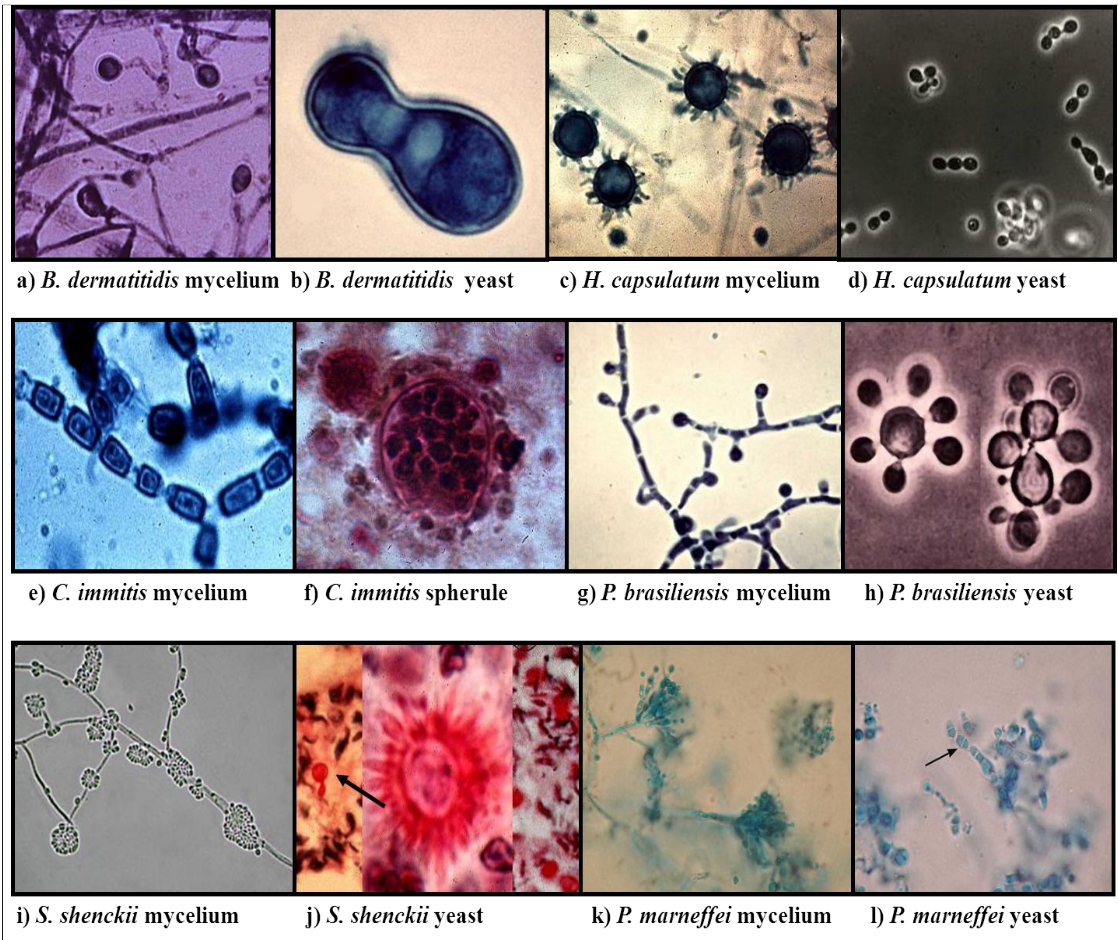


FIG. 1. Growth characteristics and morphology of dimorphic fungi. (adapted from fungus of the month web site). http://botit.botany.wisc.edu/toms_fungi/fotm.html

Histoplasma capsulatum, a dimorphic fungus closely related phylogenetically to *B. dermatitidis*, is found worldwide and can also cause chronic pneumonia or disseminated disease. The mold form grown on Sabouraud dextrose agar at 25ºC appears in 10 to 14 days, and is cottony white to tan with age. In early stage, the mycelium produces microconidia that resemble those produced by *B. dermatitidis*, except that they are not on separate branches. Later on the characteristic tuberculate macroconidia develop (FIG. 1c) that can also be confused with those produce by *Sepedonium* species (30). At 37ºC the yeast form appears as a white to tan colony. The yeast cells are slightly oval and measure 5 to 6 µm in diameter with narrow neck budding daughter cells (FIG. 1d). In tissue, the yeast cells can be found clustered within macrophages and are stained well with methenamine silver or periodic acid-Schiff stains (53).

Coccidioides immitis and *C. posadasii* are endemic to the western hemisphere including the southwestern United States, Central America and Brazil. In culture, *C. immitis/posadasii* grows rapidly in one week or less. The colonies appear velvety or cottony to granular or powdery and may change to gray with age. The mycelium consists of septate hyphae, branching and thick walled barrel shaped arthroconidia measuring 2-4 x 3-6 µm in size alternating with clear disjuncture cells (FIG. 1e). These arthroconidia are the infective form of the fungus. In tissue, the fungus produces thick-walled spherules measuring 30 to 60 µm in diameter (FIG. 1f). These spherules contain endospores, 3 to 5 µm in diameter, which are released upon the maturation and rupture of the spherule (17).

Paracoccidioides brasiliensis is endemic to South America, especially Brazil, and grows relatively slowly on Sabouraud dextrose agar. The colonies are dense and white; woolly cottony or glabrous to velvety with a yellow to brown reverse. Under the

microscope, the mycelium consists of septate hyaline hyphae with intercalary and/or terminal chlamydoconidia. The yeast form measures 5 to 15 μm and shows single or multiple buds with narrow bases (2). This appearance resembles a classical "Mariner's wheel" or "Mickey Mouse" structure that enhances diagnosis.

Sporothrix schenckii causes a clinical condition referred to as sporothricosis or "rose gardener's disease." It occurs worldwide, but is especially common in Central and South America. At 25–30°C, the colonies are moist and smooth; the color, initially white, becomes cream to dark brown with age. The mycelium form exhibits branching, septate hyphae. The conidiophores arise at right angles with an inflated base. The conidia are unicellular, oval, thin walled, and typically arranged in rosette-like clusters at the tips of the conidiophores. At 37°C, the colonies are yeast-like and creamy, cream to beige. The yeast cells are pleomorphic, round or elongated, with a cigar shape (6 - 8 μm) or fusiform (18).

The genus *Penicillium* comprises numerous species, but *Penicillium marneffeii* is the only known dimorphic species within this genus. It is prevalent in HIV patients in south Asia. The mold phase of *P. marneffei* shows phialides bearing typical conidia with thin multiple branched hyphae. A diagnostic clue to the diagnosis of *P. marneffei* is the presence of a diffusible red pigment when the fungus is grown at room temperature. At 37°C, the colonies appear cream/light to tan. Microscopically, rectangular-shaped cells are mixed with hyphae-like structures, and the cells divide by binary fission rather than budding (14).

Epidemiology of Blastomycosis. Although sporadic cases of blastomycosis have been reported in Africa, the Middle East and in South Asia, it is much more common in North America where it is endemic to the Ohio and Mississippi River Valley regions and around the Great Lakes. Unlike histoplasmosis and coccidioidomycosis, for which skin testing can reliably provide a clear picture of the prevalence and geographic distribution of human exposure, the epidemiology of blastomycosis is based on outbreak investigations and reporting of symptomatic sporadic cases (56). Based on confirmed cases, the annual incidence ranges from fewer than 1 case per 100,000 people in Mississippi, Kentucky, and Arkansas to 40 per 100,000 people in hyperendemic areas (e.g. Vilas County, Wisconsin) (13). The number of human and canine cases of blastomycosis in Wisconsin has been increasing since the 1950's, prompting public health officials to make it a reportable disease in humans in 1984 (13). Education of physicians and veterinarians concerning the distribution of this pathogen has been especially important in improving diagnosis and treatment of blastomycosis. Unfortunately, as recently as April 2007, published distribution maps displaying areas in Wisconsin endemic for *B. dermatitidis* do not accurately represent the distribution within the state when compared to actual case counts (FIG. 2) (55).

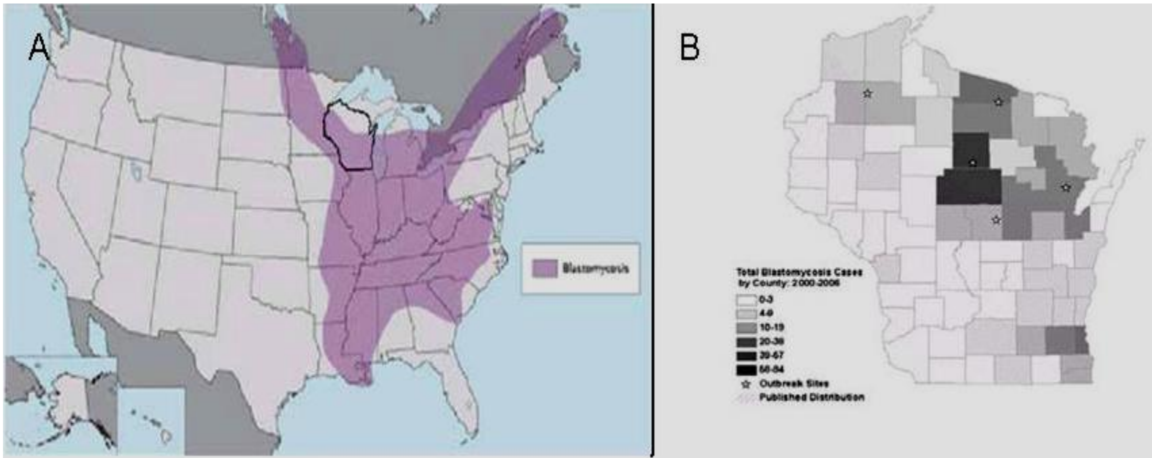


FIG. 2. Distribution map of blastomycosis in the United States and Canada. A) As recently published, distribution map shows northcentral Wisconsin (outlined by the dark line) as being non-endemic for blastomycosis. B) In contrast, data collected by the Wisconsin Division of Health and Family Services (DHFS) indicate that highest incidences of infection occur in several northcentral counties. Darker shades of gray indicate higher number of cases and lighter shades correspond to fewer cases. Stars represent sites of reported blastomycosis outbreaks. The figure is adapted from reference (55).

In Wisconsin, the Division of Health (DOH) conducted a large retrospective study of 670 cases of blastomycosis that occurred from 1986 to 1995 which resulted in a calculated statewide mean annual incidence rate of 1.4 cases per 100,000 people. Ten northern counties registered 294 cases (44% of the total), and four of them accounted for 28% of all cases statewide giving a mean annual incidence of 10.4–41.9 per 100,000 people. The overall case-fatality rate was 4.3%; this increased with the age of the patients (13).

In Canada, Crampton et al. (16) reviewed medical records of 143 patients with confirmed blastomycosis admitted to Manitoba hospitals from 1988 to 1999. The mean annual incidence was 0.62 ± 0.25 cases per 100,000 people (range, 0.26–1.00 cases per 100,000). The mean age was 38.0 ± 19.9 (range, 0–79) years with 65.0% being male.

In Illinois, 500 cases were reported to the Illinois Department of Public Health from January 1993 to August 2003. During this period the number of cases reported per year increased from 24 to 87. The median age was 43 years (range, 4–87 years), and 7% were fatal (19).

In a study done by Morris et al. in Ontario, Canada from 1994-2003, a total of 309 confirmed cases were identified in seven health regions (48). The incidence rate was higher in the northern region (2.44 per 100,000) than in the south (0.02 per 100,000). About 60% of the patients were between 30-39 years, and the majority (65%) was males. They also observed that 59% of the cases were diagnosed in the fall and winter months, suggesting that infection may occur more often in the fall.

The risk factors associated with blastomycosis are not completely understood. It is currently accepted that blastomycosis most commonly occurs among middle-aged men,

probably due to sporadic exposure to *B. dermatitidis* during occupational and recreational activities involving close contact with soil or vegetation, primarily near waterways (34). Several studies indicate that outbreak-associated case patients exhibit a much different profile of risk factors. During outbreaks, infections tend to be more equally distributed between the sexes and more cases in children and adults over the age of 45 have been observed (52). There is no evidence to suggest that ethnicity or patient genetics may play any role in susceptibility or severity of blastomycosis infection; this is in contrast to coccidiomycosis, which is clearly more severe in dark-skinned individuals such as African American and Filipino patients (21).

Ecological Niche of *B. dermatitidis*. The natural reservoir of *Blastomyces dermatitidis* in the environment is poorly defined; however sites near rivers and lakes in moist soil with acidic pH and high organic content (decaying vegetation, close to water, areas of high humidity) have been associated with outbreaks (1, 34). Outbreaks of blastomycosis have also been associated with disruptions of the soil, such as excavation that might lead to the increase number of spores and/or hyphal fragments in the air (31). Repeated attempts to isolate the organism from environmental sources in areas where the disease occurred have been a challenge and have only been successful 21 times over the past 50 years (55).

In 1984, an outbreak occurred in northern Wisconsin near Eagle River among two school groups that had visited an environmental camp. Investigations were focused on a beaver lodge that seemed associated with acquisition of infection. Investigators were able to isolate the fungus, and demonstrate the potential role of environmental conditions (i.e.,

precipitation, humidity, and soil pH) in conidia formation and aerosolization of the fungus (35).

In the summer of 1985, two outbreaks of blastomycosis occurred along riverbanks in central Wisconsin. Klein et al. (35) investigated the outbreaks and were able to isolate the fungus from soil along the Tomorrow River. It is believed that infected patients were exposed to the fungus while fishing. The soil samples obtained from the site were sandy and moist containing organic debris with an acidic pH. In addition, the samples were positive for *B. dermatitidis*. They concluded that the environment provided along waterways may support the growth of *B. dermatitidis*, and precipitation events may encourage release of conidia into the environment.

Bakerspigel et al. (3) documented the first soil isolation of *B. dermatitidis*, in Canada, from a petroleum filtering shed in south western Ontario. A 72-year old man who worked in the shed was diagnosed with systemic blastomycosis. Samples were obtained on three occasions from several areas of the shed and near his home. Mice were inoculated with processed samples and sacrificed after six weeks. Only the mice inoculated with the soil samples from the shed showed the characteristic yeast form of *B. dermatitidis* in histological examination and cultures of their tissue. Researchers concluded that the fungus was present in the earthen floor, which was sprinkled with water at least once a day, allowing aerosolization of conidia.

Hussein et al. (28) conducted two epidemiological studies of blastomycosis in Northern Tennessee. They examined occurrences for the periods from 1980 to 1995, and from 1996 to 2005. Medical records for 148 patients with culture-confirmed *B. dermatitidis* were obtained from the State of Tennessee Health Department Laboratory in

Nashville, TN. During this period, blastomycosis prevalence increased significantly from 1.23 to 1.29 cases per 100,000 population (95% CI, 1.05 to 2.15; $p = 0.03$). The geographic region is rural and adjacent to the Appalachian Mountains (elevation of 1,208 to 1,635 feet above sea level). The winter climate is harsh, and the soil varies from rocky to red clay in valleys to sandy in river and stream terraces. These findings contrast the perception that blastomycosis is uncommon in mountainous areas. In this study, high risk occupations included farming, construction work, landscaping, and truck driving.

Reed et al. (55) used a maximum entropy method (Maxent) to develop an ecologic niche model of *B. dermatitidis*. This method is based on geocoded exposure sites for predicting a species' distribution based on occurrence of the disease. Data characterizing the environment (climate, waterways, soil characteristics, precipitations) are incorporated in this model. Researchers reviewed 145 cases of human blastomycosis identified from the Wisconsin DOH records from 1 January 2005 to 31 May 2006. Cases for which the exposure was known and could be geocoded were entered into the model. A prediction map was created providing information on geographical locations that support the growth of *B. dermatitidis* (FIG. 3). It was found that blastomycosis is widely distributed across northern Wisconsin, and significant association with waterways was observed.

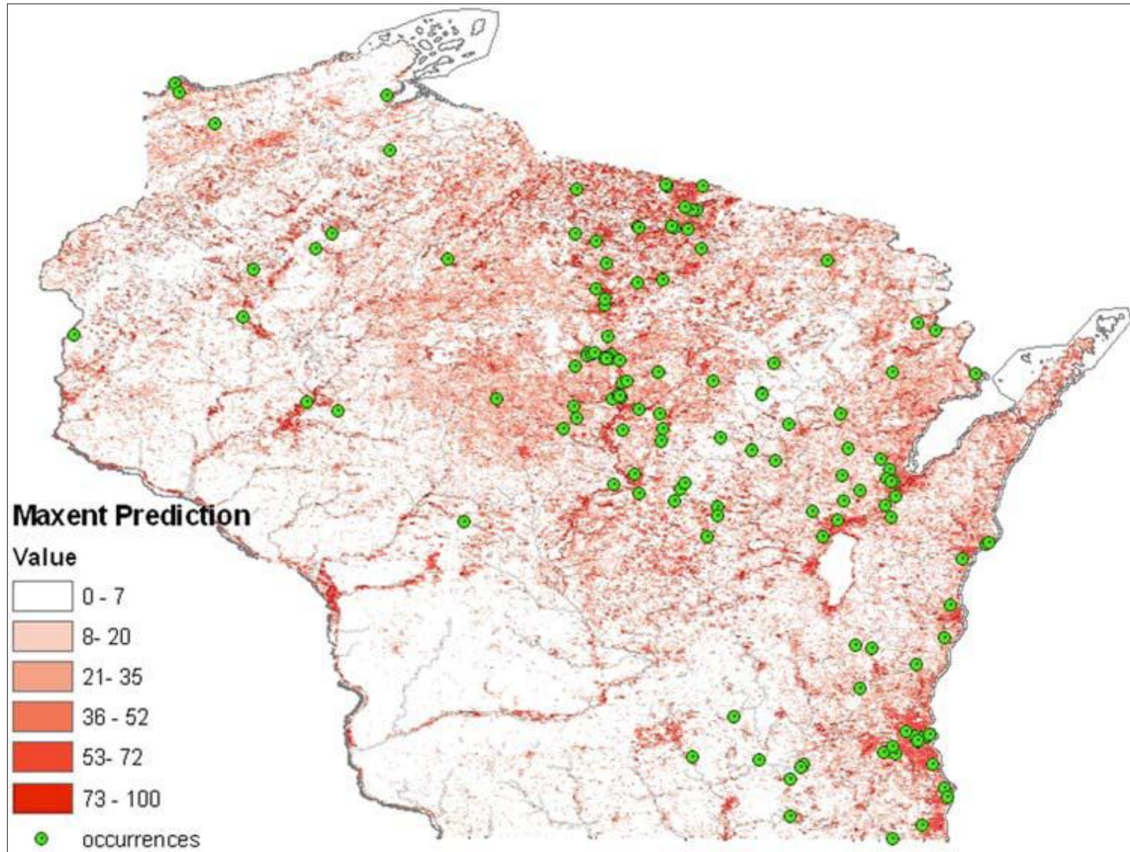


FIG. 3. Potential geographic distribution of *B. dermatitidis* in Wisconsin based on maximum entropy modeling. White and light red shades represent habitats of low suitability for the fungus while dark red shades are favorable habitats (around waterways in northern Wisconsin and along the Lake Michigan shoreline in the south (55).

Pathogenesis and Virulence. Humans acquire blastomycosis by inhaling aerosolized spores of *Blastomyces dermatitidis* present in the environment. Once in the lungs, the conidia transform into infectious yeast. This form provides a survival advantage to the fungus as the thick cell wall of the yeasts protects it from the phagocytic action of alveolar macrophages, neutrophils, and monocytes. However some natural resistance is provided by the action of alveolar neutrophils and macrophages that inhibit the transformation of conidia to the pathogenic yeast form (56). The yeast forms that successfully multiply can disseminate through the blood and lymphatics to other organs (56). The conversion from mold to yeast is essential for pathogenesis for dimorphic fungi as was demonstrated by Medoff. He treated *H. capsulatum* mycelia with p-chloromercuriphenylsulfonic acid (PCMS), which prevents conversion to the yeast form. Treated *H. capsulatum* was then inoculated into mice and no longer caused disease suggesting that factors specific to the yeast phase are required for production of disease (8).

The *Blastomyces* adhesion 1 (BAD1) (formerly WI-1) is a 120 kDa glycoprotein found on the yeast cell surface, but not the hyphal filaments or the conidia of the mold form of the fungus (8). BAD1 is an immunodominant antigen that binds the yeast to CD14 and CD18 receptors on human macrophages and complement receptors (23, 67). African strains of *B. dermatitidis* have been found to lack BAD1 or have significantly reduced production, resulting in a form of blastomycosis most often presenting as chronic cutaneous lesions (8, 33). The role of BAD1 in the pathogenesis of blastomycosis has been demonstrated in several studies. Brandhorst et al. (7) used a genetic approach to compare different strains of *Blastomyces*. They showed that a wild-type strain infected

mice died rapidly from acute pulmonary and disseminated infection several weeks after inoculation, whereas, mice infected with BAD1 knockout strains survived for a significantly longer period of time. After restoration of virulence in the knockout strain, the inoculation killed 100% of infected mice, as did the wild-type strain. The role of BAD1 in modulating host immunity by altering production of pro-inflammatory cytokines has been investigated by Finkel-Jimenez et al. (22). BAD1 appears to suppress the production of TNF-alpha by neutrophils and macrophages via a mechanism that involves upregulation and secretion of TGF-β. The production of TNF-α in mice infected with BAD1 knockout yeast was several fold higher compared to wild-type yeast.

Other yeast phase-specific factors, such as glucans, have been implicated in the pathogenesis of blastomycosis. Glucans are polysaccharide molecules of D-glucose monomers associated with the cell surface of *B. dermatitidis* yeast. About 95% of glucan components are alpha-1,3-glucan (67). The loss of yeast cell wall alpha-1,3-glucan seems to be correlated with the loss of virulence in animal and cell culture models of infection, though this finding needs to be confirmed with more studies (8). Klein (32) showed that the non-virulent strain (ATCC 60916) lacks all detectable alpha-1,3-glucan, and the attenuated strain (ATCC 60915) has greatly reduced amounts of alpha-1,3-glucan on the cell surface compared with wild-type ATCC 26199.

Clinical Features. *Blastomyces dermatitidis* not only causes acute pulmonary infection, but also chronic granulomatous disease characterized by slow clinical progression. Although the lungs and skin are the organs most often involved, the fungus can disseminate to virtually any organ including bone, prostate, and CNS. The disease

ranges from asymptomatic to rapidly fatal infection (63). The incubation period, from inhalation of the conidia to the presentation clinical symptoms, can be up to 45 days (26).

Pulmonary symptoms associated with blastomycosis, such as nonproductive cough, malaise and fatigue, can mimic various viral or bacterial respiratory infections. The acute onset is often associated with high fever and productive cough, which imitates bacterial pneumonia. Chest x-rays may be normal or display lobar infiltrates or nonspecific changes. Subacute or chronic respiratory symptoms may resemble tuberculosis or other fungal pulmonary diseases with fatigue, weight lost, night sweats, productive cough, chest pain and radiographic presentation of fibronodular infiltrates or mass-like lesions. Hemoptysis is uncommon; however, its presence makes blastomycosis difficult to distinguish from a bronchopulmonary malignancy. In fulminant infections, adult respiratory distress syndrome (ARDS) may develop due to miliary spread of the infection. This is associated with high fever, tachypnea, and diffuse bilateral pulmonary infiltrate which can progress rapidly to respiratory failure and death.
Radiographic images often show parenchymal abnormalities in one or both upper lobes. Discrete pulmonary nodules, with or without cavitation, and pleural effusion have been reported, but are seen in fewer than 30% of cases. Mediastinal lymphadenopathy is seen in less than 20% of cases (56, 63).

Disseminated Disease. Extrapulmonary involvement is seen commonly among patients with chronic blastomycosis. The skin and the subcutaneous tissues are the most common sites of extrapulmonary disease, occurring in 40 to 80% of cases (56). The cutaneous lesions are presumed to be secondary to hematogenous spread from a primary pulmonary infection. The typical lesions present in one of two forms: verrucous or

ulcerative (26, 41). The verrucous (wart-like) skin lesions usually lie above a subcutaneous abscess. They are raised and crusted with irregular sharp borders. In contrast, the ulcerative form starts as erythematous nodules or pustules that break down into one or more shallow ulcers. The ulceration spreads slowly in an asymmetric manner which can go through repeated cycles of healing and breakdown. The appearance of these lesions can mimic a number of dermatologic conditions such as pyoderma gangrenosum, squamous cell carcinoma, or chronic cutaneous infections such as sporotrichosis, nocardiosis, atypical mycobacteriosis, tularemia, anthrax, or leishmaniasis (41).

The skeletal system may be involved in 14%-60% of blastomycosis cases making it the third most common site of the disease (51). Infections involving the vertebrae, ribs, facial bones, skull, long bones, small bones, pelvis, and scapulae have been reported and are the result of hematogenous dispersal of the fungus (57). Bone lesions cause a variety of symptoms ranging from pain and swelling to abscesses, and draining sinuses. The radiographic images in bone dissemination are variable and nonspecific showing osteolysis without sequestrum, or periosteal reaction.

In cases of systemic blastomycosis, the genitourinary system is involved in 10%-30% of cases and a few cases of sexual transmission have been reported (56). In men, the disease can affect the prostate, epididymis, adrenals, kidneys, testes, and preputial skin (58). Symptoms include swelling and pain in the perineal region, dysuria, hematuria, hematospermia, and urinary retention. In females, the endometrium, fallopian tubes and the ovaries may be affected (50, 56, 58).

Central nervous system (CNS) involvement is a rare, but important secondary manifestation of disseminated blastomycosis. It occurs in 5-10% of cases in

immunocompetent patients and up to 33% in autopsy series (49). The spectrum of symptoms is diverse, ranging from subacute or chronic meningitis to encephalitis, parenchymal brain abscesses or granulomas, stroke or myelopathy (47). The differential diagnosis of CNS blastomycosis includes tuberculosis and meningeal carcinomatosis (56). Virtually any organ can be affected by disseminated blastomycosis. Other sites of infection such as the reticuloendothelial system with lymphadenopathy or abscesses in the liver or spleen, the eye, the middle ear, the paranasal sinuses, the breast tissue, the myocardium, the pericardium, the gastrointestinal tract, and the thyroid have been documented (56).

Laboratory Diagnosis. Given the nonspecific clinical presentation that mimics many bacterial or other fungal infections, the diagnosis of blastomycosis may be delayed. The laboratory diagnosis of blastomycosis can be made by visualizing the fungi in respiratory specimens or biopsies from extrapulmonary sites. Clinical specimens typically collected for testing include sputum, bronchoalveolar lavages, tracheal aspirates, pus or abscess aspirates, skin lesion scrapings, and cerebrospinal fluid. Specimens can be examined in wet mount stains, cytology and histopathology, tested for antigens or antibodies or cultured on specialized media (56).

Direct examination of clinical specimens using the fluorescent stain calcofluor white in 10% KOH or histopathology stains using periodic acid-Schiff, and/or methenamine silver are useful for visualizing the characteristic broad-based budding yeast. These are the most common rapid methods of diagnosis. The sensitivity of staining methods varies from 50%-90% on respiratory specimens (15, 56). The cytopathology shows a nonspecific inflammatory reaction with non-caseating or suppurative granulomas

containing epitheloid histiocytes and multinucleated giant cells. In early infection or in immunocompromised patients, the organisms are often numerous and easy to identify, making the diagnosis straightforward (56).

Samples submitted for direct examination should be cultured. The isolates can take 2 to 3 weeks to grow on Sabouraud dextrose agar or brain-heart infusion agar at 25ºC. Once conversion to yeast is confirmed, it provides a definitive diagnosis of blastomycosis with up to 92% sensitivity in bronchoscopic specimens (40).

Serologic tests, including complement fixation (CF), immunodiffusion (ID) and enzyme immunoassays are useful for detecting antibodies and/or antigens in biological fluids. These tests are rapid, relatively inexpensive, and have been used successfully for diagnosis. The detection of antibodies against *B. dermatitidis* using complement fixation has a 57% and 30% of sensitivity and specificity, respectively, while the immunodiffusion tests show 65%-80% sensitivity. The detection of *B. dermatitidis A*-antigen in human urine specimens has shown high sensitivity (92.9%), but low specificity because of the cross-reactivity with *H. capsulatum*, *Paracoccidioides*, and *Penicillium marneffei*. The recent use of the BAD1 protein as a target for detecting *B. dermatitidis* may increase the specificity of testing (29, 45, 56, 62).

A skin test for delayed-type hypersensitivity testing using a crude mycelial filtrate (blastomycin) has been used with low specificity and sensitivity. Two studies showed 59% and 100% of patients who were culture positive for *B. dermatitidis* were negative according to skin testing (11, 65).

Molecular diagnosis of blastomycosis, using a chemiluminescent DNA probe, has a sensitivity of 87.4% and a specificity of 100%. *In situ* hybridization, using a pair of

oligonucleotides complementary to the 18S and 28S rDNA, is another approach and reports a sensitivity of 95% and a specificity of 100% (5). Both of these methods are used to confirm the identification of culture isolates rather than direct clinical specimens.

Treatment. If left untreated, blastomycosis has nearly a 60% mortality rate (6). Currently, it is recommended that all patients diagnosed with blastomycosis be treated. The choice of the antifungal drug depends upon the clinical form, the severity of illness, the immune status of the patient, and the toxicity of the drug (15). Although there has never been a large-scale clinical trial to compare the potential treatments of blastomycosis, amphotericin B and itraconazole continue to be the drugs of choice based on the Infectious Diseases Society of America (IDSA) clinical practice guidelines (56).

Amphotericin B is a polyene macrolide antifungal obtained from a strain of *Streptomyces nodosus*. The drug acts by binding to sterols in the cell membrane of the fungus, resulting in change to membrane permeability and allowing leakage of intracellular components. Amphotericin B shows a high level of activity against many species of fungi including dimorphic and species causing hyalohyphomycosis and phaeohyphomycosis (20). Amphotericin B is usually recommended for the treatment of severe blastomycosis, such as in immunocompromised patients, life-threatening or central nervous system (CNS) disease, or in case of azole treatment failure. In addition, amphotericin B is the only drug approved for treating blastomycosis in pregnant women (15). The drug is delivered in deoxycholate or a lipid formulation which is better tolerated and limits the toxicity (54). As early as 1967, Abernathy et al. showed that a total dose of 1.0 to 2.0 g of amphotericin B cures most patients with blastomycosis. A few years later, in a comparative trial, treatment was successful for 91% of patients who

received amphotericin B, compared with 72% of patients who were treated with 2-hydroxystilbamidine (56). In another study, an intravenous amphotericin B using cumulative doses of more than 1 gram resulted in cure without relapse in 70%–91% of patients with blastomycosis (15). Another study reported an 86% response rate and 3.9% relapse rate using amphotericin to treat blastomycosis (54).

The azole antifungals are characterized by the presence of an azole ring structure. They are usually fungistatic or fungicidal at higher concentrations and act by interfering with the enzyme activity of cytochrome P-450, decreasing the production of ergosterol that damages the cell membrane by altering its permeability and functions (59). The azoles most frequently used for the treatment of blastomycosis are itraconazole, ketoconazole, and fluconazole (15).

Ketoconazole was the first azole shown to be an effective alternative to amphotericin B in the treatment of immunocompetent patients with mild to moderate blastomycosis. A cure rate of 70% and 85% among patients treated with 400 mg/day and 800 mg/day of ketoconazole, respectively, was documented in a prospective, randomized, multicenter trial. In another cohort of 46 patients, a cure rate of 76% with a dose of 400 mg/day of ketoconazole and relapse rates of 10%–14% was noted (15). In a prospective study of 48 patients with blastomycosis, treatment with 200 to 400 mg/day of itraconazole capsules was 90% effective for a median duration of 6 months (54). Another alternative for immunocompetent patients with mild to moderate blastomycosis is fluconazole. In a pilot study, fluconazole given at a dose of 200 to 400 mg/day was successful for 65% of 23 patients who took the drug for at least 6 months. A later study used 400 to 800 mg/day,

which was successful for 34 of 39 (87%) patients for a median treatment duration of 9 months (56).

The following therapeutic scheme is the current treatment recommendation of the Infectious Diseases Society of America (IDSA). For mild to moderate pulmonary blastomycosis, oral itraconazole at 200 mg once or twice daily for 6–12 months is used. The capsules must be taken with food and the serum levels of itraconazole should be monitored after the patient has received at least 2 weeks of treatment to ensure adequate drug exposure (54).

In moderately severe to severe pulmonary and extrapulmonary blastomycosis, including patients with extensive pneumonia, multiorgan disease, respiratory failure due to ARDS, other organ failure, and/or hemodynamic instability, initial treatment with a lipid formulation of amphotericin B at 5 mg/kg/day for 1–2 weeks is recommended until improvement is observed. Patients should then step down to oral itraconazole at 200 mg twice daily for 6–12 months. Serum creatinine and electrolytes (K^+, Mg^{++}) should be monitored on a regular basis. Additionally, corticosteroids have been given to patients with ARDS, with apparent success (54, 56).

In case of central nervous system (CNS) involvement, azoles should not be used as initial therapy. Lipid amphotericin B at a dose of 5 mg/kg per day for 4 to 6 weeks should be given to patients. Lipid formulations of amphotericin B are preferred because they are less likely to cause significant adverse events over the prolonged course of therapy. In addition, data from animal models suggests that liposomal amphotericin B diffuses better in the CNS than conventional amphotericin B. After 4- to 6-weeks, an azole is given for at least 12 months and until no evidence of active CNS infection (e.g.,

abnormality of cerebrospinal fluid or mass lesion). Itraconazole has better intrinsic activity against *B. dermatitidis* than fluconazole, but fluconazole penetrates better into the CNS than itraconazole. Voriconazole possesses both of these important characteristics (intrinsic activity and capability for CNS penetration), but clinical experience with this agent in patients with blastomycosis is limited. Itraconazole at 200 mg twice or thrice per day, fluconazole at 800 mg per day, and voriconazole at 200 to 400 mg twice per day are options for step-down therapy (56).

In pregnant women, lipid formulation of amphotericin B at 3 to 5 mg/kg per day is recommended. Azoles should be avoided because of possible teratogenicity (56).
In immunocompromised patients, the treatment is similar to that described above. The initial therapy consists of conventional or lipid amphotericin B for 1 to 2 weeks or until clinical improvement; thereafter, a switch to itraconazole should be made. The duration of therapy should be at least 12 months but is usually longer for patients with ongoing immunosuppression (56).

Genetic Diversity of *B. dermatitidis*. Several studies have documented genetic diversity within *B. dermatitidis*. Restriction fragment length polymorphism (RFLP) analysis of ribosomal or mitochondrial DNA, random amplified polymorphic DNA (RAPD) analysis, and more recently, microsatellite typing have emerged as sensitive techniques for the study of the population genetic structure of *B. dermatitidis* (66).
In 1995, Yates-siilata et al. (66) explored the genetic diversity of 19 clinical isolates of *B. dermatitidis* with a RAPD and grouped the isolates into three genetic classes. Similarly, in 2000, McCullough et al. (42) investigated 59 isolates collected over 35 years from 15 different regions in the world (United States, India, Africa, Canada). They combined a

RAPD typing system with restriction fragment analysis to classify the isolates into three genetic groups. At Marshfield Clinic, a study involving 112 clinical isolates from outbreaks and sporadic cases were analyzed using a novel microsatellite typing method (46). Microsatellite loci were identified in the unannotated genome sequence of *B. dermatitidis* ATCC strain 26199 (Washington University School of Medicine Genome Sequencing Center website http://genome.wustl.edu/genome.cgi?GENOME=Blastomyces%20dermatitidis). Sequences were searched for dinucleotide repeats using a computer algorithm developed at the Molecular Mycology Research Laboratory, University of Sydney (http://www.mmrl.med.usyd.edu.au/ssr.html). Primers for 27 microsatellite loci were identified. The result of the microsatellite analysis revealed two distinct populations: a clonal population (group 1) and a polymorphic population (group 2).

Sexual Reproduction. Many higher fungi have both sexual and asexual modes of reproduction. The sexual and asexual reproductive states of these fungi are named and classified independently, although genetically they are the same organism and share the same evolutionary history (38). The name *Blastomyces dermatitidis* applies only to the asexual states of the fungus classified in the division deuteromycota. In the sexual stage, the fungus is known as *Ajellomyces dermatitidis*.

Sexual reproduction in fungi is controlled by a specialized genomic region called the mating type (*MAT*) locus. Different gene combinations reside at the *MAT* locus and confer sexual identity. Some fungi exist in only two mating-types, whereas others occur in multiple mating types. Some fungi are also endowed with the ability to switch mating-type, whereas others cannot. In Ascomycetes, in which *B. dermatitidis* belongs, sexual

reproduction is regulated by a bipolar mating-type system in which cells are of two opposite mating-types (commonly called "a" and "α", or plus and minus). The plus *MAT* locus allele encodes for α-box domain transcription factor, whereas the minus encodes for high-mobility-group (HMG) domain-type transcription factor (38). For sexual reproduction to occur, mating partner cells of different *MAT* alleles must come together, hence this sexual cycle has been difficult to observe in many ascomycete species (24). Mating in *Ajellomyces dermatitidis* results in production of cleistothecia which are closed spore-bearing structures characterized by radiating hyphae in the form of spiral coils with asci having eight ascospores (43). Reproduction in *Ajellomyces* begins with the formation of a dikaryon: a cell in which two haploid nuclei from different parents are brought together and share a common cytoplasm. The dikaryotic cell develops into ascogenous hyphae and these develop into asci, in which finally meiosis, recombination and mitoses yields eight haploid ascospores. All ascospores in a cleistothecium are either of crossed or of selfed origin (FIG. 4) (9).

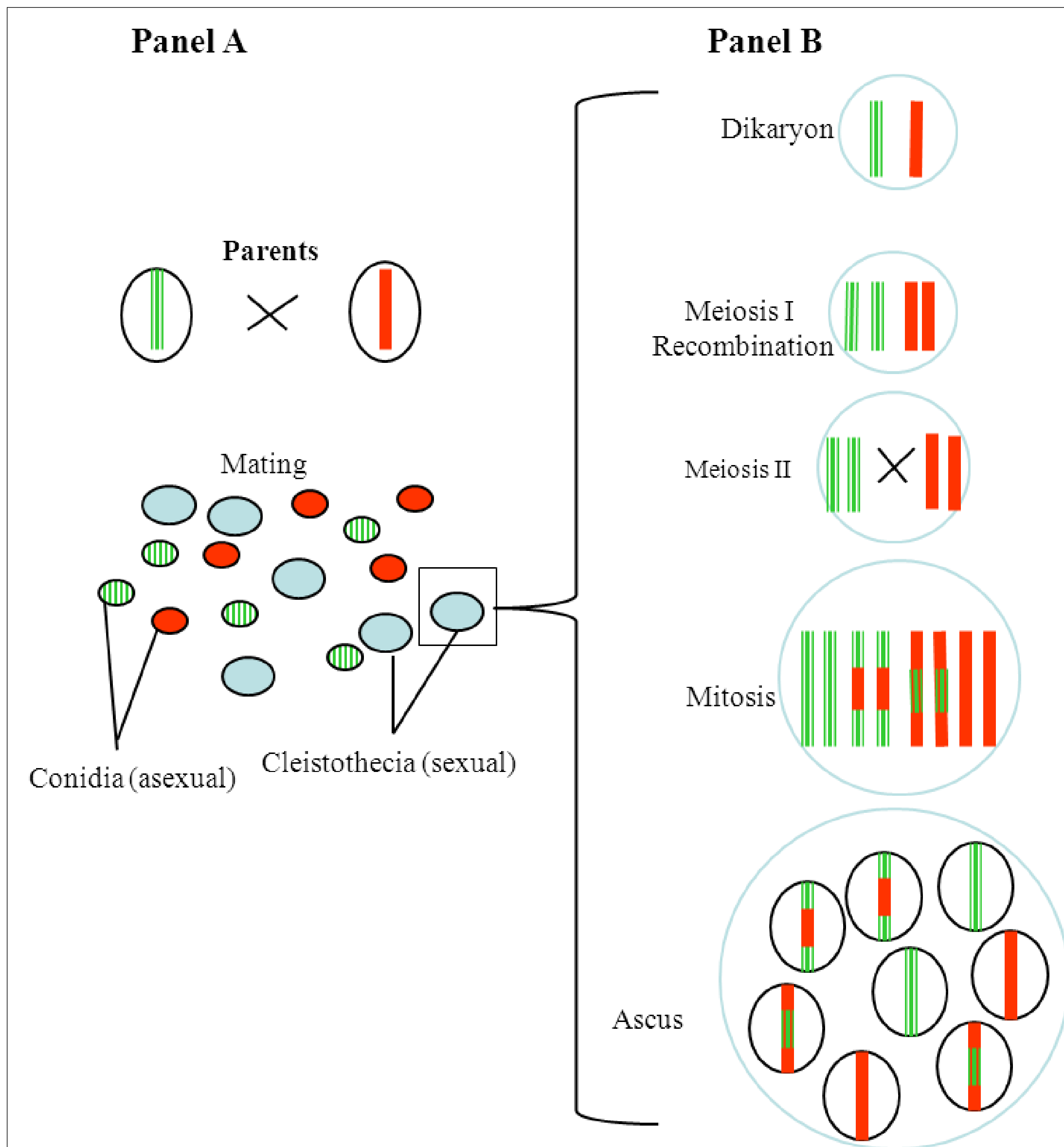


FIG. 4. The role of parental nuclei in ascospore formation between two opposite mating type strains. In panel A, the two strains (parent 1 in green and parent 2 in red) are put in contact. Both mycelia produce regular conidia (green ovals and red ovals from parent 1 and parent 2 respectively). If the two strains are compatible, mating occurs to produce cleistothecia (blue ovals). Panel B represents the process that takes place within each cleistothecium.

Several studies have been conducted on mating in *B. dermatitidis.* McDonough et al. (44) conducted a study on 206 isolates of *Blastomyces dermatitidis.* Each isolate was paired individually with each of 3 known plus (+) and minus (−) mating types. They found that 129 isolates were sexually fertile. In the remaining 77 cultures, the failure to initiate ascospore production was explained by the advanced age of the culture and/or the stress or genetic change due to multiple laboratory passages. While both mating types are thought to be equally distributed among clinical isolates, the role of mating type in the pathogenesis of the disease is not clear.

Asexual Spore Production. Asexual spore (conidia) production is an important type of asexual reproduction in many fungi, which produce a large numbers of spores throughout their mitosporic or "anamorphic" life state. This asexual reproduction is controlled by many different factors that include environmental variables (nutrient levels, CO_2 levels, light levels) and fungal genetics. Sporulation tends to spread and maximize environment colonization, and helps to guarantee species survival during one season (61). The ability to produce a large number of spores would be reproductively advantageous. In suitable growing conditions, isolates producing a large number of spores would demonstrate increased population in the environment, resulting in a greater potential for exposure to humans and other animals with increased inoculum inhaled by susceptible hosts.

Antifungal Susceptibility. Increasingly, resistance to antifungal drugs has been observed in clinically isolated yeasts; however, no standardized method is currently available for *in vitro* susceptibility testing of dimorphic fungi (15). The National Committee for Clinical Laboratory Testing Subcommittee on Antifungal Susceptibility

Testing is attempting to develop consensus standards for these pathogens, and their most recent guidelines reflect best practices based on current information. With regard to *B. dermatitidis*, comparison of antifungal susceptibility profiles among genetically diverse groups of clinical isolates could elucidate associations between genotypic groups and resistance, and allow for a rational strategy for treatment options with appropriate antifungal drugs.

PURPOSE OF THE STUDY

Genetic diversity of *B. dermatitidis* and its association with clinical variation was recently explored by Gruszka (27). In that study, microsatellite analysis was used to genotype a large group of clinical isolates of *B. dermatitidis.* The vast majority of strains segregated into one of two genetic groups. In addition, there were statistically significant differences in the clinical characteristics of the patients infected with the different groups. In the present study, we aimed to extend this work by phenotypically characterizing multiple isolates of *B. dermatitidis*, in order to determine whether or not there are specific associations between isolate phenotype and genetic type. The phenotypic traits chosen for analysis included mating locus type, ability to mate between genetic groups, quantitative spore production, and antimicrobial susceptibility to a panel of antifungal drugs. If specific phenotype/genotype associations are present it could lead to a better understanding of the virulence mechanisms for this pathogen.

RESEARCH DESIGN AND METHODS

***Blastomyces dermatitidis* Isolates.** One hundred isolates of *B. dermatitidis* were chosen from the Marshfield Clinic Research Foundation biobank for analysis. The isolates consisted of four environmental isolates, seven canine isolates, two feline isolates, and 87 human isolates. Among the human isolates, 50 were clinically associated with pulmonary infection, 28 were disseminated, and for 7 the associated clinical information was incomplete. The isolates were further classified according to the severity of the disease. Among the 87 human isolates, 37 of the patients were hospitalized, 30 were treated as outpatients, and 17 patients died as a result of the infection. Two isolates were wild-type strains from the American Type Culture Collection (ATCC 18187 and ATCC 18188), used as mating type testers. All isolates were handled in a biosafety level 3 (BSL-3) laboratory.

DNA Extraction. DNA was extracted using the QIAamp® DNA Mini Kit Tissue Protocol (QIAGEN, Valencia, CA), from isolates of *B. dermatitidis* grown on Sabouraud dextrose agar at 25°C. Mold-phase *B. dermatitidis* colonies were removed from agar plates with a sterile scalpel and placed into1.5 ml sterile microcentrifuge tubes each containing 540 µl of buffer ATL. Samples were vortexed, and then 60 µl of proteinase K was added to each sample and incubated in a 56°C heat block overnight. Samples were removed from the heat block and quickly centrifuged (4,297 x g) with an eppendorf

microcentrifuge for 2-3 sec to remove drops from the lid. Four hundred µl of buffer AL was added to each sample and vortexed. Samples were incubated at 70°C on a heat block for 1 h. The samples were removed from the heat block and quickly centrifuged to remove drops from the lid. Four hundred µl of 100% ethanol was added to each sample and vortexed. The samples were then quickly centrifuged and the supernatant was split between two spin columns. The columns were centrifuged for 1 min (4297 x g). The supernatant was discarded and 500 µl of buffer AW1 was added to each column. The columns were centrifuged for 1 min at 4,297 x g. and the supernatant was discarded. Five hundred µl of buffer AW2 was added to each column and centrifuged for 3 min at 4,297 x g. The columns were placed into a new sterile 1.5 ml microcentrifuge tube and 50 µl of buffer AE was added to each column. The columns were incubated at room temperature for 1 min and then centrifuged for 1 min at 4,297 x g. Elutions from the same isolate were combined and the resulting extracted DNA was ready for PCR or stored at -20°C.

Microsatellite Analysis. Genotyping was performed with 27 microsatellite markers according to the method of Meece et al. (46). Two microliters of previously extracted DNA was amplified with the HotStar-Taq Master Mix Kit (QIAGEN) according to the manufacturer's recommendations with a set of three primers: a T7-tagged forward primer, reverse primer, and a 6-FAM-T7 primer, all at a final concentration of 0.2 µM. The 6-FAM-T7 primer anneals to its complementary sequence and is extended, thus labeling the amplicon with fluorescent dye. Amplification conditions were as follows: 15 min denaturation at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C, followed by a 5 min final extension at 72°C. Amplified products were prepared for sizing on an ABI model 3130x1 Genetic Analyzer (Applied

Biosystems, Carlsbad, CA) by combining 1.0 µl of product with 9.0 µl of the GeneScan™ -500 ROX™ size standard, diluted 1:9 in Hi-Di formamide. Samples were denatured at 95°C for 2 min, immediately cooled on ice and loaded to a 50 cm capillary with an injection voltage of 1.6 kV for 15 sec. Electrophoresis was performed at 15.0 kV for 1.25 h at 60°C using the microsatellite default settings, and subsequently analyzed using the GeneMapper software package, version 4.0 (Applied Biosystems). DNA fragments sizes were grouped into their appropriate alleles manually using the fixed-bin method (10).

Mating Locus Determination. The nucleotide sequence for the high mobility group (HMG) domain was obtained from GenBank (XM_002623161) and primers were designed to optimize a PCR typing method. Since no sequence data was available for the α-box protein, a search was made at the Washington University website for the sequence of gene segments flanking the expected α-box region. Primers were designed and the region was sequenced by chromosome walking. The resulting sequence was BLAST searched against the database of *B. dermatitidis* contigs on the Broad Institute's website (http://www.broadinstitute.org/annotation/genome/blastomyces_dermatitidis/MultiHome.html). The search returned a 99% matching contig that was homologous to the *Ajellomyces capsulatus* (the sexual form of the closely related dimorphic fungus *Histoplasma capsulatum*) α-box sequence. Primers within the *B. dermatitidis* α-box transcript were designed; the PCR reaction was optimized and tested using the confirmed, commercially available mating types ATCC 18188 and 18187, which yielded the expected PCR presence/absence results. Mating locus type was then determined for the

100 *B. dermatitidis* isolates by showing a positive reaction for one allele and a negative reaction for the other allele using separate PCR reactions.

Briefly, 2 μl of previously extracted DNA was amplified with the HotStar-Taq Master Mix Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations with a set of two primers for each locus. For HMG, we used bdHMG forward (5'-CCTCCCGCTGATATCAACACCGT-3'), and bdHMG reverse (5'-TTGGTCTCAGCAGACTCGGCTT-3') at the concentration of 20 pmol/μl each with the following amplification conditions: 15 min initial denaturation at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C, followed by a 5 min final extension at 72°C. For α-box, we used bdalphabox forward (5'-AGTCGCTGCTCAACCAAACT-3'), and bdalphabox reverse (5'-TTGGTGTTAAAGGCCTGGTC-3') at the concentration of 20 pmol/μl each with the following amplification conditions: 15 min initial denaturation at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 63°C, and 1 min at 72°C, followed by a 7 min final extension at 72°C. The amplified products were prepared for sizing using 1.5% agarose gel electrophoresis, and then stained with 0.5 mg/L ethidium bromide. The expected HMG fragments (510 bp) and α-box fragments (228 bp) were visualized and sized using the Gel Doc 2000 (Bio-Rad Laboratories).

Mating Experiments. Mating crosses were attempted between and among selected isolates from group 1 and group 2 strains of *B. dermatitidis* as follow:

Group 1(α-box) x Group 1(HMG), 4 crosses; Group 2(α-box) x Group 2(HMG), 4 crosses; Group 1(α-box) x Group 2(HMG), 4 crosses; Group 2(α-box) x Group 1(HMG), 4 crosses. These crosses included the cross between the two testers for positive control purpose. Isolates of the same mating type were also crossed for control purpose. Group

1(HMG) x Group 2(HMG), 1 cross; Group 1(HMG) x Group 1(HMG), 1 cross; Group 2(α-box) x Group 1(α-box), 1 cross.

For mating experiments, isolates were grown first on potato flake agar at 22ºC for two to three weeks. For isolates to be crossed, a 5 mm square cut of the mycelium was made with some of the underlying agar using a sterile scalpel blade. The cut was made from the growing edge of the cultures, then the squares were placed about one to two centimeters apart on the surface of the soil extract agar. The cultures were then incubated at 22ºC for up to four weeks and monitored for cleistothecium formation. The plates were checked and pictures taken at day 7, day 14, day 21, and day 28. The cleistothecia were visualized by a tape mount with lactophenol cotton blue staining.

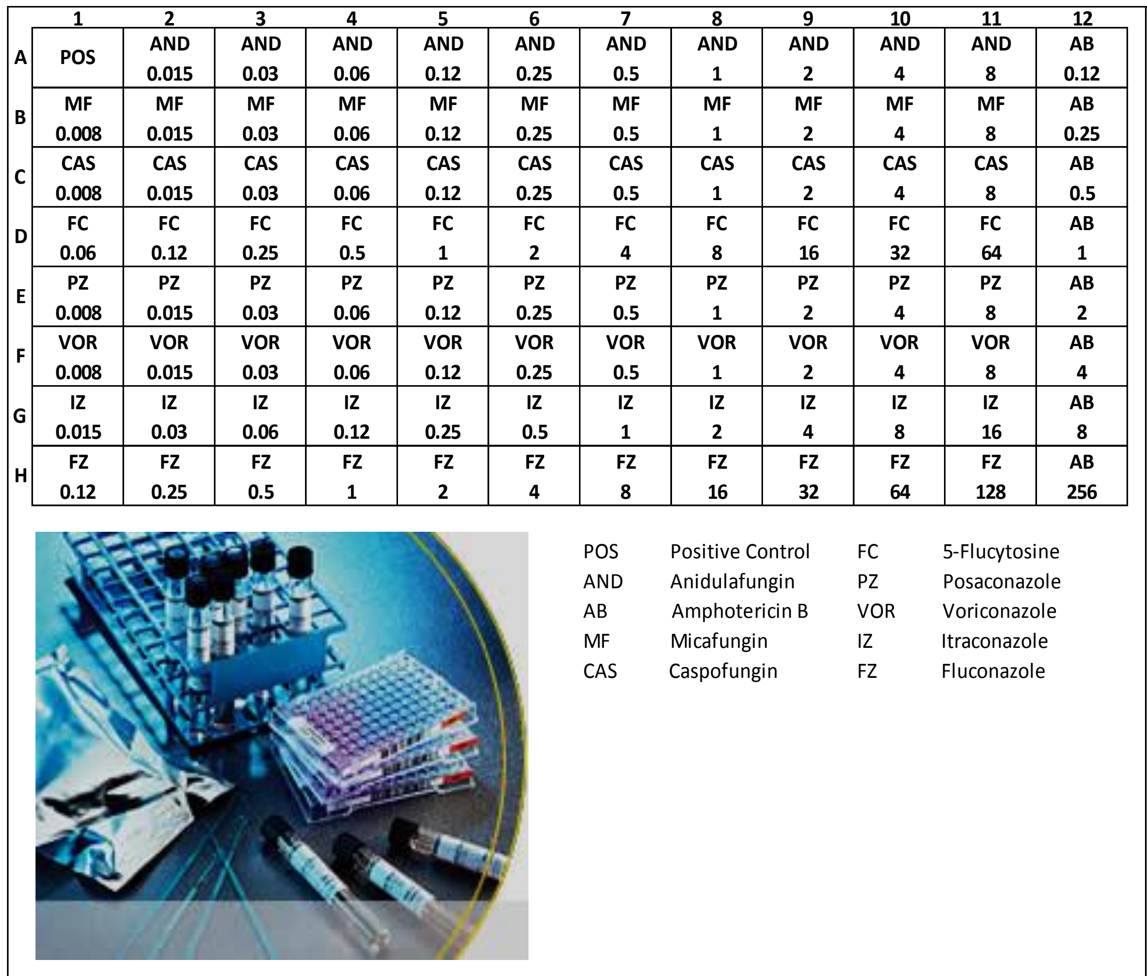
For each cross, in a 1.8 ml tube, cleistothecia were scraped from the agar with a sterile loop into 500 µl sterile PBS. The tube was vortexed to disrupt asci and release ascospores. The suspension was then plated and streaked for isolation on yeast extract agar plates. The plates were incubated at 37˚C to allow the growth of yeast-phase colonies. Ten individual colonies were picked and the genomic DNA extracted for MAT locus typing and microsatellite analysis.

Asexual Spore Quantification. Fourteen isolates were selected for spore quantification. Isolates were chosen to include human, environmental, and culture type strains. Both genetic groups were also represented. Isolates were grown first at 37ºC on yeast extract agar for yeast conversion for 7 to 10 days. A loop full of colony was resuspended in sterile Phosphate Buffered Saline (PBS) and yeast cells counted using a hemocytometer in order to standardize the initial inoculum. Approximately 1000 yeast cells were plated onto a Sabouraud Dextrose agar slant and incubated for two weeks at

25ºC, for mold conversion and sporulation. After two weeks, the slants were rinsed by adding 10 ml of PBS in order to dislodge the conidia. The initial rinse was serially diluted as follow: 900 µl of PBS were added to eight labeled 1.8 microcentrifuge tubes, then 100 µl of the initial rinse added to the first tube which will be 10^{-1} dilution. One hundred microliters of the 10^{-1} dilution was transferred to the second tube which will be 10^{-2} dilution, and so on until the 10^{-5} dilution. The initial rinse and the serial diluted isolates were plated onto yeast extract agar, and incubated at 37ºC until yeast colonies were visible. The colonies were counted on each plate and recorded. Since each conidium converts to yeast by direct budding at 37ºC, yeast colonies represent the number of spores produced in two weeks of mold-phase growth. Isolates were plated in duplicate.

Antifungal Susceptibility. A commercially available 96 well plate format colorimetric panel (Sensititre YeastOne Trek Diagnostic Systems, Cleveland, OH) using the Clinical Laboratory Standards Institute (CLSI) reference microdilution method was used to test the susceptibility of the *B. dermatitidis* isolates to a panel of nine antifungal drugs (Fig. 5). Each plate contained serial dilutions of antifungal drug and a colorimetric indicator. YeastOne endpoints are determined to be the lowest concentration at which the color in the well changes from blue (negative, indicating no growth) to red (positive, indicating growth). The isolates were grown as yeast on yeast extract agar for one to two weeks at 37ºC. A standard inoculum of 2.0 McFarland was made using a calibrated nephelometer from well-isolated colonies into 5 ml of demineralised sterile water, and then 20 µl was transferred from the 2.0 McFarland inoculum into 11 ml of inoculum broth and vortexed vigorously. The making of the 2.0 McFarland solutions and the transfer of the 20 µl into the RPMI 1640 were completed within 15 min. The inoculum

was then transferred into a sterile reagent reservoir. One hundred microliters was inoculated into each well of the Sensititre plates using a 12-channel multi-pipettor. Ten microliters was removed from the control well and plated on yeast extract agar as a purity check. The Sensititre plates were covered with adhesive seal and incubated for up to 9 days (216 h) at 35ºC without CO_2. The plates were examined at 48 h, then every 24 h. The yeast extract agar purity check plates were sealed and incubated along with the 96 well plates. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the drug which inhibits substantially the growth of the isolate.



	1	2	3	4	5	6	7	8	9	10	11	12
A	POS	AND 0.015	AND 0.03	AND 0.06	AND 0.12	AND 0.25	AND 0.5	AND 1	AND 2	AND 4	AND 8	AB 0.12
B	MF 0.008	MF 0.015	MF 0.03	MF 0.06	MF 0.12	MF 0.25	MF 0.5	MF 1	MF 2	MF 4	MF 8	AB 0.25
C	CAS 0.008	CAS 0.015	CAS 0.03	CAS 0.06	CAS 0.12	CAS 0.25	CAS 0.5	CAS 1	CAS 2	CAS 4	CAS 8	AB 0.5
D	FC 0.06	FC 0.12	FC 0.25	FC 0.5	FC 1	FC 2	FC 4	FC 8	FC 16	FC 32	FC 64	AB 1
E	PZ 0.008	PZ 0.015	PZ 0.03	PZ 0.06	PZ 0.12	PZ 0.25	PZ 0.5	PZ 1	PZ 2	PZ 4	PZ 8	AB 2
F	VOR 0.008	VOR 0.015	VOR 0.03	VOR 0.06	VOR 0.12	VOR 0.25	VOR 0.5	VOR 1	VOR 2	VOR 4	VOR 8	AB 4
G	IZ 0.015	IZ 0.03	IZ 0.06	IZ 0.12	IZ 0.25	IZ 0.5	IZ 1	IZ 2	IZ 4	IZ 8	IZ 16	AB 8
H	FZ 0.12	FZ 0.25	FZ 0.5	FZ 1	FZ 2	FZ 4	FZ 8	FZ 16	FZ 32	FZ 64	FZ 128	AB 256

POS	Positive Control	FC	5-Flucytosine
AND	Anidulafungin	PZ	Posaconazole
AB	Amphotericin B	VOR	Voriconazole
MF	Micafungin	IZ	Itraconazole
CAS	Caspofungin	FZ	Fluconazole

FIG. 5. Yeastone, Sensitre plates format and list of antifungal agents and dilution range for yeast susceptibility test. All concentrations are in μg/ml. Adapted from picture retrieved from Trek Diagnostic Systems on September 26, 2011. http://www.trekds.com/products/sensititre/c_yo9anidulafungin.asp

RESULTS

Microsatellite Analysis. The genetic relationships between isolates were analyzed by constructing an unrooted neighbor-joining tree of unique genotype based on allele-sharing distance. The result confirmed two genetically distinct groups, designated group 1 and group 2. Group 1 appears tightly clustered and contains many genetically identical isolates. Group 2 shows fanlike branching, indicating a higher number of haplotypes and greater genetic diversity (FIG. 6).

Group 1 had 51 isolates (51%) whereas group 2 had 49 isolates (49%). Among the environmental isolates, 2 were in group 1 and 2 in group 2. Five isolates out of the seven canine isolates were in group 2. Among human isolates, 47 (54%) were in group 1 and 40 (46%) in group 2. Out of the 28 disseminated cases, 13 (46.4%) were in group 1 and 15 (53.6%) in group 2. Among the pulmonary cases, 32 (64%) and 18 (36%) were in group 1 and group 2 respectively. Group 1 was more associated with fatal cases with 11/17 isolates (64.7%).

Mating Locus Determination. One hundred isolates of *B. dermatitidis* were typed for mating locus by PCR followed by gel electrophoresis (FIG. 7). Fifty two isolates (52%) were positive for the HMG locus, and 46 (46%) were positive for the α-box locus. Interestingly, two isolates were positive for both loci. In order to determine whether or not these represented dual infections within an individual patient, attempts

were made to further subculture the specimens on yeast extract agar in order to better isolate individual colonies. However, the specimens appeared nonviable.

Results indicated that the two mating types were nearly equally distributed between the two genetic groups. Specifically, group 1 had 24 isolates positive for α-box loci and 26 isolates positive for HMG loci whereas group 2 had 22 positive for α-box, and 26 positive for HMG. Among the environmental isolates, 3/4 were HMG positive. For the other nonhuman isolates, 5/7 canines and 1/2 feline were HMG positive.

For human isolates, 42/83 were HMG positive, and 41/83 were α-box positive. For disseminated cases, 16/28 were α-box positive and 12/28 positive for HMG. For pulmonary involvement, 22/48 and 26/48 were positive for α-box and HMG respectively. For fatal cases, 10/16 and 6/16 were positive for α-box and HMG respectively.

Mating Experiments. For all compatible strains, cleistothecia were observed within 21 to 28 days after pairing opposite mating locus isolates on soil extract agar (FIG. 8). Macroscopically, cleistothecia were observed on the surface of the soil extract agar at the junction of the two colonies. These structures resemble white nodules which become buff to tan with age. The cleistothecia were then visualized by tape mount with lactophenol cotton blue staining. At 600-1000x, the characteristic radiating coiling net-like hyphae were observed (FIG. 9) and numerous 1-2 μm ascospore-like structures were present. All α-box X HMG crosses between group 1 and group 2 isolates and also within the same genetic group yielded cleistothecia formation. In contrast, no cleistothecia were observed when two α-box strains or two HMG strains were paired together.

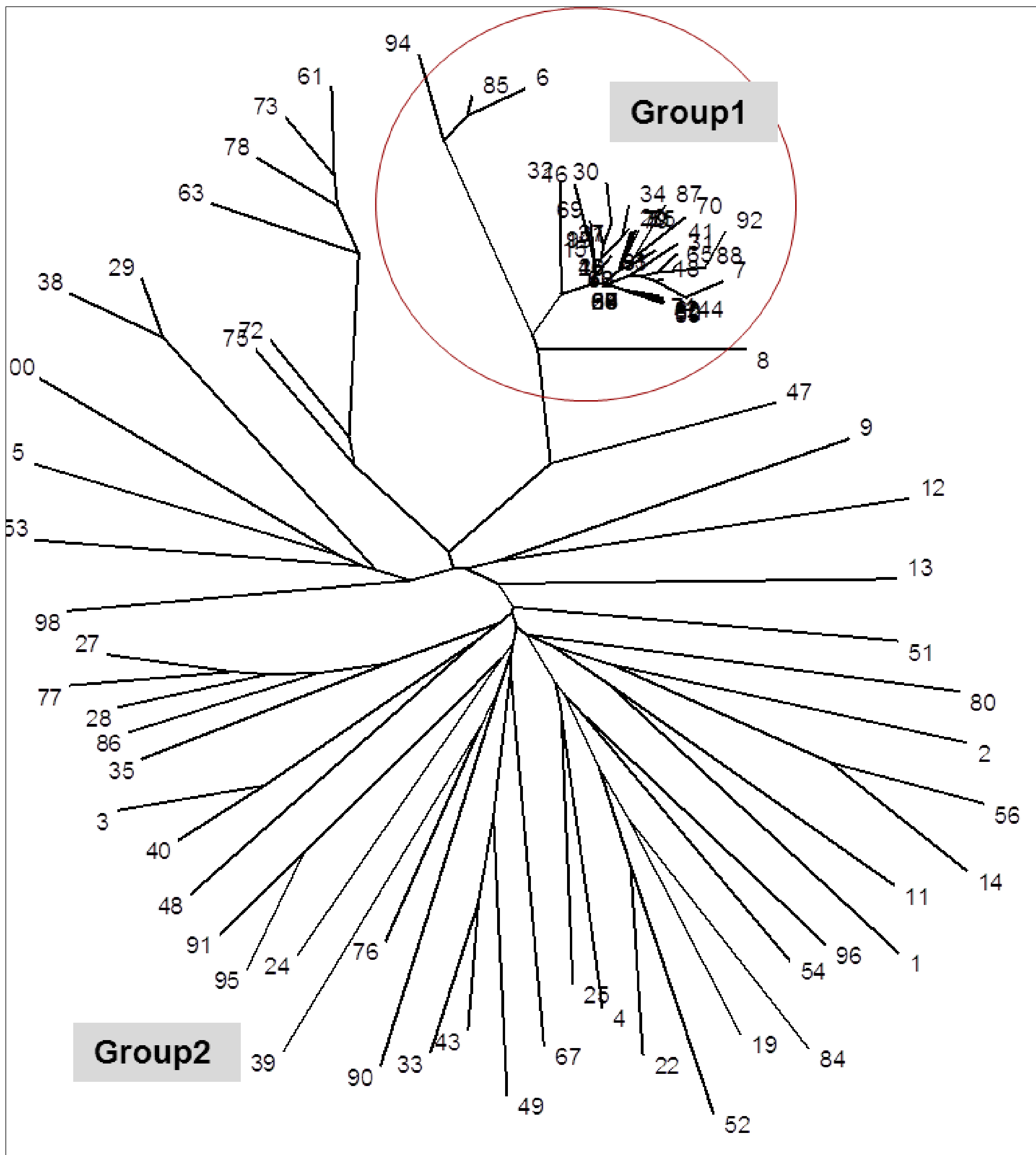


FIG. 6. Phylogeny of *B. dermatitidis* based on 27 microsatellite markers. Unrooted neighbor-joining tree of allele-sharing distance among unique haplotypes. This is constructed from 27 microsatellite loci for each 100 *B. dermatitidis* isolates. The two genetically distinct groups (a highly clonal, group clustering at the top, separated from the more polymorphic isolates that are clustering together at the bottom).

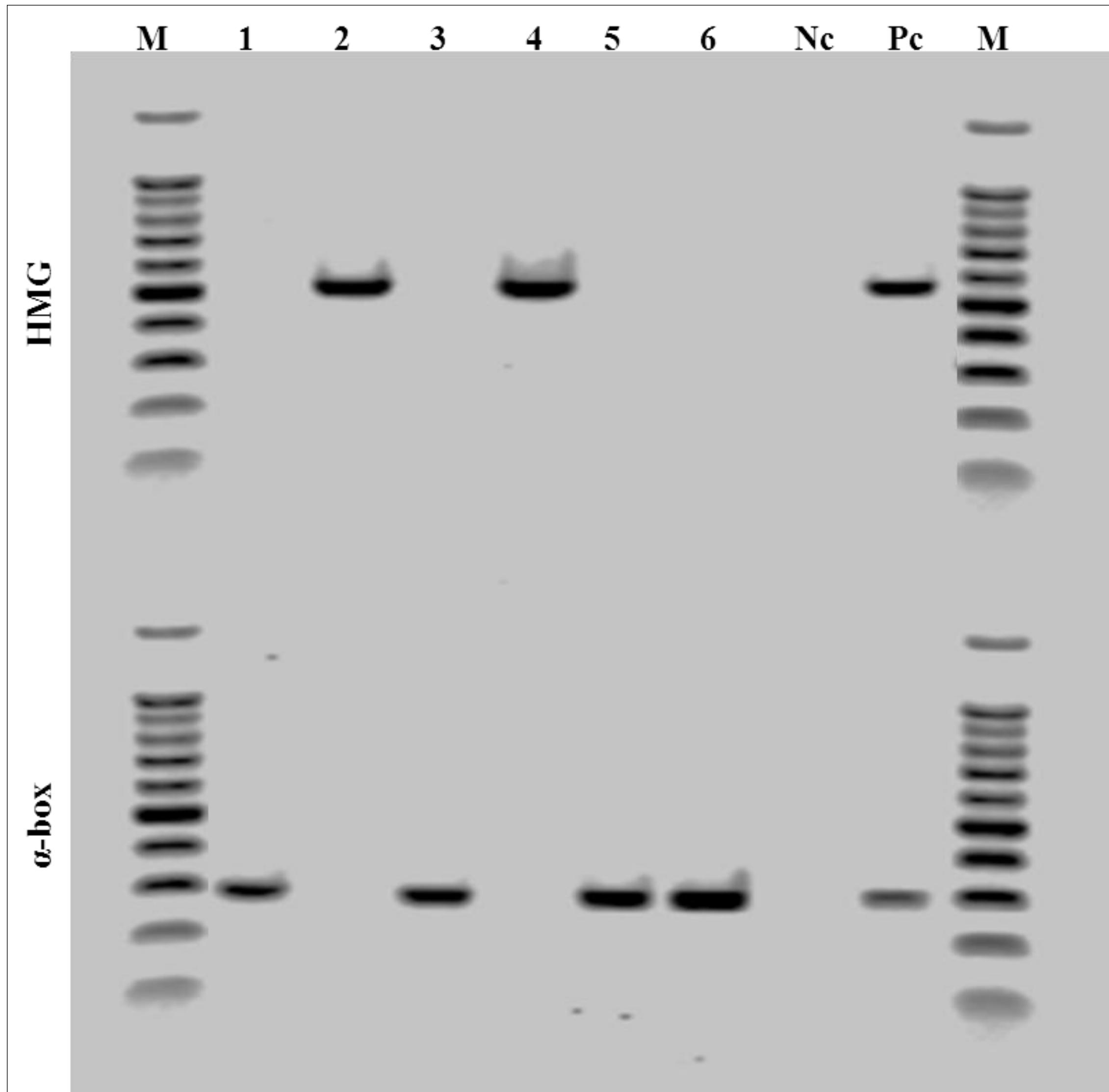


FIG. 7. Gel electrophoresis analysis of the mating type PCR product. This figure represents the PCR result for six isolates tested for the HMG and α-box alleles. The PCR product was run along with a molecular weight marker (M). The strains ATCC18188 and ATCC18187 were used as positive controls for HMG and α-box respectively (Pc). Isolate 1, isolate 3, isolate 5 and isolate 6 are negative for HMG but positive for α-box. Whereas isolate 2 and isolate 4 are positive for HMG but negative for α-box. Sterile distilled water was used as negative control in each experiment (Nc).

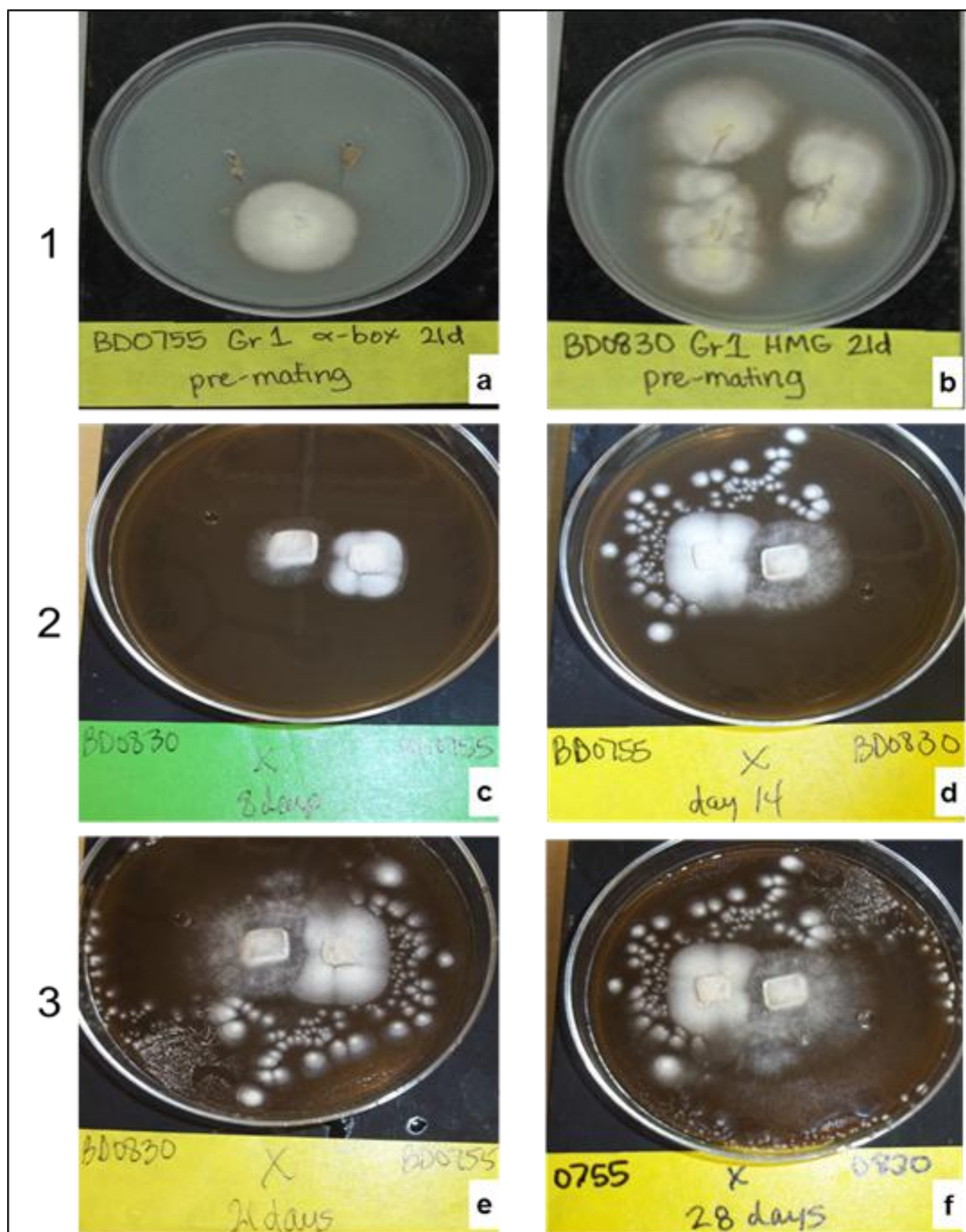


FIG. 8. Mating experiment showing sexual reproduction of *B. dermatitidis*. Line 1 represents an α-box isolate (a) and a HMG isolate (b) grown on potato flake agar plate for three weeks. In line 2, the two opposite mating type isolates are paired on soil extract agar plate at one and two weeks (c, d). In line 3, the experiment plates are monitored after three and four weeks of incubation (e, f).

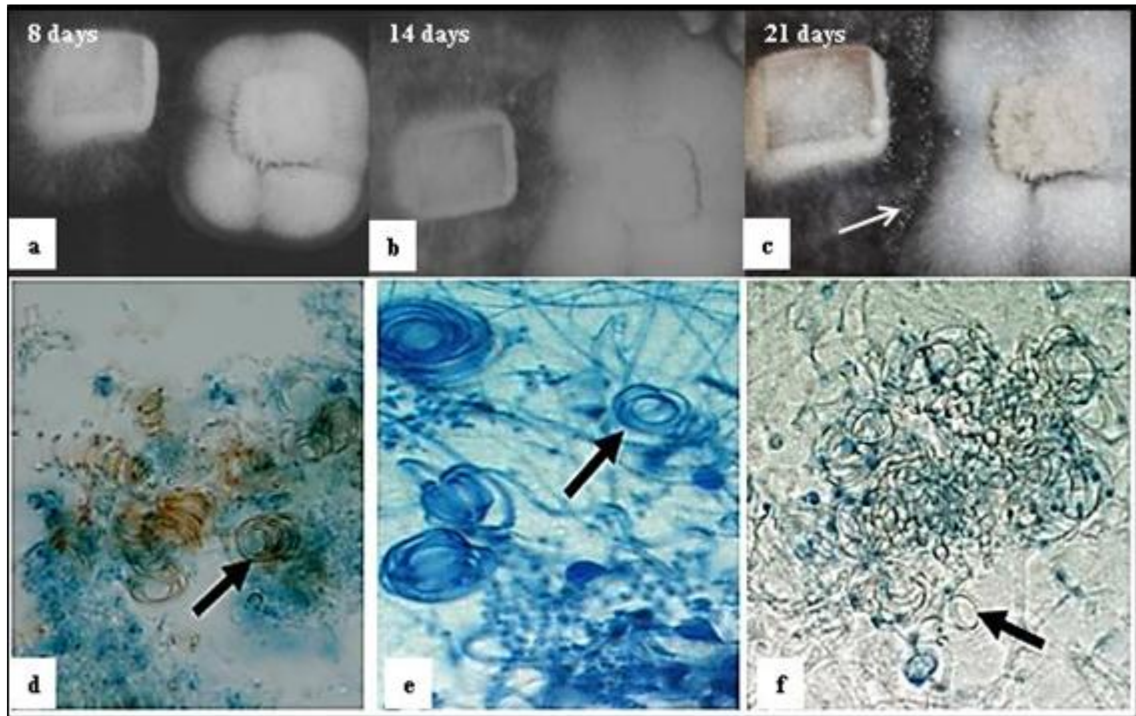


FIG. 9. Cleistothecia formation and ascospore production. Cleistothecia were observed on Yeast extract agar plates when α-box and HMG *B. dermatitidis* strains were coincubated. (a) After 1 week of incubation at 25° C, the two compatible isolates are growing toward each other. (b) At 14 days, hyphae from the two isolates are mixed together. (c) Cleistothecia formed by mating crosses. The cleistothecium is a small white structure formed on mating plates and visible with the naked eye (white arrow). (d, e, f) Cleistothecia were monitored with light microscopy. Lactophenol cotton blue staining of the produced cleistothecia showing coil of spiral hyphae. Branched hyphae forming the coiled surface are identified by black arrows.

In an attempt to provide genetic evidence that sexual recombination was occurring in the successful crosses, cleistothecia were harvested, and then plated on yeast extract agar plates for genotyping. We chose to test ten offspring for genetic recombination in three experiments. Only eight progeny from each experiment were available for testing. In addition, we were not able to test all of them for the 27 loci. TABLE 2, 3 and 4 show the results of genotyping and MAT locus typing of the parental and the progeny strains with microsatellite analysis and the α-box and HMG loci PCR reactions. In the tables, each number represents the size of the allele at the corresponding loci.

From these results, the evidence of genetic recombination between the parental strains was difficult to demonstrate and thus to confirm that the offspring were the result of sexual reproduction.

TABLE 1. Genotyping analysis of ATCC18188 and ATCC18187 and the corresponding offspring resulting from their cross

Loci	Parents		Offspring							
	ATCC18188	ATCC18187	1	2	3	4	5	6	7	8
MAT	alpha box	hmg	hmg		hmg	hmg	hmg	hmg	hmg	alpha box
Locus1	250	248		250	250	250	248	250	248	250
Locus2	211	211	211	211	211	211		211	211	
Locus3	223	223	223	223	223	223	223	223	223	223
Locus4	229	231	231	231	231	231	231	231	231	229
Locus5	208	214	214	214	214	214	214			
Locus6	254	265	265	265	265	265	265	265	265	
Locus7	257	255	253	253	253	253	253	253	253	253
Locus8	208	208	208	208	208	208	208	208	208	208
Locus9	216	216	216	216	216	216		216	216	216
Locus10	214	214	214	214	214	214	214	214	214	214
Locus11	173	173	175	175	175	175	175	175	175	173
Locus12	259	249	249	249	249	249	249	249	249	259
Locus13	184	200	200	200	200	200	200	200	200	184
Locus14	267	267	267	267	267	267	267	267	267	267
Locus15	202	202	202	202	202	202		202	202	202
Locus16	260	258	258	258	258	258	258	258	258	260
Locus17	242	242	242	242	242	242		242	242	
Locus18	222	222	220	220	220	220	220	220	220	220
Locus19	245	245	245	245	245	245	245	245	245	245
Locus20	237	237	237	237	237	237		237	237	237
Locus21	197	197	197	197	197	197		197	197	197
Locus22	206	206	206	206	206	206	206	206	206	206
Locus23	228	228	226	226	226	226	228	226	226	228
Locus24	159	159	159	159	159	159	159	159	159	159
Locus25	252	252	252	252	252	252	252	252	252	252
Locus26	167	167	167	167	167	167	167	167	167	167
Locus27	241	241	239	239	239	239	239	239	239	241

- Parent HMG
- Parent alpha box
- Different from parents
- Parent allele

TABLE 2. Genotyping analysis of BD0636 and BD0811and the corresponding offspring resulting from their cross

Loci	Parents		Offspring							
	BD0636	BD0811	1	2	3	4	5	6	7	8
MAT	alpha box	hmg	hmg	hmg	hmg	hmg	hmg	hmg	hmg	hmg
Locus1	250	250	252	252	252	252	252	252	252	252
Locus2	213	209	213	213	213	213	213	213	213	213
Locus3	199	203	203	203	203	203	203	203	203	
Locus4	219	233		233	233	233	233	233		
Locus5	214	208	208	208	208	208	208			
Locus6	279	265	265	265	265	265	265	265	265	265
Locus7	275	255	257	257	257	257	257	257	257	257
Locus8	214	210	210	210	210	210	210	210		
Locus9	194	196	196	196	196	196	196	196	196	
Locus10	206	206	206	206	206	206	206	206	206	
Locus11	155	175	175	175	175	175	175	175	175	175
Locus12	251	245	245	245	245	245	245	245	245	245
Locus13	192	202	202	202	202	202	202	202	202	202
Locus14	263	255	255	255	255	255	255	255	255	255
Locus15	198	202	200	200	200	200	200		200	
Locus16	254	258	258	258	258	258	258	258		
Locus17	232	222	222	222	222	222	222	222		
Locus18	200	194	194	194	194	194	194	194	194	194
Locus19	231	241	241	241	241	241	241	241		
Locus20	221	235	235	235	235	235	235	235	235	235
Locus21	189	201		201	201	201		201		
Locus22	200	204	204	204	204	204	204	204	204	204
Locus23	220	222	224	222	224	224	224	224	224	
Locus24	165	167	167	167	167	167	167	167		
Locus25	250	250	250	250	250	250	250	250	250	250
Locus26	179	199	199	199	199	199	199	199	199	
Locus27	235	233	239	239	239	239	241	241	239	

Parent HMG
Parent alpha box
Different from parents
Parent allele

TABLE 3. Genotyping analysis of BD0707 and BD0745 and the corresponding offspring resulting from their cross

Loci	Parents			Offspring							
	BD0707	BD0745		1	2	3	4	5	6	7	8
MAT	alpha box	hmg		hmg	hmg	hmg	hmg	hmg	hmg	hmg	hmg
Locus1	250	250		252	252	252	252	252	252	252	252
Locus2	209	213		213	213	213	213	213	213	213	213
Locus3	209	201		201	201	201	201	201	201	201	201
Locus4	229	219		219	219	219	219	219	219	219	219
Locus5	224	214		214	214	214	214	214			
Locus6	289	277		277	277	277	277	277	277	277	277
Locus7	255	275		275	275	275	275	275	275	275	275
Locus8	208	214		212	212	212	212		214	214	214
Locus9	194	194		194	194	194	194	194	194	194	194
Locus10	206	206		206	206	206	206	206	206	206	206
Locus11	183	155		155	155	155	155	155	155	155	155
Locus12	253	251		251	251	251	251	251	251	251	251
Locus13	184	192		192	192	192	192	192	192	192	192
Locus14	269	263		263	263	263	263	263	263	263	263
Locus15	202	198		198	198	198	198	198	198	198	198
Locus16	258	254		254	254	254	254	254	254	254	254
Locus17	234	232		232	232	232	232	232	232	232	232
Locus18	214	200		200	198	198	198	200	200	198	198
Locus19	241	231		231	231	231	231	231	231	231	231
Locus20	249	221		221	221	221	221	221	221	221	221
Locus21	201	191		191	191	191	191	191		191	
Locus22	216	200		200	200	200	200	200	200	200	200
Locus23	220	220		220	220	220	220	220	220	220	220
Locus24	167	165		165	165	165	165	165	165	165	165
Locus25	252	250		250	250	250	250	250	250	250	250
Locus26	183	179		179	179	179	179	179	179	179	179
Locus27	233	233		233	233	233	233	233	233	233	233

Parent HMG
Parent alpha box
Different from parents
Parent allele

Asexual Spore Quantification. Spore quantification experiments were done on fourteen isolates. Three experiments were attempted and each isolate run in duplicate. TABLE 4 and TABLE 5 show the results of the first two experiments.

In the first experiment, six strains were tested. The environmental isolate ER3 and the clinical isolate BD0901 were shown to produce more spores than the other strains tested.

In the second experiment, three strains were tested. In addition to these, we repeated two strains ran in the first experiment. The laboratory strain ATCC18187 was shown to produce more spores. But results for strain ER3 were not reproducible when compared to those from experiment 1.

In the third experiment, four strains were tested. In this experiment, results were not interpretable because of bacterial contamination.

TABLE 4. Results of the first spore quantification experiment

Experiment 1 Isolate ID	Genetic Group	Mating Type - HMG + alpha box	Isolate Source	Initial Rinse 100 µl plated CFU	10^{-1} dil 100 µl plated CFU	10^{-2} dil 100 µl plated CFU	10^{-3} dil 100 µl plated CFU	10^{-4} dil 100 µl plated CFU	10^{-5} dil 100 µl plated CFU
BD0830-1	1	-	human	6	1	NG	NG	1	1
BD0830-2				6	1	1	NG	NG	1
BD0901-1	2	-	human	4	1	NG	NG	NG	NG
BD0901-2				6	NG	NG	1	NG	NG
BD0922-1	2	-	human	66	5	NG	1	NG	NG
BD0922-2				33	3	NG	2	1	NG
BD0919-1	2	+	human	NG	NG	NG	NG	1	NG
BD0919-2				7	1	NG	NG	NG	NG
26199-1	2	-	ATCC	3	NG	1	NG	NG	NG
26199-2				1	NG	NG	NG	NG	NG
ER3-1	2	-	environment	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
ER3-2				TNTC	TNTC	TNTC	TNTC	TNTC	TNTC

TABLE 5. Results of the second spore quantification experiment

Experiment 2 Isolate ID	Genetic Group	Mating Type - HMG + alpha box	Isolate Source	Initial Rinse 100 µl plated CFU	10^{-1} dil 100 µl plated CFU	10^{-2} dil 100 µl plated CFU	10^{-3} dil 100 µl plated CFU	10^{-4} dil 100 µl plated CFU	10^{-5} dil 100 µl plated CFU
ER3-1	2	-	environment	2	NG	2	1	NG	NG
ER3-2				2	NG	1	NG	1	1
18187-1	2	-	ATCC	140	14	2	2	1	NG
18187-2				105	11	1	2	NG	cont
BD0824-1	2	+	human	1	3	2	3	NG	2
BD0824-2				36	5	1	2	2	3
18188-1	2	+	ATCC	1	1	1	2	NG	cont
18188-2				1	1	NG	2	1	3
26199-1	2	-	ATCC	4	2	1	2	NA	NA
26199-2				TNTC	TNTC	166	30		

Antifungal Susceptibility. YeastOne Sensititre® susceptibility testing was performed on six *B. dermatitidis* strains: three clinical isolates (BD0901, BD0916, BD0922), and three ATCC strains (ATCC26199, ATCC18187, ATCC18188). As recommended by the manufacturer, the minimal inhibitory concentration (MIC) was determined to be the lowest concentration of an antifungal agent that substantially inhibits growth of the organism as detected visually by observing a color change. The endpoints were analyzed at 48, 72, 96, and 192 hours of incubation for all nine drugs. The results demonstrated excellent antifungal activity within the azole group, consistent with clinical observations. Surprisingly, three of the isolates showed high MIC values for amphotericin B, a mainstay of treatment for blastomycosis and other invasive fungal infections. The observed MICs of 1.0 µg/ml are at the upper level of accepted blood concentrations in humans – higher values being associated with significant toxicity. FIG. 10 through FIG. 18 represent MIC analysis for all nine drugs at the four time points.

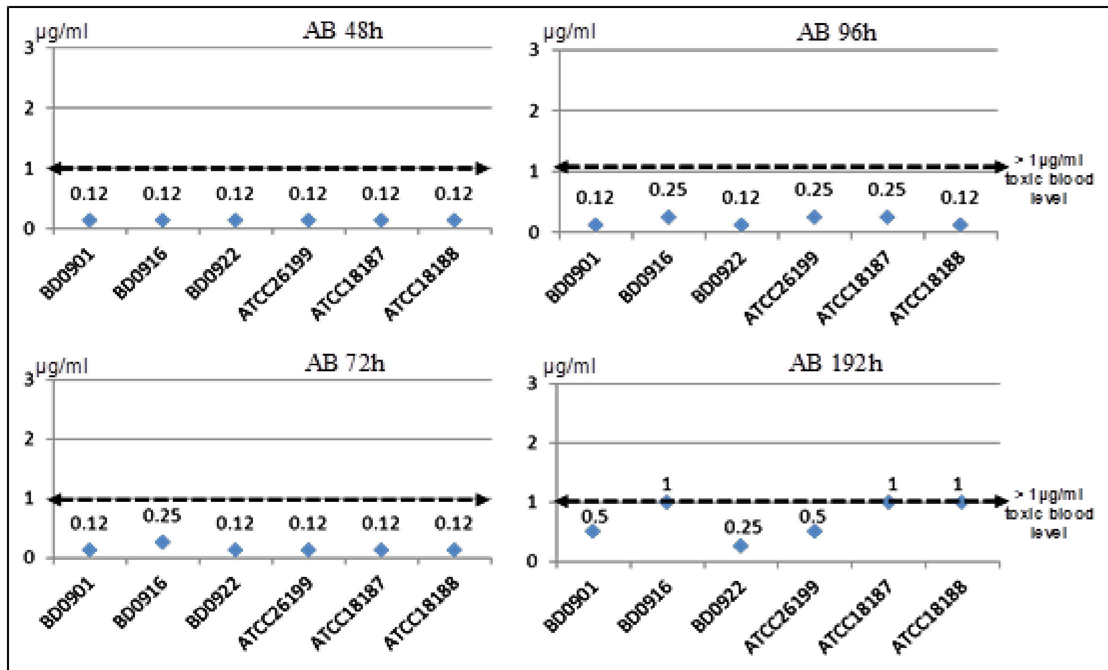


FIG. 10. MIC (μg/ml) vs. strains for amphotericin B at 48, 72, 96, and 192 h of observation. Amphotericin B showed low MIC for all isolates at 48 and 72 h with an increase with the strain BD0916. At the latest observation point, MIC high as 1 μg/ml was observed with the strain BD0916, ATCC18187 and ATCC18188.

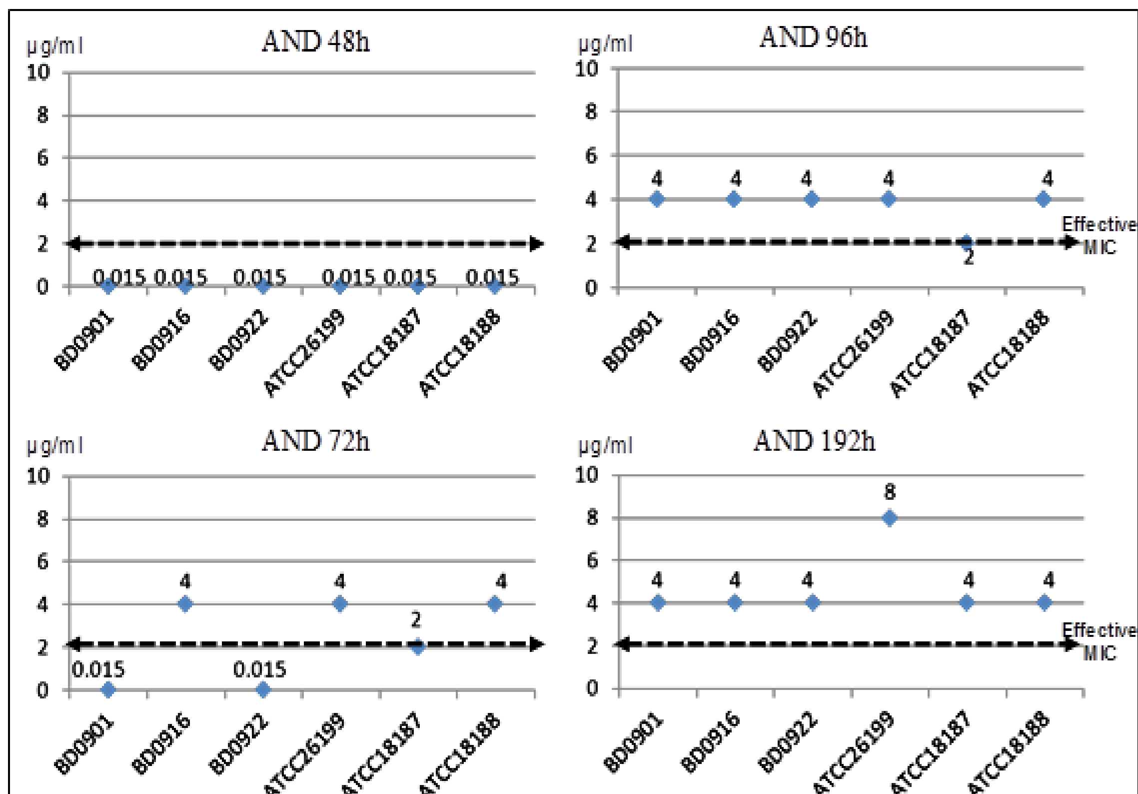


FIG. 11. MIC (µg/ml) vs. strains for anidulafungin at 48, 72, 96, and 192 h of observation. There was an increase MIC of anidulafungin over the time. The highest MIC was seen with the strain ATCC26199 at 192 h with 8 µg/ml.

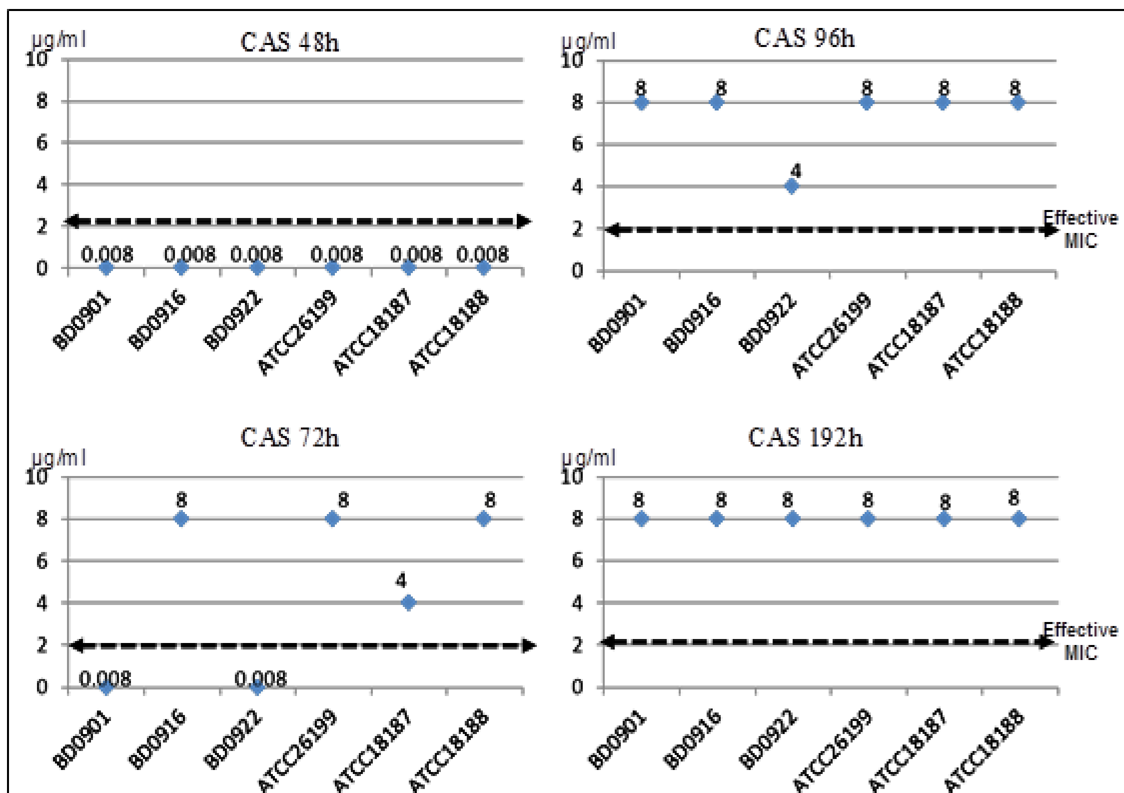


FIG. 12. MIC (µg/ml) vs. strains for caspofungin at 48, 72, 96, and 192 h of observation. Like in anidulafungin, high MICs were observed starting at 72 h for the caspofungin. All strains displayed the highest MIC at the latest observation point.

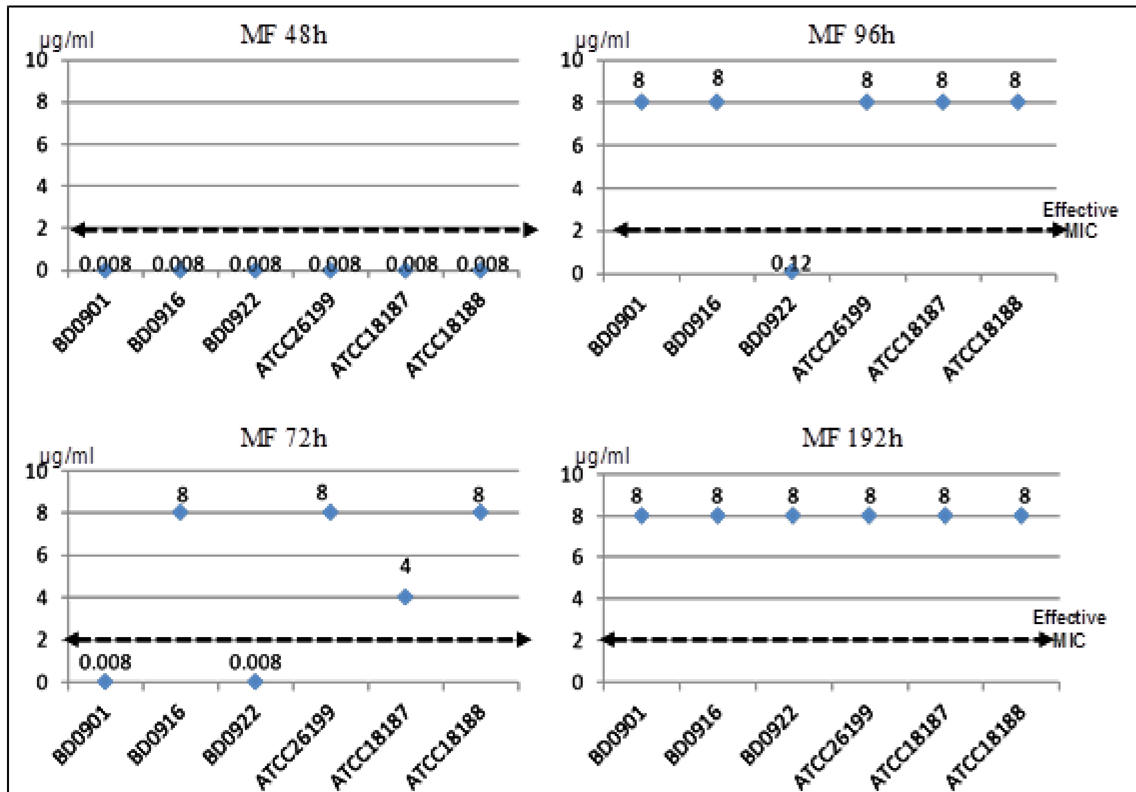


FIG. 13. MIC (µg/ml) vs. strains for micafungin at 48, 72, 96, and 192 h of observation. The micafungin displayed the same pattern as seen in caspofungin.

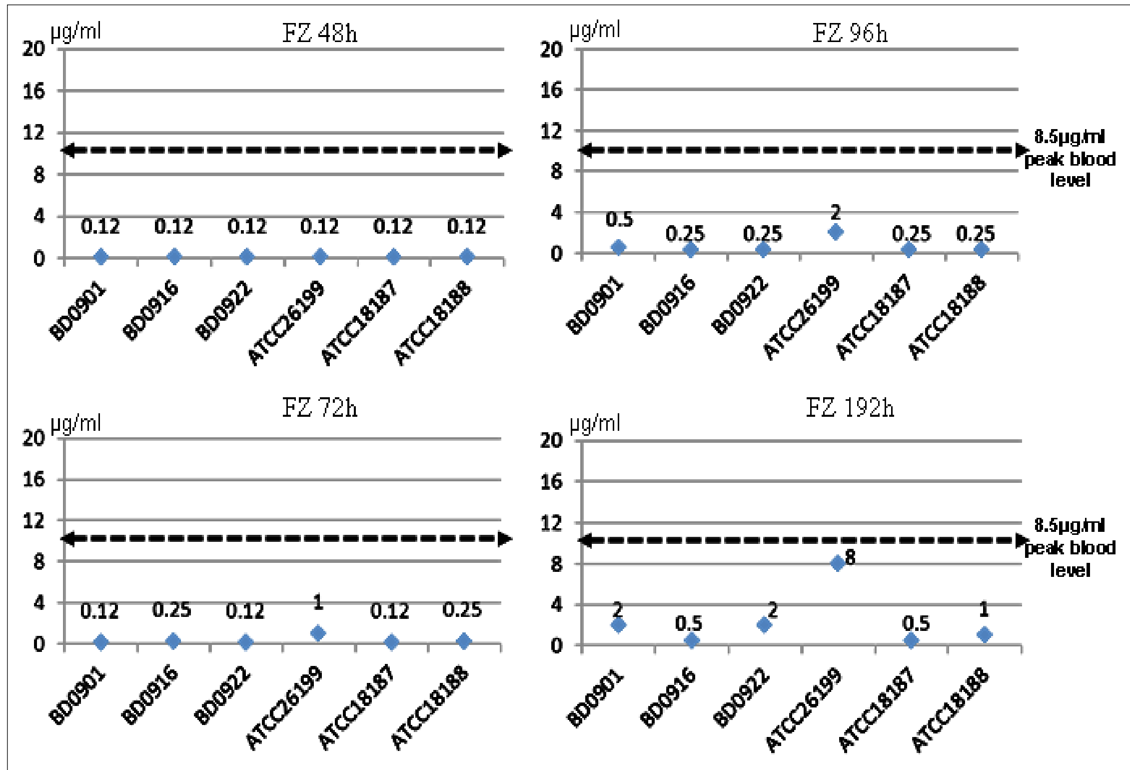


FIG. 14. MIC (µg/ml) vs. strains for fluconazole at 48, 72**,** 96, and 192 h of observation. Low MICs were observed for all strains except the ATCC26199. For this strain, the MIC was 8 µg/ml.

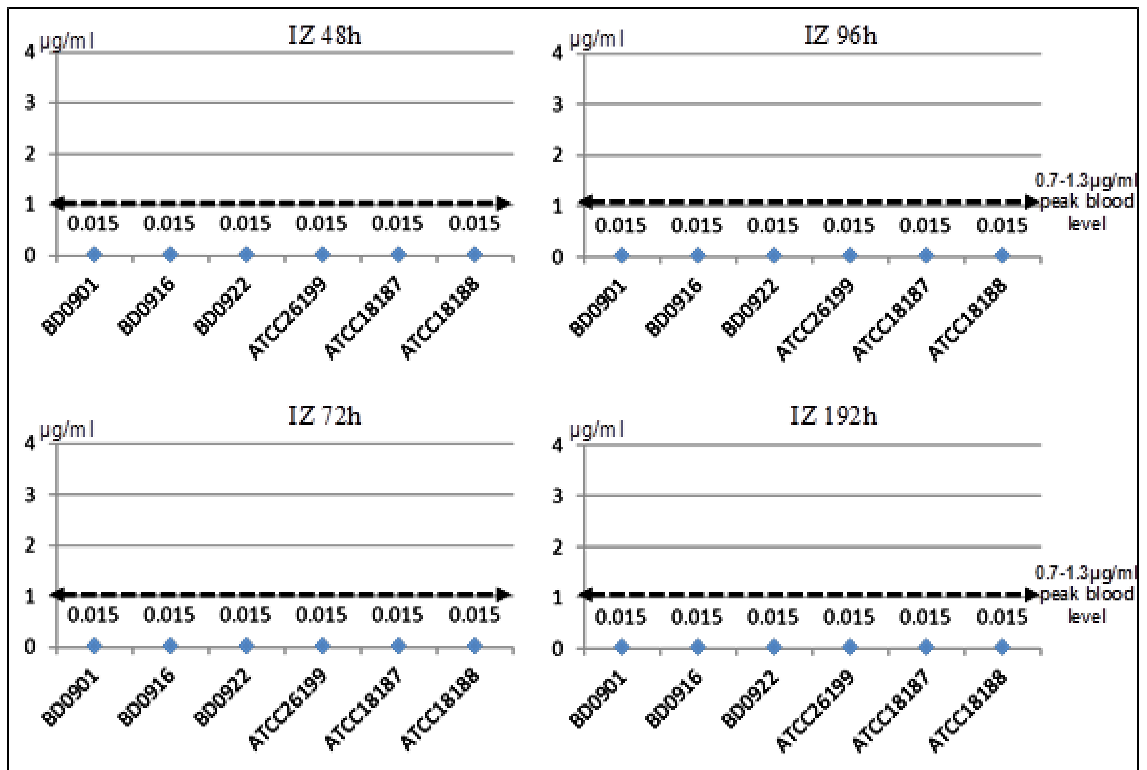


FIG. 15. MIC (µg/ml) vs. strains for itraconazole at 48, 72, 96, and 192 h of observation. The itraconazole MIC was consistently low for all strains through the time of observation.

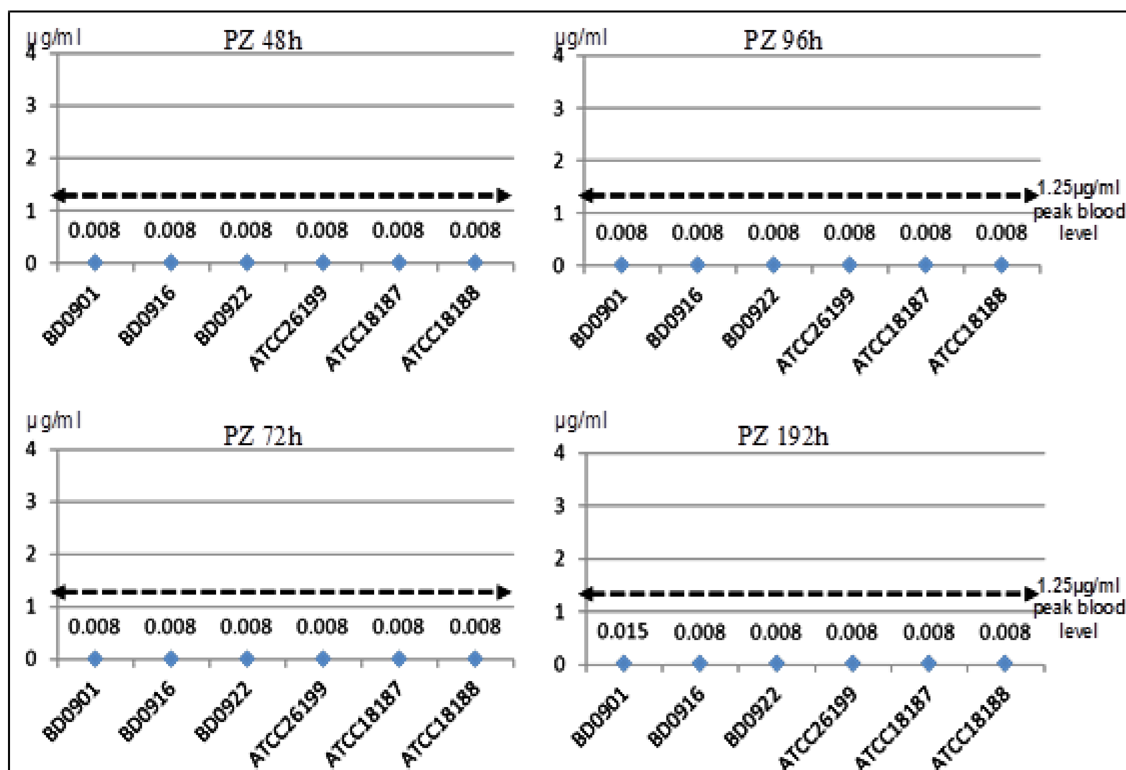


FIG. 16. MIC (µg/ml) vs. strains for posaconazole at 48, 72, 96, and 192 h of observation. Like in itraconazole, low MICs were observed over the time for posaconazole.

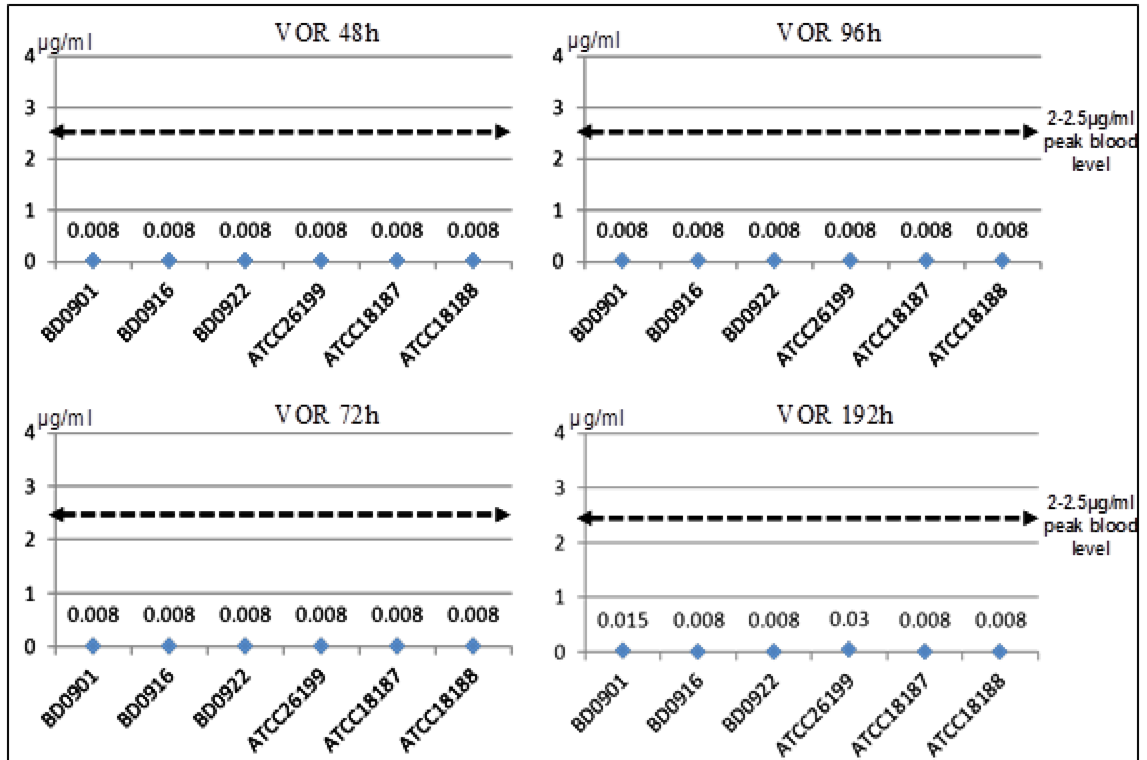


FIG. 17. MIC (µg/ml) vs. strains for voriconazole at 48, 72**,** 96, and 192 h of observation. Voriconazole displayed similar pattern seen with itraconazole and posaconazole.

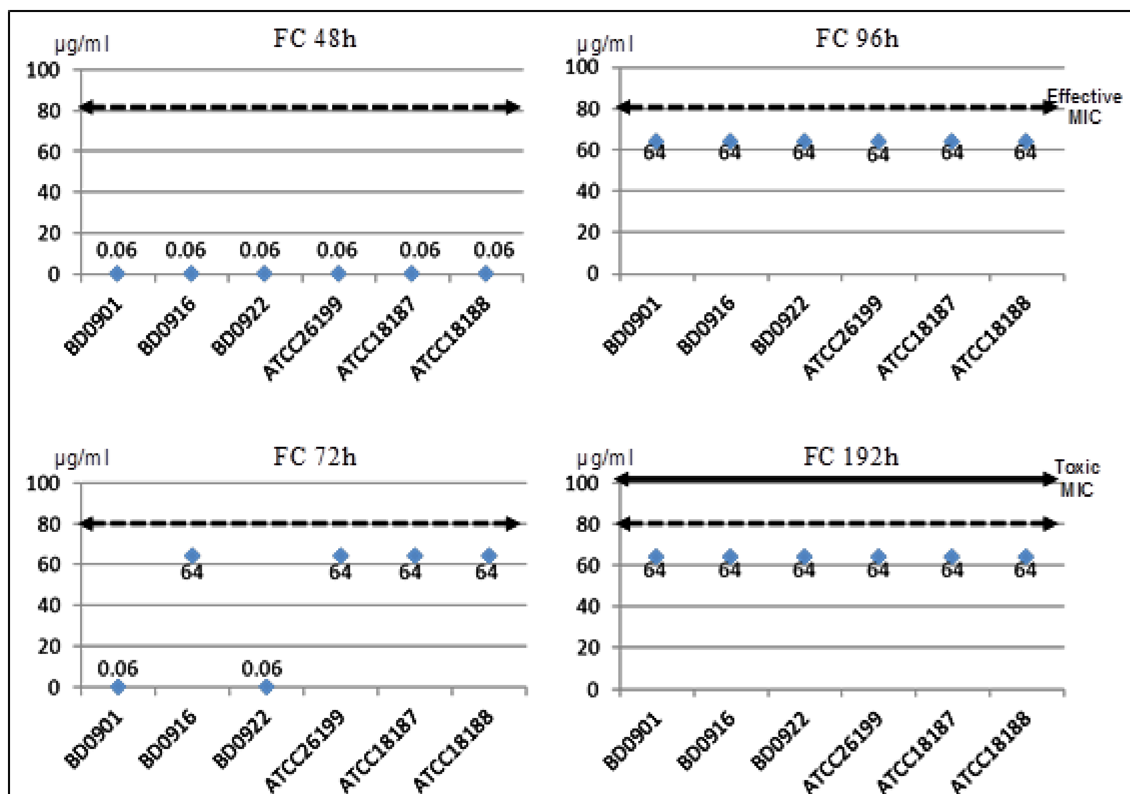


FIG. 18. MIC (µg/ml) vs. Strains for 5-flucytosine at 48, 72**,** 96, and 192 h of observation. High MICs were seen with 5-flucytosine starting at 72 h. The highest MIC was observed for all strains at 192 h.

DISCUSSION

Blastomycosis is an important public health problem in Wisconsin and other endemic areas in North America. The clinical presentation is usually a pulmonary infection that, untreated, can progress into a rapidly fatal disseminated infection. Recent genotyping studies based on microsatellites analysis have demonstrated that most *B. dermatitidis* isolates fall into one of two genetic groups and there are statistically significant differences in clinical presentation and course between the groups. In this study, we aimed to compare phenotypic characteristics of selected *B. dermatitidis* isolates from the two genetic groups.

B. dermatitidis is a heterothallic ascomycete requiring two mating types for mating. Strains of plus mating type (α-box) have the *MAT1-1* idiomorph of the mating locus while strains of minus mating type (HMG) have the *MAT1-2* idiomorph of the mating locus. When we analyzed 100 isolates for mating type using a highly sensitive and specific PCR assay, we found that the two mating types were nearly equally distributed among our samples. This is consistent with findings from a previous study (44). Interestingly, two of our samples contained both mating types, a finding that has been reported by other investigators (41). This suggests that these patients were infected in an environment that contained multiple strains of the organism. This is important from an evolutionary standpoint because it suggests that both mating types can occupy the

same ecologic niche, providing the opportunity for mating and recombination in natural settings. Some studies have suggested that the *MAT* locus may be important in modulating virulence and pathogenicity (39). For example, in *H. capsulatum*, a closely related organism phylogenetically, strains of (-) mating type may be associated with increased virulence; moreover, in *B. dermatitidis* this association is currently unknown. Moreover, in *H. capsulatum*, organisms of (-) mating type are more often associated with pulmonary histoplasmosis. However organisms of both mating types are represented equally in samples from patients with severe disseminated histoplasmosis and in environmental samples (36, 37).

Sexual reproduction has been documented in many fungal species and occurs under appropriate conditions. For many fungi, the mechanisms are very well known; but for some, it still needs to be elucidated. In *B. dermatitidis*, mating occurs when two compatible strains come together in the laboratory setting. How often mating occurs in nature is a question that has not yet been answered.

We were able to observe mating between multiple strains of *B. dermatitidis* from both genetic groups using a specialized soil extract agar. As suggested in previous studies, yeast extract agar with or without bone meal was found to enhance the hyphal growth and stimulate sporulation (43). In our study we found that all strains, when paired with opposite mating type strain produced cleistothecia with numerous ascospores. Several studies have been conducted on mating in *B. dermatitidis.* In 1977, McDonough et al. (44) conducted a study on 206 isolates of *Blastomyces dermatitidis.* The isolates were paired individually with each of 3 known "+" and "–" strains. They found that 129 isolates were reproductively fertile. In the remaining 77 cultures, the failure to initiate

ascospore production was explained by the age of the culture and the stress due to multiple laboratory passages. Storage of isolates in freezing condition was also described to diminish fertility. In our study, crosses which failed to produce cleistothecia were, as expected, associated with crosses of the same mating type.

We attempted to demonstrate evidence of genetic recombination with three crosses by doing microsatellite analysis of offspring (ascospores) recovered from cleistothcia. The results were extremely difficult to interpret; one cross resulted in offspring that were basically identical to one of the parental strains while offspring from the other two crosses showed minimal and/or inconclusive evidence of recombination. A major limitation in our study design was not utilizing microdissection equipment to isolate individual ascospores for subculture. Based on our findings it seems possible that our subcultures were contaminated with conidia from parental strains. Further research will be necessary to determine whether or not recombination is occurring between group 1 and group 2 strains of *Blastomyces*.

Many theories suggest that sexual reproduction is expensive and inefficient (12) as it requires that the two mating partners being in the same environment which can explain why asexual reproduction is more common in Ascomycete. In *B. dermatitidis*, the asexual propagules are termed conidia, which are the infectious form of the fungus. Strains producing more spores could present a greater potential of infectivity. Previously, studies have shown that the group 1 isolates are more associated with pulmonary forms of the disease which can lead one to speculate that this genetic group may produce more spores in the environment. Our finding could not confirm this trend as our experiments lacked reproducibility and were so time-consuming to perform that only a limited number

of strains could be analyzed. This lack of reproducibility in measurement of sporulation has been described by others (60).

It is generally accepted that a number of antifungal drugs are effective in treating blastomycosis infection, although occasional treatment failures raise the question of inherent antifungal resistance. Unfortunately there has been little progress in developing and standardizing methods for testing *B. dermatitis* and other dimorphic fungi in the clinical microbiology laboratory. The method used in this study was developed initially for antifungal susceptibility of non-fastidious yeast, including *Candida* species, *Cryptococcus* species, and other rapid growing yeast species in a research setting only. This method is not currently approved for clinical use.

For the six strains tested, amphotericin B MICs were between 0.25 and 1.0 µg/ml. Specifically, for three strains, the MIC was 1µg/ml, which is at the upper limit of steady state plasma concentration considered to be safe in humans. For the strains ATCC18187 and ATCC18188, the high MICs observed could be explained by the fact that control strains undergone a large number of laboratory passages which over time can be associated with the development of antimicrobial resistance. The other strain associated with a high MIC was BD0916, a clinical isolate from a patient who died of blastomycosis. This finding has clinical significance and suggests that testing of additional clinical isolates will be important in determining to what extent amphotericin resistance contributes to poor clinical outcomes in blastomycosis.

The azoles as a group were associated with consistently low MIC values. Itraconazole, fluconazole, posaconazole, voriconazole, and ketoconazole are the azole antifungal drugs that are available for the treatment of mild to moderate blastomycosis.

Ketoconazole is being widely replaced by itraconazole which is better tolerated and by fluconazole which has better CNS penetration. Indeed ketoconazole was not tested in our study because the commercially available kit we used did not contain this drug. For all isolates, MICs of itraconazole, posaconazole, and voriconazole were far below the effective blood concentration. In one isolate (ATCC26199) the high MIC seen in fluconazole could be due to the fact that, like the situation with amphotericin B, the isolate in question was a laboratory-adapted strain.

In contrast, our isolates showed increase MICs for the echinocandins group. The echinocandins act through non-competitive inhibition of the β(1,3)-glucan synthase enzyme complex of the fungal cell wall. Echinocandins differ mostly by their pharmacodynamics. While caspofungin and micafungin are primarily eliminated in the liver by hydrolysis, anidulafungin undergoes slow chemical degradation at physiologic temperature and pH. In several studies, echinocandins MIC above 2 μg/ml was considered as resistance. In our study, the MICs for anidulafungin were between 4-8 μg/ml at the latest observation point. This finding can be explained by the instability of this drug which spontaneously degrades at body temperature and physiologic pH. Similar to anidulafungin, caspofungin and micafungin displayed high MICs over the 2μg/ml effective concentration. These findings suggest that *Blastomyces* has inherent resistance to this class of antifungals, and, in fact, echinocandins are not recommended for the treatement of blastomycosis.

Our isolates showed high MICs to 5-flucytosine. This drug is used primarily in combination with amphotericin B as therapy for some selected *Candida* and cryptococcal infections. Moreover, the drug has limited spectrum of activity and significant potential

toxic effects. As a result this drug is not approved for the treatment of blastomycosis and other life threatening systemic mycoses, and our data support this guideline with regard to blastomycosis.

Overall, our study experiments were challenging to perform because of the slow, and sometimes inconsistent, growth rate of the fungal strains. Nonetheless, genetic typing and mating locus determinations were accomplished successfully for all 100 isolates, and the findings were relatively consistent with results published previously in the medical literature. Additionally, results of the mating crosses provided strong evidence that the two genetic groups are capable of sexual reproduction, at least in the laboratory setting. Whether or not recombination is occurring between these groups in natural settings remains an unanswered question. Improvements in the methodology of isolating and genotyping single ascospore cultures from group 1 and group 2 crosses will be an important goal for future research in this area.

The other phenotypic analyses (spore quantitation and antifungal susceptibility testing) were found to be more challenging than expected and resulted in only a small group of isolates being evaluated. Again, this was due mostly to the slow growth rate of the organism, but also the need to develop and continually modify techniques as the study progressed. For the spore quantification, reproducibility and presence of contaminants were major limiting factors in acquiring interpretable data. Although only a small number of isolates were evaluated for antifungal susceptibility, the new knowledge and experience acquired as part of this study provides a strong foundation for further improvements that may ultimately result in this technology being successfully applied in clinical microbiology laboratories in the future.

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